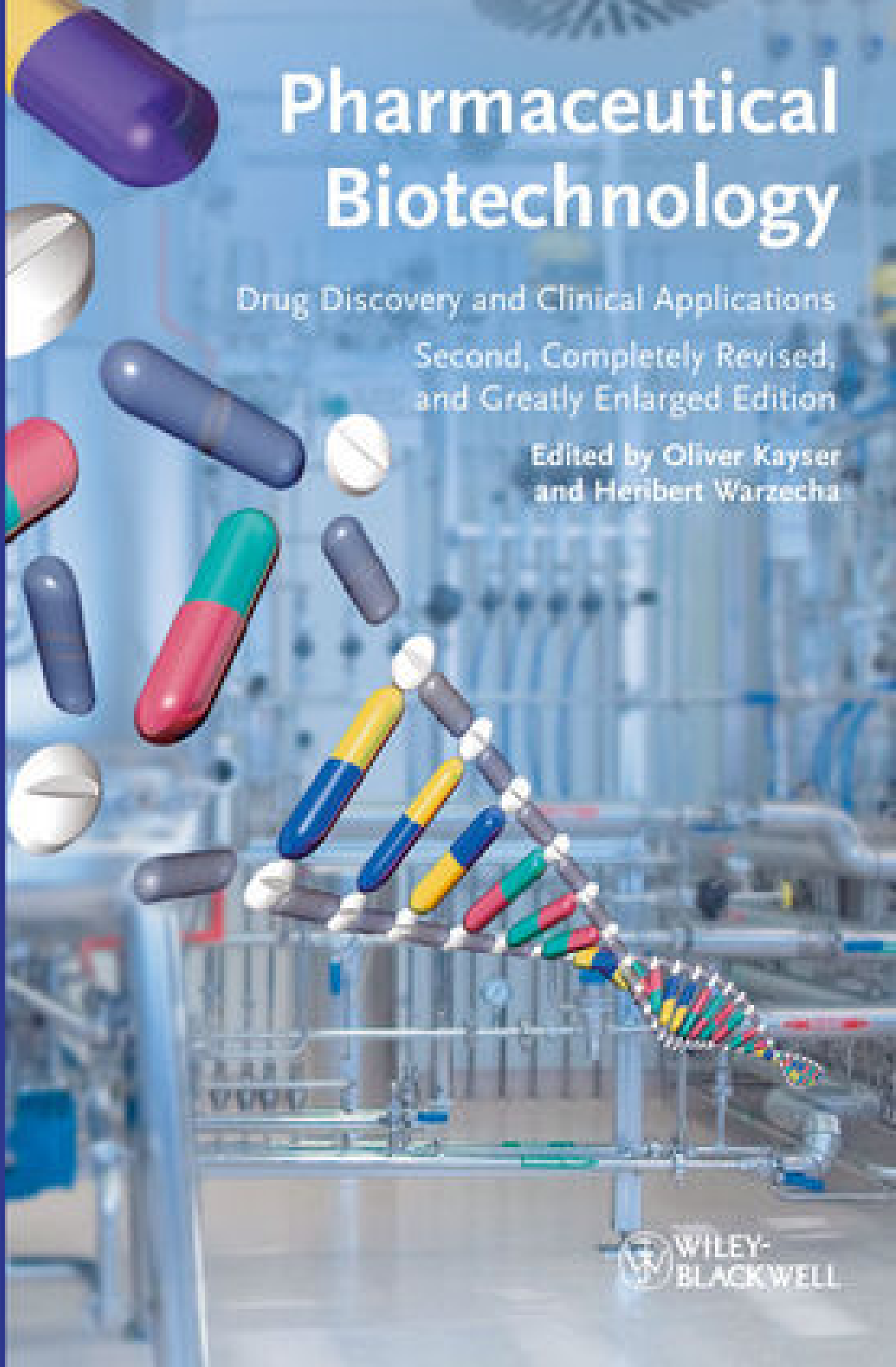
Pharmaceutical Biotechnology
Drug Discovery and Clinical Applications
Second, Completely Revised, and Greatly Enlarged Edition
Edited by Oliver Kayser and Heribert Warzecha
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Pharmaceutical Biotechnology

Drug Discovery and Clinical Applications

Second, Completely Revised, and Greatly Enlarged Edition

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Contents

Preface to the 2nd Edition

Pharmaceutical biotechnology has emerged as one of the major disciplines for drug discovery and development. In the past, the pharmaceutical branch of biotechnology – the former red biotechnology – was limited to fermentation and production of recombinant therapeutic proteins. Today, the shape and vision of pharmaceutical aspects and challenges have completely changed, and the prefix "pharma" can also be accepted as a synonym for integrated life science approaches, ranging from genetics to molecular biology to diagnostics, with the common goal of delivering the best drug to the patient by biotechnological techniques.

If we take a look at the first edition of *Pharmaceutical Biotechnology*, we see that the focus was more on molecules as potential drugs and less on the production strategies and the molecular concepts behind. The completely updated and rewritten second edition reflects the emerging trend in the pharmaceutical industry where molecular biology techniques and genetics play an increasingly important role. Today, many new biological entities can be characterized as muteins or significantly backbone-modified proteins, an exception in 2004 when we published the first edition (see insulin muteins). We are glad that we were able to attract the majority of the authors from the previous edition as experts. They reviewed the latest trends in their subjects of expertise and shared their experience and open opinion about the developments from the recent years to the near future. Pharmaceutical biotechnology and the pharmaceutical industry is a fast moving business and we all know that the future is hard to predict, but we are glad that with the selected contributors being in touch with industrial needs and challenges, we made the right choice to give answers to the readers' questions not only about new developments in protein production, host organism selection, and future platform organisms for biosynthesis and vaccine production, but also on biological generics, drug formulation, and legal aspects of biotechnology. In this textbook you will find updated facts and figures about the pharmaceutical industry and the latest drug approvals. In the first part a detailed discussion is provided about production systems for the biosynthesis of both low molecular weight drugs and proteins in prokaryotic and eukaryotic cell cultures and organisms. In the second part the drug formulation and manufacturing process is in focus, but we also want to highlight quality control and bioanalytical aspects, which have been largely neglected before. Therefore, this second part is now updated and dedicated to the

recombinant therapeutic proteins and vaccines that are already in clinical use, as well as requirements for quality control. In contrast to the first edition we recognized that drug regulation and quality assurance are becoming more important, while the legal aspects of drug patenting, and the drug approval process are again emphasized. In the third part we had a hard task of sorting and structuring the emerging diversity of research and development in this field and bring it under one single chapter. This is nearly impossible, but our aim is to guide the reader through the new upcoming lines of research impacted by genetics, synthetic biology, and nanobiotechnology. Finally we selected chapters showing exemplarily ongoing research trends that, hopefully, will find their way into clinical applications in the future or as approved drugs into the second edition of this textbook. Well-updated by authors from the previous edition, we learn about personalized medicine and xenotransplantation, and we are proud to introduce new contributors telling us about nanocarriers as future drug delivery systems, ultrahigh-throughput screening for accelerated drug discovery, and transgenic plants as future green factories.

The editors want to thank all the authors for their valuable contributions and the time they have invested in this work. We know very well that time was and is a scarce resource and that the chapters were written alongside the authors' regular duties. Special thanks also to the families behind for their patience and understanding why time was spent in this project. Special thanks to Anne Chassin du Guerny and Gregor Cichetti of Wiley-Blackwell for their professional support in the layout, proofreading, and production of this textbook.

We know that this book is far from being complete and we are aware that by the day of publishing it could be updated again. But our intention is to provide a "primer" for the interested reader to start working and to show how exciting research is in this fast moving field of life science.

Dortmund and Darmstadt, January 2012

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Part One
Concepts and Methods for Recombinant Drug Production

1
Pharmaceutical Biotechnology and Industrial Applications – Learning Lessons from Molecular Biology

Oliver Kayser and Heribert Warzecha

1.1
Introduction

To date, biotechnology has produced more than 200 new therapies and vaccines, including products to treat cancer, diabetes, HIV/AIDS, and autoimmune disorders. There are more than 400 biotech drug products and vaccines currently in clinical trials, targeting more than 200 diseases, including various cancers, Alzheimer's disease, heart disease, diabetes, multiple sclerosis, AIDS, and arthritis. These few figures demonstrate the importance of biotechnological methods and techniques, which are increasingly dominating the process of drug research and development [1].

An average approval of 10–15 products a year indicates that pharmaceutical biotechnology is a highly active sector. Amongst these, the number of genuinely new biopharmaceuticals is around 40%, indicating the high innovative character of research; some of these products are likely to be future blockbusters (Table 1.1). Examples are monoclonal antibody-based products such as Rituximab (Rituxan®/MabThera®) for the treatment of cancer with $18 billion in sales in 2009, insulin and insulin analogues ($13.3 billion/2009), and finally erythropoietin-based products ($9.5 billion/2009). The global market is growing by 7% per year for protein-based therapeutics and among all blockbuster drugs only one is a classical low molecular drug, the other four top selling drugs (Table 1.2) are derived from the biotechnology sector [3]. In addition to new drug entities (NDE), biosimilars or follow-up-biologicals will continue to increase in market value; this is the focus of Chapter 13. This trend is supported by new or adapted approved routes from the regulatory bodies such as the EMA (European Medicines Agency) and the FDA (Food and Drug Administration) (see Chapter 11).

Established molecular biology techniques for protein engineering, such as phage display, construction of fusion proteins or synthetic gene design, have matured to the level where they can be transferred to industrial applications in recombinant protein design. Traditional engineering has focused on the protein backbone, while modern approaches take the complete molecule into account. We want to discuss recent advances in molecular engineering strategies that are now

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Table 1.1 Classification of recombinant proteins for human use (according to [1]).

Category	Product
Genuinely new biopharmaceuticals	Actemra®/Roactrema®, Arcalyst®, Arzerra®, Atryn®, Cervarix®, Cimzia®, Elaprase®, Elonva®, Gardasil®/Silgard®, Ilaris®, Kalbitor®, Lucentis®, Myozyme®, Nplate®, Preotach®, Prolia®, Provenge®, Recothrom®, Removab®, Scintimun®, Simponi®, Soliris®, Stelara®, Vectibix®, Victoza®
Biosimilars	Abseamed®, Binocrit®, Biogastrim®, Epoetin-α-heaxal (Erythropoetin)®, Filgastrim hexal®, Filgrastim ratiopharm®, Nivestim®, Omnitrope®, Ratiogastrim®, Valtropin®, Zarzio®
Reformulated me-too and related	Accretropin®, Biopin®, Eporatio®, Extavia®, Exubera®[a)], Fertavid®, Lumizyme®, Mircera®, Novolog mix®, PEGintron/ribetol combo®, Pergoveris®, Opgenra®, Vpriv®, Xyntha®
Previously approved elsewhere	Increlex®, Macugen®, Naglazyme®, Orencia®, Tysabri®

a) No longer available.

Table 1.2 The ten top selling recombinant proteins for human use in 2010 (source: LaMerie Business Intelligence, Barcelona [2]).

Product	Sales value (US$ billions)	Company
Enbrel®, Etanercept	6.58	Amgen, Wyeth, Takeda Pharmaceuticals
Remicade®, Infliximab	5.93	Centocor, Schering-Plow, Mitsubishi Tanabe Pharma
Avastin®, Bevacizumad	5.77	Genentech, Roche, Chugai
Rituxan®, Rituximab	5.65	Genentech, Biogen-IDEC, Roche
Humira®, Adalimumab	5.48	Abott, Eisai
Epogen®/Procrit®/Eprex®/EPO®, Etopoetin alpha	5.03	Amgen, Ortho, Janssen-Cilag, Kyowa, Hakko Kirin
Herceptin®, Trastuzumab	4.89	Genentech, Chiguai, Roche
Lantus®, Insulin glargine	4.18	Sanofi-Aventis
Neulasta®, Pegfilgastrim	3.35	Amgen
Aranesp®/Nespo®, Darbepoetin alfa	2.65	Amgen, Kyowa, Hakko Kirin

paying off with respect to engineered proteins with improved pharmacokinetic and pharmacodynamic profiles, as reviewed in Chapter 14. In designing muteins, glycoengineering and post-translational modification with non-natural polymers such as polyethylenglycol (PEG) have affected around 80% of approved protein therapeutics [1].

1.2 Research Developments

1.2.1 Protein Engineering

The term protein engineering refers to the controlled and site specific alteration of a gene sequence encoding the transcription to a polypeptide to a mutated protein with introduced changes in the amino acid sequence. In principle, deletions and insertions of one or more triplet codes and amino acids are possible, but mostly alteration of a protein sequence is limited to exchange of amino acids at calculated sites. Since the first experiments in molecular biology to obtain insights into diseases, protein engineering has been introduced successfully into drug development of recombinant proteins to improve pharmacodynamics and pharmacokinetic profiles [4]. At the biotechnology level, tailoring of proteins has been documented for commercially relevant proteins such as insulin, erythropoietin, growth hormones, and various antibodies. Today the important objectives for protein engineering are:

- improving the pharmcodynamic profile to obtain a drug that acts faster or slower;
- alteration of the pharmacological half-life and development of controlled release kinetics;
- alteration of receptor binding specificity;
- reducing the immunogenicity of the protein;
- increasing physical and chemical protein shelf half-life.

From the 25 genuine new biological entities (NBEs) approved in Europe and the USA until 2009, 17 proteins have already been engineered. The dominant group are antibodies (11), and of these six are fully human, and one is bispecific (Revomab®); out of 25 drugs 17, or in other words around 70%, are modified from a total number of 25 NBEs, and four are humanized antibodies. Among the 25 products, two are fusion proteins (rilonacept, Arcalyst and romiplostim, Nplate). Romiplostin is a so-called *peptibody* consisting of the Fc fragment of the human antibody IgG_1 and the ligand-binding domains of the extracellular portions of the human interleukin-1 receptor component (IL-1RI). It is used for the treatment of Familial Cold Auto-inflammatory Syndrome (FCAS) or Muckle-Wells Syndrome (MWS). Interestingly the functional domain consists of peptide fragments designed by protein modeling to bind highly specifically on the thrombopoetin receptor.

1.2.2 Muteins

Based on the genetic code, a significant number of proteins, which have been approved for clinical use, are subjected to directed change and amino acid substitution to improve the pharmacokinetic and pharmacodynamic activity, and also to develop antagonist functionality. These derived proteins with site directed mutations are called "muteins" and show interesting pharmacological features, which is why a bright future is in prospect. As in classical recombinant biotechnology, insulin was the first candidate with site directed mutations. Insulin lispro was approved in May 1996 as the first mutein, and only a few months later, in November 1996, Reteplase was also approved as a tissue plasminogen activation factor. The number of muteins has since increased significantly and is now dominated by recombinant antibodies. Briefly we want to discuss the potential of muteins for analogs of insulin, tissue plasminogen activator (tPA), and humanized antibodies.

Native insulin associates from dimers up to hexamers at high local concentrations are what are usually found at the site of injection, leading to retarded dissolution and activity in the body. As a result of structure elucidation, proline and lysine at positions 28 and 29, respectively, in the B chain were identified to play a crucial role and were therefore subjected to site directed mutagenesis. Switching B28 and B29 of proline and lysine reduced the association affinity 300-fold, resulting in faster uptake and action, as well as shorter half-life [5]. In contrast, to increase the time of action towards a retarded drug delivery profile, the same concept of site-directed mutation was also applied. Insulin glargin (Lantus®) is a mutein where in the A chain A21 glycine is introduced instead of asparagine, and in the B chain two more ariginines are added at the C-terminal end [6]. As a physicochemical consequence, the isoelectric point is shifted towards the physiological pH at 7.4, resulting in precipitation and slow dissolution into the blood stream.

Tissue plasminogen activators (tPA) play an important role in the breakdown of blood clots. As with insulin, tPA is converted from plasminogen into plasmin, the active enzyme responsible for clot breakdown. tPA is manufactured by recombinant biotechnology, and is used extensively in clinics, but a disadvantage is fast elimination from the body. To overcome this problem a deletion mutant was constructed to reduce binding of the protein at hepatocytes via the EGF-domain (epidermal growth factor) encoded by an amino acid sequence starting from position 4 to 175. The remaining 357 of the 527 amino acids in Reteplase (Retavase®, Rapilysin®) showed increased half-lives of 13–16 min and, interestingly, increased fivefold activity [5, 7]. The historic development with a brief outline of the near future, for example, non-invasive delivery systems, has been described well by Heller *et al.* [8].

The beauty of antibodies can be addressed through the ability of binding to highly specific surface structures and a fairly uniform structure. Apart from vaccinations, antibodies were introduced early on in the therapy of neoplastic diseases and for the prevention of acute tissue rejection in patients with organ transplants. Muromonab CD3, with the tradename Orthoclone OKT3®, is an immunosup-

pressant monoclonal antibody that targets the CD3 receptor on the surface of T cells. It is approved to prevent acute rejection of renal transplants. As an adverse reaction, anti-mouse antibodies can be formed leading to reduced efficacy after repeated injection. To improve tolerance, chimera between mouse and humans were designed. From the protein sequence of the established murine antibodies, the genetic code was deciphered and substituted in the conserved Fc region by the respective human genetic code. These antibodies are called chimeric, in contrast to humanized antibodies where the framework regions are also substituted. Examples are Daclizumab, Zenapax (humanized) [9], Abciximab in ReoPro® (chimeric) [10], and Rituximab in Mabthera® (chimeric) [11] as antineoplastic antibodies for non-Hodgkin lymphoma.

1.2.3 Post-translational Engineering

Several approved recombinant therapeutic products are engineered post-biosynthesis. From the molecular biology background, post-translational engineering is associated with glycosylation or lipidation post-biosynthesis. Post-translational biosynthesis today is the covalent attachment of a chemical group, not a mandatory glycosylation, but attaching fatty acids or PEG-chains alteration of a pre-existing post-translational modification, and has been reviewed best by Walsh [9]. Novo Nordisk's Victoza® (liraglutid) is an example of a non-insulin once-daily medication that may help improve blood sugar levels in adults with type II diabetes. It contains the glucagons-like peptide 1 (GLP-1) analog with 97% sequence homology and with an attached C16 fatty acid (*N*-ε-(γ-Glu[*N*-α-hexadecanoyl]) at Lys26 [10].

Glycosylation is the most complex and widespread form of post-translational modification. Glycoengineering therefore becomes of greater interest, and by directed and targeted alteration of the glycosylation pattern at the protein backbone, significant changes tof the pharmacokinetic profile can be enforced. Approximately 40% of the approved proteins are glycosylated and the use of mammalian cell lines is dominating the manufacturing process (e.g., Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells). A recent trend in engineering the glycocomponent is to also use plant systems (such as carrot cells for β-glucocerebrosidase) or *Saccharomyces cerevisiae* and *Pichia pastoris*. The production of the glucocerebrosidase analog imiglucerase (Cerezyme®) for the treatment of Morbus Gaucher has been carried out in CHO cells. Alternatively, recent interesting advances by the company Protalix showed that glucocerebrosidase for oral administration can be produced in the carrot cells (*Daucus carota*, Apiaceae) (Figure 1.1) [11]. Oral glucocerebrosidase is a plant cell-expressed form that is naturally encapsulated within carrot cells, which are genetically engineered to express the enzyme. Plant cells have the unique attribute of a cellulose cell wall that makes them resistant to enzyme degradation when passing through the digestive tract, which is the main idea behind the oral administration concept. In plant systems, one of the most notable biotransformation reactions has been developed for genetically modified mosses. The Heilbronn, Germany-based company Greenovation

Figure 1.1 Production of glucocerebrosidase with *Daucus carota* plant cell suspension cultures at Protalix.

has constructed a moss (*Physcomitrella patens*) lacking in xylose and fucose transferase activity. Biolex Therapeutics, Pittsboro, NC, USA, has an alternative system in engineered duckweed (*Lemna minor*), where fucosyl and xylose transferase activity is inhibited by RNA interference (RNAi) mechanisms [12].

In contrast to the endogenous human glucocerebrosidase, imiglucerase (Cerezyme®), which is naturally glycosylated and produced in a CHO cell line and downstream processing, includes an enzyme-based processing step using an exoglycosidase. Imiglucerase must be biochemically modified by cutting off capping oligosaccharide chains (sialinic acid, galactase, and *N*-acetyl-glucosamine) down to mannose by exoglycosidases (neuraminidase, β-galactosidase, *N*-acetyl-glucosaminidase). Exposing the remaining mannose residues facilitates specific uptake by macrophages via macrophage cell surface mannose receptors. In this way the enzyme is taken up by macrophages in a very efficient way. Unmodified glucocerebrosidase, if administered, is quickly removed from the bloodstream in the liver.

A non-natural post-translational modification is PEGylation of the protein backbone. Polyethylene glycol (PEG) is a more frequently used technique to alter the physical, chemical, and biological profile of the desired protein [13]. PEGylated proteins and peptides have found promising applications in pharmaceutical biotechnology and related biomedical areas:

- to improve solubility,
- to improve thermal and mechanical stability,

- to reduce immunogenicity,
- to reduce renal excretion and clearance,
- to protect proteins from degradation such as proteolysis, and
- to optimize pharmacokinetic properties such as increased blood circulation and extend plasma half-lives.

It started initially with PEGylated interferons (Pegasys®, Viraferon®), but now four more recombinant proteins (Somavert®, Neulasta®, Oncaspar®, Mircera®) have been approved. Pegvisomant (Somavert®) is a PEGylated analogon of the human growth hormone (hGH) that is produced in *Escherichia coli*. Four to five PEG chains are attached to the protein backbone to form a hydrodynamic shell surrounding the protein, giving an improved solubility and longer half-life. Neulasta® and Oncaspar® are two more growth hormones, but Mircera is a PEGylated Erythropoietin analogon. Consequently, PEGylation has gradually become a platform technology in pharmaceutical technology. A detailed outline of the new formulation strategies is presented in Chapter 10. The chemistry of protein and peptide PEGylation has attracted more and more attention as further PEG-conjugates have reached late phase clinical trials. The discovery and development of upcoming recombinant proteins with undesirable biopharmaceutical hurdles makes PEGylation an attractive approach to drug formulation. New routes to site specific PEGylation and new reversible PEGylation are likely concepts in the near future, as discussed in Chapter 11.

1.2.4 Synthetic Biology

Synthetic Biology is a new emerging field in gene technology and system biology. In the continuation of metabolic engineering research, this new discipline tries to integrate engineering, nanobiotechnology, genetics, and bioinformatics [14]. Key enabling technologies have their roots in molecular biology and genetics. The concept behind synthetic biology is to abstract the hierarchical order and to allow standardization of biological devices, also called "biobricks," such as promotors, transcription factors for use in complex biological systems. Synthetic biologists rely on massive DNA sequencing, protein engineering, and final assembly of designed biobricks to fabricate a production host of interest. A high and constantly increasing number of genomes have been sequenced, but valuable information regarding plant and microorganism genes encoding, for example, the diverse secondary natural product metabolism, are still limited. Despite the fact that synthetic biology is in its infancy, today's achievements are impressive. In 2000, researchers at Washington University, USA, reported synthesis of the 9.6 kbp Hepatitis C virus genome from chemically synthesized 60- to 80-mers. In 2002, researchers at SUNY, Stony Brook, USA, succeeded in synthesizing the poliovirus genome from its published sequence and producing the second synthetic genome. The first bacterial genome was assembled in 2006 by scientists at the J. Craig Venter Institute. *Mycoplasma laboratorium* is derived from *M. genitalium* and

contains a minimal synthetic genome, allowing complete functionality in a living cell as host [15].

Synthetic biology is receiving more and more interest for pharmaceutical applications. In 2004, artemisinic acid, as a precursor towards the biosynthesis of artemisinin, an important antimalarial drug, was successfully transferred into *E. coli* and later in 2006 into *S. cerevisiae*. In 2007 the plant derived kaempferol and quercetin were heterologously synthesized in *E. coli* and on average two secondary natural products per year can be added to the list of combinatorial biosynthetic compounds. The role of secondary natural products is clearly highlighted in Chapter 2. Synthetic biology is still in its infancy and has not been exposed to wide use in the pharmaceutical laboratories [16]. However, the challenges and opportunities are clear and range from host design to producing non-natural chimeric recombinant proteins and also low molecular drugs (see Chapter 19), adapting the host for improved process design in pharmaceutical and chemical engineering, and to enforcing personalized medicine (see Chapter 21). Today it cannot be predicted that nucleic acids as drugs and somatic gene therapy will also benefit, and that synthetic biology may shape the road to the design of new safe gene delivery systems.

1.3 Production Hosts and Upstream/Downstream Processing

A close look at the production organisms of the approved biopharmaceuticals over the last five years reveals that out of 58 products, 32 are produced in cells derived from mammalian organisms (Chinese hamster ovarium, CHO) (see Chapter 3), 17 in *E. coli*, four in *Saccharomyces cerevisiae*, and two in transgenic animals (see Chapter 5). New production hosts have entered the stage, such as *Pichia pastoris* (Ecallanttide, Kalbitor), a baculovirus-insect cell based system (Cervarix®), and *Daucus carota* for the production of a human glucocerebrosidase (imiglucerase alpha) (see Chapters 4 and 19), and antithrombins (Atryn®, Macugen®) in goats.

To improve product efficiency of the host systems used in these times of increased competition with upcoming biosimilars, reduced budgets costs, and market-price setting by public healthcare reforms, expression levels must be improved. For recombinant proteins in mammalian production systems, a yield of 5 g/l is considered to be a standard level. In the future, yields far above the typical levels of today could be achieved by construction of high producer cell lines, where we will definitively see the impact of synthetic biology and smart metabolic engineering. System biotechnology will allow rational process design to identify and overcome metabolic bottlenecks and media optimization. From the total costs involved in a drug manufacturing process, up to 80% can be considered to be the downstream processing (see Chapter 8). This is due to extraction and purification of single compounds from a complex metabolic broth and the increasing biosafety aspects in the highly regulated GMP (Good Manufacturing Practice) Pharma environment. Process-scale columns can cost more than US$1 million, depth-filter

sets up to US$30 000, and centrifuges for biomass separation more than half a million US$.

In recent years, disposable bioreactors or single-use bioreactors have been introduced into pharmaceutical manufacturing. Disposable bags consisting of biocompatible ethylene vinyl acetate–polyethylene copolymers, are γ-radiated for sterilization and are available from 10 up to 2000 l. Apart from GE Healthcare, Xcellerex, Millipore, and Thermo Scientific, only Satorius Stedim Biotech offers a satisfying solution for a complete production line. Disposable biobags have to be certified by drug authorities and validated, as we know from experience with the traditional steel and glass bioreactors. Besides the minor ecological aspects, the eco-efficiency balance is not negative, and disposable biobags fulfill all requirements for GMP production, but it is doubtful if they will become accepted in companies who have invested heavily in running a steel-based infrastructure.

Synthetic biology has already arrived in the Pharma industry and is improving biosynthetic processes. Two examples may illustrate the potential of metabolic engineering and synthetic biology to influence bioprocesses in the future. DSM, a Dutch biotech company engaged in natural product, food additives, and antibiotic production, has improved the existing process for the commercial production of cephalexin. By cutting out 13 chemical steps and replacing them by biotransformation, a new innovative process with significant energy and cost savings has been established. The main metabolic engineering involved the introduction and heterologous expression of acyl transferase and expandase for the direct fermentation of dipoyl-7-aminodesacetoxycephalosporanic acid [15]. Sitagliptin, a dipetidyl peptidase-4 inhibitor, is a synthetic compound for the treatment of type II diabetes. Codexis, a company headquartered in the USA, has developed a biocatalytic process using transaminase for producing this compound with a higher degree of stereoselectivity than the existing organic synthesis processes, which use a metal catalyst. Codexis won the US Presidential Green Chemistry Challenge Award from the US Environmental Protection Agency (EPA), and showed how synthetic compounds can benefit from metabolic engineering and re-engineering strategies, and tailoring biocatalysts for non-natural substrates [17].

1.4 Future Outlook

The development and production of therapeutic proteins represents the first truly industrial application of recombinant DNA technology. At the beginning of the biotech revolution, the main goal was the expression and efficient production of recombinant proteins as known from humans. The success story of insulin documents this process in detail. Based on the urgent need to accommodate the demand for insulin in the world, highly sophisticated manufacturing units have been built over the last two decades. Today, sufficient amounts are available and the production is virtually free from contamination risks, from pathogens or prions for example. Advances in protein sciences, genetics, and molecular biology

have provided new opportunities to the production of tailored recombinant proteins to meet the demands of better disease management and more specific active drugs.

In the future, the design of engineered proteins will become more complex and will be specified by synthetic biology techniques, allowing de novo protein design *in silico*, and also better designed integrated manufacturing processes. Innovation for the pharmaceutical industry is based on innovative and safe drugs, but also cost effectiveness and performance, even if the product can be sold at a higher price in comparison with that from other industries. Embedding synthetic biology and integrating biotechnology and genomic sciences in the whole drug development process allow companies to save up to US$300 million per drug – about one third of the costs today – and the prospect of bringing the drug onto the market one or two years earlier. Each day lost before market entry leads to a loss of approximately US$1.5 million per day, indicating the value of efficient and optimized research and operational strategies.

Synthetic biology arose from combined activities between (bio)engineers, biophysicists, and computer scientists, but today the integration of clinicians is essential to allow successful transfer into clinical applications. Furthermore, microorganisms were the playground for synthetic biological experiments, but the move towards mammalian cells is necessary to prove developed circuits and constructs for the patient [18]. In Chapters 6 and 7 actual trends and drugs in the approval pipelines are highlighted and discussed extensively.

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2
Prokaryotic Cells in Biotech Production

Andriy Luzhetskyy, Gabriele Weitnauer, and Andreas Bechthold

2.1
Introduction

The origins of producing natural products from microorganisms extend back to individual impressive scientists and individual companies (e.g., Sandoz, Ciba, Hoechst, Bayer, Eli-Lilly). In 2010 natural products and enzymes and proteins produced by prokaryotes made up a large proportion of the pharmaceuticals production. They are most prominent in the antibiotics sector but examples can be given from nearly all disease areas. The most important producers of natural products are actinomycetes [1], myxobacteria [2], cyanobacteria [3], and marine sponges [4], and biopharmaceuticals are mostly produced by *Escherichia coli* strains.

This chapter, which is an updated and expanded version of one that was published in this book series in 2003 [5], discusses the role of microorganisms as sources of pure natural products or derivatives of natural products, and as leads for novel synthetic compounds. The impact of the increase in available genomic information and manipulation and the impact of bioinformatics are discussed in one section, followed by a section on the potential of microbes as producers of biopharmaceuticals, such as proteins, enzymes, and vaccines.

2.2
Production of Natural Products by Microorganisms

Natural products include thousands of compounds, and a high proportion of these compounds are produced by microorganisms. Among these microorganisms, actinomycetes are especially well known for their high production capacity. Many compounds produced by microorganisms are active antibiotically and are the major source for anti-infective drugs. At present about 7000 antibiotics are known and about 100 of these are produced commercially by microbial fermentation processes. A list of commercially important secondary metabolites originating from actinomycetes are given in [5].

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2.2.1 Production of Libraries of Natural Products

Genetic engineering of microbial strains either by introducing deletions into one or several genes, by heterologous expression of genes, and by combining both have become important routes for drug design [5, 6]. These technologies have been used successfully to generate "libraries" of many natural compounds (Table 2.1, Figure 2.1).

A recent successful story is about lipopetides. The story started with the cloning and sequencing of clinically useful antibiotics such as daptomycin produced by *Streptomyces roseosporus* [31], and friulimicin produced by *Actinoplanes friuliensis* [32] and by cloning the calcium dependent antibiotic CDA [33]. Genetic engineering of the producer strains, heterologous expression of complete gene clusters, and expression of sets of genes either from one or from different producer strains resulted in nearly 50 novel lipopetides, some of them with fundamental changes in properties [11, 12].

Based on its know-how in generating product libraries, a biotech company, Biotica, recently formed successful partnerships with leading pharmaceutical companies indicating the importance of the new technologies for the pharmaceutical industry. One of the key projects was the development of rapamycin analogs through genetic engineering technologies.

Table 2.1 Natural products that have been used as lead structures for the generation of natural compound libraries.

Antibiotic	Producer	Year of publication	Reference
Amphotericin	*S. nodosus*	2010	[7]
Aranciamycin	S. *diastatochromogenes* Tü6028	2008	[8]
Aureothin	*S. thioluteus*	2010	[9]
Avilamycin	*S. viridochromognes*	2004	[10]
Daptomycin	*S. roseosporus*	2009, 2010	[11, 12]
Daunorubicin	*S. peucetius*	1997	[13]
Erythromycin	*Saccharopolyspora erythreae*	1998	[14]
Landomycin A	*S. cyanogenus* S136	1998	[15]
Mithramycin	*S. argillaceus*	1999	[16]
Novobiocin	*S. niveus*	2009	[17, 18]
Nystatin	*S. nodosus*	2009	[19]
Phenalinolactones	*S.* sp. Tü6071	2010	[20]
Rapamycin	*Actinoplanes* sp.	2005, 2008	[21, 22]
Saquayamycin	*Micromonospora* sp. Tü6368	2009	[23]
Spinosyn	*Saccharopolyspora spinosa*	2006	[24]
Staurosporin	*S. staurosporus*	2009	[25, 26]
Tylosin	*S. fradiae*	2004	[27]
Vancomycin	*Amycolatopsis orientalis*	2001, 2004, 2005	[28–30]

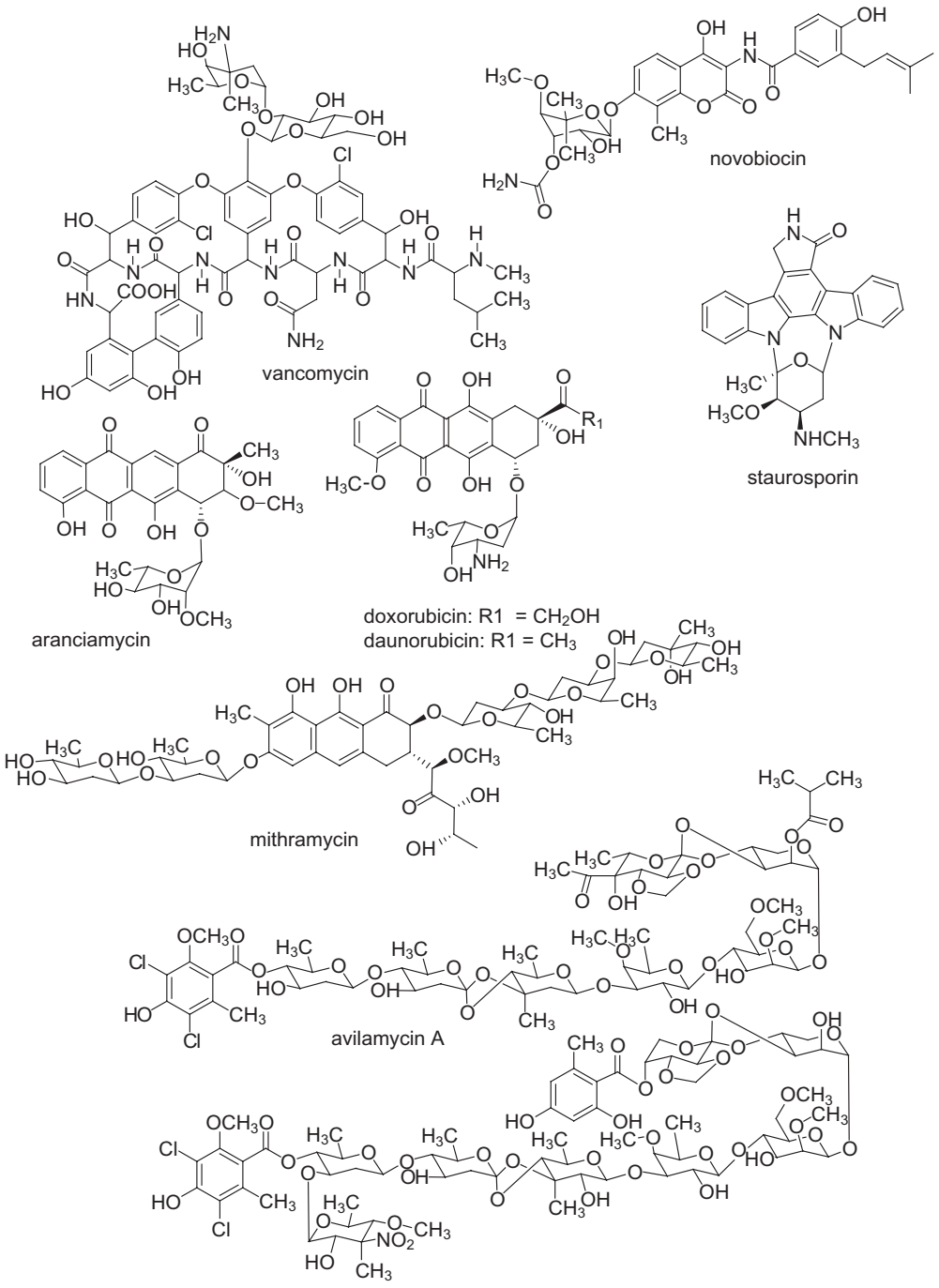

Figure 2.1 Structure of selected compounds that have been used as lead structures for the generation of novel natural compounds.

Table 2.2 Natural products produced after heterologous expression of biosynthetic gene clusters.

Antibiotic	Producer	Year of publication	Reference
Aranciamycin	*S. diastatochromogenes* Tü6028	2007	[34]
A54145	*S. ambofaciens* BES2074	2010	[35]
	S. roseosporus UA431		
Capreomycin	*S. lividans* TK24	2010	[36]
Cephamycin C	*S. avermitilis* SUKA17	2010	[37]
Chlorobiocin	*S. coelicolor* M512	2005	[38]
Cycloserine	*S. lividans* 66	2010	[39]
Daptomycin	*S. lividans* TK24 Δ*act*	2006	[40]
Dehydrophos	*S. lividans* TK24	2010	[41]
Fredericamycin	*S. albus* J1074	2005	[42]
Iso-migrastatin	*S. albus* J1074	2007	[43]
Landomycin A	*S. lividans* 1326	2001	[44]
	S. fradiae XKS	2004	[45]
Napyradiomycin	*S. albus* J1074	2007	[46]
Nikkomycn	*S. lividans* TK23	1996	[47]
Novobiocin	*S. coelicolor* M512	2004	[38]
	S. lividans TK24		
Oxytetracycline	*S. lividans* 1326	1989	[48]
Phenalinolactone	*S. lividans* TK24	2008	[49]
Puromycin	*S. lividans* 66	1992	[50]
	S. griseofuscus		
Ravidomycin	*S. lividans* TK24	2010	[51]
Staurosporine	*S. lividans* TK23	2002	[52]
Streptomycin	*S. avermitilis* SUKA5	2010	[37]
Thiocoraline	*S. albus* J1074	2006	[53]
6-dEB	*S. coelicolor* CH999	2003	[54]
	S. lividans K4-114		

2.2.2 Production of Natural Products by Cloning and Expression of Biosynthetic Gene Clusters

Biosynthetic genes are clustered in actinomycetes. As original producers often do not produce more than 1–5 mg/l of a given compound scientists started to express gene clusters in others strains to improve production rate. Examples are listed in Table 2.2. It is worth mentioning that in a few cases the production rate could be increased significantly when the cluster was heterologously expressed. The highest amount obtained was 500 mg nikkomycin produced by *S. lividans* TK23 containing the nikkomycin gene cluster.

In the last few years, microbial natural product research has been revolutionized by genomic technologies. The complete sequence of microbial genomes revealed a remarkable number of gene clusters encoding enzymes involved in the production of undetected and unknown secondary metabolites [55–58]. This finding led

to the rapid development of novel technologies to wake up these silent clusters in the original producer strain, and also to novel genomic-driven approaches for cloning and expressing these gene clusters in other strains.

2.2.3 Culture Manipulation to Wake Up Silent Gene Clusters

In 2002 the OSMAC theory (one strain many compounds) was published by Zeeck and coworkers [59]. These workers described microbial strains that are metabolically responsive to a broad range of different media, cultivation vessels, solid versus liquid fermentation techniques, and enzyme inhibitors. Following this approach, several new compounds have been detected in bacteria and other organisms. Recently the microbial coculture technique was rediscovered by several scientists. Based on the premise that bacteria live in complex communities, workers observed that cocultivating microorganisms elicitated natural product biosynthesis. One example is about bacterial–fungal interaction. When *Aspergillus nidulans* was cocultivated with actinomycetes the fungal secondary metabolism was specifically activated [60]. One major factor limiting an effective use of culture manipulation is that we do not know why natural products are produced under different conditions.

2.2.4 Genomic Driven Approaches to Wake Up Silent Gene Clusters

It was Ecopia (now Thallion Pharmaceuticals, Inc.), a small biotech company, which was the first company to develop a screening platform for searching secondary-metabolite encoding biosynthetic gene clusters in bacteria genomes. Ecopia showed that it is possible to predict structures of natural products based on genome information [61, 62].

In recent years scientists have developed heterologous expression techniques allowing the production of novel compounds in suitable host strains (see also Table 2.2). Although these techniques are still not applicable to high-throughput investigations, many novel compounds have been produced in this way. Today scientists are working on overcoming several significant problems in connection with this approach.

2.2.5 *E. coli*, an Interesting Host Also for Natural Product Synthesis

Although actinomycetes are used in industry as convenient producer strains, the advantages of using *E. coli* motivated scientists to develop this bacterium as a producer of natural products.

2.2.5.1 Production of Polyketides in *E. coli*

Engineering of *E. coli* to produce polyketides mainly focused on 6-methyl-salicyclic acid (6-MSA), 6-deoxyerythronolide B (6dEB), erythromycin, and epitholone [63–

65] As *E. coli* does not naturally produce polyketides scientists faced major problems that had to be solved.

1) Expression of polyketide synthases was only possible at low temperature (22–30 °C).
2) The biosynthesis of polyketides requires phosphopantetheinylation, which can be achieved by introducing a P-pant transferase into *E. coli*.
3) Adequate levels of precursors (e.g., malonyl-CoA, propionly-CoA, NADPH) are needed for high production. Depending on the precursor genes, encoding enzymes responsible for the biosynthesis of these precursors have to be introduced into *E. coli*.

The final amount of 6-MSA was 75 mg/l, of 6-dEB 1 g/l, of 0.4 mg/l erythromycin, and of epothilone 0.001 mg/l. The amount of erythromycin produced by *E. coli* could then be drastically increased by synthesizing artificial polyketide synthase genes with codons appropriate to the host's t-RNA and GC biases.

2.2.5.2 Metabolic Engineering of *E. coli* for Isoprenoid Biosynthesis

Many isoprenoids are found in nature, but mainly only in low abundance. Therefore, in isoprenoid research the development of renewable production processes has been much more in the focus of scientists than the generation of libraries. Beside *Saccharomyces cerevisiae, E. coli* has become the most utilized microorganism for the synthesis of isoprenoids [66].

Isoprenoid metabolic engineering in *E. coli* started with carotenoids, such as lycopene and ß-carotene. Genes of the carotenoid pathway isolated from *Erwinia uredovora* were expressed in *E. coli* resulting in the production of zeaxanthin, ß-carotene, and lycopene [67]. Recent research has focused on the diterpene taxadiene, an intermediate of paclitaxel biosynthesis [68], on monoterpenes such as (–)-carvone and (–)-limonene [69], and on amorpha-4,11-diene, an intermediate of artemisinic acid [70].

The most challenging issue for isoprenoid biosynthesis in bacteria is the limitation in the supply of the universal precursors IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate). Early attempts focused on overexpression of single or several genes of the non-mevalonate pathway, either by providing extra copies of genes [71] or by replacing native promoters [72]. Very recently, the group of Prof. Dr. J.D. Keasling engineered the mevalonate pathway from *Saccharomyces cerevisiae* in *E. coli*, resulting in a high producer of amorphadien, a precursor of the anti-malaria drug artemisinin [73, 74]. Production was even higher when yeast genes for HMG-CoA synthase and HMG-CoA reductase (the second and third enzymes in the pathway) were replaced with equivalent genes from *Staphylococcus aureus*, more than doubling production. Amorpha-4,11-diene titers were further increased by optimizing the culture conditions resulting in the production of 27.4 g/l. The conversion of amorpha-4,11-diene into artemisinin is possible by chemical synthesis with a yield of about 30%. Thus the conversion of amorpha-4,11-diene via genes and enzymes is economically desirable. A plant P_{450} enzyme

(8-cadinene hydroxylase) was found to catalyze this reaction, but attempts to express the corresponding gene in *E. coli* were very challenging. So far the artemisinic acid production in *E. coli* is around 105 mg/l. Useful approaches for increasing production rates, which were also applied, are optimizing codon usage, enhancing production of rate-limiting enzymes, and eliminating the accumulation of toxic intermediates or by-products [75]. In other approaches various methods (transposon mutagenesis, computational based genome-wide stoichiometric flux balance analysis (see also below)) were used to identify non-biosynthetic genes that influence the flux of carbons from the central metabolism of the bacteria cell towards the non-mevalonate pathway. *E. coli* strains were generated containing deleted genes and genes that were overexpressed resulting in higher production rates of lycopene [76–78].

Key enzymes in the biosynthesis of isoprenoids are synthases (e.g., taxadiene synthase, amorphadiene synthase, limonene synthase) and P450 dependent oxygenases. Engineering of these enzymes is an effective way to create novel molecules. Recently random and site-specific mutagenesis was performed to convert a (+)-δ-cadinene synthase into a germacrene D-4-ol synthase. In addition, based on the crystal structure of a P450 oxygenase involved in the conversion of (+)-campher into 5-*exo*-hydroxycamphor, a mutant was designed with altered substrate specificity. This mutant is able to convert (+)-α-pinene into (+)-*cis*-verbenol [79].

2.2.6 Global-Scale Strategies for Strains Improvement

The major objectives of pharmaceutical biotechnology include the increase in products yield, the introduction of pathways leading to new products, the deletion or reduction of byproduct formation, and the enhancement of strain tolerance. The microbial strains can be engineered for the desired phenotypes based on the global information obtained from the results of genomic (studies on whole sets of genes), transcriptomic (studies on mRNA expression levels), proteomic (studies on interactions and functional dynamics of whole sets of proteins), metabolomic (studies on the concentration of metabolites), and fluxomic (studies on the complete flux of metabolites in the metabolic network). These data are then used for computational modeling and simulation of metabolism followed by the wet lab experiments [80].

2.2.6.1 System Biology, System Biotechnology, and "Omic" Approaches

Scientists working in the area of system biology are trying to understand the function of a cell rather than the function of single components of a cell. The nature of the links that join different components are used in mathematical models to perform quantitative analysis of biological system. Data are then used for cell design, which is part of system biotechnology. The successful design requires collection, analysis, and integration of genome data and the prediction of the behavior of the biological system in response to exogenous triggers. The aim is to predict which genetic changes will lead to a desired phenotype. Recent advances

in whole-genome sequencing techniques and bio-informatic analyzes have propelled reconstruction of genome-scale biochemical reaction networks in microorganisms. Examples are *E. coli* K-12 MG1655 [81], *B. subtilis* [82], *Methanosarcina barkeri* [83], *Saccharomyces cerevisiae* [84], and *S. coelicolor* [85]. The metabolism reconstruction is based on annotated genes, physiological and biochemical information, and linear programming. The reaction sets are composed in a stoichiometric matrix with two dimensions *representing* the number of metabolites and the number of reactions. This is the starting point for various mathematical analyzes. Additional information about the enzyme complex formation, protein localization, and regulation can also be associated with the matrix. To predict the effects of gene inactivation on whole cell metabolic flux distribution, the flux value of the corresponding reaction should be constrained to zero during the simulation [86].

This simulation method has been proven to be a powerful tool for predicting targets for metabolic engineering. For instance, Nielsen and coworkers have successfully engineered an *S. coelicolor* strain to produce actinorhodin and prodigiosin at a five-times higher yield than the wild type, by combining an *in silico* gene knockout simulation and rational metabolic engineering [85]. A genome-scale metabolic model of *S. coelicolor* predicted that the deletion of the phosphofructokinase gene will lead to an increased flux through the pentose phosphate pathway, and thus accumulation of NADPH. As NADPH (nicotinamide adenine dinucleotide phosphate) is a cofactor of polyketidesynthase (a central enzyme involved in actinorhodin biosynthesis), its elevated level led to the overproduction of actinorhodin. This theoretical calculation was supported experimentally.

There are many other successful examples of *in silico* simulation and its use to predict gene targets to be knocked or overexpressed. Using these approaches, the mutant strains of *E. coli* overproducing L-valin and L-threonine have been obtained [87, 88]. Gene targets for metabolic engineering can also be predicted by comparison of the transcriptomes of the wild types and classically improved overproducers. For instance, novel genes in the *C. glutamicum* genome, NCgl0855 (putatively encoding a methyltransferase) and the *amtA-ocd-soxA* operon, which could improve the production of lysine were identified using a DNA microarray. After their overexpression, total lysine production was increased by about 40% [89]. Comparative transcriptomes profiling were performed for three different actinomycetes strain, *Saccarpolyspora erythrea*, *S. fradiae*, and *S. avermitilis* [90, 91]. Wild type strains were compared with overproducing strains. Genes that were overproduced in the superior strains were detected. These results will pave the way for manipulating strains in the future for obtaining overproducers.

2.2.6.2 **Synthetic Biology Tools**

Synthetic biology is the discipline that aims at reconstructing, designing, and building novel biological systems in the same way as engineers design electronic and mechanic systems. This discipline provides the ability to manipulate (separate and combine) modular biological components such as promoters, genes, RNA translational control devices, and the whole metabolic pathways for the production of valuable pharmaceuticals. Synthetic biology has still not achieved satisfactory

levels of precision, robustness, and reliability to become a form of engineering. Factors preventing this are incompatibility of biological parts and modules, functional overlaps between them, and the dependence of the functionality of the parts on the context. Deeper characterization of the biological components and their standardization will help to solve these problems. Despite all these difficulties, synthetic biology approaches have already been achieved in many important pharmaceuticals [92]. One notable example of a synthetic biology application is the production of artemisinin, which was discussed in Section 2.2.5.2.

Another example is the construction of synthetic operons leading to the production of activated deoxysugars, which are an important part of many commercial antibiotics and antitumor drugs. Using standard parts (promoters) and devices (genes from different biosynthetic pathways) Salas and coworkers constructed a number of modules (synthetic operons) that are responsible for the deoxysugar production. These modules have a high level of compatibility as their combination with other modules (responsible for polyketide production) and devices (different glycosyltransferases) have yielded a library of antibiotics and antitumor compounds [93].

Synthetic biology uses well-characterized, modular parts that can be put together to create new functionality in a directed, predictable manner. Therefore, one of the most important goals is to create the libraries of such modular and well characterized parts. In the following sections two examples are presented describing the construction of modular libraries.

2.2.6.2.1 **Synthetic Promoter Library**

Gene expression in bacteria is mainly controlled at the transcriptional level, and therefore the promoter is the most tunable element. A wide range of promoter strengths is necessary to express a certain gene at the desired level. Several strategies have been developed to construct synthetic promoter libraries for fine-tuning gene expression. Usually, these methods rely on random modification of existing promoters in their spacer regions between the consensus sequences, as they affect a promoter's strength most strongly. The promoter activity is tested using different reporter genes such as *gfp*, *beta-galactosidase*, and others [94]. The synthetic promoter library should represent as many variations in promoter strength as possible. For example, if only a low-range of expression is required because of gene toxicity, then a set of promoters driving low levels of expression will be used. If one needs to maximize a valuable protein, then expression from the strong promoters is necessary. For instance, a synthetic promoter library was used to assess the impact of deoxy-xylulose-P synthase levels on lycopene production, and the optimal expression level of this gene was identified for maximal desired phenotype [94].

2.2.6.2.2 **Synthetic Tunable Intergenic Regions Library**

In many cases, more than one gene needs to be expressed to produce different pharmaceuticals (e.g., antibiotics). In order to produce these molecules the genes must be expressed at appropriately balanced levels, which help to avoid the

accumulation of toxic intermediates resulting in growth inhibition or suboptimal yields. Therefore, it is important to combine multiple genes into a synthetic operon with a single promoter, and fine-tune expression of each gene at the translational level. The tunable control elements are mRNA secondary structure, RNase cleavage sites, and ribosome binding sites at intergenic regions. Libraries of tunable intergenic regions (TIGRs) were generated and screened to tune expression of several genes in an operon [95]. TIGRs can vary the relative expression of two reporter genes over a 100-fold range. The sevenfold increase in the mevalonate production was achieved using different TIGRs for the coordination of three genes expression in an operon [95]. Thus, using TIGRs is expected to be valuable for engineering of complex biosynthetic pathways in order to improve the phenotype.

2.2.6.3 Whole Genome Engineering Approaches

Rational methods for strain improvement, mainly based on a single-gene inactivation or on heterologous expression, have been developed in recent decades. However, owing to the complex nature of the biosynthetic machinery used for the production of many pharmaceutically relevant molecules, and due to the fact that not all regulatory genes involved in the biosynthesis of a compound are known, these methods have been successfully applied for the development of just a few industrial microorganisms. Many recent efforts focused on the development of global engineering approaches such as artificial transcription factor engineering [96], ribosome engineering [97], global transcription machinery engineering [98], and genome shuffling [99] for obtaining strains with desirable characteristics.

2.2.6.3.1 Protoplast Fusion and Genome Shuffling

The whole genome engineering approach is based on protoplasts fusion (Figure 2.2) and genome shuffling enable construction of many bacteria in a more global fashion. Usually protoplast fusion-based techniques are used for the improvement of phenotypes of microorganisms. Protoplasts fusion has been established since the late 1970s. There are a few reports demonstrating that protoplasts fusion allows recombination events to take place throughout the genome and at a much longer range relative to other methods. A high frequency of recombination is achieved by fusing complete genomes. Genome shuffling is a technology of recursive protoplast fusion between multi-parent strains to obtain a desired phenotype. Through this method, overproducers of tylosin, teicoplanin, natamycin, epothilone, riboflavin, and pristinamycin produced by *S. fradiae, Actinoplanes teichomyceticus, S. gilvosporeus, Sorangium cellulosum, Bacillus subtilis,* and *S. pristinaespiralis*, respectively, have been obtained [100–103]. The success of the genome shuffling strategy strongly depends on the availability of a high-throughput screening method for a desired trait. This method became popular after a publication by Stemmer and coworkers in 2002, where they described the development of a tylosin overproducer [100]. It is worth mentioning the publication of Fedorenko and coworkers from 1993. In this paper, the process of recursive protoplast fusion of multi-

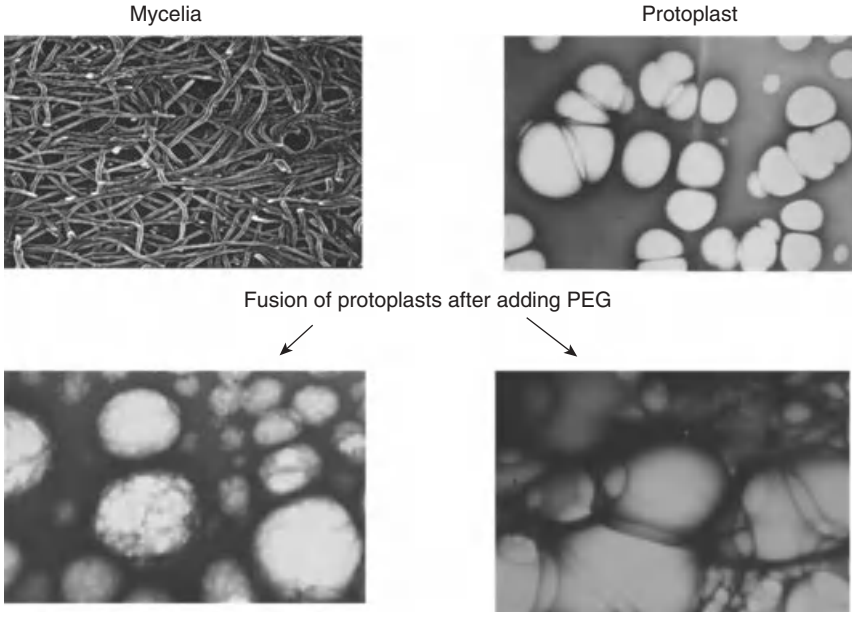

Figure 2.2 Electron microphotographs demonstrating the protoplast fusion of *Streptomyces kanamyceticus*. (The photographs were recorded by O.R. Kulachkovskyy from the Lviv National University, Ukraine).

parental populations of *Saccharopolyspora erythrea* is described. The selected mutant strains, overproducing erythromycin, were later used for the industrial production of erythromycin in the Ukraine [104]. There are a few reports on the interspecies protoplast fusion using streptomyces strains. When *S. fradiae* 261-27E (mycaminose producer) and *Streptomyces* sp. AM 4900 N3-4, (pikronolide producer) were fused, an unstable prototrophic fusant produced a macrolide antibiotic, which was not found in parent strains [105]. A novel antibiotic was also generated by interspecies protoplast fusion treatment using *S. griseus* and *S. tenjimariensis* [106]. Taking into consideration the efficiency and time for the strain development, genome shuffling will play an important role in engineering of microorganisms in the future.

2.2.6.3.2 **Engineering of Translational and Transcriptional Machineries**

Global changes in bacterial gene expression have been observed after the alteration of the ribosomal proteins and RNA polymerase subunits. These changes led to the overproduction of antibiotics and enzymes by actinomycetes and bacilli and enhanced the tolerance of *Pseudomonas* to different chemicals. The convenient and simple method of modulating ribosomes and RNA polymerases is to treat strains with low concentrations of different antibiotics (e.g., streptomycin, gentamycin, neomycin, tetracycline, paromomycin, thiostrepton, and rifampicin) [107]. This

leads to resistant strains accumulating mutations, which are located in genes encoding ribosomal proteins and the RNA polymerase subunits. A dramatic activation of natural product synthesis has been observed in many actinomycetes containing a mutation in *rpsL*, encoding the ribosomal protein S12, which confers resistance to streptomycin [108]. The beneficial effect on antibiotic production of mutations in the *rpsL* gene have been described by the isolation of mutants in *S. lividans, S. coelicolor, S. chattanoogensis, S. antibioticus, S. lavendulae*, and *S. albus* producers of actinorhodin, undecylprodigiosin, fredericamycin, actinomycin, formycin, and salinomycin, respectively [109–112]. Mutations in *rsmG*, which encodes a 16S RNA methyltransferase, also increased actinorhodin and actinomycin production in *S. coelicolor* and *S. parvulus,* respectively [113]. Combining the *rpsL* and *rsmG* mutations resulted in a further increase in antibiotic production [114]. Introduction of streptomycin resistance mutations also increased antibiotic production in *Bacillus* and *Pseudomonas*.

The simultaneous introduction of several resistant mutations to streptomycin, gentamicin, and rifampin has a synergistic effect on antibiotic production, leading to improvements in actinorhodin, salinomycin, and thiazolylpeptide GE2270 production in *S. coelicolor, S. albus*, and *Planobispora rosea* strains, respectively [115]. Mutations lead to an increase of the hyperphosphorylated guanosine nucleotide (ppGpp) level in the cells of actinomycetes [116]. ppGpp can affect gene expression by altering the selectivity of the RNA polymerase [117]. Similarly, mutations in *rpoB*, which encodes a β-subunit of the RNA polymerase, conferred resistance to rifampicin and led to overproduction of the antibiotic [118].

Another example describes the biosynthesis of actinorhodin, undecyprodigiosin, and the calcium dependent antibiotic. All three pathways were activated after introducing mutations into *rpoB*. It was shown that the mutated RNA polymerase has a better affinity to the gene promoters of the gene clusters responsible for the formation of the three compounds [119]. The introduction of antibiotic resistant mutations also improves the production of extracellular enzymes in *Bacillus subtilis* [120]. More recently, it was shown that drug-resistant bacteria produce novel natural products in contrast to their wild type strains. *Streptomyces* species that did not produce antibacterials started to produce antibacterials after selection on streptomycin and/or rifampicin. Again mutations were detected in *rpoB* and/or *rpsL* genes [121]. In one case a novel class of antibiotics, named piperidamycins, has been discovered.

2.3 Prokaryotes as Producers of Recombinant Therapeutic Proteins

Biopharmaceuticals (recombinant proteins and antibodies) are becoming more and more important as they help to overcome sicknesses that are not treatable without them. Recombinant proteins intended for the drug market are required in large amounts. Thus, the aim is to express high levels of stable, soluble, and functional proteins. Once a protein can be produced in small amounts, scaling

up the production rate is an important and difficult task. For instance, bacterial cultures of high cell density, as are frequently used in industry, show different characteristics to cultures on a small scale. Drawbacks are, among others, the limited availability of dissolved oxygen, insufficient mixing efficiency of the fermentors, carbon dioxide (CO_2) levels that can decrease growth rates and stimulate cell toxic acetate formation, or the fairly expensive use of antibiotics for selection [122]. However, several technological developments and feeding schemes can now improve the fermentation process. In this section, different strategies for successful intracellular expression of recombinant proteins, in particular mammalian proteins, in prokaryotes will be reviewed.

2.3.1 Prokaryotic Expression Systems

Today there are various expression systems available. As hosts, bacteria, yeasts, moulds, mammalian cells, plants, insects, or even transgenic animals are used. Which system is chosen mainly depends on the protein structure, production speed, and yield [123]. Non-glycosylated proteins are usually produced in *Escherichia coli* (*E. coli*) or yeasts, N-glycosylated proteins in mammalian cells, for example, Chinese hamster ovary (CHO) cells, which mimic human glycosylation. However, even these glycoproteins are not exactly the human type, and possibly need to be modified [123].

Bacterial hosts, with *E. coli* being the most important, are still widely used for heterologous protein expression. This is due to several advantages of *E. coli* expression systems in particular, including ease of handling, inexpensive culture media, very well-known genetics, availability of improved genetic tools, fast growth of the cells, and high yields of recombinant protein achieving up to 20–30% of total cellular protein [123–125]. Still much effort is being given to improve and develop prokaryotic expression systems in order to overcome deficiencies. In the following, microbial expression systems, which are employed in pharmaceutical industry, will be described.

2.3.1.1 Host Strains

2.3.1.1.1 *E. coli*

E. coli systems are most commonly used for industrial and pharmaceutical protein production and large-scale production systems are established [126]. Besides the advantages mentioned above, there are also some limitations such as acetate formation in high-density cultures resulting in cell toxicity, lack of post-translational glycosylation ability, and accumulation of endotoxins, which must be removed during the purification process. Moreover, proteins are often built as inactive inclusion bodies and require refolding and proteins containing disulfide bonds are fairly difficult to express [123].

However, many advances have been achieved to improve the *E. coli* production system. Successful measures that have been taken are, for example, the use of

different promoters to regulate expression, controlling the oxygen level, optimization of the specific growth rate, coexpression of chaperones, secretion of proteins into the periplasmic space or into the culture medium, or addition of a fusion partner [123]. Different mutant *E. coli* strains have been developed, which show specific features to meet the special demands of particular proteins (Table 2.3) [126, 127]. *E. coli* BL21 and its derivatives are the most widely used ones [126, 127]. The difference between the codon usage of different species or organisms can also cause problems, particularly for the overexpression of eukaryotic genes in *E. coli*. Insufficient tRNA pools can lead to translational stalling, formation of shortened polypeptide chains, translation frameshift, and amino acid misincorporation [126]. To enhance production of heterologous proteins that contain codons rarely used in *E. coli*, special strains were created which supply additional tRNAs under control of their native promoters (Table 2.4).

Alternatively, the codon usage can be optimized for the respective host by replacing the natural genes that are to be expressed with synthetic genes. For some proteins, for example, insulin, formation of inclusion bodies is an advantage rather than a disadvantage because of an easier isolation and purification procedure. However, others loose their functionality when falling out as inclusion bodies due to misfolding. To support the folding process certain plasmids for coexpression are available on which genes of bacterial chaperones are encoded. Different origins of replication and selection markers prevent compatibility problems with other expression vectors. The best characterized chaperone systems in the cytoplasm of *E. coli* are the DnaK-DnaJ-GrpE and GroEL-GroES systems. It is important to mention that success is by no means guaranteed and highly dependent on the nature of the overexpressed recombinant protein [126, 128, 129].

It is often desirable that heterologous expressed proteins are secreted either into the periplasm or into the culture medium. Firstly, the isolation and purification process will require less effort. Secondly, during the translocation process usually a so-called leader peptide will be cleaved at the *N*-terminus resulting in proteins without methionine at their *N*-terminus. This may be essential to maintaining the bioactivity of eukaryotic proteins. Another advantage is that correct formation of disulfide bonds can be facilitated, because in contrast to the cytoplasm in the periplasmic space a more oxidative environment predominates [126, 130–132]. Alternatively, proteins with disulfide bonds can be overexpressed in the cytosol of thioreductase-deficient (*trxB*) and glutathione reductase-deficient (*gor*) mutant strains (Table 2.4) [126, 131, 132]. The most prominent protein secretion systems used in *E. coli* strains are the Sec system and the twin-arginine translocation (Tat) system [132]. To secrete recombinant proteins into the periplasmic space, fusion with a signal sequence at the *N*-terminus is necessary. Examples of commonly used signal peptides are MalE, OmpA, OmpT, PhoA, PhoE, and Tat signal peptides [126, 127, 130, 132]. Most problems with secretion of heterologous produced proteins are caused by incomplete translocation across the inner membrane, proteolytic degradation, and a limited number of available gates. For optimized results expression, rate and transport capacity have to be balanced carefully [127, 131].

Table 2.3 Recombinant proteins that are used as human therapeutics and are approved by the EMA (status July 2010) (http://www.vfa.de/gentech; www.ema.europa.eu)

Active agent	Drug class	Host strain	Year of first approval
Aldesleukin (IL-2)	Cytokine (Interleukin)	*E. coli*	1989
Anakinra	IL-1 receptor antagonist	*E. coli*	2002
Certolizumab pegol	Antibody	*E. coli*	2009
Filgrastim (G-CSF)	Cytokine (Granulozyte colony-stimulating factor)	*E. coli*	1991
Insulin glargin	Hormone	*E. coli*	2000
Insulin glulisin	Hormone	*E. coli*	2004
Insulin human	Hormone	*E. coli*	1982
Insulin lispro	Hormone	*E. coli*	1996
Interferon alfa-2a	Cytokine (Interferon)	*E. coli*	1987
Interferon alfa-2b	Cytokine (Interferon)	*E. coli*	2000
Interferon beta-1b	Cytokine (Interferon)	*E. coli*	1995
Interferon gamma-1b	Cytokine (Interferon)	*E. coli*	1992
Mecasermin	Recombinant IGF-1	*E. coli*	2007
Molgramostim (GM-CSF)	Cytokine (Granulocyte macrophage colony-stimulating factor)	*E. coli*	1993
Palifermin	Keratinocyte growth factor	*E. coli*	2005
Parathyroid hormone (PTH)	Hormone	*E. coli*	2006
Pegfilgrastim	Cytokine (G-CSF)	*E. coli*	2002
Peginterferon alfa-2a	Cytokine (Interferon)	*E. coli*	2002
Peginterferon alfa-2b	Cytokine (Interferon)	*E. coli*	2000
Pegvisomant	Growth hormone receptor antagonist	*E. coli*	2002
Vaccine against *Streptococcus pneumoniae* infection	Vaccine	*E. coli*	2009
Ranibizumab	Antibody	*E. coli*	2007
Reteplase (rh-tPA)	Enzyme (Protease)	*E. coli*	1996
Romiplostim	Fusion protein analog of thrombopoietin	*E. coli*	2009
Somatropin	Hormone	*E. coli*	1988
Tasonermin (r-TNF-α 1a)	Cytokine	*E. coli*	1999
Teriparatid (rh-PTH 1-34)	Hormone fragment	*E. coli*	2003

Table 2.4 *E. coli* strains frequently used as hosts for heterologous protein production [126, 128, 131].

E. coli strain	Origin	Key features	Manufacturer
AD494	K-12	*trxB* mutant, facilitates cytoplasmatic disulfide bond formation	Novagen
BL21	B834	Deficient in *lon* and *ompT* proteases	Stratagene
BL21 λDE3	BL21	Contains a fragment of bacteriophage λ in the chromosome that codes for the T7 RNA polymerase and the LacI repressor protein	Stratagene
BL21 λDE3 x pLysS	BL21	Contains a plasmid for expression of T7 lysozyme, an inhibitor of the T7 RNA polymerase	Stratagene
BL 21 trxB	BL21	*trxB* mutant of *E. coli* BL21	Novagen
Origami	K-12	*trxB/gor* mutant, greatly facilitates cytoplasmatic disulfide bond formation	Merck4Biosciences
Origami B	BL21	*trxB/gor* mutant of *E. coli* BL21	Merck4Biosciences
Rosetta	BL21	Enhances expression of genes containing codons rarely used in *E. coli*, because it contains a plasmid coding for tRNAs that can recognize these codons; deficient in *lon* and *ompT* proteases	Merck4Biosciences
Rosetta-gami	BL21	*trxB/gor* mutant of *E. coli* Rosetta	Merck4Biosciences
SHuffle	K-12	*trxB/gor* mutant; overexpresses DsbC to improve correct folding of disulfide-bond dependent proteins	NEB

2.3.1.1.2 *Bacillus*

Other useful host systems are those of the Gram-positive bacilli. *Bacillus* species are often preferred for expression of enzymes such as proteases (for detergents) and amylases (for starch and baking) [123]. They are currently not used for the production of pharmaceutical therapeutics approved by the European Medicines Agency (EMA) (Table 2.3). Some advantages of *Bacillus* as the expression host are its high capability for secretion of the desired proteins into the fermentation medium, the absence of a lipopolysaccharide containing outer membrane and its metabolic robustness [126]. In addition, bacilli are also genetically well characterized, and highly developed transformation and gene manipulation technologies

are available [127]. The species most frequently used are *Bacillus* (*B.*) *megaterium*, *B. subtilis*, *B. licheniformis*, and *B. brevis* [123].

2.3.1.1.3 Other Bacteria

Besides *E. coli* and *Bacillus* some other bacteria have been tried for overexpression of proteins. An improved Gram-negative host has been developed using *Ralstonia eutropha*. This system displays a reduced inclination for inclusion body formation and seems to be amenable to high-cell-density fermentations due to its more efficient carbohydrate metabolism compared with *E. coli* systems [123, 133]. Good yields were reported for the production of a mammalian protein by *Staphylococcus carnosus* [126, 133]. *Streptomycetes* are also considered to be suitable for the development of efficient expression and secretion systems. However, the results reported so far are still not satisfactory and below cost-efficient ranges [133]. Further research is necessary to make *Streptomyces* systems competitive.

As prokaryotes show only a limited ability for post-translational modification their employability remains restricted to the production of naturally non-glycosylated proteins, for example, insulin or human somatotropin (STH), or to natively glycosylated proteins that are pharmacologically also active without glycosylation, such as interleukins, interferons or human tissue plasminogen activator (htPA). For the production of proteins that are only active when they are prost-translational modified, eukaryotic expression systems are the best choice.

2.3.1.2 Expression Vectors

As *E. coli* is the most frequently used host for the production of recombinant proteins and the only prokaryotic system employed for the production of drugs approved by the EMA, this section focuses on vector systems that are suitable for transformation of *E. coli* strains.

A variety of expression vectors are commercially available. Successful production of foreign proteins in *E. coli* depends not only on the host strain itself and on fermentation conditions, but also to a great extent on the type of expression vector. Generally, a typical expression vector contains several genetic elements that affect both transcriptional and translational steps of protein biosynthesis. Essential elements are a promoter, ribosome binding site (RBS), start codon, multiple cloning site (MCS), transcription terminator, origin of replication, selection marker, and, additionally, it may contain a fusion tag sequence.

2.3.1.2.1 Replication Rate

The origin of replication located on a plasmid determines the plasmid copy number in the *E. coli* cell. The plasmid copy number, on the other hand, determines the gene dosage accessible for expression. In many cases it was found that the higher the plasmid copy number the higher the yield of protein [122, 132, 134, 135]. However, the relation between plasmid copy number and amount of gene product is not proportional at all. In some cases it might be more successful to use medium or low copy number plasmids. Lower gene dosages lead to lower translation rates, so that proteins have an increased chance of correct folding [125].

For protein secretion slower and sustained protein synthesis is also preferred as mentioned earlier in this chapter, and high copy number may not be necessary or even desirable [136]. Depending on the target protein, low copy plasmids may have some more advantages, such as tight control of gene expression, the ability to replicate large pieces of DNA precisely, and a low metabolic burden on the host strain [135]. However, it should be noted that the final rate of protein production is the result of an interplay of many factors. It additionally depends on promoter strength, mRNA stability, and efficiency of translation initiation, which will be discussed in the following sections [136].

2.3.1.2.2 **Promoter Choice**

A variety of promoters are available by now. They differ in strength (strong/weak) and inducibility (inducable/constitutive). Several criteria have an impact on the selection of an appropriate promoter. If inclusion body formation is intended, a strong promoter may be helpful, and if the protein of interest is toxic to cell growth a tight regulation of the promoter is essential. Moreover, inducibility of the promoter to varying degrees should be possible in a simple and cost-effective manner [122, 137]. Constitutive promoters will be applicable only in a few cases. Frequently used promoters are summarized in Table 2.5. It is common to express the gene of interest from an inducible promoter controlled either by a repressor or by an activator [137]. Thereby negatively regulated expression systems are predominating. The most widely applied promoter system for research purposes is the T7 RNA polymerase system. However, the use of IPTG (isopropyl β-D-1-thiogalactopyranoside) for induction in large-scale production of recombinant therapeutic proteins is undesirable due to its toxicity and high cost.

The most widely used promoters for large-scale production use thermal (e.g., thermosensitive variants of the LacI repressor protein) or nutritional (e.g., *trp*) induction [122]. Often promoters are modified in order to optimize expression for single target genes. Prokaryotic promoters contain the so-called -35 and -10 sequences consisting of six nucleotides each and a 15–19 bp spacer in between. Among *E. coli* promoters the -35 and -10 sequences show strong homology [132,

Table 2.5 Examples of *E. coli* promoter systems used for heterologous protein production.

Promoter system	Induction
lac promoter	IPTG
lacUV5 promoter	IPTG
T7 RNA polymerase promoter-*lac* operator	IPTG
trc and *tac* promoter	IPTG
araBAD promoter	L-arabinose
tetA promoter	Tetracycline
trp promoter	Tryptophan starvation
phoA promoter	Phosphate starvation

135]. However, it is possible to change promoter strength, for example, by alteration of single nucleotides of the -35 and/or -10 boxes or by varying the spacer length [132]. In addition, there is another characteristic region present in some promoters. It is located upstream of the -35 box and is called the UP element. It consists of an AT-rich sequence that allows interaction with the C-terminal domain of the α-subunit of the RNA polymerase, resulting in the increase of promoter strength. As it functions as an independent module, it can be fused to other promoters and possibly enhance transcription [132]. Thus, many host–vector systems can be adjusted to particular requirements.

2.3.1.2.3 **Tag Fusion**

To increase protein stability and solubility and to facilitate the purification process, production of the protein of interest in a fusion form appeared to be a successful measure [127, 135]. Established fusion partners are, for example, the maltose-binding protein (MBP), thioredoxin (Trx), and the glutathione *S*-transferase (GST). Polyhistidine tags are also commonly used, although they are rather more practical to enhance protein isolation than to increase stability and solubility. One reason for improved folding and reduced degradation could be that the fusion partner rapidly reaches its native conformation, thereby promoting the folding process of the residual protein. Several theories about the detailed procedure have been reported [127, 128]. The main disadvantages of fusion tag technologies are that for liberation of the protein of interest, expensive proteases are required, that cleavage is rarely complete leading to reduction in yields, and that additional steps may be necessary to obtain a bioactive product [127, 128].

2.3.1.2.4 **mRNA Stability**

If the expression of a recombinant gene is low, insufficient mRNA stability may be one reason. Generally, mRNA molecules are relatively short-lived. Host cell exo- and endonucleases are involved in the degradation of transcripts and secondary structures, such as stem–loop structures or other localized conformations, have a detectable impact on the half-life of mRNA molecules. There are several possibilities to achieving stabilization of mRNA. One strategy could be to introduce stabilizing elements such as *omp*-like leader sequences to the 5′-end of transcripts. Optimization of the 3′-untranslated sequence of the mRNA, with the objective of blocking exonucleolytic degradation because of the formation of protective stem–loop structures, is another promising method. Also, the employment of genetically modified host strains with mutated RNase genes was shown to be a successful measure [132, 135]. However, for every protein of interest has to be found out individually, which is the best way to improve expression. There is no general rule to predict what would be the most effective.

2.3.1.2.5 **Translation Initiation**

The mRNA 5′-untranslated sequence must contain the ribosome binding site (RBS). Within this region the Shine–Dalgarno (SD) sequence, located 5–13 bases upstream of the start codon, is essential for the interaction with the 3′-end of 16S

rRNA of the bacterial ribosome during translation initiation. Efficient start codons are AUG, GUG, and UUG, with AUG being the most frequently used start codon (about 91%) in *E. coli* [134, 135].

Efforts were made to determine the optimal nucleotide sequence of the translation initiation region. Extensive studies showed that the SD sequence UAAGGAGG is superior to SD sequence AAGGA, and that for UAAGGAGG the optimal spacing is 4–8 nucleotides, whereas it is 5–7 nucleotides for AAGGA [132]. Efficiency of translation initiation depends also on the secondary structure at the RBS of the mRNA. Stem–loop structures possibly occlude the SD sequence and/or the start codon and hamper accessibility to the 30S ribosomal subunit. Mutation of single nucleotides upstream or downstream of the SD region or enrichment of the RBS with adenine and thymidine residues appeared to minimize perturbing the mRNA secondary structure, and thus improved translation efficiency [132, 134].

2.3.2 Production Steps

The production of biopharmaceutical drugs is strictly regulated and based on the directions given in the European Pharmacopeia (Ph.Eur.). Generally, the following steps are involved:

- development of a host–vector system and its detailed characterization,
- production of a validated master cell bank and derived working cell banks thereof,
- fermentation, protein isolation, and purification.
- extensive analytical testing and evaluation of the protein product, and
- detailed documentation of each single step.

The reproducibility of the whole production process and the resulting product must be guaranteed.

2.3.3 Products

By now more then 140 biopharmaceuticals have been approved by the EMA (www.vfa.de/gentech; www.ema.europa.eu); 27 of them are produced in *E. coli* (see Table 2.3). The production of human insulin and somatropin will be described in more detail in the following section.

2.3.3.1 Somatropin (Somatotropin, STH, Human Growth Hormone, hGH)

Human somatropin (STH) is a peptide hormone of 22 kDa. The active hormone consists of 191 amino acids, exhibits two disulfide bonds, and is not glycosylated. In the anterior pituitary cells it is synthesized as a larger precursor polypeptide (prohormone) containing an aminoterminal signal sequence, which is cleaved

after translocation across the endoplasmatic reticulum [138]. As somatropin is not glycosylated it can be produced using *E. coli* as a host system. Today recombinant somatropin is provided by several pharmaceutical companies. Different strategies are used to express and isolate the hormone. For instance, to make Genotropin® (Pharmacia/Pfizer) somatropin is expressed as a fusion protein containing a signal peptide to direct the protein to the periplasmatic space. The host strain is derived from *E. coli* K-12. The expression plasmid is a derivative of pBR322. It is composed of the following elements (Figure 2.3): colE1 replication origin, tetracyclin resistance gene, β-lactamase gene, fusion promoter made up of parts of the promoter/operator region of the *E. coli* alkaline phosphatase gene, and the front part of the *trp* promoter, part of the *E. coli* enterotoxin II signal sequence and the complete cDNA of the mature human somatropin [139].

As a consequence of combining parts of the two different promoters, an efficient transcription of the target gene could be achieved. The enterotoxin II signal sequence directs the fusion protein into the periplasmatic space, where it is cleaved by a membrane bound peptidase, and the active form (without the aminoterminal methionine) of natural growth hormone is built. The host–vector system was generated by transformation of calcium chloride ($CaCl_2$) competent cells, and based on one single positive clone the master cell bank was established. Subsequently, a working cell bank was made. An aliquot of that is now used to inoculate a preculture. The preculture is incubated on a shaker and expanded via a so-called seed culture. The seed culture is then used to inoculate the production fermenter. At the end of the production process, the cells are harvested and treated mainly by freezing at −20 °C and thawing, so that the cell wall ruptures releasing the periplasm containing the accumulated somatropin. Finally, the somatropin is purified by several chromatographic steps [139].

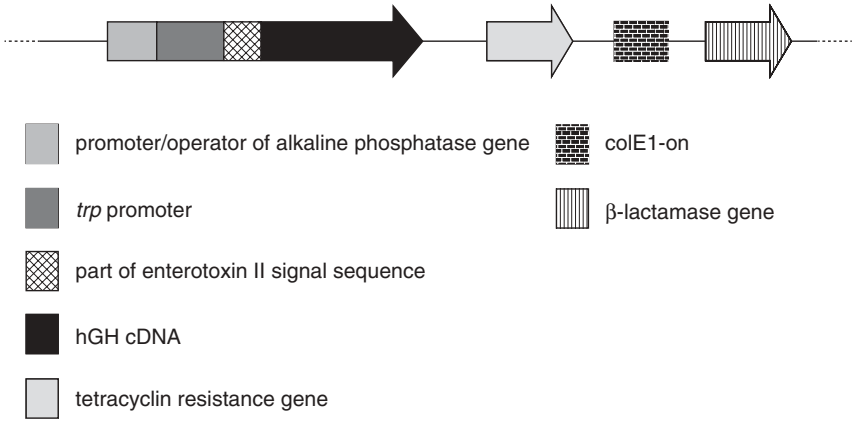

Figure 2.3 Structure of the expression plasmid used for heterologous production of human growth hormone.

2.3.3.2 Human Insulin

The mature insulin molecule is composed of two different peptide chains, the A-chain consisting of 21 amino acids and the B-chain consisting of 30 amino acids. The two chains are held together by two disulfide bonds. A third disulfide bond is located within the A-chain. Nonetheless, the whole insulin is encoded by one gene. In the beta cells of the pancreatic islets, insulin is initially synthesized as a single-chain 86 amino acid precursor polypeptide, called preproinsulin. Subsequent proteolytic processing during translocation of the nascent polypeptide into the ER (endoplasmic reticulum) removes the aminoterminal signal peptide, giving rise to proinsulin. Cleavage of an internal 32 amino acid fragment generates the C peptide and the A- and B-chain. Concurrently the disulfide bonds are formed. The mature insulin and the C peptide are stored together and cosecreted from secretory granules in the beta cells [140].

Initially the production of human insulin by recombinant DNA technology was a big challenge due to its particular structure. *E. coli* cells are not able to perform the specific posttranslational modifications in order to convert the polypeptide preproinsulin into the mature insulin with its two separated chains. Scientists followed a precursor protein approach and in the first published production technique the insulin peptide chains were made separately in two different *E. coli* strains, one for each chain, synthesized as a fusion protein using β-galactosidase [141–143]. Subsequently they were liberated, purified, and separated from the β-galactosidase and joined by chemical and chromatographic methods. However, efficiency and yield were fairly low.

Today one of the most widely used techniques is the expression of the precursor proinsulin in *E. coli* followed by extensive reprocessing. The insulin gene without the sequence coding for the signal peptide is expressed under the control of the strong prokaryotic *trp* promoter. A chimera of the aminoterminal end of the tryptophan synthetase joined to the proinsulin at a methionine residue or methionine codon, respectively, was constructed. After fermentation of an *E. coli* K-12 derived host strain containing the expression plasmid, the accumulated fusion protein is isolated. In the following step this protein is treated with cyanogen bromide, which cleaves the polypeptide chain after methionine residues, resulting in fragments of the tryptophan synthetase and proinsulin. As proinsulin does not contain any methionine moiety it is not degraded. Because of the C peptide the folding process is facilitated and the A- and B-chains are correctly oriented to form the appropriate disulfide bonds easily by oxidative sulfitolyses. The C peptide is finally removed by tryptic cleavage. The human proinsulin coding sequence is obtained either synthetically or by PCR using a human pancreas cDNA library as template [142, 144, 145].

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3
Mammalian Cells in Biotech Production

Maria J. De Jesus and Florian M. Wurm

3.1
Introduction

Stably transfected mammalian cells were first generated by non-viral gene delivery in the early 1980s [1–3]. Immortalized Chinese hamster ovary (CHO) cells were among the first hosts to be used for stable gene transfer because of the availability of auxotrophic (metabolic) CHO mutants deficient in dihydrofolate reductase (DHFR) activity. The reader needs to be reminded that immortalized cell lines differ from primary cells and cell strains with respect to their generally widely rearranged and "disturbed" chromosomal status. Their use became feasible after two critical developments: (i) the molecular cloning of genes into plasmid DNAs [4] and (ii) the chemically-mediated delivery of plasmid DNA into cultured mammalian cells [5–7]. The first CHO-derived cell lines included those that produced recombinant interferons and tissue-type plasminogen activator (tPA) [3, 8, 9]. In 1987 a thrombolytic agent for myocardial infarction, a tPA molecule, became the first FDA-approved recombinant therapeutic protein (Activase®-tPA). The market-saturating production of tPA by the company Genentech required the cultivation of suspension culture adapted mammalian cells in large-scale stirred-tank bioreactors, a technology derived and modified from large-scale batch bacterial productions in stirred-tank bioreactors [10]. The later approved Erythropoeitin (EPO) did not require deep-tank technology because the dose for this product was 1000 times smaller, 100 µg/dose, than the one for tPA. In fact, the technology of stirred tank based manufacturing has not dramatically changed in the last 25 years; CHO cells remain the major host for the generation of recombinant cell lines, and most manufacturing processes with recombinant cell lines are still performed in stirred bioreactors, ranging in scale from a few hundred liters to 20 000 liters. What then is the difference between today's technology and that of 25 years ago?

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3.2 Process Concepts and Cells

In the late 1980s a typical batch culture production run lasted about 7 days with a maximum cell density of 1–2 million cells/ml, and the usual yield was 50–100 mg/l [11]. By comparison, today's fed-batch production runs can last up to 21 days with a maximum cell density of 10–20 million cells/ml. These bioprocesses typically have specific and volumetric productivities in the range of 50–60 pg/cell/day – only about twice as high as in the 1980s – but 1–10 g/l in product concentration for antibodies and antibody-like molecules at harvest – that is, about 20–50 times higher than in the early phases of the use of CHO cells in bioreactors [11]. Surprisingly, this dramatic yield increase has come about mainly due to improvements in the media compositions, including those of complex feeds (concentrates of amino acids and other medium components), and due to targeted modifications of the handling of cells in the reactors during the production phase, resulting in more and healthier cells over a longer cultivation period.

These proprietary and largely unpublished process modifications have been developed during years of work and with the help of significant insights into the consumption of key nutrients and the production of waste products from clonal cell lines producing the required product at high rate. While some of the details of such improved processes have made it into the public domain, some of the most important information, for example, the composition of the media-formulations is largely kept as a trade secret.

Also surprising, the impact of host cell engineering, over decades claimed to promise dramatic improvements in yield and to facilitate scale up and increased robustness of host cells has been, in the opinion of us as authors of this chapter, minimal. This is most likely caused by the considerable metabolic "individuality" of clonal cell lines, fundamentally residing in the genetic instability and subsequent population diversity of CHO cells (and other cell lines also).

Recently, the genomic sequence of one ancestral CHO genome (K1) was published and showed a typical mammalian sequence composition [12]. The authors acknowledged in their paper that the sequenced genome may be in fact far from being representative for the clonal cell lines cultivated in today's large-scale bioreactors. This assessment was enforced by the accompanying commentary [13] that highlighted the complex and convoluted history of CHO cells (which includes mutagenesis, adaptations to different growth conditions, and cloning), impossible to trace back in detail. Worse, a careful analysis of a few available karyotypes of CHO cells indicates a most variable nature in chromosome structures in these cells and points to very complex genetics/genomics of both recombinant and non-recombinant CHO cell lines in use today. Thus, in our opinion, the chances to design "the" ideal mammalian host cell lines for the needs of the industry (system biology) have not emerged today and may be not achievable in the near future, since "the CHO genome" and the "CHO biology" do not exist.

However, a number of teams – mostly in large biopharmaceutical companies – working with clonal cell lines expressing different proteins and developing

the corresponding manufacturing processes for these over the years (Process Sciences, Manufacturing Science), gained deep and truly profound insights into the metabolic needs of "their" cells. This was and continues to be the basis for highly improved media and feed compositions as well as many relevant process interventions that were applied when the next protein project was "on" for development. While this did not liberate the companies from continued investment into cell line and process development for each individual protein product, it provided, however, a set of process options that greatly enhanced robustness and longevity of cells in bioreactors. In our own work – dealing with about 100 different proteins and processes over the last 30 years – we estimate that for a given clonal cell line about 100–500 different process conditions have to be evaluated/studied and fine-tuned in order to identify a few which work well. Remarkably, very small modifications in many of these process conditions can have dramatic effects.

Are further improvements in specific and volumetric productivity of recombinant mammalian cells possible? It appears that a biological limit of about 100 pg/cell/day has been achieved, at least for cells that need to grow robustly as well (which is the case for all suspension culture based processes). The volumetric yields, today at 3–6 g/l for "easy to express" antibodies in extended batch processes (10–16 days) can be pushed higher, possibly even two- to fivefold. This is achievable mainly by increasing cell densities in the bioreactor. Reports from companies disclose cell densities of up to 20×10^6 cell/ml in fed-batch processes with media whose formulations are not disclosed. At this density, cells represent about 5% of the volume of the liquid.

To achieve even higher densities more sophisticated cell culture media are needed, which are not easy to identify. In fact, they may be even difficult to formulate and to prepare: complexities in physico-chemical limits in solubility and opposing pH preferences as well as opposing chemical reaction principles of individual medium components hamper progress. It has to be seen whether designed oligo-and polypeptides (at least for the amino acid fraction of media) could play a more profound role in the future.

High-throughput cell cultivation systems for rapid and cost-effective screening of media compositions and bioprocess conditions will be necessary and used in order to identify better media, ultimately with the goal to allow for additional cell population doublings, but also for the identification of specialized feeds that assure the maintenance of a healthy, high density cell population for longer time periods.

However, let us ask a key question in this context: Are higher product yields truly an important requirement? Considering the increasing competitiveness in the market with a dramatically larger number of new protein products addressing the same disease indications, (individual) product quantities for market supply may be smaller than they were in the past. In addition, "biosimilars" and "biobetters" or "follow on biologicals" are presently entering the market, further reducing the probability for the introduction of true "blockbuster" protein drugs. Products for niche indications will become more interesting, for larger pharmaceutical

companies also, who struggle today to introduce new pharmaceutical protein products into the markets. Thus, we believe that the drive for higher volumetric yields will be a less important goal for the future.

In contrast, time to market will continue to be an overall driver for success in this highly diversified market. An important part of this strategy is the need to achieve reasonably high yields in the shortest time-frame. Today, cell line and process development take 12 months on average, but this will be shortened to 6 months or less if the target yields for production can be reduced. We have experience with our own approaches whereby a process of 1–2 g/l could be available 2–3 months after transfections of cells (data not published). The need for higher economic efficiency, will surely further increase the use of disposable container/bioreactor systems for large-scale cell culture, possibly up to the final production scale, and will also open the door for protein production using transient gene expression (TGE) systems, a new approach in protein manufacturing.

3.3 CHO-Derived Production Cell Lines

The CHO cells used in protein manufacturing originated in 1957 as a "spontaneously" immortalized cell from a primary culture of ovarian cells from a Chinese hamster (*Cricetulus griseus*) [14]. A proline-dependent clonal strain was derived from the original cell line and called CHO-K1. This cell line was mutagenized to generate CHO-DXB11 (also referred to as CHO-DUKX or CHO-**DUK-XB11**), a cell line lacking DHFR activity [15]. These cells have a deletion of one *dhfr* allele and a missense mutation in the other. Subsequently, the proline-dependent CHO-pro3$^-$strain, another derivative of the original CHO cell line, was mutagenized to yield CHO-**DG44**, a cell line with deletions of both *dhfr* alleles [13, 16]. These two DHFR-minus strains require hypoxanthine and thymidine (as well as glycine) for growth. Frequently, the non-selective medium for these cells is referred to as GHT medium (glycine, hypoxanthine, thymidine), since these components have to be added to standard cell culture media in order to allow growth of these cells.

Although not initially intended for recombinant protein manufacture, DHFR-minus CHO cells were used for a number of pioneering experiments demonstrating stable gene-transfer and repair of DHFR activity with an exogenous *dhfr* gene when growing cells in a medium lacking hypoxanthine and thymidine. This selection scheme, modified slightly when using other selection markers, remains one of the standard methods to establish stably transfected CHO cell lines for the production of recombinant therapeutic proteins.

The process begins with the molecular cloning of the gene of interest (GOI) and the *dhfr* gene into a single or into two separate mammalian expression vectors. The two plasmid DNA(s) carrying the two genes are then delivered into cells by transfection, and the cells are grown under selective conditions in a GHT-minus medium. Surviving cells will have one or more copies of the exogenous *dhfr* gene,

usually along with the GOI, integrated in its genome [1–3]. The integrated plasmid copy number varies widely from one recombinant cell to another, but there is almost always only one integration site per cell even when multiple plasmids are transfected [17].

The growth rate and the level of recombinant protein production of each cell line also vary widely. To obtain a few stably transfected cell lines with the desired phenotypic characteristics, it may be necessary to evaluate several hundred clonal cell lines. In this context, it needs to be pointed out that presently available transfection technologies result in 1–3% of exposed cells to be transformed – thus genetically not more than 1000–3000 individually different clonal cells are being generated (as these transfections occur in small volumes with less than 1 million cells).

The *dhfr* gene is not the only selection marker available for generating recombinant CHO cell lines. The glutamine synthetase (*gs*) gene, initially considered for the selection of murine NS0-derived cell lines with low or no endogenous GS activity, is also used for the selection of stably transfected CHO cells even though they have a higher endogenous GS activity than NS0 cells [18, 19]. After transfection with GOI and *gs* genes, recombinant cells are selected in a medium lacking the amin oacid glutamine. Both DHFR- and GS-systems allow enhancing the expression of the GOI by exposing the cells to a drug that blocks the enzymatic activity of the selection marker. DHFR and GS are inhibited by methotrexate (MTX) and methionine sulfoximine (MSX), respectively [18, 20, 21]. This is not a direct effect, but it is based on the selection of clonally arising cells that have, by chance, enhanced expression of the selection marker gene through a process, globally referred to as "gene amplification." For CHO-derived cell lines that express an exogenous *dhfr* gene, a majority of the cells die after 2–3 weeks of exposure to increasing concentrations of MTX. The rare survivors have a higher integrated plasmid copy number than the original cell line as the result of amplification of the *dhfr* gene and the neighboring DNA, including the GOI [2, 20, 21].

Similar observations have been made following the exposure of recombinant NS0 cell lines to MSX [19]. The process steps necessary resulting in gene amplification takes many weeks of tedious cell culture work and also contributes to significant and unpredictable genomic rearrangements of the host cells. For this reason, gene amplification is rarely used today and is routinely replaced by highly improved methods for the generation of high producing cell lines right after transfection.

3.4
Rapid Generation of High-Producing Cell Lines

The major problem with "standard" methods of cell line generation and selection is that the specific productivity of recovered cell lines is low. This necessitates the screening of hundreds if not thousands of individual cell lines to obtain a few

reasonable "clones" that have the desired phenotype combination of high productivity and high growth rate.

One way to reduce the number of irrelevant cell lines from the pool of recombinant cells is to increase the stringency of selection. With DHFR-minus CHO cells, for example, the GHT-minus medium may be supplemented with 30–100 nM MTX to increase the probability of selecting cell lines with high *dhfr* activity. As the GOI is integrated at the same site as the *dhfr* gene the number of high-producing cell lines can be increased while leaving low-producing lines aside. High producing cell lines with capacities for 1–3 g/l production (after further development work) have been identified in this way (De Jesus, unpublished data). This approach avoids the time consuming gene amplification approach utilized in the past. As indicated above, induced gene amplification is to a large degree unpredictable, and amplified DNA segments in the chromosomes of CHO cells may not be stable in the absence of MTX [17]. Also, high-throughput methods to screen candidate cell lines are being developed to reduce the time necessary for the recovery of high-producers [22].

Most of these methods are based on fluorescence activated cell sorting (FACS). For example, the GFP gene can be coexpressed with the GOI and the cells sorted for GFP-specific fluorescence [23, 24]. The GFP gene may be used as the sole selection marker or in combination with one of the selection markers described above. Alternatively, the GOI may be coexpressed with a gene encoding a cell surface protein. The recombinant cells expressing the latter are then stained with a fluorescently-labeled antibody specific for this protein and then sorted by FACS [25]. Lastly, recombinant cells selected for the presence of the *dhfr* gene have been incubated with fluorescent MTX that binds to DHFR. DHFR-positive cells can then be sorted by FACS [26]. The level of MTX-specific fluorescence is expected to correlate with the level of the recombinant protein of interest. It is not known, however, if any of the methods described in the academic literature are being actively pursued in companies, and/or whether they have entered the manufacturing approaches of approved products in the market.

Clonal cell line recovery has been automated with such instruments as the ClonePix system (Genetix Ltd., UK) and the CellCelector™ (Aviso GmbH, Germany). For these instruments, the putative recombinant cells are suspended in semi-solid medium. Under these conditions, the secreted recombinant protein remains near the cell and can be stained with a fluorescently-labeled antibody. These automated systems can detect and transfer the cell lines to another cultivation container for further analysis.

It is also possible to increase the average specific productivity of recombinant cell lines by increasing the amount of plasmid DNA delivered to cells. We have recently shown that calcium phosphate (CaPi)-mediated transfection of CHO-DG44 cells results in both a higher plasmid copy number and a higher average specific productivity compared with PEI-mediated transfection [27]. Furthermore, the specific productivity of recombinant cell lines generated by microinjection of either BHK-21 or CHO-DG44 cells depended on the amount of plasmid DNA injected per cell [28].

3.5 Silencing – Stability of Expression

Once clonal cell lines are established, they need to be characterized for the "stability" of recombinant protein production. This is necessary because expression of the integrated GOI is not always maintained at a constant level over time. Many clonal cell lines loose and/or reduce the synthesis of mRNA driven from constitutive promoters. The reasons for this are not fully elucidated and, in addition, the phenomenon is variable in its degree from clonal cell line to clonal cell line. Generally, this is referred to as gene silencing (the reduction or elimination of gene-specific transcription). One assumes that gene silencing would be influenced by the environmental DNA in the vicinity of the integrated GOI sequences. Thus, candidate cell lines for large-scale productions must be cultivated for several months to exclude stability problems.

A major determinant of gene silencing is thought to be the structure (sequence composition and modification of histones and other proteins) of the chromatin at the site of integration of the recombinant gene. In general, heterochromatin is condensed and transcriptionally inactive whereas euchromatin is relaxed and transcriptionally active [29]. The two chromatin states are associated with specific histone modifications including acetylation, methylation, and phosphorylation that function to control chromatin condensation and transcriptional activity [30]. We have observed two gene silencing effects in recombinant CHO-DG44 cell lines [27]. Rapid gene silencing occurs in about half the cell lines within days after release of the cells from selective pressure. This type of gene silencing does not appear to be correlated with the level of GOI expression. For about one-third of the cell lines, a slow and gradual reduction in GOI expression occurs within 6 months after removal of the selective pressure (data not published). Finally, about 15–25% of cell lines derived from standard transfections have a stable level of protein productivity in the absence of selection [27]. For cells treated with MTX to amplify the copy number of the integrated gene of interest, the stability of the recombinant protein production in the absence of MTX appeared to be due mainly to gene silencing rather than to loss of transgene copy number [31].

The choice of the promoter/enhancer used to drive GOI expression may also influence the extent of gene silencing. Furthermore, DNA elements such as scaffold/matrix attachment regions (S/MARs), insulators, antirepressor elements, and ubiquitous chromatin opening elements (UCOEs) have been shown to support stable protein production in recombinant cell lines and to increase the percentage of clonal cell lines with high expression levels [32–34, 35]. These DNA elements are small enough so that they can be cloned in the expression vector employed for GOI delivery. The mechanism(s) associated with their observed function, however, is not entirely clear. They may ameliorate the effects of gene silencing directly through inhibition of heterochromatin formation or they may affect plasmid DNA integration itself. For example, they may influence the integrated plasmid copy number or the site of integration.

Recently, a new approach for the generation of highly productive cell lines has been developed. With the help of a lentivirus vector system, DNA can be integrated into the CHO genome, resulting in high transcription rates for the gene of interest in clonal cell lines [36]. This approach significantly reduced the times to generated highly productive cell lines, and, as another advantage of this system, the majority of clonal cell lines generated showed good production stability in the absence of selective culture conditions.

An insect derived transposon, the PiggyBac transposon [37], has been shown to transpose in mammalian cells. While it has been used as a tool to generate knock-out mutations in transgenic animals [38], this system has now been developed for the generation of highly productive cell lines [39]. The advantage of this system is the delivery of expression cassettes into the receiving mammalian genome without any bacterial DNA remaining, as the transposase cuts the inserts out of the plasmid and delivers it, framed with two short repeats, into the receiving genome. The transposase gene is not integrated into the genome (as far as we know today – research is ongoing) and thus the delivered GOI will not be mobilized again.

3.6 High-Throughput Bioprocess Development

In the 1980s, small-scale process development studies for recombinant protein production were mainly performed in non-instrumented (without pH and O_2 monitoring/control) spinner flasks. However, they are not easily adapted to high-throughput applications since the minimal culture volume for these containers is about 50 ml, more frequently even bottles with working volumes of 250 or 500 ml were used. In these vessels small volumes of 10 or 20 ml could not be cultivated. In addition, these vessels have low volumetric mass transfer coefficients (k_La) of about 1–3 h^{-1}, limiting the maximally achievable cell densities to 2–3 $\times$ 10^6 cells/ml [40]. High-performance media allow densities of 10 $\times$ 10^6 cells/ml when the culture is performed in a fully controlled and properly oxygenated bioreactor. Therefore, the cell cultivation conditions in spinner flasks have been a very poor and frequently even entirely misleading predictor of culture performance in large-scale stirred-tank bioreactors.

In an attempt to address the urgent needs for simpler, high performance cultivation systems, we developed orbitally shaken 50-ml ventilated tubes (TubeSpin® bioreactor 50, now marketed by TPP, Trasadingen Switzerland, short "TubeSpins"). These have been found to be efficient scale-down bioreactors for mammalian cell cultivation in suspension [41]. Cell densities >10^7 cells/ml have been achieved in volumes of 5–20 ml with CHO cells [42]. At agitation speeds appropriate for mammalian cell cultivation, k_La values of 10–20 h^{-1} have been measured [43, 44]. Owing to the excellent mass transfer in these tubes, oxygen limitation has not been observed at cell densities up to 3 $\times$ 10^7 cells/ml. As these minibioreactors are used in incubator shakers that provide CO_2 in the environment, a narrow pH range is observed between pH 6.7 and 7.0. We and others have used these TubeSpins for

high-throughput screening campaigns to optimize bioprocesses for recombinant CHO cell lines. The low cost and ease of operation of orbitally shaken TubeSpins allows 100s of small-scale cultures to be run simultaneously [45]. TubeSpins are expected to significantly reduce the time necessary for medium design and the development of feeding strategies for fed-batch cultures.

Other scale-down cell culture systems have also been described recently. Examples include the SimCell microfluidics technology (Invitrogen, USA) and microbioreactors in 24- and 96-deepwell plates [46, 47]. These systems have, however, a much higher cost-basis and frequently require robotic technology.

3.7 Disposable Bioreactors

Today, large-scale mammalian cell culture is almost exclusively performed in stainless-steel stirred-tank bioreactors. However, there is a trend in the protein manufacturing industry towards the use of disposable equipment. The most successful of the disposable bioreactors is the wave-type bioreactor that was introduced about 10 years ago [48]. The cells are cultivated in disposable plastic bags of volumes of up to 500 l mounted on a rocking table. However, the k_La values in wave bioreactors are less than $4\,h^{-1}$, resulting in possible oxygen limitation at densities $>5 \times 10^6$ cells/ml when only air is used as an oxygen providing gas [48]. Modifications of the movement of a horizontally mounted bag on a platform that combines rocking (angle movement) with a horizontal forwards/backwards movement can improve the gas transfer rates ("2-D shaking" in a CELLtainer bioreactor) [49]. Disposable stirred-tank bioreactors at volumetric scales of up to 1000 l have also become available very recently from a number of suppliers.

Erlenmeyer flasks have been used in the past for both microbial and cell culture operations by applying orbital shaking as the mixing principle. However, the scale-up in these flasks is limited as the working volume is only 10–20% of the nominal volume. More recently, both cylindrical and square-shaped vessels with working volumes in the range of 100 ml to 30 l have been used, mostly in our labs, for the suspension cultivation of mammalian cells by orbital shaking [42, 43, 50–53]. Mammalian cell cultivation has also been performed in disposable plastic bags of 200 and 2000 l that were mounted within cylindrical containers on orbital shakers custom-made for this purpose [42]. Recently, the OrbShake 200X has been introduced to the market, capable of cultivating up to 250 l of cell suspension based on orbital shaking [54]. The applications of this reactor in our labs have so far shown high reliability and excellent performance, exceeding the performance of stirred tanks (personal communication by the authors).

Stirred-tank bioreactors are aerated by the sparging of oxygen or oxygen-enriched air into the culture near the bottom of the vessel. In contrast, surface aeration is the operational mode for shaken bioreactors, highlighting the importance of the surface (area) on oxygen transfer in this type of cultivation system. The flow structure at or near the liquid–air interface is the dominant factor determining the rate

of mass (gas) transfer into and out of the cell culture medium. For cylindrical shaken vessels of up to 100 l working volume, shaking at moderate shaking speeds, k_La values of 5–10 h^{-1} were obtained. So far unpublished work with a 3200-l disposable bioreactor, operated at a working volume of 2000 l, provided k_La values of 3–12 h^{-1} when operating at shaking speeds of 47–55 rpm and flushing the headspace with air only. This work extends earlier published observations with a shaken 2000-l reactor, at a working volume of 1000 l [43].

While the technology at the very large scale for any type of "disposable" reactors is very young and probably requires more improvements, notably in the field of stability of the plastic containers, the monitoring and control systems, and other issues related to the long-term provisioning of robust and consistent bag-designs, the data are highly encouraging in terms of efficiency. It is known that limiting oxygen transfer rates in bioreactors (due to low k_La values) can be overcome by aeration with oxygen-enriched air or pure oxygen, as such gas mixtures or pure oxygen have a higher driving force into the liquid.

Suspension cell culture using orbitally shaken bioreactors will become an attractive, possibly even superior option, to stirred stainless-steel tanks at scales of up to 2000 l because of increased flexibility, better mixing, and gas transfer rates and, most importantly, due to the reduced costs associated with disposable cultivation systems [42, 55–57]. Although operating conditions in large-scale orbitally shaken bioreactors require further study and in-depth analysis, the results to date demonstrate the potential of this simple bioreactor for applications in high-density mammalian cell cultivation.

3.8 Transient Gene Expression (TGE)

Large-scale transient gene expression (TGE) is a new technology that was only recently considered for recombinant therapeutic protein production [58, 59]. TGE is defined as the production of a recombinant protein over a short period (1–14 days) following highly efficient DNA transfer into single-cell suspension cultures. The recombinant gene(s) is usually cloned in a non-viral expression vector and transfected into cells with a chemical delivery agent such as calcium phosphate (CaPi) or polyethylenimine (PEI). In contrast to stable gene expression from recombinant cell lines, genetic selection is not applied to the transfected cells at any time in the process. The technology has been developed mainly with CHO and HEK-293 cells as they are easily transfected, grow in single-cell suspension, and have been used for the production of therapeutic proteins which have gained regulatory approval [60–63]. TGE is typically performed in stirred-tank bioreactors or in agitated containers including shake flasks, wave-type bioreactors, and plastic or glass bottles [64].

The main advantage of TGE over stable protein production is the saving of time. In the past, the specific and volumetric productivities achieved by TGE were significantly lower than those seen in stable cell lines. Recently with both CHO and

HEK-293 cells, however, volumetric productivities of up to 1 g/l have been achieved in a bioprocess lasting 14 days [65, 66]. Thus, significant quantities of recombinant protein can be obtained within a few days of transfection. While 1–10l scale-TGE with yields of hundreds of mg/l has been developed in the laboratories of the authors, the routine use of this approach for volumetric scales in the 100–1000l range is still not yet feasible. To date, the largest volume for TGE has been 100l [67, 68, 69].

So far no therapeutic protein has been produced by TGE that has gained regulatory approval. This must be accomplished before large-scale TGE becomes a standard method of therapeutic recombinant protein manufacturing. This is likely to be first attempted with a low-dose protein such as a vaccine requiring a relatively small amount of recombinant protein to cover the market needs. There is also a perception in the industry that large-scale TGE is not reproducible. However, as an emerging technology it needs to be addressed from all angles of science and engineering, which is a matter of time and effort. The application of high-throughput culture systems as described above will help to alleviate real or perceived (robustness/reproducibility) problems with TGE.

3.9 Conclusions

Much has been accomplished since the 1980s when considering protein manufacturing in animal cells. The typical volumetric yields from bioprocesses in large-scale stirred tanks have increased approximately 20–50-fold during this period. With (multi) gram per liter yields in highly optimized fed-batch processes the production of kilograms and even hundreds of kilogram of a desired protein in large-scale bioreactors has become a routine. Continued improvements in the efficiency of generation of high-producing cell lines, in the composition of richer and more balanced media, and in strategies for using such media are expected to result in further yield increases. Simultaneously, shorter production run times will be used than those applied today. With these trends the overall annual capacity of manufacturing plants can be increased – and fairly dramatically. It remains to be seen if the required bioreactor volumes for individual products may actually decrease over time, due to the improved yields. Surely, the time required to establish such high yielding processes, when done by experienced groups, will be reduced to a few months (3–6), offering more flexibility and choices. Overall, the improved speed for the development, the higher yields, the use of disposable reactors, and the application of "generic" processes will have a slightly positive economic impact on upstream protein manufacturing. Whether similar economic improvements can be made in down-stream processing is still largely unknown. So far, progress in this area over the last 30 years has been slow and only incremental. Dr Uwe Gottschalk, a leading expert in the field of downstream processing and a frequent speaker on this topic, has repeatedly voiced disappointment on progress in recovery of complex protein products [70].

We believe that transient gene expression will become a manufacturing technology one day, expanding the possibility to make high-value proteins in mammalian cells fast and with high yield. We do acknowledge that challenges continue to exist. However, they are mostly residing in a profoundly conservative mind-set in the industry and are not founded in fundamental problems with this technology. The technologies in cell culture are here today to deliver highest quality, highest yield for any recombinant protein for pharmaceutical applications – at a reasonable cost–benefit ratio.

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4
Biopharmaceuticals from Plants

Heribert Warzecha

4.1
Introduction

Since 1982, when insulin, as the first recombinant drug, entered the market, approximately 200 biopharmaceuticals have gained approval and are in general use worldwide [1]. However, the number of organisms in which those proteins are produced is comparably low, and only recently have new production hosts been established. Usually, bacteria are the host of choice when it comes to speed of production at low cost. *Escherichia coli* is the best established bacterial host and apart from this, very little development for the establishment of other bacteria as commercial production hosts for pharmaceuticals has been done. However, *E. coli* is only a suitable choice when the final product can be purified from all bacterial impurities and when the protein product does not require any significant post-translational modifications. For proteins of eukaryotic origin, usually a eukaryotic host is beneficial as post-translational modifications, such as processing, folding, and also glycosylation [2], will only be performed sufficiently in a eukaryotic cellular environment. The yeast *Saccharomyces cerevisiae* is a unicellular host that can be fermented easily. Hence, several recombinant therapeutics are produced in yeast [3]. However, *S. cerevisiae* produces proteins with a glycosylation pattern which differs greatly from that observed in mammalian cells, and as glycosylation is crucial for many products regarding stability, function as well as antigenicity, today mammalian cell cultures are the host cells of choice as production systems [4].

Despite several established and approved production systems, attempts are being made to either improve the current systems or to establish new platforms, some of which are discussed in more detail in other chapters of this book. This chapter will focus on higher plants as production systems for recombinant therapeutics or vaccines.

While some reasons for the use of plants as alternative production system are connected with intellectual property issues, there are also technical and scientific reasons, which are often stressed.

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i) **Cost of goods:** while traditional fermenter-based systems need a high investment for facilities and additional expenses for running the production process (e.g., heating, stirring, sterilization of nutrients), plants are believed to be a low cost production system. Once the transgenic line has been established and propagated, only low-cost agricultural maintenance has to be applied.

ii) **Low risk of infection:** to date there is virtually no evidence that pathogens could infect both plants and mammals and therefore, plant-derived pharmaceutics are thought to be safe with respect to contaminating pathogens.

iii) **Potential for oral application:** one of the first concepts of using plants for the production of recombinant therapeutics included the oral use of the final product. Indeed, if the product accumulates in a part of the plant which could be eaten raw, there is potential to deliver the therapeutic (e.g., oral vaccines or gastric enzymes) with a minimum of post-harvest processing.

All the above mentioned arguments have been repeatedly challenged since the first report of a recombinant pharmaceutical from plants was published in 1990 (recombinant human serum albumin [5]). Today, after 20 years of research there is still no recombinant pharmaceutical product from plants on the market, but there is evidence that the system is coming of age and will provide a suitable production host for proteins with specific requirements. In the following section plant-based production systems will be described on the basis of the most promising candidates that will most probably gain approval soon.

4.2 Basics in Plant Biotechnology

For bacteria, yeast, and to some extent for mammalian cell cultures, one or more "work horses" have been established, which are well characterized and have been in use as production systems for a long time. For plants, this is not the case and numerous plant species have been exploited for the purpose of recombinant therapeutic production. Only recently a few plant species, for example, tobacco, seemed to be taking the lead in the development of platform technology for plant produced recombinant therapeutics.

During the development of plant biotechnology, plants were usually chosen that were relatively easy to transform and to regenerate, which have a short generation time, and which yield reasonable amounts of biomass. Naturally, most of the well established plants are crop plants, often bred and cultivated for centuries. Among the frequently used plant species and plant tissues used are:

- cereals (rice, wheat, corn)
- legumes (pea, soybean, Lucerne)
- fruit and leaf crops (tomato, potato, lettuce, spinach)
- aquatic weeds (duckweed, *Lemna* sp.)
- single-cell cultures of the algae *Chlorella* and *Chlamydomonas*
- suspension cell cultures of a variety of plants.

From this list it becomes clear that the term "plant," includes numerous hosts which differ greatly in physiology and capacity for recombinant protein production. Moreover, there are different options to generate a transgenic plant. The most common technique is to introduce a transgene expression cassette into the nuclear genome by *Agrobacterium tumefaciens* mediated transformation. This method is widely used and can be applied to a number of plant species [6], depending on their susceptibility to infection with this soil bacterium and their ability to regenerate from a single cell. However, the process of integration of the transgene into the host genome is random regarding position and number, and, therefore, every transformation is a unique event generating individual plants differing in expression levels and viability. This fact makes an extensive screening and testing necessary as well as further subsequent breeding of individual producer lines. Since this process usually requires several months to years, this method is not suitable for fast establishment of expression systems and quick response to changing demands in candidate proteins. However, once a production line is established scale up is fairly easy by simply multiplying plants, and limits are only set by the usable area for plant growth.

As genetic transformation and selection of plant lines become more and more routine, now the focus lies on maximizing the obtainable amount of recombinant protein in a given plant tissue. One problem often observed after nuclear transformation is the relatively low amount of recombinant protein that can be obtained. Only in rare cases do amounts of >1% of the total protein consist of the recombinant protein, which usually gives enough protein for testing of integrity and functionality of the protein. Only in exceptional cases are recombinant protein levels high enough to justify the use of such plants as expression systems, for example, when the proteins are targeted to specialized protein storage compartments (see below).

Another technique that has been developed might overcome some of the obstacles of nuclear transformation, namely the integration of the transgene into the chloroplast genome [7]. Green plants contain numerous plastid organelles in their cells that are evolutionary derived from cyanobacterial ancestors. These semi-autonomous compartments comprise their own genome in multiple copies, leading to up to 10 000 copies of a gene in a single cell. Chloroplasts also exhibit prokaryotic features, which, for example, allow the targeted transgene insertion via homologous recombination, overcoming the position effect observed in nuclear transformation. Regarding recombinant protein production, plastids are really exceptional in that they allow protein accumulation of up to 70% of the total protein (as in the case of antimicrobial peptides [8]). Last but not least, plastids are maternally inherited in most crop species. Because of this feature such transplastomic plants are considered to be environmentally safe as transgenes cannot be spread by pollen and outcrossing of the transgene to other crop plants can be prohibited. However, plastid transformation is a longsome process and so far only tobacco can be used routinely. This might be the reason why to date only reports have been published showing proof-of-principle, but no product is being developed.

A third option for transgene expression in plants is the use of plant viruses for transient production of proteins. Plant viruses such as the tobacco mosaic virus (TMV) naturally infect a variety of plants and – during their infection cycle – high-jack the cellular machinery to reproduce new virus particles. Throughout this cycle some viral proteins are produced in extraordinary levels, mainly the viral coat proteins that encapsidate the genome of the virus. As the scientific background of virus infection and replication has been elucidated, methods have been developed to exploit the capacity of the plant virus to produce recombinant proteins with the help of the plant cell [9]. The most common way is to engineer viral genomes and shuffle viral coat protein genes with transgenes of interest. A subsequent infection of a plant with either the viral genome (an RNA in case of the TMV) or recombinant viruses then results in the accumulation of large amounts of recombinant proteins in the plants within days [10].

An advantage of this approach is its speed as no transgenic plant has to be developed and proteins could be produced within days in wild-type plants. A downside is that a manual inoculation of plants with viruses or with fragile viral RNA is required. Here, the combination of Agrobacteria and modified viral replicons can provide a solution. As Agrobacteria can naturally transfer DNA to plant cells, this mobile DNA element is modified to encode an engineered viral replicon. Once inside the plant cells nucleus, the viral replicon is mobilized and initiates a viral infection, which eventually affects the whole plant and generates large amounts of recombinant protein. With this technique exceptional levels of recombinant protein can be produced and even complex molecules such as monoclonal antibodies can be functionally expressed and recovered [10, 11]. Even the inoculation process of plants can be simplified in that the areal parts of the plant (*Nicotiana benthamiana*) are submerged in Agrobacteria solution and a vacuum is applied. The release of the vacuum forces the bacterial solution to enter the intercellular space in the leaves and initiates the infection process. Cell-to-cell movement of viral replicons generalizes the infection and results in uniform expression of the transgene in the plant parts.

Beside the possible variety of host plants and transformation methods there is even more variety in the plant based expression system, namely the localization of the recombinant protein in different parts or developmental stages of a given plant. For example, could a recombinant protein be expressed exclusively in a specific part like leaves, seeds, roots, or tubers by the choice of a specific promoter? The protein localization affects both protein amount as well as post-translational modification. As an example it has been shown that a recombinant antibody accumulates only at low amounts in leaf tissue but at up to 36% total protein when expressed in seeds of *Phaseolus vulgaris* (garden bean) [12]. Protein glycosylation could be different too, as exemplified by the production of the enzyme Phytase from *Aspergillus niger* [13], which showed different glycosylation patterns when produced in either the leaves or seeds of *Oryza sativa* (rice).

All the examples show that there is no such thing as a uniform plant production system for recombinant proteins, but rather there is a multitude of options. Some examples will be discussed in detail.

4.3
Plant Cell Cultures as Production System for Human Glucocerebrosidase

From all the different options of expressing genes in plants a cell suspension culture is the one system which most closely resembles the conventional fermentable cells such as bacteria, yeast, or mammalian cell cultures. For a number of plant species, tissues can be treated in that they develop undifferentiated cell growth, the so called calli. Taken into sterile nutrient solution and gently agitated, cells can be separated and maintained for an almost indefinite length of time [14]. If transformed with a transgene, those cells maintain the production of recombinant protein, almost like mammalian cell cultures, but still have the advantages of not requiring supplementation of animal products such as serum and not harboring potential human pathogens.

The suitability of the system has already been shown in 2006 when the first pharmaceutical recombinant protein gained regulatory approval in the USA: a vaccine against Newcastle disease virus to be used in poultry (produced by Dow Agrosciences) [15]. However, this product has not been marketed yet and the reasons have not been clearly communicated.

Another plant-based product has been developed in Israel (by Protalix Biotherapeutics) and is by far the most advanced plant cell culture-based product for human use, as it is in phase III trial, namely glucocerebrosidase (GCD). GCD is a lysosomal enzyme that catalyzes the hydrolysis of glucosylceramide (or glucocerebroside). Lack of this enzyme is caused by a mutation of the encoding gene and leads to accumulation of glycosylceramides in lysosomes and macrophages, followed by clinical symptoms including anemia, thrombocytopenia, and skeletal disorientation [16]. Since the identification of the encoding gene and its expression in mammalian cell cultures (Chinese hamster ovary (CHO) cells) sufficient amounts of protein can be provided for the efficient enzyme replacement therapy of Gaucher's disease. To obtain the functional enzyme from the CHO cell-produced protein, the product has to be extensively modified. Human GCD is a glycosylated protein and the terminal mannose residues of the sugar chains are essential in that they facilitate uptake of the enzyme to macrophages through mannose receptors (Figure 4.1). If the mannose residues are shielded by other sugar moieties, as is common in mammalian glycoproteins, the protein is not functional. In consequence, the glycosylated protein from CHO cells needs to be deglycosylated *in vitro* via α-neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase to obtain a functional enzyme [17]. This is how the currently commercially available GCD (INN imiglucerase, Cerezyme®) is manufactured and this extensive processing is also contributing to the high costs of the treatment.

An alternative production strategy is to express the corresponding gene in carrot cell suspension cultures and to target the protein to the storage vacuoles within the cells. Extensive investigations have shown that in carrot cells the GCD exhibits sugar chains with terminal mannose residues and that the purified protein is fully functional [18, 19]. The product (INN taliglucerase alfa) has passed a pivotal phase III clinical study and is awaiting approval.

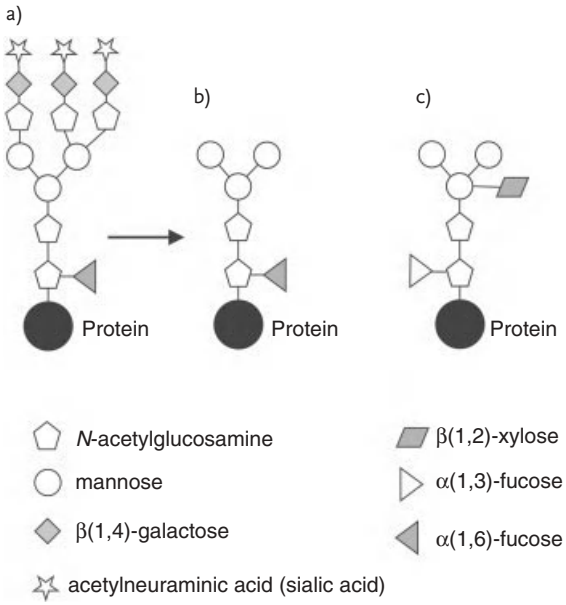

Figure 4.1 Structure of the predominant sugar residues of recombinant proteins. (a) shows the sugar structure of proteins (e.g., glucocerebrosidase) produced in CHO cells. Three enzymatic steps have to be performed to obtain the structure (b) which is necessary for glucocerebrosidase function. (c) shows the predominant sugar after expression in carrot cell suspension cultures.

In 2009 a viral contamination of a CHO cell production facility for imiglucerase caused an acute shortage in the supply of the vital therapy, threatening the therapy regime of a large number of Gaucher's disease patients worldwide [20, 21]. Owing to the fast authorization on a "compassionate use" basis for drugs which finalized phase III but had not gained approval so far, taliglucerase alfa was used in numerous patients and again proved that it is at least "biosimilar" to the traditional product. In consideration of the fact that the CHO cell-based product has to be extensively modified post-production and that the mammalian cell-based production system is prone to viral contamination, which could devastate the whole production line, the plant-based product could even be termed a "biobetter."

4.4 Insulin from Safflower—A Unique Purification Scheme

One common feature of all biopharmaceuticals is the need for extensive purification. After production, the proteins have to be separated from the complex matrix of the host cells and in the end should be virtually free from all contaminating by-products. This makes extensive down-stream processing techniques such as filtration and column chromatography necessary, which significantly contributes

to the cost of the final product. Therefore, any method that could circumvent or shorten downstream processing would be highly beneficial.

Special features of some host plants can be utilized as options for fast and easy downstream processing, an example being the production of insulin in the oilseed crop *Carthamus tinctorius* (safflower). Many crop plants produce and store in their seeds large amounts of oil in so-called oilbodies. These droplets are covered with a half-unit phospholipid membrane and an outer shell of specialized proteins, the so-called oleosins. With their hydrophobic portion these proteins are tightly associated with the triacylglycerol phase and even after extraction of the seeds the oleosins co-purify with the oil phase. If a recombinant protein is fused genetically to an oleosin portion it will allow co-purification from other components of the plant tissue by simple liquid–liquid phase separation. As a last step the recombinant protein is enzymatically cleaved from the oleosin portion to yield the pure protein. Several studies have shown that this procedure is suitable in large scale GMP conditions and yields proteins for clinical studies [22, 23].

Besides the obvious advantage of the unique purification scheme, the production of recombinant pharmaceutics in safflower has another advantage. As it is not a major food or feed plant it can be grown under open field conditions on a large scale without the concern of it entering the food chain. Hence, it poses the option of a production system that can be easily scaled up by simply increasing the acreage for growing plants, therefore reducing capital and operating costs. In the case of insulin in particular this might be a valuable alternative to classical fermenter-based technologies. Recently, safflower-based insulin termed SBS-1000 underwent phase I/II clinical studies and was shown to be bioequivalent to Humulin®R, a standard insulin in North America (http://www.sembiosys.com/pdf/fact_sheet_spring_2010.pdf).

4.5
Fast and Scalable Transient Tobacco-Based Expression Systems

The emergence of a new strain of influenza virus (H1N1) in 2009 and the subsequent pandemic have impressively demonstrated that in future there might occur the need to provide millions of vaccine doses within a relatively short timeframe. This sudden change in demand often poses problems in supply since most production systems for biologics are not easily scalable. Especially in the case of the commonly used seasonal influenza vaccines, the traditional method of using fertilized eggs for growing virus strains is intrinsically slow to adapt to changing quantities. With the recurrent influenza pandemics of the recent years and the growing demand of vaccines, large efforts have been made to develop alternatives to the virus-based split vaccines and to produce subunit vaccines in fermentable, cell-based systems [24]. Beside the traditional mammalian cell cultures for the production of the surface proteins hemagglutinin and neuraminidase of influenza viruses, also plant-based systems are under development. A boost in the development of plant-based influenza vaccine production systems came from a 2009

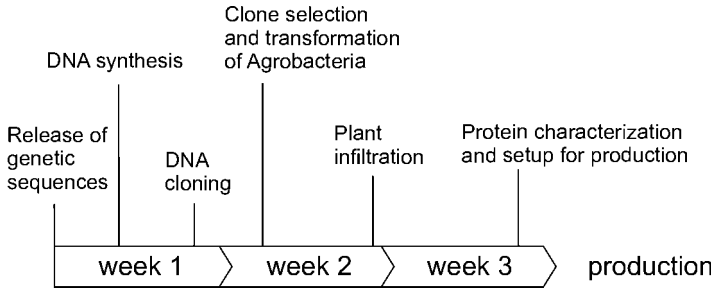

Figure 4.2 Timeframe for the establishment of a plant-based transient expression system for the production of recombinant therapeutics, as exemplified for an influenza H1-virus-like particle (VLP) experimental vaccine (modified after [25]).

DARPA initiative in the USA (Defense Advanced Research Program Agency). With the Blue Angel program, DARPA encourages the development of production facilities that are capable of delivering 100 million doses of vaccine grade recombinant protein per month as a response to threats like the influenza pandemic (http://www.darpa.mil/Our_Work/DSO/Programs/H1N1_Acceleration_(BLUE_ANGEL).aspx). Although the DARPA project also intends to develop countermeasures for potential terrorist attacks with biological weapons, this program impressively demonstrates the versatility of plant-based systems for the production of recombinant proteins for therapeutic as well as for prophylactic use.

Currently a number of facilities are under construction or already operational which can produce vaccine-grade proteins under GMP conditions in plants, using a transient expression system. The USA-based company Medicago, for example, has built a facility for the production of 120 million doses of vaccine per year based on influenza virus like particles (VLPs). Thereby the set-up for the production process can be done within three weeks (Figure 4.2). After the identification of the responsible virus strains and sequencing of the corresponding genes, the DNA sequence can be inserted into the corresponding expression system and plants already grown will be infiltrated. Within one week the protein expression in the plant is peaking and purification and formulation of VLPs can take place. With the example of the A/H1N1 influenza strain it was proven that from the release of the novel genetic sequence to obtaining purified VLPs from plants only 21 days were necessary [25].

As initially mentioned a transient expression system still has the disadvantage that manual inoculation of the plant with the vector (either viruses or Agrobacteria) needs to be performed. This obstacle has been overcome largely by highly automated procedures, which enable the fully automated growth of plants, the simultaneous infiltration of huge batches of plants in large vacuum chambers, and the automated harvesting and processing of the biomass [26]. Compared with conventional CHO-based production facilities, the costs for a plant facility including plant growth, automated infiltration, and down-stream processing are significantly lower. While costs for the former are estimated to be around US$150–200 million [27], the latter can be built for less than US$50 million.

Table 4.1 Facilities enabling the large-scale GMP production of pharmaceutical-grade proteins in plants, suitable for human clinical trials and application.

Institution/ company	Production platform	Target pharmaceuticals/ capacity	Location	
Kentucky Bioprocessing (KBP)	Transient expression in *Nicotiana benthamiana*	Various/n.d.[a)]	Kentucky, USA	http://www.kbpllc.com/
G-Con	Transient expression in *Nicotiana benthamiana*	Influenza vaccines/100 million doses per year	Texas, USA	http://www.gconbio.com/
Medicago	Transient expression in *Nicotiana benthamiana*	Influenza vaccine/120 million doses per year	North Carolina, USA	http://www.medicago.com/
Protalix	Expression in carrot cell culture, grown in disposable containers	Glucocerebrosidase/ n.d.[a)]	Israel	http://www.protalix.com/index.asp
ICON Genetics/ Bayer	Transient expression in *Nicotiana benthamiana*	Monoclonal antibodies for personalized therapy against non-Hodgkin lymphoma/n.d.[a)]	Germany	http://www.icongenetics.com/html/tech.htm
Sembiosys	Seeds from field-grown safflower	Human insulin/n.d.[a)]	Canada	http://www.sembiosys.com/

a) n.d.: not disclosed.

In addition to the production of influenza vaccines, such facilities can be utilized for the production of various pharmaceutical proteins and production processes can be easily adopted. For example, pharmaceutical grade aprotinin or a vast array of monoclonal antibodies can also be produced via automated transient expression systems [26].

In particular, monoclonal antibodies as therapeutics are of growing interest, as they represent the fastest growing sector of the biopharmaceutical market.

4.6 Conclusion

Although the number of new biopharmaceuticals has plateaued during recent years [1] there is still a high demand for production facilities and especially new production systems. Beside CHO cells and *E. coli*, the working horses for recombinant protein production, there is room and a need for the development of alternative production systems, especially as it comes to unique features of the system or the resulting product. The various plant-based expression platforms

might not only provide alternatives, but for some products they might be even the best-suited system. In the end there need to be a case-by-case evaluations for any given protein to see which expression system suits the needs best. Table 4.1 summarizes some of the most advanced facilities and their underlying technology, showing the worldwide effort in establishing production capacity. Hopefully, in the near future there will be the first approval of a plant-made biopharmaceutical to prove that plant expression systems keep their promises.

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5
Production of Biopharmaceuticals in Transgenic Animals

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5.1
Introduction

Farm animals and their products have long made valuable contributions to human nutrition, clothing, labor, research, development, and medicine, greatly improving human health and life expectancy. Transgenic technologies are further increasing the potential of farm animals to improve human health and many new areas are being explored [1]. Recent advances in genome sequencing and annotation, reproductive technologies such as nuclear transfer, and *in vitro* embryo production combined with new molecular genetic tools offer further progress. The production of pharmaceutical proteins in the mammary glands of transgenic farm animals is a prominent example of such opportunities.

Purification of human therapeutic proteins from blood or tissue extracts is an inefficient, expensive, labor- and time-consuming process that can also incur considerable risk of contamination with human pathogens. The production of human therapeutic proteins in genetically modified bacteria, yeast, or cultured mammalian cells has alleviated these problems and made numerous recombinant therapeutic proteins available for patients. However, all production systems have their limitations, and the problem with yeast and bacteria is their inability to synthesize complex human proteins carrying authentic post-translational modifications. Efforts are being made to humanize such non-mammalian systems, but current production in yeast and bacteria is restricted to "simple" proteins.

The main reason to express a particular protein in a mammalian system is because particular post-translational modifications are required for bioactivity, immunological or pharmokinetic properties. Glycosylation is perhaps the best known post-translational modification, but there are many other possible critical modifications, including propeptide cleavage, multi-chain assembly, disulfide bonding, phosphorylation, hydroxylation, amidation, methylation, hydroxylation, γ-carboxylation, acylation, and lipid attachment. The repertoire of enzymes that carry out these functions varies considerably between mammalian tissue types. Ideally, the protein processing capability of the producing cells should match the

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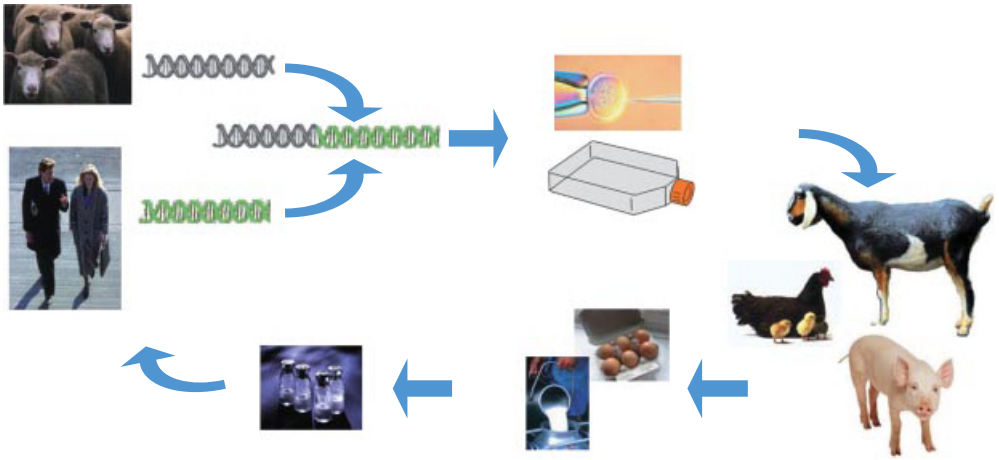

Figure 5.1 Schematic drawing of the gene pharming concept (for details see text).

requirements of the desired protein, or be readily modifiable to carry out the appropriate processing [2].

Mammalian cell culture is inherently more suitable than yeast or bacteria for the expression of complex human proteins. Cell culture has been tremendously successful in recent years, but has been restricted by limited manufacturing capacity and high marginal production costs that make scaling up production expensive. This is particularly relevant where large amounts of product are required, for example, therapeutic antibodies.

The main advantage of transgenic mammals over cultured cells is the ease with which large amounts of functional proteins can be produced and harvested. This is a consequence of the "low tech" nature of the basic production system – animals in a field rather than complex fermentation vessels, and also the biology of the producing cell. It is, for example, not unusual to obtain expression levels in milk tenfold greater than that obtained from CHO cells in culture. This multiple can be yet higher for complex proteins consisting of multiple subunits, such as fibrinogen or collagen. Farm animals such as cattle, sheep, goats, pigs, and even rabbits have the significant advantage over other production systems, that complex proteins can be produced at low running cost, with the flexibility of increasing production by simple breeding [1, 3]. These advantages led to the development of the "gene pharming" concept [4], which has now advanced to commercial application [1, 5, 6] (Figure 5.1).

Here we describe the state-of-the-art in the production of recombinant proteins in livestock, with an emphasis on the mammary gland. Then follows a brief introduction to the genetic constructs involved and an overview of current and future transgenic technologies applicable to farm animals. Finally, we briefly address quality and safety issues. Aspects particular to transgenic farm animals, such as the pros and cons of current and emerging transgenic technologies, are described

in greater detail than general features of protein purification and characterization, which are covered elsewhere in this book.

5.2 Sites of Production

Four potential sources of recombinant proteins have been studied in mammals, each of which is a body fluid: milk, urine, seminal fluid, and blood. Fluids are more suitable than solid tissues for this purpose because they are renewable and can be obtained from animals without harm or excessive invasion. Furthermore, many biomedically important proteins are themselves secreted into body fluids. Milk is by far the best-studied production system and the only method that has been examined on a large scale. Biopharmaceutical production in birds has focused exclusively on protein production in the white of eggs, specifically egg albumen.

5.2.1 Milk

The mammary gland is a promising site because of the ease of milk collection and the high quantities of protein that can be produced using specific gene promoters [5, 7]. GMP methods have been established for extraction and purification of proteins from milk

The lactating mammary gland has an enormous capacity to synthesize proteins and other biochemicals for infant nutrition. Milk is a complex and rich mixture of proteins, lipids, and carbohydrates. The dairy industry is well established and scientifically advanced, not only in cattle but also sheep and goats. The necessary equipment and expertise required for the collection, processing, and early stage purification of transgenic milk are therefore readily available.

Caseins are the major milk proteins, with five types in mice, four in sheep and cows, and two in humans. Caseins are hydrophobic and associate into spherical complexes that form a fatty suspension. The soluble, or whey, fraction of milk contains hydrophilic proteins that differ between mammalian species. Whey acidic protein (WAP) is the major whey protein in rodent milk, but is also present in pigs in low amounts; β-lactoglobulin (BLG) is the major whey protein in sheep, goats, and cows, both are absent from human milk. α-Lactalbumin has a role in lactose synthesis and is present in the whey of all milks that contain lactose.

In 1989 John Clark of the Roslin Institute, Edinburgh (UK), demonstrated that the BLG promoter could be used to direct the expression of the human blood clotting Factor IX gene in sheep and that the product was secreted during lactation [4]. Since then, promoters and regulatory sequences of almost all major milk genes have been utilized and investigated for their suitability in driving the expression of potentially useful proteins.

A large number and wide variety of foreign proteins have been expressed in the milk of transgenic animals. Expression levels as high as 35 g/l have been achieved

using the ovine BLG promoter to express human α-antitrypsin in sheep [8]. In each case, the foreign protein is secreted as part of the whey fraction. This work has included: complex multi-chain proteins, for example, fibrinogen; combinations of transgenes designed to supplement the natural protein processing abilities of the lactating mammary gland, for example, prolyl hydroxylase coexpressed with type 1 procollagen; and coexpression of transgenes to improve the stability of secreted protein in milk, for example, α1-antitrypsin protease inhibitor with fibrinogen.

The great majority of milk transgenes have been expressed in mice, mainly as pilot studies. Mouse trials provide important information on the feasibility of a large animal study and indicate whether any adverse effects to animal health can be expected, an important issue in the expression of highly bioactive recombinant proteins such as erythropoietin. It is difficult to provide a definitive list of proteins expressed in milk, because not all work has been made public. Those published, or otherwise known to the authors, are listed below:

- **Anti-microbial proteins:** Lysozyme, lactoferrin, tissue non-specific alkaline phosphatase, lysostaphin, antimicrobial peptides, for example, β-defensins.
- **Blood clotting and anti-clotting factors:** Antithrombin III, protein C, factor VII, factor VIII, factor IX, fibrinogen, tissue plasminogen activator, hementin, urokinase, thrombin-activated plasminogen.
- **Cell surface proteins expressed in soluble form:** CD4 (HIV receptor), transferrin receptor, cystic fibrosis transmembrane conductance regulator, intercellular adhesion molecule 1 (human rhinovirus receptor), pentraxins for example, serum amyloid P- and C-reactive protein.
- **Cytokines and growth factors:** Erythropoietin, Interleukin 2, Interleukin 10, thrombopoietin, insulin-like growth factor 1, nerve growth factor b, granulocyte colony stimulating factor, Interferonγ.

Guidelines developed by the US Food and Drug Administration (FDA) require monitoring of production animals' health in a designated pathogen-free (DPF) facility, sequence validation of the gene construct, characterization of the isolated recombinant protein, and monitoring of transgenic animals genetic stability. This has necessitated, for example, the use of animals from scrapie-free countries (New Zealand) and maintenance of production animals under strict hygienic conditions. Products derived from transgenic goat and rabbit milk have gained approval by regulatory bodies.

The first product from a transgenic farm animal to become a registered drug was antithrombin III (ATryn) from GTC-Biotherapeutics, USA, produced in transgenic goats. ATryn was approved for the treatment of heparin resistant patients undergoing cardiopulmonary bypass by the European Medicines Agency (EMEA) in 2006, and by the FDA in the United States in 2009. Subsequently, human recombinant C1 inhibitor (RUCONEST or RHUCIN) from Pharming BV, The Netherlands, produced in transgenic rabbit milk has been approved for treatment

Table 5.1 Examples of pharming companies (as of May 2011).

Company	Species	Products	Indication	Approved
GTC biotherapeutics	Goat	ATryn (antithrombin III)	Prevention of thromboembolic events	Yes
http://www.gtc-bio.com		Factor VII	Hemophilia	
		Factor VIII	Type A hemophilia	
		Factor IX	Type B hemophilia	
		Human α1-antitrypsin	Congenital AAT deficiency, cystic fibrosis, asthma	
		CD137 antibody	Improved immune response to tumors	
		CD20 antibody	Oncology and auto-immune indications	
Pharming http://www.pharming.com	Rabbit	C1 inhibitor	Hereditary angioedema, graft rejection	Yes
	Cow	Fibrinogen	Congenital deficiency	
		Collagen	Tissue repair	
BioProtein Technologies http://www.bioprotein.com	Rabbit	Lactoferrin	Nutritional applications	
Synageva BioPharma http://www.synageva.com	Bird eggs	Lysosomal acid lipase	Storage disease	

of acute angiooedema attacks in patients with hereditary angiooedema. The enzyme α-glucosidase (Pharming BV) from transgenic rabbit milk has orphan drug status and has been successfully used for the treatment of Pompe's disease. It is estimated that more than 12 transgenic milk derived recombinant proteins are currently in different phases of clinical testing. The overall global market for recombinant proteins from domestic animals is expected to reach $18.6 billion in 2013 [6]. Recombinant pharmaceutical proteins currently under commercial development are summarized in Table 5.1.

An interesting application of farm animal transgenesis is the production of recombinant proteins in the mammary gland of transgenic animals for use as antidotes for organophosphorus compounds used as insecticides in agriculture and chemical warfare. Butyrylcholinesterase is a potent prophylactic agent against these compounds, and recombinant butyrylcholinesterase has been produced at concentrations of 5 g/l in the mammary glands of transgenic mice and goats [9].

The recombinant enzyme was biologically active and sufficiently stable to be considered for use against organophosphorus intoxication. A small number of transgenic goats can produce sufficient butyrylcholinesterase to protect all humans at risk of organophosphorus poisoning.

Purification of the desired protein requires multiple steps, which can be costly. Protein purity is of paramount importance where the protein product is to be administered to patients on a long-term basis, especially intravenously, because even minute quantities of contaminating milk components could be immunogenic. Purification can be circumvented in those special applications where milk is to be ingested as a nutraceutical. For example, transgenic goats and cattle have been produced that secrete enhanced amounts of the anti-microbial protein lysozyme into their milk. The intention is to mimic human breast milk, which is also rich in lysozyme, to alter gut flora and combat gastrointestinal microbial infections [10, 11].

5.2.2 Urine

The mammary gland has proved to be unsuitable for the production of some highly bioactive proteins with cross-species activity, such as growth factors, cytokines or hormones, because these can enter the general circulation with detrimental effects on the physiology of the animal. In contrast, the contents of the bladder, being potentially noxious, are normally sequestered from the body.

A system to express foreign proteins in urine has been developed employing uroplakin genes [12]. Uroplakins are membrane-associated proteins expressed specifically in the differentiated uroepithelium of the bladder and urethra. The mouse uroplakin II gene promoter has been used to direct expression of human growth hormone (hGH) and also human granulocyte macrophage-colony stimulating factor (hG-CSF) in mice [12]. Production in the kidney has also been investigated using the gene promoter of Tamm Horsfall protein, also called uromodulin, which is expressed and secreted from the epithelium of the ascending loop of Henle [13].

It is not yet clear how appropriate urine is as a source of bioactive proteins. Although the body may not be exposed to the natural contents of the bladder, segregation of transgenic proteins secreted into this compartment still depends on the tissue specificity of the gene promoters. Ectopic transgene expression may lead to circulating proteins. Notably, both the uroplakin II hG-CSF mice and the Tamm Horsfall hGH mice showed evidence of transgene protein in peripheral blood.

The greatest problem with this method of production is, however, the low synthetic capacity of the bladder and kidney, which is far less than the mammary gland. Consequently, the yield of protein per milliliter is therefore very low, in the order of ng/ml. While this may be suitable for certain high value proteins, the practical usefulness of the system remains to be demonstrated. The low yields are only partially compensated by the large volume of urine obtained from animals

such as cattle. Unlike milk, urine is produced during the entire lifetime of the animal independent of age, sex, and pregnancy. Furthermore, because urine contains little protein and lipid, product purification should, in theory, be simpler than from milk. The stability of recombinant protein in urine is, however, a potential problem that remains to be fully explored. Work published so far has been restricted to mice, although there have been preliminary reports of transgenic pigs expressing hGH in urine.

5.2.3
Seminal Fluid

Porcine seminal fluid has been suggested as a suitable source for bioactive proteins [14]. The accessory male sex glands have a substantial protein synthetic capacity, semen is available in reasonably large volumes (200–300 ml per ejaculate) and, because protein secretion is strictly exocrine, bioactive proteins could be produced without adversely affecting the animal. This work is at an early stage and determination of its usefulness will require further investigation. This will include identifying appropriate genes and sequences to direct secretion into seminal fluid. Possible candidates are the spermadhesins, the major protein component in porcine semen. The protein processing capacity of the producing tissue, the stability of foreign proteins in semen, and ease of product purification are important considerations when choosing the production site.

5.2.4
Blood

The physiology and development of the animal are intimately exposed to any adverse effects of bioactive proteins circulating in the blood; therefore the range of suitable products is very restricted. Human hemoglobin for use in synthetic blood substitutes has been produced in pigs by the company DNX, of Princeton, New Jersey [15], but this was discontinued because of difficulties of purifying the human protein away from the very similar porcine protein.

Production of recombinant human proteins in the blood of transgenic livestock is likely to become more important as a source of human polyclonal antibodies. Progress is being made towards the production of humanized immune systems in cattle, pigs, and rabbits [16–18]. Such animals could be immunized against a wide range of antigens to provide an abundant source of human polyclonal antibodies, which could play an important role in passive immunotherapy and offer considerable advantages over monoclonal antibodies. For example, they are more effective than monoclonals in immune complex formation and better mimic the natural immune response; they can also disable pathogens that require neutralization of multiple epitopes, pathogens with diverse strains, and venoms with multiple toxic components. Importantly, polyclonal antibodies can only be produced in people or transgenic animals. Most applications would require large animals for production of adequate amounts of serum.

5.2.5
Bird Eggs

Chicken eggs have several advantages that make them attractive for the production of foreign proteins. The poultry industry is well developed and modern breeds of chickens are highly productive, laying about one egg per day. Egg collection is very simple and easily scaled up. Production is also very flexible, large flocks of birds can be rapidly produced from a single transgenic male. Furthermore, the use of eggs for pharmaceutical purposes is already established for the production of vaccines, providing a framework of regulatory guidelines for Good Manufacturing Practice (GMP).

Transgenic protein production in eggs was first demonstrated in 2003 [19]. Production of therapeutic proteins in eggs is, however, less advanced than production in milk, because avian transgenesis remains technically difficult and inefficient. No proteins are as yet in commercial production, but several companies are actively pursuing product development. Chickens may be more suitable than mammalian systems for certain proteins. For example, some bioactive proteins with toxic effects in mammals may not affect birds. There is also evidence that chickens and human proteins have similar glycosylation patterns, as discussed above; however current data are restricted to a few proteins and considerably more information will be required to assess the system properly. The secretory cells of the chicken oviduct certainly have a high protein synthetic capacity. Each egg contains approximately 4 g protein in the white, of which more than 54% is ovalbumin. Other major protein constituents are ovotransferrin (12%), ovomucoid (12%), and lysozyme (3.4%). This low protein complexity should simplify purification, while natural protease inhibitors present in albumin may also help stabilize foreign proteins.

Chicken ES-like cells transfected with an ovalbumin gene construct, containing 7.5–15 kb of the ovalbumin 5′ regulatory sequences that direct expression of human immunoglobulin heavy and light chains, have been used to generate somatic chimeric hens that secreted biologically active antibody into the egg white [20]. However, there was evidence of ectopic transgene expression. This was not observed in more recent experiments [21] that used lentiviral vectors containing only about 3 kb of regulatory sequences for the expression of an interferon or a mini-antibody for cancer treatment.

5.3
Transgenic Constructs

Transgenes containing sequences of milk protein genes have been successfully used to direct the expression of exogenous proteins to the lactating mammary gland. Usually, these transgenes are fusions of the target protein gene with mammary gland specific regulatory sequences (Figure 5.2). In addition to the regulatory and coding elements, transgenic constructs may harbor signal sequences

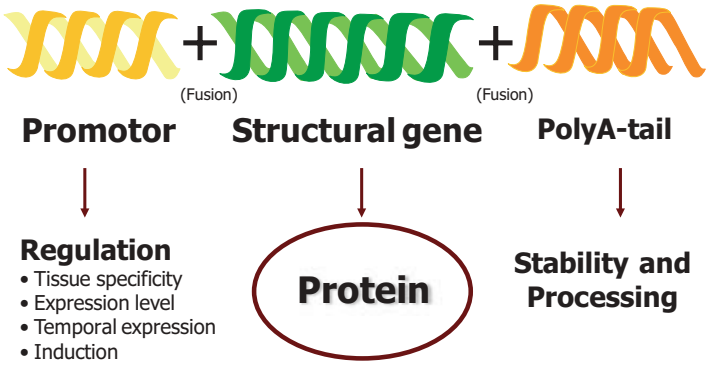

Figure 5.2 Schematic drawing of the main components of a gene construct (for details see text).

to facilitate secretion of the protein and untranslated exons and introns to enhance expression levels [22]. Ideally, the transgenes should stably integrate into the host genome, be inherited in Mendelian fashion, and direct the abundant expression and secretion of a desired protein to the target organ without affecting the health or well being of the producing animal.

The first requirement is a region of cloned DNA that encodes the amino acid sequence to be expressed (the structural gene). This may be a fragment of genomic DNA, cDNA, or chemically synthesized DNA. It may be necessary to alter the coding sequence to optimize expression in a transgenic animal. For example, if the protein is from an evolutionarily distant species, it may be necessary to alter codons to accord with those most frequently used in the host. Also, if the protein is not usually secreted, a signal peptide may be added at the *N*-terminal to direct secretion from the producing cell.

Transgenes based on genomic sequences are usually expressed more consistently and abundantly than those based on cDNA. However, many genes are too large to be conveniently inserted into plasmid cloning vectors by classical techniques, although the use of bacterial artificial chromosomes and new recombinant technologies may improve the situation. As a compromise, cDNA and genomic sequences have often been combined as a "minigene" carrying one or two introns, and 5′ or 3′ flanking regions thought to contain positive regulatory elements. However, mixing and matching in this way can sometimes lead to aberrant RNA splicing due to cryptic splice sites. Such effects are often difficult to predict. Despite considerable knowledge of gene regulation, there is no standardized method of combining disparate genetic elements that can guarantee successful transgene expression. Design of transgenic constructs is a matter of informed trial and error, frequently requiring test and refinement of successive constructs.

An important factor in the success of transgene expression is the location within the host genome. Transgenes integrated at different sites can exhibit wide

variations in expression, due to the influence of different chromatin environments. Heterochromatic regions tend to suppress expression of an adjacent transgene, while a transcriptionally active chromatin domain may support expression. The proximity of endogenous enhancers, promoters, silencers, and activation sequences may also influence the level and pattern of transgene expression. This "position effect" has been a long-standing source of inefficiency in the use of transgenic animals.

Several strategies are available to either minimize or circumvent the position effect. The level, specificity, and consistency of transgene expression can sometimes be improved by flanking the transgenic construct with insulator elements. Insulators of various types have been demonstrated to block the spread of heterochromatin effects into the transgene and to isolate transgenic promoters from the influence of adjacent endogenous enhancers and other regulatory elements. Alternatively, the uncertainties of random integration can be avoided by placing the transgenic construct in a well-characterized permissive site in the host genome using gene targeting. The Rosa26 locus in the mouse is one example of a commonly used transgene locus not subject to gene silencing. Placing recognition sites for either a recombinase, for example, bacteriophage P1 Cre recombinase, or an integrase, for example, phage ϕC31 integrase, at the locus allows transgene cassettes to be inserted at very high efficiency by so-called recombinase-mediated cassette exchange [23]. A human homolog to the mouse Rosa26 locus has been identified [24] and similar sites will probably be identified in livestock.

5.3.1 Organ Specific Expression Vectors

Production of a biopharmaceutical in a transgenic animal requires transcription and protein expression of the transgene to be directed to the site of production. This is commonly achieved using a gene promoter expressed specifically and preferably abundantly in the chosen tissue, such as the lactating mammary gland. The DNA sequences of most of milk proteins are known [25]. The bovine αs1-casein gene contains nine exons and spans 17.5 kb. The caprine β-casein gene has also been cloned and sequenced. The rodent WAP genes consist of four exons and three introns. The ovine β-lactoglobulin gene contains seven exons and six introns spanning a 4.2 kb region. The bovine α-lactalbumin contains four exons and three introns. The promoter elements from these genes have been used to direct expression into milk [25]. The ovalbumin gene promoter is used for production in the white of chicken eggs.

Some mammary gland specific gene constructs have failed to produce economically significant amounts of protein in the milk of transgenic animals indicating that the technology needs further refinement to ensure consistent high-level expression. This is particularly true for genes under very complex regulation, such as those coding for erythropoietin (EPO) or human clotting factor VIII (hFVIII) [26–28].

5.3.2
Inducible Expression

An inducible system can also be used to improve control over recombinant protein production. Transgenic mice and farm animals made with the first generation of inducible promoter elements showed expression in response to heavy metals or steroid hormones, but also high basal expression levels [29, 30]. The newer, binary expression systems based on prokaryotic control elements are responsive to exogenous IPTG, RU-486, ecdysone, or tetracycline derivatives and provide more tightly controlled expression. The first tetracycline system successfully used in mice required two DNA constructs. One gave doxycycline controlled expression of a transactivator and the other carried the target gene driven by regulatory elements that responded to transactivator [31–33]. These were typically integrated into two separate lines of transgenic mice. Crossbreeding the two lines resulted in offspring that expressed the target gene only after stimulation with doxycycline [31]. Unfortunately, the long generation intervals make this approach less practical for livestock species.

The first tetracycline controlled transgene expression in pigs was based on a bicistronic tetracycline-responsive expression cassette (NTA) in which expression was amplified by transactivator mediated positive feedback [34]. The auto-regulatory cassette was integrated at a single chromosomal site in the porcine genome after pronuclear DNA injection and was designed to give ubiquitous strong expression of a human regulator of complement activation (RCA) independent of cellular transcription cofactors. Expression from this construct could be inhibited reversibly by feeding the animals doxycycline (tet-off system).

5.3.3
Non-integrating Vectors

The position effect can be avoided by using vectors that do not integrate into the host genome, but nevertheless efficiently express the transgene. To achieve this, the foreign DNA must replicate autonomously and be stably maintained after multiple cell divisions. Artificial chromosome vectors that carry DNA regions responsible for stable chromosome behavior are promising. The major components are: a centromere that mediates chromosome segregation during mitosis, and two telomeres that stabilize the ends of the DNA molecule. Artificial chromosome vectors have the advantage that they can carry very large pieces of DNA; however, they are still in early stage development. There has been one report of "transchromosomic" cattle, designed to express human immunoglobulins in blood [16], but no evidence of transmission through the germ line.

A second form of non-integrating, or episomal, vector has been developed that contains an attachment site for the system of structural proteins within the nucleus, termed the nuclear matrix or scaffold (MAR or SAR) [35]. Such vectors are thought to achieve mitotic stability by attaching to chromosomes. Episomal vectors have not been widely used for producing transgenic livestock, although

pigs expressing a fluorescent reporter gene from an episomal vector have been reported [36].

5.4 Methods for the Production of Transgenic Animals

Mammalian oocytes and early embryos are tiny, self-contained, free-floating structures that can be obtained by flushing the female reproductive tract with fluid, cultured, and then reintroduced into recipient females to continue development up to viable offspring. This accessibility has facilitated the development of a variety of micromanipulation, culture, and embryo transfer procedures that support the production of transgenic mammals. Early stage avian embryos are far less amenable to such manipulation, being attached to a large fragile yolk and, once explanted, cannot easily be transferred back into the oviduct. Techniques for avian transgenesis have therefore taken much longer to establish.

In this section, we provide an overview of various methods of producing transgenic mammals and birds, including those in current use and emerging technologies that will significantly improve efficiency and precision of the genetic modification. The methods divide into two broad categories: DNA transfer directly into embryos and cell-mediated transgenesis.

DNA transfer into embryos, for example, by DNA microinjection or viral transduction, is more straightforward than cell-mediated transgenesis. Until recently, this approach was limited to transgene addition and allowed no control over where the transgene integrates into the genome, but emerging technologies based on highly specific DNA endonucleases (see Section 5.4.8) have now overcome this limitation. Notably, analysis of the integrated transgene must be carried out in resultant animals. Thus, the efficiency of the technique is of major importance. If only a small proportion of the embryos carries a transgene, the costs and timescales involved in transgenic livestock production are a major drawback. This motivated the development of alternate methods for improving the efficiency and reducing the cost of generating transgenic livestock.

The various methods of cell-mediated transgenesis each have in common the feature that genetic manipulation and analysis of the transgenic genotype are carried out in cells in the laboratory, rather than in animals "on the farm." These cells are then used to produce the modified genotype in whole animals. While cell-mediated transgenesis is more labor intensive than direct transgenesis, *in vitro* genetic manipulation of cells followed by detailed genome analysis offers significant advantages. Firstly, it reduces the total number of animals required to generate a useful transgenic offspring. Secondly, it increases dramatically the number of independent transgene integration events that can be screened and investigated. Thirdly, it facilitates the engineering of precisely controlled genetic alterations (gene targeting) by allowing selection and isolation of rare integration events resulting from homologous recombination. Here each method is reviewed briefly.

5.4.1 Pronuclear DNA Microinjection

DNA microinjection into fertilized oocytes was developed originally in mice and extended to transgenic livestock more than 20 years ago [37, 38]. It is technically straightforward but inefficient. Nevertheless, DNA microinjection has been used to produce a broad range of genetically modified large animals for applications in both agriculture and biomedicine. A wide variety of transgenes have been microinjected, including DNA fragments as large as 0.5 Mb and combinations of up to four different transgenes. Transgenic livestock for biopharmaceutical production are already commercialized [6, 39].

The procedure entails a number of steps as summarized below.

5.4.1.1 Collection of Fertilized Eggs

Oocytes fertilized *in vivo* can be collected from animals after spontaneous ovulation or hormonal treatment to induce a superovulatory response by means of laparoscopy or surgery under general anesthesia. Alternatively, oocytes can be withdrawn from ovarian follicles collected from commercial slaughterhouses and embryos are produced by *in vitro* maturation of oocytes and fertilization *in vitro* by co-incubation with motile sperm (IVM/IVF), followed by a variable period of *in vitro* culture. The entire process is termed *in vitro* production (IVP). IVP procedures are well established for cattle and have recently been developed for pigs [40–42]. For DNA microinjection, well defined developmental stages are required which harbor the maternal and paternal pronucleus, called zygotes. These stages are found for a short period of time (18–20 h) approximately 18–26 h after fertilization, depending on the species.

5.4.1.2 Preparation of DNA

A recombinant DNA construct used to produce transgenic animals will generally have two major components, the transgene portion and the bacterial plasmid "backbone," which comprises sequences required for selection and DNA replication in bacteria, usually *Escherichia coli*. Preparation of a DNA construct for microinjection entails purifying linear fragments of the transgene portion away from the plasmid DNA backbone, residual bacterial material, and chemical reagents.

Purified linear fragments are dissolved in microinjection buffer at a concentration between 1–6 ng/μl. Within this range, lower concentrations result in higher embryo viability but a lower proportion of transgenic embryos, higher concentrations produce more transgenic embryos but viability may be reduced.

5.4.1.3 Injection of DNA

Morphologically normal fertilized oocytes with visible pronuclei are selected for microinjection. The next developmental step is fusion of the two pronuclei with the recombination of the two parental genomes. This increases the chances that a foreign DNA is readily integrated into the host genome. In pigs and cattle, fertilized oocytes are opaque due to abundant lipid vesicles. Pronuclei can be

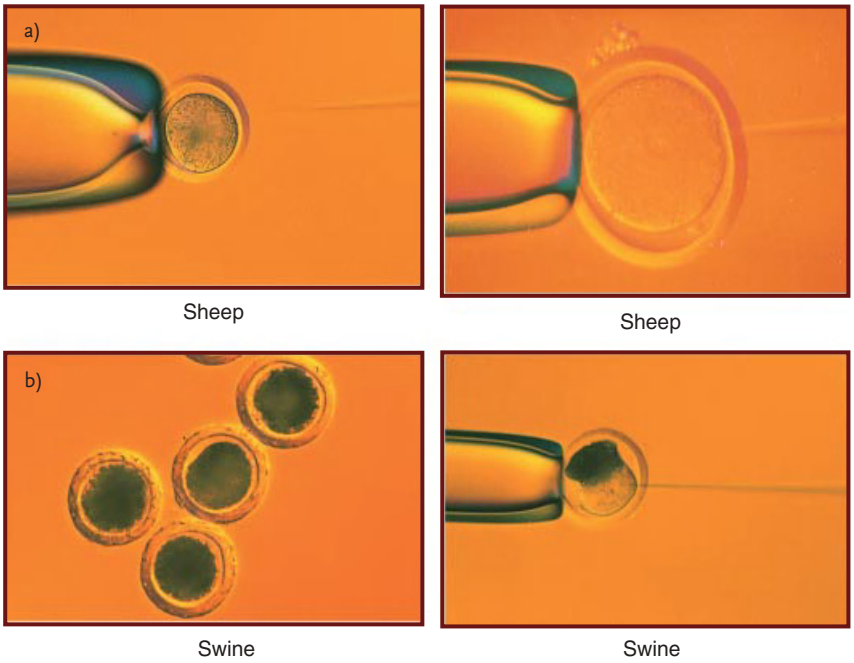

Figure 5.3 DNA microinjection into pronuclei of zygotes from sheep (a) and pigs (b). Ovine pronuclei are visualized by DIC; porcine zygotes have to be centrifuged to visualize the pronuclei.

visualized using gentle centrifugation to shift the lipids to one pole (Figure 5.3). In sheep, interference phase contrast microscopy is required to visualize the pronuclei (Figure 5.3). Microinjection is carried out on the stage of an inverted microscope at ~200× magnification, with the fertilized oocytes contained within microdrops into which are inserted a blunt-ended, glass holding pipette and a sharp, glass microinjection needle containing the DNA solution. Movements of the pipettes are controlled by micromanipulation arms. Individual zygotes are held in place on the holding pipette by gentle suction, and oriented such that one of the pronuclei is adjacent to the needle. The needle is inserted into the pronucleus and the DNA solution injected. Injected zygotes can then be either immediately transferred to recipients, or cultured *in vitro*, sometimes as far as blastocyst stage (cattle), to reveal those that would sustain further development.

5.4.1.4 **Transfer and Gestation in Recipients**

Microinjected fertilized oocytes are either immediately transferred to suitable recipients or cultured *in vitro* for a certain period of time. Viable embryos are selected and inserted into the oviducts or uterus of hormonally synchronized recipients depending on the developmental stage of the early embryos to maintain gestation. Established species specific reproductive biotechnological protocols are

available to prepare donor and recipient animals [43]. Recipient animals must be in an identical stage of the estrous cycle with the early embryos to obtain the highest possible pregnancy rate. Transgenic animals that are produced from DNA injected embryos are referred to as "founders," because they may be used to found genetically modified lines by conventional breeding.

5.4.1.5 Identification of Founders and Subsequent Breeding

Microinjection is an inefficient process. On average, between 5–20% of mice and 1–5% of large animals born after microinjection carry the transgene, and an even smaller proportion of these express the transgenic product. In mice, this inefficiency is not a major problem because it is straightforward to inject and transfer large numbers of embryos. However, this is impossible in livestock because gestation times are longer and maintenance costs far higher. This is, therefore, a strong incentive to reduce the number of animals gestating non-transgenic fetuses. One approach has been to screen embryos for the presence of a transgene prior to transfer. This can be achieved by extracting a portion of the embryo, often a single blastomere, and detecting the transgene by polymerase chain reaction (PCR) amplification. This procedure is however labor-intensive, can reduce embryo viability, and the presence of non-integrated DNA may result in false positives. Alternatively, a gene encoding a non-toxic fluorescent protein can be either co-injected, or incorporated into the transgene construct to identify intact living transgenic embryos. Expression of a non-integrated reporter construct may however produce a false positive signal, and the presence of additional DNA may be undesirable.

Transgenic fetuses can be identified *in utero* by analysis of cells obtained by amniocentesis or allantocentesis. However, these procedures carry a significant risk of inducing abortion. Some efforts have also been made to detect and analyze fetal cells or DNA in the maternal circulation, with limited success. The most common practice is therefore to screen animals shortly after birth by either PCR or Southern hybridization, using small samples taken from the blood, tail or ear tips. Transgenic animals are then analyzed in more detail to identify those most suitable for further breeding.

In the case of biopharmaceutical production, the amount and properties of the recombinant protein will be determined, and an analysis conducted to identify whether the physiology of the animal is affected in any way. It should be emphasized that the health of transgenic animals is of foremost importance for both ethical and practical reasons. Animals must be healthy to breed normally and produce sufficient quantities of milk. Preliminary data on these parameters may be gained from mouse experiments prior to starting work on transgenic livestock. However, prediction across fairly distantly related species is frequently not possible. Furthermore, the position effect means that each founder is potentially different and must be analyzed independently.

Obtaining milk expression data requires that female animals must be produced, reach sexual maturity, breed, and lactate. This is necessarily time-consuming in livestock, especially if the founder is male. Protocols for artificial hormone-induced

lactation in virgin females and even males have therefore been developed. This can accelerate the process of identifying the most suitable founders, but the quantity of milk obtained is often very low, and expression data may differ from natural lactation.

Subsequent breeding from founder animals is carried out by conventional animal husbandry. However, it is not uncommon for founders produced by microinjection to be mosaic for the presence of the transgene. This is thought to be a consequence of delayed DNA integration. Mosaicism in the germ line reduces the frequency of transgene inheritance in the first generation. Mosaic components of a founder may possibly contain independent and different integrations of the transgene that segregate in the first generation. Transgene segregation due to founder mosaicism does not indicate transgene instability.

In summary, DNA microinjection is a straightforward method of producing transgenics, and was the dominant technology for more than two decades. The limitations of random transgene addition and the inefficient use of animals are major drawbacks. Significant improvements are now being made by combining microinjection with highly specific DNA endonucleases (see Section 5.4.8), and also integration systems such as ϕC31 [44, 45].

5.4.2
Viral Mediated Gene Transfer

In 1976, Rudolf Jaenisch reported that mouse embryos could be infected with a retrovirus that stably integrated into their genome [46]. These animals incorporated the proviral DNA into their somatic tissues and germ line and passed it on to their progeny in Mendelian fashion. Since then a number of viruses have been used to produce transgenic animals with varying degrees of success. By far the most commonly used are retroviruses. These are a diverse group of viruses that share a basic common structure and replicative strategy. Each viral particle is approximately 100 nm in diameter and consists of a protein capsid containing a genome of two single-stranded 8–11 kb RNA molecules, surrounded by a lipid envelope through which glycoprotein spikes protrude.

Retroviruses infect susceptible cells very efficiently and integrate as a single copy into the host genome. This has led to the development of retroviral vectors capable of introducing foreign genes into cells, termed viral transduction. These vectors generally retain the outer parts of the viral genome, termed long terminal repeats (LTRs) necessary for the production of genomic RNA, but lack viral genes and carry other disabling mutations. Retroviral vectors are therefore "replication incompetent," being unable to produce viral particles on their own. The transfected vector generates genomic RNA transcripts within the packaging cell. These associate with the other viral proteins, become incorporated into infectious virus particles, and shed into the culture medium.

Retrovirus-mediated gene transfer has been the principle means of producing transgenic chickens, and has also been used to produce transgenic quail. Infection is most commonly carried out on embryos within freshly laid eggs. At this stage

the chick embryo comprises a multi-layered plate, or blastoderm, of about 60 000 cells overlying the large yolk. The shell of the egg is removed and retroviral particles are injected into the subgerminal cavity, which lies beneath the embryo. The shell is then resealed and the embryo allowed to incubate, sometimes being transferred to a surrogate shell after two or three days incubation. Because infection takes place at a relatively late stage of development, founder birds are mosaic for retroviral integrations and must be bred on to segregate different proviruses. Early work used replication-defective vectors based on oncoretroviruses such as avian leucosis virus, or reticuloendotheliosis virus.

The production of pharmaceutical proteins in bird eggs was first demonstrated using an avian leukosis virus vector to transduce a non-specific promoter directing the expression of interferon α-2b [19]. This resulted in extremely low frequencies of germ line integration and transgenes were frequently silenced over generations. More recent work using lentiviral vectors based on equine infectious anemia virus has been more successful, and pharmaceutically relevant quantities of proteins useful for cancer treatment have now been expressed specifically in the egg white, with no evidence of ectopic expression or transgene silencing [21].

Mammalian embryos may be infected either by removing the *Zona pellucida* and culturing in culture medium containing the virus, or a small volume of viral supernatant may be microinjected into the perivitelline space. The virus vector can infect and incorporate into the host genome, but is incapable of further replication. Procedures for embryo collection and transfer are basically those described for DNA microinjection.

Until recently, most retroviral vectors were based on the oncoretrovirus Moloney murine leukemia virus. Transgenic mice, pigs, and cattle have been produced using these vectors, but rates of transgenesis have been low and transgenes are almost always silenced by epigenetic modification during development.

In 1996 scientists at the Salk Institute developed vectors based on lentiviruses: a subclass of retroviruses that includes the sheep maedi-visna virus, equine infectious anemia virus, and the bovine, feline, simian, and human immunodeficiency viruses. Lentiviruses offer two significant advantages over oncoretroviruses. Firstly, they are less frequently subject to epigenetic inactivation by DNA methylation in transgenic animals. This is probably because, unlike oncoretroviruses, they are usually transmitted horizontally between individuals rather than vertically through the germ line. Host animals have therefore not adapted mechanisms to silence expression. Secondly, unlike oncoretroviruses they do not require breakdown of the nuclear envelope to gain access to the host genome. Lentiviral integration can therefore occur at all stages from the unfertilized oocyte onwards, in both replicating and non-replicating cells. Lentiviruses have been used successfully in a number of mammalian species including mice, rat, pig, and cattle [47]. High rates of transgenesis can be obtained using lentiviral vectors, up to 70% live born pigs carrying a fluorescent protein transgene, of which 94% expressed the transgene [48] or 31% zygotes injected with virus developing to transgenic animals, of which 95% expressed the transgene [49].

However, on the downside, multiple independent integrations and delayed retroviral integration into different embryonic blastomeres are both fairly common, and lead to a relatively high incidence of mosaic founders. This can extend the time required to establish stable transgenic lines: firstly, because mosaicism in the germ line reduces the frequency of transgene inheritance, and secondly because several generations may be required to segregate independent transgene loci.

Two features of retroviral transduction may, however, make it difficult to achieve high levels of expression. The size of the transduced gene is limited to a maximum of approximately 8 kb by the capacity of the retroviral particle. This effectively restricts their use to cDNA and small minigene constructs, and severely limits the amount of regulatory and enhancer elements that can be included. Retroviral vectors usually result in single or low copy transgenes, but high transgene copy numbers tend to result in more abundant expression.

Random integration of proviral DNA also carries the risk that genes adjacent to the integration site may be inappropriately activated, or that integration disrupts an endogenous gene with possible deleterious effects. Retroviruses have, in fact, been successfully used as experimental insertional mutagens. There is also the possibility that, despite extensive precautions taken to disable the retroviral vector, an integrated proviral transgene could somehow recombine with endogenous retroviral elements resulting in a replication-competent virus. While such events are extremely uncommon, they cannot be absolutely excluded.

Lentiviral transduction may allow more efficient production of transgenic animals. However, its usefulness for biopharmaceutical production will critically depend on the levels of expression achieved, a deeper understanding of any possible risks and public acceptance of viral-based vectors.

5.4.3
Sperm-Mediated Gene Transfer

Since 1971, it has been known that rabbit spermatozoa can associate *in vitro* with exogenous DNA and transfer it to an oocyte by fertilization. Subsequently the same has been shown for sperm cells of other species [50]. In 1989 it was reported that mouse spermatozoa exposed to exogenous DNA could be used as a vector to produce transgenic mice by artificial insemination [51]. This stimulated considerable interest at the time because the technology seemed to offer a simple approach to the production of transgenic animals. However, the method has suffered considerable problems of reproducibility and, while there have been reports of transgenic calves and pigs, transgenes introduced in this way frequently undergo rearrangement. This is consistent with findings that DNA that penetrates sperm nuclei becomes fragmented. There have also been reports that DNA introduced directly into the epididymis is transferred to offspring by natural ejaculation and fertilization [52]. The reproducibility and usefulness of this method have yet to be confirmed.

A variation of this technique is based on intracytoplasmic injection (ICSI) of DNA associated with frozen–thawed, or detergent-treated sperm. Several groups

have used ICSI to produce transgenic mice, rats, and pigs [53]. In mice it offers a lower incidence of founder mosaicism and a greater efficiency of transgenesis than standard DNA microinjection, particularly for large transgenes. In pigs, however, the efficiency of transgenesis and number of live born piglets obtained has so far been very low. One problem is failure of the injected sperm head to properly decondense and form a male pronucleus.

Sperm-mediated gene transfer by natural fertilization is not yet a reliable method of producing transgenic animals. Variations based on ICSI may offer greater success, but their potential in large animal transgenesis has yet to be fully explored.

5.4.4
Transposon-Mediated Gene Transfer

Transposons have been developed as important tools for transgenesis in flies, fish, frogs, mice, rats, and pigs [54–59]. DNA-based, or class II, transposons are mobile genetic elements that move around the host genome via a "cut-and-paste" mechanism. Most DNA transposons are simply organized; they encode a transposase protein flanked by inverted terminal repeats (ITRs), which carry transposase binding sites. It has been possible to separate the transposase coding sequence from the ITR sequences. Any DNA flanked by the ITRs will be recognized by the transposase and become enzymatically integrated into nuclear DNA. Apparently, no cellular cofactors are required. The size of the integrated foreign DNA can exceed 10 kb, but may reduce the efficiency of transgenesis. In a two-component system, the transposon is integrated solely by the *trans*-supplementation activity of the transposase.

Transposons have been successfully used in invertebrates, including *Caenorhabditis elegans* and *Drosophila*, for transgenesis and insertional mutagenesis. The first transposon sufficiently active for use in vertebrates was the Sleeping Beauty (SB) transposon [60]. Since then, several other transposons have been found to function in higher eukaryotes, including piggyBAC, Frog Prince, Tol2, and Passport. Transposition-mediated gene delivery offers efficient chromosomal integration and a high proportion of single-copy insertion events. However, the main advantage of transposon-mediated transgenesis is that the integration of foreign DNA is directed to accessible euchromatic rather than heterochromatic regions, reducing transgene silencing.

Injection of *in vitro* synthesized mRNA coding for transposase can enhance the efficiency of this technique due to the rapid availability of the transposase and circumvents the danger of integrated transposase sequences. This method has been used to produce: transgenic zebrafish, medaka fish, and *Xenopus* (transposons Tol2 and SB); transgenic chickens (transposon Tol2); and transgenic mice (transposons SB and piggyBac) and rats [59]. Transposon-mediated transgenesis for farm animal species is as yet at an early stage, but transgenic pigs produced using Sleeping Beauty and piggyBAC transposon systems have been reported [57, 58].

5.4.5 Pluripotent Stem Cells

5.4.5.1 Embryonic Stem Cells

Embryonic stem (ES) cells are pluripotent cells derived from early mammalian embryos, first isolated from certain inbred mice strains more than three decades ago [61, 62]. ES cells have three defining functional characteristics. Firstly, they can proliferate undifferentiated for indefinite periods *in vitro*. Secondly, they can differentiate *in vitro* or as tumors *in vivo* into cells of the three embryonic germ lineages: ectoderm, mesoderm, and endoderm. Thirdly, when reintroduced into a host embryo they can participate in development and contribute to all tissues of an animal, including gametes.

ES cells made it possible to engineer genetic modifications in culture and then study the effects in whole animals. For more than two decades ES cells have been powerful tools for the experimental manipulation of the mammalian genome. This work has, however, been almost exclusively restricted to mice, and only certain inbred strains of mice have an ES genotype that is transmitted through the germ line. Only relatively recently have germ line competent ES cells been reported for the laboratory rat [63, 64]. Efforts have been made to derive ES lines from other mammals and there are reports of ES-like cells in hamster, mink, sheep, cattle, pig, monkey, rabbit, and human, but none so far have been germ line competent [65]. Human ES cells remain untestable in this respect for obvious ethical reasons. Interestingly there is evidence that most human "ES" lines may in fact be epiblast stem cells [66]. ES-like cells have also been derived from chickens, but lack germ line capacity. Because of their potential advantages, work is ongoing to derive true ES cells from livestock species.

DNA can be introduced into cultured mammalian cells by a wide range of chemical, electrical, mechanical, and viral methods, often collectively referred to as "transfection." In ES cells, transfection can be used to randomly add DNA sequences to the genome, but the most potent application has been the precise modification of genes *in situ*, termed gene targeting. Gene targeting exploits the ability of cells to support recombination between exogenous DNA molecules and their cognate chromosomal sequences at regions of shared homology. Typically, ES cells are transfected with a DNA construct carrying an engineered modification flanked by 2–15 kb "arms" homologous to the target locus. At a certain, usually low, frequency transfected cells undergo homologous recombination with the construct and seamlessly incorporate the engineered modification at the target locus. Targeted cell clones can be selected and identified amongst a background of random integrants by a variety of methods.

Isolated targeted ES cell clones can be characterized and then used to produce animals. Gene targeting in mice has been used to inactivate endogenous genes by insertion or deletion, replace whole genes, precisely place transgenes in the host genome, introduce subtle gene modifications, and to delete megabase-size DNA fragments rendering large regions hemizygous. There are many variations and

refinements of the technique, and these continue to produce a wealth of information about many aspects of mammalian biology.

Several possible methods of generating mice from ES cells are available. The most common exploits the ability of an early embryo to incorporate exogenous ES cells. A small number of ES cells may be aggregated with a pre-morula stage embryo by co-culture in a microwell, or microinjected into the cavity of a blastocyst. Embryos containing ES cells are then transferred to the reproductive tract of synchronized recipients to complete fetal development. Animals produced in this way are chimeric, composed of a patchwork of ES and host-derived cells. In mice, chimerism can be readily visualized by marking the ES cells and host embryos with different coat color genotypes. Chimeras are bred to derive offspring from ES-derived germ cells. Mice derived entirely from ES cells can also be produced by aggregating ES cells with an unviable tetraploid host embryo, generated by prior electrofusion of a two-cell stage embryo. This method is more successful with either early passage, or F1 hybrid ES lines. ES cells have also been used to produce whole animals by nuclear transfer, as described below.

Chicken ES-like cells derived from blastoderm stage embryos have been cultured and transfected in a similar way to mouse ES cells. When injected into the subgerminal cavity of freshly laid eggs, some cells incorporate into the embryo and contribute to embryonic development. Transfected chicken ES-like cells have been used to generate chimeric hens with a substantial ES contribution, including the egg white producing cells of the oviduct [21], but germ line transmission has not yet been demonstrated.

Possible new routes towards the production of animals using ES cells have opened with reports that gametes can be produced from ES cells *in vitro*. Oocytes and follicular structures have been reported from differentiating mouse ES cells, but it remains unknown whether they can be fertilized [67]. Mouse ES cells can form sperm capable of producing viable offspring by ICSI [68], but the efficiency is still low and the offspring derived had a reduced lifespan, possibly indicating an imprinting problem.

In summary, the importance and usefulness of ES cells are widely acknowledged and considerable research efforts are underway to further understand and modify the genetic make-up of ES cells. This rapidly increasing body of knowledge will likely enable practical ES technology to extend to livestock in the future.

5.4.5.2 **Embryonic Germ Cells**

Primordial germ cells (PGCs) are the undifferentiated embryonic precursors of germ cells and arise outside the embryo and migrate through the body during mid-stage development to the urogenital ridges where the gonads develop. Embryonic germ cells (EGCs) were first derived from explanted mouse PGCs and shown to possess all pluripotent characteristics of murine ES cells, including the ability to re-enter the germ line [69–71]. Murine PGCs become EGCs in culture when the medium is supplemented with specific growth factors such as stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF)

[69, 72, 73]. EGCs resemble ES cells, in that they can be cultured and transfected *in vitro* and then contribute to somatic and germ cells of a chimera.

Presumptive EGCs have been isolated from humans, pigs, goats, rabbits, and chicken [65]. Reintroduction of livestock EG cells into embryos in the same way as ES cells has however not been successful. This has restricted their practical usefulness and mammalian EG cells are now rarely used.

Avian EG cells have been more successful, and have produced transgenic chickens on several occasions [74]. The typical technique has been to isolate primordial germ cells from the blood of chick fetuses two to three days after laying and convert them into EG cells. Cultured EG cells can be reintroduced into the blood stream of fetuses at the same stage, where they migrate to the gonads and form functional gametes. The efficiency of chimera production and incidence of germ line transmission is increased if recipient eggs are chemically pre-treated to deplete endogenous primordial germ cells.

5.4.5.3 **Induced Pluripotent Stem Cells (iPS Cells)**

The production of induced pluripotent stem (iPS) cells from mouse [75] and human somatic cells was a revolutionary breakthrough [76, 77]. Fully differentiated fibroblasts both from mouse and human could be reprogrammed into pluripotent state by ectopic expression of four transcription factors such as *OCT4, SOX2, c-MYC*, and *KLF4* [75, 76] or by *OCT4, SOX2, NANOG*, and *LIN28* [77]. These reports sparked worldwide intensive research on iPS cells and today such cells have been generated from various mouse and human cell types including liver cells, neural stem cells, blood cells, cord blood cells, pancreatic beta cells, mesenchymal stem cells, and stomach cells [65].

Most iPS cell lines established today have been produced using retroviral or lentiviral vectors, which integrate in the host genome [65]. This increases the risk of insertional mutagenesis and is usually associated with a high number of genomic integrations. In addition, the use of oncogenes such as *c-MYC*, *OCT4*, and *KLF4* renders iPS cells unsuitable for clinical application and production of transgenic animals. Apparently, alternative approaches have been explored, by which the virus vectors are substituted and thereby integration of viral sequences in the host genome is avoided. This has been achieved using small molecules, non-integrating adenoviral vectors, proteins, plasmids, mRNA, miRNA, or transposon based reprogramming systems [65].

iPS cells are largely identical with ES cells derived from embryos with regard to morphology, unlimited proliferation, and their differentiation potential [75, 76, 78]. Pluripotency was defined by their ability to produce germ cells [76, 79], to form teratomas after injection into nude mice, and to generate viable fertile mice through tetraploid complementation [80, 81]. However, recent analysis has revealed that substantial differences exist between iPS cells, nuclear-transfer derived ES cells, and embryonic stem cells isolated from mouse blastocysts with regard to the epigenetic DNA methylation profile [82]. The iPS cells were functionally indistinguishable from ES cells derived from *in vitro* fertilized embryos, but had retained epigenetic marks characteristic of their tissue of origin (epigenetic memory).

Efficient production of iPS cells has so far been described in mouse, human, monkey, rats, and dogs [65]. Porcine putative iPS cells have been produced from fetal fibroblasts, ear fibroblasts or primary bone marrow, or porcine MSCs, by lentiviral transduction with the four human factors (*OCT4, SOX2, KLF4*, and *c-MYC*) or using six human factors (*OCT4, SOX2, c-MYC, KLF4, NANOG* and *LIN28*) [65]. These iPS cells share characteristics of pluripotent cells, including morphology, rapid rate of proliferation, maintenance of a diploid karyotype, high level of telomerase activity, and capacity to differentiate into all three germ layers *in vitro*. Porcine iPS cells expressed the essential pluripotent transcriptional factors such as *OCT4, NANOG, REX1, SOX2*, could differentiate into cells representative of the three germ lineages, and some even formed teratomas. However, porcine iPS cells reported to date do not maintain a self-sustaining undifferentiated state and require expression of exogeneous factors. Germ line competence has also not been reported, indicating that these cells are not truly pluripotent [65]. Recently, one study reported evidence of germ line chimerism, but was based solely on polymerase chain reaction (PCR) results [83]. However, recent genomic and epigenomic analyzes of iPS cells revealed chromosomal aberrations, such as copy number variation and DNA methylation errors at the single-cell level. This warrants further studies into the safety of iPS cells [84].

A reliable iPS cell technique in pig and other farm animals will provide new options to derive pluripotent cells in those species where derivation of ES cells from re-implantation of embryos has been unsuccessful. The production of definitive porcine iPS cells will be extremely useful for studies on stem cell based therapies and the production of genetically modified animals.

5.4.6
Spermatogonial Stem Cells

Spermatogonial stem cells (SSCs) are precursors of male gametes, which reside in the seminiferous tubules of the postnatal testis [85]. The SSC population is small, ~35 000 stem cells per mouse testis, comprising 0.03% of all germ cells in testis. Mouse SSCs can be derived from adult and neonatal testis and proliferate for extended periods *in vitro* in the presence of growth factors such as glial cell line-derived neurotrophic factor (GDNF), LIF, bFGF, and epidermal growth factor (EGF) [86–88]. SSCs have been successfully used for insertional mutagenesis in mice [89]. *In vivo*, spermatogonial stem cells are unipotent, giving rise only to spermatozoa. After transplantation into seminiferous tubules of infertile mice, SSC can resume spermatogenesis and produce offspring [86, 90]. Unipotent murine SSCs can be converted *in vitro* into pluripotent cells by modifying the culture conditions, and have been termed germ line-derived pluripotent stem (gPS) cells [87, 88].

Some progress has been made in large animals, but stable SSC lines have not yet been established. Current *in vitro* culture conditions do not support long-term culture of purified pig spermatogonia [65]. After transplantation, spermatogonial stem cells can be identified in the host tissue [90]. Only spermatogonial stem cells can repopulate sterile testes when injected into seminiferous tubules of infertile

recipients. Such an assay originally developed for the mouse [90] has been successfully used in pigs and cattle and integration into the recipient testis demonstrated [65, 91].

Germ cell transplantation offers an exciting new method of producing genetically modified animals, providing culture conditions are developed under which SSCs can be expanded, undergo DNA transfection, and selection, while remaining genetically and epigenetically intact for transplantation. Because modifications are introduced directly into the male germ line, without embryo manipulation, the time required for gestation and maturing founder animals is avoided, a significant factor in large animals. However, the lack of specific SSC markers and knowledge of optimal *in vitro* culture conditions has hampered efforts to isolate these cells from large animals. SSC technology is at an early stage and the techniques required for transgenesis, such as conditions for extended culture, have yet to be developed.

5.4.7
Somatic Cell Nuclear Transfer

In 1986, the first viable mammal was produced by nuclear transfer, a sheep produced by fusion of a blastomere of an eight-cell embryo into an enucleated egg [92]. The technical aspects of nuclear transfer in mammals had already been established in the 1980s using blastomeres from early stage embryos as donor cells. At the time most developmental biologists thought that only nuclei of early embryonic cells would support successful nuclear transfer and that later cells became "determined" in their potential. This was based largely on findings in frogs, where embryonic nuclear donor cells could produce normal offspring, but adult cells only supported development to tadpoles. This view dominated biology until the mid-1990s. Cell-mediated transgenesis using nuclear transfer became possible when live sheep were produced from cells that had been cultured for several weeks [93]. The dogma of cell determination came to an end with the birth of "Dolly" in 1996, the first cloned mammal generated from an adult cell [94].

Common somatic cloning protocols involve the following steps [95]:

1) **Enucleation of the recipient oocyte:** Oocytes at the metaphase II (MII) stage of meiosis rather than any other developmental stage are the most appropriate recipients for the production of viable cloned mammalian embryos. In many domesticated species, oocytes can be obtained from abattoir ovaries, thus providing a potentially unlimited source of material for cloning experiments. Oocytes need to be matured *in vitro* to the MII stage, which in cattle requires a 24 h incubation period. *In vitro* maturation (IVM) protocols have advanced to the extent that *in vitro* matured oocytes can be used for somatic cloning with similar success rates as their *in vivo* matured counterparts. Oocytes are enucleated by sucking or squeezing out a portion of the oocyte cytoplasm closely apposed to the first polar body where the MII chromosomes are usually located (Figure 5.4).

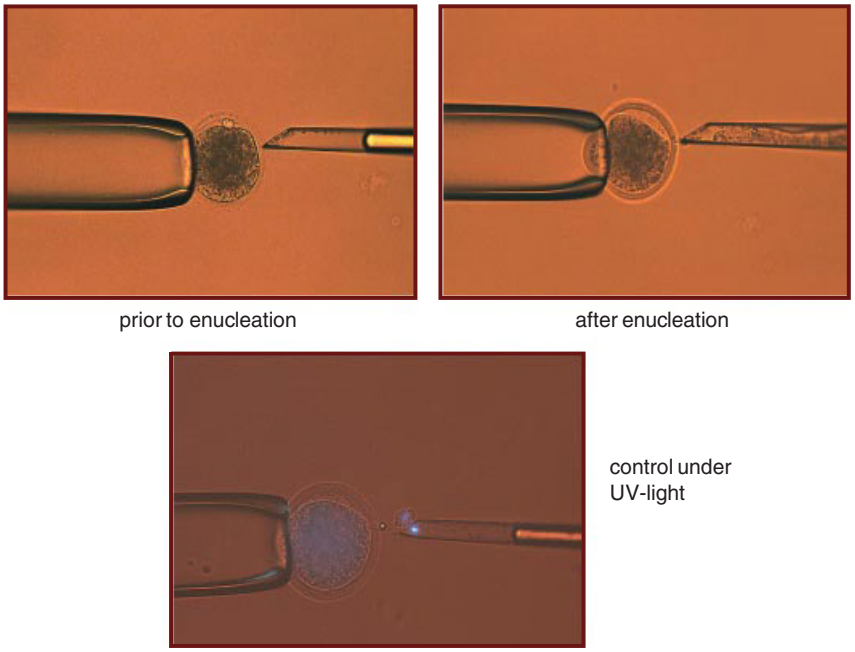

Figure 5.4 Enucleation of an *in vitro* matured bovine oocyte in the process of somatic cell nuclear transfer.

2) **Preparation and subzonal transfer of the donor cell:** The intact donor cell is isolated by enzymatic disaggregation and inserted under the *Zona pellucida* in intimate contact to the cytoplasmic membrane of the oocyte using a micropipette (Figure 5.5). Various somatic cells, including mammary epithelial cells, cumulus cells, oviductal cells, leucocytes, hepatocytes, granulosa cells, epithelial cells, myocytes, neurons, lymphocytes, and germ cells, have successfully been used as nuclear donors. Fetal cells, such as fibroblasts, have frequently been used in somatic cloning experiments, because they are thought to have less genetic damage and higher proliferative capacity than adult somatic cells. Early passage cells are usually chosen, but high rates of development have also been obtained with later passages of adult somatic cells.

Whether donor cells need to be forced into a quiescent state by either serum deprivation or treatment with cell cycle inhibitors is still a matter of debate. In most experiments, donor cells are induced to exit the cell cycle by serum deprivation, which arrests cells at the G0/G1 cell cycle stage. Specific cyclin dependent kinases, such as roscovitine, have been reported to increase the efficiency of the cloning process, although final evidence in the form of healthy offspring is lacking. Nevertheless, unsynchronized somatic donor cells have been successfully used to clone offspring in mice and cattle. There is currently a great need to develop reliable methods to select or produce donor cells that are more efficient for somatic nuclear transfer.

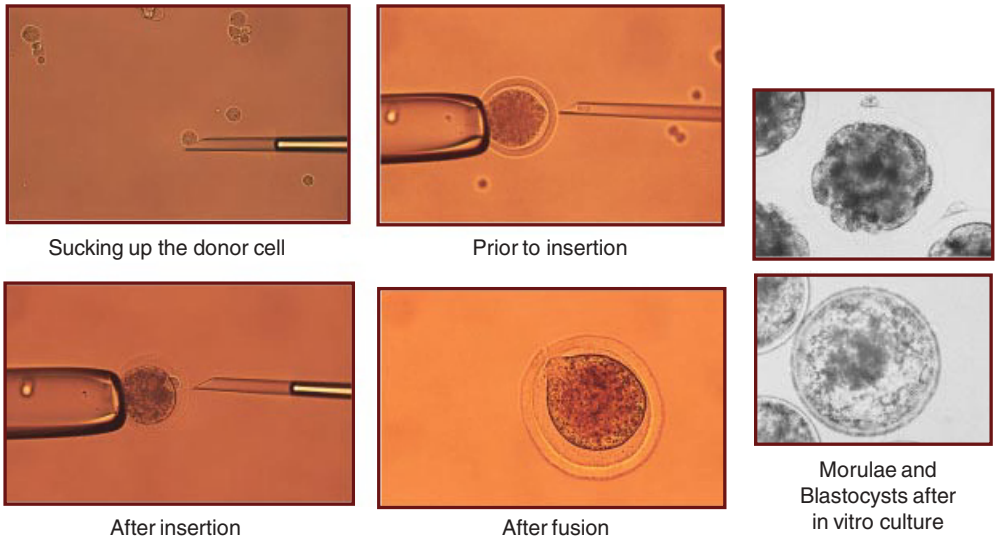

Figure 5.5 The process of somatic cell nuclear transfer; transfer of a somatic donor cell into an enucleated oocyte, fusion, and development to reconstructed morulae and blastocysts.

3) **Fusion of the two components:** The enucleated oocyte and donor cell are fused, usually by short, high voltage pulses through the point of contact between the two cells.

4) **Activation of the reconstructed complex:** Activation is achieved either by short electrical pulses, or brief exposure to calcium ionophores, such as ionomycin, or cell cylce regulators, such as dimethylaminopurine (DMAP).

5) **Temporary culture of reconstructed embryos:** Cloned embryos can be cultured *in vitro* to the blastocyst stage (5–7 days) to assess the initial developmental competence prior to transfer into a foster mother. Culture systems for bovine embryos are well advanced and allow routine production of 30–40% blastocysts from *in vitro* fertilized oocytes isolated from abattoir ovaries.

6) **Transfer to a foster mother or storage in liquid nitrogen:** Bovine embryos at morula and blastocyst stages can be transferred non-surgically to the uterine horns of synchronized recipients using established procedures. In pigs, activated nuclear transfer complexes are transferred immediately into recipients because *in vitro* embryo culture systems are not yet as effective as in cattle. This requires surgical intervention under general anesthesia [96].

To date, somatic cell nuclear transfer (SCNT) has been successful in more than 16 mammalian species and holds the greatest promise for improving the production of transgenic livestock. The proportion of live births after transferring cloned embryos to foster mothers is typically in the range of 2–5% of the transferred embryos. In cattle, pregnancy rates after transfer of cloned embryos is 20–40%

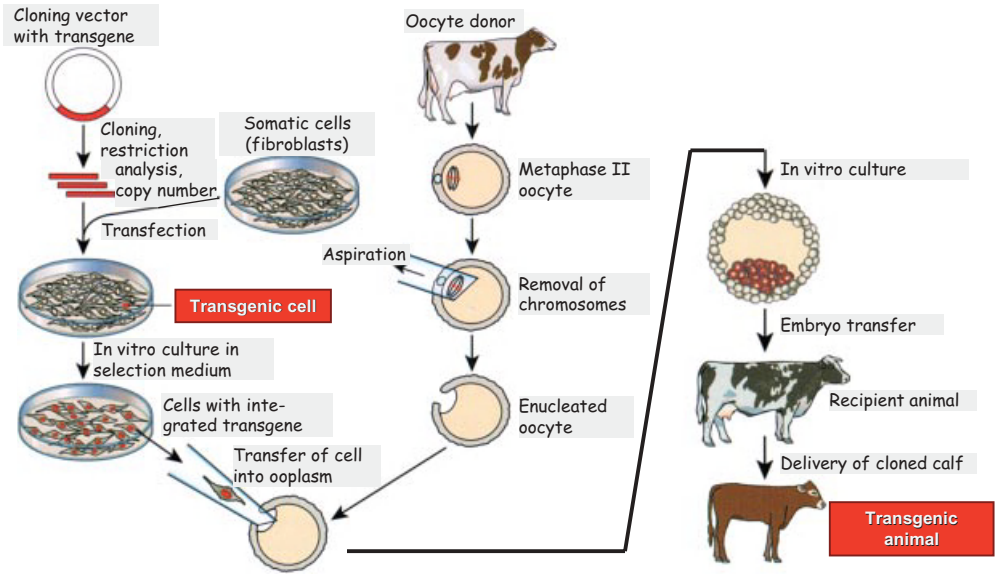

Figure 5.6 Schematic drawing of the steps involved in the production of transgenic animals (here cattle) via somatic cell nuclear transfer.

and, in pigs, pregnancy rates (the proportion of pregnant foster mothers) are as high as 60–80%, although litter size is moderately reduced when compared with conventional breeding [96].

Nuclear transfer from somatic cells opened a new means of producing transgenic livestock, lifting the requirement for ES cells for cell-mediated transgenesis (Figure 5.6). "Ordinary" cells such as primary fetal fibroblasts can be obtained in large quantities, manipulated in culture, and then converted into whole animals by nuclear transfer. This could potentially generate large numbers of genetically modified animals without conventional breeding. Initial work in the area was inspired by the possibility of producing such "instant flocks" of animals for biopharmaceutical production in milk. In 1997 lambs were reported that carried a human clotting factor IX transgene, randomly introduced into the genome by *in vitro* transfection of fetal fibroblasts [97] (Figure 5.7). Shortly after, an α1-antitrypsin transgene was placed by gene targeting into a site chosen as favorable for expression [98]. SCNT is a useful methodology for the production of transgenic farm animals and has largely replaced DNA microinjection as the method of choice.

SCNT has been used to produce random transgenic and gene targeted animals in several other species, demonstrating that sophisticated genetic manipulations are compatible with somatic cells from livestock species. However, compared with mice, relatively few gene-targeted large animals have been reported: *PRNP* in sheep, cattle and goats [99–102], α-galactosyltransferase (*GGTA1*) in pigs [103–105], *IGH* in cattle and pigs [17, 100], *IGKC* in pigs [106], *CFTR* in pigs [107], *SMN* in pigs [108], and *BRCA1* in pigs [109]. The main difficulty is that primary somatic

Figure 5.7 Photograph of human clotting factor IX transgenic lambs; the first transgenic animals produced by somatic cell nuclear transfer.

cells are short-lived in culture, allowing little time for cell transfection, selection, and clonal expansion. This obstacle can in part be overcome by "rejuvenating" cells by nuclear transfer and re-derivation from resulting fetuses. Such a procedure also allows successive rounds of *in vitro* genetic manipulation to be carried out relatively quickly, but does introduce the risk that genetic aberrations occur in the cultured cells, but remain undetected until animals are born.

Despite these reports of pathology, a critical survey of the published literature reveals that most cloned animals are healthy and develop normally [110]. This is consistent with the observation that mammalian development is rather tolerant of minor epigenetic aberrations in the genome. Subtle abnormalities in gene expression do not appear to interfere with survival of cloned animals [111]. Once cloned offspring have survived the neonatal period and reach 6 months of age (cattle, sheep), they do not differ from age matched controls with regard to numerous biochemical blood and urine parameters [112, 113], immune status [112], body score [112], somatotrophic axis [114], reproductive parameters [115], and yields and composition of milk [116]. No differences have been found in meat and milk composition of bovine clones when compared with age-matched counterparts; all parameters were within the normal range [117, 118]. Similar results have been reported for cloned pigs [119]. Offspring, including those from two nuclear transfer parents, exhibit no increased morbidity or mortality.

In summary, nuclear transfer from somatic cells is currently the major means of producing transgenic large animals and until recently was the only method of achieving gene targeting in mammals other than rodents. Clone viability remains a problem, but technical refinements may yet resolve this. Nuclear transfer requires relatively few experimental animals because oocytes are obtained from animals slaughtered for other purposes.

5.4.8
Highly Specific DNA Endonucleases

A new technology based on synthetic endonuclease enzymes such as zinc-finger nucleases (ZFNs) and transcription activator-like effector (TALE) nucleases is making a significant impact on the field of transgenic mammals. It is applicable to both direct and cell-mediated transgenesis and enables both gene inactivation by mutagenesis and precise sequence deletion, replacement, or insertion by gene targeting.

ZFNs are a fusion product between the cleavage domain of the FokI restriction endonuclease and a series of zinc-finger motifs [120]. The modular design of zinc-finger binding domains allows the production of specific binding proteins for nearly every sequence. These "molecular scissors" make it possible to introduce a double-strand break at a single predetermined site in the genome. In eukaryotes, double-strand break repair pathways often create small insertions and deletions at the break site, a useful means of inactivating genes of interest [121].

ZFN-mediated gene knockout by the introduction of ZFN mRNA directly into early embryos offers a one-step method of gene inactivation without any cell intermediate, as shown for zebrafish [122, 123], rats [124, 125], and mice [126]. The first successful use of ZFNs for a biallelic knockout in pigs after ZFN mediated homologous recombination in somatic donor cells and their use in SCNT has recently been reported [105].

ZFN cleavage can also stimulate, by several orders of magnitude, homology-directed genetic exchange between a conventional DNA gene targeting construct and the chromosomal locus. This is now significantly streamlining the production of animals carrying sequence replacements by enabling gene targeting directly in early embryos, effectively removing the need for a cell intermediate for many purposes. Efficient ZFN-mediated gene targeting by homologous recombination in embryos has been achieved in mice [127], rats [128], and most recently in rabbits [18]

TALES [129] have not yet been used for gene manipulation in mammals, but this is very likely in the near future.

5.5
Analysis of Transgenic Animals

The US Food and Drug Administration (FDA) and similar authorities in other countries have produced guidelines for potential products from transgenic sources. These include sequence validation of the gene construct, characterization of the isolated recombinant protein, and monitoring of the genetic stability of the transgenic animals.

5.5.1 Analysis of Integrated Transgenes

Each transgenic animal line destined for commercial production must be analyzed to determine the structure, integrity, copy number, and integration site of each integrated transgene. This analysis typically includes Southern hybridization of the genomic DNA to identify the lengths of various restriction fragments predicted from the construct structure. Fluorescent *in situ* hybridization of metaphase chromosome spreads can also be employed to identify the chromosomal location(s) of integrated transgenes. Molecular cloning of the integrated transgene and its proximal flanking regions may be required to determine the DNA sequence of the integrated transgene locus.

DNA introduced into mammalian embryos by microinjection tends to integrate as tandem repeats generally oriented head to tail and usually, but not always, at a single locus randomly located in the host genome. Introduction of transgenes by cell transfection has broadly similar results, but often leads to a more complex transgene array at the integration site. Lentiviral vectors also integrate randomly but as single copies at each integration site. Multiple copies of a viral transgene in a founder animal will therefore segregate in subsequent generations in Mendelian fashion.

Transgene loci produced by random cell transfection differ from those produced by DNA microinjection because selectable marker genes are necessarily introduced with the transgene; these will typically encode antibiotic resistance, for example, G418, blasticidin, or puromycin. To avoid possible gene flow from the transgenic animal to prokaryotes, bacterial gene promoters are excluded from selectable marker genes. Antibiotic resistance genes can also be flanked by site-specific recombination elements, such as *loxP* substrate sites for *Cre* recombinase, allowing their removal. However, in multiple arrays this may result in large deletions. Transgene loci produced by gene targeting are quite distinct from random events, in that a correctly targeted locus will carry a single copy of the predetermined engineered change and the rest of the genome is left unaltered. An antibiotic or other selectable marker gene is necessarily included at the target site, but again can be removed by site-specific recombination if necessary.

Multiple transgenes that are co-injected or co-transfected generally co-integrate at the same locus. Founder animals carrying high transgene copy numbers are frequently chosen to establish transgenic lines because they often express most abundantly, but such lines can undergo transgene silencing or recombination and copy loss over generations. Transgene copy loss occurs most frequently where elements in a tandem array are in inverse relative orientation. Such configurations tend to be unstable and can lead to deletions, duplications, and incomplete genes. Incomplete genes are particularly undesirable because, where breaks occur within coding sequences, shifts in the translational reading frame can lead to the expression of truncated or aberrant protein species.

Whole genome sequences are becoming available for an ever-increasing number of organisms including livestock mammals. Such databases coupled with low cost

DNA sequencing procedures will greatly facilitate the comprehensive genetic analysis of transgenic producer animals.

5.5.2 Transgene Expression Profile

The pattern of transgene expression must be characterized to determine its tissue specificity. This is primarily for the benefit of the producing animals, to assess whether any undue deleterious effects are likely to arise as a consequence of inappropriate transgene expression. Samples of a wide variety of tissue types obtained by necropsy of transgenic animals are analyzed by reverse transcriptase PCR (RT-PCR), or Northern hybridization to detect spatial, or temporal ectopic expression. The significance of any ectopic expression will depend on the level and site of expression and the nature of the protein.

Transgene mRNA expressed by the appropriate tissue should be rigorously characterized to identify the full range of mRNA species present. This is necessary to determine the integrity of the mRNA and whether it is correctly spliced. Aberrant mRNAs, even if present as only minority species, can encode aberrant proteins with possibly significant clinical consequences.

The aims of transgenic protein analysis are to determine: whether a protein is fully functional, if degradation occurs for example, in milk, and whether expression levels are sufficient for commercially viability. One then has to investigate to what extent the transgenic recombinant protein product resembles the native form, and whether any differences affect function, stability, half-life, and immunogenicity. Considerable efforts are therefore made analyzing protein products by functional assay, mass spectroscopy, peptide mapping, protein sequencing, and glycoprotein analysis. In this respect, the analysis of proteins produced by transgenic animals is the same as those produced by cell culture.

Human pharmaceutical products must be of consistent quality. Variations in expression level can affect protein structure. For example, if the post-translational modification capacity of the producing tissue is limited, high levels of expression may exceed that limit and result in partially or unmodified protein and altered bioactivity. The amount of protein produced by individuals in a transgenic herd or flock should therefore vary as little as possible. Acceptable upper and lower limits should be set to allow standardization and quality assurance of the purification process.

5.5.3 Collection, Processing, and Protein Purification

Basic collection and processing methods for large quantities of milk and eggs are well established. Collection procedures suitable for bulk collection of other fluids such as urine or semen have yet to be devised.

The purity of the protein preparation is an important factor in the assessment of any transgenic product. Producers must ensure removal of host animal proteins

and DNA, chemical reagents, and ensure exclusion of potential pathogens such as microorganisms, viruses, and prions. Large-scale recombinant protein purification has so far only been developed for milk. This is a multi-step process that combines standard methods developed for the dairy industry with procedures developed for purification of recombinant proteins produced in cell culture. The purity required of a particular product is determined by its application. If the protein is to be ingested as a nutraceutical, then skimmed milk could be suitable. If it is to be injected intravenously on a regular basis over long periods, then a very high level of purity is required.

The nature of the protein determines the specifics of its purification. As most recombinant proteins are present in the whey fraction, the first steps are removal of fat and suspended caseins by procedures that may include: centrifugation, acid or PEG precipitation or chymosin treatment, and/or microfiltration. This would usually be followed by a series of chromatography steps to isolate the recombinant protein away from the whey, remaining milk proteins, and other contaminants. Final clean-up steps might include ultrafiltration and possibly heat treatment to prepare a pharmaceutical-grade therapeutic product. Current experience indicates a final yield of purified product of between 40% and 60% of the amount in milk, depending on the nature of the protein and the required purification procedure. The greatest loss tends to be during casein removal. This may be reduced by treatment with chelating agents that deform casein micelles and release associated recombinant protein.

Standards for processing plants are equivalent to those already established for the purification of recombinant proteins from cell culture, or native proteins derived from human sources such as blood. Requirements for the process included validation for product safety and pathogen removal. All procedures have to be carried out according to GMP and using Standard Operating Procedures (SOPs). Where cell culture manufacturers are required to maintain duplicated banks of cells to ensure product continuity, transgenic manufacturers would maintain sperm banks.

5.6
Quality and Safety of the Product

In addition to the characterizations detailed above, several other factors must be addressed to ensure the quality and the safety of the product and of the procedures involved in its manufacture.

Regulations for the housing of transgenic animals vary between different countries. Veterinary health monitoring is required and, in addition, transgenic animals should be observed for any effects arising from recombinant protein expression. Generally, all animals will be kept under some type of containment regime, for example, in double-fenced fields with each animal marked by identification tags and subject to strict accounting. Procedures for disposal of waste matter and cadavers to ensure suitable containment should be observed. EU rules for general

animal husbandry also apply to transgenic herds or flocks. Animals are generally raised according to their species' needs and requirements. Some restrictions to their freedom of movement may apply due to laws regarding genetically modified organisms (GMO) or the need for safekeeping from, for example, damage by animal rights activists. As a precaution, human access might be restricted.

The health of production animals is important, not only to protect their own well being but also to avoid possible transmission of zoonotic disease. As described above, regular monitoring by veterinarians is required. Strict precautions should also be taken to prevent contact with other farm animals or wild animals, people, or equipment that have been in recent contact with either. Concerns regarding transmission of prion diseases (BSE, scrapie) also mean that land used as animal pasture should not have had contact with other farm animals for several years. For this reason, some companies have chosen to raise animals in countries free of prion diseases, for example, New Zealand. Alternatively, animals such as pigs and rabbits can be raised indoors in DPF facilities to minimize the risk of infectious diseases. Other precautions include exclusion of noxious agents, such as plant toxins or synthetic chemicals, for example, pesticides.

As with all biopharmaceuticals, production from transgenic animals must comply with current FDA or EMEA guidelines and GMP. GMP compliance is a legal requirement and includes training of personnel, validation of procedures, equipment, materials, and facilities, as well as SOPs. Production criteria must be defined at the outset, such as acceptance criteria for source material, product pooling, batch size, and the product quality and purity required at various stages during purification to ensure product consistency. Throughout, documentation is essential and meticulous records must be kept of all activities, from the production of the DNA construct all the way to the final product.

The purified product must be characterized prior to final formulation in a manner similar to other biopharmaceuticals. In this regard the concept of the "well-characterized biologic" has been very important. This was defined in the US Federal register in 1996 as "a chemical entity whose identity, purity, impurities, potency, and quantity can be determined and controlled." Biopharmaceuticals of all types have sometimes encountered problems meeting this strict definition, and it is considerably more difficult than for chemically produced products. It is conceivable that this will be revised in the future. With regard to purity, acceptable low levels can be set for such contaminants as pathogens, host proteins, DNA, and reagents used in the purification process. However, it is more difficult to set acceptable standards for "identity" because very minor differences in protein glycosylation, folding and other post-translational modifications can alter bioactivity, efficacy, and immunogenicity, possibly resulting in allergic or other adverse reactions in the patient.

Some early biological products are starting to come off patent, providing opportunities for cost-effective production in transgenic animals. These follow up products – so-called "similar biological medicinal products" or "biosimilars" – will have to adhere to equally high quality and safety standards. The necessary legal

framework has been established by regulatory authorities in the European Union. In the United States, the FDA is initiating discussion on this topic.

5.7 Conclusions and Outlook

Throughout history, farm animals have made significant contributions to human health and wellbeing, and the production of biologically active pharmaceutical proteins is a new addition to that success story. The refinement of the existing genomic maps of livestock species and the merger of the recent advancements in the reproductive technologies with the emerging tools of molecular biology will further improve efficacy and precision of the approach. The chicken, bovine, and equine genomes have already been sequenced and the pig genome is expected soon. Genetic modification requires detailed information on gene sequence, function, allelic variants, and gene–gene interactions. The derivation of iPS cells from livestock may finally deliver the long-awaited equivalent of mouse ES cell technology.

The promises of gene pharming are illustrated by the following calculations. On a yearly basis and on the assumption of average expression levels, daily milk volumes and purification efficiency, 5400 cows would be needed to produce the 100 000 kg of human serum albumin (HSA) that are required per year worldwide, 4500 sheep would be required for the production of 5000 kg α-antitrypsin (AAT), 100 goats for 100 kg of monoclonal antibodies, 75 goats for the 75 kg of antithrombin III (ATIII), and two pigs to produce 2 kg human clotting factor IX [3].

Preventing transmission of pathogens from animals to humans is critical to the safety of animal derived products. This requires appropriate maintenance systems for the production animals with strict hygienic standards as well as sensitive and reliable diagnostic and screening methods for the various types of pathogenic organisms. An important advance in this area is the derivation of *PRNP* (prion protein) knockout cattle. These animals survive and show resistance to BSE infection [102]. Elimination of the risk of BSE is mandatory for future commercial production of recombinant proteins for human medicine in transgenic cattle.

Furthermore, biomedical applications of farm animals will require strict standards of "genetic security" and reliable and sensitive methods for the molecular characterization of the "products." A major contribution towards the goal of well defined "products" from physiologically normal production animals will come from low-cost sequencing and refined array technology (cDNA, peptide, or protein arrays) which allow "fingerprint" profiles to be generated at the transcriptional and protein level [130, 131]. Low-cost sequencing ($100 per genome) will mean that once transgenic animals have been produced, their genomes, epigenomes, and organ-specific transcriptomes can be fully analyzed. This would also be required to maintain the highest possible levels of animal welfare to avoid pain or suffering due to genetic modification.

During the past two decades significant scientific and technical advances in the genetic manipulation of large animals have been achieved. During the same time period, guidelines and a coherent regulatory framework covering production and managements of transgenic herds and the purification, characterization, and administration of transgenic products have been established. The production of pharmaceuticals by transgenic farm animals significantly expands the existing panel of production methods for recombinant proteins, including cell cultures, bacteria, and plants and will be instrumental for developing production systems specifically tailored to the needs of the individual protein [132]. Transgenic animals, as with many new technologies, have been controversial. However, surveys of public attitudes to transgenic animals show that acceptance for biomedical applications is generally good [133], thus the role of livestock species in supporting human health is likely to increase.

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6
Translation of New Technologies in Biomedicines: Shaping the Road from Basic Research to Drug Development and Clinical Application – and Back Again

Michael Balls, Andrew Bennett, and David Kendall

6.1
Drug Discovery and Development

The primary goal in drug discovery and development is to identify compounds that could offer new ways of curing or managing disease, by improving the current therapies or providing treatments for currently untreatable diseases. This also involves the discovery of new targets, which, ideally, is based on an increasing understanding of the mechanisms involved in the development of the disease of concern, and of the basis of the desired and any potential unwanted effects of promising candidate compounds. This is a stepwise process, from initial target and hit discovery to drug development, then, for the very small proportion of the original candidates that survive to this stage, to clinical trials, followed by marketing at the population level, and post-marketing surveillance. Each step is more complicated, more time-consuming, more costly, more uncertain, and more prone to failure than the previous steps.

The pharmaceutical industry is in a state of crisis, because of the increased costs of drug discovery and development, and the fact that, despite the increased numbers of candidate compounds in the development pipeline, the rate of entry to the market has steadily decreased, while the rate of post-marketing withdrawal, because of lack of efficacy or unpredicted adverse effects, has increased [1].

A variety of causes have contributed to this situation. The new targets to be tackled are more difficult than those tackled in the past, and there is insufficient understanding, not only of the diseases themselves or of the mechanisms of the pharmacological and toxicological responses and effects involved, but also of the influence of human polymorphisms and the effects of a variety of contributory epigenetic factors. There has been a growing recognition that differences among patients can affect not only the efficacy or safety of a drug, but even the results of clinical trials, as a sizeable cohort of non-responders or idiosyncratic responders can throw doubt on the efficacy of a drug that is effective for the majority of the individuals in the trial.

This has led to an end to the belief that "one drug suits all," as well as to "one dose suits all," and patient-profiling before therapy begins is likely to be of increasing

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importance in the future. A further complicating factor is that the currently available non-clinical tests and testing strategies cannot adequately predict clinical effects [2]. This is partly because the models used in various testing strategies are not sufficiently related to what is being modeled, and therefore cannot be expected to be a sufficiently relevant or reliable basis for making important decisions [3].

6.2 The Nature of Models and the Need for Them

Research usually involves the establishment of a model of the system of interest, then studying the model in ways which would not be possible with the system being modeled itself (for practical or ethical reasons). By their very nature, models must differ from what is being modeled, and their value can be limited, both by insufficient knowledge of what is being modeled and insufficient knowledge of the model that is being used. Where human disease is the principal focus of research and of new drug discovery and development, the inescapable fact is that *in vivo* models other than humans themselves have insuperable inadequacies. For example, no animal model can provide sufficient insight into the genetic variant-dependent metabolism of drugs that is of vital significance in humans [4], profound anatomical and physiological differences between the respiratory systems of rodents and humans make rats unsuitable for respiratory toxicity studies [5], and differences in the responses of rodent and human cells to peroxisome proliferators (which include solvents, plasticisers, herbicides, and hypolipidemic drugs) frustrate the use of data from rodent studies for predicting effects in humans [6]. Hence, more modern, more sophisticated models, which are less dependent on the traditional use of animals, are urgently needed.

As a result of this, the need to fundamentally reappraise the value of animal studies as an essential and required background to human studies is increasingly being emphasized. The US Food and Drug Administration (FDA) put it like this in *Challenge and Opportunity on the Critical Path to New Medicinal Products* [7]:

> Despite some efforts to develop better methods, most of the tools used for toxicology and human safety testing are decades old. Although traditional animal toxicology has a good track record for ensuring the safety of clinical trial volunteers, it is laborious, time-consuming, requires large quantities of product, and may fail to predict the specific safety problem that ultimately halts development. Clinical testing, even if extensive, often fails to detect important safety problems, either because they are uncommon or because the tested population was not representative of the eventual recipients. Conversely, some models create worrisome signals that may, in fact, not be predictive of a human safety problem.

This remarkable FDA initiative presents a great challenge and an enormous opportunity, which should be welcomed and responded to by all concerned, in the

interests of good science and human benefit, as well as animal welfare. Those who have a tendency to want to protect the status quo at all costs, should note that phrases such as "animal toxicology . . . may fail to predict the specific safety problem that ultimately halts [drug] development," and, elsewhere in the document, "currently available animal models . . . have limited predictive value in many disease states" have been used by the FDA, and not by animal rights protagonists alone.

Meanwhile, in Europe, the Innovative Medicines Initiative (IMI) has been established, as a joint undertaking between the European Union and the European Federation of Pharmaceutical Industries and Associations (EFPIA), with the aim of improving the drug development process by supporting more efficient drug discovery, leading to the development of better and safer medicines for patients [8]. The IMI supports collaborative research projects and builds networks of industrial and academic experts in Europe. The focus is on the development and use of *in silico* and *in vitro* methods, and omics and imaging approaches, so that the new technologies can be used for the benefit of patients, as a result of dynamic interactions between what takes place in the laboratory and in the clinic.

It cannot be denied that many types of organisms undoubtedly have value at the research level, including plants such as onion (*Allium cepa*) and garlic (*Allium sativum*), bacteria such as *Escherichia coli*, fungi such as yeasts (*Saccharomyces cerevisiae*), coelenterates such as hydra (*Hydra magnipapillata*), nematode roundworms such as *Caenorhabditis elegans*, insects such as *Drosophila melanogaster*, lower vertebrates, including fish such as the zebrafish (*Danio rerio*), and amphibians such as the South African clawed toad (*Xenopus laevis*). These organisms are being extensively used in fundamental research on cell and molecular biology, cell death, aging, developmental biology, immunobiology, and neurobiology. However, although some pharmacotoxicological test systems involving lower organisms and aimed at predicting effects in humans have been proposed, they are unlikely to provide solutions to the problems currently facing the pharmaceutical industry, because the differences between these organisms and humans are too great for tackling other than certain highly specific questions (e.g., what is involved in DNA damage and repair).

Also, it cannot be denied that there has also been some value in historical research on higher vertebrates, including rats, mice, rabbits, dogs, and non-human primates. However, causing pain, suffering, distress or lasting harm in laboratory mammals as surrogates for humans (i.e., exposing them to procedures that would not be acceptable if applied to our own species) raises ethical questions, which are, to variable extents, dealt with in legislation which attempts to strike a balance between animal welfare and the demands of industry and science (in the service of human welfare).

Russell and Burch faced up to these issues in their seminal work, *The Principles of Humane Experimental Technique*, in which they proposed that all possible effort should be made to *reduce*, *refine*, and *replace* the use of laboratory animals in medical research and testing – now commonly referred to as the Three Rs [9]. They emphasized the difference between *high fidelity* models (with high general similarity to what is being modeled) and *high discrimination* models (with high specific

similarity to what is being modeled). They argued that a highly discriminative/poor fidelity model is more useful than a high fidelity/poorly discriminative model. A common misunderstanding of this truth leads to the *high fidelity fallacy*, which can be summarized as follows: humans are placental mammals, so members of other mammalian species are more likely to be useful as models of man than would be fish or, still more markedly, bacteria, insects or nematodes. Since the general physiological properties and pharmacological responses of other mammals are more likely to be similar to our own than are those of other organisms, mammals are always the best models to use in fundamental biomedical research, drug development, and toxicity testing, where humans are the focus of concern. The problem that is now increasingly recognized, is that, however high the overall fidelity, if fidelity in relation to a specific receptor, an enzyme pathway or a pharmacological or toxicological mechanism in the rat, dog, or macaque is not sufficiently close to the situation in humans, then reliance on the animal model is misleading and potentially dangerous. The even greater problem is that the level of fidelity is not usually known, so when an animal model is or is not relevant cannot be known.

A current example of failure to comprehend the high fidelity fallacy is the attempt to humanize animals by modifying them to carry human genes, in an attempt to obtain information of direct relevance to humans. The problem is that there is no such thing as "a human," and human polymorphism, and its implications for new drugs which go to the market, are among the most important issues confronting the pharmaceutical industry. Also, genes, cells, and tissues are influenced by complex networks of interacting neurological/endocrinological/immunological local and systemic factors. Hence, given a lack of sufficient understanding of what human characters are being transferred to the humanized animal, or of the effects of other systems in the host animal, how will it be possible to interpret and apply the results obtained? Finally, while the humanization of rodents is one matter, the humanization of non-human primates would pose some very serious ethical questions.

An opportunity to escape from some of the current problems is offered by an impressive number of new developments in the biological and computational sciences, which are focused directly on human material and human situations [10, 11]. However, they also result in new pitfalls, and there is an imperative need for well-designed experimental approaches and the application of intelligent and discriminating strategies. In addition, their use must be accompanied by the recognition that the flow from research through development to the clinic must be a two-way process, and that any temptation to believe that "one type of model suits all" must also be avoided at all costs.

6.3 New Technologies Toolbox

The enormous amount of effort invested in biological and computational technology in recent years has resulted in the availability of a vast and ever increasing array of experimental tools, which could contribute substantially to the discovery

and development of new and acceptably safe drugs. What is particularly exciting is that most of the tools are based on human material or human experience, so the limitations of non-human models can be avoided. However, the usefulness of the tools in any toolbox must rely on the skills of the craftsman who uses them. Thus, the selective, coordinated, and intelligent use of the opportunities now being created will be crucial. This will be important for a variety of reasons, including the costs and time required, and the fact that the new methods will each offer answers to a limited number of specific questions. One of the essential skills will be to combine information from different sources into patterns leading to increased understanding and to a much better basis for making important decisions.

The use of *computers* will be essential in virtually every aspect of the further development and use of the new technologies, as a means of collecting, storing, organizing, and analyzing data, and for signaling associations and correlations which deserve further attention. Therefore, while recognizing the interdependence of most, if not all, the new technologies, we will not refer to *computational pharmacology* or *computational toxicology*, preferring to use *in silico* to distinguish certain computer-based approaches from approaches described by older terms, for example, *in vivo*, *ex vivo*, and *in vivo*. That is not irrational, since all these terms are imprecisely based, for example: *in vivo* literally means "in life," but organotypic cultures are no less "living" than are organs in the intact body; *in vitro* literally means "in glass," but cultured cells are now only rarely grown on glass surfaces, but are grown on plastic surfaces or suspended in media of various types.

The same could be said of *informatics*, which applies broadly to the application of all the other technologies, but has more-precise uses in *bioinformatics* and *chemoinformatics*.

Descriptions of the new technologies could be based on various types of classification and subdivision, but the summaries in this chapter will be considered in the order shown in Table 6.1.

6.3.1 Use of Existing Knowledge

Existing knowledge should always be the starting point for any study of a novel compound, by consulting in-house data banks and more widely available resources. In the past, pharmaceutical companies have been reluctant to share such data, but that may be changing, perhaps as a result of the grave problems confronting them.

It is clearly desirable to archive as much hard-won data as possible and to make them openly available, whenever this is feasible. Prior to the 1980s, however, the standard scientific model was for laboratories to address scientific problems, then publish a proportion of their findings, while unpublished data and hard copy journal data became lost or difficult to access. With the growth of gene sequencing, it became possible, because of the relatively simple, linear nature of the data, to lodge them, in parallel with journal publication, in open access databases where they could be analyzed by using informatics tools to generate novel information

Table 6.1 New technologies and approaches that can contribute to drug discovery and development.

Use of existing knowledge
In chemico analysis and chemical interactions
In silico structure–activity methods
In vivo studies on lower organisms
In vitro methods
High-throughput screening
High-content screening
Omics approaches
PBPK models
Virtual tissue models
Human volunteer and clinical studies
Virtual patient populations
Biomarkers
Clinical imaging
Informatics
Systems biology
Integrated testing strategies

(e.g., homologies, mutations) from simple questions. Other research areas present more sophisticated challenges. Neuroscience should, possibly, be considered as a special case, given the oft-quoted, if not entirely justified, comment that the human brain is the most complex thing in the universe. The nature of neuroscience data is very different from straightforward sequencing, with the need to store massive amounts of complex information, particularly in the form of images. After much deliberation, a pilot project was funded in the United States, which envisaged a new research specialty of "neuroinformatics," combining experimental neuroscience with the development of electronic enabling technologies. This became the Human Brain Project [12], as a parallel to the Human Genome Project, with the aim of providing information to neuroscientists to produce a complete mapping of the human brain in health and disease, from the molecular to the circuit level. This has developed into the Neuroscience Information Framework, a comprehensive, dynamic inventory of web-accessible neuroscience resources constructed by a multi-site consortium [13].

6.3.2 *In Chemico* and Other Physicochemical Approaches

In chemico effects result from the covalent binding of xenobiotic molecules, including drugs, to biological macromolecules, in ways which can lead to significant effects, such as mutation or sensitization. However, effects at this level are best interpreted on the basis of organic chemistry mechanisms, rather than the properties of the biological molecules that are affected, the contributions of which can be evaluated and interpreted in other ways. Physicochemical properties are also

significant, and measurable, such as stability under various conditions, volatility, and acidity/alkalinity. For example, compounds with a low or high pH can cause severe and direct damage to the eye or the skin because of this, irrespective of the properties of the eye or skin themselves. Hence, the dissociation constant (pK_a), a measure of the strength of an acid or base, is routinely determined. Another important parameter is the partition coefficient ($\log P$), a measure of how the chemical partitions between a lipid (oil) and water, which is a particularly useful guide to the pharmacokinetics of the molecule.

Cronin *et al.* [14] considered the importance of the interface between *in chemico* and *in silico* approaches in toxicology.

6.3.3 *In Silico* Methods

In silico methods for pharmacotoxicology include many approaches, from the use of (quantitative) structure–activity relationships ([Q]SARs), category formation and read-across, through to database compilation and the integration of information from different sources. The current emphasis on *in silico* methods for predicting toxicity has primarily been in relation to the REACH system for chemicals, in Europe, and to ecotoxicology, in the United States. However, these methods can also be of great value in drug discovery and development. As with other types of methods, relatively crude and rapid approaches are applied to the screening of large numbers of compounds, whereas more-sophisticated approaches are applied as the number of candidate compounds under consideration steadily falls.

As Cronin [15] has also recently pointed out, the pharmaceutical industry not only has the financial resources to contribute substantially to the development of *in silico* pharmacotoxicology, its various companies also have the historical data that would greatly facilitate the development of new models or contribute to the evaluation of existing models. Such data tend to be jealously guarded, possibly because they are not universally of the highest quality, but a contribution in return for access policy could be valuable, as happened several years ago with the US cosmetic industry's cosmetic ingredient review scheme. The European IMI may have a role to play here.

Ekins *et al.* [16, 17] were more hopeful, and pointed out, in their oft-quoted articles on the use of *in silico* methods in drug discovery, that the public availability of data on drugs and drug-like molecules may make *in silico* analysis possible for scientists outside the private sector. For example, a number of chemical repositories, including DrugBank (http://redpoll.pharmacy.ualberta.ca/drugbank/) [18, 19] and PubChem (pubchem.ncbi.nlm.nih.gov/), contain of a wealth of target and small molecule data that can be accessed. Ekins *et al.* also pointed out that, although much of the *in silico* pharmacology research to date has been focused on human targets, many of these databases contain data from other species that could also be useful for understanding species differences, as well as assisting in understanding the significance of toxicological findings for chemicals released into the environment. They emphasized that the more successful pharmaceutical

companies manage information as a key resource and seek to integrate computational and experimental data.

6.3.3.1 *In Silico* Methods and Drug Discovery

Whatever the biological system under consideration, there are two potentially important outcomes when a bioactive compound interacts with it: activity and/or toxicity, as influenced by pharmacodynamic (PD) events (what the compound does to the system) and pharmacokinetic (PK) events (what the system does to the compound).

In the 1960s, PD and PK events began to be unraveled by using computers, and QSAR approaches were developed, in order to construct mathematical models to relate compound structure to biological effect.

As Ekins *et al*. [16] outlined, this resulted in descriptor-based methods, rule-based methods, and knowledge-based approaches, and virtual ligand screening, an attempt to apply QSAR principles to large numbers of compounds as an alternative to experimental high-throughput screening. The principal focus can be on ligands or targets, followed by virtual affinity profiling, which evaluates the pharmacological profile of the effects a molecule has on multiple targets, again with a focus on ligand-based or target-based approaches.

In a second seminal article, Ekins *et al*. [17] considered the applications of *in silico* pharmacology for application to drug discovery targets and beyond. They gave examples of the use of computational methods to discover new molecules with binding affinity for: enzymes, including drug metabolism enzymes; kinases; transporters; receptors; and transcription factors; and as antibacterials and antivirals. They also discussed the case of drug metabolism, by dividing the available models into specific/local methods for application to single enzymes and single enzyme activities, and comprehensive/global methods, which are "applicable to versatile biological systems, with the potential capacity to encompass all or most metabolic reactions and offer predictions which are much closer to the *in vivo* situation." They considered that *in silico* pharmacology would inevitably become more complicated, probably requiring the integrated use of models, as illustrated by combined metabolism modeling approaches involving QSAR, 3-D QSAR, molecular modeling and docking, quantum mechanics, meta-analysis, and expert systems.

Computational approaches to predicting drug metabolism were reviewed by Czodrowski *et al*. [20], who emphasized the need for caution in taking the applicability domains of methods into account, as well as their methodological limitations.

Combined, systems-based approaches are becoming more common, but true examples of such approaches which improve existing methods are still few and far between. One such example is the use of the Meta Drug approach by Ekins *et al*. [21] to "(i) predict metabolites for molecules based on their chemical structure, (ii) predict the activity of the original compound and its metabolites with various absorption, distribution, metabolism, excretion, and toxicity models, (iii) incorporate the predictions with human cell signaling and metabolic pathways and

networks, and (iv) integrate networks and metabolites, with relevant toxicogenomic or other high throughput data. They demonstrated the usefulness of such an approach by using previously published data from *in vitro* metabolism and microarray studies on a variety of compounds known to interact with cytochrome P-450 enzymes (CYPs), the pregnane X receptor, and P-glycoprotein, to show the predicted interactions with these entities and subsequent effects on downstream metabolites and networks of genes. The success of this approach can be gauged from the data, which indicated that it correctly identified, on average, 79% of first pass metabolites from a test set of 66 compounds.

6.3.3.2 *In Silico* Methods and Toxicology

A comprehensive consideration of principles and applications in *in silico* toxicology has recently been published [22], which deals, *inter alia*, with: finding the data needed to develop QSARs and other models and assessing their quality; molecular descriptors from 2-D chemical structure; 3-D molecular modeling of receptor-based mechanisms; the relevance and importance of the applicability domain; read-across; expert systems; exposure modeling for risk assessment; toxicokinetic considerations; weight-of-evidence approaches; and integrated testing strategies for the prediction of toxic hazard.

In a briefer, but no less useful treatment of the subject, Combes [23] has outlined the main steps involved in developing a QSAR model, including: the selection of a training set of chemicals; the analysis of molecular descriptors; the processing of physicochemical and toxicological information; the development of a model; the evaluation of goodness of fit; the definition of the applicability domain; and application to the toxicological assessment of specific endpoints. Account must also be taken of bioavailability (toxic chemicals are not hazardous if they are not bioavailable), the presence of toxicophores (the sub-structure of molecules which can specifically interact with cellular components such as receptors, enzymes, and macromolecules, e.g., proteins and DNA), and the potential production of toxic metabolites.

Computerized prediction systems involve the use of rules based on pre-existing knowledge. These can be knowledge-based systems (KBS), which use rules developed by human experts, and automated rule induction systems (ARIS), in which rules are developed by using computer-driven algorithms in ways which are especially useful for analyzing complex data. A variety of KBS and ARIS are commercially available.

The determination and rigid adherence to the *applicability domain* is vital to the use of any *in silico* predictive model: a model cannot to be used to predict the activities of chemicals that fall outside its applicability domain, that is, the physicochemical, structural or biological space, knowledge or information on which the training set of the model has been developed and for which it is applicable to make predictions for new compounds.

Enoch *et al.* [24] have provided an excellent example of how *in silico* toxicology studies are conducted, by showing how a chemical structure-based profiler for covalent binding to DNA can be used in the development of chemical categories,

also involving mechanisms of action leading to molecular initiating events, for use in read-across for genotoxicity as a way of filling in data gaps.

Recently, Vedani and Smiesko [25] discussed the use of 3-D models of small molecules binding to macromolecules for evaluating potential toxicity during drug discovery. They modeled: the binding of a small molecule ligand to a protein; flexible docking by induced fitting, as, for example, at the androgen receptor; and a mixed-model approach involving flexible docking and a 3-D QSAR. They then went on to consider toxicity predictions based on 3-D models for human CYPs, the hERG potassium channel, nuclear receptors, and the aryl hydrocarbon receptor. They concluded with a detailed description of the *Virtual ToxLab* concept and its application. *Virtual ToxLab* is an *in silico* system for predicting toxicity via a fully-automated protocol, which calculates the binding of any molecule to a series of 12 proteins known or suspected of playing a role in the triggering of adverse effects. The system has now been applied to more than 2000 compounds, including a series of drugs that have been withdrawn from the market. Vedani and Smiesko's excellent article concludes with a comment that deserves the widest possible recognition and acceptance: "The verification/validation of *in silico* techniques should not be performed by comparison with animal experiments – which they ultimately seek to replace – but exclusively with human data." This should apply to all non-animal tests, with the proviso that *in silico* methods could be used to predict and explain inter-species differences.

6.3.4 *In Vitro* Systems

A wide variety of *in vitro* systems are available, which range in complexity from the use of isolated cell fractions over a few hours to the long-term maintenance of multi-organ bioreactors (Table 6.2). By definition, "culture" is applied to cell, tissue or organ preparations that can be maintained *in vitro* in a nutrient medium for more that 24 hours. The trend is toward greater sophistication and greater humanization, which leads to greater relevance, but also to higher and higher costs in terms of time and human and economic resources. This can result in methods that are unsuitable for large-scale implementation, and which, in effect, set unachievable gold standards.

6.3.4.1 Cell Fractions

Short-term studies can be conducted with cell fractions and isolated cell components, such as nuclei, membranes, mitochondria, and receptors. This can be the basis for high-throughput screening to answer relatively specific and narrow questions.

Several drugs have been withdrawn from the market because of mitochondrial toxicity, including nefazodone, an anti-depressant that caused hepatotoxicity. It has been suggested that tests on isolated mitochondria could provide a high-throughput screening tool. It is also possible to use mitochondria to provide information on mechanisms of toxicity, for example, effects on oxidative phospho-

Table 6.2 *In vitro* systems that can contribute to the replacement of animal experimentation.

Cell fractions (including post-mitochondrial supernatant [S9], cytosolic [S100], and microsomal fractions for biotransformation and toxicity studies)
Primary cell monolayer or suspension cultures
Continuous cells lines
Immortalized cell lines
Stem cells
Genetically engineered cells
Co-cultures
Organotypic cultures
Precision-cut slices
Perfused cultures
Reconstituted tissue equivalents
Engineered tissues
Dynamic bioreactors
Multi-organ systems
Cell-/organ-/human-on-a-chip

rylation and calcium homeostasis, which would have wide implications. There is also a growing area of drug research that aims to target mitochondrial function in a variety of disease states, including Alzheimer's disease [26] and cancer [27].

6.3.4.2 Cell Monolayer or Supension Cultures

Nowadays, there is a tendency to look upon one-cell-type monolayer or suspension cultures as oversimplified and non-physiological, but it should not be forgotten that such cultures provided a basis for quantitative animal/human virology in the 1940s, which led to the production of effective polio vaccines from monkey kidney cell monolayers in the 1950s.

Cells can be isolated from the body and maintained as primary cell cultures, which can only be used in the short term, as they tend to rapidly lose their cell type-specific characteristics and functions. Some improvement can be gained, for example, by growing the cells on a surface covered with extracellular matrix components or by using a collagen sandwich method.

Primary cells can be subcultured to provide cell strains, which can lead to permanent cell lines. They can be manipulated to make them immortal, or genetically engineered so that they express selected genes and contain selected gene products.

A number of useful cell lines of human or animal origin are available, including Caco-2 cells (used to evaluate absorption in the gastrointestinal [GI] tract), HepG2

cells (for predicting phase 1 metabolism), hERG cells (to assess cardiac safety), and MDCK cells (for studies on cellular barriers). They must be used with caution, since their main disadvantage is that they are likely to differ considerably, in terms of gene content and expression, chromosome number, and specific structures and functions, from the cells in their tissues of origin.

It has been possible to produce genetically engineered cell lines, such as V79 cells, which themselves have little CYP activity, transfected with the main CYPs from humans and laboratory animals, for use, for example, in selecting the most appropriate animal model for use in drug metabolism studies. The human hepatoma cell line, HepaRG, expresses many CYPs [28], and could be very useful as a model for use in human drug metabolism studies [29].

One of the most important technical developments was that of cell hybridization, which made possible the large-scale production of monoclonal antibodies, which has led to a variety of major developments, from the research laboratory to the clinic.

6.3.4.3 Co-cultures, Organotypic Cultures, and Reconstituted Tissue Constructs

Short-term studies can be conducted with precision-cut slices of organs such as the liver or kidney, as a way of investigating drug-induced liver injury in preparations where the organ architecture and cell and tissue relationships are retained. Improvements in the maintenance and relevance of hepatocyte cultures have been achieved via co-culture with epithelial-like cells, fibroblast-like cells, Kupffer cells, and stellate cells, as a means of providing microenvironments that are more like those found *in vivo*.

Reconstructed human skin equivalents, in which multi-layers of human keratinocytes form on an appropriate base are being widely used in studies on skin irritation, and may also provide a system for evaluating percutaneous absorption. Another useful model is the human corneal air-lifted model, in which SV40-transfected human corneal epithelial cells are grown at the air–liquid interface, on corneal fibroblasts and endothelial cells submerged in the culture medium.

These procedures often involve the culture of cells on treated filter inserts, which facilitates the measurement of various properties, such as trans-membrane transport and trans-epithelial resistance.

Again, the central nervous system (CNS) poses particular problems due to its complexity and availability of tissue, but it has the advantage of being relatively stable *in vitro*. Thin slices of young rodent CNS regions can be kept in culture without significant loss of gross cellular architecture for many weeks, and the application of multi-electrode electrophysiological recording techniques [30] has the potential to generate large amounts of data of relevance to a variety of pressing problems, such as epilepsy and neurodegeneration. Thicker slices, while retaining more-realistic degrees of connectivity, suffer from internal hypoxic damage, but this can be alleviated by using recently developed microperfusion systems [31]. Accessing viable human CNS tissue is a major challenge, and although some studies have been conducted by using salvaged neurosurgical specimens [32], it is unlikely that this will ever be practical as a routine approach.

6.3.4.4 Tissue Engineering

The aim of tissue engineering is to use engineering tools to recreate *in vitro*, the biochemical, mechanical, and 3-D structural conditions under which cells and tissues function *in vivo*, with the eventual aim of restoring tissues and their function in patients. This involves various strategies, including the following: producing natural extracellular matrix models or synthetic mimics; providing appropriate gradients and cascades of specific growth factors; developing appropriate biomechanical environments; recognizing the importance of cell–cell signaling; establishing co-cultures of different cell types; and replicating tissue architecture and medium flow patterns. The materials used in the devices (polymers, textiles, glass, ceramics) are also the subject of further efforts, in order to permit down-scalability, processability and detectability, along with the use of membranes for local transport and cell adherence, and microcapillary systems and microfluidics, along with nanotechnology, for the handling of materials on the molecular scale. These types of developments are also vital to the provision of the conditions suitable for the maintenance of various tissues in *in vitro* bioreactors.

As Shakesheff and Rose have reported in a recent review [33], a degree of success has been achieved with a variety of tissues and organs, including skin, liver, skeletal muscle, cardiovascular tissue, GI tissue, the cornea, and the airway epithelium. For example, progress is being made in the development of an artificial liver, to serve as a supportive device for patients with liver failure, to allow time for their own livers to regenerate. Also, as mentioned above, reconstructed human skin equivalents have been very useful as the basis of tests for skin corrosion and skin irritation. Tissue engineering is also making significant contributions to the exploitation of stem cell technologies. Nevertheless, commercially available tissue engineering scaffolds are few and far between, making monolayer culture or static 2-D co-culture still the norm. One promising scaffold is Alvetex [34], which is now available in a 12-well format from LGC standards (http://www.lgcstandards.com).

One of the most sophisticated approaches is that of Taylor and coworkers [35], who have "decellularized" rodent hearts by coronary perfusion with detergents, which preserves the underlying extracellular matrix. This produces a heart scaffold of collagen (a "ghost heart"), which can be re-seeded with cardiac or endothelial cells. The resultant constructs have been maintained by perfusion in a bioreactor that simulates cardiac physiology, and, by day 8 in culture, these constructs can generate a pump function. With the application of autologous stem cell technology, this offers the future possibility of creating bioartificial human organs for transplantation and research.

6.3.4.5 Stem Cells

Stem cells always self-renew when they divide, but, under certain circumstances, one of the daughter cells can enter a differentiation pathway toward one or more of a variety of specific tissue types. Stem cells are found in developing embryos, but they are also involved in providing newly differentiated cells throughout life

in certain tissues, such as the skin, bone-marrow, and GI tract, or under special conditions, such as in regeneration of the liver.

The production of mouse embryonic stem cell (mESC) lines early in the 1990s led to useful test systems for *in vitro* embryotoxicology, but, as with *in vivo* reproductive toxicity studies in rodents, the relevance of the data to human embryotoxicity and teratology was uncertain. The more-recent isolation of human embryonic stem cells (hESCs) has attracted a great deal of attention, especially because of their clinical potential. Since ESCs are pluripotent, it should be possible to induce the differentiation of almost any cell type from them [36]. Indeed, it is now possible to produce hepatocytes, cardiac cells, neural cells, osteogenic cells, pancreatic islet cells, endothelial cells, and haematopoietic cells *in vitro* from hESCs [37].

hESCs also provide opportunities for dramatic developments concerning the mechanisms involved in inherited disease processes. For example, hECS from embryos related to cystic fibrosis, fragile X syndrome, and Huntington's disease are already available in the UK Stem Cell Bank.

Jalali *et al*. [38] have recently reported that they have been able to revert skin cells from normal donors and Alzheimer's Disease patients to an embryonic stem cell form, from which they have produced large numbers of cholinergic forebrain-like neurons, one of the major targets for neurodegeneration in the disease. This offers the possibility of using such cells to screen potential anti-Alzheimer's drugs and as a transplantation option.

A great deal of effort is being invested in developing stem cell therapeutics, with the hope of replacing diseased or damaged tissues, or even whole organs, in patients. A particularly important development has been that of the technology to produce pluripotent stem cells from adult somatic cells, that is, what are known as induced pluripotent stem cells (iPSCs), first produced from mouse cells and, shortly afterwards, from human cells. The use of iPSCs avoids the ethical issues related to obtaining hESCs, and, in the future, it may be possible to use iPSCs from an individual patient to provide replacement tissues for that same patient. Initially, viruses or oncogenes were used in the induced process [39], which raised some concerns about the potential carcinogenicity of iPSC-derived cells used in the treatment of patients. That problem was overcome, when Yu *et al*. [40] succeeded in inducing pluripotency by reprogramming with the proteins produced by the suspect genes.

In relation to drug discovery and development, the use of iPSCs opens up the possibility of producing normal and damaged differentiated cells of various types from individual humans, both before and after drug treatment. This would enable detailed studies to be conducted on the desired and/or adverse effects of the drug, as a means of studying genetic predisposition, dose–effect relationships, and the effects of epigenetic variables, such as treatment with other drugs, lifestyle, and occupational factors, and infections.

An industrialized process for the manufacture of iPSC-derived cardiomyocytes has recently been described [41], and functional human hepatocytes have been produced from iPSCs, which is another very important development [42]. In addition, libraries of pharmacologically active compounds are being screened,

in a search for small molecules that would promote the differentiation or survival of iPSCs *in vitro*, thus opening up the possibility of purely chemical reprogramming [43].

6.3.4.6 Examples of Some Specific *In Vitro* Systems

The MIMIC® system replicates the human immune response, and can be used to simulate a clinical trial in a human population [44]. It involves four modules, namely: (i) leukocyte collection and preservation; (ii) the Peripheral Tissue Equivalent, that is simulation of an innate immune responses (with peripheral blood mononuclear cells); (iii) the Lymphoid Tissue Equivalent, that is simulation of the adaptive immune response (via the application of dendritic cells, follicular dendritic cells, T-cells, and B-cells in the appropriate sequence); and (iv) functional assays to determine the scale and effectiveness of the immune response against the test item (including antibody production by B-cells, cytokine production, and T-cell killing).

Other systems in the course of development include: a cell-based diabetic wound assay, based on isolated healthy and diseased dermal fibroblasts from patient biopsies [45]; a 3-D *in vitro* model of breast cancer, with an emphasis on the pre-invasive stage, ductal carcinoma *in situ*, for example, by investigating the interaction between tumor cells and myoepithelial cells in collagen gels [46]; and *in vitro* method studies on cartilage repair, with an emphasis on osteoarthritis in elderly patients [47].

6.3.4.7 Dynamic Bioreactors

Bioreactors are devices designed to maintain cells or tissues under conditions where basic substrates (e.g., oxygen, glucose, amino acids) are supplied to, and waste products (e.g., carbon dioxide, ammonia, urea, lactate) are removed from, for example, liver cells maintained at high density in large 3-D constructs involving networks of hollow microfibre membranes, which provide the equivalent of a vascular network [48].

Liver bioreactors were originally developed to provide acute liver failure patients with an external artificial liver, to allow time for the self-regeneration of their own livers. Less-complex systems are now being developed, to provide higher throughput opportunities for studies on the biotransformation and elimination of xenobiotics.

Another dynamic bioreactor system is the human artificial lymph node (ALN), which provides an interface between a stationary network of antigen-producing cells and a population of suspended, highly migratory, lymphocytes, which is embedded in an appropriate environment with stromal cells and an extracellular matrix [48]. The ALN imitates the events that occur in the lymph node, when the flow of lymphatic fluid brings materials that may be antigenic and will induce a response. The input can reflect exposure to drugs, other chemicals and pathogens, and output, in terms of cytokine and antibody secretion and other relevant parameters, can be measured over an appropriate timeframe (e.g., 14 days). Theoretically, the cells loaded into the ALN could reflect human

polymorphisms, differences in susceptibility, and various epigenetic factors. The developers of the ALN are currently working on miniaturization, with a view to permitting higher throughput.

6.3.4.8 **Multi-organ Systems**

The maintenance of human organs *in vivo* relies on balanced interactions between tiny stem cell niches and surrounding, self-reliant sub-organoids, serviced by vascular networks of microcapillaries. The aim is to replicate multi-organ systems *in vitro*, in ways whereby interactions between them can be established that are relevant to what occurs *in vivo*. This includes the possibility of exposing different parts of the multi-organ system to drugs, chemicals, and other external influences, with the application of appropriate analytical techniques, so that local and systemic interactions and effects can be monitored and interpreted.

The systems in the course of development include Hurel™ [49] and the Integrated Discrete Multiple Organ Culture (IdMOC®) system [50]. Hurel involves a microfluidic circuit that links separate compartments containing cells representative of the equivalent tissues or organs *in vivo*. A culture medium circulates as a "blood surrogate," and the four compartments of a typical model contain equivalents of "liver," "lung," "fat," and "other tissues," and the physical features of the model reflect parametric values derived from PBPK models. Hurel permits pro-dug activation and metabolism, and it is possible, for example, to have an anti-cancer pro-drug metabolized in a "liver" compartment and its effects evaluated on cancer cells in another compartment, in a representation of a tumor-bearing human. As in Hurel, the different organ systems represented in IdMOC, for example, hepatocytes, kidney proximal cells, pulmonary epithelial cells, and vascular endothelial cells, are maintained independently and connected by microfluidic circulation. The system can be used to study organ-specific cytotoxicity, and also to create an *in vitro* model of a tumor-bearing man, as well as to use precision-cut slices from multiple organs, or the interactions between different cells from the same organ or *in vivo* system (e.g., small airway epithelial cells, bronchial epithelial cells and pulmonary microvascular endothelial cells).

The features of *in vitro* systems emphasized by Sbrana and Ahluwalia [51] are somewhat different. They said that:

> Researchers have only just begun to appreciate that the intricate interconnectivity between cells and cellular networks as well as with the external environment is far more important to cellular orchestration than are single molecular events inside the cell. For example many questions regarding cell, tissue, organ and system responses to drugs, environmental toxins, stress and nutrients cannot possibly be answered by concentrating on the minutiae of what goes on in the deepest recesses of single cells. New models are required to investigate cellular cross-talk between different cell types and to construct complex in-vitro models to properly study tissue, organ and system interaction without resorting to animal experiments.

They then described how tissue and organ models can be developed by using the multi-well-plate scale Quasi-Vivo® system, and discussed how such models can be used in drug toxicity studies. This system is based on the Multi Compartmental Bioreactor (MCB), and cell ratios and medium passage times are scaled to provide more-meaningful physiological relationships, avoiding some of the problems encountered when microfluidics, microfabrication, and miniaturization are pushed too far. Various cell types have now been incorporated, including hepatocytes, lung epithelial cells, intestinal epithelial cells, and endothelial cells.

6.3.4.9 **Challenge of Cells, Organs, and Organisms on a Chip**

In vitro test systems must not only be relevant and reliable, they must also be manageable and affordable, with an acceptable rate of throughput, especially if they are to take full account of human variation and contribute toward treating each patient as an individual case, rather than a member of a homogenous population. Humanization must be accompanied by miniaturization [48, 52], which means taking advantage of progress in microfluidic systems (lab-on-a-chip [LOC] or μ-TAS [micro-total analysis systems]), in the development of micro-electro-mechanical-systems (MEMS), leading to the manipulation of nanoliter to femtoliter amounts of fluids. Micro-organoids must be encouraged to develop in micro-niches, with micro-matrix equivalents and micro-vasculature, combined with micro-sensors. The costs of development will be high, but, given the imperative need for better methods for evaluating efficacy and safety in drug development, coupled with the complexity of diseases themselves and of patient populations, the rewards for success will be high. This is borne out by the huge commercial investment in these developments.

It would, perhaps, be wise to focus on medium-throughput, rather than high-throughput ambitions, since it could be said that the somewhat mindless screening of enormous numbers of compounds for highly specific properties or narrow interactions has not been very fruitful. That is the main thrust of a paper by Yuan Wen *et al*. [52], which focused on the contributions of microfabrication and chip-based technology to medium-throughput and high-throughput screening. The on-chip detections methods involve optical methods and electrochemical methods, combined with microplate technology. The advantages offered are short processing time, small space requirement, low cost, and portability.

The eventual goal is the human-on-a chip, but our reservations about the humanization of mice also apply here. Which humans will be on the chips? Ideally, there should be a lot of chips, to take full account of the variations within and between human populations, which can profoundly affect the fates of new drugs, as well as the well-being of patients.

6.3.4.10 ***In Vitro* Assays**

An *in vitro* toxicity test typically consists of a test system (i.e., a biological preparation to be used, such as a cell or organotypic culture), an endpoint to be assayed (such as inhibition of cell proliferation), an endpoint assay method (such as cell

counting or measurement of total protein or vital dye uptake), a formula for calculating the result (such as inhibition of proliferation compared with that in untreated control cultures), and a *prediction model* (PM, that is, an unambiguous algorithm for converting the result into the prediction of a pharmacotoxicological endpoint in animals or humans [53]). A straightforward example is that of using reconstituted human skin in a test for corrosivity: *if the viability of the skin preparation is <35% after treatment for 3 min,* (the PM says) *classify the test item as a severe corrosive; if the viability is <35% after treatment for 60–240 min, classify it as corrosive; if the viability is >35% after treatment for 240 min, classify it as non-corrosive*. PMs can also be used with physicochemical tests: for example, *if the pH of a 10% solution (w/v for solids, v/v for liquids) is <2 or >11.5,* (the PM says) *classify as corrosive*. The formal validation of an assay requires the establishment of the relevance and reliability of both the test system and the PM for their stated purposes.

The concept of the applicability domain, originally stated for *in silico* models, should also be applied to *in vitro* (and *in vivo*) tests. No test should be expected to provide information beyond that which it was designed to provide.

Tsaioun and Jacewicz [54] have described advances in predicting drug absorption, distribution, metabolism, excretion, and toxicity (ADMET) from *in vitro* assays. They discussed how this approach can be used early in preclinical development, to review the currently available lead-optimization and preclinical candidate selection stages, to eliminate weak candidates, and focus on a smaller number of candidates that are more likely to survive. ADMET profiling can also be used to provide relatively high-throughput screening, to identify interactions with drug-metabolizing enzymes, and, for example, to predict drug-induced liver injury via effects on mitochondria, and other mechanisms of toxicity, such as phospholipidosis, which can affect a wide range of tissues. It can also be extended to human-on-a-chip approaches, and to exploiting stem cell lineages.

6.3.4.11 Coordinated Approach with *In Vitro* Models: the Vitrocellomics Project

An excellent example of how modern developments in *in vitro* technology and other technologies can be focused on important issues in toxicology, has recently been provided by a report on the EU Vitrocellomics project [55]. The aim is to use hepatocytes produced from hESCs and human iPSCs, and human hepatocyte cell lines, such as HepG2 cells and HepaRG cells, for preclinical predictive testing for studies on ADMET drug properties. The relevance of the cells is established via a number of biomarkers and inducible CYP activities and transporters. A four-compartment bioreactor has been developed, based on a 3-D capillary network, to provide for dynamic perfusion culture of the hepatocytes. It is even possible to measure respiration in the individual wells of multi-well plates via O_2 sensors immobilized in each of the wells. The work is now being extended to incorporate other sensors, as well as metabolic flux analysis, to permit the early detection of toxicity.

6.3.5 High-Throughput Screening

Made possible by advances in robotics and computer technology, high-throughput screening (HTS) involves the rapid testing of huge numbers of compounds for selected activities or interactions with specific proteins, receptors or other cell components. Methods including drop-based microfluidics can now permit 100 million reactions to be conducted in 10 h [56]. In drug discovery, selection on the basis of HTS can be followed by lead optimization, which can involve the synthesis of new analogs with improved potency, reduced off-target activity, and properties indicative of manageable *in vivo* pharmacokinetics, as well as *in silico* analyses.

6.3.6 High-Content Screening

Originally developed as a drug discovery method, high-content screening (HCS) permits the evaluation of multiple biochemical and morphological parameters in cells [57, 58]. For example, fluorescent tags or fluorescent antibodies can be used to detect proteins of interest via the parallel use of spatially or temporally resolved methods, to provide multiple sources of quantitative information suitable for integrated analyses. Changes in cells in response to potential drugs or toxicants can be detected with high resolution in automated systems. HCS can be used, for example, to look at drug–cell surface interactions, signal transduction cascades, and effects on the cytoskeleton, and can be linked through phenotypic/visual screening to various omics and other approaches relevant to target identification and responses, and to investigating the significance of genetic polymorphism. Although slower than high-throughput screening, HCS offers much more information, and it can be linked to other approaches, such as ADMET profiling.

6.3.7 Omics Approaches

The sequencing of the human genome, and the development of a wide range of methods for application in molecular and cellular genetics has opened up dramatic new possibilities for increasing our understanding of human diseases and devising effective and relatively safe ways of managing them. Hence, *pharmacogenetics*, a rather descriptive approach to differences between individuals in terms of disease and their responses to drugs, has been supplanted by *pharmacogenomics*. This is dynamic approach to obtaining and using genetic information at the population level as a basis for drug design and development, then as a basis for the management of drug therapy by choice of drug and selection of dosing regimen, followed by monitoring of positive and adverse effects in the individual patient.

6.3.7.1 Variety of Omics

The omics approaches provide a wide range of tools with special uses, but which must be used in integrated and intelligent strategies. Just as in the world of Russell and Burch's Three Rs, where it is possible to come up with many other Rs, there seem to be an ever-expanding number of "omics," including: *cellomics* (about phenotype and functions at the cellular level); *cytomics* (distinguishable from cellomics by its application at the single cell level); *epigenomics* (about changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence); *interactomics* (about interactions and their consequences among proteins and other molecules within a cell); *metabolomics* (about the chemical processes involving metabolites), which is related to *metabonomics* (about quantitative, dynamic, and multiparametric metabolic responses); *pharmacogenomics* (a generic term, as referred to above); *phenomics* (about the functional biochemical and physiological characterization of cells, tissues, and organisms in response to genetic changes and environmental influences); *proteomics* (about the proteome, the entire complement of proteins, and about their individual production, modification, and functions); *toxicogenomics* (about responses to toxic substances); and *transcriptomics* (about all the types of RNA, including mRNA, rRNA, and tRNA, as applied to the total set of transcripts or a specific subset).

It is also possible to use the omics concept as a basis for considering the levels of organization in living material, by defining, not only the *genome*, the *transcriptome*, the *proteome* and the *cellome*, but also the *tissuome*, the *organome*, the *biolome* (about the whole set of entities in an organism; related to *biome*, the original term, which was overtaken as the generic term by genome, and hence to *biomics*); the *ecome* (about all the critical entities in an ecosystem), and even the *informatome*, and the *networkome*. The usefulness of this explosion of terms (and there are many more of them) is a matter for debate. However, it is worth noting that many biomedical scientists are not totally comfortable with the relationships between *cell*, *tissue*, and *organ*, and also that linking of considerations at the *genome* level via many of the other concepts to the *ecome* (i.e., to human populations and ecosystems involving humans) is particularly important.

6.3.7.2 Application of the Omics

The three most important omics are genomics (analysis of the full gene complement and the inherited genetic diversity), transcriptomics (what RNA is transcribed, as influenced by small interfering RNAs [siRNAs] and microRNAs [miRNAs] and many other factors, including genetic variation, and epigenetic and environmental influences), and proteomics (which also takes account of post-translational events, such as phosphorylation, glycosylation, folding, and membrane anchoring). Proteomics is less amenable to rapid, high-throughput screening, but it is potentially of the greatest use in relation to the nature and development of disease processes, whereas genomics is especially relevant in terms of human polymorphism related to susceptibility and responsiveness.

The HUPO project has been established to identify all protein isoforms in health and disease states in rodents and humans (http://www.HUPO.org). A sub-project,

the Human Brain Proteomics Project (HBPP), aims to characterize the human and mouse brain proteomes and to compare the data from mouse models of human disease with those from autopsy materials from human neurodegenerative disease patients. An important by-product of the project is to establish gold standards for the collection, exchange, and dissemination of proteomics data, in order to enhance the comparability of proteomics and biomarker studies.

The link to pharmacodynamics is especially important, and in metabolomics, a vital aspect of drug development and drug use, a number of analytical techniques can be used for metabolic profiling. Dealing with these analytical methods is beyond the scope of this chapter, but they include: Northern, Southern, and Western blotting; expressed sequence tagging; laser capture microdissection; mass spectrometry; microarray technology; molecular profiling; nanotechnology; the polymerase chain reaction; RNA interference; and single-nucleotide polymorphism. Deep sequencing should also be mentioned, as, in theory, the $100 whole genome sequence is with us, and the amount of information, both about coding regions of DNA, and possibly more importantly, about the "junk" DNA, which is increasingly being seen as important in regulating a whole variety of biological processes, will be both incredibly informative and frighteningly difficult to analyze.

Recently, Casciano [59] has reviewed the use of the omics technologies in model *in vitro* systems, with particular relevance to chemical-induced liver injury. Liver injury is one of the main reasons for the post-marketing withdrawal of drugs, largely because idiosyncratic adverse effects are not detected in preclinical testing, and in particular, in tests involving laboratory animals. Primary human hepatocyte cultures are the test system of choice at present, but their use is limited, partly by availability, and partly as a result of genotypic and phenotypic variation among the donors. Gene expression profiling by using DNA microarrays can be used to study normal gene expression in hepatocyte cell lines or in freshly isolated hepatocytes, followed by studies on alterations in gene expression in response to toxicants. For example, exposure to aflatoxin B_1 led to the increased expression of some genes in cultured hepatocytes, and to the decreased expression of others.

When there are indications that a compound could be toxic, the immediate question is *how toxic?* The answer to this question depends on many factors, among the most important of which is exposure, that is, size of dose and frequency of dosing. An interesting article by Wills and Mitchell [60] described how SimuGen are developing *in vitro* toxicogenomics systems, in order to be able to combine toxicogenomics and dose–response modeling, by combining the analysis of gene expression trends with *in vitro* assays to determine the degree of toxicity over a wide range of compound concentrations. By using compounds with well-described human safety concerns, in relation to liver necrosis, as a training set, it was possible to predict the relative doses at which the molecular events involved would become clinically significant in humans.

6.3.7.3 Handling Information Produced by the Omics

The omics technologies offer new approaches to human disease-related and drug-related processes by reducing humans and their responses to their component

parts. Each of the omics produces a different type of information, but these various types of data are inextricably linked. A key issue is how the vast amount of information so readily provided by machines can be collected, stored, and manipulated so that our understanding of fundamental processes is dramatically increased, leading to the earlier identification of adverse changes and effective means of avoiding or alleviating them.

Cluster analysis can be used to examine and collate information for each biochemical pathway. A number interaction databases have been developed, such as the Protein Structural Interactome Map (PSIMAP), the Database of Interacting Proteins (DIP), the Biomolecular Interaction Network Database (BIND), the Molecular INTeraction database (MINT), GeneQuest, Ingenuity Pathway Analysis (IPA), and GeneGo. These, in turn, provide the information for the human interactome map and other disease-specific and drug-specific biochemical maps, collated by using software such as Cytoscape. KEGG (Kyoto Encyclopedia of Genes and Genomes) PATHWAY maps also provide networks of protein interactions and information about the metabolism of xenobiotics of relevance to drug development. Thus, interactomics represents the final step in the shift from the traditional paradigm, where a reductionist view of individual biochemical pathways and events are considered in isolation, to an overview of the consequences of drug action on all the possible biochemical functions and networks in a heterogeneous cell (and human) population.

6.3.8
Systems Modeling and Simulation

6.3.8.1 Pharmacokinetic Modeling

Whatever the biological system under consideration, there are two potentially important outcomes when a bioactive compound interacts with it: activity and/or toxicity, as influenced by PD events and PK events. *In vitro* tests, *in silico* modeling, and the *omics*-based systems tend to concentrate primarily on potential pharmacodynamic events, but, if drugs are to be developed, and patients are to be helped and protected, the information provided by these approaches is of little value, unless key pharmacokinetic processes, and, in particular, ADMET, are fully taken into account.

The biological response to a compound depends on the applied (external) dose, and the concentrations that reach the target tissue for the desired effects and other tissues (tissue dose), where there might be adverse effects, how and to what extent it is altered (tissue–compound interaction), and how long the compound or its metabolites stay there to exert their pharmacological effects and/or their acute, sub-acute/sub-chronic or chronic toxic effects. Clearly, it is desirable to have information concerning the effective dose and the toxic dose and the quantitative relationship between them.

These questions are now approached through the use of biokinetic models, especially physiologically based pharmacokinetic (PBPK) models, a term which will be used to cover toxicokinetic models as well. The structure,

development, and application of such models has recently been reviewed by Lipscomb *et al.* [61].

PBPK model development takes account of ADMET on the basis of interrelationships among the critical determinants of these processes, such as tissue volumes, flow rates, rates of absorption, diffusion across membranes, tissue:blood partition, and rates and affinities for biochemical reactions. The model is designed to provide a representation of the intact organism, including routes of entry or uptake (via gastrointestinal tract, skin, lungs or blood), distribution (via bloodstream), target organs (e.g., liver, kidneys, brain), and storage sites (e.g., adipose tissue). Account can be taken of the differences between rapidly perfused tissues (liver, kidneys, brain) and slowly perfused tissues (muscle, skin). Equations can be derived for describing features such as tissue influx and efflux, hepatic metabolism, and renal clearance. The data through which the model is developed are obtained from the literature, from physicochemical considerations, from *in silico* approaches and *in vitro* tests, and from experiments on animals and, where ethically acceptable, from studies on humans or human preparations and tissues obtained from organ donors. PBPK models can be used to guide dose selection, as well as to identify responses to be looked for in later stages of the drug development process.

Once a model has been developed, it is subjected to a thorough assessment process, to evaluate its usefulness for its intended purpose. This involves consideration of its structure and parameter values, by using data not involved in the development of the model. Sensitivity, variability, and uncertainty analyses are also conducted. Lipscomb *et al.* [61] listed a number of specific aspects: the species used in model construction (e.g., the rat) versus the species of concern (e.g., man); the life-stage(s) considered; the exposure route and the exposure duration; the maximal dose considered and the plausibility of the internal dose estimations; and the specification of the model versus its intended end-use (e.g., the inclusion of an inter-individual variability factor).

Thomas [62] compared the challenge of using physiologically based simulation modeling to reduce and replace the use of laboratory animals in the discovery of new pharmaceuticals. The failure of animal studies to predict adverse effects in humans is one of the reasons for the post-marketing withdrawal of drugs, but 60–80% of the animals used in drug discovery and development (perhaps as many as 700 000 animals) are used in lead identification and lead optimization. He concluded that PBPK models are versatile simulation models, which could be ideal replacements for animal studies for predicting ADMET in humans, resulting in improvements in the prediction of human pharmacokinetics. Thomas has also advocated the more-active development and use of physiologically based pharmacodynamic (PBPD) modeling, giving as an example the benefits of using PBPK/PBPD models to elucidate the basis of the metabolism and toxicity of organophosphate pesticides in rats, mice and, humans.

6.3.8.2 **Virtual Tissue Modeling**

The first mathematical model of the working heart was produced by Denis Noble in 1960 [63], and a number of such models are now available. Holden has described

the reconstruction of the electrical activities of cardiac and uterine tissues by producing computer models in the form of virtual tissues [64]. The virtual tissues are biophysically and anatomically detailed, and provide quantitatively predictive models of the physiological and pathophysiological behavior of the tissues within the isolated organ. The cell excitation properties are quantitatively reproduced by equations that describe the kinetics of a few dozen proteins. The equations are derived from experimental measurements of membrane potentials, ionic currents, fluxes, and concentrations, some of which were taken from human cells and human ion channel proteins expressed in non-human cells, but most of which were taken from animal cells.

Data on tissue geometry and architecture are obtained from the diffusion tensor magnetic resonance imaging of *ex vivo* or *post-mortem* tissue, and are used to compute the spread of current in the tissue. Cardiac virtual tissues are well established and reproduce normal and pathological patterns of cardiac excitation within the atria or ventricles of the human heart. They can be used to increase understanding of normal cardiac electrophysiology, to evaluate the candidate mechanisms for re-entrant arrhythmias that lead to sudden cardiac death, and to predict the tissue level effects of mutant or pharmacologically modified ion channels. The human full-term virtual uterus is still in the course of development.

6.3.8.3 **Virtual Patient Populations**

Kleiman *et al.* [65] examined the novel concept of conducting virtual clinical trials for efficiently screening drug candidates, and for evaluating the prospect that they could be brought to the market successfully. The trials are carried out by using virtual patients called Optimata Virtual Patients (OVPs). The OVP is a set of mathematical algorithms that describe the main pathological and physiological dynamic processes affected by the administered drug, which has been shown to accurately predict the efficacy and safety of docetaxel in individual breast cancer patients. A test case has been reported, in which virtual clinical trials were conducted by using OVP populations for rescuing a discontinued oncology compound, ISIS-5132. The *in silico* study suggested that ISIS-5132 may be efficacious in combination with another drug, sunitinib malate, for the treatment of prostate cancer. The recommended combination treatment is predicted to result in a higher five-year Progression-Free Survival than therapy with either drug alone. This could be a way of improving the preclinical development of anti-cancer drugs, as well as unblocking the bottleneck of patient recruitment for clinical trials.

6.3.9
Biomarkers

The term "biomarker" can be used widely to encompass any indicator of biological relevance, but in the context of pharmacotoxicology, it is used more restrictively. Here, biomarkers are considered to be objectively measured and evaluated indicators of normal biological processes, pathogenic processes, and (desired) responses to or (adverse) effects of deliberate, incidental or accidental exposures to chemicals,

drugs, and other chemical products or to pathogens and biological products, such as vaccines. It is useful to distinguish between disease-related biomarkers and drug-related biomarkers, and biomarkers can also be subdivided into various categories, such as biomarkers of susceptibility, of exposure, of drug efficacy, of toxicity, or of patient response, or as diagnostic biomarkers. Another distinction is between imaging biomarkers and molecular biomarkers.

Among disease-related biomarkers are anti-citrullinated peptide antibodies (aCPAs) associated with rheumatoid arthritis, and somatic mutations in the KRas gene, which are associated with many forms of cancer. An elevated level of prostate specific antigen (PSA) has been used as a biomarker for prostate cancer, but this has not been very satisfactory, because PSA levels can vary dramatically among individuals. A promising new test has now been developed, based on the presence in the urine of Engrailed-2 (EN2), a protein specific to prostate cancer cells [66]. The test detects 60–70% of prostate cancers, but has a false-positive rate of only 4%. The PSA test detects only 40% of cancers, and also has a false-positive rate of 50%.

Drug-related biomarkers can be used, for example, as pharmacodynamic markers in early drug development studies, in proof-of-concept studies or as safety biomarkers.

Diseases involving the cardiovascular system are very common, and unsuspected cardiovascular system toxicity is the most common reason for post-marketing drug withdrawal, so a review by Vasan on biomarkers of cardiovascular disease was especially useful, both in relation to this particular clinical problem and to biomarkers in general [67]. Vasan discussed in detail the desirable features of biomarkers, and their validation and evaluation. Biomarkers are not, of course, immune from the influences of other factors, so there must be concern about false-positive associations with disease. The multi-marker concept must also be considered, since the use of a combination of biomarkers may improve the prediction of a certain risk. Hence, when a new biomarker is identified, the question may not be whether it is better than the existing biomarkers, but whether its use will improve the predictive accuracy of the best available model that incorporates previously existing biomarkers.

As Vasan made clear, since they represent biological phenomena, biomarkers are subject to variability, which can affect their performance. This is also affected, as are all the other technologies we are discussing, by the quality of the laboratory that is performing any analysis and testing involving biomarkers.

The principal way of discovering biomarkers is by the application of the omics approaches in the toolbox, linked with bioinformatics, but most importantly, also with clinical observations and analyses. Their development involves five main stages: target biomarker identification (relevant to a given disease or drug therapy); clinical characterization (in people with/without the disease or using/not using the drug); retrospective repository studies (on samples relevant to the disease or the use of the drug); prospective screening studies (to predict the occurrence of the disease or the effects of using the drug); and clinical use (in control of the disease or management of the use of the drug).

Corrias *et al*. [68] have reviewed the use of arrhythmic biomarkers for the assessment of drug cardiotoxicity, noting the variety of proposed biomarkers, the complexity of the mechanisms involved in the induction of the effects, and the existence of significant species differences. They also illustrated how computational modeling and simulation have contributed significantly to the understanding of cardiac electrophysiology and arrhythmia over the past 40 years, and how the multi-scale effects of drug-induced ion channel blocks in cells, tissues, and the whole ventricle can be simulated.

The FDA has now formally accepted seven renal safety biomarkers for use in the non-clinical and clinical stages of drug development [69].

Biomarkers should be specific to particular circumstances and processes, reliably measurable, and fit for use for a defined purpose. Potentially, they provide vital and exploitable links between all the elements in the new pharmacotoxicological technologies, from computer-aided drug design, via *in silico* modeling for efficacy and toxicity, through the use of the omics and medium-throughput and high-throughput screening, along with the use of *in vitro* systems at various levels of sophistication, to PBPK and PBPD modeling, and eventually to the investigation of polymorphism within the human population, as a basis for planning and monitoring therapies designed for the individual patient. For example, biomarker measurements in patients can be usefully linked to thresholds of positive pharmacological response and to thresholds of toxicological concern, not only to the benefit of the individual patient, but also, at earlier stages, as indicators of whether individual volunteers are or are not suitable for inclusion in clinical trials. Biomarkers are undoubtedly the key to personalized medicine.

6.3.10 Clinical Imaging

The clinical imaging of biomarkers is likely to be of increasing value, as they are non-invasive and can produce multi-dimensional results, both qualitative and quantitative, which can be used in association with information obtained by applying other technologies [70].

X-ray computed tomography (CT) has great promise, since, for example, it can produce a 3-D image of the heart and its blood supply from a series of 2-D images. There are, however, concerns about the adverse effects of radiation exposure from CT scans. It is estimated that 0.4% of current cancers in the United States are due to previous CT scans, and that this could increase to as much as 2% with 2007 rates of scanning [71]. Magnetic resonance imaging (MRI) can also be used to visualize internal structures, and is especially useful in showing what is happening in the brain tissues. Functional MRI can be used to look at the activity of the brain and to define relatively precisely what systems are working when a task is performed. It is also possible to see in which brain areas activity is altered, so it may eventually be possible to assist patients with Alzheimer's disease by selectively modulating, perhaps by stem cell transplantation, those parts of the brain that would improve their cognitive and memory function. MRI imaging can also be

used to see the type of body fat in which a drug accumulates, and this can be used to help patients to reduce excessive body weight, which is associated with a number of problems, including cancer, diabetes, and heart disease. Without this type of approach, a drug might accumulate in the wrong type of adipose tissue, so there would be little benefit to the patient.

Optical coherence topography (OCT), now typically employing near-infrared spectroscopy, can provide detailed images from within the retina; this can be used to assess axonal integrity in multiple sclerosis, and to visualize lipid-rich plaques in coronary arteries. Positron emission tomography (PET) is another way of producing images of what is taking place within the body. Used with the glucose analog, fludeoxyglucose, PET can be used to measure which cells in the body take up glucose, and this can throw light on sites of inflammation and early tumor development.

Thus, by applying clinical imaging techniques, the nature and progression of disease can be followed at the molecular level, within the human body. In drug development, it is possible to develop PET imaging strategies to visualize drug interactions with the targets on cells. This is valuable, for example, when treatments for major brain diseases are being devised, and an early question is whether the candidate drug can cross the blood–brain barrier. With modern imaging techniques, it is possible to see the molecule move into the brain and measure how much gets there. The next question is with what affinity the drug interacts with its target, and the answer can be used to work out a rational prediction of active doses. This reduces the number of subjects needed at this stage of drug development, thus contributing to a reduction of the overall costs.

Proton magnetic resonance spectroscopic imaging (PMRSI) can be used to interrogate biochemical activity in living tissues. A range of tissue constituents (e.g., *N*-acetyl aspartate, choline, glutamate, glutamine, GABA, inositol, glucose, lactate, high-energy phosphate) can be quantitatively analyzed. Measurements can now be made in volumes as small as 1 ml, allowing, for example, discrete areas of the brain to be metabolically visualized. There is great potential for using MR spectroscopy in combination with functional and anatomical imaging for the differential diagnosis and assessment of treatments for a wide range of neurodevelopmental and neurodegenerative disorders [72] and in peripheral organ disease [73].

It may soon be possible to visualize the histology within tumors, in ways which will help to guide the choice of therapy and give clues about the prognosis for the patient. At present, because anti-cancer drugs tend to be generally cytotoxic to many cells types, it is difficult to tell whether the treatment is being sufficiently effective against the cancer cells. Patients can be treated for months while gaining very little benefit. Better characterization of the cells within a tumor in a specific patient could lead to better drug selection and optimization of the treatment regimen.

Clinical imaging can also be linked to the other new technologies. For example, siRNA is an exciting new way of providing an entirely different type of treatment for patients, which could be targeted very selectively to a detrimental protein, for example, in a cancer cell. This potentially permits the therapeutic modification of a single protein, while not interfering with other parts of the cell function. New

ways of using PET may offer an approach to the quantitative measurement of how much siRNA is sticking in selected cells and where else it is going to in the body, which could allow clinical scientists to move rapidly from the consideration of dose selection to the prediction of possible efficacy.

6.3.11 Bioinformatics

Informatics, which, in its broadest sense encompasses information science and information technology (IT), is essential to the successful management of human activities of virtually all kinds, given the enormous amounts of information now available on all sorts of topics, not least through the Internet. At least three kinds of informatics technologies are involved in drug discovery, development ,and use: *cheminformatics*, the application of computer and IT techniques to problems in the field of chemistry; *health informatics*, where computer science and IT meet healthcare, and which includes *biomedical informatics* and *clinical informatics*; and *bioinformatics*, the application of computer science, IT, and statistics in molecular biology, especially in the management and analysis of data provided by the omics approaches.

Bioinformatics involves the use of computer-intensive techniques to search through vast amounts of data, on, for example, DNA sequences and protein sequences and structures, in order to increase understanding and facilitate problem solving, by identifying patterns and correlations, and creating algorithms (mathematical formulae consistent with expressions of finite lists of well-defined instructions as a basis for guiding data processing and automated reasoning). This can provide, for example, a basis for the comparative analysis of genome content, of gene expression, of gene mutation, of gene regulation, of protein expression, of network modeling, and of molecular design, in ways that facilitate the identification of applicability domains and prediction in models in *in silico* modeling and *in vitro* testing [74].

In a comment on the use of computer models in pharmaceutical safety evaluation, Boyer [75] raised some very important issues, which deserve to be taken into account in the application of all the available technologies. He emphasized that, in an environment of increasing data volume, the challenge is in the structuring and the analysis of these data, such that decisions can be made without excluding information or overstating their meaning. Informatics and modeling play a crucial role in addressing this challenge in two basic ways: (i) the data can be structured and analyzed in a transparent and objective way; and (ii) new experiments can designed with the model as part of the design process. Enhancing the use and impact of informatics and modeling on drug discovery is not simply a matter of increasing speed and memory capacity. The transformation of raw data to useable, and useful, information is a major scientific and technical challenge.

Not unexpectedly, many procedures and software tools and services are already available for application to bioinformatics, and there are many different ways of using them. Of greatest importance are the links with systems biology and clinical informatics, as a means of attempting to unravel the pivotal changes which under-

lie the etiology and progression of diseases, and to assist in the development of therapies for treating them, in ways that do not result in unacceptable side-effects and which one day will be applicable to individual patients.

6.4 Strategic Use of the New Technology Tools

The new technologies available to the pharmacotoxicologist undoubtedly offer challenging opportunities for tackling the problems confronting the pharmaceutical industry, and especially that of providing new and relatively safe ways of dealing with a number of serious and complex diseases that threaten the quality of life in rapidly aging human populations. In the evaluation of toxic hazard and the prediction of risk to humans, attention must be focused on the following: (i) the evidence that a given drug can cause adverse effect(s) at doses close to the therapeutic dose; (ii) the frequency of incidence of those effects in a given population; (iii) the degrees of severity of the effects; (iv) variations in susceptibility to and in the expression of the effects within the population and between populations; and (v) the epigenetic factors that can modulate them.

The pharmaceutical industry is not alone in having to face a challenging future. Other industries are also facing great difficulties as a result of the demand for better safety testing and less reliance on laboratory animal tests. The issue of chemicals regulation is dominated by the EU REACH (Registration, Evaluation, Authorization, and Restriction of Chemical Substances) system, which came into force in June 2007, and is managed by the European Chemicals Agency (ECHA) in Helsinki, Finland, in collaboration with the Competent Authorities in the EU Member States. The implications of the original REACH proposals caused great alarm to both chemical manufacturers and their downstream user customers. In addition, animal welfare organizations and scientists spoke out against the proposed checklist approach to hazard prediction based on tonnage, which would fail to take sufficient account of the nature of the chemicals themselves or of likely human exposure to them. There have been many political and administrative developments in relation to the REACH system proposals since they were first put forward in 2001, and there is now a vast and burgeoning literature on the subject, including thousands of pages of guidance from the ECHA. These issues were discussed in detail by Hartung [76], who said that the difficulties introduced by the REACH system were so very great that they could only be solved by the development and acceptance of non-animal tests and testing strategies.

We have long been calling for a revolution in toxicity testing based on new technologies [77], and for new approaches to risk assessment based on the different kinds of knowledge that are becoming available [78]. This is also the theme of the 2007 report of the US National Academy of Sciences on behalf of the EPA, entitled *Toxicity Testing in the 21st Century: A Vision and a Strategy*, which emphasizes the need for other industries to benefit from experience in the pharmaceutical industry, and to benefit from the emerging fields of systems biology and

bioinformatics [79]. Experience with chemicals and other products will also be of benefit to the pharmaceutical industry.

6.4.1 The Tools

The new technologies offer a variety of tools, which tend to reflect the reductionist approach on which progress in science is usually based: understanding a problem usually involves breaking it down into its component parts, then using the information gained to reconsider the whole issue or reconstruct the object of focus or concern. The biotechnology tools certainly reflect this. Most of them address issues at a lower level of organization than the intact human body, and they can offer answers to only a limited number of specific questions. Also, there are many of them, and their numbers and associated complexities and costs are continuously increasing. Thus, as when any craftsman is faced with a full toolbox of complementary tools, it is essential to use them intelligently, according to a scheme that reflects what they individually can or cannot offer. This should take into account the stage in development reached by the test item, to progress toward the completion of the job to be done, which itself has been clearly defined.

Individual tests can be duplicative or confirmatory (i.e., the result of one test can be used with a comparable result from another test to strengthen the conclusion reached about a particular toxic hazard). Alternatively, they can be additive or complementary (i.e., they can provide different kinds of information which, taken together, can support a particular conclusion, perhaps as part of a *weight-of-evidence approach*).

Tests should give clear answers to precise questions, appropriate to their applicability domains, expressed according to prediction models that link the results to the pharmacological or toxicological effect of interest.

A number of expert workshops have identified possibilities for new approaches to particular kinds of toxicity, including inhalation toxicity [80] and cardiotoxicity [81].

6.4.2 The Strategies

The strategies applied in using the tools should be clearly defined and scientifically justifiable, not least in terms of how the outcomes will be applied. The process of strategic design and application in drug discovery and development involves the selective use of *in silico*, *in vitro*, and *in vivo* systems to discern what benefits might be offered and what undesired side-effects *might* occur, if a new drug were to be introduced for use on a population scale. The pressing need is to devise better ways of discovering, during drug development, the *probability* that these benefits and adverse effects *would actually* occur.

The further optimization of this process will depend on all sorts of factors and inputs, which must be carefully considered as strategies are designed for applica-

tion in particular circumstances. They must also be manageable and affordable, and it must be recognized that there can be no standardized approach to suit all circumstances. It is particularly important that the generation of strategies for drug discovery and development is clearly different to that for other chemicals and chemical products. The body is deliberately exposed to drugs, which are powerful chemicals designed to have profound effects on body cells and tissues, in ways that are relatively well-understood, and according to dosing regimens which are defined and can be well-controlled. The *in vivo* effects of other chemicals and products range from "no effect" to "severe" or "lethal," depending on exposure, which is usually unintended, incidental, accidental, or occupational, and often difficult to quantify or control. This results in differences between consideration of benefit and risk. The side-effects of a drug may be considered acceptable if the benefit to the individual patient is sufficient, but the benefits of a pesticide in terms of food production for a human population are not so easily considered alongside the risk to the health of farm workers who apply it or to those who live in the neighborhood.

Some tests performed on industrial chemicals are intended to provide a basis for their classification and labeling, so that precautions can be recommended and exposure can be managed, limited or avoided. Thus, if potential neurotoxicity is predicted, classification and labeling can be decided, without the need to know about hepatotoxicity, nephrotoxicity, and so on. The need for further testing can also be decided according to annual production tonnage, as in the EU REACH system. It is also possible to apply other criteria, such as the use of limit tests when no observable adverse effects have been detected up to a test item concentration unlikely to be surpassed *in vivo*. Another important estimation with, for example, ingredients of cosmetics to be applied externally, is what proportions of an application are likely to be absorbed across the skin. If, as is frequently the case, the levels are low and are unlikely to approach thresholds of toxicological concern within the body, there is no need to evaluate many types of target organ or system toxicity.

These approaches would not be acceptable for pharmaceuticals, where information is needed on the potential effects of a compound on as many tissues, organs, and systems in the body as possible, as well as on the intended target. Knowing that the potential risk of, say, nephrotoxicity, was acceptable, would not be sufficient, as the risks of toxicity to other organs would also have to be evaluated.

The inescapable conclusion is that, while for chemicals and certain other products it may be possible to devise generalized integrated testing strategies [82–86], in the case of pharmaceuticals it would be more appropriate to focus on *intelligent* testing strategies, since the focus would need to be on individual purpose-designed schemes for highly specific situations.

6.4.3 Systems Biology

Discussions on drug development frequently mention a *systems biology* approach, that is, a multidisciplinary approach to considering the interactions between the components of a biological system and combining this knowledge to increase

understanding of the organism or of the phenomenon being considered [87]. In a way, this represents a holistic approach to combining the data provided by a variety of confirmatory or complementary reductionist approaches. Relatives of systems biology are *meta-analysis*, which combines parts of the results of several studies, and *evidence-based medicine* and *evidence-based toxicology* [88]. Critics of these approaches say, on the one hand, that they are obvious and already part of the normal scientific method, or, on the other hand, that they involve cherry-picking to take the best parts of inadequate studies in the hope of making the best of a series of bad jobs. Care must be taken to avoid that particular problem, but it is obviously necessary to have effective ways of analyzing all the available information and looking for clues as to what is likely to happen in the patient.

6.4.4 Involving the Patient

Most of the other chemicals and chemical products in use are not intended to be applied to or taken into the human body, so taking uncertain levels of exposure into account with potential hazard in risk assessment is a more uncertain process than is the case with drugs, where exposure is planned and controlled. In addition, whereas with other test items, there is uncertainty about the potentially exposed individuals in the population at large, the individuals exposed to drugs are readily known. This means that there should be an ever-increasing fund of knowledge about the actual benefits and side-effect costs to patients resulting from the drugs prescribed for them. This should include information on successful drug therapies, where efficacy has been balanced by manageable side-effects, and not merely on drug withdrawals. This information would provide a hugely powerful opportunity for evaluating tests based on the new technologies in the light of actual *human* experience. In the case of other chemicals and products, it has rarely been possible to escape the inadequacy of using animal test data as the "gold standard" to be met by alternative test procedures, even though it is known that the animal data are often unreliable and irrelevant for humans, thus truly representing more of a "fool's gold standard."

The new technologies are especially useful when they can be safely and ethically applied to human patients themselves, or at least to human cells and tissues, and body fluids. There is an urgent need to set up banks of various kinds, including not only human tissue banks to supply material for research use, but also banks of samples from patients who are suffering from specific diseases and/or have been treated with particular therapies. The latter type of bank would greatly facilitate the development and use of specific biomarkers of exposure, susceptibility, desired response, and adverse effect, which would greatly facilitate the development and use of *in silico* and *in vitro* systems, as well as affording a basis for treating individual patients.

The omics approaches will certainly be of increasing significance in drug discovery and development, as well as in clinical research and post-marketing surveil-

lance. However, attention must also be paid to other aspects of the patients to whom drug therapies are to be offered. For example, drug absorption and drug side-effects can be affected by other conditions (e.g., obesity), other diseases (e.g., diabetes), other drugs (via additive or antagonistic interactions), by dietary factors (e.g., alcohol, caffeine, cheese, fruit juices), and by immunological responses to allergens and infections. Ethnic, societal, geographical, and occupational factors must also be taken into account.

Vital to the technological developments and their successful application will be the translation of information from the laboratory to the clinic and back to the laboratory. In the future, population-based treatments, such as the universal use of a small number of antibiotics with a very large number of patients, will progressively be replaced by individualized treatments, for example, for cancer or cardiovascular disease patients. The drugs developed will be based on precise knowledge, rather than on intuition, and the markets for them will be smaller and themselves more targeted.

The availability of samples from individual patients could be particularly helpful, not only through the analysis of biomarkers before, during, and after treatment, but also through cell culture, including the use of iPSCs, to consider likely responsiveness to drug action and susceptibility to side-effects.

6.5 Translation as a Two-Way Process

Translational medicine involves not only the integration of research inputs from the basic sciences into improving patient care and introducing preventive measures, but also the social and political aspects of the provision of health services. Translational research investigates potential treatments, leading to tests for efficacy and safety and then to clinical trials. The new technologies offer many opportunities for improving the discovery and development process. However, many of the new technologies can also be safely and ethically applied to patients, and the flow of information from the clinic back to the laboratory is likely to be the key to progress in tackling some of the problems that currently confront scientists, industries, governments, and patients. Translation must be a two-way process.

A good example is that of drug-induced liver injury (DILI), the most common cause of the termination of clinical trials of new therapeutic compounds – the development of an estimated 33% of all new medicines is discontinued for this reason. DILI is also a major cause of the withdrawal of drugs from the market, well after they have been approved for use with patients. The failure to detect DILI at a sufficiently early stage results in both a huge financial cost for the pharmaceutical industry and a real human cost for the patient – 75% of the individuals who suffer idiosyncratic liver injury either die or require a transplant. The animal tests used in drug discovery fail to identify the potential for DILI in humans [2], and while computer modeling and advanced ADMET techniques have made great

advances over the past decade, the ability to predict DILI remains a frustratingly elusive target.

DILI is a key area of focus, not only for the FDA and the IMI, but also for a number of academic and research institutions and international collaborations. One such collaboration is the EU Vitrocellomics project [55], which involves the development of systems for preclinical predictive drug testing for metabolism and hepatotixicity, based on *in vitro* models derived from hESCs and human hepatocyte cell lines. It would be particularly useful, if hepatocytes could be routinely produced from iPSCs, since, not only could this provide a readily available source of hepatocytes on a large scale, but some of the iPSCs could be derived from human sub-populations with a greater susceptibility or greater resistance to DILI, or from patients who had already suffered adverse effects in the liver.

Another important collaboration is the International Drug-induced Liver Injury Consortium (iDILIC), which is studying the genetic susceptibility to idiosyncratic drug-induced liver injury, with a UK arm of the study, DILIGEN, funded by the Department of Health. One initiative involves collecting DNA from DILI cases and suitable controls for a Genome Wide Association Study (GWAS), with the aim of identifying polymorphisms predictive of the development of drug-related liver injury. This will open up the possibility of prevention by identifying patients at high risk of developing DILI, by means of a simple test performed before treatment with a particular drug begins. Some of the progress already being made via GWAS is summarized in Table 6.3.

There is no commonly adopted system for classifying drugs according to their DILI potentials, but Chen *et al.* [92] have now proposed a systematic and objective classification scheme, based on 287 drugs representing a wide range of therapeutic categories and daily dosages, that have been marketed for 10 years or more. They have provided a method for consistently and constantly annotating the ever-increasing number of drugs in the future, which promises to be of great value in drug discovery and biomarker development.

6.6 Concluding Comment

It is undoubtedly the case that, in relation to drug discovery and development, a large proportion of the "low-hanging fruit" has been picked, so the pharmaceutical industry is now inhabiting a more complex, more costly and more challenging environment. Nevertheless, as long as the sacred cow of animal testing is reconsidered objectively and intelligently, in the light of the rapidly developing, more human-focused, new technologies, there is every prospect that the invention and application of medicines will be re-invigorated, with consequent benefits to the industry and, more importantly, to the patients. The days of the animal-models-tell-us-all and one-drug-suits-all philosophies are over [93].

Table 6.3 GWAS as a tool for determining the cause of DILI.

Given the sporadic and idiosyncratic nature of DILI, genetic variability within the human population undoubtedly underlies a significant proportion of hepatotoxic events. GWAS allows the unbiased and simultaneous evaluation of the relationship between millions of single nucleotide polymorphisms (SNPs) and phenotypes.

The ability of GWAS to identify the underlying cause of DILI is exemplified by the results of the DILIGEN study [89]. This study determined the association of flucloxacillin hepatotoxicity with an SNP in the major histocompatibility complex region that predicted flucloxacillin DILI with an odds ratio of 80.6, $P = 9.0 \times 10^{-19}$. Such highly predictive loci are likely to increase in number over the next few years. However, while they are of great utility in screening patients prior to drug treatment, the role of GWAS analysis in drug development has not yet been explored to any great extent.

The major difficulty with such studies is the lack of any functional information regarding the majority of these SNPs. In addition, if a given SNP only predicts DILI for a single drug, the possibility of using such information for drug screening is markedly reduced.

However, recent studies have shown that, rather than increases in the likelihood of adverse drug events being due to single SNPs in the coding regions of genes, another class of SNP, the expression quantitative trait loci (eQTL), are likely to be far more influential. eQTLs are SNPs that affect the transcriptional abundance of a given gene or genes. A recent study indicated that eQTLs that regulate the expression level of a number of genes (>10) are more likely to be associated with drug-induced toxicity [90]. The identification of such "master regulator" SNPs increases the probability that certain eQTLs, by influencing a large number of genes, may be involved in DILI caused by a diverse array of drugs, rather than a single drug or drug class. The increasing availability of eQTL data in the public domain – the recently established Genevar database being a good example [91] – makes the use of these data achievable by both academic and industrial institutions. Once such influential eQTLs are identified, zinc-finger technology could be used to generate stem cell lines containing the required eQTL(s), and the required genotype could be confirmed, along with the overall genetic makeup of the cellular genome, by using next-generation sequencing. The production of bespoke cell lines containing defined eQTLs could then be used at a relatively early stage in the drug screening process.

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Part Two
Bringing the Drug into Action – From Downstreaming to Approval

7
Overview and Classification of Approved Recombinant Drugs

Theo Dingermann and Ilse Zündorf

7.1
Introduction

Recombinant drugs have come of age. Since the approval of recombinant insulin as the first recombinant drug in 1982 [1], some 120 molecules of the same class have joined the club of this new type of drugs, which has significantly enriched available therapeutic options (Table 7.1).

By definition all these drugs are proteins or peptides.

In a monograph entitled "Products of Recombinant DNA Technology" (Producta ab arte ADN recombinandorum), the *European Pharmacopeia* (7.0/0784) defines recombinant products as follows:

> Products of rDNA technology are produced by genetic modification in which DNA coding for the required product is introduced, usually by means of a plasmid or a viral vector, into a suitable micro-organism or cell line, in which that DNA is expressed and translated into protein. The desired product is then recovered by extraction and purification. . . .

According to the last word of the first sentence of this definition, recombinant products are always proteins. However, more importantly (according to the second sentence) recombinant drugs are defined based on their concrete production process. The new paradigm reads: "The product is the process."

While low molecular weight drug substances are sufficiently defined and characterized by chemical and physical properties, the definition of biotechnological drug substances includes, in addition, any relevant process parameter. These parameters have to be defined in advance, and based on these definitions the process is specified. Any deviation from these parameters causes an "out of specification (OAS)" result, which precludes approval, regardless of whether the quality of the drug substance is affected. This guarantees a remarkable quality and reproducibility of recombinant drugs, which is of utmost importance since proteins can undergo denaturation with the consequence of activity loss, just on the basis of

Pharmaceutical Biotechnology: Drug Discovery and Clinical Applications, Second Edition.
Edited by Oliver Kayser, Heribert Warzecha.

Table 7.1 Approved recombinant drugs.

No.	Brand name	INN	Molecule class	Expression system	Authentic/ modified	Fast acting/retarded
Angiogenesis inhibitor						
001	Avastin®	Bevacizumab	Antibody	CHO	Humanized	–
002	Lucentis®	Ranibizumab	Antibody	*E. coli*	Humanized/ fragment	–
Antiallergic agent						
003	Xolair®	Omalizumab	Antibody	CHO	Humanized	–
Antianemic agent						
004	Erypo® Eprex®	Epoetin alfa	Hormone	CHO	Auth.	–
005	Epoetin alfa HEXAL® Binocrit® Abseamed®	Epoetin alfa	Hormone	CHO	Auth.	–
006	Silapo® Retacrit®	Epoetin zeta	Hormone	CHO	Auth.	–
007	NeoRecormon®	Epoetin beta	Hormone	CHO	Auth.	–
008	Dynepo®	Epoetin delta	Hormone	Human cell line	Auth.	–
009	Biopoin® Eporatio®	Epoetin theta	Hormone	CHO	Auth.	–
010	Aranesp®[a)] Nespo®[a)]	Darbepoetin alfa	Hormone	CHO	Mod.	Retarded (sequence modification + additional glycosylation)
011	Mircera®	Methoxy-Polyethylenglycol-Epoetin beta	Hormone	CHO	Mod.	Retarded (Methoxy-PEG)

Antidiabetics (agents for regulating blood glucose homeostasis)

012	Insuman® Rapid Insuman® Basal Insuman® Comb 15 Insuman® Comb 25 Insuman® Comb 50 Insuman® Infusat Berlinsulin®H Basal Huminsulin Basal® Huminsulin Normal® Huminsulin Profil®	Human insulin	Hormone	*E. coli*	Auth.	–
013	Actrapid® Actraphane® Insulatard® Velosulin® Protaphane® Mixtard®	Human insulin	Hormone	*S. cerevisiae*	Auth.	–
014	Exubera®	Human insulin	Hormone	*E. coli*	Auth.	–
015	Humalog® Liprolog®	Insulin lispro	Hormone	*E. coli*	Mod.	Fast acting (sequence modification)
016	NovoRapid®	Insulin aspart	Hormone	*S. cerevisiae*	Mod.	Fast acting (sequence modification)
017	Apidra®	Insulin glulisin	Hormone	*E. coli*	Mod.	Fast acting (sequence modification)
018	Lantus® Optisulin®	Insulin glargin	Hormone	*E. coli*	Mod.	Retarded (sequence modification)
019	Levemir	Insulindetemir	Hormone	*E. coli*	Mod.	Retarded (sequence modification + fatty acid addition)

(*Continued*)

Table 7.1 (*Continued*)

No.	Brand name	INN	Molecule class	Expression system	Authentic/ modified	Fast acting/retarded
020	GlucaGen®	Glucagon hydrochloride	Hormone	*S. cerevisiae*	Auth.	–
021	Victoza®	Liraglutid	Hormone	*S. cerevisiae*	Mod.	Retarded (sequence modification + fatty acid)
Anti-infective agents						
022	Pegasys®	Peginterferon-alfa-2a	Cytokine	*E. coli*	Mod.	Retarded (PEG)
023	PegIntron® ViraferonPeg® Vitron®	Peginterferon-alfa-2b	Cytokine	*E. coli*	Mod.	Retarded (PEG)
024	Imukin®	Interferon gamm-1b	Cytokine	*E. coli*	Mod.	–
025	Synagis®	Palivizumab	Antibody	Murine cell line (NS/0)	Humanized	–
Antipsoriatic agents						
026	Raptiva®[a)]	Efalizumab	Antibody	CHO	Humanized	–
027	Amevive®	Alefacept	Fusion protein	CHO	Artificial	Retarded (AB-constant region)
028	Stelara®	Ustekinumab	Antibody	Murine cell line (SP2/0)	"Human"	–
Antirheumatic/anti-inflammatory agents						
029	MabThera®	Rituximab	Antibody	CHO	Chimerized	–

030	Remicade®	Infliximab	Antibody	Murine cell line (Sp2/0)	Chimerized	–
031	Humira®	Adalimumab	Antibody	CHO	"Human"	–
032	Simponi®	Golimumab	Antibody	Murine cell line (Sp2/0)	"Human"	–
033	Cimzia®	Certolizumab pegol	Antibody fragment	*E. coli*	Humanized	Retarded (PEG)
034	Enbrel®	Etanercept	Fusion protein	CHO	Artificial	Retarded (AB-constant region)
035	Kineret®	Anakinra	Ligand	*E. coli*	Mod.	–
036	ORENCIA®	Abatacept	Fusion protein	CHO	Artificial	Retarded (AB-constant region)
037	RoActemra®	Tocilizumab	Antibody	CHO	Humanized	–
038	Arcalyst®	Rilonacept	Fusion protein	CHO	Artificial	Retarded (AB-constant region)
039	Ilaris®	Canakinumab	Antibody	Murine cell line (Sp2/0)	"Human"	–
Antithrombotics, anticoagulants, fibrinolytics						
040	ReoPro®	Abciximab	Antibody fragment	Mammalian cell line	Chimerized	–
041	ATryn®	Antithrombin alfa	Antithrombotic	Transgenic goat	Auth.	–
042	Refludan®	Lepirudin	Hirudine derivative	*S. cerevisiae*	Mod.	–
043	Revasc®	Desirudin	Hirudine derivative	*S. cerevisiae*	Mod.	–
044	Angiox®	Bivalirudin	Hirudine derivative	Synthetic	Mod.	–

(*Continued*)

Table 7.1 (*Continued*)

No.	Brand name	INN	Molecule class	Expression system	Authentic/ modified	Fast acting/retarded
045	Actilyse®	Alteplase	Plasminogen activator	CHO	Auth.	–
046	Rapilysin®	Reteplase	Plasminogen activator	*E. coli*	Mod.	–
047	Metalyse®	Tenecteplase	Plasminogen activator	CHO	Mod.	Retarded (sequence modification)
Bone growth factors						
048	InductOs®	Dibotermin alfa	Bone morphogenetic protein	CHO	Mod.	–
049	Osigraft® Opgenra®	Eptotermin alfa	Bone morphogenetic protein	CHO	Auth.	–
Coagulation factors						
050	NovoSeven®	Eptacog alfa	Coagulation factor VII	BHK	Auth.	–
051	Recombinate® ADVATE® Helixate® NexGen Kogenate® Bayer	Octocog alfa	Coagulation factor VIII	CHO	Auth.	–
052	Refacto®	Moroctocog alfa	Coagulation factor VIII	CHO	Mod.	–

053	BeneFIX®	Nonacog alfa	Coagulation factor IX	CHO	Mod.	–
Complement factor inhibitor						
054	Ruconest®	Conestat alfa	Serum protein	Transgenic rabbit	Auth.	
Enzyme replacement in lysosomal storage disorders						
055	Cerezyme®	Imiglucerase	Enzyme	CHO	Mod.	–
056	VPRIV®	Velaglucerase alfa	Enzyme	Human cell line (HAT 1080)	Mod.	
057	Replagal®	Agalsidase alfa	Enzyme	Human cell line	Auth.	–
058	Fabrazyme®	Agalsidase beta	Enzyme	CHO	Mod.	–
059	Aldurazyme®	Laronidase	Enzyme	CHO	Auth.	–
060	Elaprase®	Idursulfase	Enzyme	Human cell line (HAT-1080)	Auth.	–
061	Naglazyme®	Galsulfase	Enzyme	CHO	Auth.	–
062	Myozyme®	Alglucosidase alfa	Enzyme	CHO	Auth.	–
Gonadotropic hormones						
063	Puregon®	r-Follitropin beta	Hormone	CHO	Auth.	–
064	Fertavid®	r-Follitropin beta	Hormone	CHO	Auth.	–
065	Gonal-f®	r-Follitropin alfa	Hormone	CHO	Auth.	–
066	ELONVA®	Corifollitropin alfa	Hormone	CHO	Mod.	Retarded (carboxyterminal peptide (CTP))

(*Continued*)

Table 7.1 (*Continued*)

No.	Brand name	INN	Molecule class	Expression system	Authentic/ modified	Fast acting/retarded
067	Luveris®	r-Lutropin alfa	Hormone	CHO	Auth.	–
068	Pergoveris®	r-Follitropin alfa/r-Lutropin alfa	Hormone	CHO	Auth./fixed dose combination	–
069	Ovitrelle®	r-Choriongonadotropin alfa	Hormone	CHO	Auth.	–
Growth hormone						
070	NutropinAQ®	Somatropin	Growth hormone	*E. coli*	Auth.	–
072	Zomacton®	Somatropin	Growth hormone	*E. coli*	Auth.	–
073	Humatrope®	Somatropin	Growth hormone	*E. coli*	Auth.	–
074	Norditropin NordiFlex®	Somatropin	Growth hormone	*E. coli*	Auth.	–
075	Genotropin® MiniQuick	Somatropin	Growth hormone	*E. coli*	Auth.	–
076	Saizen®	Somatropin	Growth hormone	Murine cell line C127	Auth.	–
077	Valtropin® Omnitrope®	Somatropin	Growth hormone	*E. coli*	Auth.	–
078	Increlex®	Mecasermin	Insulin-like growth factor 1 (IGF-1)	*E. coli*	Auth.	–
079	Somavert®	Pegvisomant	Growth hormone antagonist	*E. coli*	Mod.	Retarded (PEG)

Hemolysis inhibitor						
080	Soliris®	Eculizumab	Antibody	Murine cell line (NS/0)	Humanized	–
Immunomodulators in multiple sclerosis						
081	Betaferon®/Extavia®	Interferon beta-1b	Cytokine	*E. coli*	Mod.	–
082	Rebif®	Interferon beta-1a	Cytokine	CHO	Auth.	–
083	Avonex®	Interferon beta-1a	Cytokine	CHO	Auth.	–
084	Tysabri®	Natalizumab	Antibody	Human B-cell line	Humanized	–
Immunosuppressant for prophylaxis of acute organ rejection						
085	Orthoclone OKT®3[b)]	Muromomab	Antibody	Hybridoma cell line	Mouse	–
086	Simulect®	Basiliximab	Antibody	Murine myeloma cell line	Chimerized	–
Megakaryocyte growth and developmental factor						
087	NPlate®	Romiplastim	Peptibody	*E. coli*	Artificial	–
Mucolytic in cystic fibrosis						
088	Pulmozyme®	Dornase alfa	Enzym	CHO	Auth.	–
Osteoporosis therapeutics						
089	Forsteo®	Teriparatid	Hormone	*E. coli*	Mod.	–
090	Preotact®	Parathyroid hormone	Hormone	*E. coli*	Mod.	–
091	Prolia®	Denosumab	Antibody	CHO	"Human"	

(*Continued*)

Table 7.1 (*Continued*)

No.	Brand name	INN	Molecule class	Expression system	Authentic/ modified	Fast acting/retarded
Sepsis therapeutic						
092	Xigris®[a)]	Drotrecogin alfa	Antithrombotic agent	HEK293	Mod.	–
Tumor therapeutics						
093	Proleukin®	Aldesleukin	Cytokine	*E. coli*	Mod.	–
094	Beromun®	Tasonermin	Cytokine	*E. coli*	Mod.	–
095	Roferon®-A	Interferon alfa-2a	Cytokine	*E. coli*	Mod.	–
096	IntronA®	Interferon alfa-2b	Cytokine	*E. coli*	Mod.	–
097	MabCampath®	Alemtuzumab	Antibody	CHO	Humanized	–
001	Avastin®	Bevacizumab	Antibody	CHO	Humanized	–
098	Erbitux®	Cetuximab	Antibody	Murine cell line (SP2/0)	Chimerized	–
099	Zevalin®	Ibritumomab Tiuxetan	Antibody	CHO	Murine	–
029	MabThera®	Rituximab	Antibody	CHO	Chimerized	–
100	Arzerra®	Ofatumumab	Antibody	Murine cell line	"Human"	
101	Herceptin®	Trastuzumab	Antibody	CHO	Humanized	–
102	Vectibix®	Panitumumab	Antibody	CHO	"Human"	–
103	Neupogen®	Filgrastim	Growth factor	*E. coli*	Mod.	–
104	Biograstim® Filgrastim Hexal® Ratiograstim® Zarzio® Tevagrastim®	Filgrastim	Growth factor	*E. coli*	Mod.	–

105	Granocyte®	Lenograstim	Growth factor	CHO	Mod.	–
106	Neulasta®	Pegfilgrastim	Growth factor	*E. coli*	Mod.	Retarded (PEG)
107	Kepivance®	Palifermin	Keratinocyte growth factor	*E. coli*	Mod.	–
108	Fasturtec®	Rasburicase	Enzym	*S. cerevisiae*	Mod.	–
109	Thyrogen®	Thyrotropin alfa	Hormone	CHO	Auth.	–
Vaccines						
110	HBVAXPRO® Fendrix® Engerix®-B	Hepatitis B Vaccine	Antigen	*S. cerevisiae*	Mod.	–
111	Cervarix®	Human Papilloma virus vaccine	Antigen	Baculovirus expression system	Mod.	–
112	Gardasil® Silgard®	Human Papilloma virus vaccine	Antigen	*S. cerevisiae*	Mod.	–
113	Synflorix®	Pneumococcal conjugate vaccine	Antigen	*E. coli*	Mod.	–
114	Dukoral®	Oral cholera vaccine	Antigen	*Vibrio cholerae* strain 213 serotype Inaba	Mod.	–
Wound healing agent						
115	Regranex®	Becaplermin	Growth factor	*S. cerevisiae*	Mod.	–

a) Withdrawn.
b) Not recombinant, no longer available.

changes in the three-dimensional structure without any changes in the primary structure.

7.2 Classification of Recombinant Drugs from a Technical Point of View

It might be useful to subdivide recombinant drugs based on technological considerations into four classes:

- Class 1 recombinant drugs differ from authentic biomolecules due to technological concessions. These are the very early approved molecules, which had to be produced in *E. coli* since other expression systems were not available at that time. Typical deviations from the original are serine substitutions for cysteins in order to prevent the formation of improper disulfide bounds, an additional methionine at the protein's *N*-terminus as a eukaryotic leader peptide cannot be removed correctly in an *E. coli* expression system, and the complete absence of potentially present glycosylation structures.
- Class 2 recombinant drugs are more or less authentic copies of biomolecules. These correspond exactly to their prototypes with respect to amino acid sequence. They might, however, differ slightly with respect to post-translationally added sugar structures or other post-translational modifications.
- Class 3 recombinant drugs contain structural variations, which were deliberately introduced, mainly in order to improve pharmacokinetic properties. There are, however, also examples where pharmacodynamic characteristics are changed. An example of this type is Pegvisomant where the exchange of several amino acids in the human growth hormone results in an antagonist at the growth hormone receptor.
- Class 4 recombinant drugs are newly invented, artificial proteins.

Although this is not an official classification system, we will use it throughout this chapter to indicate the "authenticity," even though exact copies of the most common human form of a biomolecule for therapeutic use are not necessarily of value per se. In fact, most recently developed biologicals sometimes diverge remarkably from the original molecule due to challenges primarily related to the pharmacokinetics of recombinant drugs. While techniques for copying biomolecules have almost been perfected over time, the physiological delivery of these molecules has not even come close. This drawback can partly be overcome by structurally modifying biomolecules with intelligent approaches. This is possible, because we have learned that the immune system tolerates a lot more structural divergence than could have even most optimistically been foreseen.

7.3 Expression Systems

Recombinant proteins are produced in various expression systems such as bacteria, insect cells, yeast cells, mammalian cells, and even whole animals. Each expression system requires adopted expression vectors, which not only contain system specific expression signals but also signals determining the fate of the protein, whether it remains within the cell or whether it is secreted into the extracellular environment.

The first expression platform established was the gram-negative bacterium *Escherichia coli* (*E. coli*). It offers advantages such as relatively simple cultivation requirements and a short generation time. Disadvantages of this system are the lack of a glycosylation machinery and the fact that many expressed proteins accumulate in an inactive, insoluble form as inclusion bodies. This requires complex biochemical downstream processes in order to obtain active proteins. These inclusion bodies are composed of densely packed denatured protein molecules in the form of particles. Refolding of these proteins into bioactive structures is cumbersome, results in poor recovery, and accounts for a substantial amount of the downstream production cost for proteins expressed in *E. coli*.

On the other hand, *E. coli* produced proteins are much more homogeneous than proteins produced in other expression systems, mainly because of the lack of glycosylation, which always results in a complex set of slightly different structures. Therefore, some proteins, which are produced in *E. coli*, can be classified as class 3 proteins, for example, Rapilysin.

All other expression systems are able to modify proteins with sugar residues, the most important post-translational modification. Glycosylation of proteins is a highly complex post-translational modification process taking place in the Endoplasmic Reticulum (ER) and Golgi apparatus and involving more than 100 different proteins (and genes).

Although the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) adds glycostructures to *N*-glycosylation sites encoded by human genes, the resulting glycosylation patterns are so different from those of humans [2] that concerns were raised whether such an "artificial" glycosylation of recombinant products would induce a severe immune response in humans. Nevertheless, *S. cerevisiae* is meanwhile an established host organism for a number of recombinant proteins (human insulin, glucagon, the hirudin analogs desirudin and lepirudin, the urate oxidase rasburicase, the platelet-derived growth factor becaplermin, and the recombinant antigens in HBV and HPV vaccines), where the yeast glycosylation is not an issue in clinical practice.

In mammalian expression systems (human sarcoma cell line, Chinese hamster ovary (CHO) cells, Baby hamster kidney (BHK) cells, various mouse cells, whole animals (goat and rabbit)) glycosylation mechanisms are very similar, although details can vary. Also insect expression systems yield modified proteins with a mammalian type glycosylation pattern.

Some therapeutic proteins such as interferons alfa, beta, and gamma, interleukin 2, tumor necrosis factor alfa, and others, which are glycosylated in their natural human forms, turned out to be effective medicines, even though they were not glycosylated after expression in *E. coli*. Other proteins failed as therapeutics when not glycosylated. The most prominent in this group was erythropoietin, which when originally produced in *E. coli* was fully active *in vitro* but not efficacious *in vivo* [3]. The reason was an insufficient pharmacokinetic profile due to the absent glycosylation, and not a primary pharmacodynamic failure, as receptor binding itself is not entirely dependent on sugar modifications.

Mammalian cell systems have an additional advantage: recombinant proteins are secreted into the media in a natural form. This minimizes the contamination with host cell nucleic acids at the start of the purification process, and the technically demanding renaturation process from inclusion bodies is dispensable.

Frequently, a particular therapeutic protein is available from two different expression systems. Insulin and human growth hormone, for example, are expressed either in *E. coli* or in *S. cerevisiae*. Interferon beta is expressed in *E. coli* as well as in CHO cells. In this latter case, the final products even differ structurally. While the interferon beta (interferon beta-1a) produced in CHO cells is identical to the human original with respect to the amino acid sequence, the interferon beta (interferon beta-1b) expressed by *E. coli* lacks the *N*-terminal amino acid, carries a substitution from cysteine to serine, and is not glycosylated. Thus, interestingly, it is apparently not possible to anticipate superiority for the more "authentic" or "natural" version of the glycoprotein. A head-to-head clinical study, which compared the "artificial" interferon beta-1b and the "authentic" interferon beta-1a showed superiority in favor of the "artificial" interferon beta-1b [4]. However, definite proof that the one interferon molecule is indeed superior to the other cannot be deduced from this study as both drugs were given in different doses and at different dosing intervals. Interferon beta-1a induced less antibody formation and could therefore theoretically be regarded as better tolerated by the human body, however, improved safety has not been shown in clinical practice so far.

Additional production platforms ([5], and citations therein) for recombinant therapeutic proteins that are now used or will be used in the near future are given in Table 7.2. Drugs produced in mouse myeloma cell lines (e.g., several recombinant antibodies) or even in the mammary gland of a living goat (antithrombin III) or a transgenic rabbit (C1 esterase inhibitor), are already approved by the leading drug agencies (FDA, EMA). In developing countries, the first recombinant preparations are being developed or even approved by national authorities, which are produced in plant systems (tobacco, moss, carrot), in insect cell system (Sf9), or in human HEK293 or PERC.6 cells, and others are likely to follow [6, 7].

Nevertheless, expression systems used for the production of approved recombinant drugs do not reflect by far the technological potential that is available in practice. This reflects the enormous efforts that need to be undertaken in order to resolve safety concerns by the authorities. Therefore, manufacturers prefer to rely on well established, generally accepted but certainly not the most effective,

Table 7.2 Characteristics of production platforms for recombinant therapeutic proteins. Modified from reference [5].

Expression system	Classification	Development of system	Disulfide bonds	Glycosylation	Secretion	Cost of fermentation	Use of antibiotics	Safety costs	Processes developed	Product on market
E. coli	Gram-negative bacterium	Completely developed	(Yes) in the periplasm	No	Periplasmic secretion	Promoter–dependent, low to moderate	Typically required	Low costs	Industrial scale	Yes
Saccharomyces cerevisiae	Budding yeast	Completely developed	Yes	Yes; high mannose	Possible	Low	Not required	Low costs	Industrial scale	Yes
Pichia pastoris	Methylotrophic yeast	Completely developed	Yes	Yes; no terminal α1,3-mannose	Possible	Low	Not required	Low costs	Industrial scale	Yes
Hansenula polymorpha	Methylotrophic yeast	Completely developed	Yes	Yes; no terminal α1,3-mannose	Possible	Low	Not required	Low costs	Industrial scale	Yes
Yarrowia lipolytica	Dimorphic yeast	Early stage	Yes	Yes; exact features yet unknown	Possible	Low	Not required	Low costs expected	Lab scale	No
Plant cells	Higher eukaryote	Completely developed	Yes	Yes; terminal fucose	Possible; size-restrictions	Moderate	Not required	Low costs	Pilot scale; production scale	Yes (Cuba)
Mammalian cells (e.g., CHO)	Higher eukaryote	Completely developed	Yes	Yes; typically human-like	Usually	High	Not required	High costs	Industrial scale	Yes
Animals	Mammals	Completely developed	Yes	Yes; typically human-like	Usually	Farming; moderate costs	Not required	High costs	Industrial scale	Yes

expression systems rather to optimize their production with considerable risks and relevant costs.

None of the established expression systems has general advantages in all situations and for all applications, and the choice of an expression system has to be made on a case-by-case basis, sometimes with rather unexpected outcomes. Epoetin delta, for example, which is produced "authentically" in a human fibrosarcoma cell line, seems to cause notably more cardiac events than the other approved epoetins, which are produced in CHO cells and are therefore slightly different to the human epoetin [8]. Meanwhile Epoetin delta has been withdrawn from the market, which again underscores that "authenticity" is not of value per se.

From a safety point of view, it would theoretically be best to choose an expression platform that is phylogenetically most distant from humans as human pathogens cannot contaminate those systems. If glycosylation is required, mammalian expression platforms are generally favorable, such as CHO- or BHK-cells. Lately, attempts have been made to modify other platforms such as *S. cerevisiae* or plants, by metabolic engineering [9, 10]. Such modified organisms are enabled to introduce human-like sugar moieties, but molecules that are derived from these platforms have not yet been approved by authorities.

So far one protein (Cevarix, an HPV antigen) has been approved, which is produced in insect cells infected with a modified baculovirus vector. This expression system yields mostly soluble proteins, which are readily modified and processed similar to the authentic human counterparts.

The use of mammalian cells for protein production allows all post-translational modifications and protein processing steps of higher eukaryotic cells. However, cultivation of these cells is time consuming, technically complex, and therefore relatively costly.

7.4 Proteins Derived from Modified Genes

As already mentioned, authenticity of recombinant drugs is no longer regarded as an important advantage. In fact only a minority of all approved recombinant drugs correspond strictly to their "authentic" counterparts.

Besides the fact that early products, which were produced in *E. coli*, differed from the "original" due to technological concessions (e.g., lack of glycosylation, substitution of cysteine by serine, *N*-terminal amino acid addition or *N*-terminal amino acid loss), an important driver for intentionally introduced structural changes are the pharmacokinetic characteristics. Regardless of the choice of the production platform, we are still far away from (and may probably never be able to) applying recombinant proteins in exactly the same way as the body delivers them. Insulin, for example, is subtly secreted from the pancreatic beta-cells upon physiological stimuli, whereas for the treatment of diabetes it is administered by a syringe subcutaneously in a very high millimolar concentration. This concentration forces recombinant insulin into hexameric aggregates, which are unable to bind to the

receptor. Receptor binding can only occur after dilution by diffusion, with the consequence that patients are advised to anticipate an efficacy lack for 15–30 min post-injection. This problem, which clearly interferes with patient compliance, was elegantly solved by slight structural modifications at the C-terminal region of the B-chain of the protein core. The resulting second-generation (class 3) fast-acting insulins were Humalog™ (insulin lispro), NovoRapid™ (insulin aspart), and Apidra™ (insulin glulisin). These fast-acting insulins are functionally complemented by long acting insulin derivatives, again based on intelligent structural modifications, either by changing the solubility at physiological pH (Lantus™ (insulin glargin)) or by removing an amino acid at the C-terminal end of the B-chain, which allows a post-translational addition of a fatty acid at this position (Levemir™ (insulin detemir)). This in turn generates a high albumin affinity of the modified insulin from which it is slowly released. It is highly plausible that combinations of fast- and long-acting insulins will prove beneficial in the long term, not as much based on molecular superiority, but based on superior patient compliance [11].

Another example of deliberate change from "authenticity" is Tenecteplase (a modified tissue specific plasminogen activator, tPA). In Tenecteplase, the plasminogen activator inhibitor-1 (PAI-1) binding site Lys-His-Arg-Arg was replaced by an Ala-Ala-Ala-Ala sequence. This resulted in a significantly longer biological half-life compared with native t-PA due to greater resistance to biological inactivation [12].

Similarly, Reteplase is an *N*-terminal truncated tPA-version; in addition, it is not glycosylated since it is produced in *E. coli*. This second generation tPA was approved on the basis of favorable pharmacokinetic considerations, although the authentically produced human tPA Alteplase was already on the market [13].

7.5 Artificial Proteins

Etanercept was the first in a growing group of class 4 artificial proteins. Most of these proteins follow the interesting concept of applying soluble receptors in order to purge pathological proteins or to block docking sites for cell–cell interactions.

Etanercept for example, contains a soluble TNF-alfa receptor, which is indicated for the treatment of rheumatoid arthritis. This plausible concept failed until the soluble receptor part was fused to the constant region of a human antibody. Human antibodies belong to the most stable human proteins with a biological half-life of up to months. This fusion concept solved the pharmacokinetic problem associated with a pharmacodynamically well functional receptor molecule.

We will see a great variety of such third generation biotechnology products as fragments from authentic human proteins in the future, many of them being antibody fragments or antibody-derived peptides. The other approved examples are:

- **Abatacept**, a fusion protein that consists of the extracellular domain of human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) linked to a modified Fc

portion of human immunoglobulin G1 (IgG1). Abatacept selectively modulates a key costimulatory signal required for full activation of T-lymphocytes expressing CD28. Full activation of T-lymphocytes requires two signals provided by antigen presenting cells: recognition of a specific antigen by a T-cell receptor (signal 1) and a second, costimulatory signal. A major costimulatory pathway involves the binding of CD80 and CD86 molecules on the surface of antigen presenting cells to the CD28 receptor on T-lymphocytes (signal 2). Abatacept selectively inhibits this costimulatory pathway by specifically binding to CD80 and CD86.

- **Rilonacept**, a dimeric fusion protein consisting of the ligand-binding domains of the extracellular portions of the human type I interleukin-1 receptor (IL-1RI) and IL-1 receptor accessory protein (IL-1RAcP) linked in-line to the Fc portion of a human IgG1. Rilonacept binds to and blocks the activity of the cytokine IL-1 and binds both IL-1β and IL-1α, which are the primary pro-inflammatory cytokines implicated in many inflammatory diseases. Rilonacept also binds the endogenous IL-1 receptor antagonist (IL-1ra) but with a lower affinity than IL-1β or IL-1α.
- **Alefacept**, a dimeric fusion protein consisting of the extracellular domain of the human leukocyte function antigen-3 (LFA-3) linked in-line to the Fc portion of human IgG1. So far only the FDA has approved Alefacept.

Another, fairly similar molecule is Romiplostim. In this case the Fc portion of an antibody is not fused to the extracellular part of a membrane receptor or a soluble receptor but to a ligand mimetic. Romiplostim is a dimeric fusion protein consisting of a short peptide with two thrombopoietin receptor binding domains coupled via a glycine bridge and linked in-line to the Fc portion of a human IgG1. Although the receptor binding domains have no sequence homologies to human thrombopoietin, Romiplostim acts as an agonist and increases platelet production.

Corifollitropin alfa is a modified follicle-stimulating hormone (FSH), which may be sorted somewhere between proteins derived from modified genes, the totally artificial proteins, and the post-expression modified recombinant proteins (see below). Follitropin is a heterodimeric protein consisting of the glycohormone α-chain, which is also present in the luteinizing hormone (LH), the human chorionadotropic hormone (hCG), and the thyreotropic hormone (TSH) and an FSH-specific β-chain. Interestingly although very similar in their overall amino acid sequence, the biological half-life of LH and hCG differ quite a lot, due to a carboxyterminal peptide (CTP) of 28 amino acids within the β-chain of hCG. By adding the CTP-sequence to the FSH-specific β-chain, the elimination half-life of corifollitropin alfa is almost doubled compared with recombinant follitropin alfa. Within the CTP-sequence, four additional O-linked glycosylation sites are located, resulting in a higher sialic acid content within the carbohydrate moieties, providing more protection from hepatic uptake by the asialoglycoprotein receptors present on liver cells.

7.6
Post-expression Modifications of Recombinant Proteins

Not only modifications in the polypeptide chain but also post-expression modifications can provide the basis for class 3 biotechnology products. Modifying proteins with polyethylene glycol (PEG) chains, with additional sugar chains, or with fatty acids changes pharmacokinetic properties significantly, and frequently for the benefit of overall therapeutic efficacy. For example, PEGylated interferon alfa variants, with a prolonged plasma half-life, clearly proved to be therapeutically superior in treating chronic hepatitis C and B infections compared with unmodified alfa-interferons [14].

In Darbepoietin, two amino acids were replaced by asparagine residues resulting in two additional sites for *N*-linked glycosylation. The modified molecule has a lower receptor affinity compared with unmodified erythopoietins but a significantly longer biological half-life. The overall therapeutic efficacy appears to be superior for the modified erythropoietins [15, 16]. Darbepoietin has a higher sialic acid content, providing more protection from hepatic uptake by the asialoglycoprotein receptors present on liver cells.

Other modification strategies use albumin fusions, fusions with so-called PAS-polymers (peptides consisting of random sequences of amino acids proline, alanine, and serin – PASylation) [17], or conjugation with hydroxyethyl starch (HESylation).

An interesting case in terms of post-translational modification is the substitution drug glucocerebrosidase. Therapeutic efficacy turned out to be dependent on a complex but also artificial sugar modification. Glucocerebrosidase is a lysosomal enzyme, which is usually delivered through an intracellular delivery system starting at the Golgi apparatus and ending in the lysosomes. Gaucher disease is characterized by an inborn deficiency of this enzyme and it needs to be intravenously applied to reach the macrophages (the main site of pathological lysosomal accumulation of glucocerebrosides) in sufficient quantities. Surprisingly, this could not be accomplished with the natural form of glucocerebrosidase. However, as soon as the sugar moieties of the glycoprotein were trimmed to expose mannose, glucocerebrosidase modified in such a way was able to achieve therapeutic concentrations in target cells (monocytes and macrophages). Therefore, a complex biochemical modification of recombinant glucocerebrosidase sequentially using three specific glycosidases (neuraminidase, β-galactosidase, and *N*-acetyl-glucosaminidase) is required to obtain mannose-terminated glucocerebrosidase (Imiglucerase), which has proven to be a highly safe and successful therapy in patients suffering from Gaucher disease [18].

An interesting variation toward monocyte/macrophage uptake of recombinant glucocerebrosidase is achieved in case of Velaglucerase alfa [19], which is produced in a human cell line in the presence of specific glycosylation inhibitors. This ensures that glycosylation chains terminate artificially with mannose residues.

7.7 Biosimilars

Recombinant proteins are generally recognized as innovative pharmaceutical products. However, an innovation is finite; it is no longer an innovation when its patent protection expires. Although determining a definite patent expiry date for a protein product can be extremely difficult, it is clear that several recombinant drugs are currently patent-free and many others are set to follow. In principle, this provides the legal basis for follow-on products, which can be seen roughly as generic versions of original biopharmaceuticals. The question is whether they present more risks than opportunities. From a pharmaceutical point of view, they definitely offer more opportunities than risks, provided a well-designed legal framework is in place!

There is an ongoing debate about whether copying a recombinant protein for clinical use might be generally possible [20]. Of course it is! Clearly Europe is breaking the path in this respect while the United States are still struggling.

A biosimilar does not necessarily have to be a perfect copy of an original [21]. At least in Europe it is now widely understood that a biosimilar is similar but not identical to its reference molecule. This makes a lot of sense since the alternative would simply be impossible. Even originators have to make process changes and indeed they are allowed to do so. This would not be possible if the original "dogma" in its strict sense was still in place.

In addition, variations in the structure of biomolecules are nothing unusual. Even when isolated from any human source, biomolecules show remarkable structural heterogeneity if one looks carefully using up-to-date analytical tools and techniques. In fact, typically, "proteins" are more or less complex mixtures of similar molecules, particularly – but in no way exclusively – when it comes to carbohydrate modifications.

Nevertheless, one can easily identify a purified fraction as insulin, erythropoietin, beta-interferon or as an antibody with a certain specificity. There is absolutely no doubt that a molecule identified as insulin will act like insulin, a molecule identified as erythropoietin will act like erythropoietin, a molecule identified as beta-interferon will act like beta-interferon, and a molecule identified as an antibody with a certain specificity will act like such an antibody – no matter whether it is *in vitro* or *in vivo* in a patient.

On a simplified basis, the manufacturing process for biologics can be divided into six stages: host-cell development, master-cell bank establishment, protein production, purification, analysis, and formulation. Hardly any of these stages are or even can be identical between the reference and the "copy." But should they be? Is there just one optimal solution for a highly complex problem? Of course not! What is necessary, however, is that a particular solution has been rigorously tested as safe.

The challenge when setting up a manufacturing process for biologics lies in its requirement for robustness. It is usually no problem if an original and a similar

copy will differ slightly. However, it would be a problem if proteins from various batches differed significantly.

Consequently, compared with the manufacture of organic compounds, the production of biologics requires far greater stringency and documentation, including a larger number of batch records, more product quality tests, more critical process steps, and more process data entries. On the other hand, improvements in the availability and sophistication of analytical techniques allow a thorough description of all chemical and physical aspects of the molecules as well as possible contaminations and impurities.

Although the degree of sophistication of current analytical tests has improved tremendously, the fact that the safety and efficacy of biosimilars need to be defined independently in formal clinical safety and efficacy trials is still demanding. It is known that even slight structure alterations can alter key parameters such as stability, resistance to degradation, circulatory half-life, biological activity or the potential for adverse immune reactions in patients, although it is even more probable that none of these concerns will be observed. However, given the general unpredictability of biologics in humans, stringent post-marketing monitoring studies are also inevitable.

There are extremely important differences between an innovator molecule and a biosimilar:

- By definition, the innovator molecule has never been tested in humans. Even though the molecule has been developed on the basis of strong pharmacological plausibility, clinical trials nevertheless still have to prove its clinical benefit. Examples are known where new biomolecules have failed these tests.
- Many biologicals cause a complex cascade of responses and it is impossible to predict whether these will be tolerated by all patients. Consequently, side effects can always jeopardize the successful introduction of a biomedicine. A biotech product will only be successful if benefits clearly outweigh potential risks.

In the case of biosimilars, all this is known from the clinical performance of the reference drug:

- the reference drug has a long clinical history and has proven its clinical efficacy;
- the reference drug apparently has a positive benefit/risk ratio, and
- its immunological activity is acceptable.

On the other hand, what needs to be shown in the case of a biosimilar is:

- the degree of efficacy and safety of the copy, which most likely differs to some extent from the original.

Nobody realistically doubts these requirements.

Most biotech drugs are very expensive, but this can only partly be explained by the cost of the actual goods. The fact is that it is extremely risky to develop such drugs, mainly because of the uncertainty related to the drugability of a particular disease with a biotech drug. Once drugability has been demonstrated, competitors enter the market even though the original molecule is still patent protected. This is possible since in many cases a whole variety of different proteins can solve the same clinical problem. For example, five structurally very different TNF-alfa antagonists compete in the market for chronic inflammatory diseases and several more are in the advanced pipeline. All these molecules are innovations as they are all structurally very different. However, all have turned out to be safe and more or less efficacious, and consequently, all have been approved by authorities in Europe and the United States.

Why not add another molecule (or more), which is more or less an exact copy of an existing one, which has become patent-free? Such a molecule can be developed without the drugability of a particular disease being an issue and it should therefore turn out to be significantly cheaper than the original where such a risk was part of the serious calculations.

Although biosimilar proteins arise from separate manufacturing processes and differ in master cell line, processing, and purification, they can be constructed sufficiently similar to an approved product to permit the applicant to rely on certain existing scientific knowledge about the safety and effectiveness of the approved protein product.

Prices of biotech drugs must come down for the sake of patients and for the affordability of our healthcare systems, based on the solidarity principle. Competition after patent expiry is one reasonable tool.

Innovators should concentrate on innovations, and indeed the potential for innovations is enormous. Many unmet medical needs are waiting for innovative intervention options and there is always potential for improving the characteristics of specific molecules towards second or third generation drugs.

The concerns of opponents of an accelerated approval procedure for biosimilar protein products remain ill founded. They have argued that regardless of rigorous physicochemical characterization of the protein, even small and seemingly insignificant manufacturing changes could theoretically contribute to differences in protein folding, aggregates, and glycosylation, which might manifest clinically as decreased efficacy, altered pharmacokinetics, or increased immunogenicity. This could indeed happen and additional clinical testing to prove the safety and efficacy of products was, therefore, put in place before market authorization was granted.

Biosimilar products are now a reality – at least in Europe – and opponents of an accelerated approval procedure for biosimilar protein products have been proved to be wrong. Biosimilar protein drugs are marketed without unintended effects – clearly demonstrating that safety is not an issue, so long as appropriate regulatory supervision is in place.

This is an opportunity for seriously ill patients and for our healthcare systems also.

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8
Downstream Processing

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8.1
Introduction

Biomanufacturing involves an upstream production phase, typically using microbial or mammalian cells to produce a target molecule such as a protein, followed by downstream processing (DSP) in which the target product is extracted from the biological matrix and purified. This chapter provides a brief but comprehensive overview of the principles, practice, and recent trends in downstream processing, which can be defined as the part of the biomanufacturing process that begins when the biological host is no longer required. Therefore, whereas upstream production depends on biological parameters (e.g., cell density, cell division rate, and the intrinsic yield of the target product), DSP is based purely on biochemical and physical processes, aiming to separate the target product from the rest of the feed stream. DSP can therefore be defined as the series of operations that takes the complex output material from upstream production and yields a stable, pure product [1].

DSP cannot be undertaken in a single step because biological material is extremely complex, that is, each target product is mixed with a large number of non-target molecules with vastly differing properties. DSP therefore involves the stepwise removal of impurities, first according to bulk chemical and physical differences and then according to increasingly minor dissimilarities between the product and contaminants, incrementally leading to a pure and homogeneous target product. For this reason, DSP is typically divided into four stages – harvesting, clarification, capturing, and polishing. Harvesting [2] involves bulk separation of the biological material containing the desired product from the remainder of the upstream process stream. For example, where a protein is expressed in microbial cells, harvesting would involve the separation of cells from the culture medium, the latter being discarded. However, where the product is secreted, the cells would be discarded and the medium, containing the desired product, would be retained. Clarification [2] is the process of removing particulates and other bulk contaminants such as lipid droplets, so that the feed stream is a clear aqueous solution and the target product is dissolved. Where the product is secreted into the culture

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medium, clarification is integrated with harvesting and usually involves a simple filtration step. However, for intracellular products, harvesting must be followed by cell or tissue disruption, and clarification may then involve multiple steps to remove large amounts of particulate material and other contaminants. Clarification may also involve a series of renaturation and refolding steps, such as pH adjustment or the modification of feed stream conductivity, to ensure that the target molecule is soluble [3].

The next stage depends on the nature of the target product. In many cases it is appropriate to perform a capture step, which simply means that the target product is retained by binding to a particular ligand and contaminants are discarded in the flow-through. Capture ligands range in their selectivity, with affinity ligands being the most selective because of their highly specific interactions with the target. For example, many processes involve the purification of antibodies and a number of generic capture processes have been described based on Protein A chromatography [4]. This removes 99% of contaminants including excess water, but even the most selective capture steps are not 100% efficient and trace contaminants must be removed by polishing.

Polishing [5] is typically the inverse of capture; that is, the target product is released while contaminants are captured by binding to a particular ligand. Because contaminants are diverse, the best ligands for polishing are less selective than capture ligands and bind many molecules with similar properties. Where affinity-based capture is not possible, polishing may occur in several steps (intermediate polishing) to remove broad classes of contaminants. Where an efficient affinity capture step has been included, final polishing steps are used to remove trace contaminants, such as residual host cell proteins (HCPs) and nucleic acids, as well as process-derived contaminants, such as leached Protein A, and product-derived contaminants, such as aggregates and fragments. Polishing usually involves one or two orthogonal chromatography steps to remove contaminants that are very similar to the target product in either mass or charge, and this also fulfills the function of virus removal, since viruses are charged proteinaceous entities with similar properties to larger HCP and nucleic acids. However, additional virus removal and inactivation steps are included to ensure high log reduction values (LRVs) for viruses, and to make the process comply with Good Manufacturing Practice (GMP). These steps may include an acid hold (storing an acidified feed stream between processing steps) and dead-end filtration to ensure the removal of even the smallest viruses [6].

8.2 General Principles of DSP

Biomanufacturing is challenging because many small-scale purification protocols rely on the addition of expensive chemicals (especially proteolytic inhibitors) that are too expensive for large-scale processes and that in any case constitute additional contaminants that must be removed. A different approach must therefore

be taken in process-scale manufacturing, focusing on the removal of contaminants as rapidly as possible and the development of processes that employ a small number of efficient separation processes to minimize contact with potentially damaging agents. Efficient separation can be enhanced in the upstream production phase by ensuring the product is secreted, therefore avoiding the need to lyse cells and release oxidizing agents and proteases. Where intracellular expression is unavoidable, as is the case with bacterial inclusion bodies, there is generally a compromise between recovery and purity.

Depending on the host platform, a target protein may represent 0.1–5% of the total soluble protein in the biological matrix, and will be presented as a complex mixture of other molecules with a wide range of physicochemical properties [1]. DSP strategies must therefore exploit the unique properties of the target protein, such as its solubility in different environments, charge, hydrophobicity, molecular weight/size, binding affinity or the presence of specific reactive groups. Such physicochemical properties form the basis of all DSP separation methods [1].

8.3 Clarification

Clarification is the removal of particulates from the feed stream, so clarification processes exploit the solubility and small size of the target protein to separate it from larger, insoluble contaminants [2]. The solubility of the target product is more important than its size at this stage because the physical and chemical environment of the feed stream can be adjusted so that many further contaminants precipitate, allowing their removal along with cell fragments and other debris. The three major clarification methods are centrifugation, depth filtration, and cross-flow microfiltration, often used in combination with additional strategies to increase the particle size of contaminants and encourage their separation.

8.3.1 Centrifugation

Particles suspended in a less dense liquid medium tend to sediment downward under gravity while buoyant particles move to the surface. The efficiency of sedimentation depends on particle size, because as the particle size decreases, the relative impact of buoyancy forces, friction, and diffusion all increase. Sedimentation can therefore be enhanced by applying an additional centrifugal force, forcing particles to separate according to density and/or particle size, and depositing them on the walls of the centrifuge [7]. Gentle centrifugation is used to separate cells from fermentation broth to avoid breakage, but greater forces allow the removal of cell debris, the separation of protein precipitates, and even the separation of dissolved macromolecules (ultracentrifugation), although the last is too expensive to use in large- scale processes and ultrafiltration is preferred.

In most DSP scenarios, clarification exploits the principle of differential sedimentation, where the feed within the centrifuge is initially homogeneous. Here, larger and/or denser particles sediment more rapidly in the centrifugal field and thus form a pellet on the wall or floor of the rotor faster than smaller or lighter particles, which tend to remain in the supernatant. The magnitude of the applied centrifugal force and the duration of centrifugation can be used to determine the size or density of particles that are sedimented. This approach works well when the objective is to pellet solid particles, such as cells or tissue debris.

Centrifugation is typically a batch operation in the laboratory but continuous centrifuges are more efficient at the process scale. Here the feed stream is fed into the centrifuge at a constant rate and the supernatant (containing the target protein) is continuously discharged while the waste pellet is either scraped intermittently from the centrifuge walls or removed continuously by a scrolling plate, which is a feature of the bowl and scroll decanter centrifuge [7]. An alternative design is the disc stack centrifuge, which uses higher centrifugal forces than the decanter centrifuge and is therefore suitable for slurries with a lower solids concentration and/or smaller particles. The design incorporates sets of stainless-steel plates (the disc stack) which provide a greater sedimentation area and significantly accelerate the separation process. The solids deposited on the plates can be removed continuously, intermittently (semi-batch mode), or regularly (batch mode) depending on the application.

8.3.2 Filtration

Centrifuges are efficient for the removal of larger particles but for very small particles (known as "fines") and colloids, filtration is preferred. However, filters are easily clogged by larger particles, so the format of a clarification module depends on the volume of feed. Fermenter offloads of up to 3000 l often feature two depth filters in series to clarify the fermentation broth, whereas reactors with greater volumes usually employ a continuous centrifuge to remove the largest particles, followed by depth filtration and membrane filtration in series to remove smaller particles and fines.

Filtration is any process in which a liquid feed stream moves over or through a selectively permeable medium so that only certain components of the feed emerge in the permeate or filtrate, while contaminants are retained in the retentate [8]. Filtration methods are often defined on the basis of retentate size, which is largely although not entirely dependent on the maximum pore size of the filter medium. Microfiltration is the coarsest type of filtration in DSP, using media with pore sizes in the range 0.1–10 μm. This is suitable for removing suspended particulates but not for the separation of molecules (see Table 8.1). As a mechanical process, filtration requires a driving force across the filter medium, which in the case of microfiltration is usually provided by a pressure differential. Microfiltration is often used for clarification. As it can be carried out at relatively low temperatures and pressures, and it requires no phase changes or chemical additives (therefore resulting

Table 8.1 Different forms of filtration used in downstream processing.

Method	Pore size	Retained	Applications
Microfiltration	100 nm–10 µm	Cells, cell debris	Clarification, sterile filtration (fill and finish)
Ultrafiltration	10–100 nm (M_r 10^3–10^6)	Fine particles, viruses, large proteins	Clarification, virus clearance, size fractionation of proteins, concentration, diafiltration
Nanofiltration	1–10 nm ($M_r < 10^3$)	Nucleic acids, viruses, proteins	Purification of proteins, virus clearance
Reverse osmosis	0.1–1 nm ($M_r < 10^3$)	Salts, sugars	Water purification

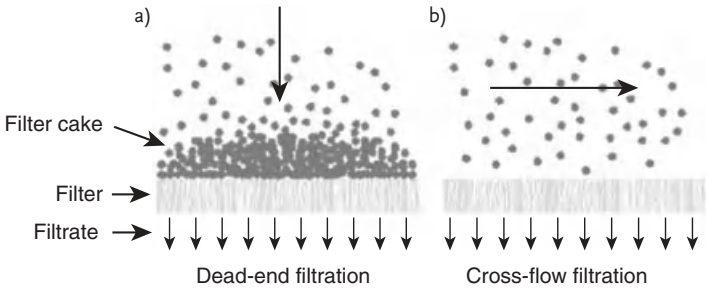

Figure 8.1 Comparison of (a) dead-end (normal flow) and (b) tangential (cross-flow) filtration. In each panel, the large arrow shows the direction of feed flow and the small arrows show the direction of permeate accumulation.

in minimal denaturation of labile target proteins), it is suitable to use throughout the process, and many devices such as chromatography modules have a pre-filter to remove particulates and reduce the likelihood of fouling.

Filter media can be divided into two major types: surface filters and depth filters (Figure 8.1). Surface filters are essentially thin membranes containing capillary-like pores. Particles or molecules that are too big to pass through the pores are retained on the membrane surface, that is, the filtration is absolute at a certain particle-size cut-off. In contrast, depth filters have a bed of filter medium rather than a thin membrane, and particles are trapped in the interstices, which describe a convoluted path from one side of the filter to the other. To increase the surface area available for filtration without increasing the footprint, depth filter pads are often supplied in a lenticular format comprising multiple filter pads pre-assembled inside a plastic casing. The materials used to construct depth filters include cellulose fibers, inorganic filter aids such as diatomaceous earth, resin binders, and synthetic polymers, offering a large inner surface area and void volumes of up to 85% [8]. Inorganic filter aids such as diatomaceous earth and perlite increase the

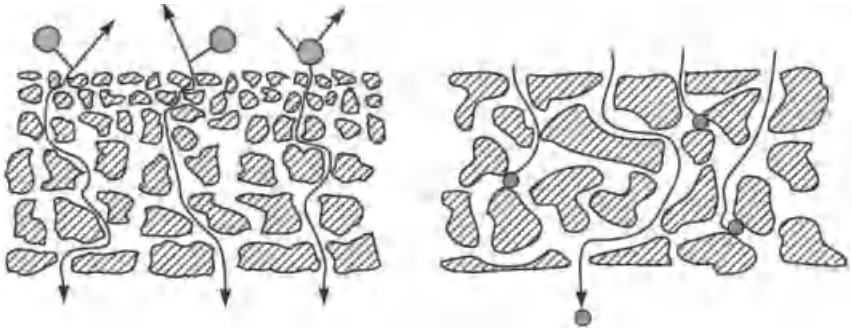

Figure 8.2 Comparison of membrane and depth filters. The filtration mechanism of membrane filters (left panel) is absolute, with particles above a certain size rejected at the surface and smaller particles allowed through to the permeate, whereas that of depth filters (right panel) is not absolute, with particles becoming trapped in the internal matrix but some getting through.

permeability and retention characteristics of the filter matrix, while synthetic polymers and resin binders increase the strength of the filter medium and generate a net positive charge that helps to trap colloids [9]. For these reasons, depth filters can trap particles much smaller than the maximum pore size. The retention mechanism of depth filters is not absolute, and can change during operation as the retentate builds up [8].

There are two main configurations of filter devices in downstream processing (Figure 8.2). In dead-end filtration (also known as normal-flow filtration) the feed stream is perpendicular to the filter device, which is usually a membrane or pad. The filter device effectively blocks the feed, which must be forced through it under pressure. Because this configuration inevitably leads to the rapid build-up of retentate on the feed-side filter surface, it is used when the retentate load in the feed stream is expected to be low (e.g., pre-filters for chromatography devices, virus filters, and also sterile filters for product filling).

The preferred configuration for clarification is tangential-flow filtration (also known as cross-flow filtration), where the feed flow is parallel to the filter medium and thus perpendicular to the flow of permeate. This allows retained species to be swept along the filter surface and out of the device, helping to maintain high flux levels even with large amounts of retentate [8]. Tangential-flow filter modules for clarification come in many designs. These differ in terms of channel spacing, packing density, cost, pumping energy requirements, plugging tendency, and ease of cleaning, so the design must be chosen on a case-by-case basis for each process depending on the implications of the above criteria in the context of the overall process train [8].

The capacity of a filtration process is usually expressed in terms of flux, which is the volume of permeate passing through a particular filter area per unit time (usually liters per square meter per hour) [8, 9]. The driving force for flux is the pressure difference between the feed and permeate sides, which is known as the trans-

membrane pressure. Opposing this is the viscosity of the liquid and the hydraulic resistance of the filter, which depends on the pore size and distribution. The permeability of a filter is operationally defined as the inverse of its hydraulic resistance. For pure water, flux increases linearly with transmembrane pressure because resistance and viscosity remain constant, whereas the flux tends to decline over time in normal filtration processes because both viscosity and resistance increase.

In cross-flow filtration, the flux declines rapidly at first but then more slowly, and eventually a steady state is achieved. This reflects two simultaneous phenomena – cake layer build-up and fouling. The former occurs in microfiltration when retentate particles accumulate on the feed side and achieve a packing density that causes them to form a defined layer on top of the membrane. This resists the flow of permeate by effectively acting as an additional filter bed, and increases local viscosity because the concentration of particles near the filter surface is higher than in the bulk feed, resulting in reduced flux. Cake layer build-up is not a permanent effect and can be reversed by back-washing.

In contrast, fouling occurs when there is physical and/or chemical interaction between contaminants and the membrane, leading to a permanent loss of function. Examples include the adsorption and deposition of macromolecules, cell fragments or small organic molecules on the membrane surface or within the pores. Fouling increases the hydraulic resistance against permeate flow, and may also increase the observed retention of the membrane as it reduces the effective pore size. Membranes used in biomanufacturing are designed to limit as much as possible the binding of nonspecific proteins, thereby reducing fouling and helping to avoid the loss of product through binding to the filter medium [8].

8.3.3
Increasing the Efficiency of Clarification

Several additional strategies can be used to increase the efficiency of clarification by persuading particles or even dissolved contaminants to form aggregates that are more likely to remain in the retentate. Flocculation is a process in which suspended particles clump together because the attractive forces between them overcome any repulsive forces caused by like surface charges, making the average particle size larger [2, 10]. Particles with like surface charges (such as whole cells or fragments of cells) can be persuaded to clump together by adding excess soluble counter-ions that shield the surface charges, or polyelectrolytes that bridge surface ions on separate particles and generate linked networks. These additives are inexpensive, and since they remain attached to the resulting floc there are no additional purification steps [10].

Whereas flocculation increases the size of particles that are already in suspension, precipitation brings soluble contaminants out of solution so they can be removed by filtration or centrifugation. Differences in solubility are often exploited at an early stage in downstream processing to separate the target protein from molecules with very different physical and chemical properties, such as carbohydrates and nucleic acids.

However, individual proteins also show differing degrees of solubility due to particular surface properties (e.g., charge distribution, juxtaposition of polar and non-polar surface patches). This means it is possible to select conditions where a desired protein remains in solution while others precipitate [11], or the desired protein is selectively precipitated while most contaminants remain in solution [10]. Proteins can be precipitated by changing the pH or temperature or by adding a mild organic solvent such as ethanol or acetone, which reduces the solvent dielectric constant. They can also be precipitated in salt solutions, by adding multivalent metal ions or specific reagents such as the detergent cetyl trimethylammonium bromide (CTAB) or the short-chain fatty acid caprylic acid [11]. Although the mechanism is not fully understood, salts are thought to remove the water of solution from the protein, thereby reducing its solubility. The efficiency of salting out can be summarized using the Hofmeister series (citrate > phosphate > sulfate > acetate $\gg$ chloride > nitrate > thiocyanate), but ammonium sulfate is usually the agent of choice because of its high solubility. Metal ions such as Mn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, and Ag^{+} are particularly useful because they bind specific functional groups and are therefore more selective than Hofmeister ions. They also act at much lower concentrations than the Hofmeister ions and are easily removed by ion-exchange chromatography or chelating agents. Solvent precipitation is typically performed at low temperature (<10 °C) because conformational rigidity then prevents irreversible denaturation. Finally, non-ionic, water-soluble polymers such as polyethylene glycol (PEG) and dextrans can induce protein precipitation by excluding water from the solvation structure, a principle also exploited in multiple phase partitioning systems (see below).

In a complex mixture of proteins, the solubilities of individual proteins will overlap considerably so precipitation is only a crude separation method. As with flocculation, however, the reagents involved are inexpensive, easily removable and do not denature the target protein, which makes them suitable for large-scale manufacturing. The Cohn process provides an excellent example, as it allows the inexpensive and consecutive extraction of albumin and other proteins from blood plasma by progressively changing the pH, ethanol concentration, temperature, ionic strength, and protein concentration. Albumin has the highest solubility and lowest isoelectric point of all the major plasma proteins, so it is the final one to be precipitated under the applied conditions.

Proteins can also be partitioned in mixtures of immiscible solvents, in which they show differing solubility. In three-phase partitioning, proteins are purified directly from cell homogenates by partitioning between a layer of butanol and a strong aqueous salt solution. Under these conditions, cell debris tends to separate into the organic phase and nucleic acids precipitate at the interphase while proteins remain in solution. The selectivity of extraction can be increased by including metal ions in the system. Aqueous two-phase extraction (ATPE) systems [12] use a mixture of aqueous polymers (e.g., PEG and dextran) and/or salts (e.g., potassium phosphate). Under ideal conditions, the target protein can be separated into the PEG phase leaving contaminants in the second phase or at the interphase.

8.4 Chromatography

Chromatography is any procedure in which the components of a mixture are separated by distribution between two phases for which they have differing affinities [13]. Although there are many different chromatography formats with solid, liquid, and gaseous phases of different characteristics, the principal form of chromatography used in biomanufacturing is process-scale liquid chromatography. This separates the components of a mixture according to their differing affinities for a fixed solid phase (typically made up of resin beads packed into a column, or a membrane enclosed in a cassette), and a mobile liquid phase that flows unidirectionally across it. The liquid phase is the process feed stream, which contains the target protein along with numerous contaminants. The solid phase can be functionalized in any number of ways, separating the components of the mixture according to properties such as size, charge, hydrophobicity, and binding affinity. All of these properties are generally related to the biological function of the target protein. The most important benefit of chromatography as a purification strategy is that two or more different operations can be carried out in series to achieve maximum separation by exploiting different separative principles, for example, separation by charge in one process, and then by hydrophobicity in another.

8.4.1 Column Chromatography

Among the many different chromatography formats available, column-based liquid chromatography has been the mainstay of DSP in biomanufacturing for several decades [14]. The clarified liquid feed stream is passed over or through a porous, solid matrix or resin held in a column and the components of the mixture become distributed by virtue of their relative affinity for the solid and liquid phases [13]. In most cases this is based on selective adsorption and desorption (i.e., reversible physical/chemical interaction with the resin).

The general procedure for adsorptive chromatography is to introduce the clarified feed stream into the column under buffer conditions where certain components bind strongly to the resin while others bind weakly or not at all. The same buffer is used to clear out all non-binding components, and then the composition of the buffer is changed so that molecules that initially bind to the resin can be eluted in subsequent fractions. Chromatography columns can be operated in various modes depending on the objective of the separation, but the most relevant for biomanufacturing are bind-and-elute mode, which aims to retain the target protein (this is often termed capture chromatography), and flow-through mode, which aims to retain contaminants and let the target protein flow through (this is often termed negative or polishing chromatography).

Additional important concepts in process chromatography include the dynamic binding capacity (DBC), selectivity, resolution, and linear flow rate. The DBC of a chromatography column is the amount of target molecule it will bind under actual

flow conditions before breakthrough (appearance in the flow through even under binding conditions). In the bind-and-elute mode, this means that the target product is wasted, and in the flow-through mode, it means that contaminants are no longer removed efficiently.

As the DBC reflects the impact of mass transfer limitations that may occur as the linear flow rate is increased, it is much more useful in predicting real process performance than the static binding capacity, which shows how much product a column can bind when saturated. The linear flow rate is the rate at which the mobile phase moves through the column, usually measured in centimeters per hour. The linear flow rate is an important determinant of process efficiency because it determines how long the feed stream remains in contact with the resin and hence the time available for the product and contaminants to interact with the resin. When scaling up a process, the linear flow rate must remain constant, which generally means the volumetric flow rate (the actual amount of liquid fed into the column per hour) must increase if a column with a wider diameter is introduced.

The selectivity of a chromatography step refers to its ability to separate two molecules, usually the target protein and a key contaminant such as nucleic acid. The greater the selectivity, the faster one species elutes compared with the other. The resolution is a related concept but also takes into account the peak widths, that is, the centers of two elution peaks may be widely separated in a selective chromatography process but the peaks themselves may be so broad that there is still a significant overlap. High resolution not only ensures that the target elutes rapidly while the contaminants are retained (or vice versa) but also that the peaks do not overlap so there is limited contamination. Where peak broadening cannot be avoided by changing the buffer conditions, continuous chromatography may help (see below).

Elution strategies also differ according to the chromatography mode and its purpose. Affinity chromatography (see below) is a bind-and-elute operation, and the interaction between the target molecule and the resin is so specific that only a few species are retained while most impurities are washed through. Elution is therefore carried out in one step with a single defined elution buffer, allowing the target protein to be recovered. The other adsorption chromatography methods exploit more general physicochemical properties of the target protein and both the retained fraction and the eluate may be complex. For this reason, elution is typically performed with a gradually changing buffer composition to produce a series of fractions whose components have gradually increasing affinity for the resin. In large-scale processes, gradient elution may be replaced with stepwise gradients, which are easier to automate, although programmable linear gradients are becoming more common. Adsorptive column chromatography is particularly applicable in DSP because short columns with a large diameter (up to 2 m) can be operated at very high flow rates (300–500 cm/h).

8.4.2 Membrane Chromatography

One of the main disadvantages of column chromatography in DSP is the requirement for cleaning and validation, both of which are expensive and time-consuming,

resulting in significant process downtime [15]. Increasingly, this is being addressed by replacing columns with equivalent disposable cassettes, since the higher costs of using multiple cassettes compared with the single purchase and installation cost of a fixed stainless-steel column can be offset by the elimination of cleaning and/or steaming in place (CIP/SIP), the validation of cleaning routines, and the associated record keeping. Disposable equipment is widely used in the laboratory (e.g., syringe filters, spin columns) and has been accepted for many years in both upstream production (disposable media bags and bioreactors) and DSP (disposable filters), but disposable membrane adsorbers have only made an impact over the last five years.

The recent adoption of membrane adsorbers in an increasing number of manufacturing processes reflects the realization that they perform as well as or better than columns in a number of settings, especially polishing, yet they have a much smaller footprint. A range of different membranes is available with functional groups equivalent to the corresponding resins, for example, membranes containing activated quaternary ammonium groups for anion exchange, or phenyl groups for hydrophobic interaction chromatography (HIC), and a relatively new variant also allows salt tolerant interaction chromatography in high-salt buffers [16, 17]. A disposable 0.5-l anion exchange membrane has been shown to remove process contaminants from a 1000-l feed stream as efficiently as a resin-packed stainless-steel column that is 100-times larger.

However, costs are only one benefit of disposable technology and one must also factor in the additional convenience and reduction in downtime. Polishing applications are likely to be dominated by disposable convective media devices in the near future as the cost and efficiency benefits become more apparent [15] but membranes are already finding use in additional settings. In a recent example described by the Italian biopharmaceutical company Philogen, membrane absorbers were substituted in the flow-through and bind-and-elute steps for the polishing of a new monoclonal antibody fusion protein in phase I/II clinical development, achieving 90% recovery and 99.9% purity [18].

8.4.3 Capture Chromatography

If protein capture by chromatography is possible, the step is incorporated as early as possible in the process, typically immediately after clarification, to remove as many impurities as possible (including excess water) and thus achieve a steep increase in both purity and concentration early in the process. Large fermenter offload volumes can be processed, and the ideal capture ligands have a high DBC, allowing high linear flow rates and low contact times. Depending on the efficiency of capture, subsequent purification may involve some intermediate processing before polishing, or (if the output from the capture stage is already highly pure) two consecutive and orthogonal polishing steps [5].

Harvesting, clarification, and capture are typically carried out in quick succession, thus removing particulates, 99% of soluble contaminants, and achieving 90% volume reduction as soon as possible [2, 13]. The use of affinity chromatography

at an early stage is advantageous because the desired product is rapidly separated from potentially damaging contaminants such as proteases and oxidizing agents. However, it is not always possible to develop ligands to trap the desired product, and in many cases the cost of affinity media becomes significant as the scale of production increases. This is certainly the case with Protein A chromatography for the capture of monoclonal antibodies [4], so a number of less expensive bulk separation operations have been considered, and are beginning to be incorporated into large-scale processes. Examples include the selective precipitation of the product [10], the use of non-affinity chromatography methods instead of Protein A chromatography [19], and the use of ion-exchange and mixed-mode chromatography [20].

The choice of capture ligand reflects the balance between cost and resolving power. The more selective ligands tend to be the more expensive, and are often related in some way to the target protein's normal function because selective interaction with substrates, receptors, cofactors, and other binding partners is an important aspect of protein activity in nature [21]. Affinity ligands exploit the complexity of the target protein and depend on electrostatic, hydrogen, hydrophobic, and van der Waals interactions – distributed spatially in a defined manner. Selective media are produced by the covalent attachment of such ligands to an inert substrate, such as agarose, glass or polymeric matrices, which can be formulated as small, porous beads. The degree of accessibility and spatial presentation of the ligand once immobilized, and the relative strength of each force, dictates the specific bonds involved in separation. Passing the feed stream through or over such media therefore allows specific binding between the target and immobilized ligand, while most other molecules remain in solution. After washing to clear as many contaminants as possible from the media, the target molecule can be released from its ligand using an elution buffer that disrupts the specific bonds involved in target–ligand binding, or a buffer that contains an excess of soluble ligand to out-compete the immobilized ligand. Methods based on this principle include immunoaffinity chromatography (using antibodies to capture their cognate antigens), Protein A chromatography for the purification of antibodies [4], ion-exchange chromatography [22], immobilized metal ion affinity chromatography (IMAC) for the purification of negatively charged or chelating proteins, dye-binding chromatography for proteins that recognize particular organic groups, and lectins to capture glycoproteins (these can be eluted by adding an excess of the appropriate monosaccharide to the elution buffer).

Although some capture ligands are absolutely specific for a given target molecule, others such as Protein A recognize families of proteins or proteins with common features allowing them to be used in different processes (Protein A recognizes most immunoglobulin G subclasses, for example). Although the interactions are not specific for individual proteins, within each process they are usually specific for an individual target because only one antibody will be produced in each process. This allows a generic capture platform to be designed and optimized and applied to many different processes, as long as the appropriate binding partner is present in the target molecule.

8.4.4 Polishing Chromatography

Polishing [5] removes contaminants (bulk contaminants during intermediate polishing, trace contaminants during final polishing) including HCPs, nucleic acids, adventitious and endogenous viruses, leached Protein A, and product-related impurities (unwanted variants of the product such as aggregates and fragments). Several chromatography formats can be used for polishing and maximum resolution is achieved with orthogonal separations. Therefore, the logical sequence of chromatographic steps should take into account the composition of the feed and the starting and elution conditions. For example, hydrophobic interaction chromatography generally begins with a high salt buffer but the eluting buffer is of low ionic strength. The converse applies in ion-exchange chromatography, so where both are used in a given process it makes sense to place them back to back.

Ion-exchange chromatography separates proteins on the basis of their net charge, which reflects the number and nature of charged amino acid residues on the protein as well as the pH of the buffer [22]. The ability to control the polarity and magnitude of a protein's charge by varying the pH is exploited in ion-exchange chromatography for the selective adsorption of target proteins onto a resin or membrane derivatized with charged groups. Anionic and cationic resins of varying strengths may be used to adsorb proteins of the opposite charge. Some examples of anionic and cationic exchangers (AEX, CEX) are listed in Table 8.2.

The process for ion-exchange chromatography is similar to that described for affinity chromatography but with important differences in column capacity and elution mechanics. Initially, the column is equilibrated with a low ionic strength buffer containing ions of opposite charge to the resin. These counter-ions are displaced by charged molecules in the feed stream, which adsorb to the resin. When run in bind-and-elute mode, the strength and selectivity of binding between the target protein and the resin are optimized by adjusting the ionic strength and pH of the buffer, and the flow rate through the column, such that maximum retention of the target molecule is achieved. However, as many proteins will share the same charge profile and p*I* (isoelectric point) value as the target, numerous

Table 8.2 Some examples of anionic and cationic exchangers.

Ion exchangers	Functional group	Comments
Anion-exchange resins		
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$	Weak
Quaternary aminoethyl (QAE)	$-O-CH_2-CH_2-N^+(C_2H_5)_2-CH_2-CHOH-CH_3$	Strong
Quaternary ammonium (Q)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$	Strong
Cation-exchange resins		
Carboxymethyl (CM)	$-O-CH_2-COO^-$	Weak
Sulfopropyl (S)	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$	Strong
Methylsulfonate (M)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$	Strong

competing molecules will cosegregate in the same fraction. This means that the DBC of an ion-exchange column is always lower than that of an affinity column. In flow-through mode, conditions are adjusted to optimize the retention of particular contaminants while allowing the target protein to wash through. In principle, it should be possible to hold the pH of the sample at exactly the p*I* value of a target protein for selective elution in both CEX and AEX, but in practice, this destabilizes the target protein and encourages aggregation. Instead, the pH of the sample is held at 0.5–1 pH unit above or below the p*I* to keep the target protein in solution. Elution from an ion-exchange column is achieved using buffers of gradual or stepped increases in ionic strength or pH to produce a number of different fractions. The resolution of ion-exchange chromatography is influenced by the sample load, linear flow rate, and slope of the elution gradient.

Hydroxyapatite (HA) chromatography can be considered as a special form of ion exchange best described as "mixed-mode," that is, involving aspects of both AEX and CEX [20]. There are nonspecific interactions between positively charged calcium ions on the HA resin and negatively charged carboxyl groups on proteins, as well as between the negatively charged phosphate ions on the HA resin with positively charged amino groups on proteins. This makes the outcome of HA chromatography difficult to predict and it must be tested case by case, but makes it useful for certain capture steps as well as polishing to remove residual nucleic acids and HCPs. Elution from HA columns is usually achieved with stepwise increases in phosphate concentration.

Hydrophobic interaction chromatography separates proteins according to their affinity for a hydrophobic solid phase, usually comprising long, unsubstituted hydrocarbons. The principle exploited by HIC is that even proteins that are generally polar and soluble in water may have hydrophobic patches allowing them to interact with other proteins with similar characteristics by excluding water from the interface. Such interactions are favored when a hydrophobic matrix is used in concert with a concentrated salt buffer so that hydrophobic interactions significantly increase the overall entropy of the system. Like ion-exchange chromatography, HIC involves the reversible adsorption of proteins to a resin and elution using a buffer that disrupts such interactions [23]. In this case the resin is equilibrated in a high-salt buffer and the feed stream similarly adjusted to high ionic strength so that hydrophobic proteins bind preferentially to the resin. Desorption is achieved by stepwise reductions in the salt concentration of the elution buffer, sometimes in combination with a gradual increase in the concentration of an organic solvent, which competes with the resin for hydrophobic interactions and therefore encourages hydrophobic proteins back into solution.

The most suitable ligands for hydrophobic interaction chromatography are C_2–C_8 alkyl groups and phenyl groups, which take part in hydrophobic interactions with most proteins but are not so hydrophobic that extreme conditions are required for elution. As in ion-exchange chromatography, the stepwise modification of elution buffer composition results in fractions containing sequentially more hydrophobic proteins. The resolution of HIC is influenced by the sample load, linear flow rate, and the slope of the elution gradient, and can be optimized by

decreasing the flow rate and increasing the gradient volume. HIC is similar in principle to reversed-phase chromatography (RPC) but the latter employs much more hydrophobic resins (e.g., C_{10}–C_{18} alkyl groups) and the elution solvents need to be stronger, which usually denatures the proteins. RPC is therefore rarely used for the large-scale manufacturing of proteins [24].

Size-exclusion chromatography (SEC) separates proteins on the basis of size and is therefore similar in application to ultrafiltration (see below). It is quite distinct from the absorptive chromatography methods described above because it does not depend on selective absorption and desorption, but instead on sieving molecules by size as they percolate through the resin [25]. The column is packed with inert porous beads and selectivity depends on the size of the pores, since larger molecules cannot enter the pores and are eluted rapidly, whereas molecules smaller than the pore size will become trapped and will move through the resin more slowly. This is known as molecular exclusion.

An important concept in SEC is that the separation medium is the pores on the beads and not the beads themselves. Therefore, 95–99% of the column volume remains unused in any operation, and feed volumes must be adjusted accordingly, representing a significant bottleneck. For this reason, SEC is a rare step in modern biomanufacturing and mostly limited to the very final stage in biopharmaceutical purification where it is used to separate the target protein from very similar molecules, such as degradation products and aggregates whose charge and hydrophobicity profiles are similar or identical to the target. The most important variables in SEC are the column length and linear flow rate. Slow mass transfer of macromolecules can cause peak broadening and loss of resolution, which can be addressed by reducing the flow rate.

SEC with resins suitable for separating molecules in the lowest size ranges ($M_r < 5000$) is used to separate macromolecules from low molecular weight compounds, and is thus useful for desalting or rebuffering protein samples. This is an alternative to filtration-based methods, such as diafiltration and reverse osmosis, and has a much higher capacity and flow rate than SEC running in fine separation mode. Sample volumes in buffer exchange may reach up to 30% of the column volume, compared with the 1–3% possible with fine separations [25].

8.4.5 Continuous Chromatography

As discussed above, it is sometimes impossible to resolve broad peaks by using different separation methods or buffers, which means either a compromise in product yield or purity. Continuous chromatography can help to address this problem by creating an environment where the separation distance is effectively infinite and thus allowing the resolution of any two products with separate peaks, no matter how much they overlap. The typical format is known as simulated moving bed chromatography (SMBC) in which the valve and column arrangement simulate the movement of the stationary phase to establish a countercurrent that can be tuned to resolve products with very similar or overlapping elution profiles [26].

In static chromatography, the two components (X and Y) would move through the column at similar rates, but the weaker-binding species (Y) would move slightly faster than the stronger-binding species (X). In SMBC, the countercurrent is set so as to be very slightly faster than that rate at which X moves through the column but not as fast as Y. The net effect is that Y is able to overcome the countercurrent and move towards the outlet, whereas X is pushed back. This works for a finite amount of the X+Y mixture, but more importantly it results in separation also if the mixture is fed into the column continuously [26].

8.5 Ultrafiltration/Diafiltration, and Virus Filtration

Whereas microfiltration is used for product clarification and as a pre-filter upstream of chromatography columns and membranes to remove potential foulants, even finer filtration (ultrafiltration/diafiltration, UF/DF) is required in the final stages of manufacturing. This is in order to separate molecules on the basis of size, clear viruses from the feed stream, concentrate the product, and remove excess salts [6, 8, 9, 27, 28].

8.5.1 Ultrafiltration/Diafiltration

Although ultrafiltration is not fundamentally different to microfiltration in mechanistic terms, the fact that one involves an insoluble retentate and the other a soluble retentate has a number of consequences. The most important of these is concentration polarization in ultrafiltration: the tendency for retentate to build up on the filter side as a gel layer, which increases the concentration of solute at the filter surface and interferes with movement across the filter by osmotic effects. This is analogous but not exactly equivalent to the cake build-up on microfiltration devices, since the former is permeable but highly concentrated whereas the latter becomes impermeable and physically blocks the filter pores.

For dead-end filtration, the sieving properties of ultrafiltration devices tend to be measured in terms of the nominal molecular weight cut-off (NMWCO), since this is more relevant than particle size for molecules in solution [8]. However, the NMWCO can only be considered approximate because the chemistry of the solute–membrane interaction and effective radius of target protein molecules determine the actual cut-off in any particular process. Stoke's radius is the best overall determinant of protein mobility across ultrafiltration membranes because this takes into account the steric effects of glycans and other modifications. Two proteins with identical molecular masses may differ significantly in their intrinsic shape and their Stoke's radius, which will in turn affect their interaction with the membrane. The base polymer of ultrafiltration membranes tends to be hydrophilic to increase flux and reduce fouling in aqueous buffer solutions, but proteins are

diverse in the physicochemical properties and may interact with membranes in unpredictable ways.

Tangential flow ultrafiltration is useful for operations where gel build-up is anticipated because the feed stream retains a significant bioburden [27], but is also used, for example, for the depyrogenation of water and buffers [8]. Membranes with an NMWCO of 300 000–500 000 are useful for the removal of viruses and large plasmids, while those with an NMWCO of 50 000 can retain large glycoproteins, such as antibodies, while most other proteins pass through the filter. Smaller proteins can be retained by a 10 000 NMWCO filter without impeding the movement of small-molecule solutes, which is useful for concentration and diafiltration. Depyrogenation and reverse osmosis can be achieved using membranes with an NMWCO of 1000–3000.

8.5.2 Virus Filtration

Mammalian cell lines may contain endogenous viruses, and other so-called adventitious viruses may be present in the culture medium or in other processing materials. ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) Q5A guidelines require at least two dedicated virus clearance steps with orthogonal mechanisms (i.e., clearance steps based on different separative principles), and the inclusion of polishing steps that remove viruses in addition to product-related and process impurities is also welcomed [6]. As discussed above, viral clearance is measured in terms of LRVs and a 4 LRV reduction is considered to be a robust removal step, such that the orthogonal application of multiple steps results in overall LRVs >10.

Low-pH inactivation is a standard virus inactivation measure usually implemented as a low-pH hold after elution from capture, and 20-nm filtration is the most common virus removal step, typically implemented by dead-end ultrafiltration. Filters are available in two pore size categories, that is, retroviral (<50 nm) and parvoviral (<20 nm), the latter now favored as an industry standard to meet regulatory requirements [6].

Parvoviral filters require a larger surface area than other filters because the small pore size results in frequent clogging, even when the feed stream has a low bioburden. The virus filtration step is therefore typically placed downstream of polishing chromatography depending on product stream volume considerations and the amount of process intermediate that can be filtered [6]. Dead-end virus filtration is performed using a conventional cartridge design with filter pore sizes in the range of 20–50 nm. Smaller pore sizes are impractical because the membrane back pressure significantly exceeds 0.3 MPa, and the flux declines due to the accumulation of aggregates and small particles. Therefore, virus filtration is preferably carried out using a 0.1-μm pre-filtered feed stream.

Typical harmful small viruses in the 20-nm range are the most difficult to separate from large protein molecules such as antibodies because the apparent

molecular weight and the effective size and shape of the protein molecules may come close to the size of the virus particles. However, 20-nm filtration is nevertheless a robust step for the removal of most viruses and is now required for the purification of almost all biopharmaceuticals produced using mammalian cells.

8.6 Crystallization

Economical, low-technology methods such as precipitation and solvent partitioning are beginning to find applications in the early stages of DSP, but even at the polishing stage, typically the territory of expensive and high-technology methods, these less costly approaches are beginning to gain popularity. Crystallization involves the separation of a solute from a supersaturated solution (mother liquor), achieved by encouraging the formation of small aggregates of solute molecules, which then grow into crystals. The crystallization process involves the formation of a regularly structured solid phase, which impedes the incorporation of contaminants or solvent molecules, and therefore yields products of exceptional purity [29]. It is this purity that makes crystallization particularly suitable for the preparation of pharmaceutical proteins, coupled with the realization that protein crystals enhance protein stability and provide a useful vehicle for drug delivery, as has been demonstrated with various protein drugs, including antibodies. Thus far, crystallization has been used solely to manufacture small and simple proteins such as aprotinin and insulin. The advantages of crystallization as a final purification and concentration step in clinical manufacturing processes include the low cost, product stability, ease of preparation, and the ability to use biopharmaceutical protein crystals as slow-release formulations [30].

8.7 Recent Developments in Downstream Processing

In the early days of biomanufacturing, product approval was more important than process efficiency and products were required in small amounts. The DSP technologies of the day were not geared towards large-scale manufacturing because they did not have to be and there was no commercial advantage in future-proofing the industry against the increased demand and much tighter regulatory scrutiny we have today.

Upstream productivity has increased over 100-fold over the last 25 years, with 10 g/l titres now becoming increasingly common in mammalian cells [31] and an increasing diversity of upstream production platforms coming on-line, including animal milk, plant cells, and whole plant tissues such as leaves and seeds [32]. Over the same time period, DSP has continued to rely on long-established technologies such as batch chromatography, with all its limitations, and this has resulted in a situation where production costs are proportional to the production

scale, because increasing yields translate linearly into higher materials costs and larger facility layouts.

To address these challenges, the industry has striven to improve product quality and reduce wastage by building quality into the manufacturing process itself. This reflects the implementation of quality by design (QbD) as a core manufacturing concept, obliging manufacturers to consider the impact of raw material attributes and process parameters on the quality of the resulting product, and to build that quality assurance into the process rather than simply testing products at the end [33].

Manufacturing processes have therefore grown more robust, streamlined, and efficient in an attempt to increase both quality and productivity [34]. A key component of this is integrated process design, where each process train is designed de novo based on the optimum arrangement and juxtaposition of different operational units to suit both the upstream production system and the product, achieving early bulk contaminant removal and volume reduction and reducing the overall process to the smallest number of modules possible [35]. The importance of the process is emphasized by the inclusion of process analytical technology (PAT), which allows the continual measurement of process parameters to ensure they stay within manufacturing tolerances [36].

As discussed in this chapter, technological innovation is also a key factor in the improvement of downstream processing, including the adoption of new technologies, such as membrane adsorbers [15], disposable modules [31], novel affinity ligands and presentation formats (e.g., viral nanoparticles displaying Protein A [37]), cutting edge chromatography formats such as STIC [17], and continuous chromatography formats, together with the reinvestigation of older, more traditional approaches such as precipitation, crystallization, and flocculation [10].

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9
Characterization of Recombinant Proteins

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9.1
Introduction

The characterization of pharmaceutical proteins comprises a broad spectrum of analytical methods, not all of which are covered in this chapter. It focuses on a selection of those methods that are routinely applied in analytical departments of small and medium enterprises (SMEs) in the biopharmaceutical arena. Major emphasis is put on the methods applied in the characterization of therapeutic glycoproteins. The range of methods covered in this chapter encompasses traditional and state-of-the-art physicochemical techniques as well as sophisticated cell-based assays.

9.2
Physical Chemical Characterization

9.2.1
Spectroscopic Methods

9.2.1.1 Ultraviolet Absorption Spectroscopy

In the 1950s, it was proposed that structural pertubations of a protein could be correlated with changes in its ultraviolet absorbance spectrum [1]. The main drivers for that correlation are the three aromatic amino acid residues tyrosine, tryptophan, and phenylalanine that show absorption maxima between 250 and 300 nm. One of the main applications today is the quantitation of liquid protein solutions using the Beer–Lambert Law:

$$A_{(\lambda)} = \log(I_0 / I) = \varepsilon(\lambda)cl$$

where

A is the absorbance intensity at a specific wavelength
I and I_0 are the transmitted and the incident light

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c is the molar concentration of the sample
l is the pathlength in centimeters
ε is the molar extinction coefficient at that wavelength (λ).

In addition, it was observed that slight differences in the spectra of protein solutions can be used to monitor subtle structural changes, for example, the exposure of the aromatic acid residues to solvent during unfolding processes, as well as to monitor for impurities in protein solutions such as particles, for example, protein aggregates. For better evaluation of structural protein changes, derivation techniques of the raw data can be used [2]. Particles whose size begins to approach 1/20 to 1/50 of the wavelength of the incident light scatter this light, hindering it from reaching the detector and increasing absorbance values [3]. For total protein determination by ultraviolet (UV) measurement, the scattering effect must be corrected as, for example, described in the *European Pharmacopoeia* (EP 2.5.33 [4]). The protein solution is measured at eight different wavelengths (nm): 280, 320, 325, 330, 335, 340, 345, and 350. The amount of scattered light is then calculated by the following equation:

Wavelength, λ_i (nm)	λ_1 (280)	λ_2 (320)	λ_3 (325)	λ_4 (330)	λ_5 (335)	λ_6 (340)	λ_7 (345)	λ_8 (350)
Decadic logarithm of λ_i	$\lg\lambda_1$	$\lg\lambda_2$	$\lg\lambda_3$	$\lg\lambda_4$	$\lg\lambda_5$	$\lg\lambda_6$	$\lg\lambda_7$	$\lg\lambda_8$
Absorption of sample A_i	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8
Decadic logarithm of A_i	$\lg A_1$	$\lg A_2$	$\lg A_3$	$\lg A_4$	$\lg A_5$	$\lg A_6$	$\lg A_7$	$\lg A_8$

$$a = \overline{\lg A} - b \cdot \overline{\lg \lambda} \quad b = \left. \frac{\sum \left(\lg A_i - \overline{\lg A}\right) \cdot \left(\lg \lambda_i - \lg \overline{\lambda}\right)}{\sum \left(\lg \lambda_i - \overline{\lg \lambda}\right)^2} \right| i = 2 - 8$$

The amount of scattered light A_{sc} at 280 nm is then calculated by

$$A_{sc} = 10^{b \cdot \lg \lambda_1 + a}$$

The corrected UV absorption value of the sample protein solution at 280 nm A_c is finally calculated by

$$A_c = A_1 - A_{sc}$$

9.2.1.2 **Fluorescence Spectroscopy**

The emission of light in the wavelength range between 200 and 800 nm by typically aromatic molecules that are in an electronically excited state is referred to as luminescence. Luminescence can be further categorized into fluorescence and phosphorescence depending on the nature of the excited energy states [5]. The primary molecules in glycoproteins that can be excited and show fluorescence phenomena are the three aromatic amino acids tryptophan, tyrosin, and phenylalanine. Typical protein structure analysis uses monochromatic excitation and measurement of the emission spectrum of the glycoprotein.

In an excitation spectrum the emission light is held at a constant wavelength whereby the excitation light is changed via a monochromator. In an emission map, different emission spectra are created from different excitation wavelengths and plotted together in a three-dimensional graph where the intensity of emission is plotted as a function of excitation and emission wavelengths. This graph is also often named a contour map. The intrinsic protein fluorescence caused mainly by tryptophan and tyrosine is used to measure the conformational state of a protein. Tryptophan fluorescence is influenced by quenching phenomena of other amino acids in the protein, and an energy transfer between tryptophan and the other fluorescent amino acids is also possible. In addition, each tryptophan residue in a protein backbone will result in its own emission spectrum due to its own local environment in the protein. This makes data evaluation complex.

In practice, fluorescence spectroscopy is often used in pre-formulation and formulation studies to examine the behavior of a protein in different environments, such as in buffer systems containing additives. If, for example, a protein containing a single tryptophan is denatured with increasing temperature, a red-shift emission spectrum will appear when the hydrophobic tryptophan moves from the inner part of the protein to a more exposed position on the aqueous outside of the protein.

Extrinsic fluorescence measurements use special dyes that bind covalently or via intermolecular bonds on the protein. These applications are very useful to measure protein–protein interactions and can also be used in the analysis of product-related substances and impurities of protein drug substance samples, such as for the analysis of aggregates in combination with a chromatographic method such as SE-HPLC (Figure 9.1). The use of Nile Red as a fluorescent dye that binds to hydrophobic macromolecules and fluorometric detection with the HPLC device (EP 2.2.21 [4]) can increase the limit of detection of this HPLC method by a factor of 1000 compared with UV analysis.

9.2.1.3 **Fourier Transform Infrared Spectroscopy**

In Fourier transform infrared spectroscopy (FT-IR), a spectrometer simultaneously collects spectral data in a spectral range between 4000 and 400 cm^{-1} in most cases (EP 2.2.24 [4]). The term Fourier transform infrared spectroscopy refers to the mathematical Fourier transformation that converts the raw data (interferogram) into the actual spectrum. For protein structure determination, the infrared active amide vibrations, especially the amide I, II, and III bands, are most important. Figure 9.2 shows the FT-IR spectrum of the oligopeptide decaglutaminylglutamate (Q11).

The protein structure information can be elucidated from these three amide regions by *band narrowing* and *curve fitting methods* that are described elsewhere in more detail [6]. Characteristic regions for deriving certain secondary structures are shown in Table 9.1.

Other techniques for structure elucidation have been developed. These comprise *pattern recognition techniques* using multivariate data analysis or neural network analysis.

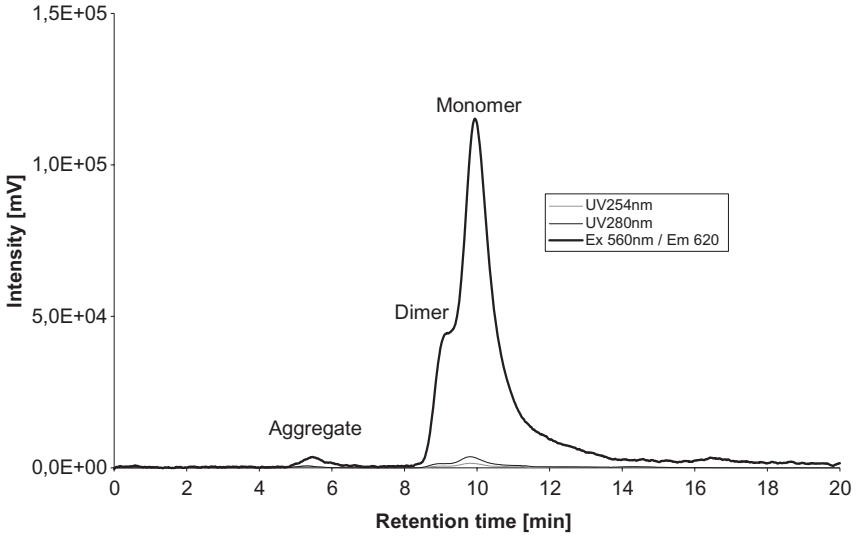

Figure 9.1 SE-HPLC chromatogram with UV and fluorometric detection using extrinsic fluorescence in direct comparison; the sensitivity of this HPLC method with regard to monomer, dimer, and aggregate detection is significantly increased using the fluorescence dye. Courtesy of ProBioGen AG.

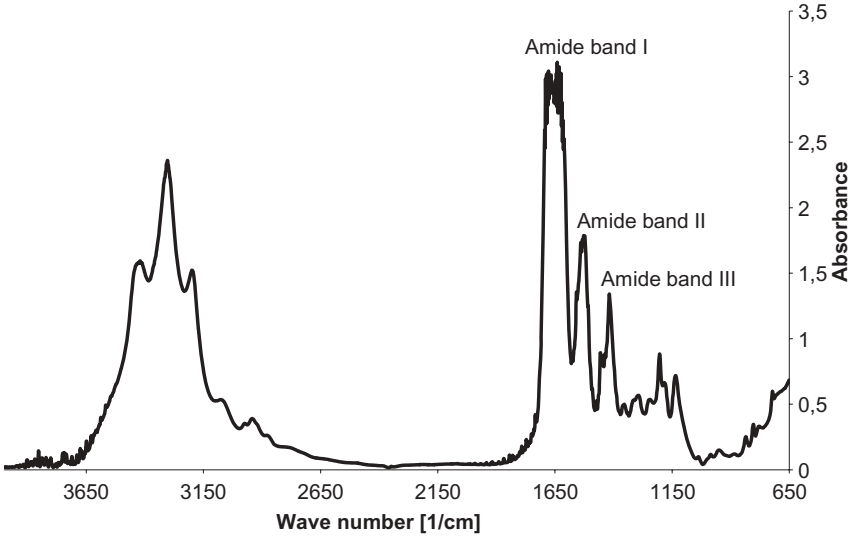

Figure 9.2 FT-IR spectrum of the oligopeptide Q11 showing the important amide bands for secondary structure characterization of proteins. Courtesy of ProBioGen AG.

Table 9.1 Characteristic regions within the amide I region for secondary structure elucidation [7].

Structure	Region
α-helix	1648–1657
β-sheet	1623–1641
Turn	1662–1686

9.2.2 Chromatographic Methods

9.2.2.1 Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) is a chromatographic separation method that separates molecules based on their size in the diluted state (e.g., hydrodynamic radius). When organic mobile phases are used, the method is called gel permeation chromatography, for aqueous mobile phases the term gel filtration chromatography is used. The sample is applied to a column that contains a gel or a porous solid material and is then transported by the mobile phase through this column. The pore sizes of the solid column material determines the molecular size range in which a separation of molecules can take place. Total permeating molecules, which means molecules of a size that can infiltrate in all pores of the stationary phase (as per the definition the eluent molecules) are eluted with the dead volume (V_d, total volume of eluent in the column). Molecules of a size bigger than the maximum pore size are only able to transfer the column in the interstationary spaces of the stationary phase particles. They are excluded by their size and are eluted with the first peak in the SEC chromatogram in the exclusion or void volume (V_0, interstationary corn volume) (EP 2.2.30 [4]).

In glycoprotein analyses, analytical SEC or SE-HPLC columns are used that possess higher maximal pressure limits compared with the preparative SEC columns used in purification processes (e.g., downstream polishing processes). The standard eluent for the analysis of glycoproteins in the native, undenatured state is phosphate-buffered saline (1xPBS). Size-exclusion chromatography is used in particular for the analyses of soluble product aggregates, a group of product-related impurities that is still viewed as one of the critical quality attributes by the biopharmaceutical industry as well as by the regulatory authorities [8–10]. Owing to the fact that soluble aggregate molecules can interact with the column matrix, due to their relatively high hydrophobicity, eluent additives, such as arginine, are used to avoid these interactions and to obtain a picture of the true amount of aggregates in the sample ([11]; see Figure 9.3). Typical detection wavelengths for glycoprotein purity determinations are $UV_{280\,nm}$, for example, for the determination of proteinogenous impurities, and $UV_{254\,nm}$, for example, for the determination of DNA-based impurities. Nowadays, diode array detection (DAD) is used to obtain the complete zero-order UV spectrum (see Section

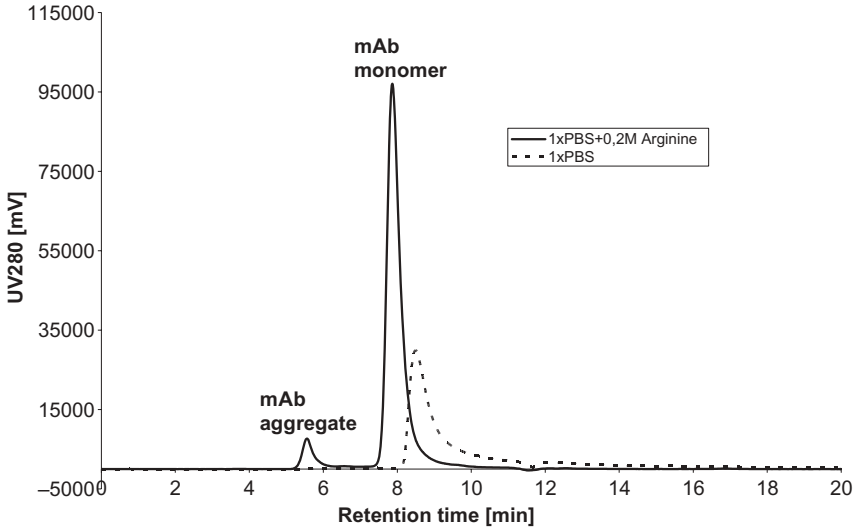

Figure 9.3 SE-HPLC chromatograms (overlay) of a partially aggregated monoclonal antibody product using two different eluents: (1) 1xPBS does not elute any aggregates and only a fraction of the antibody monomer; (2) 1xPBS + 0.2M arginine elutes a higher amount of antibody monomer in a much sharper peak and 7% of soluble antibody aggregates (monomer 93%). Courtesy of ProBioGen AG.

9.2.1.1 on Ultraviolet Absorption Spectroscopy) of each peak online in the chromatogram.

9.2.2.2 **Reversed-Phase Chromatography**

Reversed-phase chromatographic (RPC) methods use, by definition, hydrophobic stationary phases and hydrophilic eluents, in contrast to normal phase chromatographic methods that use hydrophilic stationary phases and hydrophobic eluents. Classical RP stationary phases are octyl- and octadecyl-modified silica matrices. More pH-resistant stationary phases are based on polymeric material, such as stryrene–divinylbenzene copolymer. Typical RP eluents are aqueous acetonitrile gradients.

RPC is currently used as an orthogonal purity determination method to SEC (see Section 9.2.2.1, Size-Exclusion Chromatography; see Figure 9.4).

Another important application of RPC is the peptide map characterization of the specific glycoprotein for identity determination. The method comprises the chemical or enzymatic treatment of the protein sample to create peptide fragments, in addition to the separation and identification of the resulting fragments by their retention times (EP 2.2.55 [4]). RPC is often also used in combination with mass spectrometry (e.g., LC-ESI-MS) to perform peptide mass fingerprint analyses (see Section 9.2.5 on Mass Spectrometric Analysis of Biopharmaceutical Proteins).

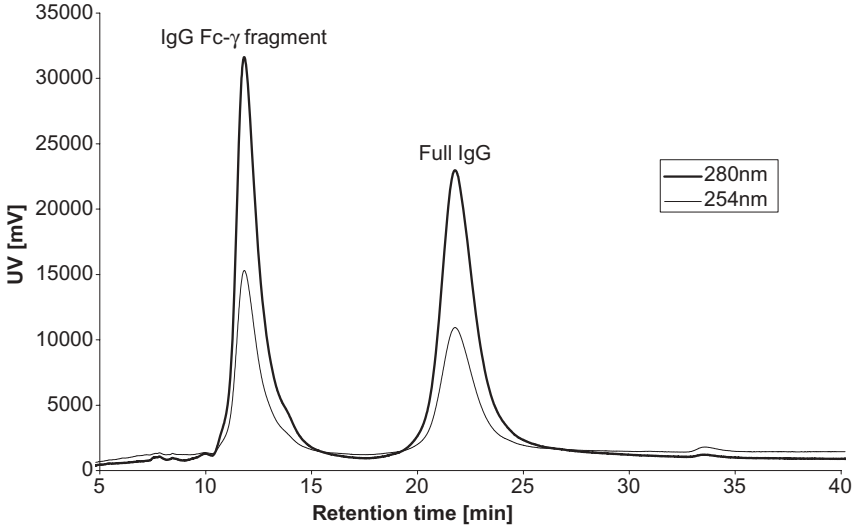

Figure 9.4 RP-HPLC chromatogram of a full IgG-mAb and its corresponding Fc-γ fragment: The Fc-γ fragment is eluted much earlier from the polymeric RP phase due to a reduced hydrophobicity value compared with the full IgG molecule; the chromatogram also shows the typical response factors of IgG-type glycoproteins: $UV_{280\ nm} > UV_{254\ nm}$. Courtesy of ProBioGen AG.

9.2.2.3 **Hydrophilic Interaction Chromatography**

Hydrophilic interaction chromatography (HILIC) is a distribution chromatography [12] that often employs a silica-based stationary phase with amino end-capping (e.g., aminopropylsilicate, APS). HILIC needs a hydrophilic stationary phase and a hydrophobic mobile phase. It is a type of "normal-phase" chromatography where elution is promoted by the use of more polar (often more aqueous) mobile phases. The order of elution is approximately the opposite of that expected for RPC. HILIC is widely used in the analysis of the glycan moiety of a glycoprotein, especially after separating the glycan part of a glycoprotein from the protein backbone, and after preparation of the glycans for chromatographic analysis. Preparation steps in glycosylation analysis often include enzymatic glycan cleavage or chemical deglycosylation techniques as well as fluorophore labeling techniques, such as 2-aminobenzamide (2-AB) labeling [13]. A typical chromatogram of a 2-AB-labeled *N*-glycan pattern of a recombinant glycoprotein can be found in Figure 9.5.

9.2.2.4 **Ion-Exchange Chromatography**

Ion-exchange chromatography, especially cation-exchange chromatography (CEX), is often used in the analysis of *C*-terminal lysine residues in monoclonal antibody (mAb) products as well as to detect deamidated product variants that indicate product degradation. Typical CEX resins for the analysis of mAbs are polymer based and use sulfonate or carboxylate functional groups as well as phosphate buffered aqueos eluents. A monoclonal antibody usually shows three lysine

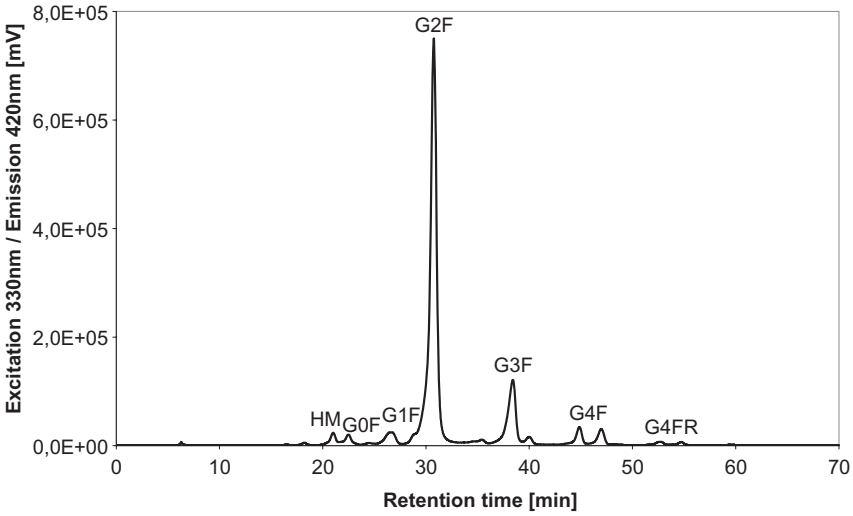

Figure 9.5 Pattern of 2-AB-labeled *N*-glycans of a recombinant glycoprotein; peaks are assigned with *N*-glycan structures from Figure 9.6 [13].

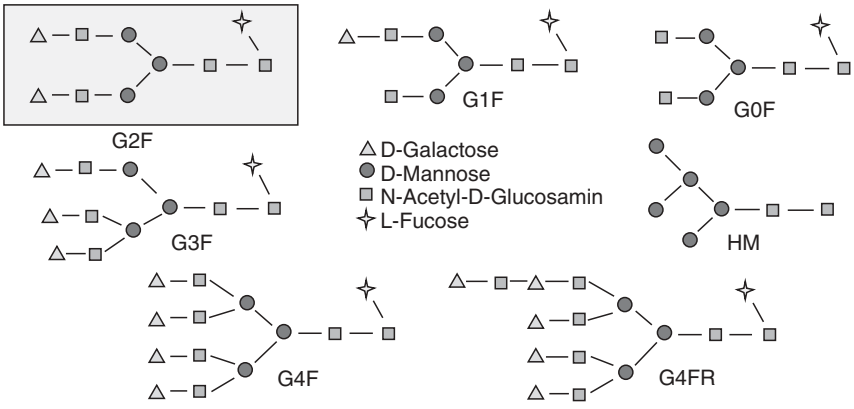

Figure 9.6 *N*-glycan structures of the annotated peaks in Figure 9.5, typical for CHO-derived glycoproteins [13].

isoforms that result in three peaks in the CEX chromatogram, corresponding to the three possible structures 0K (no *C*-terminal bound lysine), 1K (one *C*-terminal bound lysine), and 2K (two *C*-terminal bound lysines) (Figure 9.7).

9.2.3 Electrophoretic Methods

9.2.3.1 Gel Electrophoresis

Gel electrophoresis is based on the migration of dissolved charged molecules in an electrolyte solution through a gel-based matrix towards an electrode that pos-

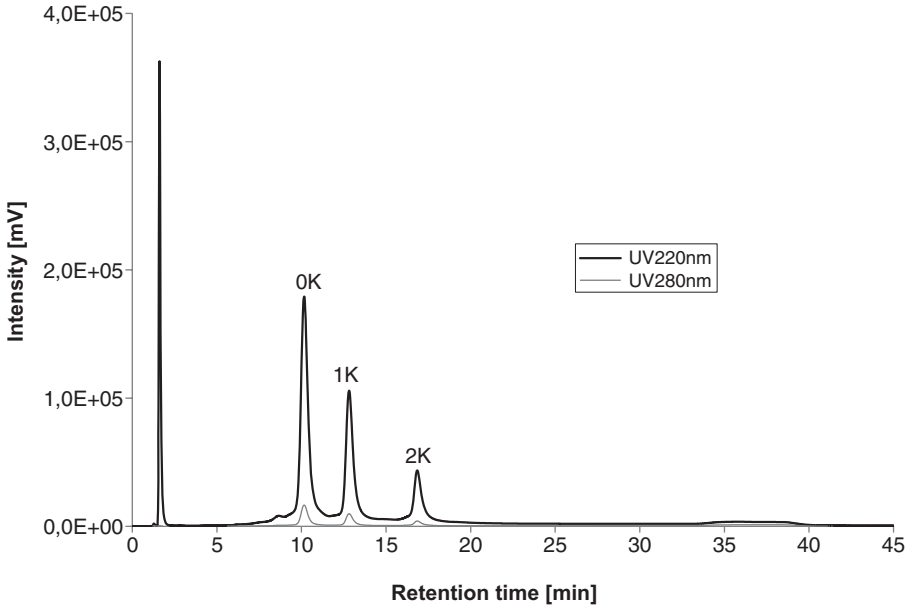

Figure 9.7 CEX chromatogram of a monoclonal antibody sample; the three typical lysine isoforms 0K, 1K, and 2K are assigned. Courtesy of ProBioGen AG.

sesses the opposite polarity to that of the molecules. Within the electric field the gel behaves as a molecular sieve that hinders larger molecules more than smaller ones to pass to the electrode (EP 2.2.31 [4]). To planish differentially charged macromolecules and realize a separation mostly based on molecular weight (hydrodynamic radius), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is used. For glycoprotein samples, the SDS-PAGEs are mainly stained by silver staining (Figure 9.8a), Coomassie staining (Figure 9.8b), or Western Blot staining (not shown).

A special gel electrophoresis for glycoprotein analysis is the isoelectric focusing technique (IEF, EP 2.2.54 [4]), which uses amphoteric electrolytes as running buffers on a polyacrylamide or agarose gel. Within the electric field the ampholytes create a pH gradient within the gel matrix. The glycoprotein samples, also amphoteric, pass through this pH gradient until their characteristic isoelectric points (p*I*) are reached and then create stainable bands in the gel. The p*I* of a glycoprotein is mainly dependent on its primary structure, but also on post-translational modifications, such as deamidated amino acid residues and acidic glycan (e.g., sialic acid) residues.

9.2.3.2 Capillary Electrophoresis

The general principles for capillary electrophoretic (CE) methods used in glycoprotein analysis are described in EP 2.2.47 [4]. Compared with classical gel electrophoresis, capillary electrophoresis offers a higher resolution and an increased sensitivity. The separation is performed by high voltage-induced migration inside

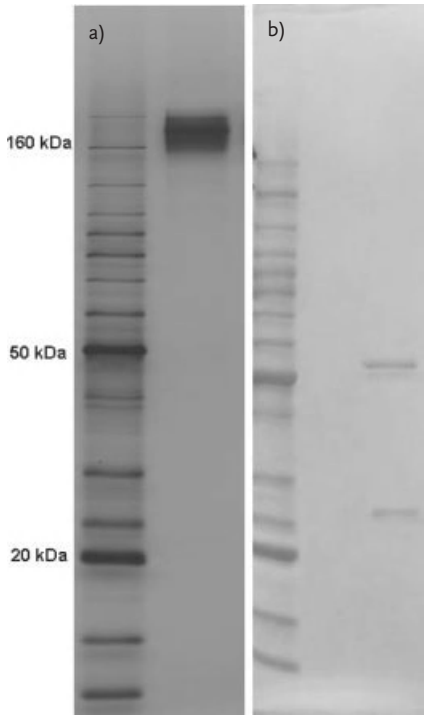

Figure 9.8 (a) Silver stained SDS-PAGE of an unreduced monoclonal antibody (IgG1) sample; (b) Coomassie stained SDS-PAGE of a reduced monoclonal antibody (IgG1) sample. Band ~25 kDa corresponds to light chain and band ~50 kDa to heavy chain. Courtesy of ProBioGen AG.

narrow-bore capillaries. CE has become a favorable technique for profiling different glycoprotein isoforms with very high resolution. CE also offers unique possibilities in assessing certain important characterization topics, such as characterizing glycoprotein macroheterogeneity (glycosylation site occupancy) in monoclonal antibody samples. With CGE (capillary gel electrophoresis) or MEKC (micellar electrokinetic chromatography), minor amounts of unglycosylated mAb can be elucidated from major amounts of glycosylated mAb within one electropherogram [14].

9.2.4 Other Physical Chemical Methods

Of course this book chapter is not designed to describe all physical chemical methods that are currently relevant for protein characterization. Other important methods not mentioned here are for example: circular dichroism (EP 2.2.41 [4]), NIR spectroscopy (EP 2.2.40 [4]), Raman spectroscopy (EP 2.2.48 [4]), analytical ultracentrifugation, field-flow fractionation, laser-light scattering techniques, and nuclear magnetic resonance (NMR) spectroscopy (EP 2.2.33 [4]).

9.2.5
Mass Spectrometric Analysis of Biopharmaceutical Proteins

The quality of a biopharmaceutical product is mainly defined by the overall product integrity as well as by the correctness of the amino acid sequence of the product, that is, product identity. Typically, quality attributes for a given product relate to product integrity issues, such as intrinsic or process-induced aggregation propensity as well as certain clone or process dependent post-translational modifications, including glycosylation, oxidation, deamidation, and so on. Since even small variations in operating conditions of an upstream process can have adverse effects on product quality attributes, quality guidelines require an extensive product analysis with respect to structural integrity. Given the fact that clones used for manufacturing of biopharmaceuticals comprise a dynamic living system, it is reasonable to anticipate a minor risk of introduction of undesired nucleotide mutations within the coding sequence of the biopharmaceutical during extended clone passaging. Regulators have addressed this concern by requiring sequence verification at the amino acid level. The quality guideline ICH Q6B [15] requires the verification of the correctness of the amino acid sequence of the product and that the identity test(s) employed should be highly specific and based on unique aspects of the product's molecular structure and other specific product properties.

Mass spectrometry methods are well suited to establish product identity down to the amino acid sequence level and to identify many critical product integrity issues. Figure 9.9 summarizes the main lines of quality assessment for biopharmaceuticals that are supported by mass spectrometry analytics.

9.2.5.1 Operating Principle of Mass Spectrometers

A mass spectrometer consists of three major parts: the ionizer and ion source, the mass analyzer, and the detector unit. At the start of a mass spectrometric analysis, a sample is introduced into the ionizing part of the instrument where it is subjected to an energy source that causes the sample to emit charged ions – this part of the instrument serves as the ion source [16, 17]. The emitted sample ions are then extracted into the analyzer region of the mass spectrometer where they are separated according to their mass-to-charge ratios (m/z) and maintained under high vacuum to allow unhindered ion travel within the instrument. The separated ions are detected and the resulting signal is recorded. The signal is transmitted to the data system where it is converted into mass-to-charge ratios (m/z) and peak relative abundance to allow presentation of the data in the format of an m/z spectrum. The m/z values are based on the equation: $m/z = (MW + n\mathrm{H}^+)/n$, where MW is the molecular mass of the sample ion, n is an integer of the number of charges per ion, and H is 1.008 Da, that is, the mass of a single proton [16, 17].

9.2.5.1.1 Modes of Sample Introduction

Depending on the type and complexity of the sample and on the ionization method used, there are two major modes of sample introduction: The sample can either

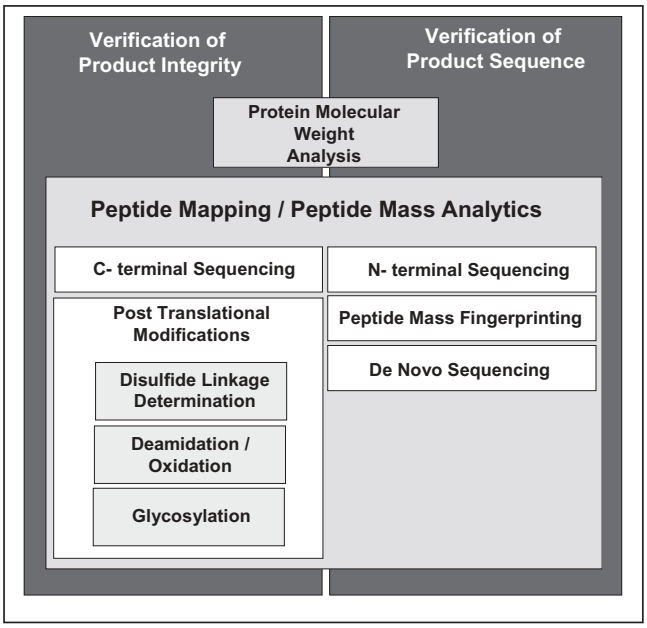

Figure 9.9 Main lines of quality assessment supported by mass spectrometry analytics.

be introduced directly into the ionization source or can be subjected to a chromatographic separation step en route to the ionization source [16, 17]. In the latter case the sample is consecutively prefractionated by coupled HPLC, GC or capillary electrophoresis into a series of components that enter the mass spectrometer one by one allowing a more detailed and intensive analysis of individual sample peaks [16, 17].

9.2.5.1.2 Ionizer Types/Ionization Sources

Ionization methods for the mass spectrometric analysis of proteins and peptides have been summarized by Ashcroft [16]. These include: matrix assisted laser desorption ionization (MALDI), electrospray ionization (ESI), fast atom bombardment (FAB), electron impact (EI), atmospheric pressure chemical ionization (APCI), chemical ionization (CI), field desorption/field ionization (FD/FI), and thermospray ionization (TSP). Since proteins and peptides are thermally unstable, their analysis by mass spectrometry requires a mild ionization technique that transfers the protein or peptide analyte into the gas phase without causing extensive loss by degradation or aggregation.

Proteins and peptides are typically analyzed in the positive ionization mode that generates protonated ions (M^+H^+) as the dominant ion species. Typical minor ion species observed in the positive ion mode include salt adducts, doubly charged molecular ions appearing at approximately half the mass over charge (m/z) value and dimeric species appearing at approximately twice the m/z value [16]. In the

case of negative ionization, deprotonated ions (M^-H^-) appear as the dominant species. Again, minor ion species include salt adducts and traces of dimeric or doubly charged ion species. Negative ionization is commonly used for the analysis of oligonucleotides and oligosaccharides.

For analysis of biopharmaceuticals, MALDI and ESI are the most commonly applied modes of sample ionization.

9.2.5.1.3 Mass Analyzers

Mass analyzers mediate the storage, separation, and resolution of the ions emitting from the ionization source. Separation and resolution of the ions within the mass analyzer is based on mass-to-charge (m/z) ratios of the sample ions. Mass analyzers all have different properties in terms of dynamic range, ion transmission, analysis speed, sensitivity, mass range, achievable resolution, and mass accuracy. Types of mass analyzers currently available include: time-of-flight (TOF) analyzers, quadrupoles, ion traps (ITs), orbitraps, ion cyclotron resonance (ICR) including Fourier transform ion cyclotron resonance (FTICR), magnetic sectors as well as Fourier transform ion traps [17, 18]. The type of mass analyzer must match the selected ionization methods within a given instrument. Mass analyzers can be applied in a consecutive fashion so that a sample ion that has passed through a first analyzer can be analyzed once again by yet another mass analyzer. Instruments with consecutively arranged mass analyzers are called tandem mass spectrometers (MS/MS). For the analysis of biopharmaceuticals, time-of-flight, ion trap, and quadrupole mass analyzers are most common [18].

MALDI, one of the two most common types of ion source for mass spectrometric analysis of proteins, peptides, and glycans, is often used in conjunction with time-of-flight (TOF) mass analyzers. Sample ions emitting from the source when activated with the same amount of kinetic energy (U_k) are differentiated by TOF analyzers based on the time elapsed between ion generation within the ion source, the ion passing through the length of the analyzer, and the time point where the ions make contact with the detector [17]. As the kinetic energy is a function of ion mass (m) and velocity (v), that is, $U_k = 1/2\, mv^2$, sample ions will be accelerated with different velocities depending on their molecular masses. Within a mass spectrometer, ions are further accelerated based on their charge (z). Thus, the time it takes for sample ions to pass the analyzer tube is proportional to their respective m/z values. The larger an ion and the lower its charge, the longer it takes until it strikes the detector. Reflectron TOF analyzers are typically applied to further increase the resolution of peptide masses, as the addition of a reflectron reduces the kinetic energy distribution of the sample ions striking the detector [17, 18]. On the flip side of the coin, however, reflectron TOF analyzers suffer from a reduced mass range and sensitivity [17, 18].

In a MALDI instrument, the sample is first mixed with an organic matrix such as α-cyano-4-hydroxy-cinnamic acid (CHCA), sinapinic acid (SA), or 2,5-dihydroxybenzoic acid (DHB), and then co-crystallized on the MALDI target, which typically consists of a stainless-steel plate. While there is a whole range of suitable matrix compounds, sinapinic acid is most commonly used as a matrix for

whole protein analysis, while α-cyano-4-hydroxycinnamic acid is a very common matrix for the analysis of peptides. The sample entrapped within the co-crystallized matrix is then applied to the instrument and immediately subjected to the high vacuum of the mass spectrometer. Once the instrument has reached its working vacuum level, the sample is bombarded with a powerful laser light, which results in efficient sample ionization.

The majority of commercially available MALDI mass spectrometers operate with a pulsed nitrogen laser with a wavelength of 337 nm [17, 18]. As the laser hits the co-crystallized sample–matrix mix, the laser energy is transformed into excitation energy for the sample. This excitation energy leads to a rapid emission of analyte and matrix ions from the surface of the sample–matrix mixture. The matrix provides an efficient energy transfer but also protects the analyte molecules from excessive direct laser energy exposure. MALDI is a soft ionization method, which means that it generates predominantly singly charged ions regardless of the molecular mass of the particular analyte [17]. The dominance of singly charged ion species in a MALDI spectrum makes MALDI spectra relatively easy to interpret as fragmentation of the sample ions does not typically occur.

ESI, the second of the two most common types of ion source for mass spectrometric analysis of proteins, peptides, and glycans is most commonly used with an ion trap or quadrupole mass analyzer [18]. In a quadrupole mass analyzer, sample ions are guided via current and radiofrequency voltages that are applied to four metal rods arranged in parallel with opposing rods carrying opposite charges [17, 18]. Sample ions are separated based on stability of the ion trajectory through the quadrupole rods. Scanning of direct current voltage and alternating radiofrequency voltage settings, while keeping their ratio constant, allows sample ions with different m/z values to pass consecutively through the quadrupole. Sample ions with different m/z values travel at different velocities and thus different trajectories through the quadrupole. At a specific voltage ratio only sample ions with a particular m/z value travel through the quadrupole while all other ions collide with the rods and never reach the detector [17, 18].

An ion-trap mass analyzer mediates an iterative operating mode of collecting, storing, and ejecting the encountered sample ions. Ion-trap analyzers support tandem MS (MS/MS), as they are able to isolate, store, and fragment trapped sample ions in a consecutive fashion. A specific sample ion is being stored within the confines of an ion trap mass analyzer when the trapping voltage setting is adjusted to eject all other ions from the trap. Once a specific sample ion is isolated and retained within the ion trap, it may be subjected to collision induced fragmentation by increasing the energy of the ions and causing them to collide with helium molecules that are present within the trap [17, 18]. Collisions of the larger sample ions with helium molecules cause the sample ions to fragment at the peptide bonds that experience the strongest collision energy effect. Fragment ions resulting from collisions within the ion trap are again trapped and emitted from the ion trap according to their m/z value, in a scanning mode that allows an identification of fragment peptide masses.

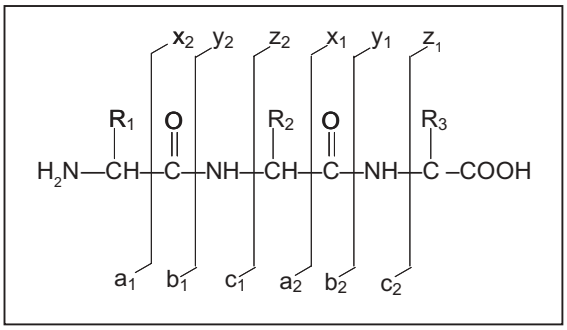

Figure 9.10 Annotation of peptide fragments in tandem MS, according to Roepstorff and Fohlman [22].

The effects of collision energy on a given peptide and the chemistry of peptide fragmentation are rather complex and thus the pattern of peaks in an MS/MS can only be predicted with certain limitations [19–21]. As of today an exact prediction of expected peak relative abundance, or even the prediction of the detectability of a given expected peak, has remained elusive [20].

In the case of peptide sample ions, two different groups of fragment ions are generated – charged fragment ions consisting of the remaining *N*-terminal part of the former precursor sample ion and charged fragment ions consisting of the former *C*-terminal part. Depending on the exact side of cleavage, these fragment ions are annotated as a_n, b_n, c_n for the *N*-terminal fragment ions or x_n, y_n, z_n for the *C*-terminal fragment ions [22]. The b- and y-ions are most common, as they arise from cleavage at the predominantly cleaved CO–NH bond. The identity of amino acid residues in a peptide sequence can be deduced from the mass difference between two adjacent b or y ions (Figure 9.10).

9.2.5.1.4 **Detectors**

The detector type must match with the type of mass analyzer. Common detector types are micro-channel plate detectors as well as electron- and photomultipliers. The detector unit records and amplifies the ion current and transmits the signal to a data system where the resulting mass spectra are compiled from the calculated m/z values and their matching peak relative intensities, which are indicative of the relative abundance of a sample ion within the complex sample [17, 18].

9.2.5.2 **Common Methods for MS Analysis of Biopharmaceuticals**

9.2.5.2.1 **Peptide Mass Fingerprinting**

Peptide mass fingerprinting (PMF) is applied to verify the identity of a manufactured therapeutic protein at early stages of drug development and later to demonstrate comparability and lot consistency during the manufacturing process. In addition, the ICH Q6B guidelines list peptide mapping as a key part of the characterization of biopharmaceuticals. PMF is a peptide mapping technique based on mass spectrometry that allows the identification of peptides which have been

obtained from enzymatic cleavage of a given protein of interest [23]. The peptide mixture is analyzed by mass spectrometry and the resulting peptide masses are then compared with a database containing absolute masses calculated for the expected peptides that would theoretically be expected from enzymatic cleavage of the biopharmaceutical. The detector signals are converted into a list of identified peaks and their cognate molecular weights and compared with a database (e.g., Swissprot, Genbank or target specific database), which contains information about protein sequences and their theoretically calculated fragments that have resulted from *in silico* cleavage of these protein sequences into peptides with the same enzyme or chemical used in the original cleavage (e.g., trypsin).

Finally, the software compares the mass list of measured peptides and all of the respective calculated peptide masses. Possible matches are identified statistically and included in the results table. Additional tandem MS enables unambiguous identification of peptides based on *de novo* sequence information. All identified peptides are then matched with the complete amino acid sequence expected for the biopharmaceutic protein under investigation and sequence coverage is calculated. In order to confirm identity by a peptide mapping approach, at least 95% sequence coverage should be achieved. Full sequence coverage may only be achieved by applying orthogonal MS techniques.

While there are several proteolytic enzymes available that allow the generation of unique patterns of peptide fragments from a given protein, trypsin has emerged as the predominant enzyme for peptide mass fingerprinting applications. The reasons for this are the efficiency of tryptic cleavage and the cleavage site specificity of trypsin, which cleaves on the carboxylic acid side of lysine and arginine residues [24], and thus results in a majority of peptides that carry either a strongly basic arginine or lysine residue at their *C*-termini. In this way it is ensured that most peptides carry at least one strongly basic charge that supports the ionization in the positive-ion mode.

In order to achieve an optimal sequence coverage for a protein of interest, there are two important sample preparation issues. Firstly, samples should be reduced and alkylated to obtain the best results. A common reagent for alkylation is iodoacetamide, which adds a carboxamidomethyl group to cystein residues. Secondly, trypsin autolysis must be avoided since interfering autolytic peaks and their ambiguous adducts may otherwise render the mass spectrum more complex and suppress the relative intensity of sample ion peaks. Trypsin autolysis peaks are avoided by using a proteomics grade trypsin enzyme that has been exhaustively processed by reductive methylation. In addition, the chymotryptic activity of proteomics grade trypsin is quenched by treatment with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) [25]. Typically, a sample : protease ratio of 50 : 1 is used to digest samples overnight. Proteins larger than 100 kDa require citraconylation, maleylation or succinylation of their lysine residues to allow cleavage of peptide bonds solely at the *C*-terminal side of arginine residues, thus generating a tryptic peptide pattern that is amenable to MS analysis [26].

In peptide mass fingerprinting, the peptide mapping approach by enzymatic or chemical cleavage is further supported by an MS analysis of the peptide mixture.

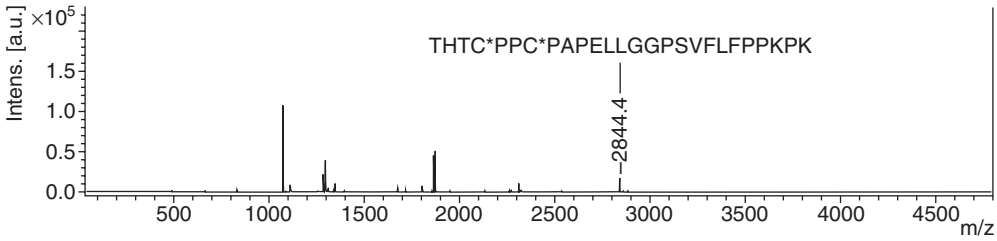

Figure 9.11 Example of a tryptic peptide mass fingerprint of a monoclonal antibody heavy chain analyzed by MALDI-TOF. Note the differences in peak relative abundance. Asterisks indicate mass alteration of cysteine residues by carboxamidomethylation. Courtesy of ProBioGen AG.

Complex peptide mixtures are only amenable to analysis by hyphenated techniques such as on-line LC-MS.

Figure 9.11 shows an example of a peptide mass fingerprint derived from tryptic fragments of a monoclonal antibody heavy chain. The peptide mass peak at 2844.4 m/z indicates the peptide shown above the peak. Both cysteines have an altered mass of +58 amu (atomic mass units) due to carboxamidomethylation by iodoacetamide. In addition, the missed cleavage at one lysine residue illustrates one of the exemptions of tryptic cleavage – when a lysine residue is directly succeeded by a proline residue.

9.2.5.2.2 *N*- and *C*-Terminal Sequencing by MALDI-ISD (MALDI In-Source Decay)

Characterization of recombinant therapeutic proteins according to the ICH Q6B guideline requires confirmation of the *N*-terminal sequence. Moreover, *N*-terminal sequencing is frequently used to monitor lot consistency and to demonstrate comparability. *N*-terminal sequencing is typically done by the classic Edman approach. In many cases *N*-termini can be blocked and thus are not freely accessible for Edman sequencing. In the case of monoclonal antibodies, pyroglutamylation of the *N*-termini of light and heavy chains is fairly common. MALDI-ISD (in-source decay) is unaffected by blocked *N*-termini and can thus be employed to obtain the *N*-terminal sequence even in cases where the first amino acid is blocked by post-translational modification. A single MALDI-ISD analysis run can result in very long sequence reads covering up to 50 amino acid residues from both the *N*- and *C*-terminal ends and can even detect unexpected post-translational modifications of the sequenced amino acids.

MALDI-ISD can be applied for proteins that have an atomic mass between 6 and 80 kDa (Alphalyse Application note: *N*- and *C*-terminal sequencing by MALDI-ISD # 2000902), for example, antibody heavy and light chains. In MALDI-ISD, the full-length protein is analyzed by MALDI-TOF and further fragmented at the terminal amino acids within the MALDI ion source, whereby the resulting fragments are again analyzed by a time-of-flight mass analyzer (MALDI-TOF/TOF approach). The MALDI spectrum that results from this approach shows mainly singly charged c- and y-ions and to a lesser extend a- and doubly charged z-ions

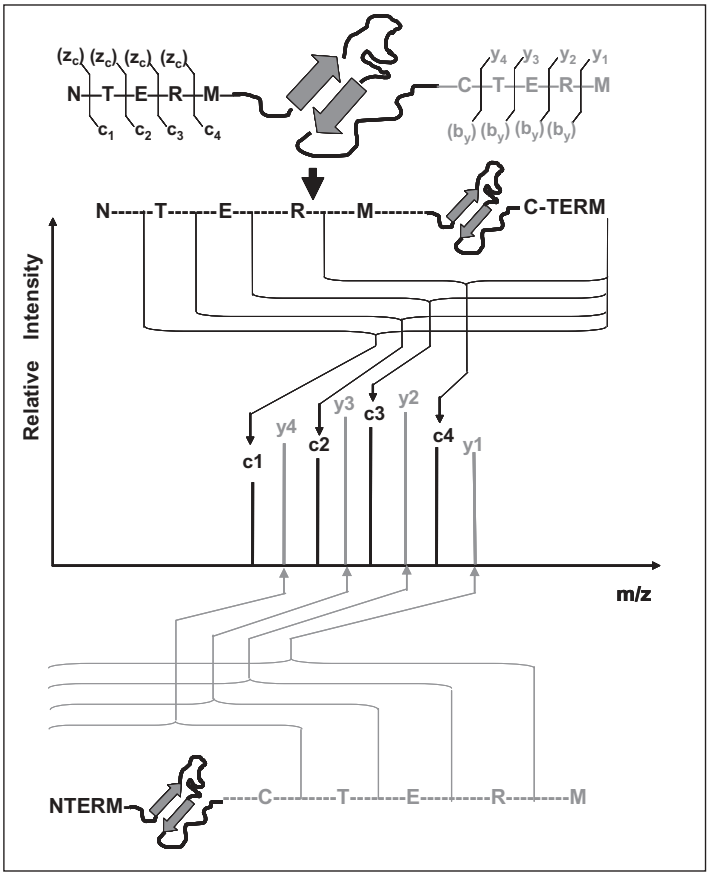

Figure 9.12 Operating principle of the MALDI-ISD method. The intact protein is fragmented at the terminal amino acids into series of c- and a-ions at the *N*-terminus and of y- and z-ions at the *C*-terminus. *N*- and *C*-terminal sequences are derived from the predominant c- and y-ion series.

(see Figure 9.12). The c- and y-ion series allow a direct assignment for both the *N*- and *C*-termini (Figure 9.12); z^{+2} ions can also be used to confirm the *C*-terminal sequence.

9.2.5.2.3 **Disulfide Linkage Analysis**

Incorrectly folded and disulfide-bonded forms of a therapeutic protein are a type of product-related impurity that is difficult to remove due to its similarity in amino acid sequence and certain associated physicochemical properties [27]. Even though mammalian cell lines such as Chinese hamster ovary (CHO) cells support the production of correctly folded and disulfide-bonded proteins, disulfide scrambling is still possible when target proteins are overexpressed in CHO cells to boost protein yield [27]. Thus, disulfide linkage as well as the location and

redox-status of unpaired cysteines are important product quality attributes that need to be analyzed as far as possible. The ICH Q6B guideline recommends disulfide linkage analysis as part of the comprehensive characterization of biopharmaceuticals.

Disulfide mapping and assignment of free sulfhydryl groups is achieved by peptide mapping strategies that allow a definitive assignment of unique mass values to the disulfide-linked peptides [28]. The correct assignment of disulfides becomes more challenging if a protein contains multiple disulfide bonds. In principle, disulfide mapping involves digestion of the target protein by an enzyme or chemical and analysis of the resulting peptide mixture by online LC-MS. Identified disulfide bonded peptides can then be confirmed either by re-analysis of the peptide masses of the reduced species or by MS/MS peptide sequencing. Powerful mass spectrometric techniques allow even the detection of very low amounts of mismatched or scrambled disulfide bonds in a given target protein sample.

Disulfide bond cleavages are more rapidly cleaved compared with the chemical bonds in the peptide backbone by electron capture dissociation and electron transfer dissociation [27]. Thus, online LC-MS strategies combined with electron capture dissociation or electron-transfer dissociation have become the current state-of-the-art in disulfide linkage analysis [9, 27, 29].

9.3 Biological Characterization of Biopharmaceuticals *In Vitro*

9.3.1 Bioassays

9.3.1.1 Introduction

The testing of bioactivity has become more important in biopharmaceutical analytics with the increasing number of very effective and highly target-specific recombinant biologics and completes the structural and biochemical characterization. Bioassays give useful information for R&D, mode of action analysis (MoA) and lead candidate selection, and are crucial for pharmaceutical release testing. Assay quality and performance is adapted to the practical needs and regulatory requirements. The spectrum of bioassays covers robust screening applications and highly sophisticated lab procedures up to qualified or fully validated QC methods. They also play an important role in comparability testing for the increasing market of biosimilars.

Bioassays are modeling pharmaceutical interactions of higher complexity beyond physicochemical and biochemical analysis. They include binding assays and cell-based assays (CBAs). Binding assays cover receptor–ligand interactions, such as ELISA (enzyme-linked immunosorbent assay) formats. Cell-based assays are targeting cellular reactivity.

The challenge of cell-based assays is given by the implementation of one or more complex biological components, “living” factors such as cells, microbes or viruses.

For most of the CBAs in biopharmaceutical testing, the single cell, cell-population or co-culture, with their complex and integrated physiology, are considered as a black box system: drug exposure as input and the single-parameter analysis of cellular response as output (Figure 9.13).

Complex bioassays bridge the gap from the lab bench to animal testing. For the very potent and highly human specific biologics in particular, CBAs based on human cells bridge the gap between animal testing and first-in-human applications also. In addition, they are applied to clinical patient monitoring, for example, for immunogenicity (see Section 9.3.1.5, Immunogenicity Testing).

CBAs are monitoring effects on a subcellular level, for example, genomic, transcriptomic or proteomic changes, the expression and presentation of cytoplasmatic or membrane markers, and also on cellular or cell population levels, for example, proliferation, secretion or cell death (Figure 9.14).

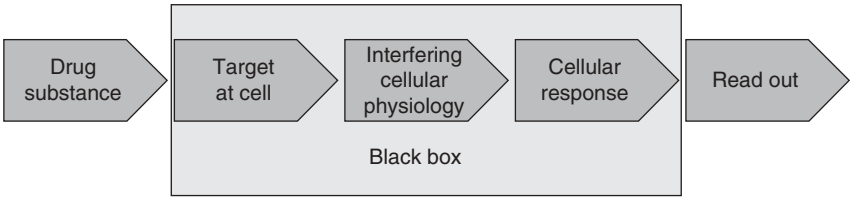

Figure 9.13 Cell-based assays use the complex and integrated physiology of a cell. For assay applications, the cell is more or less defined as a black box; only selected parameters are monitored.

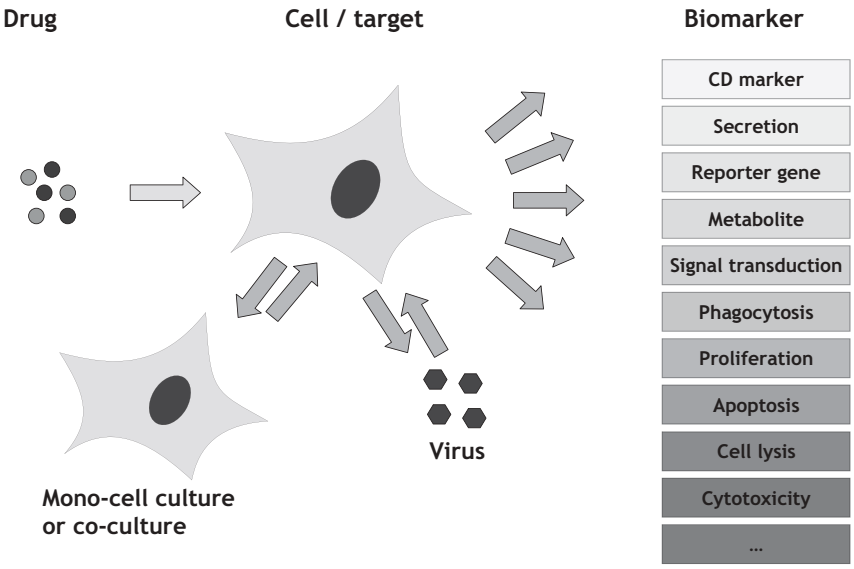

Figure 9.14 Cells and cellular reactions: selection of a suitable, robust, and relevant analytical readout parameter for cell-based assay development.

For the successful establishment of a bioassay – LBA or CBA – it is very important to pay attention to the selection and implementation of a suitable, robust, and relevant binding or cellular model.

The assay could cover, realistically, parts of the pharmacological relevant action, such as ADCC assays for antibody drugs or just a surrogate assay, such as the antiviral assay (AVA) for IFN-α products, aside from the pharmaceutical application.

9.3.1.2 **Defining Bioactivity**

The bioactivity of a pharmaceutical drug is given by the specific induction of a molecular or cellular response. The effect is selective and dose-dependent. Bioactivity is defined by the EC50 or IC50 value, the effective (or inhibitory) dose for 50% of the maximum observed effect. Application of serial dilutions of the drug will give sigmoid response curves to be fitted by 4- or 5-parameter sigmoid fit algorithms (Figure 9.15). The lower and upper asymptotes define the dynamic range. The inflection point correlates with the half-maximum effective concentration (EC50). The maximum slope and shape of the dose-response behavior could be compared with those of the reference material and is an important tool for the understanding and proofing of biosimilarity (parallelism analysis). A more simplified method uses single sample concentrations related to a standard curve. This will give bioactivity data in relation to a standard material or a reference product (activity ratio; Figure 9.16).

The EC50-derived bioactivity is specified in Units (U), as specific activity (Units per mass, e.g., U/mg) or as an activity concentration (Units per volume, e.g., U/ml).

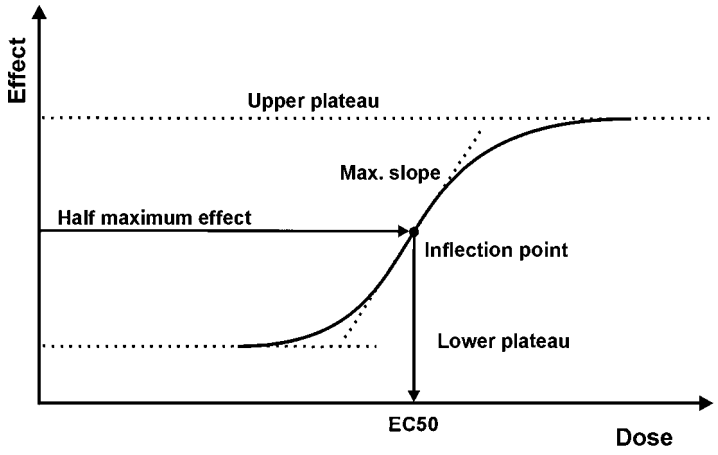

Figure 9.15 A dose-response model and a sigmoid fit analysis with a lower and upper asymptote, maximum slope, and the inflection point. The inflection point correlates with the half-maximum effective concentration (EC50). The maximum slope and shape of the dose-response behavior could be compared with those of the reference material and is an important tool for the understanding and proofing of biosimilarity.

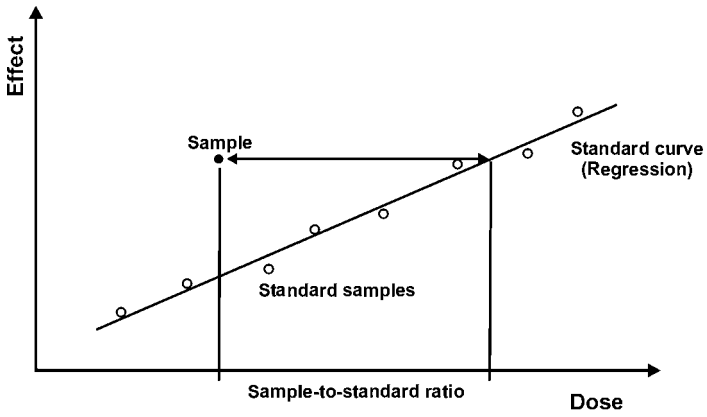

Figure 9.16 Standard curve model.

The implementation of an international WHO standard is recommended if standard material is available. This will give the opportunity of defining the bioactivity of the drug in international units (IU, IU/mg or IU/ml). The adjustment and referencing of the assay to an internationally recognized and accepted standard material will give activity data comparable to other products and other assays.

9.3.1.3 **Binding Assays**

Binding assays or immuno binding assays are commonly used for the characterization of bioactivity of therapeutic antibodies and their derivatives [30]. The target-specific binding activity is given by the affinity of the antigen binding sites in the variable domains of the antibody to the given epitope. The effector functionality, for example, natural killer cell (NK), mediated cytotoxicity or complement mediated cell killing (see ADCC and CDC), is given by the Fcγ-receptor binding site in the constant region of the antibody backbone.

9.3.1.3.1 **Ligand Binding Assay (LBA)**

Most of the binding assays are performed in the 96-well or 384-well plate ELISA format. The ELISA plate is coated with ligand by adsorption or by using tag-chelate mediated binding if a tagged antigen is available. Drug binding is detected by a direct labeled anti-drug antibody in a sandwich using ELISA. If a direct labeled anti-drug antibody is not available, or for signal amplification, a second detection antibody is used, directed against the animal species of the primary detection antibody, competitive ELISAs are described.

9.3.1.3.2 **Fcγ-Receptor Binding Assays (Fcγ-RA)**

Fcγ-receptor binding assays (RBA) are designed for the analysis of effector functionality, for example, the induction of cell mediated cytotoxicity (see Section 9.3.1.6.5).

NK cells are the most prominent cell killers of a vertebrate's immune system. NK cells carry antibody binding receptors: FcγR-IIIa/b, FcγR-II, and FcγR-I (also known as cellular markers CD16, CD32, and CD64).

For a FcγRA, the ELISA plate is coated with Fcγ-III, Fcγ-II, and Fcγ-I preparations. The to-be-tested antibody binds to the coated receptor and is quantified by a direct labeled anti-mAb antibody or a system of an unlabeled primary detection antibody and second detection antibody.

Detection Concentrations of all binding partners have to be optimized for optimal stoichiometry to ensure a high dynamic range and analytical performance. For detection, an enzymatic labeling in combination with a chromogenic substrate is commonly used, for example, alkaline phosphatase (AP) and redox substrates, such as NBT (nitro blue tetrazolium chloride) or BCIP (5-bromo-4-chloro-3-indoyl phosphate), or horseradish peroxidase (HRP, POD) and the redox substrates ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DAB (3,3′-diaminobenzidine) or AEC (3-amino-9-ethylcarbazole). Chromogenic assays are limited to two to three decades for analyte concentrations due to the low dynamic range of photometric absorption detection given by the Lambert–Beer Law. The limitations in the dynamic range are overcome by fluorescence and luminescence detection systems. Further analytical improvements are available by using electrochemical luminescence (ECL). The ECL technology uses electric-stimulated luminescence. The detection antibodies are labeled with an electric inducible luminophore. The luminescence is induced in a very short distance to the electrodes of a detection plate in a 96-well format, which gives a very low background signal with a high dynamic range of up to five decades of analyte concentrations.

9.3.1.3.3 Surface Plasmon Resonance Spectroscopy (SPR-Spectroscopy)

Surface plasmon resonance SPR spectroscopy is the standard method for the determination of binding constants of ligand and receptor interaction. All therapeutic antibodies are usually characterized by this technology for affinity and binding constants. The brand name "Biacore™" is synonymously used for SPR technology. For binding analysis, the target ligand is immobilized on a dextran layer on a gold-coated glass surface. The glass surface is part of a microfluidic system. A defined solution of the analyte is injected into the fluidic system. The analyte is deposited on the ligand–dextran layer by specific binding. A laser or diode light beam is positioned onto the glass surface from the outer side at an acute angle. Most of the light is reflected by total internal reflection. A small portion of the irradiated light is not reflected on the glass surface and induces an evanescent wave as a near-field effect in the gold layer. A defined solution of the analyte is injected into the fluidic system. The mass deposition of the analyte induces changes in the evanescent wave and changes the reflection angle or, for a given angle, the reflection light intensity. The mass deposition effect is indicated in response units (RU). The binding interaction is a dynamic process, depending on stoichiometric concentrations of binding partners, the receptor affinity or avidity, the solvent, the matrix-milieu, and other factors. The equilibrium

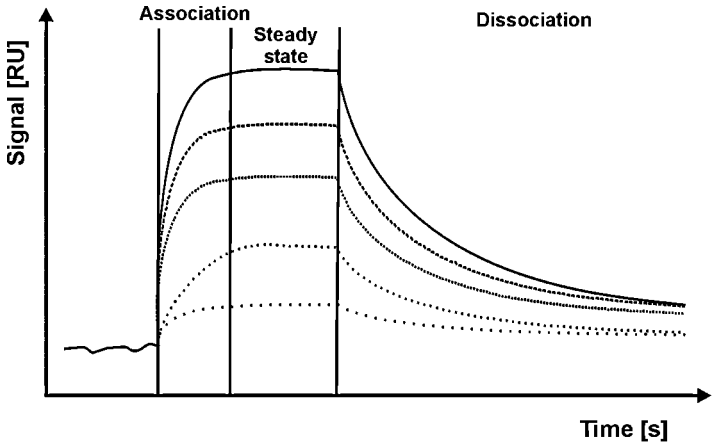

Figure 9.17 Exemplified SPR curves for antibody–ligand binding (dilution series of a to-be-tested antibody).

dissociation constant (binding constant, K_D) can be derived from the association rate (on rate, k_a) and dissociation rate (off rate, k_d). The binding constant K_D is given by the quotient of dissociation rate and association rate (Figure 9.17):

$$K_D = \frac{k_d}{k_a}$$

9.3.1.4 Bead Array Technology

To overcome the limitations of the classical ELISA format, bead-based solid phases have overrun the analytical market. The most prominent benefits are miniaturization, the reduction of sample volume, and the use of color-coded array systems for multiplexing (up to 100 parameters in parallel). Unfortunately, they are quite expensive in instrumentation and consumables and are hard to validate due to poor international harmonization and standardization and large systematic deviations between various existing technological platforms. They are well established in R&D, in clinical diagnostics, and in analytical GLP settings. They will find acceptance in pharmaceutical release testing in the future.

9.3.1.5 Immunogenicity Testing

Immunogenicity testing is used for the monitoring of unwanted anti-drug immune responses in the patients. Cellular and humoral immune responses could be induced by foreign or new active pharmaceutical ingredients (APIs) and formulations ([31–33]). The formation of anti-drug-antibodies (ADAs) or drug-neutralizing antibodies (NAbs) are the most prominent immunogenic responses observed. The NAbs capture the drug molecules, neutralize the bioactivity, and influence the pharmacokinetic profile. They may also induce unwanted effects, such as sensitization or allergy, as well as severe side effects, such as anaphylaxis or autoimmu-

nity, as described for an EPO product which has induced a red cell aplasia (RCA) in patients due to changes in the pharmaceutical formulation.

ADA formation has to be monitored during preclinical animal-toxicity studies and the clinical application of the drug to humans by *ex vivo* analyses of blood samples. ADA response is analyzed by a set of three assays, two binding assays, and a bioactivity assay: a first screening assay to catch all positives, followed by a confirmation assay to remove the wrong positives, and then a final bioactivity assay to characterize the functional neutralizing potential.

Ligand binding assays for screening and confirmation are usually used in combination with a more complex cell-based assay to assess bioactivity. Ideally, the same assays formally developed for the drug characterization are used.

9.3.1.6 **Cell-Based Assays (CBA)**

It is very important for the successful establishment of a CBA to select a suitable, robust, and relevant cell model. Modern biopharmaceutical drugs are of natural origin, slightly modified, fusion proteins or fully recombinant. They may have post-translational modifications, be processed in the glycosylation pattern or formulated, for example, PEGylated. Generic CBAs can be easily used for well described, more natural, intrinsic proteins with moderate modifications. New recombinant-modified or artificial proteins need more attention in the selection of the assay cell substrate and its molecular target, as well as in the assay procedure and analytics.

For assay purposes primary cells and cell lines from different species (mammalians, vertebrates, and non-vertebrates) and of human origin are available. Well established cell lines are preferred. Most of the cell lines are tumor derived and may have defects in the relevant genotype, relevant cellular physiology or morphology. Recombinant cell lines, so-called designer cell lines, are engineered with defined and well characterized modifications, for example, "gentle" immortalization procedures or reporter gene integrations. Even cell lines have to be well characterized, and they need this to confirm identity and functionality. Cell line preparations provided by commercial cell banking organizations such as ATCC™, ECACC™, LGC™, RIKEN™ or DSMZ™ are scientifically well described but usually not quality controlled. In review and spot tests many cell lines used in R&D and testing applications have been identified as incorrect, misidentified or cross-contaminated, for example, by HeLa cells or others [34, 35]. They may also bear the risk of hidden microbials, for example, mycoplasma infections or viral loads [35, 36]. The identity and microbial burden should be tested during isolated, quarantine cultivation [37]. To improve validity and reproducibility of cell-based models for *in vitro* testing the European Centre for the Validation of Alternative Methods (ECVAM) has recommended a set of rules for "Good Cell Culture Practice" ("GCCP") [38, 39].

The cell material has to be provided as "ready-to-use" to reduce variations in the assay application. The installation of a cell bank is strongly recommended. The number of vials and the amount of cells per vial should be adjusted to the designed assay application. It is very helpful to use single vials for every single-assay application.

The champion's league in CBAs uses primary cells. They should only be used if a suitable cell line is not available. Primary cells have a limited lifespan and they tend to dedifferentiate during culture, resulting in changes of proliferation, morphology, and physiology. They are very sensitive to cultivation handling, culture media, and supplements, freeze–thaw procedures, enzymatic and mechanical treatments, and confluency of adherent growing cells to, for example, contact inhibition.

In addition to the cellular substrates, the cultivation procedure has a strong influence on the assay performance. Optimization and standardization of storage, freeze–thaw procedures, pre-cultivation, pre-treatment, drug exposition, and analytical treatments is mandatory. Different cell culture procedures will give different results. The cells could be used as suspension cultures, as monolayers of different stages of confluency, as spheroid cultures, or matrix-assisted in 3D, perfused, and non-perfused [40]. The assay should be as simple as possible for drug release testing applications to ensure a maximum of reproducibility, robustness, and regulatory acceptance.

9.3.1.6.1 **Proliferation Assay**

Proliferation and anti-proliferation assays are designed for the testing of drugs inducing or inhibiting the proliferation of a target cell line. Type I interferons (IFN-α and IFN-β), for example, have an anti-proliferative effect on DAUDI cells (Burkitt's lymphoma-derived human B cell line). The proliferation is determined by several methods for cell enumeration and cell-viability analysis:

- Manual or automated counting of dead and viable cells by dye exclusion using Neubauer microscopic counting slides or flow chambers. Dying or dead cells incorporate Trypan blue dye or Propidium iodide, vital cells do not.
- Impedance-based cell detectors (e.g., Coulter Counter™ or Casy™ technology). When suspended cells pass through a capillary pore of cellular diameter, they can be analyzed by an impedance detector. Viable cells with an intact plasma membrane are electric insulators, dying or dead cells are not.
- Flow-cytometric devices (FACS) are usually designed to identify and quantify the proportion of certain populations of cells and particles by light scattering and cell specific fluorescent labeling (see Section 9.3.1.6.9). Modern FACS have volumetric sensors in the fluidic system or bead-based enumeration reagents are available to normalize relative signals. They can be used to count cells. In combination with a viability marker (vital dye exclusion or incorporation), the method can be used to quantify cell number and viability.
- Dispersive multi-well based method for quantification of cellular coenzyme ATP (adenosine triphosphate). Every single cell has a distinct amount of ATP. Cells are lysed and the released ATP is quantified in the lysis buffer by using ATP-dependent enzymes and fluorogenic or luminescent co-substrates.

9.3.1.6.2 Neutralizing Assay

Neutralization assays are a sub-form of proliferation or cytotoxicity assays. They are designed to test the neutralizing capacity of antibodies (NAbs) [41]. One of the most prominent neutralizing assays is designed for testing anti-inflammatory NAbs, such as Infliximab, Adalimumab or Etanercept, directed against TNFα (tumor necrosis factor alpha). A TNFα sensitive cell line, for example, KYM-1D4 or WEHI-3b, is cultivated in TNFα supplemented media. Cell proliferation is suppressed by TNFα supplementation. The antibodies neutralize the TNFα suppression and the cells proliferate. Proliferation is analyzed as described above.

9.3.1.6.3 Cytotoxicity Assay

Cells in culture die by necrosis, apoptosis or cytotoxic effects. Cytotoxicity is defined as specifically induced death of cells in culture, resulting in metabolic breakdown or cell lysis. Apoptosis, programmed cell death, is controlled by cascades of cell-borne self-killing programmes. Cytotoxicity affects the cell culture viability (specified in percent), which is defined by the ratio of the total cell number and viable cell number multiplied by 100.

Cell death can be monitored in cell cultures by cell enumeration and cell-viability methods (e.g., Trypan blue, cellular ATP), the release of vital dyes (e.g., Calcein-AM) or cytoplasmatic enzymes (e.g., proteases, LDH), or the breakdown of cellular metabolic activity (e.g., MTT, WST):

- **Cell enumeration and cell-viability methods.** See Proliferation Assay in Section 9.3.1.6.1.
- **Vital dye release.** The cells are pre-stained before treatment using a non-toxic vital dye (e.g., Calcein-AM). After treatment, the culture-supernatant is analyzed for dye-concentration released by secreting or lysed cells.
- **Release of cytoplasmatic enzymes.** Lysing cells release cytoplasmatic enzymes, such as lactate dehydrogenase (LDH) or proteases. Enzyme activity in culture supernatant can be quantified by specific chromogenic or fluorogenic substrate uptake. Advantage – no pre-staining of cell necessary. Disadvantage – high background and interference induced by other enzymes or inhibitors.
- **Metabolic breakdown.** Toxic substances may interfere or block the metabolic pathways or transporters in organelles, cytoplasm or membranes. Mitochondrial redox cascades can be monitored by the use of chromogenic redox substrates, such as the tetrazolium based MTT (dimethyl thiazolyl diphenyl tetrazolium) or XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) or other derivatives, to quantify the redox potential in cells. If the cell specific metabolic rate is set as constant, it can be used for cell enumeration. If the cell number in the culture is fixed, or is set as a constant, changes in substrate uptake can be used for the determination of cellular metabolic breakdown.

Cytotoxicity testing is, therefore, also very important in the development and certification of medical devices and compounds, as well as for consumer products,

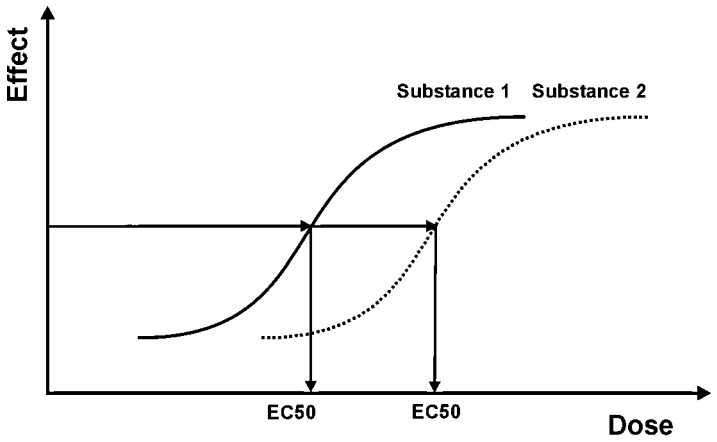

Figure 9.18 Antiviral assay (AVA). Full dose response curves for a PEGylated (Substance 2) and a non-PEGylated (Substance 1) IFN-α product. PEGylated IFNs have a reduced bioactivity *in vitro* given by a higher EC50 value.

for example, cosmetics. The testing procedures for medical devices are defined by international ISO regulations (DIN EN ISO 13485) [42].

9.3.1.6.4 **Antiviral Assay**

The antiviral assay (AVA) is used for the testing of bioactivity for Type I interferons. Type I interferons, for example, IFN-α and IFN-β play an important role in the antiviral defense of vertebrates [43]. The AVA is described as a surrogate test for potency testing of IFN-α and IFN-β in the EU and US Pharmacopoeia [4, 44].

A virus–host cell infection model is used to determine the neutralization of a virus-induced cytopathic effect (CPE). Virus infected cells die when virus particles are released. The human epithelial lung carcinoma cell line A-549 is used as a host cell and the lytical viruses Encephalomyocarditis virus (EMCV) or Vesicular stomatitis virus (VSV) are used as model viruses. A proliferating monolayer culture of A-549 cells (log-phase) is pre-incubated with serial dilutions of the interferons for 24 h and is then infected with the virus. The cytopathic effect forms plaques in the cellular monolayer and decreases the cell number. The viable cells are quantified by cell enumeration methods (see Section 9.3.1.6.1). Virus handling may be the subject of national safety regulations (e.g., biosafety or veterinary safety) (Figure 9.18).

9.3.1.6.5 **Antibody Dependent Cellular Cytotoxicity (ADCC)**

Many therapeutic antibodies induce antibody dependent cellular cytotoxicity (ADCC) by the effector domain (Fcγ). To test for ADCC performance, primary human NK cells in combination with a target specific cell line are used. The target cell line is stained with a vital fluorogenic or fluorescent dye, for example, Calcein-

AM, and co-cultured with the NK cells in a defined effector-to-target cell ratio (E : T), for example, 25 : 1 or 10 : 1. The co-culture is exposed to serial dilutions of the to-be-tested antibody. The NK cell induced ADCC is quantified by the measurement of dye release into the culture supernatant by lysed target cells.

Some commercial NK cell lines are available for ADCC applications, but primary NK cells freshly isolated from peripheral blood mononuclear cells (PBMC) perform best. NK cells are characterized by the markers CD16 and CD56. The abundance of the membrane bound CD16 (Fcγ-receptor; see Section 9.3.1.3, Binding Assays), which is down regulated during cultivation and freeze–thaw revitalization procedures of primary NK cells, is crucial for NK cell functionality. Most of the existing NK cell lines do not express the CD16 anymore. The marker CD56 is the typical marker to identify NK cells by FACS analysis. This marker is also used for the isolation of NK cells, for example, by magnetic bead separation. Purified NK cells are commonly used for ADCC testing. Alternatively, a PBMC preparation adjusted to a defined number of CD56 positive NK cells can be used.

9.3.1.6.6 Complement Dependent Cytotoxicity (CDC)

Besides ADCC, many therapeutic antibodies also induce complement dependent cytotoxicity (CDC). The complement system is a blood-borne, cell-free killing mechanism controlling tumors or infections. The drug antibodies bind to the relevant epitopes, for example, of the membrane proteins of target cells. A critical number of complex, membrane-bound antibodies induce an activation-cascade of several complement factors in the plasma. The cascade results finally in the formation of a lytical pore-complex in the target cell membrane by oligomerization of porins and the target cells die by lysis.

For CDC testing *in vitro*, the same target cells as used for ADCC are exposed to drug antibody and complementary reagents. Rabbit or human serum is commonly used as a complementary reagent. Rabbit complement gives higher signals but also a higher bias.

9.3.1.6.7 Reporter Gene Assays

Reporter gene assays play an increasing role in assay developments. They combine a cellular substrate carrying the drug target or a surrogate marker on the DNA level with a robust analytical read-out parameter on the basis of a DNA transcript. The parenteral cell line is genetically modified with a target-specific reporter gene. A fluorescent protein (e.g., Green fluorescent protein, GFP) or an enzyme (e.g., Luciferase) is inserted into the gene sequence of the target gene as a reporter. The expression of the reporter gene correlates to drug-induced gene activation. It is recommended to implement and monitor a housekeeping gene in parallel to control the drug specificity and dose dependency and to improve the signal-to-noise ratio.

Example: A reporter gene assay for Type I interferons

Type I interferons target the membrane receptors IFNAR 1 and IFNAR 2 on somatic cells. They induce interferon-stimulated response elements (ISRE) on

chromosomal DNA, for example, the MxA promoter [43], by intracellular signaling. A GFP or luciferase-enzyme integrated as a reporter gene into the ISRE will be expressed in the cytoplasm. Expression or activity of the reporter gene product is analyzed by a suitable detection system (fluorescence or luminescence).

9.3.1.6.8 Enzyme Linked Immuno-Spot (ELISPOT) Assays

The enzyme linked immuno-spot assay (ELISPOT) is a modification of the ELISA format. A defined number of cells, for example, T lymphocytes, are seeded onto a culture surface pre-coated with a detection antibody. During drug treatment, the reactive T cells secrete IFN-γ, which is locally captured on the culture surface and visualized by the second detection antibody-system. Each secreting cell will form a spot. The number of spots per well is quantified by image analysis.

9.3.1.6.9 Flow-Cytometry Assays ("FACS")

Flow cytometry is designed for the analysis of cellular markers on the cell surface (so-called CD-markers) or in the cytoplasm. ("FACS" is synonymously used; derived from "fluorescence activated cell sorting," even if there is no sorting).

In a flow cell of a microfluidic system, a cell suspension is analyzed cell-by-cell for light scattering and fluorescence intensity. Cells are stained with one or more fluorescence-labeled antibodies and injected into the fluidic system. It is a single cell based analytical method. The application of two or three lasers ("colors") allows multiple parameters to be analyzed (3–12). To have access to markers in the cytoplasm, intracellular staining can be applied after gentle permeabilization of the cells. Flow cytometry can also be used for functional testing, if a specific marker is not available. The induction of cellular activation can be monitored by the combination of a target-specific stimulation and the detection of a non-specific cellular response on the level of intracellular accumulation or secretion: application – peptide stimulation of T cells to identify T cell reactivity; advantage – multiplexed analysis of several specific markers with cell size and number, real single cell analysis; disadvantage – FACS protocols are hard to validate.

9.3.1.7 Assay Qualification and Validation

For economical reasons and to satisfy regulatory requirements, various assay formats, assay quality, and assay performances are used in the different stages of pharmaceutical developments. These range from screening applications in early R&D for a large panel of molecules, to lead candidate selection on a reduced number of candidates, for MoA analysis and in-process controls (IPC) for the manufacturing of the final candidate as well as for clinical monitoring [45]. Relevant analytical methods are selected and developed to support the economical and scientific decision-making process.

For batch release testing of the preclinical and the clinical trial material, as well as for the different levels of clinical phases, an increased level of validation of the assays is required by FDA or EMA regulations to ensure a maximum level of safety for animal and patient treatments. The regulatory requirements for biological assays have recently been described in the *US Pharmacopoeia* (USP Chapters 1032, 1033, 1034, and 111). General information about drug quality, safety, and efficacy,

including the validation of analytical procedures are described by ICH guidelines (http://www.ich.org). The qualification of an assay focuses on the optimization for best assay performance based on a Standardized Operating Procedure (SOP) using qualified instrumentation and calibrated tools. Validation goes some steps further: In contrast to an assay qualification, the validation procedure defines technical specifications that have to be defined and confirmed in the validation. Experiments are based on a pre-defined validation plan. The validation procedure is controlled and reviewed by a quality assurance system.

For the validation of analytical methods, a large panel of parameters are defined, such as specificity, selectivity, accuracy, precision, repeatability, reproducibility, intermediate precision, robustness, linearity, analytical range, limit of detection (LOD), and limit of quantification (LOQ). In contrast to other instrumental analytical methods, a reduced set of validation parameters is usually tested for a CBA. The relevant and critical validation parameters are identified in a knowledge-based risk analysis (RA), based, for example, on failure mode and effects analysis (FMEA). In particular, identity and quality of the used cell material as well as cultivation procedure, drug exposition, and incubation have to be standardized, defined, and continuously controlled to guarantee constant assay performance and comparability of the experimental data, probably throughout years of assay application.

9.3.1.8 **Outlook**

Bioassays and cell-based assays in particular will play a more and more important role in biopharmaceutical developments and analytics. The relevance of existing animal models and animal experiments for highly specific and high potent biopharmaceutical products, when discussed by the regulatory bodies, the scientific community, and the public, is controversial. Existing statutory and animal models validated over decades could fail, as was dramatically demonstrated by the TeGenero™ disaster in 2005 [46]. To improve volunteer safety in first-in-man applications, cell-based assays and more complex tissue-based *in vitro* models could bridge the existing gap [47]. The evident demands for more human relevant *in vitro* models for testing and many recent advances in bioanalytical instrumentation, for example, *in situ* imaging technologies, image analysis, and high content screening platforms, are the basis for new bioassay developments.

Advances in tissue engineering influence pharmaceutical *in vitro* testing: Tissue and organoid models are under development, not only for toxicity testing applications [40, 48].

Cell-based assays may also help to reduce the number of animal tests in pharmaceutical R&D and preclinical evaluation in the future. Following the "3R" concept of Russel and Burch ([49], re-edited by Balls [50]), animal tests could be replaced, reduced or refined by alternative methods *in vitro* and *in silico*.

Recent trends in the risk assessment and approval of new cosmetics and consumer products for the European market may influence the pharmaceutical area. A ban on animal testing for acute toxicity by the EU legislative [51] has been in place since 2009; a total ban including repeated dosing and systemic toxicity testing is scheduled for 2013.

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Legals

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10
Formulation Strategies for Recombinant Protein and Related Biotech Drugs

Gerhard Winter and Julia Myschik

10.1
Introduction

As with practically all other drugs, biotec drugs are not stored or administered as such but as formulations together with excipients. Since most of such biotec drugs need to be administered parenterally, aqueous solutions play a major role as the standard formulation type in this context. In the case of intrinsically very unstable proteins, freeze drying is applied as the standard method to achieve dry storage forms. Apart from that, specific drug delivery options, such as for example pulmonary delivery and parenteral depots, necessitate more sophisticated formulations [1, 2]. In the following we will concentrate mainly on "standard" formulation approaches, to keep the chapter focused.

10.2
Formulation and Stability of Protein Solutions

Modern protein drug formulation has a history of only 20–30 years. Prior to this, the specific aspects of insulin formulations and knowledge of the stabilization of enzymes were the major areas of this field [3]. Since then a huge number of publications, reviews, and books have appeared on the subject [4–6]. Still worth reading is the comprehensive review by Wang and Hanson from 1988 [7] where the experiences from the pre-1990s have been nicely condensed. Subsequently, formulations of the first generation of protein drug products (cytokines, hormones, growth factors) have all been validated and are on the market, and even a number of biosimilars with the same or slightly different formulations have evolved. Today formulations for monoclonal antibodies with comparably high or extremely high concentrations dominate the scientific discussion on biotec formulations [8–10].

To have a first overview of biotec formulations, the easiest way is therefore to simply analyze marketed formulations. Through this, one will immediately realize that protein formulations appear to be rather simple and based on a small number of excipients [11, 12].

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The reason behind this situation can be explained by the fact, that firstly, only a small number of excipients has been approved for parenteral use at all, and every company would do anything possible to avoid the use of new excipients. When Roche launched Herceptin™ some years ago, they did a favor to others in the field by introducing trehalose to the market as the main stabilizing excipient [13, 14]. Its valuable properties had been studied extensively, and laid down in publications and numerous patents, but everybody tried to avoid the costs of toxicity studies [15, 16]. The second reason for a rather limited panel of excipients for biotec formulations is readily derived from the general principles of formulation design and leads us directly to these strategies.

In fact, there are only a few pathways to rationale formulation design. First and most important is what we call the passive stabilization strategy, that is, testing a specific drug in solution for the relevance of typical stress factors and trying to avoid or reduce these stress factors in the formulation [17, 18]. Stress factors are temperatures (including low temperature leading to freezing), mechanical stress, light, oxygen, acids or bases (pH), salts (ionic strength), denaturants [11].

Consequently one would try to study the effect of most of these factors in so called preformulation studies, resulting in corridors or "design spaces" for a certain pH range, ionic strength, buffer choice, the need for oxygen removal by gassing, and so on [19]. At this point physical and chemical stability must be addressed in parallel [20]. A typical situation may be that a certain pH might provide the best colloidal stability due to favorable charges (zeta-potential) on the protein molecules, whereas deamidation and oxidation of certain amino acids in the protein would require a different pH [21]. It should be noted that the choice of the buffer type at a given pH can be of utmost importance too [22–24]. Cations and anions have – according to the ancient Hoffmeister series – significant differences in their salting in/salting out properties and can affect aggregation and solubility of protein. If the stability of a protein solution is not provided by optimal pH, ionic strength, buffer, and with oxygen removed by nitrogen gassing, more active measures towards stabilization have to be taken.

The two most common complications that cannot be resolved by the aforementioned steps are, in the opinion of the authors, formation of aggregates under mechanical stress and oxidation and/or fragmentation [18, 25, 26]. Typical measures taken are the addition of surfactants to cure the first, and addition of antioxidant or chelators to reduce the second problem [27–29]. Historically, addition of surfactants to protein drug products was first applied to prevent adsorption of low-dose proteins, such as interferons, on the container walls and tubings [30]. This approach works well and is still necessary in most of these cases, although Teflon coatings have reduced many of the complications on the manufacturing side. For high dose protein drugs, such as monoclonal antibodies, where concentrations in the formulation meanwhile peak as high as 200 mg/ml, adsorption plays only an academic role as the loss of concentration may not exceed 1/1000 of the dose or less [31]. Here surfactants fulfill a different role in preventing aggregation under mechanical stress, for example, shaking, and liquid–container

contact [18, 32, 33]. Although the exact mechanism is still not totally clear, the current hypothesis proposes, firstly, the replacement of the protein from the surfaces, thereby reducing its overall exposure to unfavorable void space, and secondly, the surfactant is assumed to cover more hydrophobic patches on the protein [34, 35]. Such patches may be present for more hydrophobic proteins anyway and for other proteins could be presented to the surface during partial unfolding under stress [36]. In both cases shielding of such patches would prevent them from interacting and thereby reduce the probability of aggregate formation.

To reduce oxidation and/or fragmentation, typically antioxidants, primarily methionine or chelators (EDTA, ethylenediammine tetraacetic acid) are used [26, 28]. Owing to a restrictive view by the regulatory bodies on such interactive excipients, a concept of preventive use of these additives is not feasible and they are only used in a few protein drug products. At this point, the use of citrate buffer may provide an interesting example of why simple looking protein formulations may be quite delicate when all the details are considered [11]. Citrate (like EDTA) is a chelator and can potentially prevent heavy metal induced complications [11]. On the other hand, it is a standard buffer excipient used, in particular, in combination with phosphate, preferably in the slightly acidic range [37]. Furthermore, it has already been reported, in the 1990s, that subcutaneous (s.c.) use of citrate-containing formulations is responsible for a slight burning sensation at the injection site and is therefore patient unfriendly [38].

Another stabilization strategy and related excipient groups need to be explained. Sugars, sugar alcohols, and certain amino acids, such as glycine, are often used in rather high concentrations in protein solutions [39]. At first sight, they provide isotonicity without increasing ionic strength too much. However, there is another reason behind this, the theory of preferential exclusion and thermodynamic stabilization of protein in solution. Arakawa and coworkers [40, 41] and Timasheff [42] have brilliantly worked out that proteins in solutions of the mentioned solutes will have a certain exclusion zone around their surface, where the concentration of such "excluded" solutes is lower than in the bulk solution and water molecules dominate this zone [40–42]. If we consider such an exclusion zone as thermodynamically unfavorable, that is, providing a higher ordered situation, a system tends to reduce such exclusion zones as much as possible to minimize the thermodynamic imbalance [40–42].

If we now compare protein molecules in their native and unfolded state, it is obvious that the unfolded state has a larger surface and thereby a larger exclusion zone. In return, the system would suppress denaturation thermodynamically and stabilize the native conformation [40–42]. This active stabilization mechanism will work only well with very high solute concentrations (1 M), but at least a slight effect can be expected in the range of isotonic solutions of, for example, sorbitol [43]. The use of amino acids requires a more detailed view. Arginine, for example, is not a typical representative of the group of excluded solutes, it is a solubilizer for protein drugs such as tPA, and due to its chaotropic effect is also considered to improve solubility of monoclonal antibodies [44].

10.2.1 Dry Formulations

When the concepts of stress reduction and stabilization in solution are unable to allow long-term storage of a protein drug solution, dried formulations provide a viable alternative for a marketable product [45]. The stability of the protein solution is then only required for a few hours after reconstitution of the dried formulation. Freeze drying is used as the standard process technology, whereas spray drying, although developed into a large-scale method for protein drugs, is still a niche process used only in the context of pulmonary delivery of proteins [46]. Freeze drying is no longer an "art," but, due to the combination of the process technology aspects and protein physicochemistry, it is still challenging in certain cases [47].

The basic concepts of how to freeze dry and how stabilization is achieved in dry, sugar-based cakes were worked out by Pikal [48], Carpenter *et al.* [49], Franks [50], and many other in the 1980s and 1990 [51–55]. Today we consider the water replacement hypothesis and the immobilization into glassy masses to be the key elements of stabilization in freeze-dried cakes [56]. In short, the first element of stabilization is to replace hydrogen bonds through which the protein holds the surrounding water molecules as much as possible by hydrogen bonds to molecules such as sugars, when water is removed by drying [57]. It is understandable that the molecules replacing water in the dry state must have a certain flexibility and should not be too large to allow close and complete surface coverage of the protein molecules [58].

The second aspect of stabilization in dry cakes is their rigidity and the resulting effect, that is, the reduction of molecular movement and reaction constants by orders of magnitude [59, 60]. Sugars will result in an amorphous glass-like matrix when dried properly and are thus able to form a perfect, rigid matrix having extremely low diffusion coefficients [56]. It is the task of the formulator to achieve sufficiently dry cakes that do not recrystallize during storage but which maintain their glassy matrix over many years [61]. A number of process-related details should be considered at this point but they would go beyond the scope of this chapter. Plenty of literature is available on the subject [45, 62–65].

A major drawback of freeze drying that has not been resolved so far is the long process time, and the resulting high costs of the products. More recently, we have questioned the dogma of perfectly shaped porous cakes in freeze drying and voted for more "aggressive" faster drying cycles [66]. It has been shown that under certain circumstances amorphous, dry cakes can be produced through water replacing sugars, even when the material has collapsed during the drying process. Including the concepts of Pikal and coworkers on relaxation of glassy masses by exposing them to high temperature (up to 60 °C) for a short time in the dry state, one can expect even higher stabilities than those known so far [67, 68].

10.2.2 Modern Formulation Screening Strategies

Having outlined what can be done in general to formulate and to stabilize a protein drug product, the industry concepts used today to find the right formulation, within the shortest possible time and with as low amounts of the drugs as possible, will be highlighted.

Formulation development is typically divided into the following steps: preformulation, formulation screening and short-term stress studies, process development (if necessary, e.g., for freeze-drying processes), stability studies, and finally scale up and regulatory dossier preparation [69].

Preformulation comprises the collection of all information on the drug substance that could potentially be relevant for the stability of the protein in solution and in the dried state. Such information, for example, structure, solubility, isoelectric point, sensitivity to stress factors, and resulting degradation or aggregation reactions, will in part be available from the drug candidate selection process and its preliminary production and purification processes [70]. If significant data are missing or the information is somehow imprecise and non-systematic, systematic preformulation studies must be carried out. At that point, factorial experimental design may be applied or one factor at one time studies can be carried out, for example, investigating the pH row, ionic strength, buffer type and strength, surfactant addition, and so on [71]. Basically all such preformulation studies apply short-term stresses to allow for the detection of trends over a short period of time [72].

After the preformulation data have been laid down in a "preformulation report," the optima of potential stability enhancing factors, such as pH, buffer, ionic strength, would then be combined with standard excipients to give a number of possible formulations. This number would be large and not possible to be studied in practice; fairly different approaches are taken to deal with this situation.

One concept is to describe the effect of as many factors as is practicable on the drug and to develop a theoretical design space for the formulation [73]. It might then even be possible to apply surface response curve statistics to find an "optimal" formulation and to prove the long-term stability of such a formulation in real time stability studies [71]. A different approach could be to start with only a few standard formulations that are known from the literature or to apply theoretical concepts, rules, and assumptions, leading to a small number of promising formulations, which are then studied in short- and long-term stability studies [74]. Of course looking left and right to similar proteins and their formulations may also help, but due to the individual nature of proteins and the high impact of small changes in the primary sequence and glycosylation on stability, this concept can be misleading.

A further approach that has found wider application more recently is to study a high number of possible formulations in high throughput formulation screening (HTFS) experiments [75]. HTS has been known for many years in the field of drug discovery, to select hits from a vast number of candidate molecules when testing their affinity to receptor molecules or their effect on biological test systems [76,

77]. HTFS uses part of the mature technical infrastructure that has been developed for HTS-like pipetting robots, tray handling, and so on, and, most importantly, multi-well plates, to miniaturize the entire process [78].

Although this may save a lot of material and make the preparation of hundreds of formulations possible, at least, the problem remains of how to shorten the time axis as much as possible. All formulation development strategies inherently rely on the concept that the behavior of the drug under strong, short-term stress can be correlated with long-term storage at lower stress impact, that is, "real time" stability at, for example, 2–8 °C [79]. Although this correlation is questionable, in many cases the effect of higher temperatures on chemical degradation, be it Arrhenius-like or not, is well accepted and data sets for protein drugs are published. Even the ICH guideline considers the concept as valid [80]. However, given that studies at, for example, 40 °C are relevant, they will take time, eventually up to many weeks and months, and are disturbing real high throughput concepts. Expanding the concept of elevated temperature studies towards even higher temperatures will lead to the situation that at a certain point the denaturation temperature of the protein would be reached, and storage studies would no longer be meaningful [81].

However, a different idea has evolved, namely to use the denaturation temperature of a protein in a specific environment as a marker for its long-term stability [82]. The concept is now widely applied, although it has certain limitations and only a few datasets provide true evidence for correlation with long-term stability [83]. To measure the melting temperature T_m of the protein, ultrasensitive DSC (differential scanning calorimetry) is applied as the first choice due to its precision and applicability to practically all liquid formulations [84]. Measuring T_m takes a few hours instead of weeks and brings us one step further towards HTFS. Still, DSC is not compliant with well-plate systems and therefore other methods to determine T_m have been sought [11]. FTIR and (less often used) derivative UV spectroscopy also allow for the calculation of unfolding temperatures based on the loss of secondary structural elements [85]. FTIR needs ATR (attenuated total reflectance) cells with a high chance of surface derived artifacts, and UV only works well on certain proteins, so both do not resolve our problem.

Recently, temperature induced intrinsic or (more sensitive) extrinsic fluorescence enhancement was employed successfully to determine protein unfolding in microliter-scale samples and has evolved into an new tool for HTFS [86]. It is compatible with well-plate sample management and with most formulation compositions. Other stress factors, such as freeze–thawing, mechanical stress (shaking and stirring), and light exposure can easily be simulated in short time and are all included in standard formulation screenings, either miniaturized or "normal" [18, 87, 88].

10.2.3 Analytics

Although the scope of this chapter does not allow discussion of the issue in detail, a short excursion into analytical methods applied during formulation screening is necessary. Most methods that are stability indicating for chemical degradation use

only small samples and are therefore compatible with HTFS and uncritical in terms of protein amounts needed [89]. The methods of choice, their value, and limitations are laid out in many excellent books and reviews and will not be discussed further here [90–92].

However, in many cases, physical aggregation of protein drugs is the most important problem, often the decisive parameter in limiting the stability of a certain formulation. Soluble aggregates can be fairly easily quantified by size-exclusion chromatography [20]. Insoluble aggregates appearing as particles of different sizes are not compatible with HPLC methods [93]. They are detectable by optical or other physicochemical methods [94, 95]. In particular, measuring the so called "subvisible particles" in the range of about 1–100 μm (above which the visual inspection in the closed container would be possible) uses rather high volumes of formulation and is hardly compatible with HTFS. A way out can be to measure turbidity and to apply dynamic light scattering (DLS), both in a well-plate format [96]. However, the two methods are bulk methods which do not allow real counting and sizing of particles in the micrometer range [97].

The gap between soluble and insoluble particles can be closed by analytical field flow fractionation, a chromatography-like method that applies an empty separation channel instead of a packed column, or alternatively by ultracentrifugation. Both methods have been discussed in detail with their pros and cons over recent years and are considered by the regulatory authorities as necessary "orthogonal methods" to standard HP-SEC and particle counting when complex aggregation can be assumed [98, 99].

Coming back to formulation screening, one particular type of short-term stress studies will be explained. Typically, isothermal stability studies are carried out, keeping temperatures in the range of from 2–8 °C up to 50 °C constant, and drawing samples of the stressed formulation after a certain period of exposure time [100]. Non-isothermal studies heat up samples linearly, or through other kinetics, and allow shortening of the study time significantly [101, 102]. By calculating the derivative of the degradation curve one can determine degradation rate constants for different temperatures and create an Arrhenius diagram within a few days [103]. Extrapolation to lower temperatures may be possible, if the data indicate Arrhenius-like kinetics.

10.2.4
Formulation Development for the Market

If we assume that, by whatever strategy, a formulation screening has been carried out and evaluated, a number of favorite candidates will remain and they have now to be developed further. So far we have neglected issues in our discussion such as assuring isotonicity, choice of the right packaging systems, and cost aspects.

The first aspect can be addressed in a straightforward way. Any formulation that is to be applied undiluted should be isotonic; adjustment would typically be made via the sugar/sugar alcohol/amino acid compound, which fairly often would be part of a formulation anyway [104]. If this is not applicable for any reason, inert salts such as sodium chloride could be used, assuming higher ionic strengths have been

assessed during preformulation and found to be uncritical. As for lyophilisates, high salt loads disturb the freezing and drying behavior, so tonifiers could theoretically be added to the reconstitution solution [61]. Products that have to be diluted for clinical use need not be isotonic. At this point, a comment on isohydria, that is, the neutral pH, will be made. As long as no strong buffers are used, products in the range of pH 4–8.5 can be used and have a good chance to be well tolerated, even for undiluted subcutaneous use. Several products, including for example, GCSF, are formulated at a pH as low as 4 and are well tolerated by the patients [105].

Primary packaging materials are of course an inherent part of a protein drug product. Ampoules are practically obsolete, so stoppered vials remain as the standard products plus prefilled syringes and PEN systems with single- or double-chamber cartridges [106]. With a clear trend towards self-administration in mass markets for products such as Heparin, EPO, hGH, the prefilled syringes have gained more and more market share over the last 15 years [107].

Although the production of these prefilled syringes is a mature technology with billions of units produced, one particular technical issue remains, which is siliconization [106]. To ensure low gliding forces of the plunger and good manufacturability, the inner glass wall and stopper–plunger have to be siliconized. During storage, especially for the so called "spray on" or "wipe on" siliconization, syringes sometimes loose part of the silicon oil from the walls into the protein solution [108]. Formation of droplets and particles, most of them probably loaded with adsorbed protein, takes place [109, 110]. The relevance of these pharmaceutically unacceptable species is under debate, and studies on the immunological relevance of such "heterogeneous" aggregates are under way [111–113].

The problem of rubber stopper related problems such as adsorption, extractables, and leachables, has been strongly reduced since PTFE coated stoppers have become widely available and used in many products [106]. The PTFE cover does not totally eliminate any interaction of product solution and rubber matrix, but the number and amount of extractables is well under control in a state-of-the-art product [114].

After the definition of a preliminary packaging system and some promising formulations, stability studies are started. A strategic question is whether long-term studies according to ICH guidelines over 24 months should actually be started, or if preliminary 3–6 months studies, including stress temperatures, should first used to reduce the number of candidates [115]. All details for such studies are explicitly outlined in the pertaining regulatory documents, including detailed guidance for matrixing and bracketing approaches for covering many different dosage forms, so we will not discuss this matter further.

10.2.5
Interface between Downstreaming and "Fill and Finish"

What might be of interest is the question of how the interface between bulk drug substance production and the so called "fill and finish" processes is managed today [116]. Around 10–15 years ago, educational, strategic, and geographic separation

often prohibited a close and seamless interface between the last steps of bulk drug substance production and the "pharmaceutical" production, that is, formulation and filling into primary packaging containers. It is obvious that the last steps of downstreaming typically require adjustment of certain buffers and diafiltration for up-concentration of the bulk drug substance solution, and these steps are not much different from what the first steps of formulation comprise [117]. Even more importantly, it has to be ensured that the bulk drug substance solution has a certain stability, either as a frozen solution stored, for example, at –70 °C, or as a liquid stored at 2–8 °C. In both cases, stabilization concepts need to be applied that are not much different from what needs to be considered for the final formulation [118].

With this background, it has now become quite common that surfactants and/or sugars/sugar alcohols are indeed added to the bulk solutions, and these are dialyzed into the final buffer, compatible with all the regulatory aspects for a drug product. The fill and finish group has then only to dilute and to adjust the final concentration and fill the vials or syringes. In extreme cases, the bulk drug solution is identical to the drug product formulation and nothing but filling aliquots is then necessary at the filling site.

10.2.6 Highly Concentrated Protein Formulations

In the recent years, the absolute majority of new protein projects and resulting products have been monoclonal antibodies [119]. This trend is still valid and will continue for the next 5–10 years at least, as many promising candidates are in clinical studies [119]. An interesting technical detail goes along with this, the need for highly concentrated dosage forms and the related formulation issues [120]. Many antibodies have to be given in rather high doses, up to the hundreds of milligrams range per application. On the other hand, many such products, namely those directed against immunological diseases, are applied over long periods of time and are therefore subject to self-administration and consequently used via s.c. injection. Only a maximum of about 1.5 ml can be conveniently delivered by this route, and as a result protein concentrations rush up to 200 mg/ml.

Such highly concentrated products create several problems for the formulators. Firstly, viscosity is an inherent problem [121]. Some effects have been found when using different salts and buffers in reducing viscosities, thereby playing with self-association, hydration, colloidal stability of the antibodies in solution, but, finally, for a given molecule clear limitations are given and syringeability will determine whether a product can be used or not [122]. Apart from this, achieving such high protein concentration is not at all trivial [123]. Usually tangential flow filtration will be used, but this is approaching its limits when exceeding target concentrations above 200 mg/ml. Increased aggregation levels and losses are the price to be paid if processes are not fully optimized [124]. It should be noted that at such high concentrations, the Donnan-effect leads to significant aberrations in the buffer concentration and pH before and after the dialysis [125, 126]. This may not only be a matter of correct declaration when buffer concentration is incorrect, but a pH

shift can potentially lead to degradation and/or aggregation during the diafiltration process itself and after [127]. A simple idea to increase protein concentration in the formulation to be administered is to freeze dry from a certain concentration and to redissolve in a smaller amount of water to end up with a higher, for example, double, concentration. In the United States, a patent application has been submitted based on this old trick [128].

Coming back to the concentrated solutions of monoclonal antibodies and their stability, one will find an interesting situation. On the one hand, high concentration leads to molecular crowding and a statistically high probability of interaction between one protein molecule and another. Therefore, increased levels of aggregation, high turbidity, and particle formation are observed [129]. It is actually under investigation how reversible such aggregates are, how they might dissolve rapidly when diluted or, on the contrary, whether they might even aggregate much more strongly when brought from the designed formulation into a different environment, for example, in the s.c. tissue fluid [32, 130]. On the contrary, aggregation and degradation of proteins can also be reduced at higher concentrations as relatively (to the complete dose) less protein has contact to surfaces and thereby the rate of surface induced aggregation, oxidation, and so on, should be reduced.

10.2.7
New Proteins and Related Formulation Aspects

The ongoing search for better antibodies or better "binder" molecules that mimic the effect of antibodies has also stimulated more thoughts on the interface between discovery and formulation development. Today it is understood that "drugability" should actually be a valid criterion in the selection process for new molecules besides affinity, cell toxicity, and so on, when the number of candidates must be reduced [131]. A correlation between primary sequence and pharmaceutical aspects, such as aggregation tendency, has still not been found, so candidates with poor physical stability will be eliminated from the panel as far as possible [132].

We have previously discussed the use of miniaturized and HTFS and here we find another driving force for improving such concepts. For chemical degradation, protein chemistry is already one step ahead and hot spots for potential cleavage of protein molecules can be predicted much better and avoided, even on the molecular level. Recently, Moroney and Plückthun [133], amongst, others have started to find systematic correlations between aggregation and glycosylation of antibodies [134, 135] as well as between aggregation and the presence or absence of certain short amino acid domains in the molecule [136].

10.2.8
Summary

Protein formulation is still an important aspect of overall biotec drug product development. Despite the small number of excipients used, the complexity arises from the complicated, long list of quality parameters and pertaining analytics,

especially in the area of aggregation quantification where distinction between soluble and insoluble matter is physically difficult to address. Furthermore, the interface to packaging material, the highly concentrated product solutions, and the still very unclear relevance of aggregates for immunogenicity will keep us busy for the next few years [137–139]. High throughput techniques and miniaturization on the one hand and standardization to rapidly access clinical phases on the other, have improved and will further improve development speed. Novel excipients are very rare, and it will be of interest whether, for example, cyclodextrin derivatives will become a valid alternative to polysorbates as surfactants [140]. On the process side, alternative drying technologies to freeze drying are still awaiting a breakthrough [46, 66].

10.3 Formulation of Vaccines

Preformulation approaches, excipients, analytics, and drying methods have been described for therapeutic proteins and antibodies. However, in addition to therapeutic protein formulations there is also the field of vaccine formulations. Some formulation approaches are of course similar, but for vaccines further considerations regarding formulation and analytics also come into play. The aim of this part of the chapter is to give a brief overview the different types of vaccines, formulation approaches, and to highlight the differences from proteinaceous drugs.

Vaccines can be broadly divided into the following classes: vaccines containing live-attenuated viruses, whole-killed or inactivated pathogens, and subunit vaccines that contain only fragments of the actual pathogen, such as peptides, proteins, polysaccharides or combinations thereof. This list of different vaccine types clearly highlights that formulation aspects will undoubtedly differ from those of proteinaceous drugs formulation approaches, as these will strongly depend on the product.

Vaccine formulations contain the antigen as the major component, but often it is not just the antigen that is present in the formulation. Adjuvants are added in case the antigen by itself does not sufficiently stimulate the immune system [141]. In addition, buffer salts are also present in the final formulation, as they are in the above mentioned biotec drugs. Finally, some vaccines also comprise a particulate delivery system, as there are attempts by many researchers to incorporate the antigen in a particulate delivery system [142]. What has to be considered by the formulator when looking at all these different types of vaccines?

Live-attenuated vaccine formulations often contain a mixture of alive and inactivated viral particles [143]. Because live-attenuated vaccines mimic the natural infection pathway of pathogens, they are regarded as superior in terms of efficacy when compared with subunit or inactivated vaccines. However, their major drawback is the poor physical stability, especially when exposed to elevated temperatures [143]. In addition, the presence of live viral particles may be an issue as there is a potential risk that the pathogens could revert back to their virulent forms.

Live-attenuated formulations are commonly tested in cell-based or animal testing systems, but recently new approaches have been described in the literature in preformulating such vaccines using a biophysical approach, as described by Zeng *et al.* [143]. Here, far-UV circular dichroism spectroscopy and fluorescence-based techniques were employed to preformulate a stable live-attenuated vaccine [143]. Examples for vaccines containing whole–killed or inactivated viruses–or bacteria are the polio vaccine (IPV), hepatitis A, influenza vaccine, Japanese encephalitis, pertussis, plague, cholera, and typhoid vaccines. These vaccines have a high efficacy in terms of protection.

Subunit vaccines contain only fragments of the pathogen, such as peptides, short protein sequences or DNA. These types of vaccines can be divided into inactivated toxins (e.g., diphtheria, tetanus) or acellular vaccines (e.g., pertussis). Production can be carried out using recombinant DNA technology [144], thereby making the production process of this type of vaccine well defined. However, in terms of immunogenicity subunit vaccines suffer from their high purity. This sounds puzzling at first but can easily be explained when one looks at the complex function of the immune system. Certain structures or repetitive patterns on pathogens are recognized by the immune system as dangerous [145] and an immune response is mounted by the immune system. Subunit antigens often do not provide such signals, which are necessary to activate innate immunity [146]. These types of antigens are rapidly cleared from the body before an activation of the immune system can occur. Thus the adjuvants come into play, to stimulate a stronger immune response to otherwise weakly immunogenic antigens [147]. Subunit DNA vaccines are slightly more immunogenic than peptide vaccines as bacterial plasmid DNA is generally used. These types of vaccines contain unmethylated CpG motifs that activate the endosomal Toll-like receptor 9 (TLR9) [148] and in turn trigger a potent immune response.

Adjuvants are in general a class of heterogeneous compounds, and to describe all adjuvants currently under investigation and on the market would be beyond the scope of this chapter. In depth information on this topic can be found elsewhere [149, 150]. Still, a brief comment should be given. The most commonly used adjuvants are alum salts. Alum has a long history dating back to the early 1930s. A number of new insights have been gained in recent years regarding the optimal formulation approach and in understanding the mechanism of action of alum salts [151]. More modern adjuvants include MF59 [152], Toll-like receptor agonists [153], liposomal formulations [154], immunostimulating complexes (ISCOMs) [155], and virus-like particles (VLPs) [156], to just name a few. For some of them, the mode of action has been investigated [157], but for others this is either still under investigation and sometimes a starting point where more research still has to be carried out. For many new adjuvants, systematic formulation studies are still missing or have not been published in great detail. These adjuvants differ widely in terms of chemical composition, molecular weight, solubility, and so on. Therefore, it will not be easy to find the "one fits all" method for a good formulation.

Vaccine antigens and adjuvants have also been formulated into particulate carriers [158]. This approach was taken as pathogens are fairly effectively taken up by

cells of the immune system. Extensive research efforts have been made to formulate a particulate carrier that bears features similar to those of a pathogen, for example, using surface modifications, variation of size and shape [142, 159]. It was shown that internalization of particles occurs through receptor-mediated endocytosis, phagocytosis, or macropinocytosis [160], and is influenced by particle size, surface morphology, and also shape. The antigen can be attached to the carrier surface or incorporated into the matrix in order to achieve delivery, receptor attachment, and stability. Particulate vaccine delivery will continue to be an interesting approach to improve vaccine delivery in general.

10.3.1 Analytics

Formulating a "good" vaccine is difficult, but analyzing the final product is challenging as well. One problem that complicates the analytics is the number of different compounds present in the formulation. These have to be separated prior to analysis, or methods have to be developed where several compounds can be present but do not interfere with their qualification and quantification. As with therapeutic biotec drugs, the analytics of the final product need to be monitored to make them a well-defined dosage form. This has already been suggested by Volkin and Middaugh [161], who highlighted that a number of techniques are required to characterize vaccines well.

Changes in the secondary structure can be determined by far-UV, circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR) [162], whereas changes in the tertiary structure have to be analyzed by intrinsic fluorescence, near-UV CD, and near-UV absorbance spectroscopy. Also, extrinsic fluorescence may be used to determine changes such as unfolding of protein antigens [161]. For particulate vaccines, the size of the particles has to be determined by light scattering, or microscopic techniques, for example, scanning electron microscopy or transmission electron microscopy. In addition, potential conformational changes of the antigen attached or adsorbed to the particulates must be analyzed.

A challenging aspect is the fact that the amount of the actual antigen present in the final formulation is fairly small. This is, on the one hand, beneficial in terms of vaccine costs, as the antigen can be considered to be the most expensive excipient in the formulation. However, it is difficult to detect changes in the primary, secondary or tertiary structure of the antigen in the presence of other excipients in the final formulation. Additionally, the presence of an adjuvant can alter the behavior of an antigen in the final formulation drastically. Adsorption onto alum, for example, has effects on the structure and stability of the antigen, as reported in the literature. While the antigen may be stable in solution, this could be completely different when the adjuvant is added to the formulation [163]. On top of this, adjuvanted vaccine formulations often complicate the analytics even more as they often show intrinsic turbidity, which would interfere with some spectroscopic methods. One approach has recently been described in the literature

using extrinsic fluorescence spectroscopy [164], but it was also highlighted that the presence of surfactants, such as polysorbates, poloxamers, and Triton X, can interfere with the analytics by displaying a huge background signal.

In preformulation studies for vaccines, the same aspects such as pH, IEP, buffer concentration, and so on, must to be tested as have been described for biotec drugs [165]. However, for vaccines this does not only hold true for the antigen in solution but also for the adjuvant and combinations of both, as the interaction between the antigen and the adjuvants can bring up unpleasant surprises later on [165].

Last but not least, the majority of the currently developed vaccine formulations are in the liquid state. One example is the influenza vaccines that have to be stored and distributed under defined conditions (cold-chain, certain temperature) [166], as otherwise degradation mechanisms such as denaturation, oxidation, hydrolysis, or aggregation may occur. The reason why many vaccines are still formulated in the liquid state is also due to the still preferred route of administration (mainly s.c. or i.m. (intramuscular)) as well as the increased complexity of drying processes in vaccine development. The drying of a proteinaceous formulation is a complicated task due to the potential physical or conformational instability of the protein drug. For vaccines, however, the large number of excipients including immunopotentiators/adjuvants, buffer salt, particulates, sugars, and so on, further complicates this process. Considering all this, it becomes evident why formulating a vaccine is an extremely costly and time-consuming effort.

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11
Drug Approval in the European Union and United States

Gary Walsh

11.1
Introduction

The pharmaceutical sector is arguably the most highly regulated industry in existence. Legislators in virtually all world regions continue to enact/update legislation controlling every aspect of pharmaceutical activity. Interpretation, implementation, and enforcement of these laws is generally delegated by the lawmakers to dedicated agencies. The relevant agencies within the European Union (EU) and the United States (USA) are the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA), respectively. This chapter focuses upon the structure, remit, and operation of both these organizations, specifically in the context of the approval of biopharmaceutical products for medical use.

11.2
Regulation within the European Union

11.2.1
EU Regulatory Framework

The founding principles of what we now call the European Union are enshrined in the Treaty of Rome, initially adopted by six countries in 1957. While this treaty committed its signatories to a range of cooperation and harmonization measures, it largely deferred healthcare related issues to individual member states. As a consequence, each member state drafted and adopted its own set of pharmaceutical laws, enforced by its own national regulatory authority (now known as the National Competent Authorities). Although the main principles underpinning elements of national legislation were substantially similar throughout all European countries, details did differ from country to country. As a result pharmaceutical companies seeking product marketing authorizations were forced to apply separately to each member state. Uniformity of regulatory response was not guaranteed

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and each country enforced its own language requirements, scale of fees, processing times, and so on. This approach created enormous duplication of effort, for companies and regulators alike.

In response, the European Commission (EC, Brussels) began a determined effort to introduce European-wide pharmaceutical legislation in the mid-1980s. The Commission represents the EU body with responsibility for drafting (and subsequently ensuring the implementation) of EU law, including pharmaceutical law. In pursuing this objective it has at its disposal two legal instruments: "regulations" and "directives." Upon approval, a regulation must be enforced immediately and without alteration by all EU member states. A directive, in contrast, is a "softer" legal instrument, requiring member states only to introduce its "essence" or "spirit" into national law.

By the early 1990s some eight regulations and 18 directives had been introduced, which effectively harmonized pharmaceutical law throughout the European Union. In addition to making available the legislative text, the European commission has also facilitated the preparation and publication of several adjunct documents designed to assist industry and other interested parties to interpret and conform to the legislative requirements. Collectively these documents are known as the "*Rules Governing Medicinal Products in the European Union*" and they make compulsory reading for those involved in any aspect of pharmaceutical regulation. The 10 volume (Table 11.1) publication is regularly updated and is accessible via the relevant EU website (http://ec.europa.eu/health/documents/eudralex/index_en.htm). Volume 2 is particularly noteworthy from the perspective of drug approval (i.e., seeking a product marketing authorization).

This volume is presented in three parts. Volume 2a overviews the various regulatory routes available for obtaining marketing authorization for a product. Volume 2b presents the regulatory requirements in terms of presentation and the format of the application, while Volume 2c contains various guidelines designed to assist the applicant.

Table 11.1 The 10 volumes comprising the rules governing medicinal products within the European Union.

Volume	Title
1	Pharmaceutical legislation: Medicinal products for human use
2	Notice to applicants: Medicinal products for human use
3	Guidelines: Medicinal products for human use
4	Good manufacturing practices: Medicinal products for human and veterinary use
5	Pharmaceutical legislation: Veterinary medicinal products
6	Notice to applicants: Veterinary medicinal products
7	Guidelines: Veterinary medicinal products
8	Maximum residue limits: Veterinary medicinal products
9	Pharmacovigilance: Medicinal products for human and veterinary use
10	Clinical trials guidelines

11.2.2 EMA

Harmonization of pharmaceutical law made possible the implementation of an EU-wide system for the authorization and subsequent supervision of medicinal products. Central to this was the establishment in 1995 of the European Medicines Agency (EMA, originally termed the European Medicines Evaluation Agency or EMEA) (http://www.ema.europa.eu). The function of the EMA is not to duplicate the activities of national competent authorities, but to coordinate the scientific resource base found in these competent authorities with a view to the evaluation, supervision, and pharmacovigilance of medicinal products.

An outline structure of the EMA is provided in Figure 11.1. The agency is governed by a management board, with an executive director being responsible for all operational matters. It directly employs a relatively modest number of staff (approximately 500) and these staff are largely organized into three units (pre- and post-authorization units for human medicines, as well as a unit concerned with veterinary medicines; Figure 11.1). The staff are responsible for undertaking administrative and procedural aspects of EMA activities.

The bulk of the EMA's actual scientific work is undertaken by one of six key committees:

- Committee for Medicinal Products for Human Use (CHMP)
- Committee for Medicinal Products for Veterinary Use (CVMP)
- Committee for Advanced Therapies (CAT)

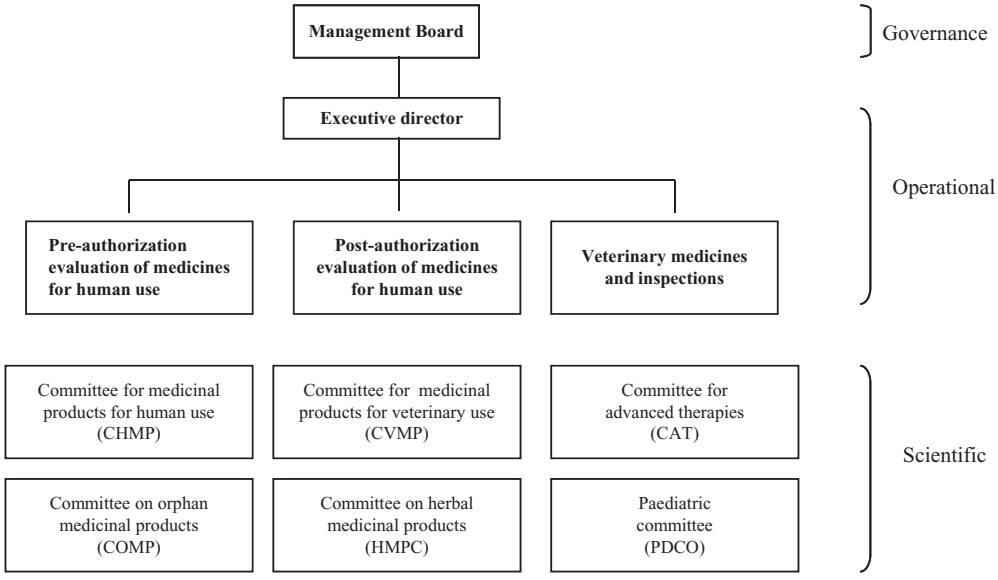

Figure 11.1 Simplified structural overview of the EMA.

- Committee on Orphan Medicinal Products (COMP)
- Committee on Herbal Medicinal Products (HMPC)
- Paediatric Committee (PDCO).

Each committee is composed of a number of (mainly technical) experts, the majority of whom are drawn from the National Competent Authorities of each EU member state. The committees meet regularly (often monthly) at EMA headquarters in London. From a drug approval standpoint, the critical committees are the CHMP and the CVMP (focusing upon human and veterinary drugs, respectively) and the function of these committees in the context of new biotechnology drug approvals will be discussed in the next section. Additionally, the EMA has at its disposal a bank of some 4500 European technical experts (the majority of whom, again, are drawn from the national regulatory authorities). The EMA draws upon this expert advice as required.

11.2.3 New Drug Approval Routes

Regulatory mechanisms exist which allow national authorization of a medicine in individual member states. The rules governing medicinal products in the European Union also provide for various routes by which new potential medicines may be evaluated with a view to gaining approval throughout the entire EU. These are termed the centralized and decentralized procedures, respectively, and the EMA plays a role in both. The centralized procedure is compulsory for biotech medicines and as such is the sole focus of the discussion below. It is also worth noting that approval and regulation of clinical trials in Europe is regulated not by the EMA, but by the National Competent Authority of the countries in which the trials are actually undertaken.

11.2.3.1 Centralized Procedure

Under the centralized route, Marketing Authorization Applications (MAAs) for proposed new medicinal products are submitted directly to the EMA. The drug sponsor will have given several months advance notice to the EMA of their intention to submit such an application. This allows some preparatory work to be undertaken, including the appointment of rapporteurs – members of the CHMP (or CVMP if the product is intended for veterinary use) who will coordinate evaluation of the application. Before evaluation begins, EMEA staff first validate the application, by scanning through it to ensure that all necessary information is present and presented in the correct format. This procedure usually takes one to two working weeks to complete. The basic regulatory fee charged for evaluating a full marketing authorization application via the centralized procedure is in the region of €259 000 (the overall EMA annual budget is in the region of €200 million, of which approximately three quarters is raised via fees).

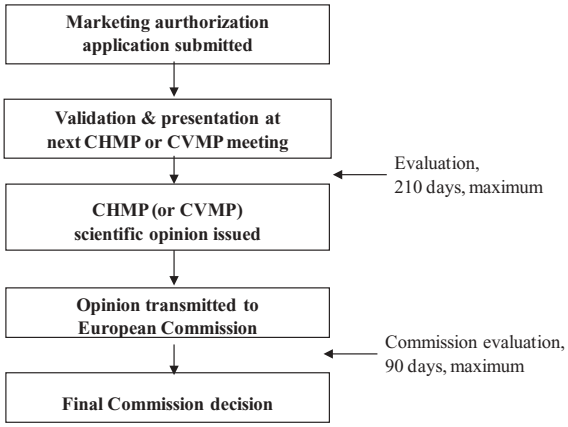

Figure 11.2 Overview of the EU centralized procedure.

The validated application is then formally presented at the next meeting of the CHMP (human medicine applications) or CVMP (veterinary medicines) and the rapporteur organizes technical evaluation of the application (product safety, quality, and efficacy). Much of this evaluation is often carried out in the rapporteur's home national regulatory agency. Another member of the committee (the co-rapporteur) assists in this process. Upon completion of the evaluation phase the rapporteurs draw up a report, which they present, along with a recommendation, at the next CPMP (or CVMP) meeting. After discussion, the committee issues a scientific opinion on the product, either recommending acceptance or rejection of the marketing application. The EMA then transmits this scientific opinion to the European Commission in Brussels (the only body with the legal authority to actually grant marketing authorizations). Legally the Commission must ensure that the potential marketing authorization is in compliance with the regulations, and it issues a final, binding decision on the product (Figure 11.2).

Regulatory evaluation of Marketing Authorization Applications must be completed within strict time limits. The EMA is given a 210-day window to evaluate an application and provide a scientific opinion. However, during the application process, if the EMEA officials seek further information/clarification on any aspect of the application this 210-day "clock" stops until the sponsoring company provides satisfactory answers. The average duration of active EMEA evaluation of biotech product applications is in the region of 160 days, well within this 210-day timeframe. Duration of clock stops can vary widely – from 0 days to well over 300 days. Upon receipt of the EMA opinion, the Commission is given a maximum of 90 days in which to translate this opinion into a final decision. Overall therefore, the centralized process should take a maximum of 300 active evaluation days.

Table 11.2 Product categories regulated by the FDA.

Foods, nutritional supplements
Drugs – chemical and biotech based
The blood supply and blood products
Cosmetics and toiletries
Medical devices
All radioactivity-emitting substances
Microwave ovens

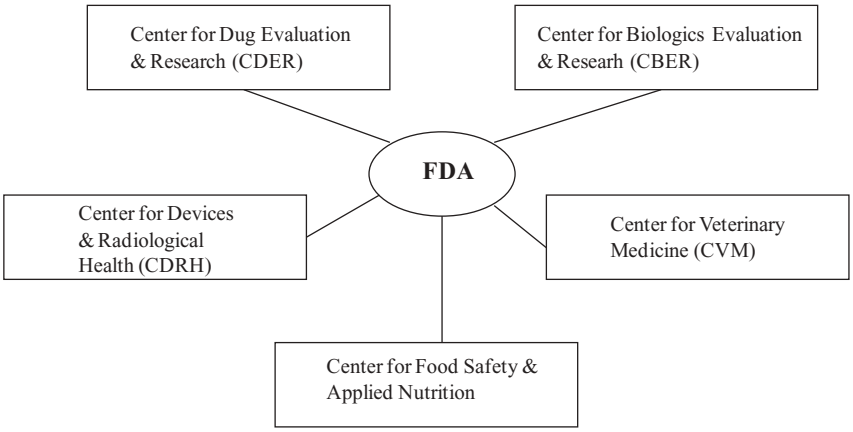

Figure 11.3 Partial organizational structure of the FDA.

11.3 Regulation in the United States of America

The Food and Drug Administration (FDA) is the US regulatory authority (http://www.fda.gov/). Its primary function is to protect public health by assuring the safety, effectiveness, and security of human and veterinary drugs, medical devices, cosmetics, products that give off radiation as well as food and selected other products (Table 11.2). Founded in 1930, it now forms part of the US Department of Health and Human Services, and its Commissioner is appointed directly by the US President.

The FDA derives its legal authority from the federal Food, Drug, and Cosmetic (FD&C) Act. Originally passed into law in 1930, the act has been updated/amended several times since. The FDA interprets and enforces these laws. Although there are many parallels between the FDA and the EMA, its scope is far broader than that of the European agency and its organizational structure is significantly different. Overall the FDA now directly employs some 11 500 people, has an annual budget in the region of $1 billion and regulates over $1 trillion worth of products annually. A partial organizational structure of the FDA is presented in Figure 11.3. In the context of pharmaceutical biotechnology the Center for Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER) are the most relevant FDA bodies.

Table 11.3 Major biotechnology/biological-based drug types regulated by CDER and CBER.

CDER regulated	CBER regulated
Monoclonal antibodies for *in vivo* use	Blood
Cytokines (e.g., interferons and interleukins)	Blood proteins (e.g., albumin and blood factors)
Therapeutic enzymes	Vaccines
Thrombolytic agents	Cell and tissue based products
Hormones	Gene therapy products
Growth factors	Antitoxins, venoms, and antivenins (antivenoms)
Additional miscellaneous proteins	Allenergic extracts

11.3.1 CDER and CBER

A major activity of the Center for Drug Evaluation and Research is to evaluate new drugs and decide if marketing approval should be granted or not. (Note the difference in regulatory terminology – the term medicinal product being used in Europe, whereas the term drug is favored in the US.) Additionally, CDER also monitors the safety and efficacy of drugs already approved (i.e., post-marketing surveillance and related activities). Traditionally CDER predominantly regulated chemical-based drugs (i.e., drugs that are usually of lower molecular weight and often manufactured by direct chemical synthesis). Included are prescription, generic, and over the counter drugs. CDER has now also been assigned regulatory responsibility for the majority of pharmaceutical biotechnology products (Table 11.3).

The Center for Biologics Evaluation and Research (CBER) undertakes many activities similar to that of CDER, but it focuses upon biologics and related products. The term "biologic" historically has a specific meaning, relating to "a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood components or derivatives or allergenic products which are used in the prevention, treatment or cure of diseases of human beings" (http://www.fda.gov/Drugs/default.htm). CBER therefore regulates products such as vaccines and blood factors, be they produced by traditional or modern biotechnological means (i.e., by non-recombinant or recombinant means). Additional "biological products," including cell, gene therapy, and tissue-based products also fall under the auspices of CBER.

11.3.2 Approvals Procedure

The overall procedure by which biotechnology and other drugs are evaluated and approved by CDER or CBER are, predictably, very similar, although some of the

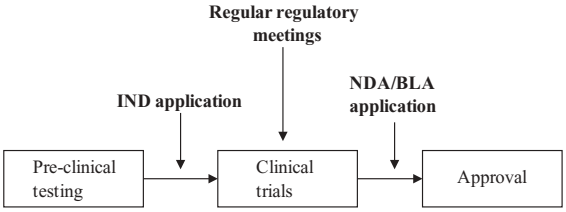

Figure 11.4 Summary overview of the main points during a drug's development at which the FDA plays a key regulatory role.

regulatory terminology used by these two centers differ. A summary overview of the main points along the drug development/approval road in which CDER/CBER play key regulatory roles is provided in Figure 11.4.

Once a sponsor (company, research institute, etc.) has completed preclinical evaluation of a proposed new drug, it must gain FDA approval before instituting clinical trials. The sponsor seeks this approval by submitting an Investigational New Drug Application (an IND) to either CDER or CBER, as appropriate. The application, which is a multi-volume work of several thousand pages, contains information detailing preclinical findings, methods of product manufacture, and proposed protocols for initial clinical trials. The regulatory officials then assess the data provided, and may seek more information/clarification from the sponsor if necessary. Evaluation is followed by a decision to either permit or block clinical trials. Should clinical trials commence, the sponsor and regulatory officials hold regular meetings in order to keep the FDA appraised of trial findings.

Upon successful completion of clinical trials, the sponsor then usually applies for drug approval. In CDER terminology this application is termed a New Drug Application (an NDA – the analogous term in Europe being the Marketing Authorization Application, MAA). NDAs usually consist of several hundred volumes containing over 100 000 pages in total. The NDA contains all the preclinical as well as clinical findings and other pertinent data/information. FDA fees in respect of evaluating a full application stand in the region of $1.5 million. Upon receipt of an NDA, CDER officials check through the document ensuring completeness (a process similar to the EMEA's validation phase). Once satisfied, they "file" the application and evaluation begins.

The NDAs are reviewed by various FDA regulatory experts, generally under topic headings such as "medical," "pharmacology," "chemistry," "biopharmaceutical," "statistical," and "microbiology" reviews. Reviewers may seek additional information/clarification from the sponsor, as they feel necessary. The regulators are allowed 60 days to ensure that the dossier is complete, and a further 180 days to conduct the scientific evaluation of the product. The review process is coordinated by a specific project manager. Upon review completion, the application is either approved or rejected.

The review process undertaken by CBER officials upon biologic and related products is fairly similar to that described above for CDER regulated products. CBER

regulated investigational drugs may enter clinical trials subject to gaining IND status. The application process for drug approval is undertaken by the sponsor subsequent to completion of successful clinical trials is termed the licensure phase in CBER terminology. The actual product application is known as a Biologics License Application (BLA). Overall, the content and review process for a BLA is not dissimilar to that of the analogous CDER NDA process, as discussed above. The bottom line is that the application must support the thesis that the product is both safe and effective, and that it is manufactured and tested to the highest quality standards.

While the majority of biotech-based drugs are regulated in the United States by either CBER or CDER, it is worth noting that some such products fall outside their auspices. Bone morphogenic proteins (BMPs) for example, function to stimulate bone formation. As such several have been approved for the treatment of slow healing bone fractures. Product administration requires surgical implantation of the BMP in the immediate vicinity of the fracture, usually as part of a supporting device. As such, in the United States, these products are regulated by the FDA's Center for Devices and Radiological Health (CDRH) (http://www.fda.gov/MedicalDevices/default.htm). Drugs (both biotech and non-biotech) destined for veterinary use also fall outside the regulation of CBER or CDER. Most such veterinary products are regulated by the FDA's Center for Veterinary Medicine (CVM) although veterinary vaccines (and related products) are regulated not by the FDA, but by the Center for Veterinary Biologics, which is part of the US Department of Agriculture (http://www.aphis.usda.gov/animal_health/vet_biologics/vb_about.shtml).

11.4
International Regulatory Harmonization

Europe, the United States, and Japan represent the three main global pharmaceutical markets. As such, pharmaceutical companies usually aim to register most new drugs in these three key regions. Although the underlining principles are similar, detailed regulatory product authorization requirements vary in these different regions, making some duplication of registration effort necessary. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (the ICH process) is an initiative aimed at harmonizing regulatory requirements for new drug approvals in these regions. The project was established in 1990 and brings together both regulatory and industry representatives from Europe, the United States, and Japan. ICH is administered by a steering committee, consisting of representatives of the above mentioned groupings. The steering committee in turn is supported by an ICH secretariat, based in Geneva, Switzerland (http://www.ich.org/). The main technical workings of ICH are undertaken by expert working groups charged with developing harmonizing guidelines. The guidelines are grouped under one of the following headings:

- **Efficacy** (clinical testing and safety monitoring related issues)
- **Quality** (pharmaceutical development and specifications)

- **Safety** (preclinical toxicity and related issues)
- **Multidisciplinary** (topics not fitting the above descriptions).

Thus far, in excess of 60 guidelines aimed at both traditional and biotechnology based products have been produced and are being implemented. One of the ICHs most significant initiatives to date has been the development of the Common Technical Document. This provides a harmonized format and content for new product authorization applications within the EU, United States, and Japan. Such regulatory streamlining will make more economical use of both company and regulatory authorities' time, should reduce the cost of drug development and also speed up the drug development procedure, ensuring faster public access to new drugs.

11.5 Regulation of Biosimilars

The advent of biosimilars possibly represents the most contentious regulatory issue to arise in recent years in the context of biotech drug approval. Once patent protection on any drug expires, alternative pharmaceutical companies are free to develop and seek marketing approval for a copy of the original product – that is, to market a generic product. Traditional targets for generic manufacturers are invariably low molecular weight organic molecules, which are manufactured by well defined, precisely controlled chemical pathways. Hence, it is possible to routinely produce a product identical in every way to the original drug. Legislative frameworks for the approval of such generic pharmaceuticals were established in many regions of the world from the 1980s onwards, and are generally non-contentious.

The first recombinant biotech products (biopharmaceuticals) entered the marketplace in the 1980s, thus many of the original products have lost patent protection only in the last decade. Collectively somewhere in the region of $12–15 billion worth of products are now off patent and it is not surprising that many such originator products would become the target of generic producers. Biopharmaceuticals, however, differ fundamentally from traditional, chemically synthesized, low molecular weight pharmaceutical products. They are hundreds, usually thousands, of times larger and are synthesized by biological processes, with all of the inherent variability that this entails. While genetic engineering can ensure the production of a recombinant protein with an amino acid sequence identical to any approved product, the exact details of manufacture (upstream and downstream processing) can and will influence the impurity profile of the product, as well as the exact detail of any post-translational modifications (e.g., glycosylation) present. Moreover, their complexity renders full analytical characterization of any such product extremely challenging.

As such, it is highly improbable that a generic version of a biopharmaceutical would be absolutely identical to the originator. What is achievable is the production of a product *very substantially similar* to the originator, hence the term biosimilar.

The EU developed legislative provisions for the approval of biosimilars almost a decade ago and the EMA subsequently developed a suite of associated regulatory

guidelines (http://www.ema.europa.eu). This has thus far facilitated the approval of 14 such biosimilar products within Europe. EU biosimilar regulations necessitate the generation of comparative data between the proposed new biosimilar product and the original (reference) product, to which it claims biosimilarity. The reference product must already be approved for general medical use within the EU. The sponsor seeking biosimilar approval must submit the data generated as a Marketing Application directly to the EMA for consideration via the centralized procedure. The Marketing Authorization Application (relative to the one for the original reference product) will contain a full quality module (containing details of product development, manufacture, specifications, and analysis, etc.), as well as reduced clinical and non-clinical data modules.

Regulators in several other world regions (e.g., Australia, Canada, Japan, Switzerland) have developed regulatory frameworks for the approval of biosimilar products. In most cases these regulatory provisions are substantially similar to European regulations. Development of a biosimilar pathway in the United States has been significantly slower, although a legal framework for such approvals was finally implemented with the ratification of the US Biologics Price Competition & Innovation Act (BPCI Act) in 2010. With the legal framework in place it is effectively now up to the FDA to implement its contents. A Biosimilar Implementation Committee has been established and an Associate Director for Biosimilars has been appointed, and the FDA are currently in the process of developing biosimilar guidelines. It is therefore likely that biosimilar products will finally become available on the US market over the next few years.

12
Patents in the Pharmaceutical Biotechnology Industry: Legal and Ethical Issues

David B. Resnik

Disclaimer: This chapter is intended to provide legal information but not legal advice. Those who are interested in legal advice should consult a licensed attorney.

12.1
Introduction

This chapter will provide the reader with an overview of patenting in the pharmaceutical biotechnology industry and summarize some of the key legal and ethical issues related to the patenting of biomedical products and processes. It will examine the legal aspects of patenting before considering ethical and policy issues. The focus will primarily be on the United States (US) patent laws, which are very similar to European patent laws. Some differences between US and European laws will be noted, and mention will be made of some relevant international intellectual property treaties.

12.2
Patent Law

12.2.1
What Is a Patent?

A patent is a type of intellectual property. All properties can be understood as collections of rights to control a particular thing. Tangible properties give the property holder rights to control tangible things, such as cars or land. Intellectual properties, on the other hand, give the property holder rights to control intangible things, such as inventions, poems, or computer programs. Tangible things have a particular location in space and time; intangible things do not. The main types of intellectual property are patents, copyrights, trademarks, and trade secrets [1].

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A patent is a private right granted by the government to someone who invents a new and useful product or process. The initial patent holder, the inventor, has the right to exclude others from making, using, or commercializing his invention. The patent holder may transfer all or part of his rights to another party, including another individual or a corporation. Researchers who work for biotechnology companies usually assign their patent rights to the company in exchange for salary, a fee, or a share of royalties. Assignment of patent rights transfers all the rights to the assignee, who becomes the new patent holder. Patent holders may also grant licenses to other parties in exchange for royalties or a fee. For example, a biotechnology company with a patent on a gene therapy technique could grant individuals or companies licenses to use the technique [1].

In the United States, a patent holder has the right to refrain from making, using, or licensing his invention, if he or she so desires. In the United States, a patent confers rights to make, use, or commercialize a thing but implies no corresponding obligations. As a result, some companies in the United States use patents to block technological development to gain an advantage over competitors. Some European countries, however, have compulsory licensing, which requires the patent holder to make, use, or commercialize his or her invention or license others to do so [1].

The term of a patent in the United States and most countries that belong to the European Union (EU) lasts 20 years from the time the inventor submits his application. A patent is not renewable. Once the patent expires, the invention becomes part of the public domain, and anyone can make, use, or commercialize the invention without permission from the inventor [1]. In the pharmaceutical industry the average interval between discovery of a new drug and its final approval by the Food and Drug Administration (FDA) for human consumption is 10 years, which includes the time required to conduct clinical research, product development, as well as FDA review. Thus, most pharmaceutical companies can expect that they will have about 10 years to recoup the money they have invested in a new drug before the patent expires. Once the patent expires, the name of the drug may still have trademark protection, but other companies can manufacture and market a generic version of the drug without obtaining permission from the company [2].

The main policy rationale for patent laws is that they promote the progress of science, technology, and industry by providing financial incentives for inventors, entrepreneurs, and investors [1]. By granting property rights over inventions, the patent system gives inventors, private companies, and other organizations the opportunity to profit from their investments of time and money in research and development. Since most new scientific discoveries and technological innovations benefit society, the public benefits from granting private rights over intellectual property. However, excessive private control over intellectual property can impede access to science and technology. Thus, patent laws attempt to strike to an appropriate balance between public and private control of inventions. A good example of this balancing is the length of a patent: if the term of a patent is too short, companies and researchers will not have enough time to obtain a fair return on

their investment; if the term is too long, the public will not have adequate access to technology.

12.2.2 How Does One Obtain a Patent?

To obtain a patent, one must submit a patent application to the patent office. In the United States, the Patent and Trademark Office (PTO) examines patent applications. The application must provide a description of the invention that would allow someone trained in the relevant practical art to make and use the invention. One or more individuals may be listed as inventors on the patent application. The application need not include a sample or model of the invention; a written description will suffice. The application will contain information about the invention, background references, data, as well as one or more claims pertaining to the invention. The claims stated on the patent application will determine the scope of the inventor's patent rights.

If the PTO rejects a patent application, the inventor may submit a revised application. The process of submission/revision/resubmission, otherwise known as "prosecuting" a patent, may continue for months or even years. If the PTO rejects the patent, the applicant may appeal the decision to a federal court [1].

The PTO will award a patent to an inventor only if he (or she) provides evidence that his (or her) invention satisfies all of the following conditions (EU countries have similar requirements [1, 3]):

1) **Originality.** The invention is new and original; it has not bee previously disclosed in the prior art. The rationale for this condition is that the public does not benefit when the patent office grants a patent on something that has already been invented. Thus, if someone else has already submitted an application for the same invention, this would qualify as a prior disclosure. Also, disclosure could occur if a significant part of the invention has been published or used [1].

2) **Non-obviousness.** The invention is not obvious to someone who is trained in the relevant practical art.

3) **Usefulness.** The invention has some definite, practical utility. The utility of the invention should not be merely hypothetical, abstract, or contrived. The rationale for this condition is self-explanatory: the public does not benefit from useless patents. Recently, the US PTO raised the bar for proving the utility of patents on DNA in response to concerns that it was granting patents on DNA sequences when the inventors did not even know the biological functions of those sequences [4].

In addition to satisfying these three conditions, to obtain a patent in the United States, the inventor must exhibit due diligence in submitting an application and developing the invention. In the United States, the person who is the first to conceive an invention will be awarded the patent unless he does not exhibit due

diligence. If the first inventor does not exhibit due diligence, the PTO may award the patent to a second inventor, if that inventor reduces the invention to practice and submits an application before the first inventor [1].

Once the PTO awards the patent, the application becomes part of the public domain, and other inventors and researchers may use the knowledge contained on the application. Indeed, the "patent bargain" is an agreement between the government and a private party in which the party agrees to disclose knowledge related to his invention to the public in exchange for a limited monopoly on the invention [1]. The public benefits from this bargain because it encourages the inventor to avoid protecting his knowledge through trade secrecy. A great deal of the world's scientific and technical information is disclosed in patent applications [5]. For example, the PTO has a large online, searchable database of patent applications [6].

12.2.3
What Is the Proper Subject Matter for a Patent?

Under US law, the PTO can award patents on articles of manufacture, compositions of matter, machines, or techniques or improvements thereof [7]. EU countries allow patents on similar types of things [3]. Although different patent laws use different terms to describe the subject matter of patents, there are three basic types of subjects for patents: (i) products (or materials), (ii) processes (or methods), and (iii) improvements. For example, one could patent a mousetrap (a product), a method for making a mousetrap (a process), or a more efficient and humane mousetrap (an improvement) [1].

One of the most important doctrines in patent law is that patents only apply to products or processes that result from human ingenuity (or inventiveness). Thus, US courts have held that one may not patent laws of nature or natural phenomena, since these would be patents on a product of nature. Over two decades ago a landmark US Supreme Court case, *Diamond v. Chakrabarty*, set the legal precedent in the United States for patents on life forms [8]. Chakrabarty had used recombinant DNA techniques to create a type of bacteria that metabolizes crude oil. The PTO had rejected his patent application on the grounds that the bacteria did not result from human ingenuity, but the Supreme Court vacated this ruling and held that Chakrabarty could patent his genetically engineered life forms [8, 9]. This decision helped to establish the legal precedent for other patents on life forms, such as patents on laboratory mice, cell-lines, and bioengineered tissues and organs [10]. EU countries have followed the United States in allowing patents on life forms that result from human ingenuity [11].

Before Chakrabarty received his patent, the PTO had also granted inventors patents on DNA, proteins, and recombinant DNA techniques [12–14]. In granting patents on organic compounds that occur in living organisms, such as animals or plants, patent agencies have distinguished between naturally occurring compounds and isolated and purified compounds [15]. For example, DNA in its natural state occurs in virtually all organisms and is unpatenable in its natural state. However, scientists can use various chemical and biological techniques to create

isolated and purified samples of DNA, which are patentable. The reason why patent agencies allow patents on isolated and purified compounds is they result from human ingenuity [16].

Another important doctrine in patent law is that patents apply to applications, not to ideas. Ideas are part of the public domain. For example, courts in the United States have ruled that mathematical algorithms are unpatentable ideas but that computer programs that use algorithms to perform practical functions are patentable [17].

12.2.4 Types of Patents in Pharmaceutical Biotechnology

There are many different types of patents that may be available to researchers and companies in the field of pharmaceutical biotechnology. Following the distinction in Section 12.2.3 between products and processes, potential patents might include:

1) Patents on pharmaceutical and biomedical products, such as bioengineered drugs, proteins, receptors, neurotransmitters, oligonucleotides, hormones, genes, DNA, DNA microchips, RNA, cell lines, bioengineered tissues and organs, and genetically modified bacteria, viruses, animals, and plants.
2) Patents on pharmaceutical and biotechnological processes, such as methods for genetic testing, gene therapy procedures, DNA cloning techniques, methods for culturing cells and tissues, DNA and RNA sequencing methods, and xenotransplantation procedures.
3) Patents on improvements to pharmaceutical, biomedical, and biotechnological products and processes.

For any of these products or processes to be patentable, they would need to result from human ingenuity.

12.2.5 Patent Infringement

Patent infringement occurs when someone uses, makes, or commercializes an invention without permission of the patent holder. In the United States, the patent holder has the responsibility of brining an infringement claim against a potential infringer and proving that infringement occurred [1]. A court may issue an injunction to stop the infringement or award the patent holder damages for loss of income due to infringement. There are three types of infringement: direct infringement, indirect infringement, and contributory infringement. Patent holders may also settle infringement claims out of court. Researchers, corporations, and universities usually try to avoid any involvement in an infringement lawsuit, since patent infringement litigation is expensive and time consuming [16].

Many EU countries have a defense to patent infringement known as the research exemption [3]. The United States also has a research exemption (also known as the experimental use exemption), which has been used very infrequently [18].

Under this exemption, someone who uses or makes a patented invention for pure research with no commercial intent can assert this defense in an infringement lawsuit to avoid an adverse legal decision. The research exemption is similar to the "fair use" exemption in copyright law in so far as it permits some unconsented uses of intellectual property [18]. There are some problems with the exemption, however. Firstly, the research exemption is not well publicized. Secondly, the research exemption is not well defined [18]. Indeed, in the United States the research exemption has no statutory basis but is a creation of case law. Some commentators have argued that countries should clarify and strengthen the research exemption in order to promote research and innovation in biotechnology and avoid excessive private control of inventions [3].

12.2.6
International Patent Law

Every country has the authority to make and enforce its own patent laws and to award its own patents. Thus, a patent holder must apply for a patent in every country in which he wants patent protection. For example, a corporation that patents a new drug in the United States must also apply for a patent in Germany, if it desires patent protection in Germany. Furthermore, complex matters relating to jurisdiction can arise when someone infringes a patent that is protected in one country but not another. For example, if someone infringes a US patent in Germany, but the invention is not protected by German patent laws, then the patent holder will have need to bring a lawsuit in a court in the United States, which may or may not have jurisdiction.

To deal with international disputes about intellectual property and to harmonize intellectual property laws, many countries have signed intellectual property treaties. Most of these treaties define minimum standards for intellectual property protection and obligate signatories to cooperate in the international enforcement of property rights. The most important treaty related to patents is the Trade Related Aspects of Intellectual Property agreement (TRIPS), which has been developed and negotiated by the World Trade Organization (WTO). The TRIPS agreement defines minimum standards for patent rights. For example, it requires that patents last 20 years. Countries that have signed the agreement agree to adopt patent laws that provide at least the minimum level of protection under the agreement. Countries must also agree to cooperate in the enforcement of patent rights. TRIPS allows countries to override patents rights to deal with national emergencies, such as public health crisis [1].

12.3
Ethical and Policy Issues in Biotechnology Patents

Having provided the reader with some background information on patenting in biotechnology, this section will briefly review some important ethical and policy issues.

12.3.1 No Patents on Nature

In the 1990s, a variety of writers, political activists, theologians, ethicists, and professional organizations opposed patents on biotechnological products and processes for a variety of reasons. Many of these critics argued that patents on living bodies, as well as patents on body parts, are unethical because they are patents on natural things [19]. They argued that it is immoral and ought to be illegal to patent organisms, tissues, DNA, proteins, and other biological materials. Some of these critics based their opposition to biotechnology patents on religious convictions [20], while others based their opposition on a general distrust of biotechnology and the biotechnology industry [21, 22]. Some of the more thoughtful critics of biotechnology patents accepted some types of patents on biological materials, but objected to patents on other types of biological materials, such as patents on genes or cell lines, on the grounds that these types of patents attempt to patent nature [23, 24].

As noted in Section 12.2.3, patents on products of nature are illegal; a product or process must have resulted from human ingenuity to be patentable. But how much human ingenuity should be required to transform something from an unpatenable product of nature into a patentable, human invention? Defining the boundaries between products of nature and human inventions is a fundamental issue in patent law and policy that parallels the tenuous distinction between natural and artificial [25]. While most people can agree on paradigmatic cases of things that are natural, such as gold, and things that are artificial, such as gold jewelry, it difficult to reach agreement on borderline cases, such as DNA sequences. On the one hand, DNA sequences exist in nature and can therefore be regarded as natural. On the other hand, isolated and purified DNA sequences do not exist in nature and are produced only under laboratory conditions. They are, in some sense, human artifacts. However, the nucleotide sequences in isolated and purified DNA are virtually identical to the sequences in naturally occurring DNA. There is probably no objective (i.e., scientific) basis for distinguishing between naturally occurring DNA and isolated and purified DNA. Likewise, there is probably no objective basis for distinctions between natural cell lines versus artificial cell lines, natural proteins versus artificial proteins, and natural organisms versus artificial organisms.

If the distinction between a product of nature and a human invention is not objective, then it depends, in large part, on human values and interests. It is like other controversial distinctions in biomedical law and ethics, such as human versus non-human and alive versus dead. The best way to deal with these controversial distinctions is to carefully consider, negotiate, and balance competing values and interests in light of the particular facts and circumstances. Laws and policies that define patentable subject matter should also attempt promote an optimal balance between competing interests and values and should carefully consider the facts and circumstances relating to each item of technology [25]. Policies adopted by the United States and the European Union with respect to the patenting of DNA appear to strike an optimal balance between competing interests

and values, because these policies disallow the patenting of DNA in its natural state but allow the patenting of isolated and purified DNA [11, 15].

12.3.2 Threats to Human Dignity

Critics of biotechnology patents also have claimed that patents on human body parts, such as genes, cell lines, and DNA, are unethical because they treat people as marketable commodities [19, 21, 22, 24]. Some have even compared patents on human genes to slavery [26]. The issues concerning the commercialization of human body parts are complex and emotionally charged. They also have implications for many different social policies, including organ transplantation, surrogate parenting, and prenatal genetic testing. This chapter will give only give brief overview of this debate.

According to several different ethical theories, including Kantianism and the Judeo-Christian tradition, human beings have intrinsic moral value (or dignity) and should not be treated as if they have only extrinsic value. An entity (or thing) has intrinsic value if it is valuable for its own sake and not merely for the sake of some other thing. A commodity is a thing that has a value – a market value or price – which serves as a basis for exchanging it for some other thing. For example, one can exchange a barrel of oil for $30 or exchange a visit to the dentist for $50. Treating an entity as a commodity is treating it as if it has only extrinsic value and not intrinsic value. Thus, it would be unethical to treat a human being as a commodity because this would be treating that person as if they have only extrinsic value and no intrinsic value. Slavery is therefore unethical because it involves the buying and selling of whole human beings. People are not property [27, 28].

Even though treating a whole human being as a commodity violates human dignity, one might argue that treating a human body part as a commodity does not violate human dignity. Human beings have billions of different body parts, ranging from DNA, RNA, proteins, and lipids, to membranes, organelles, cells, tissues, and organs. Properties that we ascribe to the parts of a thing do not necessarily transfer to the whole thing; inferences from parts to wholes are logically invalid. For example, the fact that a part of an automobile, such as the front tire, is made of rubber does not imply that the whole car is made of rubber [27]. Likewise, treatment of a part of human being, such as blood or hair, as a commodity does not imply treatment of the whole human being as part. It is possible to commodify (or commercialize) a human body part without commodifying the whole human being.

This argument proves that buying and selling hair, blood, or even a kidney is not equivalent to slavery. Even so, one might argue that treating human body parts as commodities constitutes incomplete commodification of human beings, partial commodification of human beings can threaten human dignity even if it does not violate human dignity [29]. Incomplete commodification can threaten human dignity because it can lead to exploitation, harm, and injustice, as well as complete commodification of human beings. For example, in the now famous case of *Moore*

v. Regents of University of California, the desire to patent a valuable cell line played an important role in the exploitation of a cancer patient [24]. The researchers took cells from Moore's body that over-express cytokines. The researchers did not tell Moore what they planned to do with tissue samples they took from him or that the samples could be worth millions of dollars [30]. One might argue that treating human body parts as commodities inevitably leads to abuses of human rights and dignity, as occurred in the Moore case. Although incomplete commodification of human beings is not intrinsically immoral, it can lead society down a slippery slope toward various types of immorality and injustices. In order to stop the slide down this slippery slope, society should forbid activities that constitute incomplete commodification of human beings, such as the patenting of cell lines and DNA, a market in human organs, surrogate pregnancy contracts, cloning for reproduction, and selling human gametes [31].

One could reply to this argument by acknowledging that the slippery slope poses a genuine threat to human dignity but maintain that it may be possible to prevent exploitation, injustice, and other abuses by developing clear and comprehensive regulations on practices that commodify human body parts. Regulations should require informed consent to tissue donation, gamete donation, and organ donation, as well as fair compensation for subjects that contribute biological materials to research and product development activities. Regulations should also protect the welfare and privacy of human research subjects and patients [27, 32]. These regulations should also state that some human biological materials, such as embryos, should not be treated as commodities because they pose an especially worrisome threat to human dignity. Although an embryo is not a human being, it should be illegal to buy, sell or patent a human embryo. However, it should be legal to buy or patent embryonic stem cells, provided that society has appropriate regulations [33]. Although selling organs is illegal in many countries, including the United States and many European nations, some have argued that organs could be bought and sold, provided that appropriate regulations are in place [34, 35].

12.3.3 Access to Technology

One of the most important ethical and policy concerns raised by critics of biotechnology patenting is that patenting will have an adverse impact on access to materials and methods that are vital to research and innovation in biotechnology as well as medical tests and treatments. The negative effects of patenting on science, industry, and medicine will constitute a great social cost rather than a social benefit. In Section 12.2.1 we noted that the primary rational for the patent system is that it benefits society by encouraging progress in science, technology, and industry. However, this argument loses it force when patenting has the opposite effect. If patenting does more harm than good, then we should forbid or greatly restrict patenting [24, 36]. The issue of access to materials and methods in biotechnology, like the issues discussed in Sections 12.3.2 and 12.3.3, is very complex and controversial. This chapter will not attempt to explore these issues in great

depth, but it will attempt to provide the reader with an outline of the arguments on both sides.

Concerns about access to materials and methods stem from potential problems with the licensing of patents on products and processes that are useful in research and innovation biomedicine and biotechnology [37]. Firstly, if a researcher or company wants to develop a new product or process in biotechnology and biomedicine, then he or she may need to negotiate and obtain dozens of different licenses from various patent holders in order to avoid patent infringement. The researcher or company might need to fight through a "patent thicket" in order to develop a new and useful invention. For example, DNA chip devices test for thousands of different genes in one assay. If dozens of companies hold patents on these different genes, then one may need to obtain dozens of different licenses to develop this new product. Although larger biotechnology and pharmaceutical companies are prepared to absorb the legal and administrative transaction costs associated with licensing, smaller companies and universities may find it difficult to navigate the "patent thicket" [38].

Secondly, "blocking patents" in biotechnology could prevent the development of downstream products and processes [39]. In industries with many different interdependent products and processes, someone who holds a particular invention may be able to affect or control the development of subsequent inventions that depend on that prior invention. These prior inventions are also known as "upstream" inventions, and the subsequent inventions are also known as "downstream" inventions. Some companies may obtain patents for the sole purpose of preventing competitors from developing useful inventions in biotechnology. In the United States, these companies would have no obligation to use, make, market, or licenses such inventions. They could use their inventions to block the development of downstream products and processes. In countries that have compulsory licensing, companies would have a legal duty to make, use, commercialize, or license their inventions, but they could still use other means to prevent the development of downstream technologies, such as setting very high licensing fees.

Thirdly, high licensing fees could impose a heavy toll on research and innovation in biotechnology and biomedicine [37]. Companies with patents on upstream inventions might issues licenses on the condition that they receive a percentage of profits from downstream inventions. While downstream patent holders have no legal obligation to share their profits with upstream patent holders, upstream patent holders may try to acquire a portion of downstream profits by issuing these "reach through" licenses. Even companies that do not issue "reach through" licenses may still set high licensing fees. For example, many commentators have claimed that Myriad Genetics' high licensing fees for its tests for BRCA1 and BRCA2 mutations, which increase the risk of breast and ovarian cancer, have had a negative impact on research and innovation, and diagnostic and predictive testing [40].

These aforementioned problems related to licensing – the patent thicket, blocking patents and high licensing fees – could undermine not only research and innovation but could also have an adverse impact on health care by undermining

access to new medical products and services, such as genetic tests. For example, if a company is unable to develop a genetic test, due to licensing problems, then patients will not benefit from that test. If a company develops a genetic test but charges a high fee to conduct the test or charges a high fee to license the test, then many patients may not be able to afford the test. In either case, problems related to the licensing of biotechnology products and processes could prevent the public from benefiting from new developments in biomedicine.

On the other hand, many commentators and industry leaders have rebutted these criticisms of biotechnology patenting by arguing that the free market, patent offices, and the legal system will keep potential licensing problems in check [41–43]. Companies will not have any major difficulties negotiating and obtaining licenses because they will all understand the importance of cooperation in the biotechnology industry. Few companies will develop blocking patents because these patents will usually prove to be unprofitable: one can make much more money from marketing or licensing a new invention than from keeping it on the shelf. Finally, high licensing costs will decline in response to lower consumer demands, especially if competitors are able to enter the market by developing new inventions that work around existing ones. (A "work around" invention is an improvement on a patented invention or an alternative to a patented invention.) Industry leaders also point out that the potential licensing problems faced by the biotechnology industry are entirely new because many other industries have faced – and solved – similar problems [41]. For example, many different companies in the semiconductor industry have worked together to develop licensing agreements [44]. There are many interdependent products and processes in the semiconductor industry and many different patent holders, but companies have managed to avoid licensing problems and the industry has thrived. Indeed, the semiconductor industry is one of the most successful and innovative industries the world has ever known.

Some commentators have argued that societies should reform the patent system to prevent licensing problems from occurring and to ensure that new biomedical technologies are affordable and accessible. These proposed reforms, some of which have been mentioned above, include the following:

1) Banning patents on particular types of products or processes, such as patents on genes that are associated with diseases or patents on genetic tests [23].
2) Expanding and clarifying the research exemption in biotechnology [3, 16].
3) Raising the bar for the various conditions for awarding patents, such as novelty and utility [3, 16].
4) Restricting the scope of biotechnology patents in order to allow for "work around" inventions and to promote competition [3, 16].
5) Applying anti-trust laws to the biotechnology industry to promote fair competition [16].
6) Conducting an ethical review of patent applications to address ethical and policy issues before awarding patents [3, 45].
7) Developing a patent pool in the biotechnology industry to promote efficient licensing [46].

Most of these proposed reforms, with the exception of banning some types of biotechnology patents, would probably promote research and innovation in biotechnology and biomedicine without undermining financial incentives for researchers and companies. Many of these reforms could be enacted without any additional legislation, since patent offices and the courts already have a great deal of authority to shape patent law and policy through their interpretation and application of existing statutes [47].

12.3.4 Benefit Sharing

The final issue this chapter will consider involves the sharing of the benefits of research and innovation in biotechnology. Some critics of biotechnology patents have claimed that the distribution of the benefits of research and innovation is often unfair [22, 24, 48, 49]. According to these critics, pharmaceutical and biotechnology companies benefit greatly from research and innovation by earning large profits, but individual patients or research subjects, populations, or communities benefit very little. For example, to study a genetic disease, researchers need to take tissue samples from patients/subjects. Very often, researchers do not offer to pay subjects any money for their tissue samples or promise them any royalties from the commercialization of their research or its applications. If a company develops a profitable genetic test from free genetic samples, patients/subjects could argue that the company is not sharing benefits fairly. Unequal distributions of benefits could also occur between companies and entire communities or countries. For example, some pharmaceutical and biotechnology companies are now developing drugs based on knowledge obtained from indigenous populations concerning their medicinal plants. If a company develops a profitable medication from this indigenous knowledge and does not offer the population any compensation, the population could argue the company has not shared the benefits of research fairly. Unequal distributions of benefits could also take place between developed nations and developing nations. For example, if researchers, patients, and companies from the developed world benefit a great deal from biotechnology, but people in the developing world do not, one might argue the benefits of biotechnology have been distributed unfairly.

Several writers and organizations have called for the fair distribution of the benefits of research in biotechnology [50–53]. Some writers appeal directly to theories of justices, such as utilitarianism, egalitarianism, or social contract theory, to argue for a fair distribution of research benefits [47, 54]. Others appeal to the concept of a common heritage relating to human biological materials, such as DNA [52, 53]. Regardless of how one justifies a general principle of benefit sharing in biotechnology, the most important practical problems involve determining how benefits should be shared. What would be a fair sharing of benefits between researchers and companies and subjects/populations/communities? Should researchers and companies offer to give subjects/populations/communities financial compensation for providing research materials and methods, such as tissue samples or indigenous

knowledge? Should researchers and companies offer to pay royalties for the commercialization of research to subjects/populations/communities?

Although financial compensation might be useful and appropriate in some situations, such as giving communities royalties for indigenous knowledge or providing some subjects with compensation for their valuable tissues (as in the Moore case, discussed Section 12.3.2), in other situations direct financial compensation may not be very useful or appropriate. For example, if a company collects thousands of tissue samples from subjects and uses knowledge gained from those samples to develop a commercial product, the financial benefit offered to any particular subject might be miniscule, since the benefits would need to be divided among thousands of subjects. Moreover, it may be impossible to estimate the potential benefits to subjects prior to the development of the product, since most new products are not profitable. Furthermore, subjects in some cultures might not be interested in financial rewards for participation. Perhaps the best way to share benefits in situations like these would be to offer to provide the population or community with non-financial benefits, such as improvements in healthcare, education, or infrastructure. In any case, these complex questions cannot be addressed in depth in this chapter. To answer questions about the fair distribution of research benefits in any particular case, one needs to apply theories and concepts of distributive justice.

Even though there is little consensus about the how to distribute the benefits of research and innovation in biotechnology, almost everyone with an interest in the issue agrees that subjects should be informed about plans for benefit sharing (if there are any) [55]. For example, the researchers in the Moore case should have told Moore that they planned to develop a cell line from his tissue and that they were not planning to offer him any financial compensation. If researchers conduct a study that involves an entire population or community, they should discuss benefit-sharing plans with representatives of the community or population [56]. Indeed, respect for human dignity requires nothing less than fully informing subjects of the material facts related to their research participation, including facts pertaining to the commercialization of research [57, 58].

12.4 Conclusion

This chapter has provided the reader with an overview of the legal, ethical, and policy issues relating to the patenting of products and processes used in pharmaceutical biotechnology. Although it has attempted to provide the reader with up-to-date information, it is possible that some of this information may soon be out-of-date, due to changes in technology, case law, legislation, and international treaties. Since most of these issues are very complex and constantly changing, those who are interested in learning more about this topic should review the relevant documents, guidelines, and policies relating to their particular areas of research and development.

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13
Biosimilar Drugs

Walter Hinderer

13.1
Introduction

Biopharmaceuticals, and especially therapeutic proteins, represent a prominent and still exceptionally fast growing segment within the global pharmaceutical market, reaching US$773 billion in 2008. Up to March 2010, in total 103 recombinant protein drugs had been approved in Germany, which is the largest market in Europe, and thus is a good indication of the European market situation. Of these, 20 are recombinant antibodies, which have already gained 28% of the total volume of biopharmaceutical sales. The recombinant drug products are the most prominent area of biopharmaceuticals and they comprise 16% of the total turnover of pharmaceuticals. Today around 4% of all approved drugs are of recombinant origin. Moreover, considering new pharmaceuticals coming to approval, the participation of recombinant proteins has increased 25% [1–3].

Among these recombinant therapeutic proteins many are blockbusters and most of them are high-priced. It was calculated that in the United States some of these drug products cost 10–20 times more per daily dose than chemical drugs. Twelve protein products have sales greater than US$1 billion. In addition, it is expected that for the years 2013–2015 biotechnology-derived products with annual sales in the region of US$20 billion will lose patent protection. This is half of all sales for 2006 in this market segment [4].

In 2003 when I wrote the respective chapter for the first edition of this book (*Biogeneric Drugs*) the first wave of the therapeutic proteins had began to run off patent protection and the market segment had just opened-up to the generic drug suppliers. At that time neither a regulatory framework in Europe nor a clear development pathway for these biogeneric drugs had been established. Hence, the companies followed different strategies. Subsequently, along with the release of the European guidelines, the term "biosimilars" emerged and is commonly used today.

Up to June 2010, in total 16 biosimilar products (including epoetin theta from Ratiopharm, which was in fact not approved via the biosimilar route) had received approvals by the centralized European procedure. They captured significant market shares and the prices dropped considerably. The cost savings for the health systems

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are evident and further benefits will result from subsequent approvals of additional biosimilars.

Taking all these facts together, the biopharmaceuticals are increasingly attractive to the generic industry. Further opportunities lie ahead for the second wave of biosimilars. It is believed that a cluster of recombinant antibodies offer a multibillion Euro marketplace in the near future.

A similar situation to that which existed in Europe in 2003 is currently occurring in the United States. A regulatory framework for biosimilars or "follow on biologics" (FOBs), respectively, is under way, accompanied by a lot of public discussion, and it remains to be seen how difficult it will be for the existing European biosimilar products to adapt their market authorizations and data records to the requirements of the US Food and Drug Administration (FDA). For the second wave of biosimilar products, a dual development in order to comply with US and EU requirements from the beginning will preferentially be followed by companies. This is driven by the huge US market volume, which is 37% of the global pharmaceutical market. More than two thirds of the global turnover is made in Europe and the United States combined [3].

This chapter will narrow the subject of biosimilar drugs down to practical and regulatory aspects, supported by published data from the first approved biosimilars in Europe and by our own experience with biosimilar drug development. In contrast to the chapter in first edition of this book, I will focus more on the second wave of biosimilar drugs, in particular on recombinant antibodies, Fc-fusion proteins, and proteins conjugated with polyethylene glycol (PEG). Finally, it must be emphasized, that this chapter is directed predominantly at the European situation due to lack of an elaborated regulatory pathway in US for biosimilars or FOBs, respectively.

13.2 Recombinant Therapeutic Proteins

The term "biopharmaceuticals," despite having a broader definition, is often used equivalently to "therapeutic proteins." The first generation of these potent drug substances were derived exclusively from human or animal sources and applied in the replacement therapy of hormone or blood clotting factor deficiencies in chronic diseases. Examples of this first generation of proteins to be used as pharmaceutical drugs are human growth hormone (hGH), isolated from cadaveric human pituitary glands, insulin, extracted from pig or bovine pancreas, and several human blood proteins, derived from pooled plasma fractions, for example albumin, immunoglobulins or coagulation factors. These natural sources, have, however, been linked to many safety problems in the past, and the resulting protein products caused the transmission of infective agents, leading to Creutzfeld–Jacob disease in the case of hGH [5], and chronic hepatitis B, C, and AIDS in the case of coagulation factor VIII and IX [6]. Further problems emerged from the immunogenicity of non-human proteins, for instances in the case of bovine or porcine

insulin [7], which can lead to allergic or other immune reactions such as neutralization of the drug activity by antibodies in some cases.

Along with the emergence of methods of genetic engineering in the 1970s and 1980s, such as recombinant DNA technology and hybridoma technology, these natural proteins have been replaced more and more by recombinant versions or at least by proteins produced *in vitro* with cell cultures. Only a few exceptions exist, for example insulins and blood-derived proteins, such as albumin, immunoglobulin, factor VIII and IX, where therapeutic proteins derived from natural sources have maintained a significant role in the market. The first human healthcare product derived from recombinant DNA technology was Eli Lilly's insulin (Humulin®), approved in the United States and Europe in 1982 [8]. This was a milestone for the biotechnology industry. The continuous improvements in these new technologies has been the foundation for the successful development of the then young biotechnology industry. Some of the originally small Biotech start-ups, such as Genentech, Amgen, or Biogen, have grown into large pharmaceutical companies over the last three decades and today they represent a significant portion of the pharmaceutical industry.

Besides the safety aspect, the recombinant DNA technology provides further advantages. Most important are: (i) the large-scale production of high amounts of protein with defined and homogeneous quality at lower costs; (ii) the development of novel drugs, directed at new targets, which could not be isolated in sufficient amounts and qualities from natural sources; and (iii) creating protein variants, muteins or fusion proteins, with properties that are actually improved relative to the natural polypeptides. Today it would be a very difficult approach to apply for a market authorization of a natural protein, derived from living organisms or organs, in view of existing recombinant versions, the biological sources of which are regarded as less inferior by the regulatory authorities.

Considering all these aspects, one can easily understand that the product portfolios of biogeneric companies exclusively consist of recombinant proteins. Therefore, this overview is restricted to recombinant therapeutic proteins, including monoclonal antibodies and PEGylated proteins, and thus excludes natural proteins and synthetic peptides.

13.3 Definition of Biosimilars

The previously used term "biogenerics" was not appropriate and nor was it accurate in the eyes of regulatory authorities, because these types of products differ from classical generics in several aspects. The directives of the European Parliament and of the Council and the guidelines of the Committee for Medicinal Products for Human Use (CHMP) refer to "biological medicinal products similar to a reference product" or "similar biological medicinal products" (Table 13.1). Colloquially, these phrases are reduced to "biosimilars." Hence the definition is simple: a biosimilar is a product that has been approved by the appropriate

Table 13.1 Relevant CHMP/CPMP guidelines for the development of biosimilars.

Guideline ID	Content	Title	Status
EMEA/CPMP/BWP/3207/0/Rev1	General, quality	Comparability of Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance – Quality Issues	In force 12/2003
EMEA/CPMP/BWP/3097/02/Final	General, non-clinical, clinical	Guideline on Comparability of Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance – Non-clinical and Clinical Issues	In force 04/2004
CPMP/ICH/5721/03 ICH Topic 5QE	General, quality	Step 4 Note for Guidance on Biotechnological/Biological Products Subject to Changes in their Manufacturing Process	In force 06/2005
CHMP/437/04	General	Similar Biological Medicinal Products	In force 10/2005
EMEA/CHMP/49348/05	General, quality	Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance – Quality Issues	In force 06/2006
CHMP/42832/05	General, non-clinical, clinical	Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance – Non-clinical and Clinical Issues	In force 06/2006
CHMP/32775/05	Insulin	Annex to CHMP/42832/05: Guidance on Similar Medicinal Products Containing Recombinant Human Insulin	In force 06/2006
CHMP/94528/05	Somatropin	Annex to CHMP/42832/05: Guidance on Similar Medicinal Products Containing Somatropin	In force 06/2006
CHMP/31329/05	G-CSF	Annex to CHMP/42832/05: Guidance on Similar Medicinal Products Containing Recombinant G-CSF	In force 06/2006
CHMP/94526/05	EPO	Annex to CHMP/42832/05: Guidance on Similar Medicinal Products Containing Recombinant Erythropoietins	In force 06/2006

CHMP/BMWP/101695/06	General, non-clinical, clinical	Comparability of Biotechnology-Derived Medicinal Products after a Change in the Manufacturing Process – Non-clinical and Clinical Issues	In force 11/2007
CHMP/BMWP/14327/06	General, immunogenicity	Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins	In force 04/2008
CHMP/BMWP/102046/06	IFN alpha	Non-Clinical and Clinical Development of Similar Medicinal Products Containing Recombinant Interferon Alpha	In force 10/2009
CHMP/BMWP/118264/07	LMW Heparins	Similar Biological Medicinal Products Containing Low-Molecular-Weight-Heparins	In force 10/2009
EMEA/CHMP/BMWP/301636/08	EPO	Similar biological medicinal products containing recombinant Erythropoietins	In force 09/2010
CHMP/BMWP/7241/2006	IFN alpha	Annex to CHMP/42832/05: Similar Medicinal Products Containing Recombinant Interferon Alpha	Concept paper Consultation 04/2006
CHMP/BMWP/496286/06	LMW Heparins	Similar Biological Medicinal Products Containing Low Molecular Weight Heparins – Clinical and Non-clinical Issues	Concept paper Consultation 01/2007
EMEA/CHMP/114720/2009	Monoclonal abs, immunogenicity	Immunogenicity Assessment of Monoclonal Antibodies Intended for *In Vivo* Clinical Use	Concept paper Consultation 03/2009
CHMP/BMWP/632613/09	Monoclonal abs	Development of a guideline on similar biological medicinal products containing monoclonal antibodies	Concept paper Consultation 10/2009
CHMP/BMWP/94899/10	FSH	Similar biological medicinal products containing recombinant follicle-stimulating hormone	Concept paper Consultation 03/2010
CHMP/BMWP/86572/10	IFN beta	Similar biological medicinal products containing recombinant interferon beta	Concept paper Consultation 03/2010

authority, which in Europe is the European Medicines Agency (previously EMEA, and since December 2009 EMA), as a similar biological medicinal product in compliance with the respective guidelines.

However, it may be helpful to give some further descriptions of the attributes of biosimilars and their differences from chemical generics. At least three aspects seem uniform and helpful for a general definition of generics. Generics are pharmaceuticals that: (i) appear after patent expiry; (ii) are sold with a price reduction compared with the original product; and (iii) which have an active substance essentially similar to that in the originator. Since chemical generics have a well established and considerably simplified route of approval, this regulatory feature contributes substantially to their definition. Classical generics are typically approved on the basis of an abridged filing dossier, which makes use of the possibility of referring to the originator's dossier of approval. The applicant proves essential similarity utilizing comparative analytical data and shows bioequivalence in a small pharmacokinetic study, usually performed with healthy volunteers. It is also common that generics are sold under an international non-proprietary name (INN) often without any product-specific promotional activities. Branded generics, however, are exceptions and they have a significant market share, especially in Europe.

What is accomplished for biosimilars? The aforementioned general three items, patent expiry, lower price, and essential similarity are fulfilled except for the last, although the price differences from the proprietor products are more moderate compared with chemical generics, where substantial price erosion can occur within a few months after the end of patent protection. The "essential similarity" is not required for biosimilars, instead they have to be "biosimilar." What this means will be elucidated in the Section 13.4.2. Briefly, in contrast to classical generics, toxicity studies in animals and clinical studies proving efficacy and safety are mandatory. For this type of product, own brands and specific promotions are more frequent than for the classical generics.

In summary, biosimilars are best defined at the regulatory level. They are copies of therapeutic proteins, launched after patent expiry of the active pharmaceutical ingredient, and sold with a moderate price reduction. They have to be approved via the route of the centralized procedure in Europe in compliance with the existing guidelines and require a complete dossier, including a comparability exercise on quality, safety, and efficacy to demonstrate biosimilarity to a reference product.

13.4 Regulatory Situation

13.4.1 Basic Principles

To understand the position of the regulatory authorities it is necessary to consider the specific differences between low-molecular weight substances and complex macromolecules such as polypeptides. In contrast to synthetic small molecules,

large proteins, and especially glycoproteins, have a complex tertiary structure, which is sensitive to modifications in solution. Slight conformational changes may reduce efficacy and/or lead to an increase in immunogenicity. Degradation or oxidation of amino acid residues are examples of such undesired alterations, which can occur during the process or later on during the shelf-life of the product. Aggregation, often favored by oxidation, can be a critical parameter during the production process and for the storage of bulk substance or final product. Sophisticated formulation is necessary to keep the protein stable in solution as a monomer. Aggregation is correlated with an increase in immunogenicity. It is known that immunogenicity can cause severe clinical consequences up to life-threatening complications.

Unfortunately, the analytical methods established today, cannot fully predict the biological and clinical properties of a protein and cannot establish whether the structures of two biopharmaceuticals are completely identical [9]. Another panel of arguments against a pure generic and essential similarity approach results from the specific impurity profile related to process and biological sources. This is the reason for the link of the Market Authorization Application (MAA) to the product, process, and site of production, which builds an inseparable package for the approval. Changing the process or transfer to another production site would require a new registration for a process variation. With the help of batch record data a comparability approach is possible in such cases and the guideline released in 2001 on comparability, which was updated in 2003 [10], is appropriate. Undoubtedly, for a biosimilar product the companies have to apply new manufacturing processes and have neither access to the innovator's methods nor to reference material from intermediate steps. Thus, an exercise for comparability is difficult and has to be limited to highly purified product.

13.4.2 European Guidelines

It must be emphasized that the first biosimilar developments entered clinical stages in the absence of existing European guidelines. Therefore, the respective sponsor companies followed different strategies for seeking regulatory approval. Usually an agency will not draft product-specific guidelines before they are approached by applicants with the respective questions. Suchlike questions are addressed to the national agencies at interested party meetings or, by the more formal and expensive scientific advice procedure, to the CHMP directly. Biosimilar-specific questions have been addressed to the agencies at least since 2001. It was of crucial importance for the biogeneric industry how the EMA would set the extent of preclinical and clinical testing for the targeted products, because this has a strong impact on the time lines and overall costs. However, according to the scientific arguments raised above, a substantially abbreviated clinical program for biosimilars, in accordance with the classical generic route for small molecules, was not expected.

In 2004 a new perspective for the requirements of a biosimilar approval was provided with the EU Directive 2004/27/EC [11], which is an amendment to the

basic Directive 2001/83/EC. This directive was a precursor for a new comprehensive legal framework to establish the regulatory pathway for biosimilar products in Europe. It introduced new terminology in terms of "biological medicinal products similar to a reference medicinal product" and gave initial guidance for the use of a reference product and the need for appropriate preclinical tests and clinical trials for safety and efficacy. References to results of the original product are not permitted. Thus, in contrast to classical generics, biosimilars require a full dossier.

In 2005–2009 further guidelines came into force, have been adopted or are still in consultation to date. Table 13.1 summarizes the relevant guidelines for biosimilars, which one can obtain from the homepage of the EMA [12].

The biosimilar legislation is structured into two levels. The upper level consists of general guidelines starting with those for "Similar Biological Medicinal Products," which came into force in October 2005, followed by general guidance on quality issues and non-clinical/clinical issues in June 2006. Further general guidance is provided by the comparability guidelines and by an immunogenicity guideline, which came in force in April 2008. The second level is more downstream and product-specific. An initial panel of four annexes to the general biosimilar guideline ruled on the products insulin, somatotropin (hGH), erythrpopoietin (EPO), and granulocyte-colony stimulating factor (G-CSF) and came into force in June 2006. Further product-specific biosimilar guidelines came in force in October 2009: interferon (IFN) alpha and low-molecular weight (LMW) heparin. In addition there are three concept papers still under consultation for follicle-stimulating hormone (FSH), IFN beta, and monoclonal antibodies (mAb). It should be mentioned that the relevant guidelines in Table 13.1 make multiple references to further guidelines. In addition, biosimilars, like other biological products, have to comply with the general ruling for biotechnology-derived products provided by the ICH guidelines and other CHMP guidelines.

Within the scope of this chapter it is not possible to exhaust the details of all these guidelines. Nevertheless, I will summarize briefly the most important practical consequences and add some personal views on the development of biosimilars in the following paragraphs.

The biosimilar pathway is scientifically based and focused on the safety of the patients. The agency gave a clear refusal to the essential similarity approach of generics. Following the guidance on comparability for biotechnology-derived proteins, the biosimilarity has to be demonstrated on the level of comparability exercises for the chemistry, manufacturing, and controls (CMC) section throughout the development and for both levels, drug substance and drug product. A full quality dossier (module 3) is required. The applicant has to select one reference product which is authorized in the European Union. A centralized approval for the reference product is not mandatory. The amino acid sequence has to be identical for both the biosimilar and the reference product. The expression host has to be of the same category, whenever possible (*E. coli*, yeast, CHO, etc.) to avoid an extended non-clinical and clinical program. Post-translational modifications, for example, glycosylation, is not expected to be identical, nevertheless any differences have to be investigated regarding a possible risk to efficacy and safety.

The same holds true for the impurity profile. As for generics, the pharmaceutical formulation can differ from the reference product. Toxicity studies and immunogenicity studies in animals have to be performed in comparison to the reference product. Fewer animal studies are needed for the biosimilar route than for a stand-alone approach. For example, instead of the normal two, only one animal species is sufficient. Moreover, studies on safety pharmacology, reproduction toxicology, mutagenicity, and carcinogenesis are waived. Key elements are the comparative pharmacokinetic (PK) and pharmacodynamic (PD) studies (phase I) in humans to demonstrate clinical comparability or bioequivalence, respectively. In general, one selected dose is sufficient. Not surprisingly, the classical dose finding studies (phase II) are waived. These are not necessary at all for a product claimed to be biosimilar. However, comparative efficacy and safety studies (phase III), to demonstrate equivalence, are normally indispensable. One aspect for the pivotal study is to demonstrate "the same effect at the same dose." In general, the biosimilar has to apply the same pharmaceutical form, dose-strength, and route of administration as the reference product. Studies in one key-indication are sufficient and in the best case the approval can then be extended to additional indications of the reference product by data extrapolation without the need of further clinical studies.

It has to be emphasized, that all applications will be handled case-by-case by the authority and the more differences in the CMC part that occur the higher will be the risk for additional demands in the non-clinical and clinical sections. The CHMP has to be convinced by state-of-the-art analytical methods of high sensitivity and quality. The same holds true for stability data.

Finally, the guidance unequivocally weights the aspect of immunogenicity, and the applicants are advised to develop appropriate sensitive assays to detect anti-drug antibodies and also neutralizing antibodies. The assessment on immunogenicity may significantly prolong the clinical studies as late follow-up samples from the patients are required. Moreover, because on the one hand the immunogenicity is not predictable from the analytical data, and on the other hand the safety database in terms of total patient numbers of the clinical studies, usually several hundred, is too small to allow statistical assessments on immunogenicity, the applicants are obliged to make post-marketing studies. The extent and duration of the risk management programs will be decided case-by-case. This is not a specific requirement for biosimilars but a general obligation for all therapeutic proteins.

What changes can be expected in the near future? First, the already drafted concept papers (Table 13.1) will be finalized and will come into force and further new product-specific guidelines will be issued. In particular, the guidelines for recombinant antibodies are long-awaited and several specific aspects have to be clarified (see Section 13.7.2). In view of the successful introduction of the first wave of high-quality biosimilars, which even exceeded the quality of the reference products in some cases, stipulations have been raised to drop the obligations of the comparability exercises in order to make it easier to develop more complex biosimilars, such as monoclonal antibodies or vaccines [13]. The

pressure for easing the regulatory requirements in the near future will increase with more biosimilars coming to market and with more successfully closed pharmacovigilance investigations. This will produce further evidence that biosimilars are safe and effective drugs, showing in the long term similar profiles of adverse reactions, including the immunogenicity, as known for the reference products.

13.4.3 Regulatory Situation in the United States

In the United States, until recently the term "follow-on biologics" (FOBs) was used more frequently than biosimilar wording. Although a legal framework became effective in 2010 (see below) there is still no practical guidance for obtaining a biosimilar-type of approval for a biotech drug in the US. Historically there are two different legal frameworks for approval of pharmaceuticals effective in the United States: (i) the Federal Food, Drug and Cosmetic (FD&C) Act and (ii) the Public Health Service (PHS) Act.

Small-molecule drugs, but also some less complex proteins such as insulin or somatotropin, are licensed under the FD&C Act, whereas biologics, including most proteins, are regulated by the PHS Act [14]. Small-molecule generics are approved according the FD&C Act, Section 505(j) for an "Abbreviated New Drug Application" (ANDA) known as the "Hatch–Waxman Act of 1984." This route of approval does not apply to biologics. In contrast, new small molecule drugs are approved according to Section 505(b)(1) for "New Drug Application" (NDA). There are a few exceptions of simple, non-glycosylated protein drugs, such as calcitonin, glucagon, hyaluronidase, and somatropin, which have been approved under the FDC&C Act by using Section 505(b)(2) of the NDA route. Finally, most biologics apply for a "Biological License Application" (BLA) under the PHS Act, which until recently did not contain provisions for abbreviated applications such as in the FD&C Act with the ANDA pathway.

Interestingly, despite the lack of an entry pathway for FOBs in the United States, Sandoz' biosimilar somatotropin, Omnitrope® (see Section 13.6.2), was approved by the FDA through section 505(b)(2) of the NDA route. This was enabled by the fact that the reference product Genotropin® was approved according to section 505(b)(1) of the same act. To date, Omnitrope® is the only biosimilar product to receive dual approval, by the EMA and the FDA. Thus, for drugs more complex than somatotropin, there was no legal framework in the United States to approve FOBs and there was an urgent demand to fill the gap with the respective legislation, as has been established in Europe.

The political influence of the different lobbies, including citizen petitions, put pressure on for the emergence of a clear regulatory outline for FOBs. The economic pressure is high. In 2008, the Congressional Budget Office estimated that enactment of biosimilars legislation would save costs of US$25 billion over the 2009–2018 period [14]. The FDA recognized the need to implement new regulations for the abbreviated approvals of FOBs several years ago. Scientific meetings

and workshops were held in 2004 and 2005 [14]. A rather long period of political debate followed and to make the story short, the current status is best summarized as follows.

On 23 March 2010 President Obama signed the Health Reform Bill titled "Patient Protection and Affordable Act." This bill included the "Biologics Price Competition and Innovation Act of 2009" (Section 7002 "The Biosimilar Act"), which amended the PHS Act.

What does this act contain? The Biosimilar Act provides an approval pathway for biosimilar and interchangeable biological products, allows the submission of a BLA for such products, and gives some general principles and definitions. It also gives the legal permission for the specific guidance documents to be issued by the FDA, the patent resolution procedures, the data exclusivities, and the pediatric extensions. In some aspects this legislation followed the European one. However, it is obvious, that besides the European biosimilar terminology it contains a second feature "Interchangeable Biological Products." Biosimilar products are defined as "highly similar to the reference product notwithstanding minor differences in clinically inactive components" and for which "there are no clinically meaningful differences between the biological product and the reference product in terms of safety, purity, and potency of the product," whereas interchangeable biological product means a biological that "may be substituted for the reference product without the intervention of the health care provider who prescribed the reference product" [15].

One could believe that these two definitions supplement each other and there is no real difference from the European guidelines, where interchangeability, although not literally mentioned, is a logical consequence of the clinical and other requirements. However, this is not the case. Interchangeability (with the reference product) is an additional claim of biosimilars and the FDA might request extra clinical data for approval. It remains to be seen what will be written in the FDA guidelines and if harmonization will be possible between the EMA and FDA requirements. There seems to be an agreement at the two cornerstones, the need for clinical data and the comparability on quality, safety, and potency/efficacy. A legal basis for approval of biosimilars in the United States exists now, a compromise between the two political parties, but the practical procedures and the data requirements have to be developed by the FDA as soon as possible. According to the European experience, this can take rather a long time. Until then, an uncertain situation exists for biosimilar applicants and it is not clear how the FDA will handle submissions in this interim period.

Finally, some other legal aspects of the biosimilar act should be mentioned briefly. For more details refer to recent publications [15, 16]. The patent resolution procedures appear rather complicated and cannot be discussed within the scope of this chapter. I want to emphasize, that biosimilars in contrast to generics do not have to deal with an Orange Book. However, there are analogous patent resolution procedures to the Hatch–Waxman Act (ANDA, "paragraph IV certification"). The complex implications were reviewed recently [15]. The act defines several data exclusivity periods. Submission of a biosimilar product is prohibited until four

years after the reference product was first licensed. Furthermore, a 12-year data exclusivity period is guaranteed to the sponsor, this means a biosimilar will not be approved until 12 years after the reference product was first licensed. Reference products with orphan drug status can be protected for longer. An exclusivity period of seven years after the license of an orphan drug status has to be considered too. This can overrule the 12-year period. All these periods will be prolonged by an additional six months when the BLA holder has to perform pediatric investigations ("pediatric exclusivity"). Finally, the first interchangeable product is also afforded a market exclusivity period with regard to subsequent interchangeable products, which is 12 months after the first commercial marketing or 18 months after approval. In cases of patent disputes with the BLA holder, this exclusivity period can be even longer [15].

13.4.4 Regulation in Other Territories

Europe was the first region in the world with a comprehensive legislative and regulatory pathway for the introduction of biosimilars [13] and served as a standard for many other countries. Following the European model, during recent years many countries have drafted, adopted or issued biosimilar-like guidelines. In addition, the World Health Organization (WHO) drafted a guidance, which contains besides the EU-like biosimilar pathway a second "alternative pathway." This has led to controversial discussions. In the following those countries are listed that have released or drafted legislation on abbreviated licensing pathways for biosimilars. Public information is referred to that was obtained from a perspective of the European Generic Medicines Association (EGA) presented in 2009 [17].

- **Japan:** The final guideline was issued on March 2009. Reference product has to be approved in Japan.
- **Canada:** Health Canada issued finalized guidelines on "Subsequent Entry Biologics" (SEB) in April 2010. An SEB is defined as "a biologic drug that enters the market subsequent to a version previously authorized in Canada and which demonstrated similarity to a reference biologic drug." A reference product not authorized for sale in Canada may be used provided that certain criteria are satisfied.
- **Australia:** The Therapeutic Goods Administration (TGA) adopted the EMA guidelines in August 2008. Omnitrope® was approved in 2005 on the basis of generic regulation.
- **Turkey:** Final guidelines on "Biosimilar Medicinal Products" were issued in 2008 by the General Directorate of Pharmaceuticals and Pharmacy.
- **Near and Middle East:** The consensus group recommended the implementation of the EMA guidelines as the basis of regional guidelines for the registration of "Biosimilars."

- **India:** Final guidelines on "Biotechnological/Biological Products" were issued in July 2008.
- **Taiwan:** Final guidelines on "Biological Products" were issued in November 2008 by the Department of Health.
- **Malaysia:** Final guidelines on "Biosimilars" were issued in July 2008 by the Ministry of Health.
- **Mexico:** Guidelines on "Medicamentos Biotecnológicos" were drafted in October 2008.
- **Brazil:** Final guidelines on "Biological Products" were issued in October 2005.
- **Argentina:** The National Administration of Food, Drug, and Medical Technology (ANMAT) drafted guidelines on "Biological Medicinal Products" in July 2008.
- **Saudi Arabia:** Draft guidelines on "Biosimilars" were issued in August 2008.
- **Venezuela:** Final guidelines on "DNA recombinant products, monoclonal and therapeutic antibodies" had already been issued in August 2000.
- **Columbia:** Guidelines on "Biological Products" were drafted.

13.4.5 Final Remarks

At least in the European Union, United States, and Japan the reference product has to be authorized in the respective country or community. However, different versions of the reference product are usually sold in the European Union, United States, and Japan. Although the drug substances and often also the pharmaceutical compositions and forms are identical, they are legally different. As biosimilars have to be developed for the global market, due to the high investments, companies are faced with this complication when approaching the different authorities. It would be unethical, uneconomical, and scientifically dispensable to perform animal studies and clinical trials against three comparator products. Therefore, the generic industry requests that duplicated preclinical and clinical testing should be waved, for example by accepting the equivalence of the country-specific approved reference products [17].

13.5 Patent Situation

Patents are the most relevant and the most effective means of intellectual property (IP) protection. Obviously, knowledge of relevant patents is of crucial importance for the generic business. The leading companies in this field have established strategies to launch their products immediately after patent expiry. Thus, they have

to be sure, country by country, when corresponding patents will expire. The search of relevant patents, the understanding of the scope, the analysis of the patent family, and examination of the legal status, including supplementary protection certificates (SPC), and other term extensions has to be performed thoroughly.

The patent situation is considerably more complicated in the field of biotechnology than for chemical drugs. Some of the specific problems should be mentioned here. The total number of patents dealing with a specific protein can be incredibly high. Patent searches for potential biogeneric target products can reveal up to 10 000 hits for a given therapeutic protein. The huge number of documents has to be restricted to those few which are really relevant. This requires specific biotech knowledge along with patent know-how. Patent analysis for a generic development is mainly a survey of the past, a historical work-up. If a patent expires in Europe today, it was filed 20 years ago. Although the methods are then free for use, they are in most cases old-fashioned and not suitable for a state-of-the-art production process. Moreover, since patents are rapidly filed in the earliest stage of development the methods described, in general, are of a laboratory scale and far removed from the final biopharmaceutical manufacturing process.

The majority of the relevant biotechnology patents are related to specific methods for production, sometimes even restricted to a specific expression system, so as to appear as broad substance patents. Many patents claim a recombinant protein in combination with modes of production, including DNA vector constructs, expression hosts, and purification methods, or more importantly, together with an application. It is obvious, that for almost all the examples, which are mentioned in Table 13.2, there is no monopoly market situation. Typically, parallel developments using distinct solutions lead to two or more competitor products.

Besides basic patents, which cannot be circumvented and their expiry dates have to be waited for, the originators file follow-on patents, which can manifest as severe hurdles. One example is the pharmaceutical formulation of therapeutic proteins, also a dangerous minefield for classical generics. Numerous patents claim distinct product-specific formulations. These inventions are related to a more advanced development stage and they usually expire several years later. Again it requires specific experience to find a gap within a dense net of formulation patents. Biosimilar products (Table 13.3) with modified formulations that differ from the original products, have appeard on the market. Otherwise the generic competitors have to wait some more years for launch or would undergo the risk of being sued for patent infringement. In addition, there is increasing generic competition in the field of biosimilars. The companies who developed biosimilar-specific pharmaceutical compositions nearly always protect their formulations by filing their own patent applications. The same is true for the processes of biosimilars, which differ from the unknown procedures used for the reference product. Biosimilar manufacturers use state-of-the-art technology, whereas the processes of the original products are often locked in to old technologies, because changes are linked with high costs [13].

Another important category of follow-on patents are applications claiming the therapeutic use of the drug for a medical indication, including methods of use, specific patient groups, dosage regimes, and combinations with other drugs. This

Table 13.2 First wave of targets of biosimilar developments in the European market.

Recombinant human protein	Branded product (EU market)	Originator companies	Launch (EU)	Active pharmaceutical ingredient (INN)	Major indications	Expression system (host organism)	EU status of biosimilars
Erythropoietin (EPO)	Erypo, Eprex	Kirin, Amgen, Johnson & Johnson	1988	Epoetin alfa	Renal anemia (dialysis patients),	CHO	Approved
	NeoRecormon	Genetics Institute, Boehringer Mannheim, Roche	1992	Epoetin beta	CT-induced anemia (oncology)	CHO	Approved
Granulocyte colony-stimulating factor (G-CSF)	Granocyte	Chugai	1993	Lenograstim	CT-induced neutropenia (oncology), Bone marrow transplantation	CHO	None
	Neupogen	Amgen	1991	Filgrastim		*E. coli*	Approved
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Leucomax	Genetics Institute, Schering-Plough, Sandoz, Novartis	1992	Molgramostim	CT-induced neutropenia (oncology),	*E. coli*	None
	Leukine	Immunex, Amgen, Schering AG, Bayer	US only	Sargramostim	Leukemia, lymphoma	*S. cerevisiae*	None
Human growth hormone (hGH)	Genotropin	Genentech, Pharmacia-Upjohn	1988	Somatropin	Growth deficiency in children, growth hormone deficiency in adults (1994)	*E. coli*	Approved
	Humatrope	Eli Lilly	1988	Somatropin		*E. coli*	Approved
	Norditropin	Novo Nordisk	1988	Somatropin		*E. coli*	None
	Zomacton	Ferring	1994	Somatropin		*E. coli*	None
	Saizen	Serono	1989	Somatropin		C127	None

(*Continued*)

Table 13.2 (*Continued*)

Recombinant human protein	Branded product (EU market)	Originator companies	Launch (EU)	Active pharmaceutical ingredient (INN)	Major indications	Expression system (host organism)	EU status of biosimilars
Insulin	Huminsulin	Eli Lilly	1982	Insulin human	Diabetes mellitus	*E. coli*	Withdrawn
	Insuman	Aventis	1999	Insulin human	Diabetes mellitus	*E. coli*	None
	Insulin Actrapid	Novo Nordisk	1991	Insulin human	Diabetes mellitus	*S. cerevisiae*	None
Hepatitis-B virus surface antigen (HbsAg)	Engerix-B	GlaxoSmithKline	1987	HBV surface antigen	Prophylaxis of HBV infection (active immunization)	*S. cerevisiae*	None
	Gen H–B-Vax	Aventis Pasteur, Chiron. Behring	1986			*S. cerevisiae*	None
Factor VIII	Kogenate	Genentech, Bayer	1994	Octocog alfa	Hemophilia A	BHK	None
	Helixate	Genentech, Aventis Behring	1994	Octocog alfa	Hemophilia A	BHK	None
	Recombinate	Genetics Institute, Baxter	1992	Rurioctocog alfa	Hemophilia A	CHO	None
	ReFacto	Pharmacia-Upjohn, Wyeth-Ayerst	1999	Moroctocog alfa	Hemophilia A	CHO	None
Interferon alpha (IFN-α)	Intron A	Biogen, Schering-Plough	1986	IFN-α-2b	Chronic hepatitis B and C	*E. coli*	None
	Roferon A	Genentech, Roche	1989	IFN-α-2a		*E. coli*	Rejected
	Infergen	Amgen, Yamanouchi	1999	IFN alfacon-1		*E. coli*	None
Interferon beta (IFN-β)	Betaferon	Chrion, Schering AG	1995	IFN-β-1b	Multiple sclerosis, relapsing-remitting and secondary progressive forms	*E. coli*	None
	Avonex	Biogen	1997	IFN-β-1a		CHO	Rejected
	Rebif	Serono	1998	IFN-β-1a		CHO	None

Table 13.3 Granted EU market authorizations for biosimilars.

Protein	Biosimilar product	Company	INN Biosimilar	Host	Production	Ref. product [a)]	Approval	Grouping [b)]
hGH	Omnitrope	Sandoz	Somatropin	*E. coli*	Austria	Genotropin	2006	1
	Valtropin	Biopartners	Somatropin	Yeast	South Korea	Humatrope	2006	2
EPO	Epoetin alfa Hexal	Hexal	Epoetin alfa	CHO	Germany	Eprex/Erypo	2007	3
	Binocrit	Sandoz	Epoetin alfa	CHO	Germany	Eprex/Erypo	2007	3
	Abseamed	Medice Pütter	Epoetin alfa	CHO	Germany	Eprex/Erypo	2007	3
	Silapo	Stada	Epoetin zeta	CHO	Germany	Eprex/Erypo	2007	4
	Retacrit	Hospira	Epoetin zeta	CHO	Germany	Eprex/Erypo	2007	4
	Eporatio[c)]	Ratiopharm	Epoetin theta	CHO	Germany	NeoRecormon	2009	5
	Biopoin[c)]	CTArzneimittel	Epoetin theta	CHO	Germany	NeoRecormon	2009	5
G-CSF	Ratiograstim	Ratiopharm	Filgrastim	*E. coli*	Lithuania	Neupogen	2008	6
	Filgrastim Ratiopharm	Ratiopharm	Filgrastim	*E. coli*	Lithuania	Neupogen	2008	6
	Biograstim	CTArzneimittel	Filgrastim	*E. coli*	Lithuania	Neupogen	2008	6
	Tevagrastim	Teva Generics	Filgrastim	*E. coli*	Lithuania	Neupogen	2008	6
	Filgrastim Hexal	Hexal	Filgrastim	*E. coli*	Austria	Neupogen	2009	7
	Zarzio	Sandoz	Filgrastim	*E. coli*	Austria	Neupogen	2009	7
	Nivestim	Hospira	Filgrastim	*E. coli*	Croatia	Neupogen	2010	8

a) For details on reference products see Table 13.1.
b) Group refers to a single development leading to several parallel market authorizations (filing of duplicates).
c) These products have been approved on the basis of a complete and independent application.

category is more relevant to the second wave of biosimilar products. The inventive step of such patents can be very small. The Enlarged Board of Appeal of the European Patent Office (EPO) recently decided that claiming a new dose of a known pharmaceutical in a known indication is patentable [18]. Suchlike indication patents can prevent the market entry of biosimilars for a long time.

In contrast to the first edition of this book I decided to omit the putative patent expiries of the various products within the tables. This is justified by two reasons. Firstly, for the first wave (Table 13.2) all the products are already off-patent in Europe. Secondly, the putative expiries of patent protection of the second-wave products (Tables 13.5 and 13.6) depend on the estimation of scope and enforceability of follow-on patents in connection with their own applied methods. This will lead to individual risk assessments. Moreover, many such follow-on patents are still under prosecution or legal dispute and the outcomes are difficult to predict. However, for readers who are interested in the putative patent expiries, there is the possibility of an estimate. I can recommend a very simple method, which gives, with a precision of ±1 year, a practical approximation to the European patent expiry and which is accurate in more than 80% of cases: Take the year of approval (Tables 13.4 and 13.5) and add 15 years. The rationale behind this simple approach results from the SPC lifetime calculation, which at most grants 15 years exclusivity to a product.

Finally, two important changes in the European IP environment which are of relevance to pharmaceuticals should be mentioned. These are the Euro-Bolar clause and the pediatric extension of SPCs.

The EU legislation on pharmaceuticals included the long-awaited Roche–Bolar provision ("Euro-Bolar") along with the update of the legal framework, which was completed in spring 2004. The so-called Euro-Bolar clause is introduced by Article

Table 13.4 Rejected or withdrawn submissions for EU market authorization of biosimilars.

Protein	Biosimilar product	Company	INN Biosimilar	Ref. product[a)]	Outcome	Grounds	Year
IFN alfa	Alpheon	Biopartners	Interferon alfa-2a	Roferon A	Rejected	CMC part insufficient	2006
IFN beta	Biferonex[b)]	Biopartners	Interferon beta-1a	Avonex/Rebif	Withdrawn	Not comparable	2009
Insulin	Insulin Rapid Marvel	Marvel	Insulin	Humulin	Withdrawn	*Inter alia* not biosimilar	2007
	Insulin Long Marvel	Marvel	Insulin	Humulin	Withdrawn	*Inter alia* not biosimilar	2007
	Insulin 30/70 Marvel	Marvel	Insulin	Humulin	Withdrawn	*Inter alia* not biosimilar	2007

a) For details on reference products see Table 13.2.
b) This product was filed as an independent application and not as a biosimilar.

Table 13.5 Second-wave targets for biosimilar developments: approved PEGylated biopharmaceuticals.

Company	INN	CAS no.	Product	Active component	Mode of PEGylation	PEG per molecule	PEG size	Indications	1st US approval	1st EU approval
Enzon	Pegademase bovine	204565-76-4	Adagen	Bovine adenosin deamidase (ADA)	Random amino-PEGgylation	11–17	5 kD	ADA deficiency leading to severe combined immunodeficiency (SCID)	1990	None
Enzon, Medac	Pegaspargase	130167-69-0	Oncaspar	*E. coli* L - asparagine amidohydrolase	Random amino-PEGgylation	Several	5 kD	Acute lymphoblastic leukemia (ALL)	1994	1997
Enzon, Schering-Plough	Peginterferon alfa-2b	215647-85-1	PegIntron	Interferon alfa-2b	Random amino-PEGgylation	1–2	12 kD	Hepatitis C, monotherapy and in combination with Ribavirin	2000	2000
Roche	Peginterferon alfa-2a	198153-51-4	Pegasys	Interferon alfa-2a	Random amino-PEGgylation	1	44 kD branched	Hepatitis C, mono and combination therapy with Ribavirin, Hepatitis B	2002	2002
Amgen	Pegfilgrastim	208265-93-3	Neulasta	rMet(hu)-G-CSF (filgrastim)	Site-specific N-terminal amino-PEGylation	1	20 kD	Prophylaxis of neutropenia in cancer chemotherapy	2002	2002

(*Continued*)

Table 13.5 (*Continued*)

Company	INN	CAS no.	Product	Active component	Mode of PEGylation	PEG per molecule	PEG size	Indications	1st US approval	1st EU approval
Pfizer	Pegvisomant	218620-50-9	Somavert	Growth hormone recpetor antagonist	Random amino-PEGylation	4–6	5 kD	Acromegaly	2003	2002
Enzon, OSI, Pfizer	Pegaptanib	222716-86-1	Macugen Macuverse	RNA oligonucleotide (28mer, anti-VEGF)	Site-specific phosphodiester PEGylation using aminolinker	1	40 kD branched	Age-related macular degeneration	2004	2006
Roche	Pegepoetin-beta	677324-53-7	Mircera	Erythropoietin (epoetin-beta)	Random amino-PEGylation	1	30 kD	Renal anemia	2007	2007
Enzon, UCB (Celltech)	Certolizumab pegol[a)]	428863-50-7	Cimzia	Humanized, Fab (anti-TNF alfa)	Site-specific sulfhydryl-PEGylation	1	40 kD branched	Rheumatoid arthritis	2009	2009

a) For more details see Table 13.6.

10.6 of Directive 2004/27/EC [11] and refers to both generics and biosimilars. Meanwhile the directive has been adopted by the national legislations of the member states and by Switzerland as well. What does the Bolar provision mean? The term "Roche–Bolar" arose from a legal procedure held 1984 in United States, *Roche Products Inc. v. Bolar Pharmaceutical Co., Inc.* [19]. Briefly, a Bolar provision allows all development, testing, and experimental work required for the registration of a generic medicine to take place during the patent period of the original product. The purpose of such a provision is to ensure that generic medicines are on the market immediately after patent expiry. Bolar-type provision exists in United States, as a part of the Hatch–Waxman Act, Canada, Japan, Australia, Israel, and many other countries. The European-Bolar provision was a breakthrough for the generic industry in Europe.

The pediatric extension goes back to a regulation of the European Parliament and of the Council of December 2006 [20]. The ruling is thought of as an incentive to perform studies in children. If an application includes the results of all studies conducted with an agreed pediatric investigation plan, the SPC shall be extended by six months. The same term extension will be rewarded in the United States under the Biosimilar Act (see Section 13.4.3).

13.6 First Wave of Biosimilars in the EU

13.6.1 General Remarks

All information listed in the tables were collected from the following sources: the published European Product Assessment Reports (EPAR) [21], the online version of the German pharmaceutical compendium "Rote Liste®" [22], the Canadian database "DrugBank" [23], and Wikipedia (wikipedia.org and wikipedia.de). All product names in this chapter are designated as registered trademarks (®), without verifying whether these trademarks are registered or not.

Table 13.2 summarizes potential target proteins for a first wave of biosimilar products and was based originally on expectations in the year 2003. It must be emphasized that at that time this was a personal selection. Owing to the high costs for the development of a biosimilar, only top-selling products are considered by companies. Meanwhile, all the protein drugs of Table 13.2 have run-off patent protection. The last column of Table 13.2 indicates if the EMA has so far been approached with a biosimilar submission.

Except for three categories of products, granulocyte-macrophage colony-stimulating factor (GM-CSF), coagulation factor VIII (FVIII), and hepatitis-B virus surface antigen (HBsAg), all the other categories are represented by at least one biosimilar candidate. The lesser attractiveness of these three drugs is explained readily. GM-CSF is a second choice compared with the more successful granulocyte colony-stimulating factor (G-CSF). Both CFSs have a similar potential in

cancer, however G-CSF, especially regarding the product Neupogen®, has a larger market volume. Therefore, the European generic companies preferred G-CSF. On the other hand, FVIII is a large-sized and complex structured protein and requires special experience in manufacturing and in the highly competitive market as well. Obviously, FVIII was not a first choice candidate for a portfolio of biosimilars. Likewise, some other first-wave candidates are not attractive due to difficult clinical development, for example the plasminogen activators. Finally, the biosimilar players concentrated exclusively on therapeutic proteins and seemed to have little interest in prophylaxis as provided with the subunit HBsAg vaccine for hepatitis B. Along with the highly attractive opportunities offered by the second wave of biosimilar target products, it is questionable, if biosimilar products for GM-CSF, FVIII, and HBsAg will appear within this decade.

In the following, the EU-approved biosimilar products will be presented (Table 13.3). In addition, products that were initially rejected or withdrawn (Table 13.4) will also be discussed.

13.6.2 Human Growth Hormone (hGH)

Recombinant hGHs are well established products, having been launched in Europe in 1988. The market is highly competitive, as five different non-generic products are available for a limited number of patients (see Table 13.2). The spectrum of indications of hGH ranges from growth deficiency in children, caused by the lack of or insufficient endogenous production of the hormone, to adolescents and adult patients having secondary weight losses or muscular atrophy, with or without hGH deficiencies. All five available non-generic recombinant growth hormones, four expressed in *E. coli*, one in murine cells (Saizen®), have an identical amino acid sequence and are not glycosylated. These products have the same INN, somatropin, and the same clinical efficacy. This is a good example of where independent developments led to very similar products.

Two biosimilar hGHs were approved in April 2006 by the EMA (Table 13.3). Omnitrope®, marketed by Sandoz, was compared to Genotropin® as the reference product, whereas Valtropin®, marketed by Biopartners, was proved to be biosimilar to Humatrope®. It should be mentioned that the applications for both biosimilar products were submitted before the biosimilar guidelines were adopted at the EMA. Moreover, both approvals were granted in April 2006, Omnitrope® at first followed two weeks later by Valtropin®, shortly before the specific biosimilar guidelines came in force in June 2006 (Table 13.1). It is worth mentioning that Omnitrope® had been submitted in May 2001 to the EMA. However, the approval was initially denied by the European Commission, despite a positive opinion by the CHMP because neither a legal basis nor a scientific concept existed at that time. The approval was then granted on a resubmission with additional data [24]. Shortly after its EU approval, Omnitrope® was also approved in the United States in May 2006, according Section 505(b)(2) (the NDA pathway) of the FD&C Act (see Section 13.4.3). The use of the NDA route is in agreement

with other hGH products, including Genotropin®. This means Omnitrope® was treated as a non-generic in the US and is formally not a biosimilar and interchangeable product.

A second biosimilar hGH, Valtropin®, was developed and is manufactured in South Korea by LG Life Science. In contrast to all other somatropins Valtropin® is produced with the yeast *Saccharomyces cerivisiae*, despite the use of *E. coli* for production of the reference product Humatrope®. This makes the Valtropin® process unique among growth hormones [25]. The difference in type of host cells between biosimilar and reference product in the case of Valtropin® might provide a basis for using other expression systems than those of the reference products. However, for proteins more complex than hGH this could be a risky approach. The appearance of these first two biosimilars in 2006 was recognized as significant in the industrial and medical community, and frequently reviewed in connection with general issues on biosimilars. For more details on the development programs in particular and the approval of Omnitrope® and Valtropin® refer to recent reviews [24–28].

13.6.3 Erythropoietin (EPO)

Erythropoietin is clinically applied to treat anemia in patients with chronic renal failure and in cancer patients receiving cytostatic chemotherapy or radiation. These are the two main indications. EPO was exceptionally attractive for the generic industry. It shows the highest market volume among the first wave therapeutic proteins and is one of the top blockbusters. In 2006 global sales reached US$12 billion [28]. Most biogeneric companies considered EPO for their product portfolio.

Until the first biosimilar EPOs appeared (Table 13.3), three products dominated the European market: (i) Eprex®/Erypo® (epotin alfa) from Johnson & Johnson, (ii) NeoRecormon® (epoetin beta) from Roche, and (iii) Aranesp® (darbepoetin alfa) from Amgen. The former product is marketed in Europe by Janssen-Cilag and is available in two brands – Eprex® and Erypo®. The latter, Amgen's Aranesp®, is a second-generation product, based on a mutein with prolonged bioavailability. This was achieved by introduction of additional glycosylation sites. Aranesp® was not in the current focus of first wave biogeneric developers and will not be outlined in this chapter.

A fourth EPO, Dynepo® (epoetin delta), which originates from a cooperation of Sanofi-Aventis with Transkaryotic Therapies (TKT), passed into the ownership of Shire Pharmaceuticals through the acquisition of TKT in 2005. It had been approved in 2002. However, Shire did not launch the product until 2007 and surprisingly started sales with a prominent markdown of 30% over the average price. Two years later, in 2009, Shire voluntarily withdrew the marketing authorization for Dynepo® for commercial reasons [21]. The commercial failure might have been influenced by the immediate appearance of the EPO biosimilars from 2007 onwards, but problems in maintaining cost-effective manufacturing were also

discussed. Additionally, a fifth innovative EPO appeared in the market in 2007, Mircera® (pegepoetin beta), owned by Roche. This product is PEGylated and will be discussed later (see Section 13.7.1).

The first EPO biosimilar was approved in August 2007. The EPO product (epoetin alfa) was developed by Hexal and came into the possession of Sandoz along with the acquisition of Hexal in 2005. The manufacturer is Rentschler Biotechnology, Germany. Three authorizations have been granted, Epoetin alfa Hexal® to Hexal, Binocrit® to Sandoz, and Abseamed® to Medice Pütter (Table 13.3). The duplication or multiplication of market authorizations reflects comarketing by several companies, is a typical strategy to obtain a better market penetration, and applied not just for the sales of biosimilars. Four months later, in December 2007, the EPO product (epoetin zeta) of Bioceuticals Arzneimittel AG, a subsidiary of Stada Arzneimittel AG, received grants of two EU market authorizations, one for Stada Arzneimittel AG (Silapo®) and the other one for the licensee Hosipira Enterprises B.V. (Retacrit®) (Table 13.3). The products are manufactured by Norbitec, Germany. The CHMP concluded that each product, epoetin zeta and epoetin alfa (Hexal), have been shown to have comparable quality, safety, and efficacy profiles to Eprex®/Erypo®. Based on the review of data on quality, safety, and efficacy the CHMP considered by consensus that the risk–benefit balance was favorable [21]. An independent analysis of the physicochemical characteristics of both biosimilar epoetins found the quality of these biosimilars to exceed the original product [13].

Of note, in 2007 five EPO biosimilar products entered the EuropeanUnion market. Considering also Dynepo® and Mircera®, the EPO market was flooded with seven new products within one year. One can imagine that this strongly increased competition and had a severe impact on price levels. Finally, in 2009 the EPO product (epoetin theta) developed by BioGeneriX AG, a subsidiary of Ratiopharm, was approved and two market authorizations were granted for Ratiopharm (Eporatio®) and for CT Arzneimittel (Biopoin®), respectively (Table 13.3). The product is manufactured by Merckle Biotech, another subsidiary of Ratiopharm.

It must be emphasized, that epoetin theta was approved on the basis of a full and independent application and strictly speaking is not a biosimilar. Moreover, BioGeneriX used NeoRecormon® (epoetin beta) as reference product, whereas Bioceuticals and Hexal both used Eprex®/Erypo® (epoetin alfa). The stand-alone approach of BioGeneriX was reflected by a rather high number of clinical studies and patients. In the pivotal phase III studies, 1428 patients were investigated [21] compared with 1183 for Silapo® and 593 for Binocrit® [28]. In clinical studies, epoetin theta seems to be as well tolerated as epoetin beta by both i.v. and s.c. routes. Overall the safety profile of epoetin theta is consistent with the known safety profile of epoetins. There were no new or unexpected findings [21]. The efficacy in pre-dialysis patients was reported to be similar as well [29]. Since epoetin theta will behave like a biosimilar, in the market it is justified to add this product to the genuine biosimilars in Table 13.3.

In summary, the three big generic companies in Germany, Ratiopharm, Hexal, and Stada, sequentially launched their follow-on EPOs between 2007 and 2009. In

total seven different brands have been approved, which are each marketed by different companies. It is worth mentioning that all these products are manufactured in Germany (Table 13.3).

The field of EPO biosimilars is a good example for discussing the policy of assignment of the generic name (INN), which was debated in connection with the approval of the first biosimilars [28]. The innovative biotech industry argued strongly for biosimilar-specific INNs. Hexal achieved the INN of the reference product, epoetin alfa, whereas Stada and Ratiopharm decided to file product-specific new INNs, epoetin zeta and epoetin theta, respectively. Obviously this is inconsistent. One reason is that the INN assignments are directed by the World Health Organization (WHO), independent of regulatory processes [14, 28].

As a first step in the process of obtaining a new INN through the WHO, the applicant has to request a registry number from the American Chemical Society (CAS number), and to submit the molecular and structural data of his protein. Owing to its diversified glycosyl structures, EPO is not a homogenous product consisting of one single molecule. EPO products marketed today contain a very complex mixture of many different isoforms, which differ in their glycosylation. The capability for sufficient glycosylation is provided by the host cell. In the case of EPO, these are CHO cells.

However, upstream and downstream production procedures have a considerable influence on the final isoform composition. During purification, the isoforms have to be at first separated and then selectively pooled to match the desired specifications. This is necessary in order to comply with the relatively high demands on molar sialic acid content and the *in vivo* activity in the bioassay. The various epoetins differ in the quantitative composition of these isoforms rather than showing significant qualitative differences. Even the analytical and biological differences between the reference products epoetin alfa and beta seem not of clinical relevance [30]. Nevertheless, a manufacturer of an EPO usually applies for its own INN, because differences in the gylcosylation pattern cannot be avoided. In the field of EPO this is accepted. With the exception of Hexal's product, all other EPOs attach different Greek letters behind the term "epoetin" thus producing specific INNs. It should be mentioned that such inconsistent INN policy also applies for other glycoprotein categories. For instance, the interferon beta (IFN-β) products Avonex® and Rebif® have a common INN (IFN-β-1a) without being biosimilar (Table 13.2). On the other hand, for non-glycosylated proteins such as hGH or human insulin, the same INN for all products is commonly used, provided that the amino acid sequence is identical.

Extended post-marketing studies were imposed on the EPO biosimilars. The risk management plans include the various proposed pharmacovigilance activities and are summarized in the respective EPARs [21]. The CHMP is concerned particularly with the problem of immunogenicity.

In the field of EPO the most significant risk is associated with the so-called "Pure Red Cell Aplasia" (PRCA) syndrome. Since 1999 there has been growing concern about case reports of PRCA observed in patients with chronic renal anemia treated with Eprex® (epoetin alfa). Neutralizing antibodies induced by

EPO therapy cross-neutralize natural endogenous EPO leading to severe transfusion-dependent anemia. Between 1999 and 2004 the number of reported PRCA cases increased from 12 to 191 patients worldwide [31].

There were two coincidences for the emergence of PRCA: (i) with the reformulation of Eprex®, mainly the withdrawal of albumin under pressure of the CHMP, and (ii) with the shift from intravenous to subcutaneous administration. Procrit®, the US version of Eprex®, is still formulated with human albumin and seems not to be correlated with PRCA. Detailed investigations identified that the causes are with the pharmaceutical components. The formulation of Eprex® and the primary packaging material were both suspected to be in causal connection with PRCA. The use of the detergent polysorbate 80, which was introduced instead of albumin, may have increased the immunogenicity of Eprex® by eliciting the formation of EPO-containing micelles.

Another explanation is related to the use of uncoated rubber stoppers in the pre-filled syringes. Nowadays synthetic rubber materials, such as chlorobutyl or bromobutyl rubbers, when in direct contact with the protein solution are coated, that is, Teflonized. This prevents leaching of undesired compounds out of the rubber material. Such leachables were detected in Eprex® syringes having uncoated rubber stoppers and were shown to have adjuvant immunogenicity effects in mice, that is, induced PRCA in the animals. With the use of coated stoppers for Eprex® the incidence of PRCA decreased from 3.4 to 0.2 per 10 000 patients a year [31]. Since the biosimilar manufacturers have developed their own formulations and usually purchase different types of syringes than those applied for the reference products, a post-approval, long-term surveillance on immunogenicity and other safety data over several thousands of patients seems to be justified. This strategy is a cornerstone of the biosimilar guidelines and keeps the patient numbers in the pivotal comparability studies on a much lower level. Obviously, the occurrence of the PRCA syndrome had an impact on the biosimilar guidelines. On the other hand, the companies have learned from the PCRA phenomenon and avoid the use of uncoated stoppers. No increased PRCA incidences or other safety issues have been reported for the biosimilar EPOs in Europe after more than two years of sales.

13.6.4 Granulocyte-Colony Stimulating Factor (G-CSF)

The endogenous growth factor G-CSF regulates the proliferation and differentiation of progenitor cells within the bone marrow and the release of mature neutrophilic granulocytes (neutrophils) into the peripheral blood. Cancer chemotherapy, which affects rapidly dividing cells, frequently leads to a side effect termed "neutropenia." Neutropenia is a decrease in counts of neutrophils in the peripheral blood and affects more than one in three patients receiving chemotherapy for cancer. Patients driven into neutropenia can develop fever and are prone to infections. Life-threatening gastrointestinal and pulmonary infections occur, as does sepsis.

Recombinant human G-CSF is an effective pharmaceutical substance and has been successfully applied to treat chemotherapy-induced neutropenia. It restores the number of neutrophils in the blood and keeps it above the critical level [24]. The dominant product on the market is Amgen's Neupogen® containing filgrastim, an *E. coli*-expressed, recombinant human methionyl-G-CSF. The second G-CSF product, Chugai's Granocyte®, is derived from recombinant CHO cells, free of the artificial *N*-terminal methionine, and glycosylated. INN is lenograstim. With the exception of France, this product has only little market share. This is explained by the respective marketing power of the pharmaceutical companies rather than by scientific reasons. In 2002, in addition Amgen launched Neulasta®, a second generation G-CSF using PEGylated filgrastim. This product will be discussed below (see Section 13.7.1).

The first biosimilar G-CSF received EU approval in September 2008. This product was co-developed by BioGeneriX (Ratiopharm) and Sicor Biotech (Teva). The drug substance is manufactured by Sicor Biotech in Lithuania [21, 24]. In total, four authorizations have been granted, Filgrastim Ratiopharm® and Ratiograstim® to Ratiopharm, Biograstim® to CT Arzneimittel, and Tevagrastim® to Teva Generics (Table 13.3). Since Teva acquired Ratiopharm very recently all these brands came under Teva's control. Five months later, in February 2009, the G-CSF product of Sandoz (filgrastim) were granted two EU market authorizations, one for Hexal (Filgrastim Hexal®) and the other one for Sandoz (Zarzio®). The products are developed and manufactured by Sandoz in Austria (Table 13.3). Hence, within a period of a few months six biosimilar G-CSF brands appeared in the EU.

Very recently in June 2010 the EMA granted approval to Hospira's biosimilar filgrastim, named Nivestim®. The development origins are from Pliva in Croatia and were sold to Hospira by Teva after the acquisition of Barr/Pliva. The production site is in Croatia. All products used Neupogen® as reference for comparability exercises in quality, non-clinical, and clinical studies. They were determined to have comparable quality, safety, and efficacy profiles to Neupogen®.

Curiously, the submitted clinical data in terms of total patient numbers differ significantly between the three developments of a biosimilar filgrastim. Whereas Ratiopharm/Teva performed extended phase III studies in 348 breast cancer patients [32], 240 lung cancer patients [33], and 92 non-Hodgkin lymphoma patients [34], Sandoz investigated just 170 breast cancer patients in the phase III study [35]. Moreover, Sandoz compared the results in the breast cancer study with historical data only and did not apply Neupogen® as an internal standard in a comparator arm [21]. Finally, Hospira performed a 2 : 1 randomized double-blind comparator phase III study against Neupogen® in 250 breast cancer patients [21]. Thus in contrast to Zarzio® and in agreement with Ratiograstim®, a complete comparability exercise, including the efficacy in a phase III study, was provided by Nivestim®. Prima facie, the clinical data package of Zarzio® seems to be in conflict with the comparability philosophy of the biosimilar guidelines. However, according the G-CSF-specific guideline CHMP/31329/05 and the non-clinical and clinical guideline CHMP/42832/05 (Table 13.1), which under certain conditions

allows alternative models to demonstrate clinical comparability, for instance provided by PD studies in healthy volunteers, this can be justified. The CHMP considered the clinical comparability exercise of Zarzio® in phase I, which consists of four PK/PD studies in healthy subjects, to be sufficient to prove comparable clinical efficacy and denoted the phase III breast cancer study as a supportive study. It was literally stated in the EPAR that "The supportive trial was non-comparative and therefore of limited usefulness for the assessment of the comparability of the test and reference products" [21].

Nevertheless, the reasons for this unequal treatment of the three sponsors by the CHMP are unclear. Although filgrastim is a small non-glycosylated protein and known to have a very low risk for immunogenicity, if any, the approval of Filgrastim Hexal®/Zarzio® on the basis of the light clinical data set, without performing a comparability exercise in cancer patients, was very surprising. Perhaps this might be a precedent and indicates that in future the authorities are willing to make more advances such as this for followers of non-glycosylated biosimilar products.

13.6.5 Rejected or Withdrawn Applications: Interferons and Insulin

Three biosimilar product developments failed to meet regulatory EU requirements and were documented by the EMA either to be withdrawn or rejected (Table 13.4). The negative opinions by the EMA are discussed briefly in the following.

By June 2006 a biosimilar version of Roche's Roferon A® (IFN-α-2a, Table 13.2), Alpheon® from Biopartners, had already been rejected by the EMA, although just two months earlier the EMA had granted approval to Biopartners' hGH biosimilar Valtropin® (see Section 13.6.1). Several deficiencies of Alpheon® were mentioned. Besides the differences between the test and the reference product in quality and clinical studies, inadequate data on stability, process validation, and validation of immunogenicity tests were also criticized [13, 24].

In February 2009, Biopartners received another negative opinion by the CHMP for their follow-on IFN-β-1a version designated Biferonex®, which is produced by Rentschler Biotechnology, Germany. The product was submitted as a stand-alone application and hence is not a genuine biosimilar development. References were made to both marketed IFN-β-1a products, Avonex® and Rebif® (see Table 13.2). However, the pivotal phase III study compared Biferonex® with a placebo only. The CHMP recommended refusal and Biopartners withdrew the application in May 2009. The assessment report was subsequently published. The main concerns of the CHMP were the differences between the active substance of Biferonex® and other interferon beta products. Thus the historical data of the reference products do not support the use of Biferonex®. In addition, the therapeutic efficacy has not been demonstrated. The overall benefit–risk assessment was negative [36].

Finally, the Indian company Marvel LifeScience filed a biosimilar version of human insulin with three different standard formulations (rapid, long, mixed) for EU market authorization (Table 13.4). The reference products were the corre-

sponding Humulin® formulations from Eli Lilly (Table 13.2). Marvel withdrew the application in December 2007 upon a list of questions and severe concerns that they received on day 120 of the approval process. According to the opinion of the CHMP, the comparability of Marvel products and Humulin® insulins had not been shown. In addition, process-related data were not sufficient and processes have not been validated. Additional objections were raised on the results of the clinical studies [13, 24].

13.6.6
Conclusions on the First-Wave Biosimilars

To draw a balance, since 2006 eight biosimilars or follow-on biologics in the case of Ratiopharm's EPO, respectively, had successfully passed the hurdle of the centralized EU approval. By duplication and multiplication of the dossiers, in total 16 different brands appeared on the market. In contrast, three biosimilars, or follow-on biologics in the case of Biopartners' Biferon®, respectively, failed to receive a positive opinion by the CHMP. This indicates that there is still a risk in demonstrating successfully the biosimilarity and to convince the CHMP about the quality of the CMC section. Not many more first wave biosimilar pursuants (Table 13.2) will be approved within the next few years. The hGH and EPO markets are fairly saturated by the numerous products and profits to be expected for latecomers will be small. The prominent price competition will continue further. The same is true for the G-CSF market, although a fourth biosimilar from Bioceuticals/Stada, may still be pending.

Moreover, some of the first-wave products already have second-generation counterparts in the market (see Section 13.7.1), which are more attractive for biosimilar developers. Considering the huge cluster of recombinant therapeutic antibodies it is easy to predict that the companies have switched already or will switch to the second wave of biosimilar products. These products will be presented in the next section. There is one important aspect left for the first-wave products. It remains to be seen how the European companies will pass the regulatory hurdles in the United States and Japan and if and when they will enter these markets with their biosimilars. For the pioneer drug Omnitrope® a US market authorization has already been achieved, although it was not approved as a biosimilar (see Section 13.6.2).

13.7
Biosimilar Targets: Second Wave of Future Biosimilars

13.7.1
PEGylated Protein Drugs

PEGylation, which means conjugation with polyethylene glycol (PEG), is a widely used type of modification of biomolecules for improving bioavailability of drugs and thus leading to less frequent dose applications. Instead of daily dosing weekly,

bi- or tri-weekly dosing is possible. In addition, physicochemical properties such as solubility and stability are improved. Finally, PEGylation reduced the immunogenicity of the polypeptide in some cases [37]. Owing to their function as regulatory messengers, hormones, cytokines and other inter-cellular factors have a relatively short *in vivo* half-life. This is a main drawback of unmodified protein drugs using the natural amino acid sequences of these factors.

For two decades the applicability and safety of PEG conjugates, including the corresponding linker chemistries, have been proven by use of various PEGylated pharmaceuticals (Table 13.5). The first PEGylated product, Adagen® from Enzon Pharmaceuticals, was approved by the FDA as early as 1990. The active molecule is an enzyme of bovine origin. It is not approved in Europe. To date, seven further PEGylated proteins and one PEGylated RNA (Macugen®/Macuverse®) have been approved by the FDA and the EMA. Cimzia® from UCB Pharma SA is the latest PEGylated product and contains an Fab antibody fragment that is PEGylated at the free sulfhydryl group of the hinge region. It was approved by the EMA in October 2009. This product is a good example of modern drug design. On one hand a large polypeptide was replaced by a truncated version consisting of the functional domain only. On the other hand, the physicochemical and pharmacokinetic properties are restored or even improved by PEGylation. In contrast, all the other protein drugs in Table 13.5 are PEGylated full length polypeptides.

A few important general aspects of commercial protein PEGylation should be noted. For more details refer to a recent review [37]. There are several chemistries available to attach a PEG moiety onto a protein. Besides amino groups of the polypeptide, provided by the *N*-terminus or by lysine, also hydroxyl (serine, threonine), and sulfhydryl (cysteine) groups are used mainly for a covalent attachment of PEG. In addition, the carbohydrate chain of a glycoprotein can serve as an anchor for PEG moieties. Only a few suppliers exist who offer activated PEG of the required pharmaceutical quality. In order to obtain a significant long lasting effect of the drug *in vivo*, a large overall molecular size has to be generated. Some proteins are PEGylated with multiple smaller PEG molecules of 5 kD, such as Adagen®, Oncaspar®, and Somavert®, others are monoPEGylated with one large PEG molecule, such as Pegasys®, Neulasta®, Mirecera®, and Cimzia® (Table 13.5). Linear PEG is available up to a size of 30 kD only. However, for some proteins larger PEG sizes are more favorable and the proteins then have to be conjugated with branched PEG molecules, such as applied for Pegasys® and Cimzia®.

For most products of Table 13.5 the patent expiries lie within this decade. The interest of biosimilar developers in PEGylated products is obvious, however it will be limited to a few products only. As already mentioned, the market volume mainly triggers this interest. The most attractive products are believed to be PEG-G-CSF and PEG-IFN-α. These products have already been on the market for 6–8 years and have prominent sales figures.

In contrast to the corresponding first-generation products, the PEGylated versions of EPO (Mircera®) and hGH (Somavert®) should be less attractive for different reasons. Mircera®, although providing advantages to the patients,

behaves just like any other EPO in the highly price-sensitive EPO market and has disappointing market shares. According a recent press release from Roche [38], in the third year of sales Mircera® reached only CHF179 million, which is just about 11% of global turnover of Roche's first generation EPO NeoRecormon®. Both EPO products have not been launched in the United States so far, due to a patent blockade of Amgen. The PEG-hGH candidate, Somavert® from Pfizer, is a mutated protein and approved to treat patients with acromegaly. This is a rare disease and an orphan drug status was granted to Somavert®.

In 2002 Amgen launched Neulasta®, a mono-PEGylated filgrastim. A PEG moiety of 20 kD in size is attached by site-specific *N*-terminal amino coupling. This product has a prolonged pharmacokinetic half-life profile and requires bi-weekly (that is, once per cycle of chemotherapy) instead of daily application. The use of a body weight independent fixed dose (6 mg) is another improvement. Finally, Neulasta® is claimed to have a somewhat improved efficacy [21] compared with Neupogen®. Not surprisingly, Amgen did not substitute Neupogen® by Neulasta® in the market. Nevertheless, Neulasta® has displaced Neupogen® to a significant extent and reached prominent sales figures. In 2006 Neulasta® yielded three times the turnover of Neupogen®. Both products together made US$3.9 billion [4]. Since 2008 with the appearance of the biosimilar G-CSFs in Europe (see Section 13.6.4) Neulasta® and Neupogen® have had to compete with biosimilar products of lower price. Nevertheless, it is assumed that a pegfilgrastim is highly attractive for the biosimilar companies. At least those companies which had already developed a biosimilar filgrastim (Table 13.3) have certainly calculated the profitability of pegfilgrastim development. Hospira has already announced the ongoing development of such a product.

Emphasis must be given to two essential developments in hepatitis-C (HCV) therapy of the past. Initially, Schering-Plough introduced a combination therapy with IFN-α and ribavirin (Rebetol®), a classical chemical antiviral compound of ICN, up to that time only used for treatment of pulmonary infections of the respiratory snycitium virus (RSV). This combination therapy was a breakthrough for treatment of chronic HCV infections and set a new standard within a short time. Secondly, in 2000 Schering-Plough launched PegIntron®, a PEGylated IFN-α-2b based on Enzon's technology for PEGylation (Table 13.5). This product offers an advantageous once-weekly administration, but more importantly, the clinical efficacy has been improved. Again, within a short time the market switched almost completely to the second-generation product.

Two years later Roche launched a PEGylated IFN-α-2a product, Pegasys®, using the PEGylation technology of Shearwater. This product is marketed together with an own-brand ribavirin (Copegus®) for combination therapy. The introduction of PEGylated IFN-α rendered it difficult if not impossible to sell non-PEGylated biosimilar versions in HCV therapy. Both products are randomly amino-PEGylated. For the manufacture of PegIntron® a 12 kD linear PEG is used. The product consists of mono- and di-PEGylated species. In contrast, for Pegasys®, branched PEG of approximately 40 kD is applied and the product is mono-PEGylated [21]. Because of a cross-licensed, strong intellectual property

position, it is assumed that Roche and Schering-Plough will dominate the huge HCV market from now on. Roche reported a turnover of Pegasys® in 2009 of CHF1.655 billion [38].

To conclude, owing to their exceptional blockbuster status, PEGylated IFN-α and G-CSF are believed to be attractive candidates for a second wave of biosimilars. It should be mentioned that for PEGylated G-CSF there are also two non-biosimilar follow-on developments in advanced clinical stages. One of these is from Maxygen, based on a G-CSF mutein bearing three PEG molecules, and the other one is from BioGeneriX, which make use of enzymatic glycoPEGylation, developed by Neose Technologies, to generate a mono-PEGylated filgrastim conjugate, bearing the PEG molecule at threonine-133, which is the natural glycosylation site of human G-CSF [37, 39]. For such types of improved drugs, terms like "biosuperiors" or "biobetters" were proposed.

13.7.2 Recombinant Antibodies and Fc-Fusion Proteins

All therapeutic monoclonal antibodies (mAbs) and Fc-fusion proteins that possessed effective EU market authorizations in June 2010 are presented in Table 13.6. For some mAbs initially approved, the authorizations have subsequently been revoked or voluntarily withdrawn by the authorization holders and the products withdrawn from the market. These products are not included in the table. The same is true for radioisotope-conjugated antibodies used as *in vivo* diagnostics, which are of little interest, if any, for biosimilar developers. On the other hand, palivizumab (Synagis®), which is considered in Table 13.6, is in fact not a therapeutic but a prophylactic antibody. Three products based on the Fc-fusion technology, Enbrel® (etanercept), Orencia® (abatacept), and Arcalyst® (rilonacept), are integrated into Table 13.6 and combined with the cluster of antibodies within this chapter because of their similar structural and physico-chemical properties to immunoglobulins. This close relationship is also seen by the regulatory authorities (see below).

Antibody-based drugs have higher specificity and are more effective than chemical reagents in depletion of target cells, particularly diseased cells such as tumor cells, viral-infected cells, and other pathogenic cells [40]. One disadvantage compared with small molecules is the relatively high price for an antibody-mediated therapy, thus often restricting the application to high risk patient groups, which do not respond well to standard therapies, or to patient groups showing the highest benefit. Therefore, the use of reasonably priced biosimilar antibodies may expand their application to more patients.

The history of therapeutic antibodies is full of milestone inventions and Nobel Laureates and can briefly be summarized by sequentially listing the key stages. Citations are taken from the review of Strohl from 2009 [41]. (i) Polyclonal antisera (serum therapy, end of the 19th century, Robert Koch, Paul Ehrlich, Emil von Behring); (ii) purified polyclonal immunoglobulins from blood donations (IVIG, 1950s); (iii) hybridoma technology to generate and isolate specific monoclonal

Table 13.6 Second-wave targets for biosimilar developments: EU approved therapeutic antibodies and Fc-fusion proteins.

Company	INN	CAS no.	Product	Construction	Class/ Subclass	Expression host	Specificity/Target	Indications	Application route	1st US Approval	1st EU Approval
Ortho (Janssen–Cilag)	Muromonab CD3	140608-64-6	Orthoclone OKT3	Murine	IgG2a	Murine hybridoma	T-cell receptor CD3	Transplant rejection	i.v.	1986	1987
Centocor Eli Lilly	Abciximab	143653-53-6	ReoPro	Chimeric, Fab	IgG	Mammalian cells (not disclosed)	Inhibition of platelet aggregation, binds to gp IIb/IIIa (CD41)	Cardiac ischemic complications	i.v. infusion	1994	1995
Genentech Roche	Rituximab	174722-31-7	Rituxan/ MabThera	Chimeric	IgG1, kappa	CHO	CD20	Non-Hodgkin lymphoma (NHL)	i.v.	1997	1998
Novartis	Basiliximab	179045-86-4	Simulect	Chimeric	IgG1, kappa	Murine myeloma	IL-2Ralpha (IL-2-Receptor = CD25)	Renal transplant rejection	i.v. bolus	1998	1998
Centocor (Johnson & Johnson) Schering-Plough	Infliximab	170277-31-3	Remicade	Chimeric	IgG1, kappa	Murine myeloma	TNFalpha (inhibits TNFalpha signaling)	Several autoimmune disorders (RA, Morbus Crohn)	i.v. infusion	1998	1999
MedImmune Abbott	Palivizumab	188039-54-5	Synagis	Humanized	IgG1, kappa	Murine myeloma	Respiratory syncytial virus (RSV) F protein	Prophylaxis for RSV infections	i.m.	1998	1999
Amgen Wyeth	Etanercept[a)]	185243-69-0	Enbrel	TNFR-IgG Fc-fusion	IgG1 (Fc)	CHO	TNFalpha (inhibits TNFalpha signaling)	Rheumatoid arthritis and other autoimmune disorders	s.c.	1998	2000

(*Continued*)

Table 13.6 (*Continued*)

Company	INN	CAS no.	Product	Construction	Class/ Subclass	Expression host	Specificity/Target	Indications	Application route	1st US Approval	1st EU Approval
Genentech Roche	Trastuzumab	180288-69-1	Herceptin	Humanized	IgG1, kappa	CHO	ErbB2 (Her-2)	Breast cancer (Her-2 positive)	i.v. infusion	1998	2000
Bayer-Schering	Alemtuzumab	216503-57-0	MabCampath	Humanized	IgG1, kappa	CHO	CD52 (T-lymphocytes)	Chronic lymphocytic leukemia (CLL), T-cell lymphoma, MS	i.v. infusion	2001	2001
Abbott	Adalimumab	331731-18-1	Humira	Human	IgG1, kappa	CHO	TNFalpha (inhibits TNFalpha signaling)	Rheumatoid arthritis and other autoimmune disorders	s.c.	2002	2003
Biogen-Idec Bayer-Schering	Ibritumomab tiuxetan	206181-63-7	Zevalin	Murine Ig-Y^{90}, tiuxetan = chelator	IgG1, kappa	CHO	CD20	Non-Hodgkin lymphoma (with ^{90}Y or ^{111}In)	i.v. infusion	2002	2004
Bristol-Myers-Squibb Merck KGa	Cetuximab	205923-56-4	Erbitux	Chimeric	IgG1, kappa	Murine myeloma	Epidermal growth factor receptor (EGFR)	Cancer, coloreactal, head and neck	i.v. infusion	2004	2004
Genentech Novartis	Omalizumab	242138-07-4	Xolair	Humanized	IgG1, kappa	CHO	Immunoglobulin E (IgE)	Allergy-related asthma and some other allergy indications	s.c.	2003	2005
Genentech Roche	Bevacizumab	216974-75-3	Avastin	Humanized	IgG1, kappa	CHO	anti-VEGF-A, angiogenesis inhibition	metastatic colorectal cancer, NSCLC, etc.	i.v. bolus	2004	2005

Biogen Idec	Natalizumab	189261-10-7	Tysabri	Humanized	IgG4, kappa	Murine myeloma	alpha-4 integrin (VLA4, CD49d)	Multiple sclerosis and Crohn's disease	i.v. infusion	2004	2006
Bristol-Myers-Squibb	Abatacept[a)]	213252-14-3	Orencia	CTLA-4-IgG Fc-fusion	IgG1 (Fc)	CHO	CD80/86 receptors on antigen-presenting cells	Rheumatoid arthritis	i.v. infusion	2005	2007
Amgen	Panitumumab	339177-26-3	Vectibix	Human	IgG2, kappa	CHO	EGFR (epidermal growth factor receptor)	Colorectal cancer	i.v. infusion	2006	2007
Genentech Novartis	Ranibizumab	347396-82-1	Lucentis	Humanized, Fab of bevacizumab	IgG1, kappa	*E. coli*	Vascular endothelial growth factor A (VEGF-A)	Wet (age-dependent) macular degeneration	Intravitreal	2006	2007
Alexion	Eculizumab	219685-50-4	Soliris	Humanized	IgG2/4, kappa	Murine myeloma	Complement protein C5 (CD59)	Paroxysmal nocturnal hemoglobinuria	i.v. infusion	2007	2007
Janssen-Cilag Centocor	Ustekinumab	815610-63-0	Stelara	Human	IgG1, kappa	Murine SP2/0	IL12/23	Plaque-Psoriasis	s.c.	2009	2008
Chugai Roche	Tocilizumab	375823-41-9	RoActemra	Humanized	IgG1, kappa	CHO	IL-6R (myeloma receptor antibody, MRA)	Rheumatoid arthritis	i.v. infusion	2005	2009
Regeneron	Rilonacept[a)]	501081-76-1	Arcalyst	IL-1RI/ IL-1RAcP-IgG Fc-fusion	IgG1 (Fc)	CHO	IL-1α and -β	Cryosporin-associated periodic syndromes	s.c.	2008	2009

(*Continued*)

Table 13.6 (*Continued*)

Company	INN	CAS no.	Product	Construction	Class/ Subclass	Expression host	Specificity/Target	Indications	Application route	1st US Approval	1st EU Approval
UCB (Celltech)	Certolizumab pegol	428863-50-7	Cimzia	Humanized, Fab, PEGylated[b)]	IgG1	*E. coli*	TNFalpha (inhibits TNFalpha signaling)	Rheumatoid arthritis	s.c.	2008	2009
Centocor (Johnson & Johnson) Schering Plough	Golimumab	476181-74-5	Simponi	Human	IgG1, kappa	Murine SP2/0	TNFalpha (inhibits TNFalpha signaling)	Rheumatoid psoriatic arthritis and active ankylosing spondylitis	s.c.	2009	2009
Novartis	Canakinumab	914613-48-2	Ilaris	Human	IgG1, kappa	Murine myeloma	IL-1β	Chronic inflammatory diseases (CAPS,MWS, NOMID,FCAS, FCU)	s.c.	2009	2009
Genmab/ GlaxoSmithKline	Ofatumumab	679818-59-8	Arzerra	Human	IgG1, kappa	Murine NS0	CD20	Chronic lymphocytic leukemia (CLL)	i.v. infusion	2009	2010
Amgen	Denosumab	615258-40-7	Prolia	Human	IgG2, kappa	CHO	RANKL (activates NFkappaB)	Osteoporosis and bone loss	s.c.	2010	2010

a) Fc-fusion protein.
b) For type of PEGylation see Table 13.5.

antibodies (Köhler and Milstein, 1975); (iv) phage display technology (George Smith, 1975); (v) chimerization of monoclonal antibodies (Cherrie Morrison, 1984); (vi) humanization of monoclonal antibodies (Jones, Winter and colleagues, 1986); (vii) full human antibodies from phage display libraries (McCafferty and research group 1990). The molecular display technologies and the future trends of mAbs have been reviewed recently [40].

Since the first approval of a monoclonal antibody for human therapy by the FDA in 1986 (Orthoclone OKT3®, muromonab-CD3) extensive research conducted by pharmaceutical companies and medical institutes has concentrated on the development of more immune compatible recombinant antibodies. Muromonab, a monoclonal mouse hybridoma antibody, produced in ascites of mice, is a forerunner rather than a prototype of a therapeutic antibody. Several failures with hybridoma antibodies clearly indicated that the use of murine mAbs is not justified in chronic or less severe diseases. Mouse IgG elicits a strong humoral immune response along with cytokine-mediated effects. The latter led to investigations on the various Fc-related effector functions [41]. One second exception of an approved murine antibody should be mentioned, that is ibritumomab tiuxetan (Zevalin®). This product, from Biogen-Idec, is conjugated with a chelator (tiuxetan) for complexing ^{90}Y and used for a limited timely and cell-specific radioisotope treatment of CD20+ lymphoma. Immunogenicity in such a therapy is of little relevance.

After the launch of OKT3® there was a rather long lean time until the next mAb product, ReoPro® (abciximab), was approved in the EU in 1995. The approval was not of the centralized type, but was obtained first in the United Kingdom and then in the other EU countries according the mutual recognition procedure. Abciximab was developed by Centocor as an Fab fragment of a mouse-human chimeric antibody, thereby reducing the immunogenic potential substantially. Abciximab was constructed by using recombinant technology and produced with mammalian cell culture in a continuous perfusion mode.

Four further chimeric IgG antibodies obtained EMA approvals between 1998 and 2004, rituximab (MabThera®), infliximab (Remicade®), basiliximab (Simulect®), and cetuximab (Erbitux®). The ending "ximab" in the INN indicates the chimeric nature of an antibody. Chimerization technologies maintain the murine V_L and V_H domains while exchanging the constant regions C_L, C_H1, C_H2, and C_H3 by human counterparts. Thus chimeric antibodies still possess 30–35% murine sequences [41]. Since the approval of Erbitux® in 2004 only humanized or fully human mAbs have been approved.

The first marketed Fc-fusion protein was etanercept (Enbrel®) from Immunex, which was approved in the European Union in 2000. Two subsequent Fc-fusion proteins (ending "cept") received EU authorization in 2007 (abatacept, Orencia®) and 2009 (rilonacept, Arcalyst®).

The first commercial humanized mAb was daclizumab (Zenapax®) from Roche, which was approved in February 1999 by the EMA. However, the market authorization was voluntarily withdrawn by Roche in 2008, due to commercial reasons [21]. No less than ten further humanized mAbs (ending in "zumab") have been approved up to June 2010 (Table 13.6). One of them, eculizumab (Soliris®)

became the first mAb with a non-natural IgG consisting of IgG2–IgG4 chimeric domains. Humanization technologies also render the variable regions more human. Processes have been developed to graft the binding sites, the so-called complementary determining regions (CDR), amended by additional mutagenesis at the DNA level.

Finally, the first approved completely human mAb and concurrently the first mAb from a phage-displayed library, was adalimumab (Humira®) developed by Cambridge Antibody Technology. In 2003 the EMA granted market authorization for this product to the license holder, Abbott. So far, six further human mAbs (ending in "umab," exluding "zumab") were approved in the European Union between 2007 and 2010, panitumumab (Vectibix®), ustekinumab (Stelara®), golimumab (Simponi®), canakinumab (Ilaris®), ofatumumab (Arzerra®), and denosumab (Prolia®). It is worth mentioning that panitumumab was the first mAb from a transgenic humanized mouse.

With the latest entries of Arzerra® and Prolia®, both approved in June 2010, in total 24 therapeutic antibodies and three Fc-fusion proteins have been authorized for market application in the European Union (see Table 13.6) and more than 100 candidates are known to be in clinical development or already in approval procedures. It has been estimated that by the year 2018 there could be a total of 135 mAbs and Fc-fusion proteins on the market, which is five times the number for today. In 2008 more than 1500 clinical trials testing mAbs were registered [41]. This incredible pipeline indicates an exceptional future dominance of mAbs within the cluster of biopharmaceuticals or pharmaceuticals in general, respectively.

Some technical and medical aspects should be mentioned (see Table 13.6). The majority of the antibodies ($n = 17$) are of the subclass IgG1, having a kappa light chain. Five are of IgG2 or IgG4 subclass and three are Fab fragments. Except for two Fab fragments (ranibizumab and certolizumab), which are produced in *E. coli*, the mAbs and Fc-fusion proteins are expressed in mammalian cell cultures, either in CHO cells ($n = 13$) or in murine cells ($n = 10$).

There are two fields of indication that are predominantly targeted by the mAbs and Fc-fusion protein products: (i) Oncology (MabThera®, Herceptin®, MabCampath®, Zevalin®, Erbitux®, Avastin®, Vectibix®, and Arzerra®) and (ii) rheumatoid arthritis (Remicade®, Enbrel®, Humira®, Orencia®, RoActemra®, Cimzia®, and Simponi®). The other applications are spread over many indications (see Table 13.6). Interestingly, no less than five products are directed to inhibit the tumor necrosis factor (TNF) functions (Remicade®, Enbrel®, Humira®, Cimzia®, and Simponi®) indicating the "me too" strategies of the companies. This is also seen for other molecular targets such as CD20 (MabThera®, Zevalin®, and Arzerra®), epidermal growth factor receptor (EGFR; Erbitux® and Vectibix®), and interleukine-1 (IL-1; Arcalyst® and Ilaris®). The competition between products targeting the same preferential molecules will increase further with the launch of more mAbs, which are in late-stage development and directed to the same targets [41].

In view of these multiple opportunities for biogeneric companies, the question arises: which will be the first biosimilar mAbs and Fc-fusion proteins coming onto

the European market? As already mentioned, the most interesting targets for biosimilar developers are always those products which have the largest market volumes. The total market volume for mAbs and Fc- proteins was projected for 2010 to reach US$41.2 billion. Four outstanding blockbusters attract attention, each of which made more than US$3 billion in turnover in 2006. These are Enbrel® (US$4.4), Remicade® (US$3.8), MabThera® (US$3.8), and Herceptin® (US$3.0 billion).

Three additional blockbusters that had turnovers above US$1 billion should be mentioned, namely Avastin (US$2.4), Humira (US$2.0), and Erbitux (US$1.1 billion) [41]. For today one can expect much higher figures for these products. Roche reported the following sales for 2009 (in billions Swiss francs, for conversion into US dollars, take a figure approximately 10% lower): Avastin® (CHF6.2), MabThera® (CHF6.1), and Herceptin (CHF5.3 billion) [38].

Considering also the market lifetime of these products (see Table 13.6), which indicates indirectly the order of the patent expiries (see Section 13.5), it can be assumed that rituximab, infliximab, etanercept, and trastuzumab are at present the hot candidates for biosimilar developments. Presumably they will be the first coming to the EU market. Owing to the enormous attractiveness of these products a strong biogeneric competition can be expected worldwide. Local approvals in India (rituximab), South Korea (abciximab), and China (etanercept) [24] already exist and it cannot be excluded that some Asian companies, through alliances with American or European partners, will penetrate the European Union and United States markets with biosimilar products in the future. In Europe, Teva very recently announced phase I clinical studies with a biosimilar version of rituximab. Teva, after the acquisition of Ratiopharm (including the affiliate BioGeneriX) and Sandoz (after acquisitions of Hexal and Lek), are believed to be the dominant companies and strong rivals in the biosimilar business in Europe. Anyhow, the huge market and the high number of products should offer adequate chances for further companies.

Some practical and regulatory problems related to biosimilar mAb products have to be emphasized in the following. mAbs are highly complex, large-sized glycoproteins consisting of four polypeptide chains, two heavy and two light chains, not comparable to simple proteins such as somatropin or filgrastim. The products are often characterized by micro-heterogeneity and contain subtle mAb variants [42]. Furthermore, glycosylation can be critical for the various biological effects of antibodies and the stabilization of the C_H2 domain as well.

IgG bears a family of diantennary complex-type oligosaccharides at the conserved glycosylation site of asparagine-297. Antibody dependent cellular cytotoxicity (ADCC), which is dramatically enhanced in the absence of a single fucose unit on IgG-Fc, antibody-induced inflammation, which is mediated by sialic acid, and complement activation, which leads to complement-dependent cytotoxicity (CDC) are just three examples where glycosylation plays an important role [43]. This structural and biological complexity requires special know-how and demands in cell culture technology and for *in vitro* and *in vivo* analytics. Moreover, mAbs require high titers and high volumes in production caused by the relatively high

dosing of patients. For example, Herceptin® is dosed at minimum with 2 mg/kg every week and MabThera® with 375 mg/m^2 (around 550–750 mg/patient on average) up to eight times. Other mAbs are dosed with similar amounts. Therefore, multi-grams of drug substance per patient and therapy are needed. The demands are orders of magnitude higher than for first-wave biosimilars, which are dosed with micrograms (EPO) or at most with milligrams (G-CSF, hGH). The wholesale prices for mAbs and Fc-fusion proteins ranged between US$2000 and 20 000 per g in 2008 with an average price of approximately US$8000 per g. Top-selling products need more than 1000 kg/year to achieve the market requirements. The total demand for 15 products in 2009 was estimated to be approximately seven tons [44]. Without doubt the pressure on cost of goods for mAb production is high and will further increase with price reductions provoked by biosimilars.

During the last two decades the manufacturers have steadily raised the production titers in the cell cultures up to 5 g/l, optimized downstream processes, and improved economies of scale. Almost all the manufacturing of blockbuster products such as Enbrel® or MabThera® was linked to the construction of large bulk manufacturing plants containing multiple bioreactors with volumes of 10 000 l or larger [44]. Multi-kilogram batch sizes are yielded by such large-scale processes. Moreover, the originators additionally occupy a major part of the existing large scale contract manufacturing capacity worldwide.

Taken all together, it is a difficult environment for the biosimilar companies, who are forced to invest in production infrastructure to a higher extent than they did for first-wave products. The return of investment and the degree of future commercial success with mAb products will also depend on excellence in manufacturing. Alternatively, generic companies may seek alliances with experienced contract manufactures, as Teva did by entering a joint venture with Lonza in 2009 [45].

The regulatory guidance for biosimilar antibodies at the EMA is still at the level of concept papers and discussions. Drafts on biosimilar mAbs and on immunogenicity assessment of mAbs were released for consultation by the CHMP (Table 13.1). The suitability of the regulatory framework on biosimilars, consisting of the general guideline, the quality guideline, and the non-clinical and clinical guideline (see Section 13.4.2) are called into question for mAbs by the paper of Schneider and Kalinke in 2008 [42]. The authors (Schneider is member of the CHMP and chairman of the BMWP) discussed the challenges for the comparability exercises in analytical, non-clinical, and clinical development and recommended that companies intending to develop biosimilar mAbs seek regulatory scientific advice in the EU early in process. The concept paper for biosimilar mAbs discusses several problems and provides recommendations for the final guidance. In particular, the existing non-clinical and clinical guideline is believed not to be adequate for mAbs. As expected, the principles of the concept paper will also apply to the related Fc-fusion proteins. In addition, the concept paper on immunogenicity of mAbs provides the basis for a specific guideline to handle the "unwanted immunogenicity," and will supplement the general guideline on the immunogenicity of therapeutic proteins.

The methodology of measuring antibodies against antibodies is a real challenge and more sophisticated assays than for the detection of anti-drug antibodies have emerged by non-immunoglobulin proteins. Although this concept paper is apparently not directed primarily at biosimilars but at the forthcoming flood of novel mAb products, it will also be of high relevance for biosimilars. At the moment the biosimilar companies are waiting wishfully for the release of the first guidelines on biosimilar mAbs.

13.8 Biosimilar Developments and Requirements

The CMC requirements for the development of a biosimilar therapeutic recombinant protein are equal to those of a novel protein drug except for the comparability exercise on quality. The comparative analysis of biosimilar and reference product is an add-on for the quality dossier. The ICH guidelines provide a framework that is indispensable for an EMA approval. The ICH tripartite documents define "good manufacturing practice" (GMP), which specifies the requirements and conditions for manufacturing the API or final product. Furthermore, the ICH guidelines give instructions, for example, on the analysis of the expression constructs, cell hosts and substrates, viral safety evaluations, analytical procedures and their validation, and stability testing. In addition, the CHMP guidelines have also been considered. As already outlined in Section 13.4.2 the regulatory prerequisites for approval of a biosimilar recombinant protein are far removed from the abridged pathway applied for chemical generics. Biosimilars are more similar to new drugs.

The product development of a biotech product is characterized by three main sections: (i) process development, (ii) development of analytical methods, and (iii) non-clinical and clinical development. The central theme throughout the development is the comparability exercise. It has to be executed stepwise at various levels and starts with the qualities of the drug substance and the drug product, followed by the non-clinical and clinical parts. Table 13.7 gives an overview of the various pieces, which in the end have to be put together to obtain the market authorization under the legalities of drug approval.

Non-compliance may have severe impact on the time schedule and in the worst situation, might even be irreversible. Non-comparability to the reference product, depending on its degree, can lead to a more extended non-clinical or clinical program justified by the case-by-case principles of the CHMP. It is obvious that much less difficulty occurs for biosimilar developers than for the originators. Many problems have been solved in advance and there is no need for a proof of principle. This results in abbreviated programs and time lines in the non-clinical and clinical part. A product launch can be realized 6–8 years after starting the project with molecular biology. By using *E. coli* as the expression system the project could be finished approximately one year earlier. In the following, emphasis is given to the special features of biosimilar development.

Table 13.7 Development steps for a biosimilar recombinant protein.

I. Process development	II. Analytical development	III. Non-clinical and clinical development
Defining the DNA sequence	Characterization of the reference product	Comparative non-clinical *in vitro* studies (Bioanalytics)
Cloning of the gene	Definition of standards	Real time binding assay and cell-based activity assay
Construction of the expression vector	Bioassays (cell-based *in vitro* assays, *in vivo* assays)	*Comparative animal studies* (one mammalian species):
Transfection of the host cell	SDS-polyacrylamid gel electrophoresis (SDS-PAGE)	Pharmacodynamic effect (relevant for clinical indication)
Selection of stable clones	Western blot (WB)	Repeated dose toxicity, including toxicokinetic measurements
Optimization of expression, culture media selection	Isoelectric focusing (IEF)	Immunogenicity (anti-drug Abs, neutralizing Abs, quantitative)
Master cell bank (MCB)	Capillary zone electrophoresis (CZE)	Local tolerance (case-dependent)
Working cell bank (WCB)	Reversed-phase HPLC (RP-HPLC)	Normally not required: Safety pharmacology, reproduction toxicology, mutagenicity, and carcinogenesis
Characterization and safety of cell banks	Size-exclusion HPLC (SEC-HPLC)	*Comparative PK/PD study in humans (Phase I):*
Upstream procedures: Fermentation process (USP)	Product-specific ELISA	In healthy volunteers or patients
Downstream procedures: Purification scheme (DSP)	Host-cell-protein-specific (HCP) ELISA or Treshold	Single dose comparator study, pharmacokinetic, pharmacodynamic, safety, important touchstone of biosimilarity
Pharmaceutical development: Formulation, container	Residual total DNA detection (picogreen, Treshold)	*Not required: Phase II studies:* (patients)
Development of fill and finish	*N*-terminal sequencing (Edman degradation)	Dose-effect relation and dose finding is waived for biosimilars
Optimization of individual process steps	*C*-terminal amino acid composition	*Comparative efficacy study (Phase III)* (in patients):
Stability and robustness of the process	Peptide mapping	Controlled safety and efficacy in one selected indication
Virus safety validation	Total amino acid content (upon hydrolysis)	Typically two arms crossover study, biosimilar vs. reference therapy, double blinded, matched patient groups Immunogenicity assessment is of high relevance, late follow-up samples required
Introducing GMP	Carbohydrate analyses (total sugar, antennarity, sialic acids)	
Stability studies (holding steps, bulk material, final product)	MALDI-TOF spectroscopy (molecular weight)	
Consistency batches	Detection of free sulfhydryl moieties	
Process validation (full scale and down scale)	Circular dichroism spectroscopy (CD-spectra)	
	Surface plasmon resonance (SPR, BIAcore)	

13.8.1 Process Development

Prior to all practical work, the reference product has to be defined. The EMA will accept EU-authorized products only. The same is true for the United States and Japan (see Section 13.4.5). The reference product must not to be changed during the entire development. The required gene and its sequence are usually in the public domain. If not, the protein has to be sequenced. Identical amino acid sequence to the reference product is a premise. Typically, the DNA will be chemically synthesized. The selection of the host cell and the construction of useful expression systems is standard technology of molecular biology. The same type of the expression host as used for the reference product is advisable to lower the risk of non-similarity. Biogeneric companies have to deal mainly with two expression systems, *E. coli* and CHO (see Tables 13.2, 13.3, and 13.6). Occasionally, *Saccharomyces* and murine myeloma cells (for mAbs) might be alternative expression hosts.

The first milestone for the project is completion of the master cell bank (MCB) and the manufacturing working cell bank (WCB). Comprehensive analytical work is necessary, especially for the mammalian cells, to obtain all the safety data required for release of the cell banks into production. It should be mentioned that even in the earliest step of the development, for example for clone selection, it is indispensable to quantify and to analyze the product. In many cases this will be easier to establish for biosimilar developments because specific antibodies or complete ELISA products for clinical diagnosis are available commercially for several products. Likewise, reference material can be obtained in high purity from the sales product. However, the reference product is a complete pharmaceutical composition and ingredients can interfere with the analytical methods. Therefore, the active ingredient has to be isolated from the reference product in order to perform the comparability exercise on the level of drug substance.

The development of the upstream procedures does not differ for a biosimilar. This has to be done independently. There is no access to any experience from the reference product. However, the fermentation techniques applied are standardized and routine in the hands of experienced manufacturers. Nevertheless, this is an important and time-consuming section, and difficulties may occur along with scale up. The downstream process (DSP), which always contains a sequence of different chromatographic steps, accompanied by some filtration procedures, can be deduced from published literature, supported by experience in protein chemistry. In most cases biosimilar developments will end up with their own, unique, and state-of-the-art processes. There are many alternative methods available. The necessity of DSP is the removal of impurities, such as host cell proteins (HCP), DNA, endotoxins/pyrogens, and other process-related substances. The requirements on purity for a therapeutic grade protein are high. As discussed in Section 13.5, processes are an area for secondary patent applications and often the development has to be performed along a circumventing strategy. The same is true for the development of the pharmaceutical formulation (see Section 13.5).

Much work and high costs are related to the production of substances for use in clinical trials, especially for phase III. The authorities always recommend taking the clinical materials from the final process only. However, this is often in conflict with time lines and the economy of a project. Owing to significant costs one should like to introduce the full scale and GMP status as late as possible. In the best case, the phase III material will be taken from the commercial scale.

Production of clinical material requires introduction of GMP and a huge amount of validation work later on. In contrast to the first developers, who had to walk more carefully step by step, considering feedback from preclinical or clinical phases, biosimilar companies have the possibility of moving on rapidly and performing many steps in parallel. In addition to the demands on comparability provided by the biosimilar guidelines, there are additional comparability exercises between the necessary process stages, and the comparability guideline for biotech products is appropriate (Table 13.1). If different processes were applied to produce pivotal materials for stability, toxicology, phase I, phase III, and validation, they have to be bridged by such comparability exercises.

The development of the final product, which is a liquid within a pre-filled syringe or within a vial in most cases, shows no biosimilar-specific aspects. Because of patents, most biosimilar products will appear with their own formulations, which differ more or less from the original product. The formulation of the reference product, with respect to stability, always serves as the gold standard. In addition, the compatibility of the various surfaces of the container (rubbers, siliconized glass, metal of the needle, glues, etc.) that are in permanent contact with the protein solution has to be investigated. The worst case scenario of what can happen is known from the PRCA syndrome caused by Eprex® (see Section 13.6.3). As already mentioned, a comparability exercise is also necessary for the drug product.

Finally, I want to emphasize that all materials and raw materials used for the development and manufacturing of a biotech product have to be of non-animal origin, wherever applicable, to provide against safety concerns regarding transmissible agents. Moreover, it is also recommended, wherever possible, to use materials and raw materials that comply with the European and US Pharmacopoeia (Eur. Pharm. and USP grade). If compounds have to be used that are not specified in the pharmacopoeias they should be classified by the FDA as "generally recognized as safe" (so-called GRAS substances).

13.8.2
Development of Analytical Methods

Table 13.7 presents a selection of assays frequently taken for the characterization of a therapeutic protein. The field is complex and I can mention only some important aspects. The assays are applied for in-process controls (IPC), batch releases, extended characterization of the purified protein, comparability exercises, process validation, or stability studies. Most of the assays are standard biochemical methods, which have to be adapted and validated for the specific protein. Biosimilar developers will relate their standards to the reference product and it is manda-

tory to use the same reference product throughout the development. In many cases there are also defined reference standards available from the European Pharmacopoeia (Biological Reference Preparations, BRP), the World Health Organization (WHO) or the British National Institute for Biological Standards and Control (NIBSC), which are required for calibration of the assays.

Of special importance is the potency of the protein, analyzed by specific biological assays. These assays determine the biological activity, mostly in terms of international units (IU). Typical bioassays are: (i) *in vivo* animal systems, for example mice (EPO), or (ii) cell-based proliferation assays (CSF) or anti-viral assays (IFN). The biological assays have to be calibrated according to European Pharmacopoeia requirements. The potency to bind to the natural receptor can effectively be demonstrated with so called "reporter gene activation assays," which transduce the receptor-binding signal via gene activation to an easily detectable marker. Alternatively, real-time binding kinetics may be measured with surface plasmon resonance methods such as the BIAcore® platform.

For the characterization of a biosimilar one has to consider purity, impurity profiles, product-related substances, biological activity, and physicochemical properties. This has to be compared to the reference product by side-by-side testing and differences have to be justified. Process-related impurities can differ, but have to be specified. Several sophisticated state-of-the-art methods are needed and have to provide structural evidence for a biosimilar product in comparison to the reference product. The *N*-terminus and *C*-terminus have to be intact. Free sulfhydryl groups and disulfide bridges have to be in the right position. Altered versions of the proteins, which are termed as "product-related substances," for instance caused by methionine oxidation or deamidation, truncated species, dimers, and aggregates, have to be characterized and quantified at very low levels.

Additional analytical methods are utilized for glycoproteins, which appear with a broad range of glyco-variants often referred to as "isomers." The analysis of the carbohydrate composition is mandatory. The antennarity structure and the specific number of sialic acids have a strong influence on potency and on the pharmacokinetic behavior *in vivo*. Moreover, for mAbs the glycosylation triggers effector functions of the Fc domain to a considerable extent (see Section 13.7.2).

For the immunological detection of HCP impurities a source- and process-specific test has to be developed based on mock material used for immunization. Mock material is a type of control protein preparation, without the product, just modeling the impurities. Alternatively, a commercial generic HCP assay, if available with appropriate sensitivity, can be used, especially for the comparability exercise.

Of particular importance are the stability investigations of drug product and drug substance. Stability-indicating methods have to be defined by using accelerating or stress conditions, meaning higher temperatures. The specific degradation pathways of the biosimilar and the reference product have to be compared and characterized.

Preceding stability studies are needed to support the clinical studies. Dedicated stability batches have to be produced and put on real-time stability. From such

preceding and further ongoing stability studies, the shelf life of the study medication is justified (use by date).

Although it is a tremendous amount of work to establish and validate all the analytical methods, the biogeneric developers can refer to publications, the European Pharmacopoeia in many cases, and consult specialized service laboratories that offer these methods. However, most of the assays required for the routine quality control, IPC, and batch release need to be installed at the site of manufacturing. Validation of the analytical methods has to be finalized before the first batches can be released for clinical trials, including the preceding stability batch. The specifications for the quality control have to be fixed stepwise and justified according the appropriate ICH guidelines.

13.8.3 Non-clinical and Clinical Development

This is the most significant part of the total development costs of a pharmaceutical. Biosimilar developers, however, undergo low risk for failure in clinical studies. This is a major difference from the development of a new protein drug. Depending on the type of product, its medical indication, and the degree of analytical comparability, the extent of the clinical studies will vary for biosimilars (case-by-case). This has been discussed in Section 13.4.2 in more detail.

The non-clinical study program for a biosimilar drug differs from a stand-alone approach. Routine investigations for safety pharmacology, reproduction toxicology, mutagenicity, and carcinogenesis are normally not required. The applicant has to perform comparative toxicity studies in animals. One pivotal repeated dose toxicity study in one established species can be sufficient. The study should be designed to see differences from the reference product rather to work out toxicological effects per se. The comparator study should address the pharmacodynamic effect (related to the clinical indication), the toxicokinetics, and the immunogenicity. The last could also be elaborated in a separate study, which then would allow specific dosing and sampling. In cases where the final formulation of the drug product is very different to the reference product, a local tolerance study, usually in rabbits, might be necessary. Generally, published data are available for the type of toxicological effects in established species and for other non-clinical results of the reference drug substance. These can be used as supporting material.

It is important to realize that to measure PK and antibodies in animal and human sera a cascade of assays is needed, which can mean a long lead time for development and validation. However, many synergies between the animal and the human testing can be utilized. Numerous different serological assay methods have been described for these applications: enzyme-linked immunosorbent assay (ELISA), Western blots (WB), radioimmunoprecipitation assay (RIPA), surface plasmon resonance (SPR, BIAcore®), electrochemiluminescence (ECL) assays, and xMAP fluorescence immunoassays (Luminex®).

Besides the quantitative detection of drug and anti-drug antibodies (ADA), also neutralizing antibodies (nAbs) have to be analyzed. The neutralizing activity is

normally measured as an inhibitory effect in the potency assay. It is recommended to contract well-experienced research organizations for state-of-the art development and GLP-conform testing. It is for sure that the authorities will have a very deep look at the quality and compliance of the immunological assays and the test runs. The immunoassays will also be needed for post-marketing immunogenicity surveys as part of the pharmacovigilance program.

As summarized in Table 13.7 the clinical study program for a biosimilar drug differs considerably from a stand-alone approach. The clinical comparability exercise is a stepwise procedure that should begin with PK/PD studies followed by clinical efficacy and safety trials. In exceptional cases just PK/PD studies might be sufficient (see Zarzio® in Section 13.6.4).

Before entering clinical studies, the formulation has to be fixed and stability data for at least three months are required. Most of the biosimilar products are liquid formulated parenterals presented as pre-filled syringes. There are some important differences for the clinical trials of biosimilars. All studies are designed as comparator studies to demonstrate comparability in PK, PD, efficacy, and safety. Phase I, which consists of at least one PK/PD study, will be performed with healthy volunteers or occasionally even with selected patient groups depending on the ethical situation. This study has to show comparability or in the best case even bioequivalence to the comparator product. The study will be performed with one selected dose. To achieve bioequivalence, very precise dosing per kilogram of body weight or per square meter of body surface is essential. In addition, the phase I study can also address safety aspects. There is a residual risk for missing clinical comparability and thus showing non-biosimilarity (see Marvel insulins, Section 13.6.5). This would have severe impact on further clinical development.

The classical phase II studies for dose-effect relations and dose finding are not reasonable for a biosimilar product. Once proven analytical similarity, non-clinical similarity, and equivalent PK/PD responses, a dose finding or a general proof of efficacy is not necessary at all.

The comparability exercise for efficacy will be necessary in most cases and has to be performed by a phase III study in one key-indication. Further indications, if granted to the reference product, could be obtained by extrapolation without clinical data. It is strongly recommended to confirm the study design in time by a scientific advice procedure at the EMA. Agreement should be obtained with respect to the indication, patient inclusion, and exclusion criteria, total number of patients, basic or supplementary medications, primary and secondary endpoints, immunogenicity assessment, randomization, and degree of blinding. The studies have to be blinded as much as possible. A typical biosimilar phase III study is designed as a multi-center, randomized, double-blinded, comparator study with two arms and crossing over. The study aim is to show a comparable efficacy to the reference product and provide sufficient evidence that both products are interchangeable. In addition, the phase III study has to provide safety data based on a statistically calculated number of patients. The safety matter is the most important aspect throughout the different non-clinical and clinical stages and has to be continued by the pharmacovigilance program after approval.

13.9 Conclusions

Recombinant therapeutic proteins have been in the sights of the generic industry for more than a decade and several product developments have been successfully finalized. The differences between chemical generics and biosimilars are dominated by the type of approval, the long time lines, and in the overall high costs for a project. In these aspects, biosimilars resemble new drugs rather than classical generics. Significant investment and lack of biotech know how were the two major barriers to be conquered by the generic industry for this type of business. Biosimilars have been a reality in Europe since 2006. Up to June 2010, eight developments from different biosimilar companies had received EMA approvals and in total 16 brands are registered today. The biosimilars have become a reliable and respected group of products with steadily increasing market shares in the EU.

However, the first wave of biosimilars covers three product categories only, hGH, EPO, and G-CSF. Developments in three further categories, insulin, IFNalfa, and IFNbeta, have been withdrawn or rejected. It is not known if the sponsors will try again. Most of the biosimilar developments have been performed in the absence of an existing regulatory framework. The EMA guidelines came into force between October 2005 and June 2006 in a phase where non-clinical and clinical developments of most of the developments were finalized. This explains the different size of data packages of the companies when submitting for market authorizations.

Although the regulatory pathway and legal framework for biosimilars in the United States was set up recently, many questions and the practical requirements are still open. The biogeneric industry needs clarity urgently. The US biosimilar act revealed some drawbacks related to complicated patent dispute procedures, various exclusivity periods, and the interchangeability terminology. Further amendments seem inevitable. The degree of harmonization between the European Union and the United States is also seen to be critical. The legislations in the most important markets, the United States, the European Union, and Japan, insist on locally approved reference products, and this can induce costly bridging studies.

Special emphasis has been given to the second wave of future biosimilars, which target PEGylated proteins, mAbs, and Fc-fusion proteins. A huge panel of commercially successful products is on the market and a rich pipeline behind them will breed many further blockbusters within the next few years. These products will lead to further expensive therapies and the legitimate question appears as to how this can be financed by the healthcare systems in the near future. Pressure to reduce healthcare expenditure and increase patient access to treatment will drive the development of cheaper biosimilars. However, similar to the belated EU biosimilar guidelines for the first-wave products, biogeneric companies are, for the second-wave products, again in the situation of developing biosimilars in the absence of specific guidance. However, it is expected that the EMA will adopt specific guidelines for mAbs and Fc-fusion proteins soon.

Consolidation in the generic field in Europe led to two dominant players and also severe competition in the biosimilar business. Sandoz and Teva each acquired

one of the two biggest generic companies in Germany, Hexal and Ratiopharm, respectively, and they will certainly dominate the European biosimilar market in the future. On the other hand, the competition between originators and generic companies put pressure on both sides to innovate. This results in benefits for patients (improved therapy) and the health system (lower costs).

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14
Pharmacokinetics and Pharmacodynamics of Therapeutic Peptides and Proteins

Yi Zhang and Bernd Meibohm

14.1
Introduction

Biotech drugs have played increasingly important roles in applied pharmacotherapy by offering improved treatment modalities for many chronic medical conditions, such as rheumatoid arthritis, multiple sclerosis, cancer, hepatitis C, osteoporosis, and diabetes. Overall revenues for biologic drugs reached approximately $125 billion worldwide in 2008 [1]. It is estimated that during the next few years, the fraction of all newly approved drug products in the United States will be more than 50% for drugs of biological origin and the demand for biotech drugs and spending on these will keep rising substantially throughout this decade [2]. Meanwhile, annual treatment costs for biologics can be 5–200 times higher than those from non-biologic medicine treatment depending on the various medical conditions [3], further creating incentives for the development and regulatory approval of biogenerics or biosimilars.

The major distinction between biotech drugs and classical small molecule based therapeutics is based on their production: While biotech drugs are manufactured from living organisms (cells) through biotechnology-derived processes, small-molecule drugs are chemically synthesized [4]. Thus, biotech drugs are defined by their production process, whereas small-molecule drugs are defined by their chemical structure. In this chapter, we will focus on peptide and protein therapeutics as the largest and most important group of biotech drugs.

The basic paradigm of clinical pharmacology is the fact that drug effects, desired as well as undesired, are a function of the drug concentrations within different organs and tissues in the human body. Thus, drug concentrations are the driving force for the spectrum of drug responses observed in a drug-treated patient. Drug concentrations in blood or plasma are used as a surrogate for drug concentrations at the effect site, or site of drug action, under the assumption that under pharmacokinetic steady-state conditions there is a constant relationship between the free, unbound drug concentration in the plasma and at the site of action. Even though this assumption is frequently not accurate, it has proven to be a generally very useful approximation to achieve the desired effect levels via modulation of the

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plasma concentration, especially during prolonged pharmacotherapy with multiple-dose regimens. However, it is usually limited to small-molecule drugs, for which passive diffusion is a major mechanism in the drug disposition process.

In contrast, this concept might be less applicable for protein therapeutics, as their distribution is largely determined by convective transport rather than diffusion due to their high molecular weight and charge. Nevertheless, drug concentrations in blood and plasma are frequently also used for protein therapeutics as surrogates for effect-site concentration as the latter are usually not easily accessible in human subjects.

The dose–concentration–effect relationship is defined by the pharmacokinetic and pharmacodynamic characteristics of a drug. Pharmacokinetics comprises all processes that contribute to the time course of drug concentrations in various body fluids, generally blood or plasma, that is, all processes affecting drug absorption, distribution, metabolism, and excretion. In contrast, pharmacodynamics characterizes the effect intensity and/or toxicity resulting from certain drug concentrations at the assumed effect site. Simplified, pharmacokinetics characterizes "*what the body does to the drug*," whereas pharmacodynamics assesses "*what the drug does to the body*" [5]. Combination of both pharmacological disciplines by integrated pharmacokinetic–pharmacodynamic modeling (PK/PD modeling) allows a continuous description of the effect–time course directly resulting from the administration of a certain dose (Figure 14.1) [6, 7].

Similar to conventional small molecule drugs, protein therapeutics are characterized by well-defined pharmacokinetic properties that form the basis for the design of therapeutic dosing regimens as well as drug delivery strategies. Potential

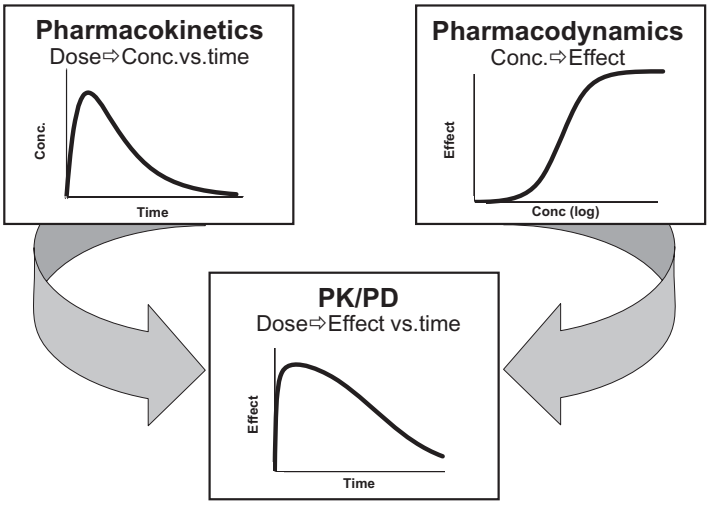

Figure 14.1 The concept of pharmacokinetic–pharmacodynamic (PK/PD) modeling as combination of the classic pharmacological disciplines pharmacokinetics and pharmacodynamics. From [6]. Reprinted with permission of Springer.

differences, caveats, and pitfalls, however, may arise from their similarity to endogenous and/or dietary molecules with which they share common drug disposition pathways, as well as their interaction with endogenous regulatory feedback pathways, especially if they are analogs of hormones or other tightly regulated endogenous substances [8].

The widespread application of pharmacokinetic concepts in drug development has repeatedly been promoted by industry, academia, and regulatory authorities [9–11]. It is believed that the application of pharmacokinetically based concepts in all preclinical and clinical drug development phases may substantially contribute to a more scientifically driven, evidence-based development process. In addition, it provides the pharmacologic basis for dosage selection and dosage regimen design. Thus, in-depth knowledge of a compound's pharmacokinetic characteristics for protein therapeutics will also continue to form a crucial element to achieve their fullest therapeutic potential for an optimal use with regard to efficacy and safety in the target patient population.

Since peptide and protein therapeutics are frequently identical or similar to endogenous substances, however, they oftentimes exhibit unique pharmacokinetic and pharmacodynamic properties. These may pose extra challenges and questions during their preclinical and clinical development that are different from small molecule drug candidates and may require additional resources and unique expertise. Some of these problems and challenges will be discussed in the following.

14.2 Pharmacokinetics of Peptides and Proteins

The *in vivo* disposition of endogenous peptide and protein drugs may often be predicted to a large degree from their physiological function. Peptides, for example, which frequently have hormone activity, usually have short elimination half-lives, which is desirable for a close regulation of their endogenous levels and thus their function. Insulin, for example shows dose-dependent elimination with a relatively short half-life of 26 and 52 min at 0.1 and 0.2 U/kg, respectively. Contrary to this, proteins that have transport tasks, such as albumin, or long-term immunity functions, such as antibodies, have elimination half-lives of several days, which enables and ensures the continuous maintenance of necessary concentrations in the blood stream [12]. This is, for example, reflected by the elimination half-life of antibody drugs such as the anti-epidermal growth factor receptor antibody cetuximab, for which a half-life of approximately 7 days was reported [13].

14.2.1 Protein Absorption

Protein drugs, unlike traditional small-molecule drugs, are generally not therapeutically active upon oral administration. Therefore, most protein drugs are currently formulated as parenteral formulations to allow for complete absorption and to

avoid presystemic degradation [12, 14, 15]. Major routes of administration include intravenous (i.v.), subcutaneous (s.c.), and intramuscular (i.m.) administration. In addition, other non-oral administration pathways are utilized, including nasal, buccal, rectal, vaginal, transdermal, ocular, and pulmonary drug delivery. Nevertheless, different methods are currently being developed to enhance the oral bioavailability of protein drugs due to the general advantages of oral administration compared with other routes of administration.

14.2.1.1 **Parenteral Administration**

For i.v. administration, protein drugs are usually given as either a bolus dose or a continuous infusion. This results in immediate drug concentrations in the systemic circulation and maximum bioavailability. Protein therapeutics administered by the i.v. route include, for example, the tissue plasminogen activator (tPA) analogs alteplase and tenecteplase, the recombinant human erythropoietin epoetin-α, recombinant activated factor VII (rFVIIa), and the granulocyte colony-stimulating factor filgrastim [16]. However, i.m. or s.c. injections may be more appropriate alternatives when the desired concentration–time profiles of protein drugs cannot be achieved through i.v. administration. For example, a prolonged absorption phase and longer terminal half-life of epoetin-α have been observed following s.c. administration compared with i.v. administration, which likely allows for a convenient once-weekly s.c. dosing of epoetin-α in chemotherapy patients [17, 18].

Another similar example has been reported in the study of leuprorelin, a luteinizing hormone releasing hormone (LHRH) agonist. While LHRH in bursts stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), a continuous baseline level of LHRH will suppress the release of these hormones [19]. Thus, to avoid the high peaks following an i.v. administration of leuprorelin, a long acting monthly depot injection of the drug has been approved for the treatment of prostate cancer and endometriosis [20].

One of the major drawbacks of s.c. and i.m. administrations is the presystemic protein degradation frequently associated with these administration routes, resulting in a reduced bioavailability compared with i.v. administration. The pharmacokinetically derived apparent absorption rate constant k_{app} for protein drugs administered via these administration routes is thus the combination of absorption into the systemic circulation and presystemic degradation at the absorption site, that is, the sum of a true first-order absorption rate constant k_a and a first-order degradation rate constant. The true absorption rate constant k_a can then be calculated as:

$$k_a = F \times k_{app}$$

where F is the bioavailability compared with i.v. administration. A rapid apparent absorption, that is, a large k_{app}, can thus be the result of a slow true absorption and a fast presystemic degradation, that is, a low systemic bioavailability [21].

Other potential factors that may limit bioavailability of proteins after s.c. or i.m. administration include variable local blood flow, injection trauma, and limitations of uptake into the systemic circulation related to effective capillary pore size and

diffusion. Several peptide and protein therapeutics including anakinra, etanercept, insulin, and pegfilgrastim are administered as s.c. injections.

Following s.c. injection, peptide and protein therapeutics may enter the systemic circulation either via blood capillaries or through lymphatic vessels [22]. In general, macromolecules larger than 16 kDa are predominantly absorbed into the lymphatics whereas those under 1 kDa are mostly absorbed into the blood circulation. There appears to be a linear relationship between the molecular weight of the protein and the proportion of the dose absorbed by the lymphatics [23]. This is of particular importance for those agents whose therapeutic targets are lymphoid cells (i.e., interferons and interleukins). Studies with recombinant human interferon α-2a (rhIFN-α-2a) indicate that following s.c. administration, high concentrations of the recombinant protein are found in the lymphatic system, which drains into regional lymph nodes [24]. Clinical studies show that palliative low-to-intermediate dose s.c. recombinant interleukin-2 (rIL-2) in combination with rhIFN-α-2a can be administered to patients in the ambulatory setting with efficacy and safety profiles comparable to the most aggressive i.v. rIL-2 protocol, against metastatic renal cell cancer [25, 26].

14.2.1.2 **Inhalation Administration**

Inhalation delivery of peptides and proteins is a novel approach to treating systemic diseases. It offers the advantage of ease of administration, the presence of a large surface area (75 m^2) available for absorption, high vascularity of the administration site, and bypass of hepatic first-pass metabolism.

The success of inhaled peptide and protein drugs can be exemplified by inhaled recombinant human insulin products, with Exubera® being the first approved non-injectable insulin (in 2006). Inhaled insulin potentially provides better patient compliance as a noninvasive alternative administration approach. In addition, inhaled insulin is absorbed more rapidly and exhibits a shorter duration of action as compared with s.c. administered regular insulin. Thus, it mimics the physiological appearance of insulin secretion after a meal as well as providing a better postprandial glucose control to reduce the risk of inappropriate hyperinsulinemia in the circulation [27]. A 6-month phase III study involving 328 patients with type I diabetes mellitus treated with basal insulin showed that inhalation insulin treatment exhibited comparable glycemic control to that with subcutaneous administered regular insulin, as assessed by glycosylated hemoglobin (HbA1c) levels [28]. In addition, several other clinical studies also reported that inhaled insulin showed a comparable efficacy with type II diabetic subjects as compared with the conventional s.c. insulin regimen, thus providing a promising alternative administration approach for insulin [29, 30].

These clinical trials demonstrated that the noninvasive administration of inhaled insulin offers similar efficacy, but better patient compliance, as s.c. administered insulin. However, clinical use of the inhalation delivery has been limited by the low bioavailability due to the presence of certain proteases in the lung, potential local side effects of the inhaled agents on the lung tissues (i.e., growth factors and cytokines), and molecular weight limitations [31, 32]. Approximately one year after

introduction into the marketplace, Pfizer announced it would be discontinuing Exubera, citing that the drug failed to gain market acceptance. This experience highlights that there are challenges beyond technical and scientific problems that oftentimes need to be addressed when protein therapeutics intend to utilize novel administration pathways.

14.2.1.3 **Intranasal Administration**

Intranasal administration of therapeutic proteins also offers the advantages of noninvasive administration, delivery to a surface area rich in its vascular and lymphatic network, rapid absorption, as well as the bypassing of hepatic first-pass metabolism [33]. Molecular weight was found to be a major factor affecting bioavailability via the intranasal route. Polypeptides with a molecular weight of up to 2000 Da were found to be pharmacologically active after nasal administration, while peptides and proteins with molecular weights of 2000–6000 Da (i.e., insulin, calcitonin, and LH-RH) required the addition of absorption enhancers in order to reach adequate bioavailability [19].

A variety of peptide and protein drugs have been extensively investigated for nasal administration, including calcitonin, oxytocin, LH-RH, growth hormone, interferons, and vaccines. Intranasal administration has recently been proposed as a means to deliver protein therapeutics directly into the central nervous system (CNS), thereby bypassing the blood–brain barrier [34]. In particular, one study reported achieving a higher concentration of ^{125}I-labeled recombinant human insulin-like growth factor-I (^{125}I-rhIGF-I) in the CNS after intranasal administration rather than i.v. administration [35]. However, some drawbacks of the intranasal administration, such as high variability associated with the site of deposition, the type of delivery system, changes in mucus secretion and mucociliary clearance, as well as the presence of allergies, hay fever, or the common cold in the target population may limit the clinical use of the intranasal administration [36].

14.2.1.4 **Transdermal Administration**

Transdermal drug delivery also offers the advantages of bypassing metabolic and chemical degradation in the gastrointestinal tract as well as first-pass metabolism by the liver. Sonophoration and iontophoresis are two frequently used approaches in transdermal drug delivery. Skin permeability to ionic compounds has been shown to be greatly enhanced by either applying low-frequency ultrasound as in sonophoration or by applying a low-level electric current as in iontophoresis. Successful transdermal delivery of therapeutic doses of insulin, interferon-γ, and epoetin-α have been reported via sonophoresis [37]. Additionally, transdermal iontophoresis has been used in the delivery of various proteins and peptides including insulin [38], growth hormone releasing factor [39], calcitonin [40], and parathyroid hormone [41]. Furthermore, some other approaches have also been investigated for transdermal drug delivery, including painless microneedles and skin perturbation devices [42, 43]. For example, Martanto *et al.* reported that insulin could be successfully delivered to the hairless diabetic rat using solid microneedle arrays, resulting in decreased blood glucose levels [44].

14.2.1.5 Oral Administration

The lack of systemic exposure of protein drugs following oral administration is mainly caused by two factors: (i) high gastrointestinal enzyme activity and (ii) low permeability through the gastrointestinal mucosa. In fact, the substantial peptidase and protease activity in the gastrointestinal tract makes it the most efficient body compartment for protein drug metabolism. In addition, the gastrointestinal mucosa presents a major absorption barrier for water-soluble macromolecules such as peptides and proteins [12]. Thus, although various factors such as permeability, stability, and gastrointestinal transit time can affect the rate and extent of absorption of orally administered proteins, molecular size is generally considered the ultimate obstacle [45].

Oral delivery of protein drugs would be a highly desirable route of administration if bioavailability issues could be overcome, as it offers the advantages of convenient, pain-free administration. Thus, numerous strategies to improve the oral delivery of proteins have recently been explored. Coadministration of absorption enhancers may be used to either temporarily disrupt the intestinal barrier so that drug penetration is increased or to serve as transport carriers for the protein via complex formation. The oral coadministration of parathyroid hormone, an 84 amino acid protein, with *N*-amino caprylic acid, a transport carrier, resulted in positive bioactivity as demonstrated in a rodent model of osteoporosis [46]. While parathyroid hormone has no oral bioavailability when administered alone, coadministration of this absorption enhancer resulted in 2.1% oral bioavailability relative to s.c. administration in monkeys [46]. Meanwhile, coadministration of protease inhibitors has also been suggested for the inhibition of enzymatic degradation [15, 47].

Other strategies of oral protein delivery include micro- or nanoparticles, chemical modifications such as amino acid backbone modifications, and chemical conjugations to improve the resistance to degradation and the permeability of the protein drug.

14.2.2 Protein Distribution

The distribution volume of protein drugs is determined largely by their molecular weight, physiochemical properties (e.g., charge, lipophilicity), protein binding, and by their dependency on active transport processes. Most therapeutic proteins have small volumes of distribution limited to the volume of the plasma or the extracellular space, primarily due to the fact that most protein drugs are large in size and have limited mobility, secondary to impaired passage through biomembranes [33]. Active tissue uptake and binding to intra- and extravascular proteins, however, can substantially increase the volume of distribution of protein drugs, as reflected by the relatively large volume of distribution of up to 2.8 l/kg for interferon beta-1b [48]. Additionally, binding to other endogenous proteins may also affect the distribution of protein drugs.

In contrast to small-molecule drugs, protein transport from the vascular space into the interstitial space of tissues is largely mediated by convection rather than

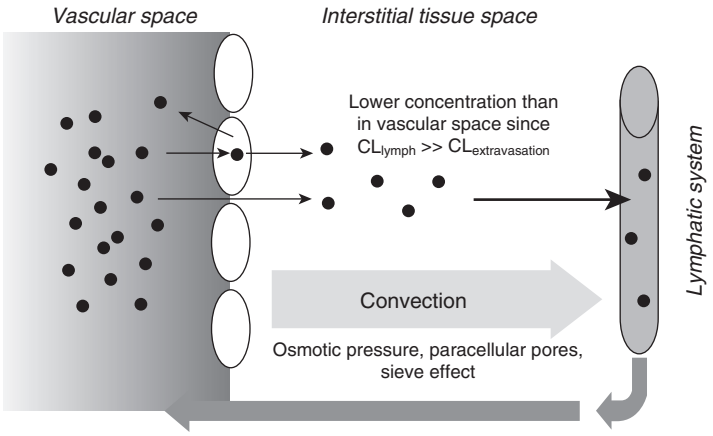

Figure 14.2 Distribution mechanisms of therapeutic proteins: convective extravasation rather than diffusion.

diffusion, following the unidirectional fluid flux from the vascular space through paracellular pores into the interstitial tissue space (Figure 14.2). The subsequent removal from the tissues is accomplished by lymph drainage back into the systemic circulation [49]. This underlines the unique role the lymphatic system plays in the disposition of protein therapeutics, as already mentioned in the previous section. Another, but much less prominent pathway for the movement of protein molecules from the vascular to the interstitial space is transcellular migration via endocytosis [50].

The plasma concentration–time profiles for proteins after i.v. administration usually follow a biphasic decline that can be best described by a two-compartment pharmacokinetic model [21]. The central compartment in this two-compartment model represents primarily the vascular space and the interstitial space of well-perfused organs with permeable capillary walls, especially the liver and kidneys, while the peripheral compartment comprises the interstitial space of poorly perfused tissues such as skin and inactive muscle [12]. Generally, the central compartment volume of protein drugs after intravenous administration is typically equal to or slightly larger than the plasma volume of 3–8 l. The steady-state volume of distribution frequently comprises 14–20 l, not more than twice the initial volume of distribution [21, 51]. For rFVIIa after i.v. administration, the central volume of distribution (Vc) has been reported as 0.089 l/kg (6.2 l for a 70 kg subject) and the steady-state volume of distribution (Vss) as 0.130–0.165 l/kg (9.1–11.6 l for a 70 kg subject) [16, 52]. Similarly, this distribution pattern has been reported for SB 249417, a humanized anti-factor IX antibody, with a Vc of 0.092 l/kg (6.4 l for a 70 kg subject) and a Vss of 0.15 l/kg (10.5 l for a 70 kg subject) [53], and for the tPA analog tenecteplase with a Vc of 4.2–6.3 l and a Vss of 6.1–9.9 l [54]. In addition, the central volume of distribution for epoetin-α and golimumab, a human anti-tumor necrosis factor alpha monoclonal antibody, following an i.v. administration

has been reported as 0.056 l/kg (3.9 l for a 70 kg subject) and 3.1 l, respectively, similar to the plasma volume [55, 56].

It should be stressed that pharmacokinetic calculations of volume of distribution may be problematic for many protein therapeutics [12, 57]. Noncompartmental determination of volume of distribution at steady state (Vss) using statistical moment theory assumes first-order disposition processes with elimination occurring from the rapidly equilibrating or central compartment [58, 59]. These basic assumptions, however, are not fulfilled for most protein therapeutics, as proteolysis and receptor-mediated elimination in peripheral tissues may constitute a substantial fraction of the overall elimination process. Thus, Vss values reported for protein drugs in the literature should be interpreted with caution.

In contrast to conventional drugs, distribution, elimination, and pharmacodynamics are frequently interrelated for peptides and proteins. The generally low volume of distribution should not necessarily be interpreted as low tissue penetration. Receptor-mediated specific uptake into the target organ, as one mechanism, can result in therapeutically effective tissue concentrations despite a relatively small volume of distribution [60]. Nartogastrim, a recombinant derivative of the granulocyte colony-stimulating factor (G-CSF), for example, is characterized by a specific, dose-dependent and saturable tissue uptake into the target organ bone marrow, presumably via receptor-mediated endocytosis [61].

14.2.3 Protein Binding

It is a general pharmacokinetic principle, which is also applicable to peptides and proteins, that only the free, unbound fraction of a drug substance is accessible to distribution and elimination processes as well as interactions with its target structure (e.g., receptor) at the site of action. Hence, the activity of a drug is better characterized by its free rather than total concentration if there is no constant relationship between free and total drug concentration.

Physiologically active endogenous peptides and proteins frequently interact with specific binding proteins that are involved in their transport and regulation. Furthermore, interaction with binding proteins may enable or facilitate cellular uptake processes and thus affect the drug's pharmacodynamics. Specific protein binding has been reported for a variety of protein drugs, such as growth hormone [62], recombinant human DNases for use as mucolytics in cystic fibrosis [63], and recombinant human vascular endothelial growth factor (rhVEGF) [64]. Protein binding may affect the disposition properties of protein therapeutics, but many times it may also either prolong the circulation time of proteins by acting as a storage depot or enhance the elimination of proteins. For example, various cytokine-binding proteins including soluble cytokine receptors and anti-cytokine antibodies, have been identified for the binding of recombinant cytokines following i.v. administration [65].

Six specific binding proteins were identified for IGF-1, with one binding at least 95% of IGF-1 in plasma. Since the binding affinity of IGF-1 to this binding protein

is substantially higher than to IGF receptors, the binding protein is assumed to have a reservoir function that protects the body from insulin-like hypoglycemia. Furthermore, the elimination half-life for bound IGF-1 is significantly longer than for free IGF-1, as only the unbound IGF-1 is accessible to elimination via glomerular filtration or peritubular extraction [60, 66].

Somatotropin, another example, has at least two binding proteins in plasma [67]. This protein binding substantially reduces somatotropin elimination with a tenfold smaller clearance of total compared with free somatotropin, but also decreases its activity via reduction of receptor interactions.

Apart from these specific bindings, peptides and proteins may also be non-specifically bound to plasma proteins. For example, metkephamid, a met-enkephalin analog, was described to be 44–49% bound to albumin [68], and octreotide is up to 65% bound to lipoproteins [36].

14.2.4 Protein Elimination

Protein drugs, such as endogenous or dietetic proteins, are generally eliminated through the same metabolic pathways, leading to amino acids that are reutilized in the endogenous amino acid pool for the *de novo* biosynthesis of structural or functional proteins. Therefore, detailed investigations on the metabolism of protein drugs are usually not performed since myriads of potential molecular fragments may be formed.

The elimination of peptides and proteins can occur unspecifically nearly everywhere in the body or can be limited to a specific organ or tissue. Locations of intensive peptide and protein metabolism are liver, kidneys, gastrointestinal tissue, but also blood and other body tissues. Molecular weight determines the major metabolism site as well as the predominant degradation process [21, 69] (Table 14.1). Owing to the unspecific degradation of many peptides and proteins in blood, clearance can exceed cardiac output, that is, >5 l/min blood clearance and >3 l/min for plasma clearance [21].

Non-metabolic elimination pathways such as renal or biliary excretion are negligible for most peptides and proteins. Amino acids, as well as some peptides and proteins such as immunoglobulin A, however, are excreted into the bile [70]. For octreotide, biliary excretion is an important elimination pathway, at least in rat and dog [36]. If biliary excretion of peptides and proteins occurs, it generally results in subsequent metabolism of these compounds in the gastrointestinal tract [21].

14.2.4.1 Proteolysis

Proteolytic enzymes such as proteases and peptidases are ubiquitously available throughout the body, but are especially localized in blood, in the vascular endothelium, but also on cell membranes and within cells, for example in lysosomes. Thus, intracellular uptake is *per se* more an elimination rather than a distribution process [71]. While proteolytic enzymes in the gastrointestinal tract and in lysosomes are relatively unspecific, soluble peptidases in the interstitial space and

Table 14.1 Molecular weight as the major determinant of the elimination mechanisms of peptides and proteins. As indicated, mechanisms may overlap. Endocytosis may occur at any molecular weight range. Modified from [60, 70].

Molecular weight	Elimination site	Predominant elimination mechanisms	Major determinant
<500	Blood, liver	Extracellular hydrolysis Passive lipoid diffusion	Structure, lipophilicity
500–1 000	Liver	Carrier-mediated uptake Passive lipoid diffusion	Structure, lipophilicity
1 000–50 000	Kidney	Glomerular filtration and subsequent degradation processes (see Figure 14.2)	Molecular weight
50 000–200 000	Kidney, liver	Receptor-mediated endocytosis	Sugar, charge
200 000–400 000		Opsonization	α_2-macroglobulin, IgG
>400 000		Phagocytosis	Particle aggregation

exopeptidases on the cell surface have a higher selectivity and determine the specific metabolism pattern of an organ [70]. In addition, molecular weight is one of the major determining factors for metabolic rate of peptides and proteins drugs, as the metabolic rate generally increases with decreasing molecular weight. Other factors include size, overall charge, lipophilicity, functional groups, glycosilation pattern, secondary and tertiary structure, and propensity for particle aggregation [72]. The proteolytic activity of subcutaneous tissue, for example, results in a partial loss of activity of the subcutaneously compared with the intravenously administrated interferon-γ.

14.2.4.2 **Gastrointestinal Protein Metabolism**

Major factors limiting systemic bioavailability of orally administered protein drugs include acidic degradation and protein metabolism in the gastrointestinal tract. Dietary proteins and protein therapeutics are equally digested to amino acids by proteolytic enzymes. The metabolic activity of the gastrointestinal tract, however, is not limited to orally administered proteins. Parenterally administered peptides and proteins may also be metabolized in the intestinal mucosa following intestinal secretion. At least 20% of the degradation of endogenous albumin takes place in the gastrointestinal tract [21].

14.2.4.3 **Renal Protein Metabolism**

The kidneys constitute a major site of metabolism for many smaller sized proteins that undergo glomerular filtration. Glomerular filtration is generally the dominant, rate-limiting step in renal metabolism of protein drugs, with a cut-off value of

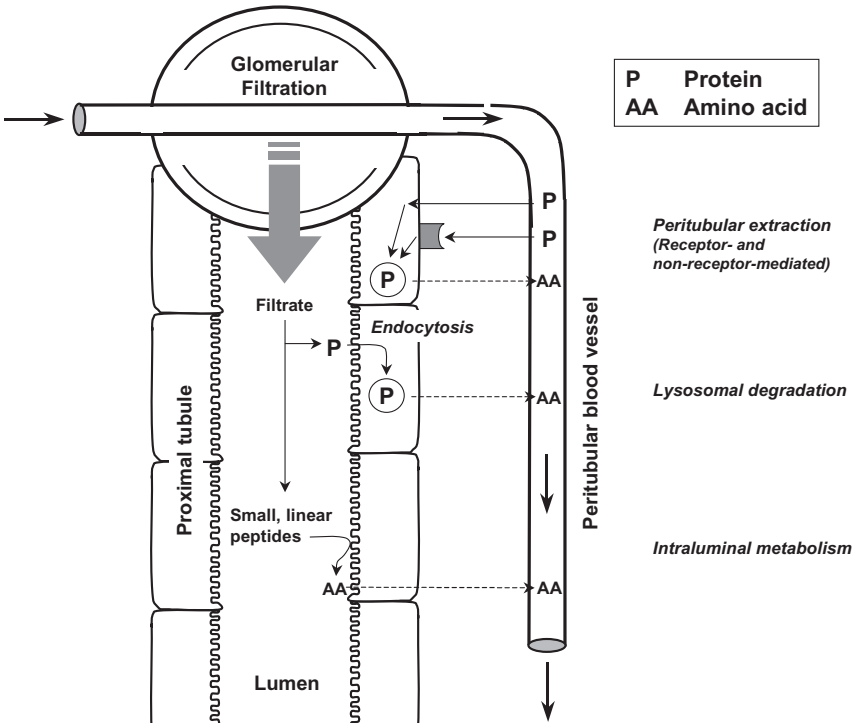

Figure 14.3 Renal elimination processes of peptides and proteins: glomerular filtration, intraluminal metabolism, tubular reabsorption with intracellular lysosomal metabolism and peritubular extraction with intracellular lysosomal metabolism. Modified from [75].

approximately 60 kDa for molecular weight. In addition, molecular conformation and charge of proteins also contribute to the selectivity of glomerular filtration. For example, cationic macromolecules pass through the capillary wall more readily than neutral macromolecules, while neutral macromolecules pass through more readily than anionic macromolecules [73, 74].

Various renal processes contribute to the elimination of peptides and proteins (Figure 14.3). For most substances, glomerular filtration is the dominant, rate-limiting step as subsequent degradation processes are not saturable under physiologic conditions [75]. Hence, the renal contribution to the overall elimination of peptides and proteins is reduced if the metabolic activity for these proteins is high in other body regions, and it becomes negligible in the presence of unspecific degradation throughout the body. In contrast to this, the contribution to total clearance approaches 100% if the metabolic activity is low in other tissues or if distribution is limited. For recombinant IL-10, for instance, elimination correlates closely with glomerular filtration rate, making dosage adjustments necessary in patients with impaired renal function [76].

After glomerular filtration, small linear peptides such as bradykinin or glucagon undergo intraluminal metabolism, predominantly by exopeptidases in the luminal

brush border membrane of the proximal tubules. The resulting amino acids are transcellularly transported back into the systemic circulation [60]. Larger peptides and proteins, such as IL-2, IL-11, and insulin, are actively reabsorbed in the proximal tubules via endocytosis. This cellular uptake is followed by addition of lysosomes and hydrolysis to peptide fragments and amino acids, which are returned to the systemic circulation by proton driven peptide transporters PEPT2 and to a lesser degree PEPT1 [77]. Therefore, only minuscule amounts of intact protein are detectable in urine. An additional renal elimination mechanism is peritubular extraction from post-glomerular capillaries with subsequent intracellular metabolism, which has for example been described for vasopressin, calcitonin, and growth hormone [70, 75, 78, 79].

14.2.4.4 **Hepatic Protein Metabolism**

Apart from general proteolysis and the kidneys, the liver substantially contributes to the metabolism of peptide and protein drugs. Proteolytic degradation usually starts with endopeptidases that attack in the middle part of the protein, and the resulting oligopeptides are then further degraded by exopeptidases. The ultimate metabolites of proteins, amino acids, and dipeptides, are finally reutilized in the endogenous amino acid pool. The rate of hepatic metabolism is largely dependent on the specific amino acid sequences in the protein [60].

As proteolytic enzymes in the hepatocytes are mainly responsible for the catabolism of proteins in the liver, intracellular uptake of proteins into the hepatocytes is a prerequisite for hepatic protein metabolism. While small peptides may cross the hepatocyte membrane via passive diffusion if they have sufficient hydrophobicity, various carrier-mediated energy dependent membrane transporters and receptor-mediated endocytosis are usually responsible for the uptake of larger peptides and proteins. An example of such a receptor mediated uptake has been reported in radio-iodinated tissue plasminogen activator (tPA) studies. Mannose and asialoglycoprotein receptors were identified to facilitate tPA uptake and clearance in the liver. In addition, the low density lipoprotein receptor-related protein, a hepatic membrane receptor, was suggested to contribute to the overall tPA clearance [80, 81].

14.2.4.5 **Receptor-Mediated Protein Metabolism**

While receptor binding is usually negligible for conventional small molecule drugs and rarely affects their pharmacokinetic profiles, a substantial fraction of a protein drug can be bound to receptors. This binding can lead to elimination through receptor-mediated uptake and subsequent intracellular metabolism. The endocytosis process is not limited to hepatocytes, but can occur in other cells as well, including the therapeutic target cells.

Although any tissue that expresses these generally high affinity, low-capacity binding receptors for the drug can contribute to the protein drug's elimination, the limited capacity of the protein drug receptors can usually result in saturable and dose-dependent clearance of protein drugs within therapeutic concentrations, or more specifically at relatively low molar ratios between the protein drug and

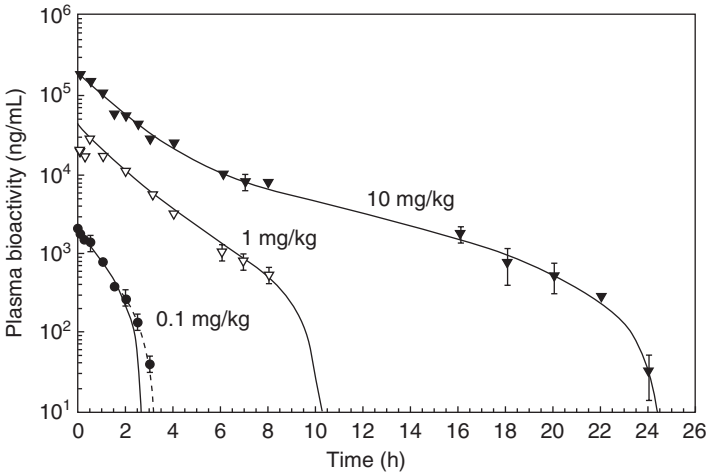

Figure 14.4 Nonlinear pharmacokinetics of M-CSF, presented as measured and modeled plasma concentration–time curves (mean ± SE) after intravenous injection of 0.1 mg/kg ($n = 5$), 1.0 mg/kg ($n = 3$), and 10 mg/kg ($n = 8$) in rats. From [84]. Reprinted with permission from the American Society for Pharmacology and Experimental Therapeutics.

the receptor [82]. Thus, receptor-mediated elimination contributes as a major factor to the nonlinear pharmacokinetic behavior of many peptide and protein drugs, that is, a lack of dose proportionality [8].

An example of such receptor-mediated elimination is demonstrated in the studies of the granulocyte colony-stimulating factor (G-CSF) derivative nartograstim. Nonlinear clearance by the bone marrow and spleen might be due to the down-regulation of G-CSF receptors on the cell surface with increasing doses of nartograstim [61, 83].

Another example is the nonlinear elimination clearance of recombinant human macrophage colony-stimulating factor (M-CSF). At low concentrations, dose proportional pharmacokinetics of M-CSF was observed with a combination of linear renal elimination and a receptor mediated uptake into macrophages, while at high concentrations, nonlinear pharmacokinetic behavior was observed due to the saturation of the nonrenal elimination pathway (Figure 14.4) [84, 85].

14.2.5
Role of the Neonatal Fc-Receptor in the Disposition of Proteins

Immunoglobulin G (IgG) based monoclonal antibodies and their derivatives constitute one of the most important classes of protein therapeutics, with many members currently being under development or in therapeutic use. Interaction with the neonatal Fc receptor (FcRn) constitutes a major component in the drug disposition of IgG molecules and their derivatives [86]. FcRn has been well described in the transfer of passive humoral immunity from a mother to her fetus

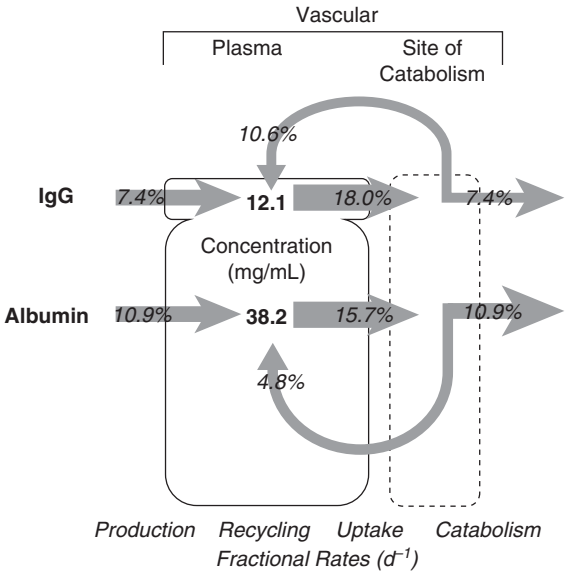

Figure 14.5 Effect of FcRn mediated recycling on IgG and albumin turnover in humans expressed as fractional rates. Shown are homeostatic plasma concentrations (12.1 and 38.2 mg/ml), fractional catabolic rates (7.4 and 10.9%/day), the FcRn-mediated fractional recycling rates (10.6 and 4.8%/day), and the fractional production rates (7.4 and 10.9%/day). The figure is to scale: areas for plasma amount and arrow widths for rates. From [89]. Reprinted with permission from Elsevier.

by transferring IgG across the placenta in humans and the proximal small intestine via transcytosis in rodents. More importantly, interaction with FcRn in a variety of cells, including endothelial cells, monocytes, macrophages, and other dendritic cells, protects IgG from lysosomal catabolism and thus constitutes a salvage pathway for IgG molecules that have been internalized in these cells types. This is facilitated by intercepting IgG in the endosome and recycling it to the systemic recirculation [87]. The interaction with the FcRn receptor thereby prolongs the elimination half-life of IgG, with a more pronounced effect the stronger the binding of the Fc fragment of the antibody is to the receptor.

Based on the affinity of this binding interaction, human IgG1, IgG2, and IgG4 have a half-life in humans of 18–21 days, whereas the less strongly bound IgG3 has a half-life of only 7 days and murine IgG in humans has a half-life of 1–2 days [88]. Similar to IgG, FcRn is also involved in the disposition of albumin molecules. The kinetics of IgG and albumin recycling is illustrated in Figure 14.5. For IgG1, approximately 60% of the molecules taken up into lysosomes are recycled, for albumin 30%. As FcRn is responsible for the extended presence of IgG, albumin and other Fc- or albumin-conjugated proteins in the systemic circulation, modulation of the IgG–FcRn interaction or the albumin–FcRn interaction will deliberately control the half-life of these molecules [89].

14.3 Immunogenicity and Protein Pharmacokinetics

Immunogenicity is the capacity to elicit an immune response and lead to antibody formation against the therapeutic protein, and is one of the major safety concerns for protein drugs. Various clinical manifestations include: either increased or decreased efficacy, neutralization of endogenous proteins and generalized immune effects, such as allergy, anaphylaxis or serum sickness, even life-threatening events [90–92]. Immunogenicity is distinguished into: (i) classic vaccine-type immune responses resulting in anaphylactic reactions with memory-effect and (ii) immune responses secondary to chronic exposure that develop slowly over time, have no memory effect, and are related to breaking of the B-cell tolerance towards endogenous proteins.

Antibody formation is a frequently observed phenomenon during chronic dosing with protein therapeutics, especially if the drugs were derived from animal proteins. The presence of antibodies can obliterate the biological activity of a peptide or protein drug. Also, complexation with anti-drug antibodies (ADA) may modify its pharmacokinetic profile. Administration of monoclonal antibodies of murine origin can lead to the development of a human anti-mouse immunoglobulin antibody (HAMA) response, which is in most cases directed against the constant regions of the monoclonal antibody. Genetically engineered mouse–human chimeric antibodies, humanized antibodies or fully human antibodies minimize the fraction of the molecule that is of murine origin, thereby trying to minimize the immunogenicity of these compounds in humans [93]. Nevertheless, even for fully human monoclonal antibodies, ADA formation has been reported.

Extravascular injection is known to stimulate antibody formation more than intravenous application, most likely due to the increased immunogenicity of protein aggregates and precipitates formed at the injection site [94]. The presence of antibodies can obliterate the biological activity of a protein drug. In addition, protein–antibody complexation can also modify the distribution, metabolism and excretion, that is, the pharmacokinetic profile, of the protein drug. Elimination can either be increased or decreased. Faster elimination of the complex occurs if the reticuloendothelial system is stimulated. Elimination is slowed down if the antibody–drug complex forms a depot for the protein drug. This effect would prolong the drug's therapeutic activity, which might be beneficial if the complex formation does not decrease therapeutic activity [60, 95]. Furthermore, antibody binding may also interfere with bioanalytical methods, such as immunoassays.

The development of the anticancer agent pegaspargase is a successful example of reduction of immunogenicity towards L-asparaginase using PEG conjugation techniques [96]. Through steric hindrance, PEG conjugation can shield antigenic determinants on the drug from detection by the immune system [97].

Although some important factors that contribute to the immunogenicity of biopharmaceuticals have been identified, many other factors certainly remain unknown. As the occurrence of immunogenicity remains unpredictable, it is

impossible to adequately power clinical studies to identify immunogenicity *a priori* [92]. Despite the ever-expanding toolbox of analytical methods for evaluating biopharmaceuticals, the immune system appears more sensitive to alterations in products than the array of physical tests and bioassays available. In view of the limitations of the analytical methods, the lack of a standard immunogenicity assay for many biopharmaceuticals, and even the lack of consistent results between laboratories using the same assay, immunogenic safety can only be assessed thoroughly on the basis of clinical data, including post-marketing data.

14.4 Exposure–Response Correlations for Protein Therapeutics

Pharmacokinetic–pharmacodynamic modeling (PK/PD modeling) is a technique that combines the two classical pharmacologic disciplines of pharmacokinetics and pharmacodynamics. The combination of pharmacodynamics with pharmacokinetics by so-called integrated PK/PD modeling allows deriving pharmacokinetic and pharmacodynamic model parameters that characterize the dose–concentration–effect relationship and allows a continuous description of the time course of effect intensity for a specific drug based on measured concentration and effect data following a certain dosage regimen (Figure 14.1) [6, 7].

Since peptide and protein therapeutics are usually highly potent compounds with steep dose–effect curves, a careful characterization of the dose–concentration–effect relationship should receive particular emphasis during the preclinical and clinical drug development process. Integrated PK/PD modeling approaches have been widely applied to describe the dose–concentration–effect relationship for protein drugs [98, 99]. PK/PD modeling not only allows for a continuous description of the time course of effect as a function of the dosing regimen and a comprehensive summary of the available data, but also allows untested dosage regimens and trial designs in simulation-based exercises to be tested.

The application of PK/PD modeling is beneficial in all phases of preclinical and clinical drug development, with a focus on dosage optimization and identification of covariates that are causal for intra- and inter-individual differences in drug response and/or toxicity [10], and has been endorsed by the US Food and Drug Administration [100]. Mechanism-based PK/PD modeling, appreciating the physiological events involved in the elaboration of the observed effect, has been promoted as a superior modeling approach as compared with empirical modeling. In particular, this is because it not only describes observations but also offers some insight into the underlying biological processes involved, and thus provides flexibility in extrapolating the model to other clinical situations [6, 101, 102]. Since the molecular mechanism for the action of peptide and protein drugs is generally well understood, it is often straightforward to transform this available knowledge into a mechanism-based PK/PD modeling approach that appropriately characterizes the real physiological process leading to the drug's therapeutic effect.

In the following, the application of the four common PK/PD modeling classes for proteins will be discussed in more detail: direct link models, indirect link models, indirect response models, and cell life span models.

14.4.1 Direct Link PK/PD Models

While drug concentrations are usually analytically quantified in plasma, serum, or blood, the magnitude of the observed response is determined by the concentration of the drug at its effect site, the site of action in the target tissue [7]. The relationship between the drug concentration in plasma and at the effect site may be either constant or undergo time-dependent changes. If equilibrium between both concentrations is rapidly achieved or the site of action is within the plasma, serum or blood, there is practically a constant relationship between both concentrations, with no temporal delay between the plasma and the effect site. In this case, measured concentrations can directly serve as input for a pharmacodynamic model (Figure 14.6). The most frequently used direct link pharmacodynamic model is the sigmoid E_{max}-model:

$$E = \frac{E_{max} \cdot C^n}{EC_{50}^n + C^n}$$

where

E_{max} is the maximum achievable effect
C is the drug concentration at the effect site

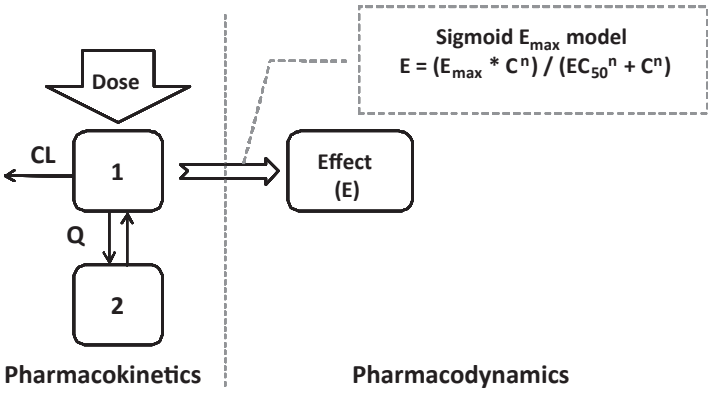

Figure 14.6 Schematic of a typical direct link PK/PD model. The PK model is a typical two-compartment model with a linear elimination clearance from the central compartment (*CL*) and a distributional clearance (*Q*). The sigmoid E_{max}-relationship is characterized by the pharmacodynamic parameters: E_{max}, the maximum achievable effect; EC_{50}, the concentration of the drug that produced half of the maximum effect; and the Hill coefficient *n*.

EC_{50} is the concentration of the drug that produces half of the maximum effect
n is the Hill coefficient, a shape factor that allows for an improved fit of the relationship to the observed data

Thus, a direct link model directly connects measured concentration to the observed effect without any temporal delay [5, 7].

Racine-Poon *et al.* provided an example for a direct link model by relating the serum concentration of the anti-human immunoglobulin E (IgE) antibody CGP 51901 for the treatment of seasonal allergic rhinitis to the reduction of free IgE via an inhibitory E_{max}-model [103]. Radwanski *et al.* used a similar approach to assess the effect of recombinant interleukin-10 on the *ex vivo* release of the pro-inflammatory cytokines TNF-α and interleukin-1β in LPS-stimulated leukocytes [104].

14.4.2
Indirect Link PK/PD Models

The concentration–effect relationship of many protein drugs, however, cannot be described by direct link PK/PD models, but is characterized by a temporal dissociation between the time courses of the plasma concentration and effect. In this case, the concentration maxima would occur before the effect maxima, effect intensity would increase despite decreasing plasma concentrations and would persist beyond the time the drug concentrations in the plasma are no longer determinable. The relationship between measured concentration and observed effect follows a counterclockwise hysteresis loop. This phenomenon can be caused by either an indirect response mechanism (see Section 14.4.3) or by a distributional delay between the concentrations in the plasma and at the effect site. The latter can conceptually be described by an effect–compartment model, which attaches a hypothetical effect compartment to a pharmacokinetic compartment model. The effect compartment does not account for mass balance and only defines the changes in concentration at the effect site via the time course of the effect itself (Figure 14.7) [5, 105].

An effect-compartment approach was, for example, applied by Gibbons *et al.* to quantify the reduction in mean arterial blood pressure by the anti-adrenergic peptoid CHIR2279 [106]. The same concept was used by Pihoker *et al.* to characterize the relationship between the serum concentrations of the somatotropin releasing peptide GHRP-2 and somatotropin [107].

14.4.3
Indirect Response PK/PD Models

The effect of many biotech drugs, however, is not mediated via a direct interaction between drug concentration and response systems, but frequently involves several transduction processes that include, at their rate-limiting step, the stimulation or inhibition of a physiological process, for example, the synthesis or degradation of a molecular response mediator such as a hormone or cytokine. In these cases,

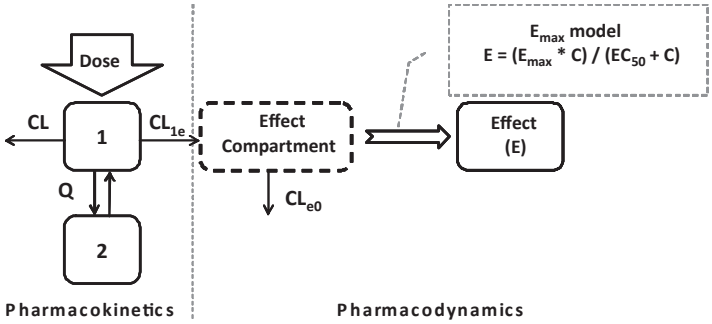

Figure 14.7 Schematic of a typical indirect link PK/PD model. A hypothetical effect compartment is linked to the central compartment of a two-compartmental pharmacokinetic model. The concentration in the effect compartment (CL_e) drives the intensity of the pharmacodynamic effect (E) via an E_{max}-relationship. CL_{1e} is the transfer clearance from the central to the effect compartment, CL_{e0} the equilibrium clearance for the effect compartment. All other PK and PD parameters are identical to those used in Figure 14.6.

mechanism-based indirect response models should be used that appreciate the underlying physiological process involved in mediating the observed effect. Indirect response models generally describe the effect on a representative response parameter via the dynamic equilibrium between increase or synthesis and decrease or degradation of the response, with the former being a zero-order and the latter a first-order process. Each of these processes can the stimulated or inhibited in four derived basic model variants, as outlined in Figure 14.8 [108–110].

An indirect response model was used, for example, to describe the delayed effect of TRX1 on CD4 receptors on T-cells (Figure 14.9) [111]. TRX1 is a humanized anti-CD4 IgG1 monoclonal antibody developed to induce immune tolerance by blocking CD4-mediated functions. After administration by intravenous infusion, TRX1 binds to the CD4 receptors on T-cells, thereby leading to their blockade and down-regulation (Figure 14.10). The elimination of TRX1 from the central compartment (serum) is mediated through both a nonspecific elimination pathway (K_{el}) and a specific CD4 receptor-mediated internalization (K_{int}). The CD4-mediated internalization has been suggested as one pathway by which CD4 is down-modulated.

14.4.4 Cell Life Span Models

Cell life span models are mechanism-based, physiological PK/PD models that assume the sequential maturation and life span-driven cell turnover of their affected cell types and progenitor cell populations. These models have been widely used for characterizing the dose–concentration–effect relationships of hematopoietic growth factors that modulate erythropoiesis, granulopoiesis, or thrombopoiesis [112, 113]. Protein therapeutics can regulate blood and/or immune cell types

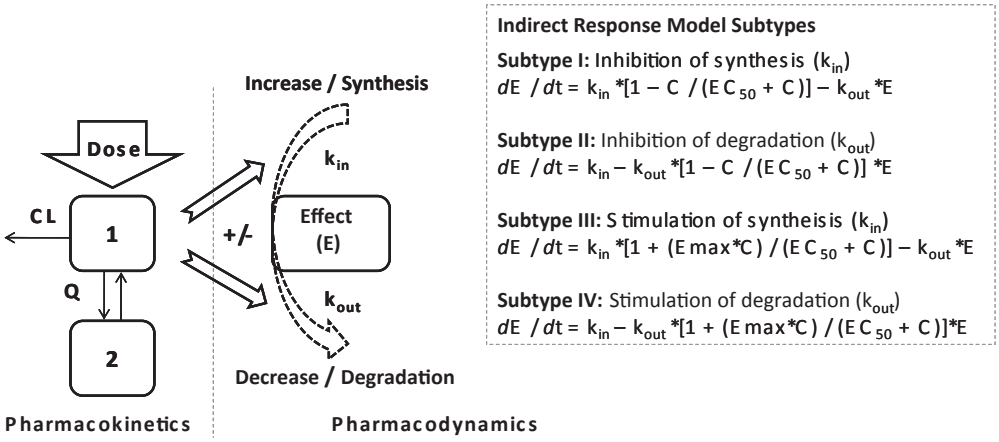

Figure 14.8 Schematic of a typical indirect response PK/PD model. The effect measure (E) is maintained by a dynamic equilibrium between an increase or synthesis and a decrease or degradation process. The former is modeled by a zero-order process with rate constant k_{in}, the latter by a first-order process with rate constant k_{out}. Thus, the rate of change in effect (dE/dt) is expressed as the difference between synthesis rate (k_{in}) and degradation rate (k_{out} times E). Drug concentration (C) can stimulate or inhibit the synthesis or the degradation process for the effect (E) via an E_{max}-relationship using one of four subtypes (model I, II, III, or IV) of the indirect response model. The pharmacokinetic model and all other PK and PD parameters are identical to those used in Figure 14.6.

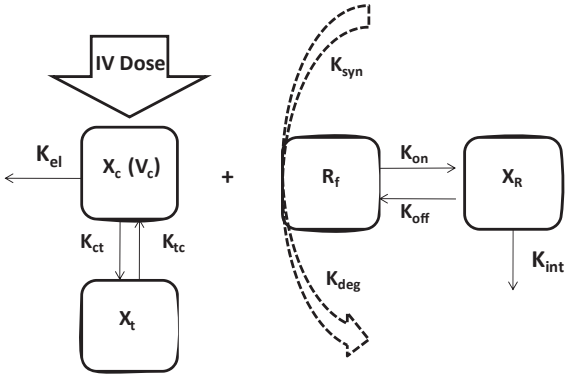

Figure 14.9 A PK/PD model describing the relationship between TRX1 concentration and CD4 receptors on T-cells. X_c and V_c are the amount and volume of distribution of TRX1 in the central compartment, respectively. X_t is the amount of TRX1 in the tissue compartment. K_{tc} and K_{ct} are the first-order distribution rate constants. The elimination of TRX1 from the central compartment has been suggested to be through both a nonspecific elimination pathway (K_{el}) and a specific CD4 receptor-mediated internalization (K_{int}). The zero-order synthesis rate and first-order elimination rate constants of free CD4 receptor (R_f) is described as K_{syn} and K_{deg}, respectively. The free CD4 receptor interacts with TRX1 to form a TRX1–CD4 receptor complex (XR), which is followed by cellular internalization. K_{on} and K_{off} are the association and dissociation rate constants for the reversible TRX1–CD4 receptor binding, respectively. Modified from [111].

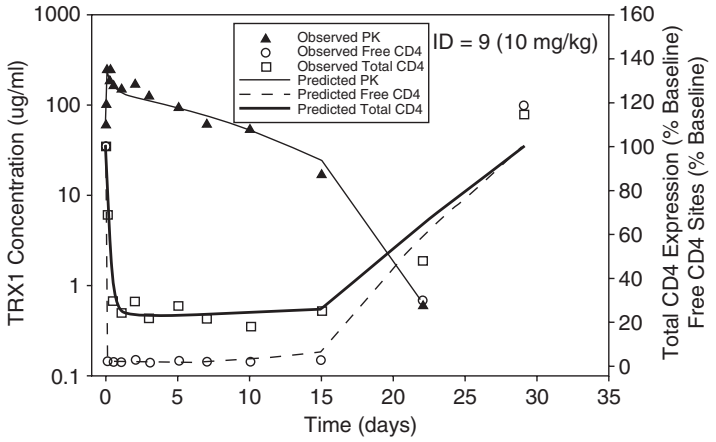

Figure 14.10 Measured and model predicted time courses of TRX1 serum concentrations and free and total CD4 after intravenous administration of 10 mg/kg TRX1 in a representative subject. Modified from [111] with permission from Springer.

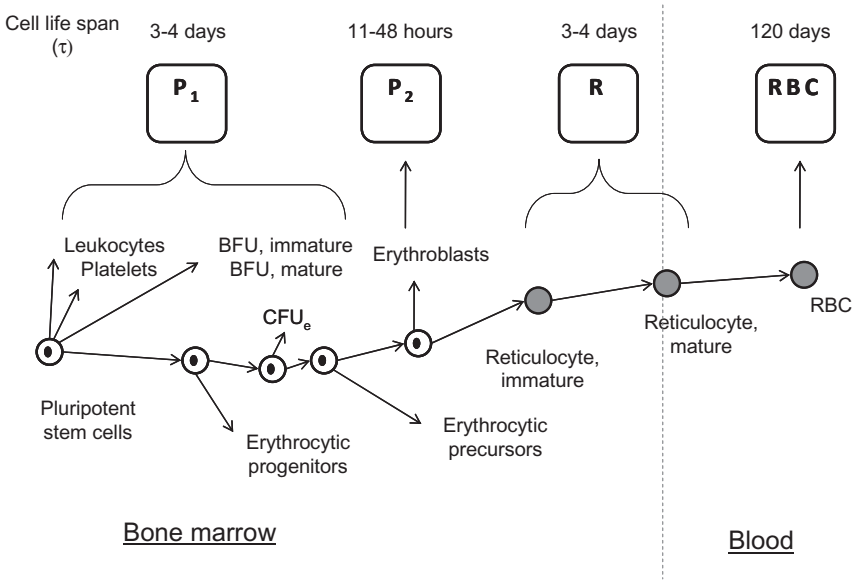

Figure 14.11 Process of erythropoiesis. Erythropoietin stimulates the proliferation and differentiation of the erythrocyte progenitors (BFU, burst-forming unit erythroid; CFUe, colony-forming unit erythroid) as well as the erythroblasts in the bone marrow. The life spans (τ) of the various cell populations are indicated at the top. See explanations of P1, P2, and RBC in Figure 14.12. Modified from [55].

through direct or indirect modulation. A prolonged delay usually exists between protein drug administration and the observed response, that is,, change in the cell count in peripheral blood. As the fixed physiological time span for the maturation of precursor cells is the major reason for this delay, cell life span models accommodate this sequential maturation of several precursor cell populations by a series of transit compartments linked via first- or zero-order processes with a common transfer rate constant.

The effect of recombinant human erythropoietin (rhEPO), which can stimulate the production and release of reticulocytes from the bone marrow, has been described by a cell life span model [55]. The process of erythropoiesis and the applied PK/PD approach including a cell life span model are depicted in Figures 14.11 and 14.12, respectively. In the study, rhEPO was given as a multiple dose

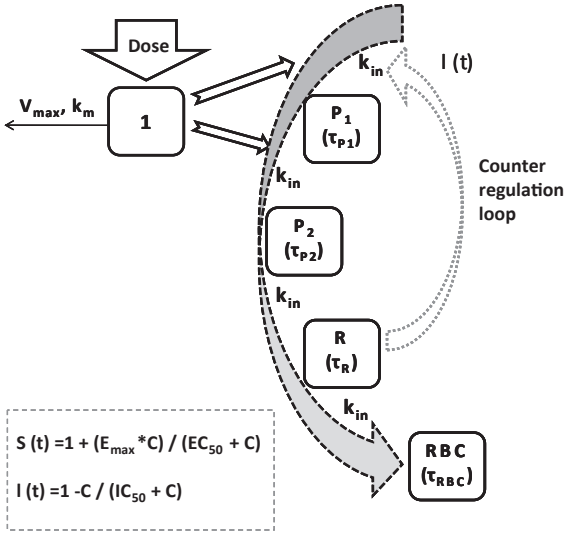

Figure 14.12 A PK/PD model describing the disposition of recombinant human erythropoietin and effects on reticulocyte count, red blood cell count, and hemoglobin concentration. The PK model is a one-compartment model with Michaelis–Menten type elimination (k_m, V_{max}) from the central compartment. The PD model is a cell life span model with four sequential cell compartments, representing erythroid progenitor cells (P1), erythroblasts (P2), reticulocytes (R), and red blood cells (RBC). τP1, τP2, τR, and τRBC are the corresponding cell life spans, k_{in} the common zero-order transfer rate between cell compartments. The target parameter hemoglobin in the blood (Hb) is calculated from the recticulocyte and red blood cell count and the hemoglobin content per cell. The effect of erythropoietin is modeled as a stimulation of the production of both precursor cell populations (P1 and P2) in the bone marrow with the stimulation function $S(t)$. E_{max} is the maximum possible stimulation of reticulocyte production by erythropoietin, EC_{50} the plasma concentration of erythropoietin that produces half-maximum stimulation. A counter-regulatory feedback loop represents the feedback inhibition of reticulocytes on their own production by reducing the production rate of cells in the P1 compartment via the inhibitory function $I(t)$. IC_{50} is the reticulocyte count that produced half of complete inhibition. Modified from [55].

regimen of once-weekly dosing at 600 IU/kg by s.c. injection. The stimulation of the maturation of two progenitor cell populations (P1 and P2 in Figures 14.11 and 14.12) by rhEPO and a feedback inhibition between erythrocyte count and progenitor proliferation were included in this model. The corresponding measured and modeled time courses for reticulocytes, red blood cells, and hemoglobin are shown in Figure 14.13.

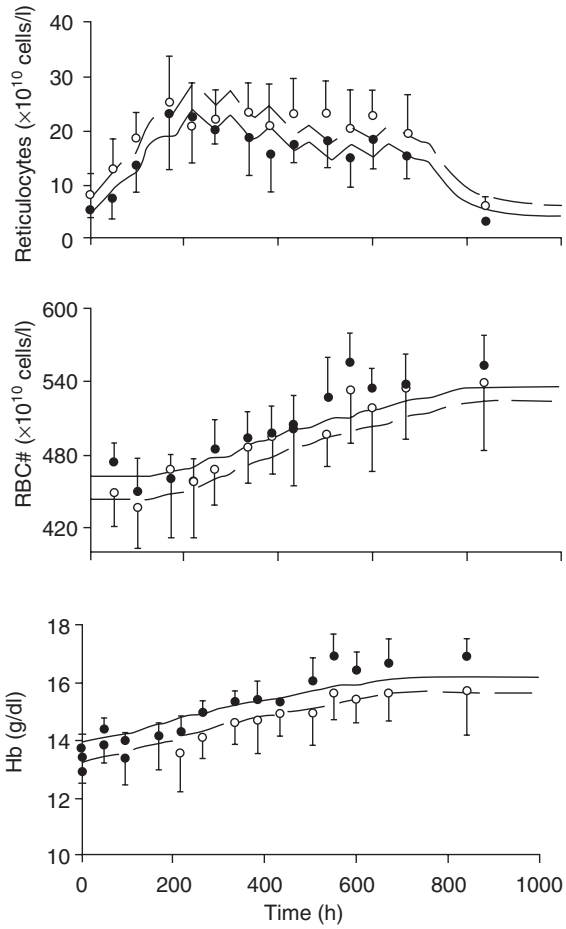

Figure 14.13 Reticulocyte, red blood cell (RBC), and hemoglobin (Hb) responses after multiple subcutaneous (s.c.) dosing of 600 IU/kg/week recombinant human erythropoietin (rHuEpo). Solid and open circles represent data for males and females, whereas the solid and broken lines for the reticulocytes are model fittings. The solid lines in the RBC and Hb panels are the predictions using the model-fitted curves for the reticulocytes and the life span parameters. Modified from [55]. Copyright © 2004 American College of Clinical Pharmacology, with permission from SAGE Publications.

14.5 Summary and Conclusions

In analogy to conventional small-molecule drugs, protein-based therapeutics are characterized by well-defined pharmacokinetic properties that form the basis for the design of therapeutic dosing regimens as well as drug delivery strategies. Potential caveats and pitfalls, however, may arise from their similarity to endogenous and/or dietary molecules with which they share common drug disposition pathways, their oftentimes high specificity for endogenous target structures, and their immunogenic potential. Additional resources may be necessary during the drug development process to overcome some of these obstacles.

The widespread application of pharmacokinetic and exposure-response concepts in drug development has repeatedly been promoted by industry, academia, and regulatory authorities [10, 100]. It is believed that the application of PK/PD-based concepts in all preclinical and clinical drug development phases may substantially contribute to a more scientifically driven, evidence-based development process. Model-based drug development is a new paradigm based on a combined mathematical and statistical approach to integrate knowledge from all aspects of drug development, from drug discovery to post-marketing, thereby serving as a key decision-making tool, enabling rational, scientifically based choices at critical decision points [11]. Exposure-response assessments form the backbone for the model-based drug development approach [114]. Thus, in-depth knowledge of a compound's pharmacokinetic and pharmacodynamic characteristics will also continue to form a cornerstone in a rapid, cost-efficient, and successful drug development program for protein-based therapeutics.

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Part Three
Vaccines

15
Scientific, Technical, and Economic Aspects of Vaccine Research and Development

Jens-Peter Gregersen

15.1
Introduction

Vaccine research and vaccine development are commonly combined by the term research and development (R&D) because, in practice, these two different disciplines cannot be easily separated. Vaccine research and development have much in common: they both use the same technical language, apply very similar methods and tools, and have the same ultimate goal, but there is also a fundamental difference, as the underlying motivating factors, working habits, and the final output and results are entirely different.

Research is mainly motivated by, and aimed at, scientific publications, which are best achieved by new methods, inventions, and discoveries. As soon as these have been published, a researcher's attention must turn to another, and new, subject. Developers normally start their work when new discoveries or inventions have been made and may well work on one and the same objective for an entire decade without publishing anything. They are not aiming at inventions; their intention must be to arrive at innovations, that is, products that will have an impact on our daily life. For the researcher, a vaccine could be an antigen or a preparation that has the potential of eliminating or inhibiting microorganisms. In order to convert this into a useful vaccine, developers must then add several other dimensions to the research product, namely, quality, safety, a specifically defined clinical efficacy, and practical utility. Building practical utility into a product is probably the most demanding or far-reaching one of these four categories. It encompasses and combines almost any aspect of the product, including its local reactivity, acceptable application schemes with only a few vaccinations, a proven and perceived effectiveness, comfortable presentation forms, formulations that guarantee good stability and shelf-lives, and, of course, adequate product prices.

Pharmaceutical Biotechnology: Drug Discovery and Clinical Applications, Second Edition.
Edited by Oliver Kayser, Heribert Warzecha.

15.2
From the Research Concept to a Development Candidate

Concepts for new vaccines arise from research and are based on combined scientific findings collected over many years and by various scientific institutions and disciplines. New vaccine concepts are regularly presented and proposed in large numbers by scientific publications or patent applications, but these concepts rarely result in new vaccines. After being tested in mice, most concepts slowly fade, as the original results cannot be reproduced under more practical conditions, or turn out to be insufficiently effective to justify additional work. On the other hand, there are also organizational and financial aspects that represent serious hurdles. Most academic institutions and scientists simply do not have adequate resources to perform vaccine studies in specific models or even in monkeys or primates. Whereas vaccine antigen candidates can be designed and made by only one or a few individuals, studying these more intensively would normally require other specialists, specific facilities, and, of course, much more money. The initial research project now competes for scarce resources and needs very convincing data to make it to the next stage.

Scientific collaborations across institutional walls are an almost absolute prerequisite for continuing projects beyond testing in small laboratory animals, and in order to proceed into a more intensive and application-oriented research phase. During this secondary research phase, promising concepts are taken up, reproduced, and improved until finally – and in only very few cases – a viable product and development concept can be put together. Almost invariably the efficacy of the candidate vaccine needs to be improved and made more reliable. For many new indications, even the tools and models must be established first, by which immunological effects or protection can be adequately measured.

For those few candidates that remain attractive after being studied in a more reliable way or in better models, it will then be important to assess the technical and economic aspects of the vaccine candidate very carefully. As these strongly depend on available facilities, general expertise, and specific experience with certain techniques needed, these aspects are normally evaluated by the developing organization during a project evaluation or "predevelopment" phase. At the end of this phase, a development concept should be available, which at least fulfills the following three criteria:

1) There should be sufficient evidence that the vaccine candidate is effective and protective in humans or in the target animal species. This normally presupposes that meaningful animal models have been established and that the vaccine has been tested successfully.

2) There should be a defined technical base or verified options by which the vaccine can be reliably and safely produced on a large scale. This includes, for example, cell culture or expression systems, purification schemes, and formulations that are qualified for the production of pharmaceutical products and

do not contain hazardous components that cannot be removed during later process steps.

3) The expected product cost and the resulting sales prices must be in balance with the envisaged benefit of using the vaccine and expected revenues should be able to recoup the development cost in a reasonable period of time.

Thus, there should be fairly clear ideas as to how the vaccine is to be manufactured and how its main qualities are characterized. If this base is not yet known or is based only on assumptions, a targeted product development in its strict sense is not possible, as neither the way to go nor the target or end result are known. In this case, the project should still be considered to be a research project. However, particularly in the case of vaccines, development projects are frequently begun with many uncertainties, assumptions, and compromises, as vaccines are highly complex compositions, which cannot be characterized entirely and completely by analytical methods.

Vaccines are products that are defined to a great extent by the process by which they are made, by the analytical tools by which they are tested, and even by the facilities in which they are manufactured. As a consequence, most vaccine development projects have no clear starting point and the research and process development activities run in parallel. Although not quite impossible, this should in fact be avoided as far as possible, as development activities need many more people and are considerably more expensive than research. No developing organization has sufficient resources to run numerous complex development projects in parallel or to change the direction of a development again and again. By defining adequate criteria and by proper project organization, critical aspects of a development project can be identified early, so that these are evaluated during the applied research phase prior to the onset of product development.

15.3 Vaccine Research Projects

An excellent overview of ongoing research activities for vaccines is provided by the Jordan Reports issued at intervals by the US National Institutes of Health [1]. According to the latest issue of these reports, the number of vaccine R&D projects in the United States in the year 2007 amounted to about 500. A list of the main target indications pursued by recent vaccine research and development efforts is given in Table 15.1. Various efforts to develop vaccines against AIDS represent the largest number of vaccine projects for one indication.

The top positions of the vaccine research "hit list" have not changed very much over the past few decades. Well-known viral and bacterial infections continue to occupy the most prominent positions. However, the number of individual projects for many of these vaccine indications has increased considerably during the past two decades. The simple reason for this is that, previously, complete microorganisms or subfractions thereof, but rarely purified single antigens, had to be used

Table 15.1 Main infectious agents or targets for new vaccines in advanced R&D.

Viruses	Bacteria	Parasites	Tumors
HIV/AIDS[a)]	*Streptococcus*	Malaria	B-cell lymphomas
Hepatitis C virus	*Helicobacter pylori*	*Leishmania*	Melanomas
Herpes simplex viruses	*Borrelia/Lyme disease*	*Schistosoma*	Prostate carcinoma
Cytomegalovirus	*Salmonella*	*Trypanosoma*	CEA-tumors[b)]
HRSV[c)]	Enterotoxigenic *E. coli*		
Parainfluenza	*Mycobacterium leprae*		
Dengue			

a) HIV/AIDS, human immunodeficiency virus/acquired immunodeficiency syndrome.
b) CEA, carcino-embryonal antigen, an antigen that is frequently found on colorectal, bronchial, and breast cancer cells.
c) HRSV, human respiratory syncytial virus.

as vaccine candidates. Modern molecular biology and recombinant techniques now result in individual antigens or even single epitope peptides, which may be varied or combined by almost endless options. Of course, this increases the number of candidates significantly and offers many new opportunities and possibilities, but it does not necessarily increase the chances of success for each individual approach. Molecular biology has opened up various new potentials to approach antiparasite vaccines and tumor vaccines but also, in these particularly complex fields, the number of projects dealing with conventional "whole" organisms or cells is quite remarkable. Antitumor vaccine projects indicate that vaccines should no longer be regarded only as infection prophylaxis. Immunizations can, and will in future, also be used as therapeutic measures. Vaccine research even covers approaches that attempt to induce temporal infertility by the induction of antihormonal antibodies.

In comparison with current vaccine R&D projects, the number of newly licensed vaccines is extremely small. Most newly licensed products are improvements or combinations of existing vaccines; real vaccine novelties are very rare. Thus, the chances that a vaccine project in advanced research finally ends up as a vaccine product is minimal and is certainly below 1%. These low success rates in research inevitably lead to long research phases. Short time intervals of around five years between the first publication or patent application of a new vaccine concept and the start of full development are extremely short, applied research phases for vaccines. These may be applicable to some veterinary vaccines, for which vaccine protection of a candidate vaccine can be measured directly in the target species. For vaccines against human diseases, ten or more years appear to be a more realistic average estimate for this phase. If one adds those further 10–12 years that it takes on average to develop a vaccine product, one must assume that after the basic concept has been published or patented for the first time, about 20 years are needed to successfully develop a new vaccine product. Those who consider these figures as unrealistic estimates are reminded that the average time interval between

concept and first appearance on the market for various innovative technical products developed over the past 100 years (including, e.g., not only complex products, such as antibiotics, the pacemaker, and radar, but also presumably simple products such as the zipper, dry soup mixes, powdered coffee, ballpoint pens, and liquid shampoo) was also 20 years [2, 3]. At any time, innovations have to overcome hurdles, such as scientific challenges, technical difficulties, and usually financial limitations also.

15.4 Scientific Challenges of Vaccine R&D

Science and technologies are the driving forces that enable us to develop new vaccines. Regarding the basic technologies, there are few discoveries that could be cited to have had a significant positive influence and resulted in new vaccines. Cultivation of pure bacterial cultures is still the fundamental base for most bacterial vaccines. A remarkable breakthrough came with the invention and development of cell culture techniques in the 1950s, which led to several new or significantly improved antiviral vaccines, including the currently still exclusively used "state-of-the-art" vaccines against poliomyelitis, mumps, measles, rubella, and cell culture rabies vaccines. Compared with these technologies, molecular biology and recombinant techniques up to now have had a rather limited success and are represented by two new, recombinant human vaccines for hepatitis B and, more recently, vaccines for human papillomaviruses. Cholera and diphtheria strains, detoxified by recombinant methods and used to manufacture conventional vaccines, or recombinant toxoids, may also be counted as results of the new technologies.

DNA vaccines may be regarded as yet another new and basic technology for new vaccines, but within less than two decades after their discovery they certainly have not yet had enough time to mature to practical applications. Monoclonal antibodies or anti-idiotype antibodies, however, did not lead to new vaccines as expected, although these basic techniques were initially considered a major breakthrough in vaccine research.

Apart from a few essential technologies, continuous research in virology, microbiology, parasitology, and immunology are the foundations for vaccine research. However, even the most detailed knowledge about cytokines and their regulation of immune responses, or of fundamental genetic mechanisms controlling the growth and replication of microorganisms cannot be expected to bring any direct or immediate success. For the past, and also for the foreseeable future, it seems that it is more the pragmatic, application-oriented trial-and-error approach that primarily fosters vaccine development. Complex immunological hurdles must be overcome in order to arrive at a new vaccine target, and that is mainly done by establishing suitable animal models and by testing all sorts of vaccine candidate antigens in these models in a very pragmatic way for their protective effects.

Current efforts to develop a vaccine against AIDS serve as a good example of illustrating the importance of suitable models for vaccine development. The Jordan

Report 2000 [1] listed 135 different AIDS vaccine projects. Only 10% of these were considered to be basic research and development (R&D) projects, that is, they are mainly in a phase of selecting, constructing, and making the desired antigen. The remainder of them were allocated to preclinical testing phases in animals or to clinical testing in humans. Less than one-third of these projects seemed to have passed small animal testing successfully and appeared to be worth testing in monkeys or primates. A substantial proportion, 61 projects (44% of the vaccine candidates), were tested in humans for safety and efficacy, however, only two were already in phase III clinical trials, indicating that these different vaccine candidates appear interesting enough to go into widespread field-testing for efficacy.

Seven years later, by 2007, there was still no AIDS vaccine licensed and the number of projects in human clinical trials was reduced to 43 projects, of which only one was in phase III clinical trials. The low number of projects in basic R&D shows that after three decades of AIDS research, there are not too many new antigens or entirely new approaches to be discovered. In the absence of reliable animal models, the relatively high number of projects in early human clinical trials and the low number in later stage clinical trials very clearly demonstrate that in this case research is essentially performed in human clinical trials – with all the inherent limitations. Consequently, the chances of success are low, while at the same time the cost of such research is extremely high.

What are the scientific challenges and difficulties to be overcome on the way toward an effective AIDS vaccine? As summarized in Table 15.2, infectious microorganisms and parasites have developed various mechanisms by which they effectively prevent their elimination by the host's immune response. All of these negative attributes have been found to be associated with HIV infections. HIV evades the immune responses by presenting itself as different subtypes, by varying its main immunogenic antigens during the protracted course of infection in an infected individual, or by hiding itself in a non-accessible form by integrating its genome into the host cell's genes. In addition, it even interferes actively with several important immune functions and modifies these for its own benefit and support. Of particular relevance is the selective preference of HIV for CD4 immune cells, as disturbance of their function can result in numerous deleterious effects. The ability of HIV to persist and replicate in macrophages enables HIV to convert the migrating immune cells into an efficient vehicle across normal barriers. HIV is not only insufficiently neutralized by antibodies, it even uses bound antibodies to get access to immune cells, such as macrophages, which carry receptors for the Fc fragment of antibodies.

Whereas AIDS and HIV were only chosen as an example that contributes any imaginable difficulty to vaccine development, Table 15.2 also lists many other current vaccine projects and their specific difficulties. A limited number of different serotypes may still be overcome by making and combining several similar vaccines, once a successful vaccine against one of these has been accomplished. Thus, vaccines against parainfluenza infections appear reasonably feasible but are of low clinical and commercial relevance. Other indications, such as malaria, herpes virus infections or Lyme disease/*Borreliosis*, however, represent quite

Table 15.2 Immunological challenges on the way toward new vaccines.

Attribute	Examples	Detail
Different serotypes or subtypes to be covered by the vaccine	Parainfluenza	3 major pathogenic subtypes
	Dengue	4 subtypes
	Malaria	4 major pathogenic plasmodium species
	Borreliosis	4 genetic and immunological types
	Hepatitis C	6 major genotypes and >100 subtypes known
	HIV/AIDS[a)]	>10 subtypes known
Antigenic variation of major immunogens	Malaria	High variance of major antigens within the parasite [4–6]
	HIV	Antigens vary during the course of infection even within the same patient [7]
	Trypanosoma	Periodic switching of major surface glycoproteins [8]
Genetic restrictions of immune recognition and immune responses	Malaria	Multiple HLA[b)] restrictions for recognition of Plasmodium falciparum CTL[c)] epitopes even within the same individual [5, 6]
	HIV	HLA-restricted CTL escape mutations associated with viral load and disease progression [9, 10]
Microoganism not accessible to immune responses	HIV	Virus genetically integrated in host cell genomes [11]
	Herpesviruses	Virus persists in a latent state in neuronal cells [12]
Microorganism persists in immune cells and may spread with these into tissues or across blood–brain barrier	HIV	Persistence and active replication in, for example, macrophages [13]
	Herpesviruses	Can infect endothelial cells and macrophages [14]
	Borreliosis	Borrelia survive in macrophages. Complement membrane complexes and macrophages in the endoneutrium of Lyme neuroborreliosis [15, 16]
	Hepatitis C	Macrophages and T-cells found to be infected by hepatitis C virus (HCV) [17]
Immune-enhancement and immune-mediated disease	HIV	Antibody and Fc receptor-mediated enhancement of infection and disease [18, 19]
	Dengue	Antibody-mediated enhancement of infection [20]
	Borreliosis	Immune-mediated neuropathology and arthritis [15, 21]
	Respiratory Syncytial virus	Inactivated vaccine induced high serum antibodies and aggravated disease upon infection [22]

a) HIV/AIDS, human immunodeficiency virus/acquired immunodeficiency syndrome.
b) HLA, human leukocyte antigens.
c) CTL, cytotoxic T-lymphocytes.

significant immunological challenges, because the responsible microorganisms combine many unfavorable immunological characteristics.

Finally, the example of a respiratory syncytial virus (RSV) vaccine developed and tested in the late 1960s may serve as an example to illustrate the difficulties and practical effects that some of these imponderable aspects can have. This RSV vaccine turned out to enhance a later disease, rather than preventing it [22]. More than 30 years after those results were published, there is still no real explanation for the underlying mechanism and many further efforts to develop a new vaccine were stuck in a preclinical phase.

Another important aspect, which seems to be underrated in many current vaccine research projects, is the fact that most vaccines are not sufficiently effective if they are based on only single antigens. Controlled vaccine studies performed under ideal conditions in genetically homogenous or inbred animals quite often lead to the false assumption that a fully protective vaccine antigen has been identified. However, when the same vaccine is then studied under more practical conditions, by fewer numbers of immunizations and in the presence of acceptable and better-tolerated adjuvants, it becomes evident that the selected antigen candidate alone is simply not effective enough.

Table 15.3 summarizes the experiences made with different foot-and-mouth-disease (FMD) experimental vaccines. Results from model studies with this type of vaccine can be correlated reasonably well with the protective response in the target species. The FMD virus consists of only three structural proteins and the most relevant virus-neutralizing antigenic epitopes are known to be located on virus protein 1. Thus, FMD vaccines appeared to be an excellent target for new vaccines based on recombinant technologies. The standard vaccine, made of inactivated whole virus particles, required a relatively low amount of antigen and only one immunization in order to confer protection. Efforts to make smaller subunit or single-protein vaccines resulted only in a similar protection if several immunizations and/or massively increased antigen doses were given. These results had been established fairly early on in the molecular biology vaccine era, but despite intensive further research, a commercially viable recombinant FMD vaccine has never been achieved. Meanwhile, conventional whole virus vaccines were successful enough to allow measures to be taken to eradicate the disease in those countries where the vaccine has been used intensively.

Table 15.3 Protectivity of different forms of vaccine antigens. Type of antigen, amount of antigen, and frequency of immunization required to achieve protection against foot-and-mouth-disease virus infection in the guinea pig model [23].

Type of antigen	Amount of antigen (μg)	Vaccinations
Purified whole virus particles	1	1
Virion subunits (12 S)	10	2
Virus protein 1	200	3
Oligopeptide (N-142-160-C)	200	1

15.5
Technical Aspects of Vaccine Development

In an ideal situation, vaccine development commences with a proven, protective, and well-defined antigenic composition. Successful vaccine development then normally takes ten or more years, but only a small proportion of all development candidates finally end up as a licensed product; the vast majority remain stuck in early development phases or are abandoned [24, 25]. Figure 15.1 summarizes the essential tasks of a vaccine development project and may give a rough impression of what is to be expected. For the sake of clarity, several time dependencies and overlaps during the preclinical phase have been neglected in this graphic overview. Figure 15.1 also shows that during the clinical phase, numerous technical tasks have to be accomplished and completed, which require substantial resources. Not only the clinical studies themselves, but also these technical operations greatly increase the project cost during the clinical phase.

An extensive range of national and international rules and guidelines exist, covering almost any aspect of pharmaceutical and vaccine development and registration [26–28]. These guidelines describe standards that are not binding in a legal sense, but adherence to these is strongly recommended, as during later registration and licensing, the product will be judged by the same rules. Deviations from guideline recommendations may be inevitable for certain aspects and particularly for vaccines, but these should only be considered if convincing reasons for doing so can be presented. A summary of relevant guideline requirements along with specific interpretations and applications for biopharmaceuticals and

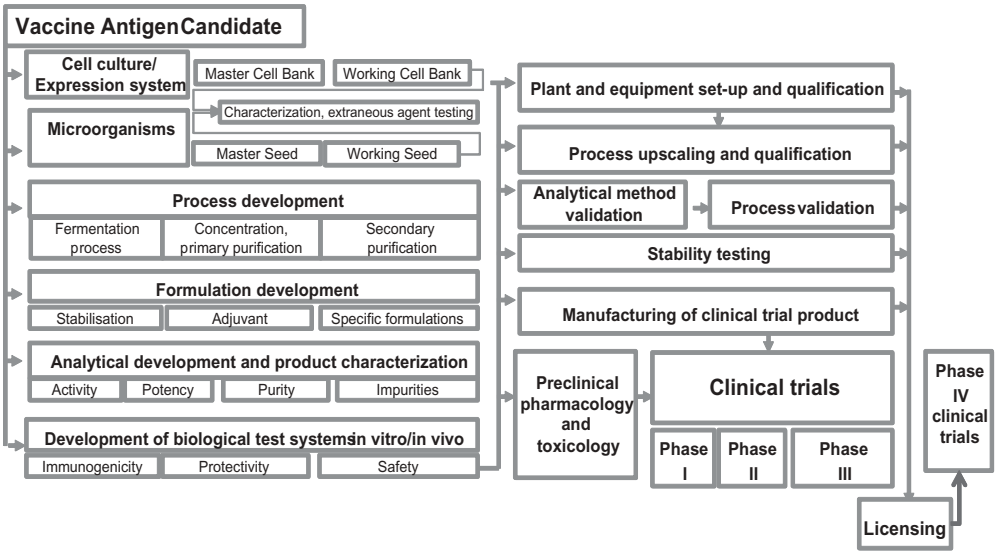

Figure 15.1 Schematic diagram of a vaccine development process.

vaccines is given in reference [29] and may be helpful for prospective developers in order to get a reasonable understanding of the guiding principles.

15.5.1 Preclinical Development

Preclinical development comprises the technical and scientific elaboration of a process to manufacture the desired product on a large scale. Firstly, the cell cultures and microorganisms that are to be used must be established as Master Cell Banks and Working Cell Banks, or as Master and Working Seeds, respectively. These ensure a constant supply of well-characterized, uniform, biological starting materials. Numerous tests *in vitro* and *in vivo* are required to guarantee the absence of undesired adventitious agents and to confirm the identity of these cell banks and microbial seeds.

Starting with a single aliquot of the Working Cell Bank and/or the Working Seed material, a process is then established and brought up to a final scale. The term "upstream process" typically means a cell culture or fermentation process up to some 100l, but for very common vaccines larger scales may be chosen. Downstream processing summarizes activities during the purification process and typically includes recovery and concentration steps, followed by a secondary purification or "polishing" to remove specific impurities and process-related impurities introduced during earlier steps. Inactivation of bacteria or viruses or detoxifying steps for toxoid vaccines is usually included after an initial concentration step.

Formulation development includes the design of adequately buffered and well-tolerated, stable formulations, adjuvantation, the development of specific application forms, combination of vaccines into compatible vaccines, and, particularly for live attenuated vaccines, the development of a lyophilization process. Formulation development also extends to the selection or design of final syringes or other presentation forms and to filling and packing processes. Stability monitoring programs for intermediate and final products are of adamant importance for any development work and should be started as early as possible to avoid difficulties at a late development stage.

Analytical development encompasses all activities related to the design and use of adequate methods to control and specify all parts of the process and the product. This includes testing of starting materials, intermediate products, and the final product, for example, for identity, specific activity, conformation, purity, and impurities. For a vaccine developed according to today's standards, a range of about 100 different tests and methods will be required. Most of these tests need to be validated for their specific purpose in order to assess the methods specificity, sensitivity, statistical exactness, and its limitations.

In parallel with process development, biological tests and model systems must be available to monitor the vaccine's potency, immunogenicity, or protectivity at any stage, as vaccines are particularly labile products and minor modifications of the process can have a significant – mainly negative – influence on the vaccine

antigen. Likewise, biological models must be on hand to study the vaccine's basic pharmacological, immunological, toxicological, and potential immunotoxicological characteristics. As far as this can be adequately studied, these include dose responses, characterization of induced humoral and cellular immune responses or of their major contributing protective mechanisms, longevity of immune responses, and potential immunological side effects. Although vaccines rarely present severe tolerability or toxicological risks, abbreviated classic toxicological testing is mandatory before the onset of studies in humans. Most vaccines need to be tested only in local and systemic tolerance studies and in repeated dose studies in standard toxicology models, but for new adjuvants and certain new excipients, representing a significant part of the vaccine composition, even a complete toxicology program, including two years of carcinogenicity studies, may be needed. Further toxicology and safety studies addressing specific risks, such as embryonal, fetal, or peri- and neonatal toxicity may be required for certain vaccines and applications or if risks are expected or known. The withdrawal of a newly licensed Rotavirus vaccine, which was suspected to cause intussusceptions and fatal bowl obstructions in vaccinated children [30], may serve as an example that such studies may be required not simply on hypothetical grounds.

Owing to the biological origin of many starting materials, risks associated with prions and potentially contaminating viruses must be addressed. Organizational measures must be put in place to avoid risk materials in addition to testing for adventitious agents. Potential risks from starting materials or process contaminants can be further evaluated and assessed by model studies with various viral and microbial agents. If specific risks are identified and if safety margins appear low, specific countermeasures should be included in the process. As far as possible, within the technical limitations of these safety studies, a residual risk of less than 1 in 1 000 000 should be aimed for. In practical terms this means, for example, that an unnoticed contaminating virus is inactivated or eliminated by the process to a degree that no risk would be associated with a vaccine volume equivalent to one million doses. Examples of how such safety margins can be achieved and how to demonstrate these are presented in reference [31]. For live attenuated vaccines, viral safety must also be assured, by assessing the genetic and phenotypic stability of the vaccine virus and by evaluating the chances and consequences of transmissions of the virus to unvaccinated individuals.

15.5.2
Manufacturing Facilities

Facilities and equipment for the manufacturing of a vaccine are an immanent part of the registration dossier for the product. Any major change would have to be approved by the regulating authorities. Thus, at least for the later clinical phases, the product should be made in a specific plant and with dedicated equipment. For a development project, this means that after the process has been defined, large investments in buildings, facilities, and equipment are to be expected. Owing to

the inherent risks of these investments, pilot plants should be available to produce initial clinical trial vaccine lots on an intermediate scale. A developing organization may even choose to go into phase III clinical trials with a vaccine that has been produced in a pilot plant and to seek registration for this "preliminary" product. This approach delays the investment decision to a later point in time when all development risks have been abolished, but inevitably requires a process transfer, new process validation, and may even necessitate new clinical trials for the vaccine that is subsequently made in the final plant. For large-scale products, this extends the time to the market by several years. The sum to be invested in manufacturing facilities greatly depends upon the scale of operation. For a complete vaccine plant including all auxiliary functions, the total investment may well accumulate to far above 100 million US dollars or Euros. Vaccine producers who can use their existing infrastructure, such as buildings, filling and packaging facilities, raw material and media production areas, and quality control laboratories, would have to invest significantly less. For small or start-up companies, outsourcing and outlicensing may be chosen to reduce risky capital investments, as only vaccines with high market expectations justify establishing a complete, own manufacturing operation.

15.5.3 Clinical Development

The clinical development of a new vaccine is done in three phases and lasts 3–7 years. A time period of three years may be an exception and only applicable for a replacement of existing vaccines that are being evaluated for effectiveness solely by serological tests. As for drug development, the average time span of 6–7 years may be taken as a more realistic estimate for a successful clinical development of a new vaccine [32, 33]. The duration depends not only on the novelty and complexity of the vaccine indication to be explored but also on the availability of measurable immunological surrogate markers of protection. If the vaccine's efficacy must be evaluated by comparing randomly occurring cases of the disease in test groups and in alternatively treated control groups, clinical studies can be extremely long lasting, demanding, and risky.

Prerequisites of all clinical trials are adequate preclinical pharmacological and toxicological safety assessments, including animal studies, to justify tests in humans. On the basis of the available safety data and documentation, approval for clinical trials must be obtained by the relevant ethics committees and health authorities. Trials will only be admitted if these are conducted according to pre-established, systematic, and written procedures for the organization and conduct of the trials for data collection, documentation, and statistical verification of the trial results. The "informed consent" of all participating trial subjects and medical personnel is essential. For trials involving children or mentally handicapped persons, the informed consent must be given by parents or by the person responsible. Clinical trials are to be planned and conducted according to "good clinical practice" standards that require controlled

and randomized trials where possible. Control groups are to be treated by established products or treatments. Placebo treatments are normally only admitted where no alternative treatment exists.

During the initial phase I, the basic safety features of the vaccine candidate are intensively studied in a limited number (<100) of patients or healthy volunteers. The main purpose of these studies is to confirm the vaccine's local and general tolerance before it is applied in further clinical trial subjects, but phase I vaccine studies can also partly be used for a first dose-finding, and immunological evaluations for adequate immune responses. During phase I trials, vaccines rarely fail due to safety concerns, but more frequently due to insufficient or inconsistent immune responses below expected levels.

Phase II clinical studies usually comprise no more than several hundred subjects and are normally done as controlled studies comparing the test vaccine along with an alternative prophylactic or therapeutic treatment. Clinical evaluations mainly address the vaccine's effectiveness and safety, doses, application schemes, and possibly also different target groups selected by age, specific risks, countries, or by epidemiological criteria.

Phase III clinical studies are expanded, controlled, or uncontrolled trials on efficacy and safety in various clinical settings and under practical conditions. Altogether several hundred to several thousand trial subjects are enrolled at various trial sites, which are often distributed over several countries in order to study different epidemiological situations, ethnic populations, and deviating local medical practices. Phase III studies can also be evaluated for risk–benefit relationships and address practicability aspects as well as interactions by other products or concomitantly applied medical treatments. Post-marketing clinical trials of the licensed product, often referred to as phase IV clinical trials, are nowadays, fairly often, also requested as part of a conditioned licensing of pharmaceutical products, mainly in order to specifically investigate those aspects that can only be assessed by large statistical cohorts.

For live attenuated or vector vaccines, specific safety aspects must also be studied clinically. As the live viruses or bacteria replicate in the vaccinated host and may be shed into the environment, the potential transmission of vaccine microorganisms to unvaccinated subjects must be studied. If transmission is possible or likely, the vaccine's genotypic and phenotypic stability must be carefully studied and confirmed.

15.5.4
Licensing and Registration of Vaccine Products

The formal aspects of pharmaceutical product licensing has been dealt within another chapter of this book (see Chapter 11 in this volume). On the basis of past experience and evaluations, the process of getting a vaccine through the evaluation at different national licensing authorities on average takes about two years, which includes time periods for working out and answering questions not adequately covered by the registration dossier. The introduction of defined timelines for

specific licensing steps now seems to have reduced the licensing period to 16–18 months [32, 33].

As vaccines and other biological pharmaceuticals are particularly complex compositions that cannot be adequately characterized by specific quality control methods, the entire process, manufacturing facilities, analytical methods used to specify the product and its starting materials, and all ingredients, are considered as being an inherent characterizing part of the product. Any change to these affects the product's license and requires approval by the licensing authority. Changing essential elements, such as production cell substrates or microbial strains, critical test methods such as potency assays, purification methods, or formulations would almost inevitably be seen as a change to the product that needs to be verified by new clinical trials. Furthermore, each individual batch produced must be approved and released by the authorities.

15.6 Economic Aspects of Vaccine Development

Without any doubt, the development of vaccines is a very costly and long-lasting process that bears a significant risk of failure. The following paragraphs are intended to provide some deeper insights into the specific risks and chances, cost, and time requirements to develop a new vaccine, as the knowledge of these basics, drawn from experience, may by helpful in decision making. After all, successful vaccine development depends not only on good science and technical methods but also, to a great extent, on adequate management decisions.

15.6.1 Vaccine Development Cost

The number of successful vaccine projects is fairly low and retrospective evaluations of the specific costs incurred by these development projects over a time period of ten or more years are difficult. However, cost evaluations covering developments from the late 1960s until 2006 exist, which summarize the development cost of various pharmaceutical developments [32–37]. These evaluations show that pharmaceutical development cost initially tended to increase by a factor of two within a decade, whereas the more recent investigations noted costs that rose by a factor of four within ten years. As demonstrated by a simple graph (Figure 15.2), increasing regulatory demands caused exponentially rising cost, while average consumer prices rose much slower and on a linear scale.

Although chemical drug products dominated these figures, several vaccine projects were also assessed. With all the inherent variability, we can reasonably assume that these figures also give adequate estimates for new and complex vaccine products. However, the average vaccine project, which includes "me-too" products, might incur lower cost. The favorable or known safety profile of those vaccines often requires less demanding pharmacology and toxicology studies, and

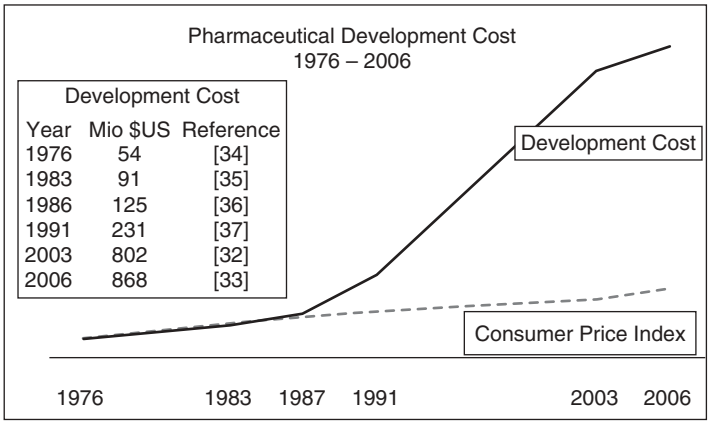

Figure 15.2 Pharmaceutical development cost. Development cost for various pharmaceuticals, mainly chemicals but also including vaccine projects. Consumer Price Index (All Urban Consumers, CPI–U, US city average). Graph shows relative figures adjusted to a uniform scale.

may also reduce the number of clinical trials and trial subjects. A lower number of unsuccessful projects (compared with drug development, see below) may also account for lower overall cost. Furthermore, most vaccines are manufactured on a lower scale than many drugs, which also reduces complexity and cost.

Based upon evaluations for numerous successful development projects, which were published along with exact descriptions of the applied methodology, the studies by DiMasi and colleagues [32, 37] are most often referred to in order to describe the cost and risks of pharmaceutical development projects. However, quoting these figures without mentioning important details about the original calculations is misleading. These figures (US$231 million published in 1991 and US$802 million in 2003) include, to a large extent, opportunity cost (calculated by an interest rate of around 10% of the invested capital over a period of 12 years) and the cost of many unsuccessful or abandoned projects (assuming a success rate of around 20% during the defined preclinical and clinical development period); furthermore, tax credits were not accounted for. All in all, the underlying out-of-pocket expenses must be assumed to be only about 30% of the total sum and according to today's standards, direct cost around 200–300 million US dollars or Euros may be assumed as a realistic estimate for the development of a new pharmaceutical product. If, however, there is no suitable infrastructure and if investments into completely new production facilities are to be made, this could easily double the cost.

Apart from capital investments, personnel is the most relevant cost factor to be considered. Owing to the high number of persons involved in the preclinical and technical development phases and to the long duration of these activities (on average about four years until the start of clinical trials and more than seven years beyond until registration), preclinical and technical activities account for about

one half or more of the development cost. Clinical development normally leads to about one-fourth of the development cost; the remaining quarter is evenly spread throughout the developing organization and covers overheads, technical support functions, quality control, and quality assurance, as well as various other specialists, for example, for patenting, regulatory affairs, and market research.

Based upon a detailed task–personnel–cost estimate from 1997, an average vaccine development project required about 170 man-years of work with average total expenditures per person and workplace in the pharmaceutical industry at that time being around €180 000–200 000 or, for the United States, around US$220 000–240 000. This resulted in roughly 30–40 million US dollars or Euros for personnel and workplace expenses [38]. External cost of around 20% and highly variable capital investments into plant and facilities must then to be added on. Considering the rising standards, and as explained above, those sums must at multiplied by four to arrive at a figure that better applies to current times.

15.6.2
Risks and Opportunities

The success of a project during and for the entire development process can be estimated by the numbers of projects that make it to the next development phase and finally end up as commercial products. Of products developed during the preceding decades until 1994, only 50% of the preclinical vaccine development projects entered the clinical phase and another 50% were abandoned during the clinical trials. Having passed all preceding hurdles, product registration seemed to be uncritical, as only a loss of one out of 100 vaccine projects was noted. For pharmaceutical drug products, overall success rates of 11% were found, that is, 100 product candidates entering the preclinical development resulted in only 11 licensed products. Vaccine projects appeared to be more successful with an average of 22% licensed products per 100 projects [24, 25]. However, the conditions seemed to have changed since these data were accumulated. The figures presented above for AIDS vaccine projects show that average figures can also be grossly misleading.

Many current vaccine candidates are dealing with quite “difficult” infectious diseases, which under natural conditions do not induce a lasting protective or sterile immunity, thus doubts about the applicability of those earlier evaluations to current vaccine projects are justified. Even if AIDS vaccine projects are not considered, a snapshot view of more recent vaccine developments supports the suspicion that success rates for today’s projects and particularly for new vaccine indications are much lower. As shown in Figure 15.3, the success rates of preclinical development since 1994 and until 2007 appeared to be below 50% and only 13–17% of all projects were found in phase III clinical trials. Whereas preclinical projects represented a very wide spectrum of entirely new vaccines, the majority of phase III clinical trials were covering alternatives to already existing vaccines, such as competitor’s developments, combinations, or improved formulations. Only 2.4–4% of these advanced projects were approaches to developing entirely

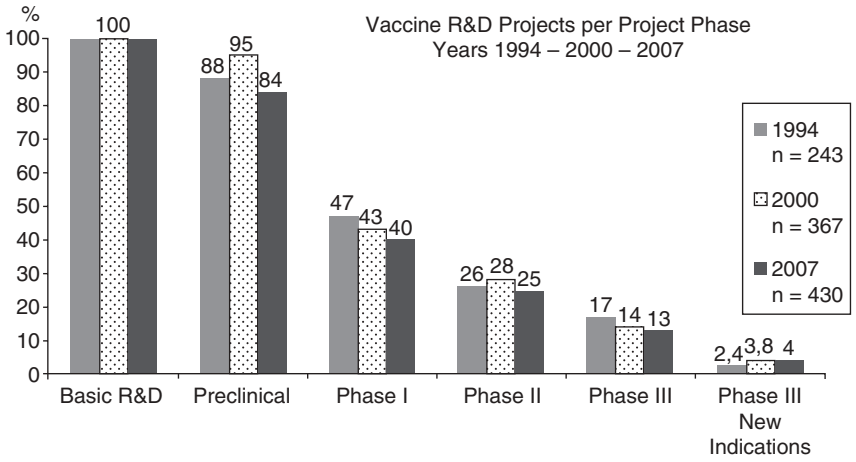

Figure 15.3 Human vaccine development projects in the years 1994, 2000, and 2007. Relative figures in percent with basic R&D projects set as 100%. Data extracted from listed vaccine projects in the years 1994, 2000, and 2007 [1] without consideration of AIDS vaccine projects. The number of AIDS vaccine projects in clinical trials in 2007 was 43. Vaccine projects in phase III clinical trials for new indications include vaccines against Rotavirus (now licensed), human Papillomavirus (now licensed), Herpes simplex virus, *Borreliosis*, *Coccidioides immitis*, *Streptococcus pneumoniae*, *Plasmodium falciparum*, *Leishmania major*, and *Mycobacterium leprae*.

new vaccines. Although the figures for each year represent only a static view upon the vaccine development, Figure 15.3 summarizes and describes rather similar situations for a time period spanning 13 years. During that time period, which covers little more than the average development time, only two new vaccine types (new disease indications) were licensed, two vaccines against Rotavirus infections of infants, and two vaccines against human papilloma virus infections causing cervix carcinomas.

Nowadays – and particularly for really new vaccines – development success rates clearly below 5% appear more realistic than earlier estimates that were above 20%.

15.7 Conclusions

Judging by the number of scientific publications, vaccine R&D appears to have a great attraction for scientists from all pertinent scientific disciplines. Whenever new methods and technologies became available, these have always and immediately triggered a high number of new vaccine research projects and stimulated research into formerly hopeless vaccines. Along with the good reputation that vaccines enjoy, this scientific enthusiasm is an excellent base for new vaccines, and a good base to attract the required capital as well. However, considering the

high risks and the long duration of vaccine R&D, there must also be other reasons why investors and pharmaceutical companies invest in this field.

Vaccines represent only a small proportion of the pharmaceutical market, but, nevertheless, they are extremely successful products. Firstly, vaccines effectively prevent diseases, rather than just curing them. Owing to these advantages, vaccines have often created their own markets and have even defended their market shares against competition by effective therapeutics or antibiotics. Secondly, many vaccines are recommended by public health authorities and thus enjoy a fairly safe position in the market. Furthermore, there are usually only rather limited numbers of competitive products because vaccines are far too complex to become an easy target for producers of generic imitations. Finally, vaccines usually have a very long life span. As long as vaccine products are not neglected and become outdated, but are constantly adapted to better state-of-the-art, vaccines do not lose their market position, unless they are too successful and by and by eliminate the need to use the vaccine.

Thus, vaccine R&D can be very rewarding for both scientists and investors. Regarding the risks, however, the investor has quite a different perspective than the scientist. The investor may contain risks by putting capital into many different projects and enterprises, thus participating in the statistically successful "average vaccine." To a limited extent, large companies who develop vaccines can also apply the same strategy. However, small enterprises and individual scientists working for only one or a few R&D projects have only few options to manage and reduce risks. They often choose a high-risk approach by aiming only for "blockbuster" products. In this case, they must be aware that competition in this field will also be very strong, which increases the risks even more. Nevertheless, within the given financial limitations, risks could also be spread over a certain number of projects in early R&D phases, preferably by approaching different indications and concentrating on an attractive new or improved technology.

In any case, vaccine R&D is certainly not the play ground for those who expect fast success and revenues. Any organization that intends to invest in vaccine R&D should be prepared – both mentally and financially – to endure it for at least 10–20 years.

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16
New Nanobiotechnological Strategies for the Development of Vectors for Cancer Vaccines

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16.1
Introduction

The application of nanobiotechnology to identifying, curing, abating or resisting human disease is a well established and yet constantly evolving field. As our knowledge of the physiology of healthy versus diseased states increases, so does the potential for effectively designed curative strategies that exploit the vulnerabilities of disease while inflicting minimal damage on healthy tissue. Nanotechnology in medicine has a plethora of applications that can be subcategorized into the three main areas of: drug delivery, disease therapy, and, finally, diagnostics and imaging. Here we are concerned with the therapeutic application of nanotechnology with specific regard to nanocarrier cancer vaccines (NCVs).

Cancer is a malignant disease that is characterized by the unregulated proliferation of genetically aberrant and transformed cells. Despite significant advances made in screening and treatments over the past five decades, cancer is still the second leading cause of mortality in the United States, with one in four deaths attributed to it [1]. Well established treatments for cancer, such as chemotherapy, radiotherapy, and surgery, have variable cure rates that often depend on the type of cancer and its stage of diagnosis. Radiotherapy and surgery are procedures that fail to eliminate metastases, while chemotherapy has the disadvantage of indiscriminately targeting proliferating cells, thus resulting in killing off both tumor and healthy cells. In addition, cancer cells can develop resistances to chemotherapeutic agents. Hence, the need for more efficacious and less harmful cancer therapies still exists.

Cancer vaccines are a promising alternative, or addition, to conventional cancer treatments and their study has increased significantly over the past two decades [2, 3]. The principle aim of tumor immunotherapy is to manipulate the patient's own immune system to recognize and destroy cancer cells. The successful application of a cancer vaccine would have three significant advantages over current treatment modalities. Firstly, a successful vaccine would generate tumor-specific killing, with minimal damage to healthy cells. Secondly, a cancer vaccine would generate a systemic antitumor immune response that would target primary and

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secondary metastases. Thirdly, tumor vaccinations would ideally generate long-term protection against possible future tumor recurrences, due to the induction of immunological memory [4–8].

Relatively recent key discoveries in the fields of cancer and immunology have provided significant impetus for laboratory and clinical research into therapeutic and prophylactic vaccination strategies for cancer. These findings include: (i) understanding the biochemical nature of protein processing and presentation; (ii) recognition of the role of dendritic cells (DCs) as the primary antigen presenting cells (APCs) in initiating adaptive immune responses; and (iii) the discovery of tumor-associated antigens (TAAs).

It has been recognized since the 1980s that endogenous versus exogenous proteins have different fates within the cell post-translationally and post-engulfment, respectively [9, 10]. A fraction of the pool of each endogenously produced protein is broken down into small peptide fragments of 8–10 amino acids and expressed on the cell surface in association with major histocompatibility complex (MHC) class I proteins [10]. This process was evolutionarily designed so that the host's immune system could monitor for "foreignness" in the form of intracellular pathogens. MHC class I proteins are recognized by receptors expressed by CD8+ cytotoxic T lymphocytes (CTLs). Upon engagement of this T-cell receptor (TCR), and along with appropriate costimulatory signals, CTLs become activated and a cytolytic response ensues, leading to target cell death [10].

Soluble or exogenous proteins, as opposed to endogenously produced proteins, are usually pinocytosed or phagocytosed by professional APCs, such as DCs. Subsequently, a portion of the peptide fragments is processed and presented on the cell surface in the context of MHC class II proteins, which can be recognized by TCRs expressed by CD4+ T helper (T_H) lymphocytes [10]. T_H lymphocytes are usually not cytotoxic but instead provide paracrine activation signals in the form of lymphokines that CTLs often require to be fully functional and proliferative [11].

TAAs are defined here as proteins that are expressed by tumors that can potentially be exploited as targets for the immune system either because they are expressed abundantly in tumors but not by healthy adult tissues, or their expression is limited to tumor tissue and healthy tissue that is considered expendable [12]. Endogenously produced TAAs are processed and presented in the context of MHC class I on tumors but usually remain undetected or tolerized by the host's immune system, despite the presence of tumor antigen-specific CTLs. This is usually because additional activation signals are required to trigger an effective CTL response. These additional signals come either directly from DCs or, as is generally recognized, indirectly from DCs via T_H lymphocytes [11, 13]. Since DCs are the initiators of immune responses, many cancer vaccine strategies involving nanocarriers have been aimed at specifically targeting DCs with payloads of antigen that favor its presentation in the context of MHC class I with the goal of generating more effective CTL responses [14–16]. The process by which exogenous antigens become processed and presented in the context of MHC class I is known as "cross-presentation" and requires the antigen to be released into the cytoplasm either through the plasma membrane or by endosomal escape subsequent to

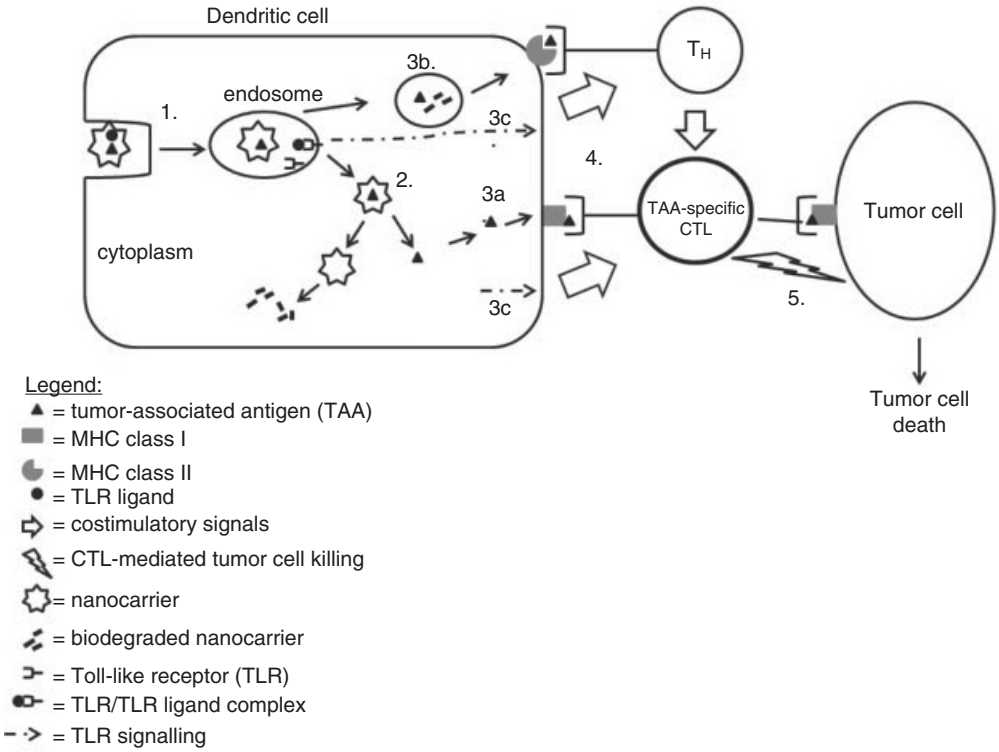

Figure 16.1 Schematic of NCVs proposed mode of stimulation of anti-tumor immunity. 1, Engulfment of NCV by phagocytosis, pinocytosis or receptor-mediated endocytosis; 2, endosomal escape of antigenic cargo (TAA) into cytoplasm; 3a, processing and presentation of TAA epitope in association with MHC class I; 3b, processing and presentation of TAA in association with MHC class II via recycling endosome; 3c, upregulation of costimulatory signals via TLR signaling; 4, activation of TAA-specific CTL and T_H lymphocytes; and 5, release of cytolytic factors by CTL resulting in tumor cell death.

engulfment. Various nanocarrier systems are capable of promoting either or both of these two modes of cytoplasmic entry and are discussed below (see Figure 16.1 for schematic).

In recent times, alternative vaccination strategies have been investigated in order to improve efficacy, reduce the number of subsequent boosts, and broaden the spectrum of disease targets [17]. Vaccination strategies, particularly against diseases such as cancer, generally require the activation of the cytotoxic or cellular arm of the immune response. This necessitates a more considered and refined approach compared with conventional vaccine strategies that simply induce antibody production by B lymphocytes but generate negligible CTL responses [18]. The stimulation of antibody responses alone is usually ineffective against cancers as most TAAs are not expressed on the cell surface of cancer cells in a form recognized by antibodies. Hence the need for NCVs that have the potential to activate

CTLs that can specifically recognize TAAs in the context of MHC class I on the tumor cell surface.

In addition to promoting CTL responses to their antigenic cargo, NCVs offer other unique advantages over the administration of the soluble form of the antigen. These include: (i) protection of the drug–antigen–adjuvant from premature enzymatic and proteolytic degradation; (ii) enhanced absorption of the drug–antigen–adjuvant into targeted tumor tissue either by the enhanced permeability and retention (EPR) effect or via active targeting with the use of ligands; and (iii) ability to control the pharmacokinetic and drug–antigen–adjuvant tissue distribution profile and enhance cellular uptake by DCs to trigger a strong immunostimulatory cascade. Furthermore, these nanoscale carriers offer the unique advantage of multi-component loading, which is of considerable significance particularly in immunotherapy where simultaneous delivery of antigens, immunoadjuvants, and targeting ligands is optimal [19–21].

In addition to carrying antigenic material such as TAA peptides, it is considered crucial that NCVs co-deliver adjuvant signals. Promising adjuvants that have received a large amount of attention in the last decade are the Toll-like receptor (TLR) ligands [22, 23]. These are molecules that contain specific pathogen-associated molecular patterns that include, but are not limited to: nucleic acids, lipopolysaccharides, lipopeptides, or peptidoglycans. Some NCVs, such as archeosomes and viruses, possess intrinsic adjuvant properties capable of potent DC stimulation, while other NCVs have negligible DC-stimulating properties and require an adjuvant to be co-loaded.

There are many other important attributes that NCVs should possess. Of these attributes, particle size is of paramount importance when considering either drug or vaccine delivery as it can influence circulation time, release kinetics, and tissue targeting. For consistent biological outcomes, it is generally advisable to use particles of uniform size. In terms of vaccine applications particle sizes in the range of 0.1–10 μm are considered optimal as these are dimensionally comparable to viral or microbial pathogens and are readily phagocytosed by APCs [24]. It has recently been demonstrated that particle size influences the rate and manner in which particles reach local draining lymph nodes as well as the efficiency with which they are taken up by various DC subpopulations. Thus, the implications on immune outcome and therapeutic benefit that particle size could have may be profound and requires serious consideration when designing NCVs. Other factors that play a major role in determining the ultimate applicability of various NCVs include: loading capacity, biodegradability, biocompatibility, safety, cost of manufacture, storage stability and ease of upscaling for commercial or widespread use.

It is important that NCVs do not accumulate within specific organs in the body because of their potential to cause physical damage or physiological disruptions. Furthermore, breakdown products of NCVs should be non-toxic and readily cleared. Of final consideration is the hurdle posed by necessary sterilization procedures for NCVs that could significantly affect the physical and functional integrity of the cargo being delivered [25].

Table 16.1 A comparison of salient properties of various nanocarrier systems. An overview of the relative degree to which nanocarriers possess three important attributes often desired of NCVs, along with one undesired characteristic (potential for toxicity).

Nanocarrier	Cross-presentation capacity[a)]	Intrinsic DC-stimulating capacity[a)]	Potential toxicity[a)]	Loading capacity[a)]
Conventional liposomes	+	−/+	−/+	++
Cationic liposomes-DNA complexes	+/++	+++	+++	++
Long-circulating liposomes	+	−/+	+	++
Archeosomes	++/+++	+++	−/+	+
Fusogenic liposomes	+++	++	+	++
Viruses	+++[b)]	++++	++++	N/A[c)]
Virus-like particles	+	++++	+++	N/A
Virosomes	+++	++++	+/++	+
PLGA	++	+	−	++/+++
Acid-degradable hydrogel-based particles	++++	−	−	+++
Gelatin nanoparticles	+/++	−	−	+++
Sub-micron emulsions	+/++	−	−	+++
γ-PGA nanoparticles	+/++	++	−	++

a) + = low, ++ = medium, +++ = high, ++++ very high, and − = negligible. This table is only meant as a general guide and, rather than being based on comparative empirical studies, has been deduced by the authors based on published data for each of the vector systems.
b) Viruses are often used as NCVs such that they infect cells with genetic material encoding TAAs resulting in expression of TAA epitopes in association with MHC class I. This is not strictly "cross-presentation" but achieves the same desired result.
c) N/A = not applicable.

Ultimately, all of these factors need to be carefully considered if NCVs are to be successfully introduced into the clinic. Table 16.1 summarizes the relative potencies of various nanocarrier systems with respect to four of the aforementioned factors: "cross-presentation," DC-stimulation capacity, loading capacity, and potential for toxicity.

This chapter reviews recent nanobiotechnological advances that have resulted in colloidal and particulate formulations designed primarily for cancer immunotherapy and the potential for translation into clinical cancer vaccines.

16.2 Biodegradable Nanoparticles

16.2.1 Poly(*D*,*L*-lactic-co-glycolic Acid) (PLGA) and Polylactic Acid (PLA)

Poly(D,L-lactic-co-glycolic acid) (PLGA) is a biodegradable copolymer of lactic acid and glycolic acid that has received widespread attention as a vaccine vector. Such interest stems from the ability of PLGA to release its cargo in a sustained and pulsatile manner, therefore making it a good replacement for current vaccine strategies that require multiple boosts. In addition, PLGA can be designed to target a specific tissue. PLGA can be readily synthesized with varying ratios of lactic acid : glycolic acid, although 50 : 50 is most commonly used. Polymerization occurs through the formation of ester linkages catalyzed by tin(II) 2-ethylhexanoate, tin(II) alkoxides or aluminum isopropoxide, resulting in an amorphous aliphatic product [26]. PLGA is biocompatible, has received FDA approval for human use and has a well established safety profile in humans [27]. The degradation products of PLGA are easily metabolized and eliminated *in vivo* [28].

PLGA nanoparticles and microparticles can be fabricated from PLGA polymers using a number of solvent-based techniques that involve emulsification (with evaporation), nanoprecipitation or interfacial polymerization [29–31]. The most frequently used method for vaccines, which include proteins, peptides, and other water soluble molecules, is a double emulsion solvent evaporation method which involves water-in-oil-in-water emulsion [32]. Several studies have shown that careful control over the formulation parameters, such as surfactant concentration and stirring speed, can be used to optimize loading levels and particle sizes [20, 33–37]. To attain uniform size, however, further centrifugation or filtration steps are necessary, which can result in substantial material loss [38]. More recently a novel fluidic nanoprecipitation system has been developed to manufacture PLGA particles of uniform sizes [39].

PLGA particles are highly versatile in that: (i) they can be delivered through many different routes ranging from inhalation to intramuscular; (ii) they are capable of carrying diverse cargo (DNA, siRNA, proteins, and drugs); (iii) they can be readily engineered to carry various surface molecules such as DC-specific antibodies; and (iv) they can be produced to uniform sizes ranging from 50 nm to >5 μm.

Independent murine studies have shown that a single subcutaneous administration of PLGA-ovalbumin (OVA) particles (3–4 μm), or PLGA-bovine serum albumin (BSA) particles (1 μm), was more effective at inducing immunogen-specific antibody responses than complete Freund's adjuvant (CFA) combined with soluble OVA or BSA [40, 41]. Altering the ratio of lactic acid to glycolic acid affects biodegradability and, therefore, release kinetics. As the ratios of the monomers move away from 50 : 50, the biodegradability decreases and so the cargo is retained for longer time periods. However, other parameters such as hydrophilicity of the cargo may prove to be the rate-limiting step in terms of release kinetics. Thus, a slowly degrading PLGA nanoparticle carrying a water soluble peptide is likely to have

released all of its cargo through diffusion well before the particles have completely degraded.

PLGA particles possess intrinsic adjuvant properties that are assumed to result primarily from: (i) the protection of the immunogenic cargo from rapid *in vivo* degradation and clearance; (ii) the sustained release of the immunogenic cargo, which can potentially endure for greater than 30 days; and (iii) the efficiency with which the PLGA particles target and are taken up by DCs. It is also possible that empty PLGA particles themselves have modest, yet significant, adjuvant properties, as they are capable of upregulating a costimulatory molecule (CD80) on *in vitro* cultured bone marrow-derived murine DCs [20]. Nanoparticulates, including PLGA, are capable of escaping the phagosome–endosome compartment and releasing their contents into the cytoplasm, resulting in cross-presentation of the antigenic cargo to CTLs in the context of MHC class I [42]. Finally, PLGA particles (350–450 nm) are efficiently phagocytosed by DCs and can significantly enhance a TLR agonist's capacity for DC activation when loaded into PLGA particles compared with delivery of the agonist in soluble form [43].

In terms of potential use of biodegradable particles in tumor immunotherapy, the focus has mostly centered on PLGA particles. One group performed a prophylactic vaccination study with PLGA particles (2.4 μm) loaded with tumor antigens (from a murine melanoma cell line B16) and CpG ODN (a TLR-9 agonist) [3]. The PLGA particles were fabricated using a double emulsion solvent evaporation technique and the average size of the particles was 2.4 μm (see Figure 16.2). Vaccination with these loaded particles resulted in protection against subsequent tumor challenge, in a murine tumor model, when combined with diminution of regulatory T lymphocytes (Tregs). In a separate therapeutic study it was shown that

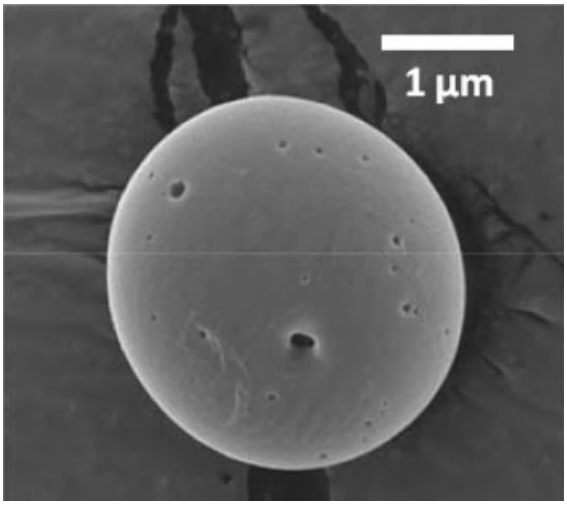

Figure 16.2 PLGA particle. Electron micrograph of PLGA particle prepared using the water-in-oil-in-water double emulsion solvent evaporation method [3].

mice vaccinated, and boosted twice, subcutaneously with PLGA nanoparticles, encapsulating both a TAA peptide, tyrosinase-related protein 2 (TRP-2), and 7-acyl lipid A (a TLR-4 agonist), could control tumor growth through the induction of TRP-2-specific CTLs. Thus it was shown that such a vaccination strategy could break immunotolerance to a normally poorly immunogenic self antigen such as TRP-2 [44].

Polylactic acid (PLA) is a homopolymer of lactic acid that is more crystalline than PLGA. PLA has been less intensively investigated than PLGA with respect to vaccination formulations and tumor immunotherapy. A recent study showed that PLA particles (150 nm) encapsulating OVA were not capable of inducing an OVA-specific antibody response greater than soluble OVA, when administered transcutaneously or subcutaneously [45]. These results are in stark contrast to those obtained with PLGA-OVA particles mentioned above [40, 46]. Two possible explanations for this discrepancy are: (i) the higher antigen-retention capacity inherent to PLA resulting in sub-optimal immunostimulation; or (ii) the smaller particle sizes used in the PLA study were less effective at targeting or activating DCs. A lack of published material on PLA-based NCVs obscures their potential in cancer immunotherapy. That PLGA has received substantially greater focus than PLA may reflect a lack of promising, and therefore published, data where PLA carriers are concerned. Studies that directly compare PLGA and PLA NCVs as tumor vaccines would help to elucidate potential benefits and deficits of each.

PLGA/PLA particles were heralded as the future vaccination method for a wide variety of diseases due to many of the properties mentioned above, particularly because of their burst and slow release kinetics [25, 47]. However, PLGA/PLA has proven to be problematic in that protein degradation can occur for some proteins during the encapsulation process [47]. The fabrication of PLGA/PLA carriers often requires the use of organic solvents, heat, and high speed mechanical agitation. Proteins are highly susceptible to denaturation upon exposure to any of the previously mentioned conditions and may result in loss of antigenic epitope recognition, thereby failing to trigger an immune response. These concerns can be addressed by a more careful fabrication design and stringent control of formulation process parameters including: (i) volume of organic solvents; (ii) assessing the extent of residual solvent content; and (iii) use of protein stabilizing additives including various sugars and polysaccharides. Another possible major stumbling block is the financial consideration of upscaling of production for commercial use and also the clean-up procedure to ensure aseptic and uncontaminated delivery [25].

16.2.2
Acid-Degradable Hydrogel-Based Particles

Acid-degradable hydrogel-based particles (ADHPs) were designed to remain stable at physiological pH but to degrade under the acidic conditions of the endosome (~pH 5) [48]. Upon acid degradation of ADHPs the generation of a large number of molecules within the endosome triggers a rapid influx of water molecules, which causes endosomal ablation and release of contents into the cytoplasm.

These particles therefore have an advantage over PLGA/PLA particles in that they are more potent at cross-presentation of antigen. In fact, ADHPs were specifically designed for the purpose of tumor immunotherapy and were tested as such by the same group that generated them, using OVA as the model tumor antigen [48]. Synthesis of these ADHPs was performed by copolymerization of an acid-degradable cross-linker and a 3′-methacrylamide monomer, using an inverse emulsion polymerization technique, in the presence of OVA. The particles were then further modified by surface conjugation of a long hydrophilic oligoethylene glycol layer, which was done to improve syringability for parenteral administration. The modified particles possessed enhanced colloidal properties due to greater hydration and steric stabilization. An initial murine study revealed that vaccination with ADHPs encapsulating OVA (ADHP-OVA) led to better protection against subsequent EG.7 tumor (OVA-expressing thymoma) challenge compared with vaccination with soluble OVA alone.

Follow-up studies were aimed at improving the immunogenicity of ADHP-OVA by co-loading an adjuvant, the TLR-9 agonist, CpG [49, 50]. This was performed by covalently attaching the CpG to a modified form of the 3′-methacrylamide monomer prior to the copolymerization step. This group established a high antigen loading efficiency (70%), high acidic degradability of the ADHPs, and also demonstrated *in vitro* that these particles were readily phagocytosed by murine dendritic cells. Prophylactic vaccinations in mice revealed that the presence of ADHP-OVA-CpG enhanced immune protection against OVA-expressing B16 melanoma cells compared with ADHP-OVA alone. In addition, murine immunization studies demonstrated potent stimulation of OVA-specific CD8+ T lymphocytes [49, 50]. The therapeutic potency of these acid degradable particles in clinical studies is yet to be determined.

16.3 Liposomal Nanovectors

Liposomes are spherical unilamellar or multilamellar lipid vesicles usually comprising one or more phospholipid bilayers and possessing an aqueous center [51–55]. They can range dramatically in size (unilamellar 50–250 nm, multilamellar 1–5 μm) and are generally classified on the basis of structure, composition, and mode of manufacture. Discovered in the 1960s, their potential as drug nanocarriers had been realized by the 1980s because of their potential for tissue targeting and reducing drug toxicities [56]. However, it was not until the 1990s that liposomes were formulated in such a way as to avoid rapid clearance by the liver and spleen. Now liposomes are considered as part of mainstream nanotechnology for drug delivery with some formulations (e.g., Doxil) having been approved by the US Food and Drug Administration (FDA). The manufacturing methods of liposomes are varied but mostly involve hydration and spontaneous formation as part of the process. These techniques include mechanical processes such as film rehydration, methods based on replacement of organic solvents with aqueous

media, and methods based on detergent removal (reviewed in reference [57]). The method used is primarily dictated by the lipid characteristics as well as end use. Large- scale preparation of liposomes is an ongoing concern where quality and sterility are still big issues [57].

The design of liposomal cancer vaccines is directed toward antigen delivery to, and activation of, professional APCs, such as DCs. In general, liposomes are capable of carrying their antigenic cargo in the aqueous core, embedded in the lipid bilayer, or adsorbed/engrafted to the outer surface. Liposomes are capable of fusing with the membranes of DCs or being pinocytosed. Upon membrane fusion, antigenic cargo (proteins/peptides) is released into the cytoplasm and processed, and presented via the MHC class I pathway. One well recognized drawback of liposomes is their low entrapment efficiencies for water soluble antigens [58]. In order to improve entrapment efficiencies, it is often incumbent on the researcher to optimize the manufacturing method and/or alter the antigen properties, without affecting antigenicity. A variety of liposomes with diverse biochemical properties and biofunctional attributes has been tested as potential vaccine vectors, and are discussed here.

16.3.1
Conventional Liposomes

Conventional liposomes are composed of neutral lipids and phosphatidylcholine and are relatively non-toxic, however they have little or no intrinsic immunoadjuvant properties and in such situations co-loading of antigen with an adjuvant, such as a TLR agonist, is desirable. BLP25 (or Stimuvax®) is a conventional liposome vaccine that has undergone phase I and phase II clinical trials for an advanced form of non-small cell lung cancer (NSCLC) [59]. BLP25 comprises conventional liposomes (cholesterol, dimyristoyl phosphatidylglycerol, dipalmitoyl phosphatidylcholine), a TAA (MUC1 peptide), and the immunoadjuvant monophosphoryl lipid A (MPL) [60]. MPL is a less toxic derivative of lipopolysaccharide that can stimulate DCs through TLR-4 [61, 62]. MUC1 is a mucinous transmembrane glycoprotein that becomes overexpressed and hypoglycosylated in many types of cancer, exposing normally cryptic epitopes in the MUC1 protein core (reviewed in reference [63]). BLP25 is a 25-mer lipopeptide, possessing tandem repeats representing the exposed core of MUC1, which has been palmitoylated to facilitate insertion into the liposome. The results from clinical trials have thus far revealed the vaccine to be of low toxicity and to enhance survival for a subset of patients [59]. Based on these findings, phase III clinical trials are currently underway to establish the potential for BLP25 as a maintenance treatment for patients with unresectable stage III NSCLC who have successfully undergone chemotherapy [64].

16.3.2
Long-Circulating Liposomes

A major limitation of conventional liposomes is their short systemic half-life as they are readily opsonized and cleared by the mononuclear phagocytic system

(MPS) [65]. Long-circulating liposomes (LCLs), also known as stealth liposomes, are sterically stabilized and therefore less readily cleared by the MPS. This steric modification is often performed using a hydrophilic polymer such as polyethylene glycol (PEG). PEG-modification of lipids not only increases circulation times of liposomes but also improves the CTL response to antigenic cargo [66, 67].

The mechanism by which steric stabilization of liposomes extends their circulation half-life is discussed in detail elsewhere but is mainly explained by steric hindrance of binding of blood plasma opsonins [68]. To date no LCL formulations have been used as cancer vaccines in clinical trials. LCLs are more commonly used to target tumors with chemotherapeutic drugs [69], however, there is potential for their use as cancer vaccine vectors in the future. Owing to their increased circulation times, LCLs are known to be more effective than conventional liposomes at passively targeting tumors undergoing aberrant and rapid angiogenesis. Such tumors have more permeable blood vessels amenable to infiltration by nanoparticles in general. This accumulation within the tumor is known as the enhanced permeation and retention effect and has been described in detail by others [70]. However, because of their PEGylated surface, LCLs are less readily capable of interacting with cells in general and require further modifications to enhance association with, and delivery of their cargo to, specific cell types [71].

In one preclinical study it was shown that vaccination of mice intravenously with LCLs carrying adjuvant and a model tumor antigen was capable of generating antigen-specific IFN-γ producing CTLs [72]. These LCLs were prepared from a mixture of disteroyl phosphocholine (DSPC), cholesterol and disteroyl phosphoethanolamine (DSPE) with surface grafted PEG 750. In addition, the adjuvant, derived from the high-mobility box (HMGB1) protein, was engrafted onto the liposomal surface to function as a DC-targeting ligand that also induced activation and maturation of DCs. The same group was capable of generating an anti-tumor response that protected against lung metastases by OVA-expressing B16 melanoma cells, after intravenous vaccination of mice with DC-targeting LCLs (encapsulating OVA +/− LPS or IFN-γ) [73].

Recent findings have highlighted a serious drawback of LCLs that needs to be addressed if these liposomes are to be continued to be used in clinical settings. It was shown that LCLs, and even conventional liposomes, are capable of generating anti-PEG antibody responses *in vivo* that result in accelerated clearance of subsequent systemic PEGylated liposome administrations [74]. These findings pose important questions regarding both the safety and efficiency of PEGylated liposomes despite some formulations being approved by the FDA.

16.3.3 Positively Charged Liposomes

Cationic liposomes are composed of cationic lipids of which the most commonly used include 1,2-dioleoyl-3-trimethyl ammonium propane (DOTAP), dimethyl dioctadecyl ammonium bromide (DDAB), and *N,N*-dioleyl-*N,N*-dimethyl ammonium chloride (DODAC). Cationic liposomes are readily internalized by DCs, and induce strong CTL responses *in vivo* (reviewed in reference [66]).

Cationic liposomes–DNA complexes (CLDC) can induce innate and adaptive anti-tumor activity in various mouse tumor models [75]. It was demonstrated that the co-encapsulation of OVA with CpG in cationic liposomes led to efficient uptake by DCs and macrophages and resulted in preferential localization to draining lymph nodes and generated OVA-specific CTLs as well as *in vivo* anti-tumor immunity [76]. The liposomes used were neutral at physiological pH but contained an ionizable amino lipid 1,2-dioleoyl-3-dimethyl ammonium propane (DODAP) that facilitated encapsulation of CpG. This vaccination procedure was effective against B16 melanoma pulmonary metastasis when the immunogen was switched from the xenogeneic OVA to the less immunogenic syngeneic TAA, TRP-2.

Allovectin-7® is an example of a CLDC currently being used as an immunotherapeutic in phase III clinical trials in patients with metastatic melanoma [77]. Allovectin-7 is a cationic liposome made from (±)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide and dioleoylphosphatidylethanolamine, which is combined with a bicistronic plasmid encoding human leukocyte antigen-B7 (HLA-B7) and beta-2 microglobulin, which combine to produce MHC class I. Allovectin-7 was injected intratumorally into patients with late-stage melanoma in a phase II clinical trial and yielded a 9% overall response rate (complete and partial remissions) [78]. When a dose increase was made in a subsequent phase II clinical trial there was an 11.8% overall response rate (reviewed in reference [77]). It is believed that this vaccine acts through three compounding mechanisms that involve adaptive and innate arms of the host's immune system and are discussed in detail elsewhere [79].

One major problem with the clinical application of cationic liposomes is the potential for dose-dependent cellular toxicity [80, 81]. Furthermore, it has been reported that CLDCs can induce excessive macrophage-mediated pro-inflammatory cytokine production [82].

16.3.4 Archaeosomes

Archaeosomes represent a unique group of liposomes made from lipids derived from the nonpathogenic microbes, *Archaea*. These glycerolipids provide membrane stability due to the presence of an ether-linked isoprenoid phytanyl core and consequently promote potent immune memory. In addition, the variable head domains of these glycerolipids have powerful and unique TLR-independent APC-stimulating properties (reviewed in reference [83]).

Similar to the manufacturing of many other liposomes, fabrication of archaeosomes involves an initial extraction of lipids using a chloroform–methanol–water treatment of frozen–thawed *Archeae* [84]. Total polar lipids are precipitated using cold acetone and resuspended and stored in chloroform–methanol. The lipids are then dessicated and hydrated in the presence of antigen(s), which become encapsulated in multilamellar archaeosomes in the size range of 100–150 nm. These can be stably stored in suspension in contrast to most other types of liposomes.

Archaeosomes are not suitable for oral or intravenous administration since they are rapidly cleared, however, they do form prolonged depots when injected subcutaneously. Consequently, they are capable of promoting immune responses with long cellular memories [85]. It has been noted that altering the relative amounts of a particular archaeal lipid, known as cardarchaeol, within the archeosome dictates the type of immune response induced [86]. In particular, archaeosomes made from the strain of *Archeae* known as *Methanobrevibacter smithii,* which are favorably rich in cardarchaeol, are potent TLR-independent activators of CTLs and thus good candidates for tumor immunotherapy [86]. In murine tumor models, prophylactic vaccinations with archaeosomes encapsulating OVA, or TAAs, resulted in increased survival times due to the stimulation of OVA-specific, or TAA-specific, CTLs [86, 87]. Another advantage of *M. smithii*–derived archaeosomes loaded with antigen is their capacity for cross-presentation, a trait not usually possessed by more conventional liposomes [88]. In a therapeutic setting where the OVA-expressing EG.7 tumor was used, increased survival was shown for mice that had been administered twice post-tumor challenge with OVA-baring archaeosomes [86, 87].

In order to augment the intrinsic adjuvant properties of archaeosomes, a series of novel synthetic archaeal glycolipids were recently manufactured and trialed in murine vaccine studies [89]. Using archaeol as the core lipid, a suite of disaccharide archaeols was generated, possessing minor variations in their carbohydrate head groups. Particular synthetic diglycosylarchaeols, such as beta-gentiobiosylarchaeol and beta-lactosylarchaeol, were found to be capable of inducing potent CTL activity. Therefore, synthetic archaeosomes have the potential to be tailor-made to generate a specific type of immune response. Clinical cancer trials using archaeosomes, synthetic or otherwise, are yet to be initiated, however, given the promising preclinical findings discussed above, and their proven biocompatibility, they appear worthy of further evaluation [90].

16.3.5
Fusogenic Liposomes

Fusogenic liposomes (FLs) are a hybrid of conventional liposomes and the replication deficient Sendai virus. Utilizing the fusion (F) protein derived from the Sendai virus, FLs are capable of fusing with DCs (and other cell types), and emptying their cargo directly into the cytoplasm [91]. The basic method of preparation of FLs involves the fusion of conventional liposomes with ultraviolet light-treated Sendai virus at a neutral pH at 37 °C [92]. Any unfused material is removed by sucrose density gradient centrifugation and the resultant purified FLs are unilamellar and approximately 380 nm in diameter.

Most studies using FLs as tumor vaccines have been limited to murine models at this stage. In one prophylactic study the protective effect against B16 melanoma challenge in mice vaccinated with FLs encapsulating tumor cell lysate was significantly enhanced compared with mice vaccinated with conventional liposomes [93].

16.4
Gelatin Nanoparticles

Gelatin is a pure natural polymer that is biodegradable, biocompatible, and has proven to be safe and protective of its cargo in clinical settings [94]. These factors, combined with the fact that gelatin possesses several functional groups open to diverse covalent attachments or modifications, have recently made gelatin a source of attention in the tumor immunology field. In order to develop uniformly stable gelatin nanoparticles a two-step desolvation technique was developed [95]. This involves dissolving gelatin in water under constant stirring and heating, followed by sedimentation in acetone. The sediment is re-dissolved and re-sedimented at a pH of 2.5 to yield positively charged gelatin that can be further used to fabricate nanoparticles. Gelatin is easy to cross-link and can be sterilized using a wide range of chemical and heat sterilization methods. In addition, gelatin is pyrogen-free and possesses low immunogenicity.

In one tumor protection study, gelatin nanoparticles, loaded with OVA, were surface modified using cholamine hydrochloride in order to gain a net positive charge. This was done for two reasons: (i) positively charged particles are more readily phagocytosed by DCs than negatively charged or neutral particles; and (ii) these researchers wished to adsorb the negatively charged TLR-9 agonist, CpG, to the surface of the gelatin nanoparticles in order provide an adjuvant effect [96]. The payload of CpG was 5–10% w/w of the nanoparticles, which still rendered them stable under physiological conditions while also retaining their positive charge. Comparison of immunizations that co-delivered OVA and CpG in gelatin nanoparticles to those that delivered particulated antigen with soluble CpG, showed augmented antigen-specific CTL responses and enhanced tumor protection for the former treatment. As yet, however, no clinical trials appear to be currently underway to address the therapeutic potential of gelatin nanoparticles as cancer vaccines.

16.5
Sub-micron Emulsions

Sub-micron emulsions, also known as nanoemulsions are thermodynamically stable colloidal dispersions of nanosized (20–200 nm) droplets of oil and water stabilized with a surface active film. In terms of vaccine potential, sub-micron emulsions are capable of delivering immunogens, such as peptides, either systemically or locally and triggering potent humoral and cellular immune responses. As with other nanocarriers already mentioned, such as PLGA, sub-micron emulsions can efficiently encapsulate antigen during preparation and subsequently protect cargo against *in vivo* enzymatic degradation as well as provide a sustained release of the antigenic cargo. The release of cargo occurs upon lipolysis of the continuous phase forming the emulsion. In recent years, considerable effort has been directed towards developing sub-micron emulsions as vaccine carriers. Some of these have recently been described for the mucosal route vaccination against infectious diseases, such as hepatitis B [97], HIV [98], and influenza [99].

The most favorable method for production of sub-micron emulsions for vaccine use is high-energy emulsification, primarily due to the ease of manufacture and industrial scale up. High-pressure homogenization or ultrasonic emulsification under magnetic power produces very stable and homogenous dispersions relatively quickly. The nanometer size range of sub-micron emulsions results in a decreased propensity to sediment and cream during manufacture, which are problems commonly associated with conventional emulsions.

Sub-micron emulsion vaccine delivery of tumor-specific antigens, in mice, induced strong tumor-targeted antibody and CTL responses, which ultimately conferred protection against tumor growth [100–104]. One group developed a sub-micron emulsion vaccine co-loaded with CpG and a gastric cancer TAA, MG7 [100, 105, 106]. The sub-micron emulsion carrier was prepared by vacuum high shear ultrasonic emulsification resulting in high payloads for the antigen (70%) and the CpG (90%). Formation of the sub-micron emulsion involved mixing an aqueous phase consisting of 0.8% surfactant mixture of Span-80 and Tween-80 and an oil phase consisting of the same ratio of surfactant in soybean oil combined with antigen and CpG solubilized in PEG2000. After homogenization of the oil phase, final size reduction was performed using an ultrasound generator. Mice vaccinated with these sub-micron emulsions showed significant protection against challenge with MG7-expressing carcinoma cells compared with sub-micron emulsions containing peptide alone. The tumor protection directly correlated with MG7-specific antibody and IFN-γ production.

Using a similar manufacturing technique, an independent group developed a melanoma-targeted sub-micron emulsion vaccine that encapsulated the TAAs, MAGE-1, and/or MAGE-3, as well as adjuvants, heat shock protein 70, and Staphylococcal enterotoxin A [101–104]. This product exhibited high storage stability and no evidence of creaming or sedimentation under shelf-storage conditions. Vaccination of mice with sub-micron emulsions encapsulating MAGE-1/HSP70/SEA resulted in enhanced tumor-specific responses and enhanced resistance to subsequent melanoma challenge when compared with non-encapsulated delivery. Comparison of various routes of administration (i.e., intravenous, intraperitoneal, subcutaneous or peroral) revealed no difference in the ability of the sub-micron emulsion vaccine formulations to generate anti-tumor responses [103, 104]. These promising tumor protection data, along with the demonstrated safety and stability of sub-micron emulsion formulations, provide a strong incentive for their use in clinical trials for cancer patients [97].

16.6 Amphiphilic Block-Graft Copolymers

Another interesting area in the use of nanoscopic systems for tumor immunotherapy is the application of amphiphilic block-graft copolymers as antigen/protein carriers using the biodegradable polymer poly(γ-glutamic acid) (γ-PGA). γ-PGA is produced by several Bacillus species as an extracellular polymer. It is frequently referred to as a pseudo-amino acid with the glutamate repeat units in γ-PGA

containing linkages between the α-amino and γ-carboxylic acid functional groups. γ-PGA is entirely biodegradable and non-toxic to humans, and is degraded *in vivo* by γ-glutamyl transpeptidase, which is widely distributed in humans and catalyzes the hydrolysis of the polymer to its constituent amino acids. These self-assembled amphiphilic nanocarriers typically possess a hydrophobic corona and are commonly referred to as core-corona type polymeric particles. The hydrophobic microdomains of these self-aggregates can be used for encapsulating proteins.

The use of biodegradable γ-PGA nanoparticles was recently shown to be effective for delivery of protein antigen [107, 108]. Nakagawa and coworkers developed such a self-assembly system using γ-PGA in which the L-phenylalanine ester (PAE) was introduced as a hydrophobic residue on the α-position carboxylic acid groups of the γ-PGA in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [109]. The antigenic protein of interest, OVA, was encapsulated within this carrier using an electrostatic interaction mechanism. These particles entrapping OVA exhibited a mean size of 250 nm with a 60% protein loading efficiency. These workers further showed a controlled release of the entrapped antigenic protein for a period of 30 days. Preliminary studies with these nanoparticles indicated their potential for use as cancer vaccines. Specifically, these particles were efficiently phagocytosed by DCs *in vitro*, which induced maturation, as evidenced by cytokine production and up-regulation of co-stimulatory molecules [110]. Furthermore, the signaling pathways involved in the γ-PGA nanoparticle-induced maturation of DCs were found to be MyD88-dependent, and ultimately resulted in NF-κB activation.

In vivo, mice immunized with OVA-loaded γ-PGA nanoparticles responded with strong OVA-specific T- and B-lymphocyte responses, as measured by lysis of OVA-expressing target cells, production of IFN-γ by OVA-restimulated splenocytes, and production of anti-OVA IgG antibodies. Uto *et al*. further demonstrated that mice previously immunized with γ-PGA nanoparticles with immobilized bacterial antigen on the surface were significantly protected after an *in vivo* challenge with a lethal dose of *Listeria monocytogenes*, a model for CD8+ T lymphocyte-mediated protection against intracellular pathogens. These findings were followed up by a brief study that further demonstrated the effective delivery of peptide antigens and induction of CD8+ T lymphocytes responses when the γ-PGA nanoparticulated peptides were targeted to the ER [111].

Tumor protection studies employing γ-PGA nanoparticles as cancer vaccines have followed these initial reports. Yoshikawa and colleagues first reported that this system could be used to deliver antigenic vaccines that target APCs and promote the MHC class I presentation pathway, ultimately conferring significant tumor protection in models using OVA-expressing tumors [109, 112]. Based on their findings, they reported that preliminary clinical trials were planned for the near future. Most recently, γ-PGA nanoparticles loaded with a known TAA, EphA2, were used to vaccinate mice to determine the level of protection induced upon EphA2-expressing tumor cell challenge [113]. For this study, tumor cells were injected into the livers of mice to simulate a model of tumor metastasis. Mice vaccinated with EphA2-γ-PGA nanoparticles exhibited enhanced EphA2-specific

CD8+ T lymphocyte activation, target cell lysis, and decreased overall liver size as a measure of tumor protection. Importantly, immunization and induction of responses against the liver tumors did not result in liver pathology or any toxic effects on liver or kidney function, indicating this system is safe and a good candidate for clinical applications. Based on these findings this group also reported that they were currently preparing their γ-PGA nanoparticle vaccine formulations for clinical testing.

16.7 Iron Oxide Nanoparticles

Magnetite (Fe_3O_4) is a ferrimagnetic mineral and is the most magnetic of all naturally occurring minerals on Earth. Magnetite cationic liposomes were first described for hyperthermia cancer therapy, or "heat immunotherapy," a little over a decade ago [114, 115]. Since then these nanoparticles have repeatedly been demonstrated to induce significant tumor regression via induced heat shock protein (HSP) expression, which ultimately leads to enhanced presentation of TAA in association with MHC class I and the generation of tumor-specific CTL-mediated immunity [116–126]. This phenomenon has been studied in several animal tumor models using magnetite nanoparticles alone, and in combination with additional immunotherapies or chemotherapies, such as immunoliposomes [121, 127] and intratumoral cytokine or DC injection [116, 122, 123].

The most commonly utilized methods for synthesis of magnetite nanoparticles are a co-precipitation method and a thermal decomposition method [128]. The co-precipitation method is an inexpensive method that results in magnetite nanoparticles in the 15–50 nm size range. The procedure involves the addition of a strong base to a solution of Fe^{2+} and Fe^{3+} salts in water. The thermal decomposition method results in magnetite particles ranging in size from 5 to 40 nm. This method involves the application of high temperatures to a high boiling point solvent containing an iron-oleate and a surfactant under an inert atmosphere.

Recently, preclinical therapeutic murine studies showed that melanoma-targeting chemo–thermo–immunotherapy with magnetite nanoparticles (~10 nm) coated with a melanogenesis substrate could prevent melanoma tumor growth [126]. These functionalized nanoparticles were injected intratumorally and were detrimental to tumor growth through two mechanisms. The first was a biochemical mechanism where the melanogenesis substrate, *N*-propionyl cysteaminylphenol, a tyrosine analog, caused cytotoxic oxidative stress. The second mechanism involved cancer cell disintegration, caused by the application of an alternating magnetic field, to mice injected intratumorally with the magnetite particles, which induced the production of heat shock proteins. This treatment not only inhibited tumor cell growth but protected against subsequent rechallenge with similar tumor cells (B16), implicating the involvement of adaptive immunity. The preparation of these coated magnetite nanoparticles involved coating the particles with

aminosilane and then conjugating the melanogenesis substrate using maleimide cross-linkers [129].

Preliminary phase I and II clinical trials focusing on using these magnetite nanoparticles began in Japan, with remission for more than 24 months observed in two of the four patients enrolled [126, 130]. However, an important limitation of this system to consider is that in all cases the particles were delivered via intratumoral injection. The research group that has primarily studied this system previously achieved successful targeting of magnetite particles to Her-2/neu-overexpressing breast cancer cells *in vitro* [121] and *in vivo* [127] by combining them with anti-Her-2/neu immunoliposomes [121], but systemic delivery was not examined. This promising therapy might be more universally applicable in the clinical setting if it could be modified for systemic delivery with preferential targeting of tumor cells. Another important drawback is that inorganic particles, such as gold and magnetite, are not biodegradable or cleared easily, resulting in potential accumulation in the body, which may cause long-term toxicity.

16.8 Viruses–Virus-Like Particles–Virosomes

16.8.1 Viruses

Viruses are infectious agents, ranging in size from 15–400 nm, whose genome is contained within a protective protein coat that may be further enclosed in a host cell-derived lipid envelope. The use of live attenuated viruses as vaccines has proven extremely successful in controlling or eradicating numerous viral borne diseases (reviewed in reference [131]). However, most cancers (>80%) are not of viral origin and therefore recombinant DNA encoding specific TAAs usually needs to be introduced into the viral genome prior to vaccinating against tumors.

To date very few viruses have been used in clinical cancer trials primarily due to the potential for immune-mediated toxicity as a result of undesirably immunogenic viral components of the vaccine. Triggering long-term adaptive CTL-mediated anti-tumor responses often involves repeated vaccinations, rendering first generation viral vectors generally unsuitable due to the greater potential for a morbidly high and inappropriate immune response [132].

In phase II studies on patients with metastatic androgen-independent prostate cancer, one group has attempted to overcome this problem by vaccinating using a prime–boost regime that involved antigenically distinct viruses [133, 134]. They primed with a recombinant vaccinia virus vector containing four transgenes that included prostate specific antigen (PSA), which is a known TAA, and three T-cell co-stimulatory molecules, B7.1, ICAM-1, and LFA-3. The boost was performed using recombinant fowlpox virus vector containing the same four transgenes. The results of these studies indicated improved survival times for those patients with less aggressive disease.

Another vaccine currently used in clinical trials is TroVax® (MVA-5T4), a modified vaccinia Ankara virus (MVA) encoding the TAA, 5T4 (reviewed in reference [135]). MVA is highly attenuated, replication deficient, nonpathogenic, and has a good safety profile. 5T4 is a cell surface oncofetal antigen highly expressed by many adenocarcinomas but rarely in healthy adult tissues other than the placenta. Clinical trials with MVA-5T4 have thus far established its safety and immunopotency with regard to 5T4-specific CTL production, but have not yet observed significantly improved patient survival [136].

Adenoviral (Ad) vectors are the most commonly used viral vectors, and one of the most efficient methods for gene delivery *in vivo,* for vaccine applications and gene delivery [137]. Ads are non-enveloped, double stranded DNA viruses that can potentially enter cells by exploiting multiple independent molecular tropisms involving ubiquitously expressed cellular receptors [137]. Importantly, Ads are capable of directly invading DCs through mechanisms that depend both on the type of DC and the serotype of the Ad used. For instance, it has been shown that Adenovirus serotype 5 (Ad5), which is commonly used in research and clinical therapy, is capable of infecting bone marrow derived DCs by lactoferrin-mediated binding of Ad5 to a C-type lectin receptor, DC-SIGN, expressed on the DCs [138]. Once Ads are endocytosed by DCs, and other cell types, they can escape the endosome and subsequently inject their genome into the nucleus, where it can be stably maintained and expressed without host genomic integration. That Ads fail to integrate into the host genome substantially limits their oncogenic potential and makes them more favorable vaccine options over certain other viruses, such as retroviruses. Another advantage of recombinant Ad vectors is that they can be grown to high yields under conditions suitable for manufacture for clinical use.

Owing to the prevalence of Ad infections in the community, a high proportion of the population already have neutralizing antibodies to the more commonly used Ad vectors such as Ad5 and Ad2. Such a problem can be overcome through the use of human Ads with rare serotypes, or using non-human Ads, as cancer vaccine vectors. In a preclinical study, one group, using a second generation chimpanzee Ad vector (ChAd) carrying DNA encoding the TAA, Her-2/neu, was able to break tolerance in neu-transgenic mice resulting in antitumor activity that led to significant levels of protection [139].

Another way of circumventing the neutralizing effect of Ad antibodies is through the use of Gelfoam®. Gelfoam is a purified gelatin powder that is water insoluble, porous, and capable of absorbing fluids at many times its own weight. Although established for other clinical or surgically related purposes due to its clot-promoting abilities [140], Gelfoam has recently been trialed as an adjuvant for Ad vaccinations. In preclinical studies, vaccination of Ad-primed mice with Gelfoam admixed with Ad5-PSA could induce stronger PSA-specific CTL-mediated immune responses than aqueous Ad5-PSA vaccinations [136]. Thus, Gelfoam provides protection against antiviral antibodies already present in the host. The mechanism of protection is as yet unknown. Phase I clinical trials in prostate cancer patients revealed that subcutaneous Gelfoam–Ad5-PSA vaccinations were safe and more efficient at generating CTL responses than aqueous Ad5-PSA [141].

As is the case for viruses in general, Ad vector use has been limited in clinical cancer vaccine studies due to the potential for the activation of unwanted cellular, humoral, and innate immune responses (reviewed in reference [142]). The hepatic toxicity caused by Ads, however, can be avoided if they are delivered non-systemically.

16.8.2 Virus-Like Particles (VLPs)

Virus-like particles VLPs are self-assembling non-infectious spheres comprising a viral coat but lacking the viral genome (reviewed in reference [143]). Their safety profile is superior to that of attenuated viruses since they are non-infectious and incapable of replication. VLPs can be cheaply and efficiently produced for therapeutic use in a variety of expression systems, including yeast, green plants (e.g., tobacco mosaic virus) and attenuated gut flora [143]. VLPs are readily taken up by DCs via macro-pinocytosis and endocytosis, and trigger both the innate and adaptive arms of the immune response without the requirement for additional adjuvants (reviewed in reference [144]).

One particularly successful VLP vaccine to have been licensed in the last decade is Gardisil®, a quadrivalent vaccine against human papilloma virus (HPV). HPV is a primary cause of cervical cancer, which is responsible for over a quarter of a million deaths worldwide annually. Gardisil® is made from a mixture of four recombinant HPV type-specific VLPs comprising the L1 major capsid proteins of HPV 6, 11, 16, and 18, which are synthesized in the yeast *Saccharomyces cerevisiae* [145]. Both phase II and III clinical trials involving susceptible females demonstrated the vaccine to be highly protective [145, 146].

VLPs are likely to be limited to prophylactic use for cancers of viral origin (<20% of total cancers). Although the potential for them to form sustained and effective neutralizing antibodies has been well documented, their capacity to induce strong cellular-based effective antitumor responses has not been thoroughly addressed. It has been reported that VLPs containing envelope proteins possess enhanced MHC class I presentation capacity due to increased receptor-mediated fusion, thus implicating such VLPs as potentially effective CTL-inducing vaccines [147].

16.8.3 Virosomes

Virosomes are virally derived unilamellar lipid envelopes that retain both the fusogenic and antigenic properties of the virus but lack the viral genome. Some of the more promising virosomes gaining attention in the field of cancer immunotherapy are the immunopotentiating reconstituted influenza virosomes (IRIVs) (reviewed in reference [148]). IRIVs are approximately 150 nm in diameter and are composed of both natural and synthetic phospholipids along with influenza surface glycoproteins [149]. One of these viral proteins, haemagglutinin, plays a crucial dual role in: (i) attaching the virosome to the DC via sialic acid residues;

and (ii) promoting the fusion of the engulfed virosome with the endosome, resulting in release of virosomal contents into the cytoplasm [149]. To date only two virosome vaccines have been licensed and these are both IRIVs, which protect against hepatitis A (Epaxal®) or influenza (Inflexal®) [150, 151].

Manufacture of IRIVs often involves dissolving phosphatidylcholine and phosphatidylethanolamine in an octaethyleneglycol (OEG) solution and mixing with haemagglutinin (purified from influenza/Singapore), also dissolved in OEG. This mixture is ultrasonicated then ultracentrifuged (100 000×*g*) and the supernatant sterile filtered (0.22 μm). Virosomes are then formed by detergent removal [152]. IRIVs can be manufactured to favor the generation of TAA-specific B lymphocyte responses or TAA-specific CTL responses, depending on whether the TAA is surface attached to, or encapsulated within, the virosome, respectively [149]. Nevertheless, IRIVs are inherently capable of stimulating T_h1-biased CTL responses independent of TAA loading, as was shown for the melanoma TAA, Melan-A/$Mart1_{27–35}$ [152].

In a separate study, an IRIV vaccination protocol based on those used for hepatitis A and influenza has recently undergone a phase I trial in patients with breast cancer [153]. Here, IRIVs were generated that incorporated Her-2/neu B cell epitopes coupled to phosphatidylethanolamine and hemagglutinin and were integrated into the virosome during reconstitution. The primary and secondary endpoints of this phase I study, which were safety and immune responsiveness, respectively, were both met. In terms of immune responsiveness, it was established that anti-Her-2/neu antibodies were generated in 8 out of 10 patients. Generation of a CTL-mediated response was not presented, although it was demonstrated that the vaccinations resulted in a significant lowering in the percentage Tregs.

A major disadvantage of the more conventional virosomes, such as the IRIVs mentioned above, is the limited loading capacity for antigenic peptides [154]. It was demonstrated that the fusion of epitope-loaded conventional liposomes with IRIVs generated chimeric IRIVs (CIRIVs) that possessed a 30-fold greater loading capacity. The immunoadjuvant properties of the IRIV were retained and the CIRIVs could generate TAA-specific CTL responses *in vitro* [155]. These chimeric carriers were capable of being stably stored at 4 °C for at least 1 month. CIRIVs represent a promising mode of generating antitumor immune responses to TAAs and future clinical studies are anticipated.

Another of the virosomes to receive attention recently is the Sendai virosomes. These are similar to fusogenic liposomes mentioned earlier, but different in that they are not produced by fusion with conventional liposomes. These are vesicular nanoparticles (50–200 nm) that are reconstituted from Sendai viral envelopes or intact Sendai virions. The manufacture of Sendai virosomes involves the solubilization of the virions in detergent followed by detergent removal using SM-2 BioBeads® [156, 157]. Similar to IRIVs, Sendai virosomes express sialic acid binding proteins, which allow for APC attachment. In addition, Sendai virosomes possess a fusion protein that promotes the fusion of the virosome with the plasma membrane resulting in release of the virosomal contents directly into the cytoplasm. Very few studies have been performed to date using Sendai virosomes as

NCVs. One preclinical study has shown that vaccination of mice with Sendai virosomes loaded with proteins derived from B16 melanoma cells results in the generation of B16-specific CTLs [156].

16.9 Conclusion

NCVs are primarily designed to generate strong adaptive cytotoxic antitumor responses that are systemic. With some novel exceptions the majority of nanocarriers reviewed here were designed to achieve this goal by specifically or passively targeting dendritic cells (DCs) such that: (i) the tumor antigens delivered by these nanocarriers are at least partially presented in the context of MHC class I (i.e., cross-presented) and, thus, a trigger for antigen-specific cytotoxic CD8+ T lymphocytes is provided; and (ii) the delivery of a second signal ("danger signal") is achieved usually in the form of various intrinsic or co-encapsulated adjuvants, which result in activation and maturation of the targeted DCs. The majority of the treatment modalities have demonstrated promising preclinical results in terms of prophylactic and/or therapeutic tumor models. Some have progressed as far as phase I, II, and III clinical trials that demonstrate the safety and potential therapeutic benefits of these nanocarrier systems. It is difficult at this early stage to prognosticate as to which systems are most likely to progress as established vehicles for cancer immunotherapy. Nevertheless, aside from the empirical therapeutic benefits, other strongly determining factors are likely to be biocompatibility, the ease and cost of manufacture and storage, along with a nanocarrier's malleability with regard to design features and routes of administration.

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17
Recombinant Vaccines: Development, Production, and Application

Luke R. Le Grand, Michaela White, Evan B. Siegel, and Ross T. Barnard

17.1
Introduction

This chapter reviews the place that recombinant vaccines have achieved in the therapeutic armory since they first emerged on the market in mid-1980s. It notes the current challenges faced in the development, manufacture, and marketing of these agents and the emerging solutions to these challenges. Since their inception, vaccines have been the best disease prophylaxis and most cost effective medical measure available.

Until the launch of Engerix-B® (GlaxoSmithKline) in 1986 [1], all vaccines on the market were based on the administration of killed or attenuated disease vectors. However, this process was not ideal. Although scientific evidence has debunked many public misconceptions about vaccine safety [2], the best known example of the problems relating to this traditional form of vaccine was the reversion of the attenuated Sabin oral polio vaccine (OPV) to an active or partially active neurovirulent form after inoculation. In these exceptionally rare cases, 1 in 2.5 million patients in the United States between 1980 and 1989 acquired vaccine-associated paralytic poliomyelitis (VAPP) [3, 4].

With recombinant technologies, specific antigenic epitopes can be isolated, amplified in a cellular expression system, combined with other immunogenic effectors, and delivered to immune cells, greatly reducing the risk of iatrogenic infection. Biotechnology and the development of recombinant vaccine technology has revolutionized the approach of the biomedical community to infectious disease prevention.

For the sake of brevity the scope of this chapter has been limited to select examples of vanguard technologies, illustrating the key issues in vaccine research and development.

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17.2
Range of Recombinant Vaccines on the Market and in Development Today

In the two and a half decades since recombinant vaccines first became available, at least 34 recombinant vaccine formulations have been released onto the global market [5–8]. This figure does not include the derivative products that result from the re-badging of vaccines for the purpose of marketing the vaccines in different nations nor in discriminating between divergent dosing regimes optimized for either children or adults. However, recombinant vaccines on-market only address a narrow range of diseases; two compounds immunize against HPV, one recombinant vaccine targets *Vibrio cholerae* (cholera) and enterotoxigenic *Escherichia coli* (ETEC), with the rest shielding against hepatitis B [5]. With seven products currently licensed for immunization and distribution by the FDA, recombinant vaccines account for just over 10% of the 63 vaccines on the US market [6]. Figure 17.1 provides an overview of the current range of recombinant vaccines on the market and in development.

While the attrition of vaccine candidates from preclinical trials through to launched products follows much the same path as other pharmaceuticals, peculiar to the recombinant vaccine development process is how compounds appear to readily move through *in vitro* testing only to stall at preclinical trials, with only one in five vaccine candidates proceeding beyond this juncture. This is often a result of the failure of vaccines to elicit a protective immune response or economic factors related to the extensive testing and time required for characterization of

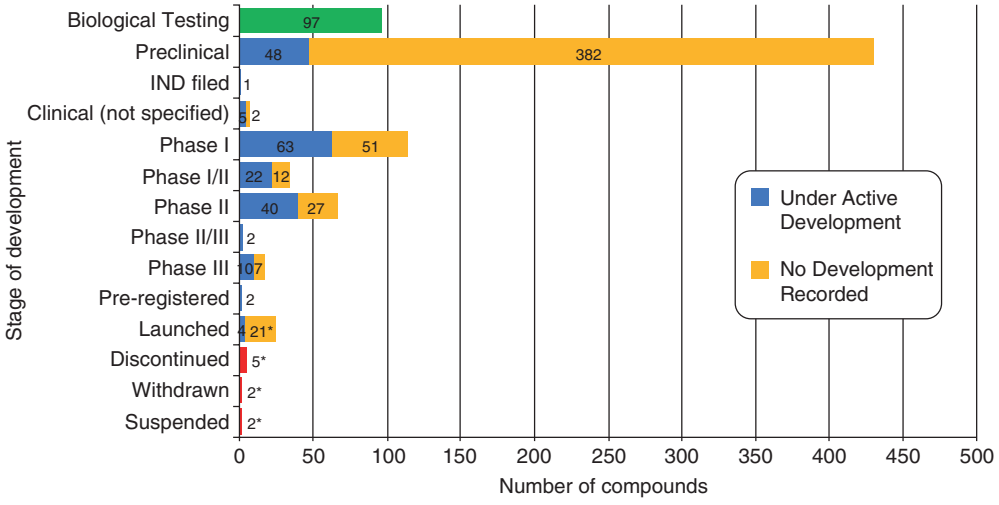

Figure 17.1 An overview of the current range of recombinant vaccines on the market and in development, May 2011. For a compound to qualify as *Under Active Development*, announcement of active work must have been made in the past 18 months otherwise it is filed as *No Development Recorded*. Data source: *Thomson Reuters Integrity* SM [5]. *Data supplemented using FDA, EMEA and TGA [6–8].

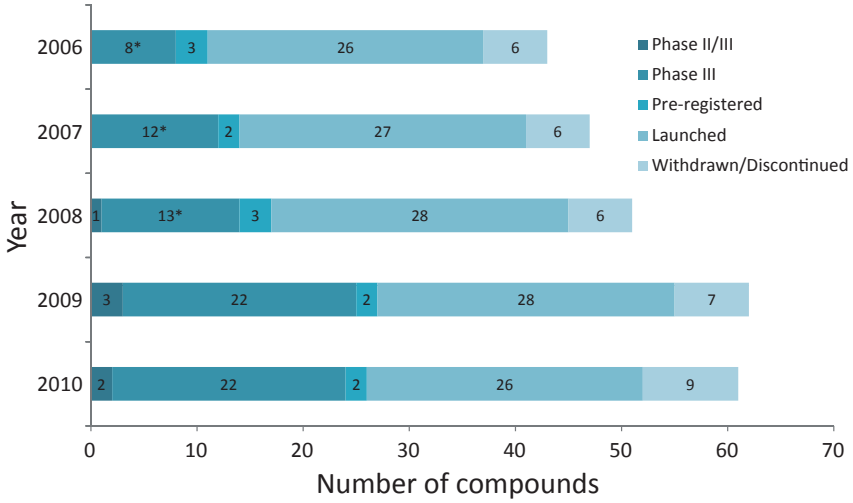

Figure 17.2 Longitudinal study of recombinant vaccines for infectious disease over the past five years to the end of 2010. Data source: *Thomson Reuters Integrity* SM [5]. *Data supplemented using clinicaltrials.gov.

the vaccine *in vivo*. The challenges of maintaining adequate delivery, distribution, and targeting of the vaccine to establish a memory immune response (both mucosal and systemic), ensuring a balanced Th1/Th2 response and eliminating potential toxicity, significantly impedes progress to market.

All vaccine development programs must establish field sites early, prepare to meet regulatory and manufacturing standards, standardize the diagnostics required to measure response to the vaccine, obtain burden of disease estimates in target populations, and address safety concerns early in the basic research phase.

As a consequence of the challenges faced, and highlighted in this chapter, there was little change between 2009 and 2010 in the number of vaccines entering late-stage development. Further, recombinant vaccines on the global market have returned to the number available in 2006 as a result of the suspension of two hepatitis B vaccines. Figure 17.2 provides a longitudinal view of recombinant vaccines in development for infectious disease over the past five years. It will be interesting to observe trends in the migration of compounds through the various stages of development over the next five years. Despite this plateau, there have been some exciting developments in the vaccine development pipeline, including advances by several groups developing Dengue vaccines (see Table 17.1).

Of this group of vaccines, one particularly promising candidate is the Sanofi Pasteur chimeric virus platform, which uses a recombinant YF 17D live virus carrying the prM and E genes from each of the four dengue serotypes. When combined as a tetravalent vaccine and given as a single dose there was a 92% protection rate observed in monkeys. In human trials, three doses were required at 6 month

Table 17.1 Development pipeline for dengue vaccines (modified from Halstead [10]).

Developer	Producer	Process development	Evaluation		
			Phase I	Phase II	Phase IIB-III
Acambis	Sanofi Pasteur	→			2009
WRAIR	Glaxo Smith Kline	→			?
		→		mid 2009 tetravalent	
NIH	Biological E	→	?		
	Butantan	→	2010		
	Panacea	→	?		
CDC	InViragen/Shantha	→	mid 2009		
Hawaii Biotech	Hawaii Biotech	→	mid 2009		

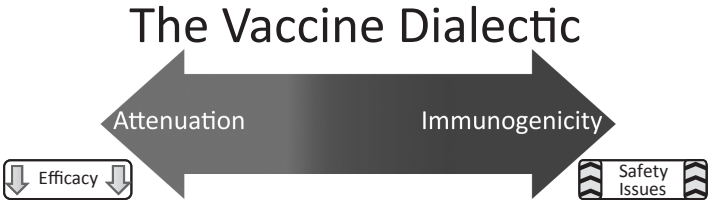

Figure 17.3 The vaccine dialectic showing the opposing pressures for vaccines to raise an immunogenic response but not at the expense of safety.

intervals to produce 100% tetravalent neutralizing antibodies. Phase II–III trials are now in progress across Asia and the Americas monitoring 30 000 adults and children [9, 10].

17.3 Vaccine Dialectic

At the heart of vaccine design and development there is a fundamental tension: how can a pathogen be sufficiently attenuated to avoid producing a disease state after administration without destroying the ability of the active principle to elicit a long-lasting and protective immune response? (Figure 17.3)

Successful vaccine development requires both objectives to be met. Unfortunately, cases of poor tolerability have plagued development efforts in conventional vaccines (see Section 17.5.1) while, conversely, recombinant vaccines have often struggled to reach sufficient levels of immunogenicity (see Section 17.5.2) [11].

However, as our understanding of the immune system and disease processes deepen, recombinant technology continues to expand and diversify with new and exciting rationally designed vaccines approaching the market.

17.4
Comparing Vaccine Efficacy

Comparing the efficacy of different vaccines is complicated by the inability to make direct comparisons between experiments carried out using different animal strains. For example, Atkins *et al.* attempted to immunize against Melioidosis using an *ilv1* mutant into a BALB/c murine model [12]. Mice from this strain eventually succumbed to infection. However, in a similar study aiming to induce protective immunity, conducted by Srilunchchang *et al.*, the C57BL/6 mouse strain showed efficient antigenic clearance after immunization [13]. The difference in immune protection is very likely a consequence of genetic difference between murine strains, not limited to differences in major histocompatibility antigens.

The discrepancies in immunogenicity observed between strains of the same species are further compounded when extrapolating the pharmacodynamic effects of the vaccine candidate between species [14]. The traditional approach to preclinical testing of therapeutic compounds has been to carry out *in vivo* testing on murine models followed by testing on lower-order primates. However, there is a trend emerging at institutions such as the National Institute of Health (NIH), towards reducing emphasis on lower-order primate tests in favor of more rigorous studies in mice and omission of the intermediate primate testing [10] (see Figure 17.4). From a regulatory perspective the establishment of a qualified, reproducible and, eventually, validated potency assay in a cellular or *in vivo* environment, is important in fostering licensing by the regulatory authorities and assuring batch to batch consistency in manufacturing.

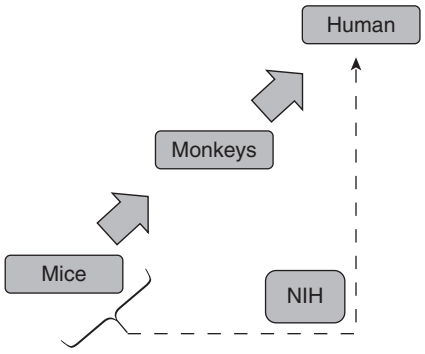

Figure 17.4 Shift in National Institute of Health (NIH) strategy towards a reduced emphasis on testing of therapeutics on lower order primates before human clinical trials commence.

17.5
Vaccines: A Brief Overview

There are several types of vaccine available. Although these vaccines can be grouped under various headings, it must be recognized that there is intersection between the various groups. These groups are based in part upon the terms articulated in the literature by researchers and have become terms of art rather than terms of scientific precision. However, as these terms are encountered frequently in the literature an attempt at a broad definition is useful and provides context for the discussion that follows. Over the last two centuries of their use, vaccines have gone through many generations of refinement.

A conventional categorization of all vaccines places them in the following groups.

17.5.1
Conventional Vaccines

17.5.1.1 Whole Organism

The whole organism was the original type of vaccine dating back to Jenner's work in 1796 with cowpox (*Orthopoxvirus bovis*) [15], and is manufactured using relatively crude extracts of the biological material capable of inducing an immune response in the human subject. The effectiveness of this class of vaccines is dependent on the range of antigens present and the ability of these to stimulate both a Th1 (cell mediated) and Th2 (antibody mediated) response. In the case of attenuated vaccines it is important to ensure that there is no reversion of the attenuated pathogen back to an active state [16]. These vaccines are often referred to as *first-generation* vaccines.

17.5.1.2 Toxoid

The toxoid vaccine class is often identified as a *second-generation* vaccine technology. A well-known example of this group is the multi-component diphtheria, tetanus, and pertussis (whooping cough) vaccine (DTaP) [17]. Here, toxins expressed by bacterial pathogens are administered to patients at sub-toxic levels allowing for both cellular and humoral immune recognition of the pathogen upon reinfection.

17.5.2
Recombinant Vaccines

17.5.2.1 Subunit Vaccines

An underlying principle for the design of recombinant vaccines is to present an easily recognizable and generally conserved antigenic epitope to the immune system without exposing patients to the risk of contracting the very disease the vaccine was designed to prevent. The presentation of a few epitopes rather than the entire organism is the essential difference from conventional vaccines and lies at the heart of recombinant vaccine technologies. Theoretically, these vaccines are

more elegant, consistent in manufacture, and allow for engineering of their components such that unwanted immune and autoimmune responses in the inoculated patients will occur with less frequency than those induced by conventional vaccines. From a regulatory point of view, their manufacture may be considered to be more easily controlled and assessed at key stages of production.

A recent example of this approach is the work of Shattock and colleagues [18] who carried out repeated mucosal immunization of rabbits using soluble recombinant trimeric HIV-1 clade C gp140 protein. A single immunization cycle induced immunoglobulin G antibodies in serum and the female genital tract, and titers were boosted on further immunization. Vaccine-induced serum antibodies neutralized the infectivity of a pseudovirus carrying a heterologous clade C envelope. The importance of route of administration and the challenges inherent in moving from animal models to humans was evidenced by the observation that the intravaginal route was ineffective in humans. This work has been extended to non-human primate trials in which an intramuscular injection, followed by an intravaginal immunization produced systemic and mucosal responses.

17.5.2.2 **Conjugate Vaccines**

In conjugate vaccines (a category which includes fusion constructs), poorly immunogenic proteins or polysaccharides are paired with toxins that cause the immune system to respond more vigorously to the poorly immunogenic subunit. These protein arrangements often act as *self-adjuvanting* compounds (see Section 17.8.3.2), potentially allowing for an easier and often faster drug approval process. In these instances, only one substrate needs to be analyzed, removing complications associated with dynamic interactions and concentration ratios in multicomponent vaccines [19]. Thus, the analytics required for production are simpler and the move from small-scale trials to full-scale production, with demonstrable chemical equivalence, is likely to be easier [20]. From the regulatory point of view, the fate of the toxin component is important with regard to patient safety.

17.5.2.3 **DNA Vaccines**

DNA vaccines are classed as *third-generation* vaccines and work by delivering a segment of DNA derived from the infectious agent into host cells. These DNA fragments are typically highly conserved regions of the infecting pathogen. Epitopes of the pathogen are expressed by the transformed host cells and are presented to the immune system. By this means it is hoped that an immunological memory with induction of CD4+ and CD8+ T-cells specific for the pathogen will be elicited. DNA vaccines are inherently safe but face the very large technical problem of delivery to target cells (see Section 17.7).

For DNA plasmid vaccines, the US Food and Drug Administration is concerned with both changes to the vector and the genetic sequence of the inserted gene(s) during and after manufacture. This should be taken into account in the assessment of structure and sequencing of the vaccine during the manufacturing process.

17.5.2.4 T-Cell Receptor Peptide Vaccines and T-Cell Epitope Conjugate Vaccines

The failure of many trial vaccines to elicit an effective immunogenic response to a target antigen is a critical hurdle that must be overcome if a vaccine candidate is to be successful. Fusing antigenic epitopes to T-cell receptor peptides may help facilitate an immune response to a vaccine, as the MHC class II recognition sites present on antigen presenting cells (APC) interact with the T-cell receptor ligands. This directed targeting of vaccines to APCs can boost vaccine effectiveness, extend circulation half-life, and may allow for a reduction in the administered dose so that vaccine stocks can be stretched further.

The principle of T-cell directed vaccines can be extended to a range of small antigenic epitopes that may fail to induce an adequate immunogenic response if presented in isolation. The ligand epitope antigen presentation system (LEAPS) converts small peptides (as low as eight amino acids) into immunogens. It is a novel technology that may improve the immunogenicity of these peptides [21]. Antigenic epitopes are fused to immune cell binding ligands (ICBL), which are peptides containing a T-cell epitope, via a small glycine (G3) linker. When paired with a peptide from herpes simplex virus (HSV) type 1 ICP27 protein the entire construct is a mere 30 amino acids [22]. The heteroconjugate compound was administered subcutaneously to mice in an oil-in-water adjuvant emulsion. Inoculation did not produce antibodies in mice, instead generating a cellular Th1 response that was sufficient to protect against a lethal HSV dose. The LEAPS vaccine activates cellular precursors of IL-12 + p70 + dendritic cells, which, in turn, deliver antigen and stimulate a Th1 response [22, 23].

17.5.2.5 Virus-Like Particles

The incorporation of virus-like particles (VLPs) in modern therapeutics has become so common that the technology is now considered mainstream. Currently marketed VLP vaccines include Gardasil® (Merck & Co.) and Cervarix® (GlaxoSmithKline) for vaccination against types 6, 11, 16, and 18 [24, 25] and types 16 and 18 [24, 25] of human papilloma virus (HPV), respectively, and also the hepatitis B vaccines, Hepavax-Gene® (Crucell), Recombivax HB® (Merck & Co.) and Engerix®-B (GlaxoSmithKline) [5].

Refinement of this technology will accelerate the development of vaccines to combat the rapid onset of infection encountered in a pandemic – most recently experienced with the emergence of the novel H1N1 2009 "swine flu." Comparative studies of antibody responses in children through to the elderly between the A/New Jersey/8/1976 (A/NJ/76) H1N1 strain and seasonal H1N1 viruses showed only limited cross-reactivity in all age groups for the A/California/04/2009 clade of the 2009 H1N1 virus [26, 27]. Genetic drift of the pandemic 2009 H1N1 flu was thought to be a contributing factor to the reduced cross-reactivity attained through exposure to seasonal H1N1 strains [28, 29].

One possible solution is the linking of conserved T-cell epitopes to antigenic portions of the influenza A virus antigens in a VLP. A recent example of VLP conjugate vaccines are those that utilize T-epitope "*kites*" – so named because of the similarity the molecular diagrammatic representation has to a kite (see

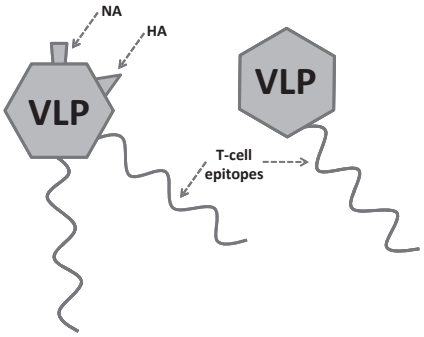

Figure 17.5 Diagrammatic representation of T-cell epitopes linked to influenza A virus antigens—"*kites*."

Figure 17.5) [30]. T-epitope kites have been developed for co-expression with surface antigens, namely haemagglutinin (HA), neuraminidase (NA), and matrix (M1) proteins [31].

Recombinant baculoviruses with genes encoding for HA, NA, and M1 surface antigens have been used to create self-assembling VLPs. Challenge studies conducted on BALB/c mice have shown that the VLPs are protective against the matching influenza subtype (H9N2) [32].

17.5.2.6 **Viral Vectors**

This vaccine technology works by packaging and delivering genes that contain specific antigens of particularly virulent pathogens in attenuated viruses. Once the host has been inoculated with the recombinant viruses, expression of the inserted gene commences, with viruses expressing high levels of the antigenic protein. Several organisms have been used in the development of recombinant viral vectors and include the modified Ankara vaccinia (MVA) virus (*vide infra*), replication-defective forms of poxvirus [33, 34], attenuated poliovirus [3, 4, 34], and highly immunogenic adenoviruses of various serotypes [34, 35]. Changes in both the vector itself and inserted gene(s) should be carefully assessed and monitored during various stages of the manufacturing process and batch to batch consistency assured.

17.5.2.6.1 **Recombinant Vaccinia Virus**

The highly conserved nucleoprotein (NP) and matrix (M1) protein of influenza A generates strong cross-recognition in CD4+ and CD8+ T-cells [36]. Gilbert hypothesized that an NP–M1 fusion inserted into a recombinant MVA could induce a protective immune response since most adults have some T-cell memory against NP and M1 through previous exposure to influenza A. By targeting the highly conserved regions of influenza A, Gilbert and colleagues hope to produce a vaccine that would give broad protection and not require reformulation each season. The candidate vaccine is cultured in suspension cells, preliminary safety

and immunogenicity studies have been conducted and challenge studies in humans are progressing. It is intended that the final formulation will be lyophilized for stability at room temperature [37].

NYVAC-Pf7 is another example of a vaccine based on a recombinant *vaccinia* vector. Like MVA, it too is a highly attenuated vaccinia virus with seven *P. falciparum* genes inserted into its genome [38]. However, this multivalent vaccine was poorly protective [39]. This has prompted a change in strategic direction using a single invariant epitope on self-assembling nanoparticles (see Section 17.8.3).

17.5.2.6.2 **Fowlpox Virus**

The safety of fowlpox (FP) virus vectors has been well established. The fowlpox live recombinant vaccine TROVAC-H5 was widely used in Mexico in 1995–1996 to immunize against avian influenza (AI) [33]. The vaccine contains live recombinant FP expressing the H5 haemagglutinin gene. This recombinant vaccine was licensed in the United States for emergency use in 1998 and registered in Mexico, Guatemala, and El Salvador, where over 2 billion doses were safely administered. A single injection protected chickens against AI induced mortality and morbidity for at least 20 weeks, and decreased virus shedding after challenge with a wide panel of H5-subtype strains. Subsequently, protection was demonstrated against 2003 and 2004 highly pathogenic H5N1 isolates from Asia. Efficacy was not inhibited by anti-AI or anti-FP maternal antibodies (passive immunity), however, protection against AI was significantly decreased in chickens previously vaccinated or infected with FP. It was simple to differentiate, by ELISA, between infected and vaccinated chickens. Thus it is an ideal vaccine for administration to chickens in hatcheries [33].

In pioneering work, Taylor *et al*. showed that recombinant FP vectors expressing rabies glycoprotein could be used to produce specific, protective, anti-rabies immune responses in six mammalian species [40]. More recently, Webster *et al*. have demonstrated that prime–boost regimes, using these vaccines against liver-stage malaria, are immunogenic in humans and produce complete or partial protection against stringent experimental malaria challenge. DNA priming followed by an MVA boost induced a high proportion of malaria-specific lymphocytes (predominantly CD4+) and led to partial protection against malaria challenge, whereas two FP9 priming vaccinations (at 1×10^8 pfu) boosted by MVA led to complete protection [41].

17.5.2.6.3 **Recombinant Retrovirus Vectors**

There has been a resurgent interest in disabled retroviruses as vaccine delivery vehicles, due to their ability to generate long-term expression from transgenes integrated into the genome of target cells. The downside is that integration can result in undesirable *integration-induced insertional mutagenesis* [42]. Integration-deficient lentiviral vectors (IDLV) are attenuated by disrupting the function of integrase (via a D64V point mutation). The lentivirus vectors (a member of *Retroviridae*) are particularly efficient at transducing dendritic cells (DC) but cannot induce a protective immune response on their own [43]. Hu *et al*. have encapsulated

IDLV in a mutant Sindbis virus glycoprotein envelope (SVGmu). This IDLV–SVGmu vector complex selectively binds to DCs through the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and may offer a safe and highly efficient vector platform for dendritic cell targeted vaccines [44].

17.5.2.7 Recombinant Attenuated Bacterial Vaccines

Other forms of recombinant vectors include attenuated strains of *Salmonella* (see Section 17.9.1).

Although the genetic basis of the attenuation of *Mycobacterium bovis* BCG strain (which has been in use for more than 50 years) has been elucidated [45] this vaccine was not produced using recombinant DNA technology.

17.6 Recombinant Vaccine Development

It is crucial that research into novel recombinant vaccines focuses initially on the ultimate viability of the product in the field. For example, researchers working on vaccines for application in tropical, developing world environments should bear in mind that the inability to provide storage at low temperatures for prolonged periods is likely to prevent the practical use of a vaccine unless it is heat stable. Vaccines that require repeat administration might also be impractical for some regions. The requisite transport and storage infrastructure may simply not be available, especially in the areas where the disease is most prevalent. However, recent trial vaccination programs by Australian researchers in Vanuatu, using VLPs to protect against human papilloma virus (HPV) have shown that, at least in small countries, these barriers can be surmounted. Stability of the vaccine is not only important for considerations of potency, but also safety, which can be tied to aggregation, sequence, physicochemical consistency, and other key parameters.

17.6.1 Manufacturability

Manufacturability encompasses the central issue of whether a process developed within a controlled laboratory environment can subsequently be translated into a viable industrial process. Under this heading falls the simplicity and reproducibility of the process, and the cost of manufacture, in particular the scale and cost of the required bioreactors, purification processes, storage conditions, and distribution networks.

The current manufacturing practice for the development of flu vaccines is a cumbersome process devised over 65 years ago requiring the fertilization, inoculation, incubation, candling, harvesting, clarification and purification, splitting, inactivation, and sterile filtration for every one of the 700 million embryonated chicken eggs cultured each year [46]. This laborious means of production requires long-term planning for an annual production cycle and is not easily scalable with

vaccine stocks constrained by the limited availability of specific pathogen-free (SPF) chicken eggs [47]. Hence, much attention is being directed towards cell-based production for this vaccine.

One example is the Novavax, Inc. trivalent seasonal influenza vaccine candidate that recently underwent clinical trials in Mexico. The phase II studies indicated that the VLP self-adjuvanted and may only require a dose of 5–15 μg, which affords one million doses per 1000l batch, reducing the burden of production scale up (see Section 17.6.2) and improving accessibility of the vaccine (see Section 17.6.3). The VLP is expressed in Vero cells, which are passaged through robotic roller bottles and fed into fermenters on polymer bead microcarriers. Purification of the cell lysate was a reasonably simple two-step process of UV and formalin inactivation [48].

The prospect of cell-based flu vaccines promises a global solution with: (i) greater simplicity and chemical characterizability of the formulation; (ii) cheaper, more reliable, and a practically inexhaustible supply of reagents; and (iii) a far more rapid batch cycle with cell cultures moving to bulk production in just three weeks [48] as opposed to the current SPF egg based market cycle of 4–5 months [49].

17.6.2 Scalability

Scalability implies retention of bio-equivalence by the final industrialized product, meant for market distribution, retaining both the biological and chemical properties of the tested and clinically proven laboratory compound.

The best solution for scale up is not necessarily very large, centralized reactors. A distributor network of smaller scale reactors can avoid some of the thermodynamic and engineering problems associated with very large reactors and, at the same time, address the challenge of vaccine accessibility in countries most affected by the target diseases. This *distributed manufacture* model has been the approach of a Novavax, Inc.–Cadila Pharmaceuticals Ltd. partnership. The companies are able to rapidly construct small factories with a relatively modest capital outlay of $5–10 million per facility [48].

Furthermore, as alluded to in Section 17.2, it is prudent to not only consider the scalability of the proposed production process but also to identify suitable analytical tests for both small- and large-scale operations. Congruent analytical tests between the small- and large-scale operations are desirable as this arrangement gives a greater degree of uniformity between test results and improved confidence in the final product.

17.6.3 Deliverability and Accessibility

Delivery and accessibility encompass stability of the compound, administration of the vaccine, and its distribution to the end user. These issues are commonly considered late in vaccine development but are of such critical importance to the

success of a vaccine candidate that they should be addressed before development of the compound begins in earnest [20]. The vaccine may have been approved for human application, however, its viability can still be challenged by limitations of accessibility and deliverability.

Immunization regimes that require booster shots (see Section 17.8.2) to be administered weeks and months after the initial dose can be impossible in regions where there is little infrastructure, nomadic or transient populations, inadequate access to healthcare professionals, and a lack of follow up support.

Vaccine delivery remains a critical challenge for recombinant vaccines. For example, constraints on mass inoculation may arise due to an inability to supply the antigenic component of the vaccine in a timely manner, or logistical considerations that preclude the implementation of large volume inoculation programs due to inability to maintain a cold chain during transport and storage. This is particularly important at a time of vaccine stockpiling. Lyophilization may be a solution if low cost, effective, and safe stabilizers can be developed, removing the need for refrigeration [50].

Ward and colleagues at, Imperial College London [51], have undertaken research on performance of a panel of low-cost, nontoxic, water-soluble excipients that are effective in long-term stabilization of live viral vaccines and biopharmaceuticals. The individual components in these storage formulations have been safely used previously in clinical settings, and can be readily integrated into existing cGMP manufacturing processes. Some of these stabilizers are being developed for biodefense vaccines, to stabilize both adenovirus vectored and adjuvanted vaccines for potential use against bacterial pathogens.

Another solution is a distributed network of medium-scale factories for production of vaccines closer to the end user. This distributed manufacture approach is being pioneered by Novavax. Local GMP factories for production of VLP vaccines have been be built in 3 months, with very short lead times (around 11 weeks) for production of millions of doses of influenza VLPs [48] (see Section 17.6.2).

17.7
Delivery Systems

Delivery systems can be broadly categorized as viral and non-viral.

Viral systems allow for highly specific antigenic targeting (see Section 17.5.2.6) and are currently the most efficient means of directing therapeutic DNA to target cells, but are unfortunately constrained by immunotoxicity, the potential for insertional mutagenesis, and a recurring failure to acquire site specificity [52]. Concurrent development of non-viral systems aims to compliment viral delivery.

In non-viral delivery systems, intra-muscular injection does not distribute well, with the vaccine pooling at the site of injection. The gene gun does not deliver high doses although both cellular and humoral responses have been induced in human clinical trials [53, 54]. Other limitations for ballistic vaccine delivery, such

as the Biojector™, include the induction of significant cell death at the point of administration [55].

Other non-viral systems that may address the problems associated with intramuscular or intra-peritoneal injection are the micro-nanoprojection arrays. These systems may offer a rapid antigen diffusion process. The Nanopatch™ vaccine delivery system adheres to the skin and allows for the direct delivery of vaccines at various depths from the epidermis to the dermis [56].

Further means of vaccine delivery that may warrant consideration from inception of research and design of a vaccine program include *in vivo* electroporation and sonoporation. Potentially, these technologies could be used in concert with viral delivery systems to significantly enhance transfection efficiency of vaccines by improving the range and extent of gene delivery [57]. Electroporation techniques subject the tissue surrounding the injection site to electric pulses in an effort to temporarily raise the permeability of cell membranes to facilitate the uptake of the recombinant DNA (see Section 17.5.2.3) [58]. However, the lack of portability of this technology into areas without electricity is a drawback.

Sonoporation offers another mode of physical vaccine delivery. Of particular note is the development of gas-encapsulated microbubbles as an acoustic-based, non-viral transfection technique [59]. Microbubbles resonate within an acoustic field created by ultrasound in a technique known as ultrasound and microbubble targeted delivery (UMTD). As the microbubbles vibrate, kinetic energy is transferred to cell membranes and can induce endocytosis of macromolecules (such as DNA) once the poration threshold is met [60].

The diversity of delivery systems, both viral and non-viral, is wide and varied. This has implications for the therapeutic strategy, which must be compatible with the delivery method to ensure stability of the compound. Whatever system is chosen must also meet the key challenges of recombinant vaccine research: that is attenuation/safety and immunogenicity (see Section 17.3).

17.8 At the Vanguard

There are three technological directions in the vanguard of recombinant vaccine research. These are:

1) targeting antigen to dendritic cells
2) combinations of different technologies in prime–boost vaccine administration regimes
3) multivalent presentation of antigen.

Examples from each of the recombinant vaccines (see Section 17.5.2) will be used to illustrate these new technological directions. Some of these examples have been chosen because they illustrate more than one of the above technological directions, integrated into one vaccine.

17.8.1 Antigen Targeting

As early as 1984, it was reported that hepatitis-B surface antigen (HBsAg) when added to T-cells along with IgG2a antibody to hepatitis B lowered the required antigen concentration for antigen specific proliferation of T-cell clones cellular response by 100-fold [61]. As noted previously (see Section 17.7), directed targeting of vaccines to APCs can greatly enhance the effectiveness of a compound in orchestrating Th1 and Th2 responses. Viral vectors employ a form of antigen targeting by encasing genes into recombinant viruses, and are discussed at length in Section 17.5.2.6.

17.8.1.1 Resident DCs Crucial to Humoral Immune Responses

There has been a recent and fundamental shift in our understanding of which cells are crucial to the generation of humoral immune response to viral infection. The work of Gonzalez and colleagues has shown that dendritic cells resident in the lymph node medulla, but not macrophages, are essential for production of an antibody response against influenza viruses. Dendritic cells use the lectin receptor SIGN-R1 to capture lymph-borne influenza virus and promote humoral immunity. This mechanism is also likely to be relevant to infection by any viruses with an envelope containing glycoprotein. A great deal of research has gone into what cell types we should target with vaccines. This, and much earlier work by Corley *et al.* [62] and Hart and Fabre [63], suggests that dendritic cells should be the target [64].

Gosselin and colleagues have developed vaccines targeted to Fc-γ receptors on antigen presenting cells (APC). In the case of the *Francisella tularensis* vaccine, this was done by utilizing monoclonal IgG2a antibody-bacterium complexes (not fusions) [65]. More recent versions of this work involve recombinant fusion proteins that link single-chain antibody fragments (ScFv) recognizing Fc-γ R1 to *S. pneumoniae* recombinant antigen (see Figure 17.6).

For the purpose of antigen targeting, Demento *et al.* [66] are developing flavivirus vaccines that consist of antigens encapsulated in polymer nanoparticles (180 nm in diameter). The capsule of these nanoparticles consists of palmitic acid and PLGA in an emulsion. The wall of the capsule contains ligands recognizing Toll-like receptors (TLR) on dendritic cells, for example the ligand might be bacterial CPG (a TLR-9 ligand) or bacterial lipopolysaccharide (LPS, a TLR-4 ligand). Nanoparticles are carried into the cell by the endocytosing TLR, then break through the endosome wall. By this means they can produce both MHCI and MHCII mediated immune responses. This approach is similar in principle to the *multivalent* fusion constructs being developed by other groups (see Section 17.8.3) for antigen targeting to APCs. Vaccination of mice with West Nile virus (WNV) antigen loaded TLR-ligand modified PLGA nanoparticles provided protective immunity in mice [67].

These complexes are thought to act via multiple mechanisms:

1) Increased antigen binding and internalization by APC.
2) Increased maturation of CD86+ DEC205+ dendritic cells.

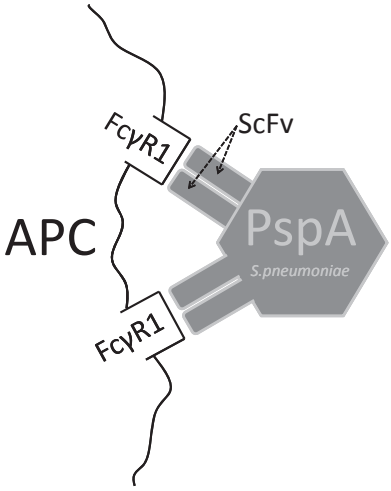

Figure 17.6 Fusion construct consisting of ScFv anti-Fc-γ R1 and *S. pneumoniae* (Psp) A-antigen.

3) Increased half-life of antigen/MHC complexes on the APC surface.
4) Increased trafficking of antigen to nasal associated lymph tissue (NALT) *in vivo*.
5) Increased cellular immunity characterized by both Th1 and Th2 cytokines.
6) Increased humoral immunity mediated by IgA and IgG.

17.8.2 Prime–Boost Staging

Prime–boost regimes can be integral to the success of a vaccine. However, immunization strategies that require booster shots to be administered weeks and months after the initial dose can be complicated in regions where there is little infrastructure, nomadic or transient populations, inadequate numbers of healthcare professionals, and a lack of follow-up support. These factors can lead to a lack of efficacy of the vaccine due to lack of compliance with the proven dosing regime.

17.8.2.1 A Case Study – RTS,S

The recombinant VLP vaccine, RTS,S, is currently the most advanced drug candidate against malaria caused by *P. falciparum*. RTS,S has been trialed using two different adjuvant systems, AS01B and AS02A (GlaxoSmithKline proprietary adjuvants) [68]. To date, phase III clinical trials have been largely devoted to RTS,S/AS02, in which a lipid emulsion is used to induce an immune response. However, studies on RTS,S/AS01, where a liposomal vehicle replaces the lipid emulsion, appear to induce both greater humoral and cellular responses against the CS protein [69, 70]. These vaccines are expressed using transgenic yeast (*Saccharomyces cerevisiae*) cells [68].

In phase IIa clinical trials, RTS,S/AS01B and RTS,S/AS02A delivered protection in up 50% and 32%, respectively, in malaria-naïve adults [66]. The RTS,S vaccine targets infected hepatocytes through the expression of circumsporozoite (CS) epitopes presented by the hepatitis B surface antigen. This arrangement has the added effect of inoculating patients against hepatitis B [71].

Heterologous prime–boost combination regimens, whereby different vaccine platforms are used for priming and boosting, could provide enhanced immune protection against malaria. RTS,S is able to stimulate a humoral immune response but fails to induce detectable cytotoxic CD8+ cellular responses [72]. However, human immunization trials involving a *P. falciparum* CS protein (PfCSP) prime followed by RTS,S/AS02A boosting increased the CD4+ response and generated a CD8 T-cell response to the CS protein. While encouraging, challenge studies were not conducted, and so the level of protection remains to be determined [73].

More recent work along these lines has been carried out using prime–boost combinations of replication deficient adenovirus 35.CS and RTS/S [74]. This resulted in T-cell responses but poor antibody responses. The subsequent use of Ad35.CS in combination with a lower seroprevalence adenovirus (Ad35.CS prime, Ad5.CS boost) improved the T-cell and specific antibody responses [75]. The most recent work in rabbits, builds on this mixed-platform prime–boost strategy, and is utilizing a series of injections of CS protein, followed by a boost with adenovirus 35 CS or Ad26.CS.

17.8.2.2 Targeting the Conserved/Invariant Region and Multimerizing the Construct

Generation of high levels (>100 μg/ml) of antibodies to the PfCSP repeat epitope, could provide protection [70]. However, so far the only protective, long lasting vaccination strategy has been inoculation through bites of over 1000 gamma-irradiated mosquitoes containing "attenuated" sporozoites. In this model it is clear that the protective role is played by CD8+ T-cells and not antibodies. Thus, new strategies are needed to induce protective, cell-mediated immunity, with a strong CD8+ response involving multimeric presentation of conserved epitopes (see Section 17.8.3.1)

17.8.3 Multivalent Antigen Presentation

The poor affinity between a *single* antigenic epitope for surface receptors on APCs often results in a failure of the immune system to mount an appropriate response. It has long been recognized that IgM can act as an adjuvant and dramatically reduce the amount of antigen necessary to produce an immune response [62, 63]. In our recent work, we are exploiting this characteristic of IgM by developing IgM-like scaffolds to present antigens multivalently to APCs (Barnard and Le Grand, in preparation). From the regulatory point of view, multivalent vaccines must be supported by data that show the increased utility of the various antigens presented to the treated patient, as well as an acceptable safety: efficacy ratio.

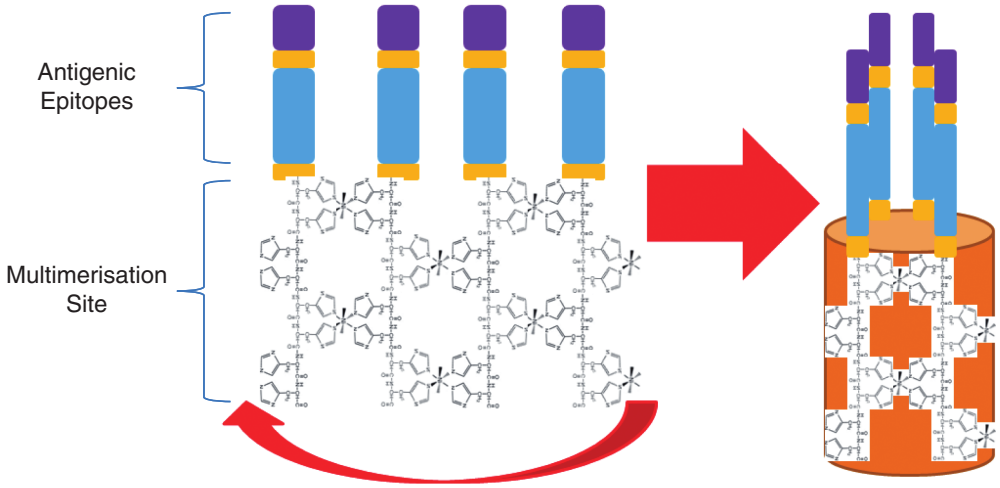

Figure 17.7 Tetrameric structure through His-tag–metal-ion binding (under development by Barnard and Le Grand).

A simple way of multimerizing protein strains is by linking His-tags together with metal ions. Histidine is a tridentate molecule capable of binding to transition metals such as nickel and copper. When linked together in a His-tag, only a single binding site remains on each histidine residue [76]. The metal ion (in this case nickel) is able to link the imidazole rings of the same His-tag while retaining four more valencies [77]. The vacant valencies may allow for another construct His-tag to bond with the metal ion and linking the two constructs. It is conceivable that molecular biologists could exploit this property by rafting His-tags together using metal ions to form multimerized complexes. In Figure 17.7, the concept is extrapolated to a possible tetrameric structure.

17.8.3.1 Self-Assembling Peptide Nanoparticles for Multivalent Antigen Presentation

In view of the less than optimum performance of NYVAC-Pf7 in trials, the US military malaria vaccine program is redirecting their strategic focus towards a simple vaccine that can generate a strong, sustained response against a single invariant epitope.

To this end, a strategy of multivalent antigen presentation on self assembling peptide nanoparticles (SAPN) is being used. The surface sporozoite invariant epitope will be displayed as repeats on an icosahedral SAPN [11]. These 25–30 nm particles can be taken up by dendritic cells. SAPN induce T-cell dependent antibodies without the need for adjuvants [39].

Another example of fusion constructs for multivalent antigen presentation is the work of Shattock (see Section 17.5.2.6) and colleagues [18] who used soluble recombinant trimeric HIV-1 clade C gp140 protein with chitosan adjuvant (see Section 17.8.4) in rabbits.

17.8.3.2 Self-Adjuvanting Agents

The incorporation of auto-assembling molecules or multivalent components into recombinant vaccines can act as a self-adjuvanting agent, the 69 amino acid B-subunit of *E. coli* O157:H7 Shiga-like toxin is one such example of a multimerizing agent that also self-adjuvants [78]. This eliminates the need for the addition of an extraneous adjuvant such as alum.

17.8.4 Adjuvants in Recombinant Vaccines

The typically poor immunogenicity of recombinant antigens is a major limiting factor in the development of effective subunit vaccines. It follows that discovering adjuvants that elicit a proficient cellular (Th1) or humoral (Th2) response is of critical importance. The challenge is to discover formulations that enhance the immune response to the vaccine antigens, without excessive reactogenicity or toxicity. Newer adjuvants used in humans, including MF59, AS02, and AS04, have higher local reactogenicity and systemic toxicity than alum [79]. Even alum, despite being FDA-approved, is associated with adverse effects including injection site pain, inflammation, lymphadenopathy, and less commonly, injection-site necrosis, granulomas, or sterile abscess [80].

Adjuvants can be serendipitously, or by design, intrinsic components of a vaccine. These may be pathogen-associated molecular patterns (PAMPs) such as unmethylated CpG sites in the measles, mumps, and rubella vaccine. Alternately, added-adjuvants are delivered with the antigen, as is the case with the addition of AS02A/AS01B adjuvants to RTS,S malaria vaccine candidate (see Section 17.8.2.1) or the incorporation of alum in hepatitis A and B vaccines.

Chitosan is increasingly recognized as a versatile adjuvant. A chitin-derived polyaminosaccharide, chitosan is well tolerated when administered to humans and mice, is an effective absorption enhancer and functions as a mucoadhesive. Co-formulation of chitosan with Th1 response inducing cytokine IL-12 has elicited greater antigen specific responses in both CD4+ and CD8+ T-cells in mice [81]. The adjuvanting ability of chitosan appears to be linked to the NLRP3-inflammasome pathway [82].

Alum was the first adjuvant constituent of vaccines approved by the FDA for human use. Approvals have been obtained in Europe for the squalene based MF59 adjuvant (first registered in Italy in 1997) in the flu vaccine for elderly patients, Fluad® (Novartis), and for the adjuvant AS04 (a combination of alum and MPL, GlaxoSmithKline) as the adjuvant for viral vaccines against hepatitis B and HPV. There is considerable evidence [83] that organic compounds, such as squalene, provide greater potentiation of the immune response than alum. MF59-adjuvanted influenza vaccine has been shown to provide stronger and broader immunogenicity than non-adjuvanted vaccine, with good tolerability and safety, even in vulnerable groups [84] Although MF59 is approved for use in Europe, the only adjuvant currently approved for human use in the USA is alum [76, 85].

The adjuvant system used in the malaria vaccine, GaxoSmithKline's AS02, and in AS04, contains saponin and the Toll-like receptor (TLR) agonist monophosphoryl

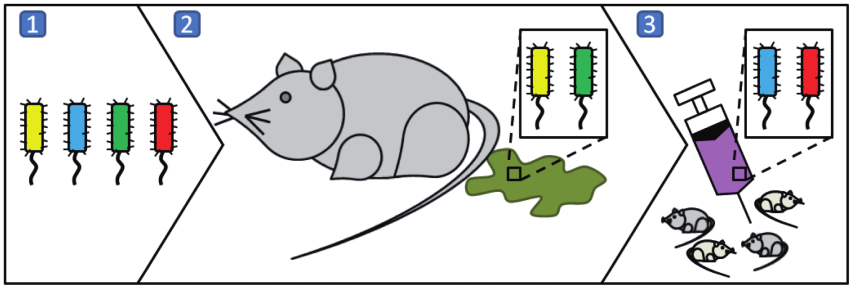

Figure 17.8 Sequence tag mutagenesis (1), different strains of *Salmonella* are characterized through the use of molecular barcodes and inserted orally into mice. (2) Feces of mice are screened for *Salmonella*. Strains present in feces are associated with pathogenesis and (3) deleted strains are associated with attenuation. Deleted strains were candidates for live-attenuated (SPI-2 deleted) genetically modified vaccines.

lipid A (MPL). Both saponin and MPL are necessary to induce a modest level of protection in immunized individuals [86]. MPL is a chemically detoxified derivative of lipid A from *Salmonella minnesota*.

Careful selection of specific adjuvants may allow for induction of the most suitable antibody isotype. It may, for example, be feasible, by incorporating a particular adjuvant into influenza vaccines, to bias the humoral immune response towards IgG2a and IgA isotypes [87].

There is much interest in the use of cytokines to potentiate immune response. Research over the last ten years in mouse models has demonstrated the ability of IL-12 to markedly increase protective immunity to respiratory pathogens such as pneumococcus and influenza virus. Moreover, IL-12 is a potent adjuvant for protective neonatal immunity. IL-12 stimulates both Th1-associated cellular immunity and humoral immunity to T-dependent and T-independent antigens in mice [88]. This is an exciting frontier in vaccine research, but much additional work is needed to establish the efficacy and safety of this approach in humans.

The ideal solution, simultaneously addressing safety concerns, and easing transition through the regulatory pathway, is to develop vaccines that do not require exogenous adjuvant. Some of the recombinant technologies discussed above (see Section 17.8.3) have the potential to provide just such a solution, via multivalent antigen presentation or targeting antigen to antigen presenting cells.

17.9
Novel, Recombinant DNA Approach to Identifying Attenuated Vaccine Strains

17.9.1
Combining the Biological with the Molecular

As an alternative to rational attenuation based on detailed knowledge of molecular pathogenesis, Lewis and colleagues have developed an ingenious method, called

"sequence tag mutagenesis." The method entails random gene knockouts by insertion of molecular barcodes into the genome of pathogenic *Salmonella* [20, 89, 90]. The "library" of mutated organisms is injected into mice. The absence of particular barcodes from the shed population of *Salmonella* in feces enabled the discovery of deletions associated with attenuation (see Figure 17.8). These randomly discovered deletions will be useful in the rational design of new, attenuated, vaccine strains of *Salmonella typhi* and *Salmonella typhimurium,* which may contain combinations of the attenuating mutations.

17.10 Clinical Trials

While the bulk of research, development, and its associated funding are conducted and sourced from the developed world, it is the developing world that bares the greater share of the infectious disease burden. This creates a peculiarity for the regulation of recombinant vaccines for infectious disease. The bulk of Investigational New Drug (IND) applications for the development of the RTS,S malaria vaccine candidate (currently the most advanced vaccine candidate targeting the clinical disease caused by *Plasmodium falciparum*) had been filed with the FDA. However, the program unexpectedly met with licensure difficulties when it was recognized that the drug was never intended for administration in the United States and thus not eligible for FDA approval [68].

The lack of FDA approval could have jeopardized the commencement of phase III clinical trials in sub-Saharan Africa, as countries in this region, which lack strong regulatory bodies often defer to the FDA for the approval of new drug candidates. However, recent cooperative ties between the European Medicines Evaluation Agency (EMEA) and the WHO goes some way to reassuring these regulatory agencies, by allowing for the Committee for Medicinal Products for Human Use (CHMP) through Article 58 to assess a drug candidate and offer a Scientific Opinion on its efficacy [68]. This could help to avoid future incidents relating to bureaucratic hurdles unnecessarily exaggerating the time-to-market for a given therapeutic.

17.11 Conclusion

Emerging *viral* disease targets for which there are no current vaccines, no vaccines close to market launch, or inadequate vaccines (e.g., those that provide inadequate duration of protection, or those that require reformulation each year) are:

1) **Chikungunya:** A crippling disease with origins in the developing world that is now making incursions from Reunion and South-East Asia into Europe [91, 92].

2) **West Nile virus:** There are no antiviral drugs or vaccines. Between 2001 and 2006 there were approximately 10 000 cases in the United States with a 10% fatality rate [93]. WNV has been present in Europe since 1998 [94].

3) **Multivalent BTV vaccine (blue tongue virus – an animal virus):** The challenge for recombinant vaccines for animal disease is to produce them cheaply enough for farmers and to avoid the need for multiple dosing.

4) **A universal influenza vaccine:** The current vaccines require reformulation when a new reassortant emerges.

5) **An inexpensive HIV vaccine**.

To meet these challenges, investment in the core recombinant vaccine technologies will be necessary. Recombinant vaccines, when combined with new stabilizer and adjuvant technologies (when necessary), will deliver enhanced vaccine safety, immunogenicity, and stability. There will need to be private or government investment in the infrastructure for local production, in distributed facilities (the Novavax model is instructive), in those countries where these diseases are most prevalent. In the world of emerging diseases and rapid movement of viruses, we need to have technologies on hand that can respond quickly to the appearance of unanticipated pathogens. The surprise emergence of Usutu virus [95], West Nile virus, and Chikungunya in temperate regions are recent examples. Technologies that are adaptable and rapidly scalable will be crucial if we are to respond in a timely manner to those surprises.

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Part Four
Recent Applications in Pharmaceutical Biotechnology

18
In Silico and Ultrahigh-Throughput Screenings (uHTS) in Drug Discovery: an Overview

Debmalya Barh, Shoaib Ahmad, and Atanu Bhattacharjee

18.1
Introduction

18.1.1
High-Throughput Screening

Modern high-throughput screening (HTS) techniques use liquid handling systems, robotics, high end computational data processing, and sensitive detectors, to quickly conduct several hundreds or millions of biochemical, genetic, or pharmacological tests. HTS allows rapid identification of disease genes, proteins, RNA, miRNA, antibodies, and so on, from omic based experiments using microarray and robotics. Similarly, HTS are frequently used as starting points in identification of signaling pathways, drug targets, active compounds, and functional assays.

Chemical libraries screening using HTS was started in the mid-to-late 1990s with initial attempts to screen natural products from plant extracts. During that time the compound library sizes were ~50 000 compounds and most assays were developed in 96-well format without automation. In general, microtiter plates of 96, 384, or 1536 wells containing nanoamounts (2.5–100 µl) of either compounds to be screened or targets were used in screening. During the past two decades due to the strong increase in number of available compounds and molecular targets, a considerable advancement in technologies in terms of assay miniaturization, automation, and robotics have enabled us to use high-throughput and ultrahigh-throughput screening (uHTS) levels.

HTS generally can screen 10 000–100 000 chemical compounds per day whereas in uHTS, the compound numbers are greater that 100 000 per day. Therefore, both the HTS and uHTS are now an integral part of the drug discovery pipeline for screening conventional and new chemical entities on mass. The trend toward miniaturization is represented in Figure 18.1. In further minimization, 3456-well microtiter plates that can use total assay volumes of 1–2 µl are sometimes used, there are technical difficulties in routine assays, so therefore 384-well and 1536-well microplate formats are currently used in industry. Progress is also being made towards screening outside the microtiter plates at ultralow volumes of <0.1 µl.

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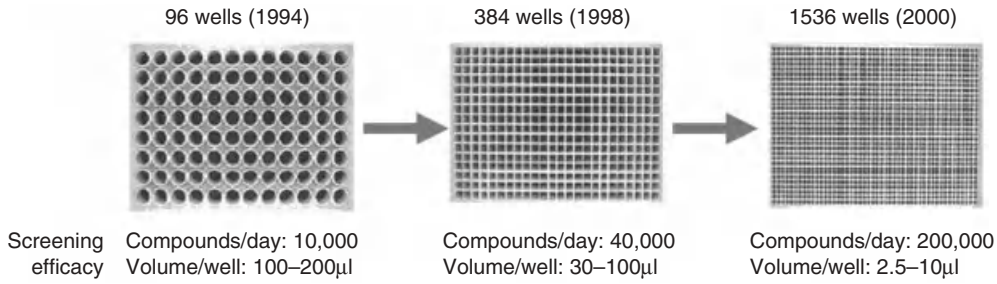

Figure 18.1 Miniaturization of HTS towards uHTS. Adapted from Mayr and Fuerst [1].

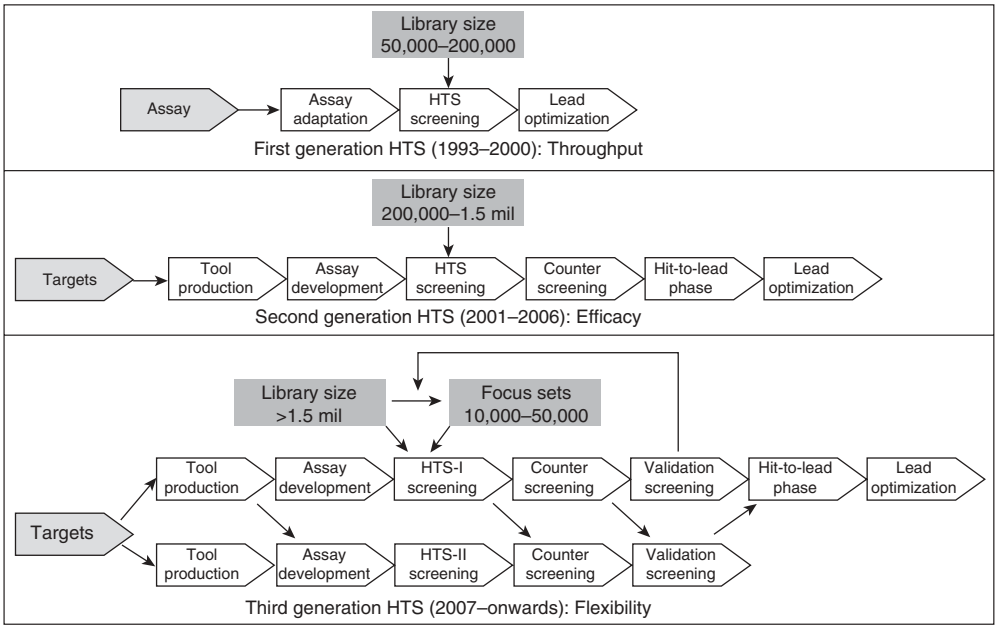

Figure 18.2 Evolution of HTS strategies. Adapted from Mayr and Fuerst [1].

18.1.2
Ensuring Quality, Quantity, and Cost Effectiveness Using Integrated Approaches

With the increases in library size over time, in addition to the increase in the number of molecular targets, assay miniaturization, automation, and quality concerns with respect to screening have become relevant. This has created a huge shift in the time process of HTS in terms of assay development, tool production, adaptation, detection tools, counter and selective screening, quality assurance, hit-to-lead, and lead optimization, and so on. The technical and process evolution of HTS over the last 15 years is represented in Figure 18.2.

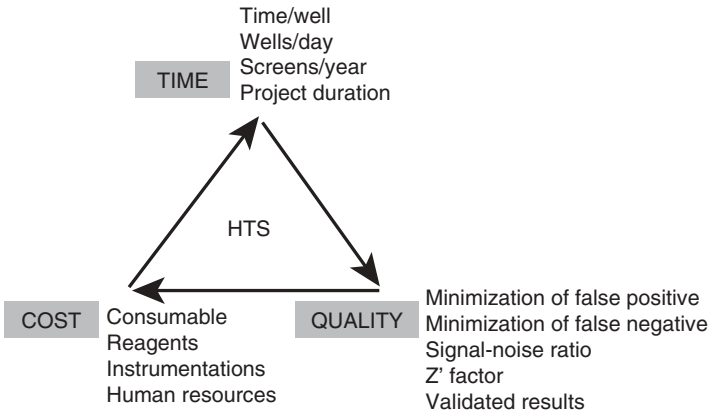

Figure 18.3 Optimized HTS process in modern lead discovery. (Adapted from Mayr and Bojanic) [2].

The time, cost, and quality of the process ("the magic triangle of HTS") are important parameters in any HTS technique. Assay development, data analysis and interpretation, hit-list validation, and for secondary assays the follow-up hit results, are the main time-consuming steps and thus contribute to the cost of today's HST screening. Major expenses are involved in the robotics, liquid handling systems, sensitive detection techniques (such as highly expensive readout technologies – TR-FRET, time-resolved fluorescence resonance energy transfer), and reagents (including antibodies, enzymes, cells, and membranes, etc.) of any given biological test samples. Similarly, the costs incurred also depend on the quality of the assay, by minimizing the number of false positives and false negatives, signal-to-noise ratios, and optimization of Z′ value of the statistical quality of an HTS output. Therefore, cost and quality are interlinked and should be considered before starting an HTS. Figure 18.3 represents the correlation between these three key factors for developing an integrated approach to be adopted in modern lead discovery by HTS.

18.1.3
Traditional versus Novel Targets in Lead Discovery

Conventional targets are very well suited for screening large compound collections in today's HTS-based lead discovery, and are generally grouped into classes, such as: (i) enzymes – kinases, transferases, phosphatases, oxidoreductases, proteases, and phosphodiesterases; and (ii) cell-based targets – G-protein-coupled receptors (GPCRs), voltage and ligand-gated ion channels, and nuclear hormone receptors, transporters, and transmembrane receptors. Although the human genome has been characterized in terms of functional genomics (>25 000 genes) and disease associated SNPs (>50 000), to date only 324 targets have been identified for which

there is an approved drug. Data from these genomics and other omics (proteomics, transcriptomics, methylomics, interactomics, etc.) and newly developed uHTS have tremendous potential for future lead discoveries, based on targets such as signal pathway, microRNA, protein–protein, protein–DNA, and protein–RNA interactions, and so on, which may help in the exploration of low molecular weight compounds for superior novel classes of pharmaceutical leads. Among these new-generation targets, protein–protein interaction based targets that modulate intracellular pathways are most attractive in developing novel leads using modern technologies such as sub-cellular imaging (high content screening-HCS). For further reading, see references [1–4].

In the following sections, we will briefly discuss various computational methods and uHTS techniques, along with omics-based approaches currently applied or in developmental stages for next generation drug target and lead discovery.

18.2
In Silico Pharmacology and Virtual Ligand Screening for Drug Discovery

In a broad sense, *pharmacology* is the branch of science dealing with the effects of drugs on biological systems (micro-organisms, animals, and human beings). Pharmacology involves a composite study of *pharmacodynamics* (what the drug does to the biological system) and *pharmacokinetics* (what the biological system does to the drug) (Figure 18.4). Traditionally, pharmacology has relied on the use of animals (e.g., rats, mice, guinea pigs, frogs, cats, dogs, and monkeys) for over 100 years.

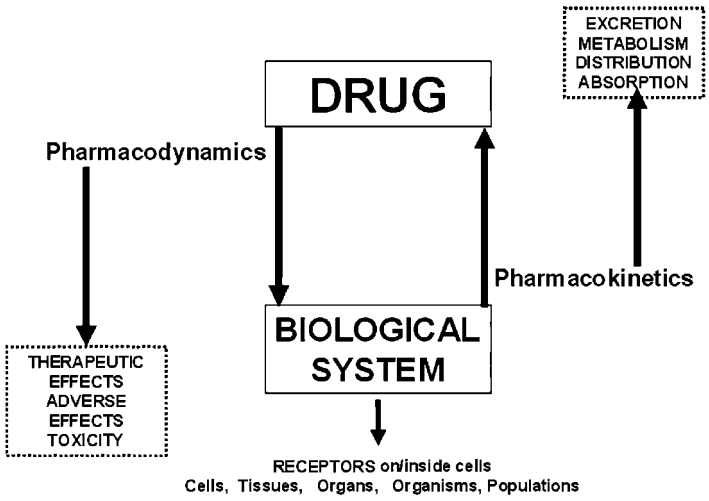

Figure 18.4 Concept of pharmacology as an interaction between the drug and the biological system. (Adapted from Ekins *et al*. [5]).

However, in the last two decades or so, computing technologies have revolutionized the world and pharmacology is no exception. The term "*in silico*" denotes the usage of computational technologies for experimentation in a particular field of study. "*In silico* pharmacology" is sometimes referred to as *computational therapeutics* or *computational pharmacology*. It is a rapidly developing science covering software usage techniques for capturing, analyzing, and integrating biological and medical data originating from different sources. In a strict sense, it may be defined as the science dealing with the use of biomedical information in creating computational models or simulations that can be used for making predictions, suggesting hypotheses, and hence, leading to discoveries or advances in medicine and therapeutics.

Pharmacological screening of drugs is an essential part of the drug discovery process. It involves pharmacodynamic as well as pharmacokinetic studies. Pharmacodynamic studies largely depend on the study of interactions between *receptors* (a type of structural proteins usually present on cell surfaces) and *ligands* (any chemical substance that attaches or tries to attach to a receptor site on/inside a cell). The ligands can be virtually any substance capable of binding to a receptor by chemical or physical interaction. The ligands may be chemically synthesized drugs (e.g., paracetamol), hormones (e.g., esterogen and progesterone) or proteins. Pharmacological screening is largely dependent on the behavior of ligands at receptor sites. The receptors are basically proteins and can undergo structural changes when ligands arrive at them for binding. The attachment of ligands on receptors may produce a biological effect, provided the ligands have a *pharmacophore* (part of chemical compound responsible for eliciting a pharmacological effect). The biological effects include absorption, distribution, metabolism, excretion, and toxicity, commonly referred to as ADME, ADMET or ADME/Tox. There are several approaches to virtual screening of drug molecules, which are summarized in Table 18.1.

Table 18.1 Virtual screening approaches.

Common approaches to virtual screening of drug molecules
Quantitative structure–activity relationship (QSAR)
1) Descriptor-based methods
2) Rule-based methods
3) Knowledge-based methods
Virtual ligand screening
1) Ligand-based methods
2) Target-based methods
Virtual affinity profiling
1) Ligand-based methods
2) Target-based methods
Data visualization

18.2.1 Quantitative Structure–Activity Relationship (QSAR)

A clear relationship exists between the chemical structure and biological effect or activity of a compound. As expected, the physicochemical properties of a compound are also a deciding factor of its biological activity or range of biological activities. Some of the biological activities are beneficial in clinical scenarios (therapeutic activity) and some are harmful (toxicity). *In silico* pharmacology exploits this relationship (structure activity relationship, SAR) to construct mathematical models and enables the quantification of SAR. This is the main domain of *in silico* pharmacology – QSAR or the quantitative structure–activity relationship. Over a period of the last 40 years or so, several QSAR models have been generated and they are stored in the C-QSAR database. There are three main types of QSAR based approaches as following.

Descriptor-based Methods These methods rely on use of the molecular descriptors as numerical representations of individual chemical structures. There are a large number and types of molecular descriptors. These descriptors are classified as per the dimensionality of the descriptor. One-dimensional (1D) descriptors are computed from the generic properties of the chemical compound (e.g., molecular weight and partition coefficient). Some of these descriptors are responsible for the drug-like character of the molecule. Two-dimensional (2D) descriptors are computed from topological representations of the molecules usually resulting in so-called 2D-QSAR methods. 2D models are quite helpful in reasonable predictions of physicochemical properties and biological effects of the molecules under consideration. Three-dimensional (3D) descriptors are computed from 3D structures of the molecules and result in 3D-QSAR methods. 3D descriptors are largely dependent on molecular conformations. 3D-QSAR methods are helpful in better QSAR predictions but they require alignment of molecules prior to the construction of the model.

Rule-based Methods These methods rely on constructing a QSAR model using a smaller set of molecules and applying it to a larger and external set of molecules. An example of this approach is the knowledge available on the biotransformation of ligands for predicting the sites liable to drug metabolism. Here, the algorithm recognizes the target sites for a particular molecule followed by a listing of all possible metabolic transformations a site can undergo, and then prioritizing the metabolites on the basis of pre-existing knowledge. Such systems are aptly identified by programs such as MetabolExpert, META, and METEOR.

Knowledge-based Approaches These approaches rely on ligand–protein complex potentials for estimating the free energies of molecular interactions when docking of ligands, that is, orientation of ligand into protein cavities, takes place. BLEEP-2, DrugScore, PMF, and SMoG2001 are examples of such potentials used for understanding ligand–protein complexation and the atomic and energy factors affecting this phenomenon.

18.2.2
Virtual Ligand Screening

Virtual ligand screening is the process of scoring and ranking molecules in large chemical libraries as per their probability of having an affinity for a specific target. It can also be considered to be an attempt to extend the concept of QSAR along the chemical dimensions as appropriately defined by existing synthesized as well as theoretically synthesizable molecules. Virtual screening gained much popularity in the late 1990s when experimental high-throughput screening (HTS) techniques were displaying poor performances and higher costs in comparison with what had originally been anticipated for the concept. The pharmaceutical industry has accepted virtual screening as an efficient complement to HTS. The industry belief in virtual screening has grown to such an extent that today it has become an integral part of lead generation and drug discovery processes. Virtual screening is basically a knowledge-driven process and requires structural information on either the ligand or the target protein, hence the *virtual* ligand screening.

Ligand-Based Methods These methods rely on the central similarity–property principle. According to this principle, similar molecules should exhibit similar properties. Hence, chemical similarity calculations form the core of ligand-based virtual screening. All the molecules in a particular database are sorted in relative order of similarity to one or more bioactive ligands, and then listed to reflect decreasing probability of bioactivity. The top scoring molecules can then be prioritized to enter into the experimental testing phase. The ligand-based screening is a cost-effective strategy in drug discovery.

Target-Based Methods These methods rely on the availability of structural information regarding the target. This information can be determined experimentally or derived computationally. The aim is to provide a good approximation of the expected conformation and orientation of a ligand into a protein cavity (docking) as well as a fair estimation of its binding affinity (scoring). Even after more than 20 years of studies, target-based screening remains a challenging process.

18.2.3
Virtual Affinity Profiling

Virtual ligand screening has extended the concept of QSAR in the chemical sense and virtual affinity profiling has extended it in the biological sense. Virtual affinity profiling has enabled the estimation of pharmacological effects of a single molecule on multiple target sites. Both ligand-based and target-based methods are available for virtual affinity profiling.

Ligand-Based Methods These methods have benefited immensely from the construction of annotated chemical libraries incorporating literature-based pharmacological data into traditional chemical repositories. For example, WOMBAT

database provides information on biological effects of 120 400 compounds reported in different medicinal chemistry journals across the world for a period more than 30 years. Another database PASS provides prediction for around 500 biological activities based on QSAR of more than 35 000 compounds.

Target-Based Methods These methods have taken advantage of the functional coverage of protein families provided by growth of experimentally determined protein structures. Owing to technical problems, 3D structures of all therapeutically important protein families are not known. One of the protein targets, enzymes, are structurally the most populated family with over 20 000 members. This is in sharp contrast to information on receptors (around 200 structures are known for nuclear receptors and ligand-gated ion channels) and G-protein coupled receptors (GPCRs, only a few structures are known). Many applications of target-based virtual profiling (also referred to as inverse docking) have been reported in recent years. Three libraries have been screened against a panel of six purine phosphoribosyltransferases (PRTs) from different species leading to the discovery of micromolar inhibitors of *Giardia lamblia* guanine PRT (GPRT) [5].

18.2.4 Data Visualization

Computational technologies can be used for generating predictions of pharmacological and physicochemical properties for each molecule structure. The analysis of such data requires multidimensional methods and sophisticated visualization tools for proper and effective data mining. Diva and Spotfire programs are widely used for analysis of ADME and physicochemical properties. Programs and databases routinely used in virtual screening of drug molecules have been summarized in Table 18.2.

Table 18.2 A partial list of available programs and databases for virtual screening.

Virtual screening for drug molecules	Programs and databases
QSAR	
1) Descriptor-based methods	C-QSAR
2) Rule-based methods	MetabolExpert, META, METEOR
3) Knowledge-based methods	BLEEP-2, DrugScore, PMF, SMoG2001
Virtual affinity profiling	
1) Ligand-based methods	WOMBAT, MDL Drug Data Report or MDDR, AurSCOPE, MedChem, Target Inhibitor, PASS
2) Target-based methods	Protein Data Bank or PDB, sc-PDB
Data visualization	Diva, Spotfire, Ingenuity Pathways Analysis

18.2.5
Applications of *In Silico* Pharmacology

Virtual screening has become popular in lead drug identification through computational screening of a vast array of chemicals against protein targets. Fluorescently tagged drug molecules are successfully utilized for visualization of the location of their receptor targets particularly GPCRs. Recently, a novel ligand-based screening process (MTree approach) has been reported. This method utilizes a new concept of combining query molecules into a multiple feature tree (MTree). These MTrees can be used to identify new leads for chemo-optimizations. MTrees have been generated for angiotensin-converting enzyme and alpha1a receptor. Acyl groups have been incorporated as P′ ligands in azacyclic urea. The extensive SAR studies have led to a series of *N*-acyl azacyclic ureas with HIV-1 protease inhibitory activity. These compounds have shown high potency against wild and multiple drug resistant viral strains of human immunodeficiency virus. The radiolabelled ligands have an ability to interact with molecular targets of importance in etiology or treatment of cancer and endocrine disorders.

18.3
Lead Discovery Using Integrative Virtual Screening

Virtual screening technologies appeared for the first time in 1997, and within a short period of just three years became part of the mainstream drug discovery process. Virtual screening has become a complimentary technology for bioactivity screening. It has gained popularity in the recent years in the pharma industry because it can help in the implementation of "fail-early" strategy. The industry can terminate drug discovery projects at an early stage prior to great financial investment in studies of molecules that may not turn up in the commercial usage market for one scientific reason or another. Virtual screening has traditionally been classified into target-based and ligand based virtual screening. When sufficient structural information is available regarding the target (protein), the target-based virtual screening is the method of choice. Otherwise, ligand-based virtual screening is preferred. Virtual screening applications (Table 18.3) focus largely on evaluation of ADMET properties of the molecules.

Table 18.3 Virtual screening application software.

Computational tools for virtual screening	
Approach	Implementation
Target-based virtual screening	AutoDock, DOCK, FRED, ICM, FlexX, GOLD, ICM, PDB, SMoG, Muegge's, Drug-Score
Ligand-based virtual screening	ALMOND, ROCS, CATS

18.3.1 Target-Based Virtual Screening

This relies on docking and scoring for evaluation of potential candidate molecules for further action in drug discovery process. Some of the software used for this purpose are AutoDock, DOCK, FRED, ICM. Most of these computer programs can provide scoring information based on one of the following four functions [6]:

1) **Knowledge-based methods** using Boltzmann-weighted potentials. These potentials of mean force are usually derived from statistical analyses of ligand–receptor inter-atomic contacts and are largely based on study of the complexes available in Protein Data Banks (PDBs). SMoG, Muegge's, and Drug-Score programs are based on this approach.
2) **Master equation approaches** use estimations of energetic contributions of various interaction types. This is a semi-quantitative approach.
3) **Regression-based methods** rely on available biological activity for training sets of ligand–receptor complexes taken from PDB.
4) **Poisson–Boltzmann equation solvers** address electrostatics and solvent effects.

Consensus scoring can be used in case a particular scheme meets failure.

18.3.2 Ligand-Based Virtual Screening

This can be used for the ranking of novel ligand molecules by using either pharmacophore pattern matching or 3D similarity searching. However, this ranking is only possible if detailed information regarding confirmation is available either from structural methods (X-ray crystallography and NMR spectroscopy), or from molecular modeling. Similarity search as well as pattern matching requires appropriate software for querying large databases of hypothetical/virtual or existing chemicals as possible ligands. Regardless of the chemotypes, all ligands show similar steric and electrostatic characteristics recognizable at target-binding sites. These characteristics are responsible for the typical biological activity of the ligand molecules. This whole notion forms the core theme of the pharmacophore concept. ALMOND is a program based on this concept. On the other hand, 3D similarity searching assumes that molecules with similar features are likely to have similar biological activity or set of biological activities. Tanimoto's "distance-between-patterns" and Tversky's asymmetric "contrast model" form the basis for similarity measures. In a search program, the database queries initiate the comparison of the molecules (steric, electrostatic similarity) with the 3D information derived from known actives. ROCS is an example of programs used for similarity search.

18.3.3
Application of Integrative Virtual Screening

In the target-based virtual screening, three human carbonic anhydrase II inhibitors have been identified using a set of hierarchical filters and the FlexX Docking. These inhibitors have been identified from a database of nearly 100 000 compounds. Similarly, thyroid receptor antagonists have been identified using ICM. In the ligand-based screening, CATS program has been used to identify clopimozid as a novel T-type calcium channel blocker. The same program has been used to identify a structurally new glycogen synthase kinase-3 inhibitor. Using the pharmacophore matching technique, 12 nonpeptidic urotensin II (U–II) receptor antagonists have been identified.

18.3.3.1 Case of Cisplatin in Tumor Therapy

Cisplatin is used for testicular, ovarian cancer, and head/neck tumors. Many a time, cases of resistance to cisplatin therapy surface. Cisplatin is believed to act by binding of a *cis*-$Pt(NH_3)_2$ unit to DNA at two neighboring guanine bases. Novel platinum anticancer drugs developed after understanding the mechanism of platinum resistance are based on either changing the coordinated nitrogen ligand or altering the leaving groups. This has resulted in a decrease in cisplatin resistance cases.

18.4
Application of Microarray Technology in HTS and Drug Discovery

18.4.1
DNA Microarray

Microarray refers to a piece of glass, plastic or silicon with a large number of biosensors at known, specific locations. These microarrays are often referred to as "biochips" or "DNA chips." They can be used for testing a single biological specimen for a range of effects or attributes. Several chemical moieties of variable nature can be used for detecting proteins – largely on the basis of a specific physical or chemical interaction and/or resultant phenomenon. These protein-detection molecules include ligands, dyes changing color, fluorescing, or causing electronic signals when they come in contact with specific protein molecules. If these protein-detector molecules are placed onto a microarray, protein expression and expression levels of genes in the given biological specimen can be evaluated. DNA microarrays were initially developed and described by Patrick Brown during the 1990s [7]. They are utilized in the analysis of gene expression levels in an organism and for comparing gene expression levels (e.g., between diseased and healthy tissues). This is accomplished by passing samples with DNA (e.g., in liquid) over the array surfaces. As a result, messenger RNA (mRNA) hybridizes to its counterpart DNA sequence.

18.4.1.1 DNA Microarray Fabrication

DNA microarray fabrication methods include inkjet and microjet deposition or spotting technologies, *in situ* or on-chip photolithographic oligonucleotide synthesis, and electronic DNA probe addressing processes. DNA microarray hybridization applications include gene expression analysis, genotyping for point mutations, single nucleotide polymorphisms (SNPs) and short tandem repeats (STRs).

The two commonly used and relatively simple approaches for manufacturing DNA microarrays are described below:

1) Using the reverse transcriptase polymerase chain reaction (RT-PCR), cellular mRNA is utilized to prepare approximately 500–5000 base pair long segments of complementary DNA (cDNA) which are later attached to a nylon or glass surface at known spots. When hybridization of sample DNA takes place, the location of the spot specifies the DNA in the sample. Process flow in a typical system has been depicted in Figure 18.5.

2) In another method, oligonucleotides or peptide nucleic acids of known sequence are attached at known spots on the nylon or glass surface. Then the biological sample with DNA (e.g., in liquid) is passed over this surface to identify the DNA contained in the sample.

DNA microarrays are an example of functional genomics. Complete genomic signals guiding the cellular processes are recorded by using DNA microarrays. High-density microarrays have been devised for HTS applications and lower-density microarrays for diagnostics. Recently DNA microarrays have been classified into three types on the basis of functionality with their applications by Chavan *et al.* [8]: (i) *Comparative genomic hybridization* for detection of chromosomal aberrations; (ii) *expression analysis* for gene expression analyses; and (iii) *mutation or polymorphism analysis* for detection of occurrence of mutations or polymorphisms

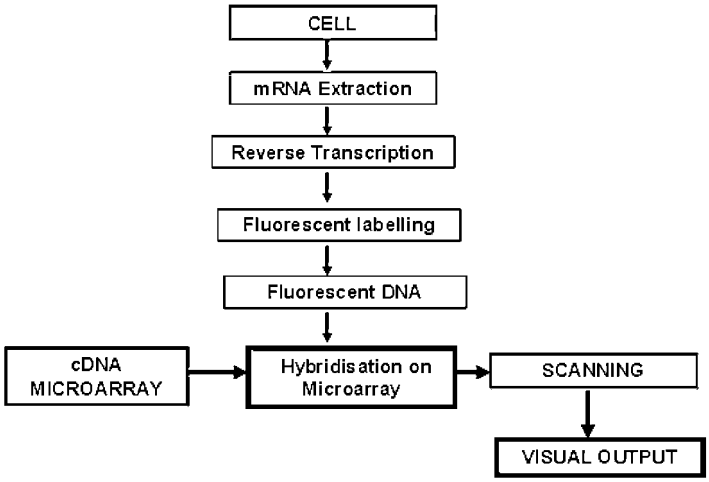

Figure 18.5 Process flow in a DNA microarray system.

in a gene sequence. Table 18.4 [8] provides an application summary of these three types of microarrays.

18.4.1.2 Application of DNA Microarrays in HTS and Drug Discovery

DNA microarrays have the distinction of being the first array technique that are close to entering routine laboratory diagnostics. In the near future they may provide information about activated oncogenic signaling pathways and other cell functions in malignant tissues. They may also help in predicting drug response or metastasizing potential. DNA microarrays are being manufactured by several companies specializing in biomedical or analytical applications (Table 18.5) and

Table 18.4 DNA microarray classification and applications. (Adapted from Chavan *et al.* [8]).

Application	Microarray type		
	Comparative genomic hybridization	Expression analysis	Mutation or polymorphism analysis
Tumor classification	✓		
Disease diagnosis	✓		
Drug development		✓	✓
Drug response		✓	
Mechanism of action of drug		✓	
Disease progression tracking		✓	✓
Genotyping			✓

Table 18.5 Manufacturers of DNA microarray and related products. ✓ indicates that a particular manufacturer is producing a specific product related to microarray technology.

Company	Scanners	Arrayers	Pre-spotted slides
Applied Precision	✓		
Gene Focus	✓		
Gene Machines		✓	
Genetix		✓	
Clontech			✓
Origene			✓
Agilent	✓	✓	
Motorola	✓	✓	
Hitachi	✓	✓	
RoboDesign	✓	✓	
Virtek Vision	✓	✓	
Affymetrix	✓	✓	✓
Genomic Solutions	✓	✓	✓
MWG	✓	✓	✓
PerkinElmer	✓	✓	✓

can be used for a variety of applications for example, pharmaceutical research (pharmacogenomics and drug discovery), toxicogenomics, diagnostics (infectious, genetic disease, and cancer), and forensics (genetic identification). DNA microarrays and proteomics technologies can be combined together to study the gene regulation process in diseases and identify drug discovery and diagnostics targets. The combined application of these technologies holds the potential to revolutionize areas of biology particularly in molecular medicine. Peptide (probe sets are made up of peptides) and antibody (probe sets are made up of peptides) microarrays are similar to the DNA microarray with respect to the basic technology, except that the probe sets can also be used for proteomics based uHTS [9, 10].

DNA microarrays have also been used successfully in herbal drug research, for example in validating the role of triterpenes from the tropical herb *Centella asiatica* in wound healing and treatment of connective tissue disorders. Similarly, antiproliferative activity of another medicinal herb *Coptidis rhizoma* and its constituent berberine has been established in human pancreatic cancer cell lines. DNA microarrays have also been utilized in discovering novel molecular targets for a combination therapy (docetaxel and estramustine) in prostate cancer cells. These microarrays have also helped to illustrate the mechanism of action of *Ginkgo biloba* leaf extract in neuroprotection. DNA microarrays have proven their worth in anticancer and antibacterial therapy and prediction of drug toxicity.

18.4.1.2.1 **Anticancer Drug Discovery**

DNA microarray analyses may change the future of cancer therapy. DNA microarrays provide a deep insight into carcinogenesis at a cellular level. When combined with tissue specific microarrays, DNA microarrays can help in rapid validation of gene targets for cancer therapeutics. The use of global RNA and protein expression-profiling technologies including DNA microarrays, has resulted in identification of cellular targets in pancreatic cancer with considerable potential for clinical implications. Needless to say, these microarrays can help in understanding the pathogenesis of pancreatic malignancies and help in devising better therapeutic strategies.

18.4.1.2.2 **Diagnosis and Therapy of Bacterial Diseases**

Bacterial DNA microarrays can be utilized in studying bacterial pathogenicity, genomic complexity, and antimicrobial resistance. Such an understanding will definitely help in devising novel therapeutic tools for clinical management of bacterial diseases.

18.4.1.2.3 **Prediction of Drug Toxicity**

DNA microarrays can be used for predicting toxicity of drugs through practical gene expression profile screens (GEPS). However, cost, reproducibility, and efficient data analysis are some of the hurdles in realizing the potential of GEPS for compound prioritization.

18.4.2 Chemical Microarray for Drug Screening and Discovery

Although there are 10 000 druggable targets, estimated using various genomic and proteomic approaches, less than 500 can be targeted with FDA approved drugs. Therefore, HTS is the only powerful tool for rapid screening and identification of new active compounds and pharmacophores against this large number of druggable targets, which also reduces time and cost. Owing to the generation of large numbers of new chemical compounds, HTS methods are also rapidly evolving, and microarray technology that is mostly used in genomic and proteomic studies is being implemented for rapid screening of drug compounds. Automation and miniaturization of the technology provides a leading edge that makes it suitable for this purpose.

A chemical microarray is a form of chemical combinatorial peptide library first described by Geysen *et al*. in 1984 [11], and is a powerful tool in drug screening and discovery. A large chemical library can be screened against hundreds of biological targets using chemical microarrays or small-molecule microarrays in solution phase on an automatic liquid handling platform. However, isolated wells are required for individual reactions when compounds are of different structures and properties, and currently available liquid handling systems are not capable of processing thousands of reactions individually in a solution phase. Therefore, several versions of this technology are currently under development [12].

18.4.2.1 Chemical Microarray with Immobilized Technology

The first type of chemical microarray technology where new DNA microarray technology merged with the receptors conjugated to fluorescent dyes or reporter enzymes screened from the libraries has been created. This platform uses various linking techniques to immobilize chemical compounds to the surface of the glass and then screen against the chosen biotargets.

There are many techniques by which compounds can be immobilized, the simplest type of immobilization being through surface adsorption, which is specifically used for proteins. In addition, molecules can also be tagged and immobilized through non-covalent interaction between the tag and the immobilized molecule for the tag. Although nonspecific physical adsorption is mainly used for generating a microarray for macro-molecules. it is less useful when concerned with small molecule or small peptide microarrays. Though these small molecules can be immobilized by non-covalent interaction, an alternative method is by immobilization of the covalent attachment to a functional group on the solid surface. The solid surface undergoes a chemical change, which is necessary to create a functional group for the covalent interaction to achieve a homogenous immobilization.

18.4.2.2 Small Molecule Microarray (SMM)

A small molecule microarray (SMM) is a chemical compound microarray that uses various linking techniques to immobilize organic molecules or chemical

compounds to the surface of the slide. This was the first chemical compound microarray that came out of DNA microarray technology and developed through different immobilization techniques, as described by the group working with Schreiber at Harvard University. The important requirements for this form of microarray are particular reaction groups on both the microarray surface and the library. Libraries were initially screened against the receptors conjugated to fluorescent dyes or reporter enzymes. However, many limitations arise from immobilization of a large chemical library. Therefore, a fragment chemical compound microarray system was created by scientists from Griffinity Pharmaceuticals, which immobilizes thousands of drug fragments or pharmacophores. On this platform, the drug compounds are synthesized and then immobilized with a specially designed self-assembled monolayer (SAM) and a thin gold layer providing the base for the SAM and surface plasmon resonance (SPR) detection. This microarray chip is then treated with the biological target.

Immobilized chemical microarrays (SMM and fragment chemical microarray) are useful in identifying targets, but there are certain disadvantages to these platforms. The microarray involves specific chemistry for the coupling, thus limiting the use of the existing chemical libraries. Thus, factors such as the length and flexibility of the linker, compound binding orientation, spatial hindrance, and microarray surface properties also affects the target binding.

18.4.2.3 **Dry Chemical Microarray**

A dry chemical microarray developed by scientists from Abbott Laboratories, and licensed by Discovery Partners International for commercialization, has generated a new form of chemical microarray that avoids the limitations of immobilization, by putting compounds dissolved in dimethyl sulfoxide (DMSO) on polystyrene sheets and drying them. These microarrays are then processed by diffusing the immobilized target in the agarose gel. This process is also known as microarrayed compound screening (μARCS). Agarose gels containing the biological targets are laid on top of the compound sheet, thus allowing the compounds to dissolve and diffuse into the gel and interact with the target. A second agarose gel containing radioactive ATP (adenosine triphosphate) was then placed on top of the target gel containing the substrate, to initiate the biological reaction after a short incubation. The final reactions are then detected using a standard phosphorimager. The main advantage of this format is that the chemical compound library in a dry form can be deposited on to a polystyrene sheet, and also biochemical assays can be performed in any aqueous-rich environment. However, a problem with this approach is that the rate of resolubilization and the diffusion of different classes of dry compounds hinders the effect of the dynamic range of arrayed compounds against the bio-available compounds.

18.4.2.4 **Solution Phase Chemical Microarray**

A solution phase chemical microarray using glycerol as an anti-evaporating reagent mixed with chemical compounds that were arrayed on the microarray surface was created by Diamond's group [13–15] from the University of Pennsylvania, and is

currently the most advanced microarray for HTS. Reaction Biology Corporation has licensed and developed this into a commercial product, Discovery plot platform, and are endeavoring to use this technology as a new tool for HTS. This technology microarrays more than 6600 organic compounds on a standard glass slide (1×3 inch) in a nonvolatile glycerol-based format compatible with −80 °C storage. The aerosol deposition technology was used to start the reaction, which was then detected with a scanner or imager. Both the chemical compounds and the targets are in solution throughout the process, thus allowing any existing chemical library to be screened by this platform.

18.4.3 Cell Microarrays in Screening and Drug Discovery

Chemical microarrays have entered a new area of high-throughput screening. Stockwell and Sabatini's group [16] developed the first cell-based microarray format for cell-based high-content screening using biodegradable poly(lactic-co-glycolic acid) (PLGA). These assays are useful to evaluate potential drugs by functionally characterizing their effects in cells, the specificity and efficacy of the drugs, and also to identify drugs of unknown mechanism of action.

18.4.3.1 Types of Cell Microarrays

Based on the design, efficacy, and methods, cell microarrays can be broadly classified into two types.

18.4.3.1.1 Positional Cell Microarrays

In this type, cells are arranged on a surface and assigned *x*- and *y*-coordinates. In position cell microarray, different substrates are arrayed producing the many cell-based microarrays that are based on polymers, glycans, peptides, antibodies, extracellular matrix, and membranes. The cells are deposited onto the array and are attached to the printed substrate. The main principle here is that the *x*- and *y*-coordinates are specifically characterized and physically separate the clusters of the cells.

18.4.3.1.2 Batch or Non-Positional Cell Microarrays

Here cells are encoded. In this system, phenotypes are assayed in lots or batches and cells of potential interest are subjected to decoding. Batch cell microarrays are also known as non-positional microarrays, that do not depend on the *x*- and *y*-coordinates but are associated with a different code. There are two main approaches. The first technology uses codes that rely on colored bars that are physically associated with the cells. Another coding system uses a nano-size semiconductor crystals and quantum dots that are incorporated with the cells. The quantum dots fluoresce according to their size at different wavelengths.

Some workers have classified the cell microarrays on the basis of substrate arraying [17]. Accordingly, several types have been recognized (Table 18.6).

Table 18.6 Types of cell microarrays on the basis of substrate arraying.

Cell microarrays based on polymers (chains of epoxy monomers)
Cell microarrays based on carbohydrates (glycans)
Cell microarrays based on peptides (fluorescent peptides)
Cell microarrays based on antibodies (for identifying specific cell surface antigens)
Cell microarrays based on extra cellular matrix (proteins and proteoglycans)
Cell microarrays based on membranes (support membranes of glass coated with phospholipids bilayer with anchored proteins)
Cell microarrays based on RNA interface

18.4.3.2 **Cell Microarray Formats**

Cell assays have been miniaturized by using 96-, 384-or 1536-well microtiter plates for growing cells or by mixing cells with one-bead one-compound type-chemical libraries. Cell microarrays can hold at least 5000–6000 spots and are definitely better than traditional microtiter plates. Several formats are available in cellular microarrays. The standard monolayer two-dimensional culture and three-dimensional organotypic cell cultures are more popular than other formats. Recently, reversely transfected cell microarrays (RTCMs) have been introduced. They have been envisaged as a parallel HTA method for gene functions in mammalian cells. This technique allows transfection of up to thousands of different recombinant DNA or RNA molecules into different cell clusters at the same time on a single glass slide. Several sophisticated detection systems have been devised to quantitatively analyze the effects of transfected molecules on cell phenotypes. 2D and 3D cell microarrays have been reported and 3D microarrays can substitute "*in situ* like" condition during HTS, and therefore can potentially substitute the *in vivo* assays [18].

18.4.3.3 **Applications of Cell Microarrays**

Cell microarrays offer an opportunity to measure parameters on hundreds of individual cells used in the microarrays. The results can then be averaged. These results are superior to those obtained for the corresponding parameters for a whole cell population. This is the main advantage of using cell microarrays. Cell microarrays have been used for identifying the biomaterial and polymer for growing human embryonic stem cells, which can be used for a variety of purposes (described later in this chapter in detail). Cell microarrays based on antibodies have produced better results than flow cytometry in clinical studies on prostate cancer and leukemia. These microarrays have also been used to screen anticancer drugs in the presence of different double-stranded interfering RNA and to show how the fate of cancer cells is decided if loss of function of a gene can sensitize these cells to anticancer drugs.

18.5
Chemical Proteomics for Drug Discovery and Development

According to the human genome project (HGP) data there are 25 000–30 000 protein coding genes present in the human genome, and the study of proteomics reflects the understanding of function, interaction, modification, localization, and regulation of all protein expressed by a cell or in an organism. Typical expression levels of proteins maintain normal physiology and any alteration in protein structure or expression causes diseased phenotypes. Therefore, functional and structural proteomics are very important and, recently, new technologies such as shotgun proteomics, reverse proteomics, top-down proteomics, and chemical proteomics have been developed to eleviate this intimidating task.

Chemical proteomics is a powerful tool for isolating and identifying novel cellular receptors at the level of enzymatic activity using both *in vitro* and *in vivo* chemical probes, and thereby has potential to accelerate the discovery of new drug targets and often providing precious selective information regarding biochemical and cellular process. Chemical proteomics was first used by Taylor *et al.* in 1965 [19] to identify tubulin as the cellular receptor for colchicines. However, currently it is emerging as a powerful tool for drug discovery as it only requires a known drug to start with and can be defined as application of synthetic organic chemistry, bioinformatical analysis, biochemistry, and mass spectrometry to the field of proteomics.

Chemical proteomics can help us design specific protein modifying reagents that can be used for functional studies of a distinct enzyme family within a proteome. These chemical probes are designed to covalently modify a target enzyme in such a way that it can be subsequently identified and/or purified. Chemical proteomics parallel functional proteomics except that a tagged small molecule is used to isolate a single protein or a family of proteins from an entire proteome. The tag can consist of either a radioactive/fluorescent label to allow visualization of bound protein on an electrophoresis gel, or a solid phase bead/surface, to allow affinity purification of the protein. Nevertheless, as this is a probe-based technique so before continuing the discussion it is necessary to understand the anatomical component of a probe to make this study easier.

18.5.1
Structure of a Probe

Regardless of their mechanism of action chemical probes are finding increasing use in the field of proteomics and have great potential to aid in the process of target identification, target validation, and drug discovery. A probe is a small molecule used in chemical proteomics can be designed chemically via synthetic organic chemistry to react with an enzyme.

Anatomically a probe is made up of three basic elements: the *reactive group* consists of a functional group and is covalently attached to the active site of an enzyme; a *tag*, which can be used for identification and purification of modified

enzymes; and a spacer, the spacer/linker connects these two elements and can alter the reactivity and selectivity of the reactive group. The probe may be a drug, natural compound, synthetic chemical compound, peptide reactive group or any other element that can react with the receptor enzyme covalently or non-covalently, and has a specific reactivity towards the probe.

There are two class of probe: activity-based probes (ABPs) and affinity-based probes (AFBPs). Activity-based probes are designed to isolate a specific class of enzyme and include modification of the residue present on the active site of the enzyme in a manner that requires enzymatic activity of a target. On the other hand, affinity-based probes do not rely on protein enzymatic activity, but on the recognition of a particular motif, which requires highly selective tight binding to the target for probes to be useful for a distinct protein/enzymatic family.

The reactive group is conceivably the most vital component of a probe and has its own significance, as is conveys essential covalent alteration of the target enzyme. However, the choice of a suitable reactive group is a challenging task, because it should have specific reactivity for a specific protein residue, but also non-reactivity towards other reactive elements in the proteome. Most reactive groups take the form of a suicide/mechanism-based inhibitor or affinity labeling reagent. These drugs have been use for decades (e.g., aspirin), as tools to identify the active site residue in an enzyme and to understand the mechanism and function of enzyme catalysis *in vitro*.

A spacer not only works likes a connective link between the reactive group and the tag but can also provide enough of a gap between the reactive group and the tag to avoid steric hindrance. This can block access of the reactive group or the approachability of the tag to the receptor enzyme and manipulate the specificity of some probes (e.g., activity-based probes). In most cases polypeptides are used as a spacer because they are easy to prepare and attuned with solid-phase chemistry, but in addition, long-chain alkyls or polyethylene glycol (PEG) are also well known examples, due to the specificity to modulate hydrophobicity and to allow entry into live cells or tissues. The use of very large peptides or proteins to provide a high degree of target specificity to a chemical probe also holds much promise. However, almost all enzymes are made of protein and therefore bind probes with peptide character. Furthermore, information regarding substrate specificity of a given enzyme can be used to design a probe that displays selectivity for distinct enzyme targets.

A tag is an essential component of a probe and is used to identify the modified protein. The most commonly used tags are biotin, fluorescent, and radioactive tags. Biotin is used frequently as the tag as it allows both affinity purification, using a streptavidin resin, and gel visualization, using streptavidin to couple to the receptor enzyme, such as horseradish peroxide. Compared with biotin, fluorescent and radioactive tags have several advantages. They are faster to use, more sensitive, have enormous dynamic range, are easy to visualize as they can be visualized directly by scanning the gel with a fluorescent scanner, and, lastly, one can use probes with different colored fluorescence tags in various experiments and readily obtain all the result on a single gel.

18.5.2 Strategies in Chemical Proteomics in Drug Discovery

Chemical proteomics is a powerful tool for isolating and identifying cellular receptors for biologically active products, therefore facilitating subsequent rational drug design and providing valuable information regarding underlying biochemical and cellular processes. There are two strategies for chemical proteomics techniques: (i) activity-based probe profiling (ABPP), which is based on enzymatic activity of a particular class of enzyme, and (ii) the fragment-based approach, which facilitates the assembly of a large library of diverse molecules from a smaller number of building blocks [20].

18.5.2.1 Activity-Based Probe Profiling

Activity based probe profiling is a functional proteomics technology used to identify new proteins with respect to their functional state. It uses active site directed chemical probes to monitor enzyme function in complex biological systems. The basic unit of ABPP is the probe, which typically consists of two elements: a reactive group (RG) and a tag, typically a fluorophore or biotin for detection and/or enrichment of the probe labeled protein. Additionally, some probes may contain a binding group that enhances selectivity. The reactive group usually contains an electrophile that becomes covalently linked to a nucleophilic residue in the active site of an active enzyme. An enzyme that is inhibited by enzyme inhibitors or post-translational modifications will not react with an activity-based probe. It can also be used to determine the selectivity profile of a drug targeting an enzyme family via pretreatment of lysate with the drug of interest, and then subsequent labeling and identification of the remaining enzyme using an appropriate reactive probe. There are multiple levels of annotation for enzymes. The most basic level is assignment to a specific mechanistic class based on the general chemical reaction catalyzed by the enzyme (e.g., hydrolase, kinase, oxidoreductase, and others).

Covalent bonding is involved between the probe molecule and the protein in this technique, which occurs via irreversible attachment of an inhibitor to a moiety in the active site of the enzyme, but in some cases a reversible inhibitor can also be coupled. In order to proceed to the next step, a probe is designed by connecting a reactive warhead using a linker to a reporter tag, such as biotin. After the cell lysate of interest is incubated with ABP, targeted proteins are captured on an affinity matrix followed by digestion with trypsin, prior to MS, protein database mining, and further bioinformatics analysis.

A new technique known as chemo or compound centric profiling is more popular nowadays, and is a modification of activity-based profiling. This technique is based on classical drug affinity chromatography in combination with high resolution mass spectrometry analysis and bioinformatics for identification of the binding protein. The chemo centric approach results in the positive identification of hundreds biochemicals in a single sample. This technique has advantages over activity based probe profiling as it delivers a characterization of the target profile

of the bioactive compound, that is, it provides direct information about the activation state of the identified protein.

18.5.2.2 **Fragment-Based Approach**

The fragment-based approach is based on the identification of small chemical fragments, which may bind only weakly to the biological target and then growing or combining them to produce a lead with a higher affinity. However, there are many approaches that determine the interaction of "full size" ligands with their protein complements. Analysis of the outcome of HTS and approved drug molecules show recurring moieties in the active compounds. These moieties are separated from their parent molecules and are termed "privileged fragments." This approach assists in the assembly of large libraries of diverse molecules (totaling $N1 \times N2$) from smaller numbers of building blocks (totaling $N1 + N2$), where N is the number of each type of building block. A molecule fragment with as little as 10–12 heavy atoms could theoretically lead to a nanomolar inhibitor or ligand.

The fragment-based approach includes click chemistry and amide formation reactions. "Click chemistry" is a term coined by Sharpless *et al.* in 2002 [21], and is one such fragment-based tool that is becoming increasingly popular because of its ease of use and high efficiency. It is a new approach to synthesizing drug-like molecules and its applicability ranges from lead identification to proteomics research. Using conjugation reaction of bio compounds, click products may be used as inhibitors or detector probes. It utilizes combinatorial chemistry and target –template *in situ* chemistry, and is applicable to modification of the peptide function with triazole, modification of natural products and pharmaceuticals. One important "click" reaction is the Cu^{I}-catalyzed 1,3-dipolar cycloaddition reaction between azides and terminal alkynes. This makes use of a biologically orthogonal reaction, which is both efficient and specific, that is, the reaction may proceed even in the presence of complex biological specimens or extracts.

The Cu^{I}-catalyzed "click" reaction between an alkyne and an azide was also applied in the synthesis of both affinity-based probes and inhibitors for metalloproteases by Cravatt and coworkers [22]. They developed mixture-based hydroxamate probe libraries for the identification of metalloproteases within complex cell lysates. Besides the hydroxamic acid ZBG (zinc-binding group), click chemistry was applied to incorporate rhodamine-based ZBG inhibitors. The 1,3-dipolar cycloaddition chemistry has also been applied in the synthesis of inhibitors against many other enzymes, including caspases and phosphatases.

Another important reaction is amide-bond formation between an amine and a carboxylic acid. The reaction is highly efficient and generates the desired products with quantitative yields, hence facilitating modular fragment based assembly and *in situ* screening. Wong *et al.* [23] have successfully applied this in the solution phase for enzyme inhibitor discovery. The *in situ* solution-phase approach is convenient and easy to implement but may introduce byproducts that affect the biological screening, it is not amenable to multistep reactions without purification, and cannot always be driven to completion through use of excess starting materials.

18.5.2.3 Applications of Chemical Proteomics

Chemical proteomics is a new and promising tool for drug discovery, as it requires only a known drug as the starting point, and leads to the identification of a suitable target whose biological activity can be directly linked to a pathological process. Minor imbalance in enzyme activities, either through mutation, expression changes, or regulatory dysfunction, are known to cause debilitating diseases and even promote cancer or tumor metastasis. Several diseases such as cancer, rheumatoid arthritis, and osteoporosis are associated with elevated levels of proteases activity. In this modern age, doctors and scientists are giving more emphasis to natural druggable compounds, as this is more effective and has less side effects.

Although many valuable natural compounds have been annotated and recorded in databases, identification of their appropriate partner targets is still in progress. Annotation of new druggable targets is a necessity for modern pharmaceutical research, and in this area, chemical proteomics again seem to be a useful tool for the prediction of suitable interacting targets for natural compounds.

Identification of new drug target is also essential for solving drug resistance problems. Nowadays drug resistance is a well known problem, from bacterial infections (such as *Mycobacterium tuberculosis*, HIV, etc.) to cancer, most of cases of which involve the structural modification of drug targets. In addition, probes are able to monitor and profile activity of different enzymes throughout the various stages of a disease and this information can be used to understand the relationship of a particular enzyme expression with the corresponding disease. This means that this technique is able to retrieve suitable markers for the particular diseases. Detailed understandings of molecular configurations that are recognized and accepted by an enzyme active site facilitate not only the design of a potent and selective inhibitor but also discovery of its biological function and downstream targets.

18.6 Target and Drug Discovery Using Lipomic Profiling

Lipid metabolism dysregulation is involved at the onset of several diseases and adverse drug responses. Changes in lipid metabolism lead to a simultaneous long-term shift in lipid metabolite concentrations. The application of "omic" technologies and HTS/HCA technologies has rapidly led to the emergence of a new body of knowledge dealing entirely with the role of lipids in molecular events resulting in pathologies. This new branch of study has come to be known as lipomics [24]. Lipomics can be considered an offshoot of metabolomics, as it deals with metabolome data of one particular class of chemicals – lipids. Lipid profiling is considered to be the most developed and focused area of metabolomics. Metabolomics itself is a developing branch of science. Lipomics also plays an important role in systems biology, as the concept of systems biology involves integrative "omics" (i.e., combining genomics, transcriptomics, proteomics, lipomics, and metabolomics)

as well as bioinformatics and modeling. Lipomics can provide a much deeper understanding of lipid metabolism in the human body, its regulation, and involvement in pathologies and malignancies.

18.6.1 Applications of Lipomic Profiling

At present, lipomic profiling is not particularly popular but is gaining ground as the discovery process continues. It is, nevertheless, an important tool in drug discovery. Its practicality can be illustrated by the following example.

18.6.1.1 Case of Rosiglitazone in Diabetes Mellitus Treatment

A study has been conducted to determine the effects of the peroxisome proliferator-activated receptor gamma (PPARgamma) agonist rosiglitazone on structural lipid metabolism in a type 2 diabetes mellitus mouse model. Dietary supplementation with rosiglitazone (200 mg/kg diet) has resulted in experimental suppression of type 2 diabetes in obese F1 male mice. Chronic supplementation, however, exacerbated hepatic steatosis. It has been concluded that rosiglitazone induces hypolipidemia through liver–plasma lipid exchange dysregulation. Rosiglitazone induces *de novo* synthesis of fatty acids and also decreases lipid biosynthesis within peroxisome. This drug has been found to alter free fatty acid and cardiolipin metabolism. It elicits an unusual adipose tissue accumulation of polyunsaturated fatty acids. This single study has demonstrated the potential and importance of lipomics in drug discovery and profiling.

18.7 Drug Discovery Using Integrative Genomics

Systematic analysis of candidate gene sequences for determination of specific sequence variations linked with disease remains a key step in the discovery process. Approaches to candidate and disease gene identification include whole genome-based methods (e.g., integrative genomics) and functional genomics-based methods. The analysis of whole complex systems can provide a further insight into complex gene variability patterns and genotype–phenotype relationships [25]. Progress in robotics and HCS/HCA methods have made it possible to use transcriptional profiling as a comprehensive chemical genomics platform for drug discovery and development. Transcriptional "fingerprints" indicate the mechanism-of-action of compounds used in the treatment of cells [26].

18.7.1 Applications of Integrative Genomics

Integrative genomics has several applications in the diverse fields of biomedical sciences. Some of these applications are summarized in Table 18.7.

Table 18.7 Illustrative biomedical applications of integrative genomics.

1) Infectomics and antimicrobial drug resistance
2) Antifungal drug discovery
3) Genomics and proteomics in antidiabetic drug discovery
4) Human Genome Project
5) Anticancer drug discovery
6) Toxicogenomics of hepatotoxicity

18.7.1.1 Infectomics and Antimicrobial Drug Resistance

An integrative study of structural and functional genomics and proteomics of microbial infections (infectomics) is expected to help overcome present-day challenges. Genome-wide approaches to genotyping and phenotyping result in a better understanding of microbial pathogenesis as well as rapid diagnosis of infectious diseases. These approaches can also help in the development of novel anti-infective agents of therapeutic relevance.

Microbes have a tendency to develop resistance to particular classes of antibiotics. This type of drug resistance results in reduced efficacy of current antimicrobial therapies. As a result, the newer classes of antibiotics are required to overcome the cases of clinical resistance to existing antibacterial and antifungal drugs. The availability of information pertaining to the bacterial genome has added impetus to the search for newer antibiotics. The rapid advancements in sequencing and analysis of prokaryotic genomes have enriched our understanding of antibiotic resistance and helped to identify and evaluate novel antibiotic classes.

18.7.1.2 Yeast as Tool for Antifungal Drug Discovery

Saccharomyces cerevisiae is an important model system for new drug target identification, drug screening, and detailed analysis of drug effects at the cellular level. Closeness to human pathogenic fungus (*Candida albicans*) makes yeast a favorite study system for antifungal drug discovery.

18.7.1.3 Genomics and Proteomics in Antidiabetic Drug Discovery

Genomics and proteomics provide better insights into the molecular mechanisms of diseases. Genomics aims at defining a static genetic substrate whereas proteomics helps in the proper understanding of structure and function of proteins expressing under specified conditions. These techniques may play an important role in various aspects related to drug discovery (e.g., biomarker development, target validation, diagnosis, prognosis, and optimization of treatment). Proteomic technologies have also contributed towards the discovery of new biomarkers for pathophysiology and the clinical outcome of diabetes.

18.7.1.4 Human Genome Project and Anticancer Drug Discovery

The outcome of the human genome project has the potential to transform cancer therapy scenarios. The merger of genomics with pharmacology will allow the

development of new therapeutic agents. Single-nucleotide polymorphisms (SNPs) are indispensable tools in the pharmacogenetic approach to cancer therapeutics. SNPs can be utilized as disease markers and help in identification of certain populations facing increased risks for different cancer types. SNP genetic screening may facilitate therapeutic modalities and reveal genetic profiles of importance in drug efficacy and toxicity. DNA and tissue microarray analyses may also contribute to a changed course of cancer therapy. DNA microarrays, for example, can help to study gene expression changes taking places in the development of cancers through carcinogenesis. Tissue microarrays can identify candidate genes against specific tumor types. Both types of microarrays can help in the rapid validation of gene targets. Certain antitumor compounds (e.g., anthracyclines) can trigger a potent T-cell-dependent antitumor response. Proteomic analyses of anthracycline-treated tumor cells have shown involvement of calreticulin in the immunogenicity of dying tumor cells.

18.7.1.5 **Toxicogenomics for Hepatotoxicity**

Hepatotoxicity is not only a cause of failure in drug discovery but also a potent adverse drug reaction source. Better prediction and understanding of drug-induced hepatotoxicity can aid efficient development of safer drugs. Toxicogenomics is the latest tool for predicting toxicity and for mechanistic understanding of toxic changes taking place; for example, in the liver after administration of particular drugs. It has been used for investigating drug-induced hepatotoxicity in *in vitro* systems and *in vivo* animal models (see Section 18.8).

18.8
Toxicogenomics in Drug Discovery and Development

18.8.1
Toxicogenomics

Toxicogenomics is a branch of toxicology that deals solely with the reactions involving toxins/drugs and the different responses of various individual (due to differential DNA/genomic makeup) to such toxins. Different people have different genetic setups and, hence, they respond differently to toxins of plant, animal, or microbe origins. The study of genetics underlying the differential responses in these conditions is termed toxicogenomics. For example, plants of the genus *Rhus*, such as *R. toxicodendron* and *R. diversiloba*, produce simple phenolic compounds (urushiol derivatives). These phenolic substances cause severe skin problems (dermatitis). In this case, watery blisters are formed on the skin, which burst and spread quickly over the whole body, making the life of the person who has come in contact with these plants miserable. Most people react to these plants and urushiols in this way only. However, some people are either resistant to typical effects or develop very mild symptoms on contact with such allergenic substances. These differences arise because of the different genomic setups of the different people.

A human genetic variation makes some colorectal cancer patients seven times more prone to a toxic reaction with irinotecan – a drug used to treat colorectal cancer. If this variation is known to physicians treating colorectal cancer patients, a drug other than irinotecan can be prescribed so that the chances of complications in the cancer therapy are minimized.

Drugs with pyrimidine rings are degraded inside the human body under the influence of a specific enzyme. Rarely some human beings lack the gene that codes the production of this enzyme and, hence, cannot be given drugs such as 5-fluorouracil for treating cancer. The objective of the science of toxicogenomics is to minimize the occurrence of toxic reactions on the basis of genomic studies. Thus, it is fairly clear that genetic testing prior to drug administration or initiation of therapy can minimize toxic reactions. People whose bodies can resist the effects of a toxin, or are unable to degrade other toxins are known as haplotype. A haplotype may be an individual or a small group of individuals. The resistance-to-toxin results from one single nucleotide polymorphism (SNP).

18.8.2
Toxicogenomics in Drug Discovery

Poor pharmacokinetic profiles and drug toxicity are the biggest obstacles in developing a new drug, as opposed to appropriate ADMET results in the testing stages. Toxicogenomics, the study of the relationship between genomic constituents and an adverse drug effect, came as a boon to resolving this hurdle to some extent. Using toxicogenomics approaches, adverse drug effects can be mapped at interactome and signalome/signaling pathway levels, thereby an effective solution to the adverse effects can be easily achieved. The usefulness of toxicogenomics is represented in Table 18.8.

Long-term effects of drugs can be predicted in short-term assays using toxicogenomic approaches, therefore the drug discovery and development process can be accelerated many folds. Using an HTP/cDNA microarray, the effect of a drug at the transcriptome level along with gene expression patterns are mainly analyzed to identify the toxic responses on genome in most of the toxicogenomic experiments.

In general, an organ specific gene expression profile of a well characterized drug is obtained to provide a gene expression signature (clusters of genes that are expressed in a specific condition) of the adverse effect of the drug. The signature is then compared with the transcriptome profile of a new drug to predict possible

Table 18.8 Utility of toxicogenomics in drug discovery and development.

Time course of exposure
Determination of target-organ toxicities
Determination of target-cell toxicities
Relationship between transcription and protein expression

degrees of toxicity of the tested drug. Such toxicity signature profiles, for example hepatotoxicants and nephrotoxicant, are now of current interest to several research groups. Toxicity-related candidate biomarkers (early and late) can also be predicted using these signature data and various computational approaches. Therefore, toxicogenomics is useful in the classification of drugs based on gene expression profiles.

18.8.3
Toxicogenomic Studies Using *In Vitro* Models

In vitro toxicogenomics that can provide reproducible data is used for HTS (to screen small amounts of candidate compounds in relatively small assays) at early stages of drug development. For example, *in vitro* hepatoxicity is evaluated using liver biopsies, isolated hepatocytes, or hepatic cell line systems from human/mouse/rat with an appropriate candidate test drug and identification of the signature gene profile. However, when test systems are different, it is obvious that the expression profile will also vary, which is a big concern when using such tytpes of heterogeneous systems. Another problem is the failure to produce chronic toxicity symptoms of such systems due to multiple passaging of cell lines. Other concerns are the culture microenvironment, data compilation, and interpretation from various similar systems to standardize the signature profile [27]. Therefore, current trends is to use embryonic stem cells or specific adult stem cells (discussed later) for such experiments. However, variable systems such as these are currently in use and several good results have been produced from them.

18.8.4
Toxicogenomic Studies Using Animal Models

As discussed in earlier sections on the various drawbacks of *in vitro* systems, the best option is therefore to use animal models for such preclinical studies if a positive result is generated from any *in vitro* assay. Direct uses of transgenic or knockout mouse or rat disease models are beneficial and are mostly used in current toxicogenomic studies. Such model systems have successfully been used to screen and identify various chemopreventive agents, such as epigallocatechin gallate, curcumin, sulforaphane, for cancer chemoprevention, except for the fact that the dose–response standardization can only be fixed using primate or human clinical trials.

18.8.5
Toxicogenomics and Gene Polymorphisms

Idiosyncratic toxicity and its prediction and also detection are other challenges in drug development due to single nucleotide polymorphisms (SNPs) in drug metabolizing enzymes in various human populations. Such SNPs plays a crucial role in ADMET, therefore a single and universal dose may not be applicable to various human population or ethnic groups. Hence, pharmacogenomics or personalized

medicine is a recent trend for various drug discovery and pharmaceutical companies.

18.8.6 Application of Toxicogenomics

Toxicogenomics can be applied for the following purposes.

1) Time course of exposure to a chemical agent/drug.
2) Toxicities in target-organ, for example, liver – the main site of drug metabolism.
3) Toxicities in target-cells, for example, tumor or cancer cells in specified loctions or in body fluids such as blood and lymph.
4) Relationship between transcription and protein axpression.
5) Saving time in clinical research.
6) Cutting the cost of drug discovery and development.
7) Solving ADME/Tox problems in clinical situations.
8) Reducing the chances of occurrence of adverse drug reactions.
9) Identifying the populations at high risk to certain chemical or drug exposures.
10) Improving existing therapeutics for life-threatening toxic reactions.
11) Looking into post-marketing surveillance problems related to drug usage in specific areas.

The practicality of toxicogenomics lies in reduction of animal requirements for preclinical experimentation; and minimization of undue risk in volunteers participating in clinical trials. Some examples of toxicogenomics based identified drug toxicity are listed in Table 18.9. For further reading, readers can go through references [28, 29].

Table 18.9 Toxicogenomic approaches to identifying testicular and renal toxicity.

Techniques	Toxicity	Drug
RT-PCR	Germ cell toxicity	Mono-(2-ethylhexyl) phthalate and 2,5-hexanedione
cDNA microarray	Sertoli cells toxicity	2,5-Hexanedione
cDNA microarray	Spermatocytes toxicity	Ethylene glycol monomethyl ether
cDNA microarray	Spermatogonia toxicity	Cyclophosphamide
RT-PCR	Vasoconstriction and decreased renal blood flow	Aspirin, ibuprofen, naproxen, indomethacin, diclofenac
RT-PCR	Renal necrosis, anuria	Heavy metals
RT-PCR	Interstitial nephritis leading to fibrosis, reduced kidney size, and kidney tumors	Mycotoxins (Ochratoxin A)

18.9
HTP RNAi Screening for Targeted Drug Discovery

Genes involved in phenotypes of interest can be directly identified using functional genomics approaches. RNA interference (RNAi) provides a powerful tool for screening genes based on loss-of-function. Thus this technology can also be used in high-throughput screening of genomic targets for drug development and discovery, based on the understanding that induced expression of screened genes using a suitable antagonist will overcome the defective phenotype due to the loss of function of the screened genes that cause the disease [30].

RNAi is a widely accepted technology for various purposes such as gene therapy, knowout experiments, and so on. In this technique, a small single stranded noncoding RNA (ncRNA) molecule that is complementary to certain mRNA can bind to the mRNA, therefore inhibiting the translation process of the particular mRNA so that the corresponding protein is ultimately involved in expressing a certain specific phenotype.

18.9.1
HTS Using RNAi Libraries

Owing to the immense potential of RNAi, today it is also used for HTP screening. Various RNAi libraries are available based on experimental systems, or experiments or diseases of interests. Various forms of ncRNAs that are mainly used in HTP screening include: small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), short hairpin RNAs (shRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs). siRNAs or shRNAs are designed specific to mRNAs to be used in RNAi and they may be natural but are mostly artificially synthesized. Synthetic siRNAs from some companies, for instance Invitrogen, Qiagen, Ambion, can be used to screen specific targets, such as GPCRs, kinases, or can also be applicable to screen a whole genome using appropriate protocol provided by manufacturers. The biggest difficulties in RNAi screening are the proper delivery and expression system of the RNA into the screening cells and off target silencing.

Loss-of-function HTP screening using RNAi can be performed using either pool based library selection or array based screening approaches.

In pool based library selection, in general, a viral vector mediated small library of RNAi (shRNA having inducible promoters) is transfected to a pool of cells. The pool is then divided into two sub-pools and in one sub-pool the expression of shRNA is induced. Thereafter, each sub-pool is subjected to stress, drug treatments, and so on. After a certain period of culture, both the sub-pools are harvested and differential screening of gene expression in terms of barcodes is generated using microarray and PCR. Increased representation of a barcode from an induced sub-pool is indicative of selection of the corresponding shRNA, and therefore the target gene that is responding to the stress. If the stress correlates to a disease condition, an antagonist to the gene might be a good candidate drug for the disease. Similarly, if an shRNA induces some gene expression (causative to a disease) by targeting an inhibitor of the gene, the target of the shRNA can be

antagonized to induce the inhibitor gene expression to inhibit the expression of disease causing gene.

Array based RNAi screening is the most common method, and multiple different trigger deliveries are possible. In general, each well of a microtiter plate with a single RNAi trigger is incubated with a transfection medium and cells. After a defined incubation period, flow cytometry analysis, high content imaging, ELISA, and various homogeneous endpoint assays are performed to identify the loss of gene function.

18.9.2
Applications of RNAi in HTS

Screening using RNAi technology is a cost-effective and rapid process. RNAi libraries have successfully been used to discover "opportunistic" drug targets. Brummelkamp *et al.* [31] used a library to deliver sets of siRNAs against 50 human cancer-related de-ubiquitinating enzymes that are involved in cell signaling and regulation of protein stability. Among the tested targets CYLD, which is mutated in skin cancer, was identified as a key regulator of the NF-κB pathway. Patients with such cancer are now being treated with NF-κB blockers [32].

18.10
High-Throughput Screening with Stem Cells

More than 90% of candidate drugs tested in clinical trials fail to get approval due to high toxicity or lack of sufficient efficacy. This is because these drugs are mostly screened using different *in vitro* systems, such as various tissue explants or cell lines that cannot represent the exact physiology of the tissue or cell under its *in situ* conditions. Similarly, screening using animal models sometimes will not give the proper result because an animal model cannot always substitute the actual disease condition of a human. Therefore, a new strategy is to use disease specific human embryonic stem cells, which have the potential to give rise to all disease specific as well as normal cell types, so that the deregulated physiology of the disease will be more attractive for candidate drug screening and for better prediction of efficacy and toxicity in drug discovery.

18.10.1
Stem Cell

Stem cells are cells of multi-cellular organisms having the ability to renew themselves and the potential to be differentiated into various specialized cell types. Based on the potential for differentiation, stem cells are of two types, that is: embryonic stem cells (ESC) and adult stem cells. The prime importance of stem cells is their therapeutic applications in various diseases. ESC are pluripotent (they can differentiate into all cells of the body), and therefore can be used to treat any type of degenerative disease. However, adult stem cells are multipotent (can

differentiate to only a few types of cells), for example hematopoietic stem cells can be used for only a few diseases. There are ethical issues related to ESC and in case of multipotent stem cells, their therapeutic applications are limited due to their restricted differentiation potential.

18.10.2
Advantages of Stem Cells

Stem cells have several advantages over transformed/tumor cells. These include normal genetic composition, normal growth, easy genetic transformation, uniform cellular physiology, and pharmacology. Stem cells have emerged as important tools in human health and related research in the recent past. However, stem cell therapy alone may not produce miracles to repair tissue and organ damage occurring in diseased states, particularly in degenerative diseases. A judicious combination of stem cell therapy, HTS, and 3D tissue engineering is now almost mandatory in preclinical drug discovery process. Genomic technology has enabled the researchers to seek vital information or clues to genes, signaling pathways, and whole genomes. RNAi is an important tool for studying loss-of-function of select candidate genes in stem cells. The application of RNAi library screens at the genomic scale for stem cell phenotypes will certainly bring therapeutic opportunities [33].

18.10.3
Applications of Stem Cells in Screening and Drug Discovery

The recent development of induced pluripotent stem cells (iPSCs), that are created by reprogramming a skin fibroblast which can act like an embryonic stem, has revolutionized the applicability of stem cells. iPSCs created from a patient's specimen can represent the exact disease model (at the cellular level) for the specific disease that the patient has. Therefore, these disease models of iPSCs can be utilized in therapy as well as routine HTP drug screening and ADMET/Tox in drug development. They can be particularly useful for evaluation of cardio- and hepato-toxicities of drugs among others, which are of major clinical concern. Hematopoietic stem cells can be used for toxicity testing of drugs. Stem cells derived liver technologies are held in high esteem in regenerative medicine, for their possible role in liver transplant related research.

iPSCs can be included to develop midbrain dopaminergic neurons. These neurons are among the cells lost in Parkinson's disease (PD). Therefore, these cells can be utilized to develop drugs for PD. Multipotent adult stem cells are induced to differentiate into specific cell types with minimal variation for specific screening purpose and to develop novel assays. Hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and bone marrow-derived mesenchymal stem cells (BM-MSCs) are mostly used for this purpose. BM-MSCs can be differentiated into adipocytes, osteocytes, and chondrosites and an *in vitro* differentiation (IVD) screening can point the way to identifying lead molecules that are responsible for

differentiation switching. Therefore, modulators identified in such a way might be prospective therapeutics for diseases where the cell differentiation process is defective. Small molecules such as purmorphamine, are successful at producing osteoblasts from human mesenchymal stem cells. Similarly, the use of a small coded drug (LY-294002) in the mouse embryonic stem culture has resulted in the formation of pancreatic beta-cells [34]. Cancer stem cells may help in the anti-cancer drug discovery process.

18.11
Systems Biology in Drug Discovery

In recent decades, the biological sciences have seen a paradigm change in the understanding of life and associated processes, such as disease. This has happened largely due to the emergence of a variety of HTP data pertaining to organisms at sub-cellular and molecular levels. In turn, this has created the need to integrate the diverse data into useful information using sophisticated computing technologies. As such, a new science known as systems biology has emerged. Systems biology can be described as the science resulting from the integration of omics such as genomic, proteomic, transcriptomic, and metabonomic data using computational methodologies and mathematical modeling followed by simulation to mimic the natural biological systems *in silico* [35].

18.11.1
Industry Approaches to Systems Biology

In practice, the pharmaceutical industry uses three approaches to systems biology [36].

1) **Omics** (the bottom-up approach)**:** A traditional approach for target identification and validation for generating hypotheses and for experimental analysis by mining literature and HTP omics data. This approach can lead to targeted and personalization drug discovery.

2) ***In silico* modeling and simulation** (the top-down approach)**:** This method is employed for developing integrative computational/mathematical disease or organ modeling to gain a better insight into human physiology in health and disease conditions using integromics approaches and *in silico* simulation. This approach is helpful in target selection, ADMET, and in clinical trial design. Such models are available for asthma. diabetes, cardiovascular diseases, and so on.

3) **Complex cell systems:** An intermediate approach, for bridging omics, modeling, and simulation all together to generate profiling data (e.g., biologically multiplexed activity profiling) from high-throughput assays with incorporated biological complexity at various levels: multiple interacting pathways, multiple intercommunicating cell types, and multiple different environments. In this

highly complex approach, the need arises for data on cell responses to physiological stimuli as well as therapeutic agents.

All the three approaches are complimentary in nature and require integration so that they can achieve the real objective of systems biology.

The human genome project (HGP) can be seen as a major accomplishment for systems biology. Similarly, a large amount of data emanating largely from preclinical research has already been gathered and lies underutilized. Statistical techniques have almost failed to contribute to proper functional analysis of these data. Such types of analyses require a fundamental understanding of human biology – at the tissue, cellular, sub-cellular, and molecular levels.

The utility of system biology lies in the help it offers in the meaningful interpretation of data originating from molecular-level studies and simplification of the otherwise diverse molecular networks involved in the normal and abnormal physiological processes. Interrelated and overlapping cellular metabolic pathways or networks hold a key-step to creating system level representations of biological systems. Such networks are largely dependent on a wide range of general or organism-specific enzymes, cofactors/catalysts, enzyme coding genes, and an array of reactants or substrates. A number of databases are available to aid the understanding of systems biology towards drug discovery. Some of these databases are listed in Table 18.10.

18.11.2
Simulation Models

Several simulation models have been proposed and created for understandable simplification of human and microbial processes. Modeling the whole organs (e.g., heart for prediction of action potential, activation spread, and blood-flow calculations) and cellular processes (e.g., calcium wave in neuronal cell) and software such as E-CELL (e.g., for biochemical and genetic processes in *Mycoplasma genitalium*) have contributed – to a considerable extent – to the understanding of diverse activities taking place in organs and cells. Several hypotheses have been proposed to understand drug–protein binding in cardiac cells and the fate of xenobiotics in the human body. Induced abnormalities in the functioning of these models can also help in the understanding of the role of various factors in the progression of disorders and diseases such as hypertension, cancers, and Alzheimer disease. Indirectly, a chance is offered to predict abnormalities in the human body or a disease causing organism, and, hence, a valuable opportunity to validate therapeutic targets and outcomes [35]. A pipeline of systems biology in drug discovery and ADMET/Tox [37] is represented in Figure 18.6.

18.11.3
Applications of Systems Biology

Scientists have developed cell-based models using brain endothelium. Some of these models are used for medium to high throughput permeability screening and

Table 18.10 Databases for systems biology.

Database	Name	Website/webpage
BIND	Biomolecular Interaction Network Database	http://www.bind.ca/
BRENDA	BRaunschweig ENzyme DAtabase	http://www.brenda.unikoeln. de/
CSNDB	Cell Signaling Networks Database	http://geo.nihs.go.jp/csndb/
DIP	Database of Interacting Proteins	http://dip.doe-mbi.ucla.edu/
EMP	Enzymes and Metabolic Pathways	http://emp.mcs.anl.gov/
GenMAPP	Gene MicroArray Pathway Profiler	http://www.genmapp.org/
GeNet	Regulatory gene network	http://www.csa.ru/Inst/gorb_dep/inbios/genet/genet.htm
HPRD	Human Protein Reference Database	http://www.hprd.org/
KEGG	Kyoto Encyclopedia of Genes and Genomes	http://www.genome.ad.jp/kegg/
PKR	A database of protein kinase	http://www.sdsc.edu/kinases/
SPAD	The Signaling Pathway Database	http://www.grt.kyushuu.ac.jp/eny-doc/
STKE	Signal Transduction Knowledge Environment	http://www.stke.org/
UMBBD	The University of Minnesota biocatalysis/biodegradation database of xenobiotics	http://umbbd.ahc.umn.edu/

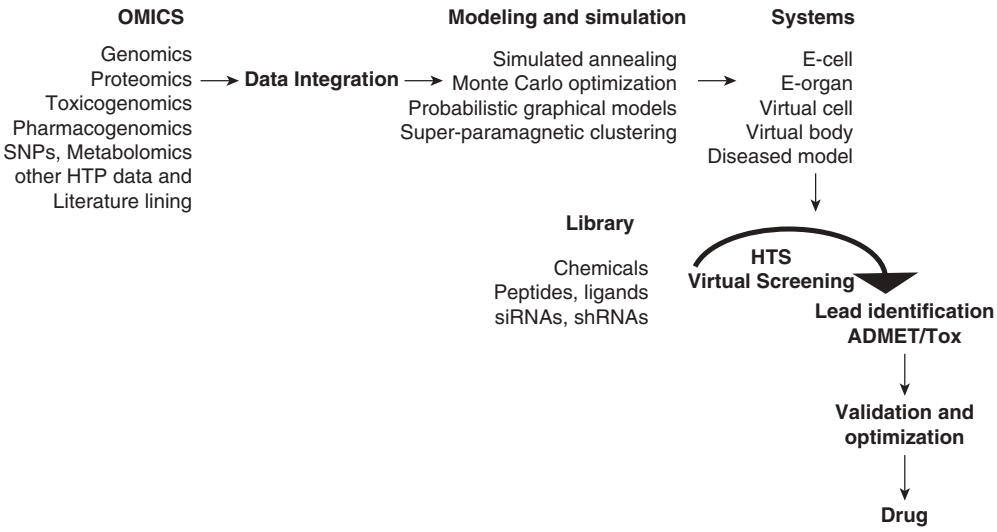

Figure 18.6 Steps used in systems biology for drug discovery. Based on Ekins *et al*. [37].

Table 18.11 Applications of systems biology in drug discovery.

Identification of surrogate markers for disease detection, or for monitoring of therapies. Complex biological systems are designed to identify many pharmacological targets relevant to inflammation.
Omics applied to cell systems designed to incorporate meaningful biological complexity. This biological complexity encodes useful information about drug and protein function, and suggests that it can be used for smarter, faster, cheaper industrial-scale functional screening and profiling.
Systems–ADMET/Tox methods might help to determine the toxicity of molecules and the genes that might be involved by reconstructing functional networks and to screen toxic compound.

toxicology. Such models based on a real blood–brain barrier, cellular anatomy, and physiology are advantageous in the sense that they can reveal and incorporate previously undiscovered properties, such as new transporters, metabolic enzymes and modulation, and also allow extrapolation of physiologically-based pharmacokinetics (PBPK) from animal models to humans.

Systems biology has helped in developing drugs for treating neuropathological lesions, such as neurofibrillary tangles (NFTs), and beta-amyloid senile plaques (SPs), found in the brains of Alzheimer's disease (AD) patients. These anti-aggregation drugs are devoid of typical secondary effects of non-steriodal anti-inflammatory drugs. Targeted small-molecule therapies (such as monoclonal antibodies and tyrosine kinase inhibitors) have proven their worth against several tumor models including metastatic bladder cancer.

Unbiased mutation analyses of human breast, colon, brain, and pancreas tumors have shown that a few frequently mutated genes are outnumbered by a multitude of infrequently mutated genes. Limited central molecular pathways are emerging from infrequently mutated genes. A systems biology approach is required for better identification and proper definition of such pathways. Downstream genetic analyses, scalable prediction, and experimental determination methods for mutant allele phenotypes and pathways are a must for improved understanding of cancer as well as future drug discovery. A list of applications is given in Table 18.11.

18.12 Conclusion

In this chapter we have discussed various approaches currently available for HTS, except for methods of automation, robotics, and microfluidics. Among the several techniques discussed, chemical and cell microarrays are of high potential for uHTS. Use of iPSC in screenings are highly recommended to obtain better screens

compared with any other *in vitro* systems. Similarly, integrative genomics approaches are also beneficial in terms of target identification and toxicogenomics for ADMET/Tox prediction. RNAi screening is also powerful for identifying drug targets as well as the candidate drug. *In silico* pharmacology and omics based systems biology have generated *in silico* virtual cells, and disease models are promising approaches with uHTS for rapid identification of targets as well as the lead compounds and the subsequent predictive ADNET in drug discovery.

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19
Metabolic Engineering of Medicinal Plants and Microorganisms for the Production of Natural Products

Oktavia Hendrawati, Herman J. Woerdenbag, Jacques Hille, and Oliver Kayser

19.1
Introduction

Plants are a rich source of bioactive compounds. Compounds of plant origin are used as drugs and precursors of semisynthetic drugs, and may provide valuable leads for novel drug design. Furthermore, plant extracts have been and are still used to prevent, and to treat, a number of diseases although the mechanism of action is frequently unknown. Finally, there is a global demand for "greener" manufacturing processes, which are economically attractive, to be available in a timely manner [1].

Worldwide more than 50 000 plant species are used for medicinal purposes [2]. The World Health Organization (WHO) estimated that more than 80% of the population in the world in less developed countries depend primarily on herbal medicine for basic healthcare needs [3]. The current herbal drug market has reached a level of US$62 billion, which is forecast to grow to US$5 trillion in 2050 [4]. The world market for herbal medicines shows an annual growth of 5–15% [5]. In the United Kingdom, more than 25% of the population use herbal medicines on a regular basis [3].

In the past 30 years, more than 25% of the new drug entities approved were based on a molecule of plant origin and about one third of the approximately 980 new pharmaceuticals originated from, or were inspired by, natural products [6, 7]. About 50% of the top-selling chemicals are derived from knowledge of plant secondary metabolism [7]. About 40% of the pharmaceuticals in the United States and Europe use plants as raw source material [8].

Besides plants and plant extracts, pure compounds derived from plants play an important role in contemporary pharmacy and medicine. Typical plant compound (Table 19.1 and Figure 19.1) commonly used drugs are terpenoids, alkaloids, polyketides, phenylpropanoids, and flavonoids. For examples, morphine and codeine from *Papaver somniferum* L., artemisinin from *Artemisia annua* L., paclitaxel from *Taxus brevifolia* Nutt, genistein from *Glycine max* L. (Merr.), scopolamine from *Dubosia species*, campothecin from *Camptotheca acuminata* Decne, and podophyllotoxin from *Podophyllum* species.

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Table 19.1 Overview of the production of the plant-derived and medicinally relevant compounds with their metabolic engineering strategies.

Compound	Activity/ function	Plants source	Demand (tons/yr)	Current production	Price US$/kg	Reason for combinatorial biosynthesis	Ref.
Dihydroartemisinic acid (Terpenoid)	Anti-malarial	*Artemisia annua* (0.01–0.86% in aerial parts)	120	*E. coli*	1000	Availability in nature is limited Chemical synthesis–economically not feasible	[9]
Paclitaxel (Terpenoid)	Antitumor	Taxus species	0.3	Plant cell culture Idem ESCA genetics (USA); Phyton Catalytic (USA/ Germany); Nippon Oil (Japan); Samyang Genex (Korea)	28000	Low production in nature Slowly growing tree The bark is non renewable–harvesting the bark results in the death of the tree Chemical synthesis–economically not feasible	[10–12]
Vanillin (phenylpropanoid)	Flavor	*Vanilla planifolia*	>10000	Chemical synthesis	12 (synthetic) 30–120 (vanilla pods)	Green manufacturing	[13]
Genistein (flavonoid)	Fiber	*Gossypium hirsutum*	36000	Isolation from plants	Unknown?	Cottonseed free from gossypol for protein source of human diet	

Podophyllotoxin (Lignan)	Antitumor	*Podophyllum* species (4.3% DW)	–	Plant cell culture (Nippon oil – Japan) From which starting compound/	1490	Endangered species Chemical synthesis – economically not feasible	[10, 14]
Scopolamine (alkaloid)	Anticho-linergic	*Duboisia* species (1.2 –2.4% in leaves)	–	Transgenic hairy root culture of *Hyoscyamus niger* (HnH6H and NtPMT) – 411.2 mg/l *Duboisia* sp. Sumitomo Chemical Industries (Japan)	Unknown?	Multiple chiral centers – chemical synthesis – economically not feasible	[10, 15, 16]
Morphine (alkaloid)	Analgesic	*Papaver* (20% DW in latex; 1.23–2.45 % DW whole plant extracts)	27.8	Isolation from plants	Unknown?	Multiple chiral centers – chemical synthesis – economically not feasible	[12, 17]
Vincristin (alkaloid)	Antitumor	*Catharanthus roseus* (0.0003 % DW in whole plant)	0.3	Isolation of plants	1400	Multiple chiral centers – chemical synthesis – economically not feasible Higher yield	[18]

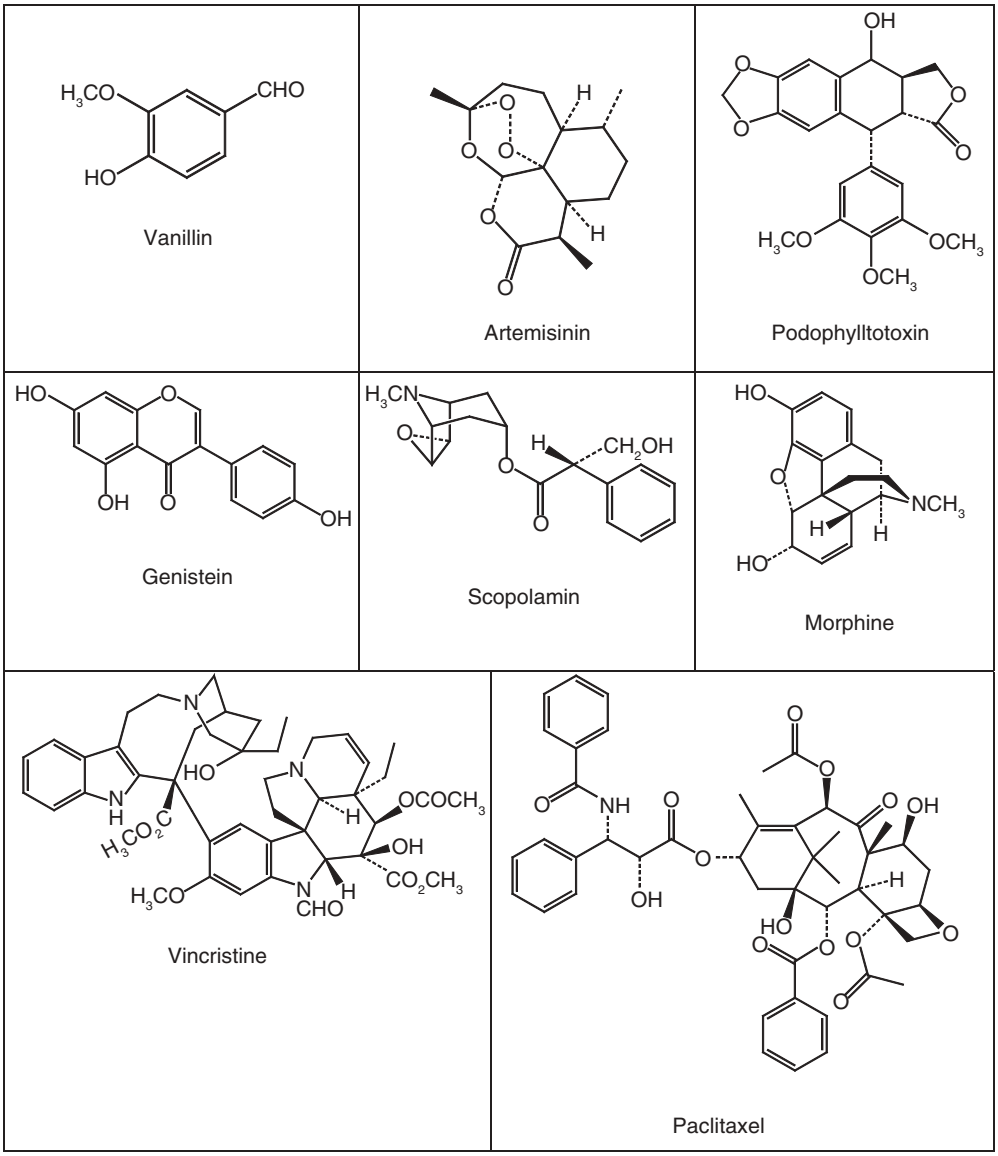

Figure 19.1 Chemical structures of the important plant- derived compounds discussed in this chapter.

Despite the use of and demand for plant-derived compounds, their availability is a major bottleneck in supplying the pharmaceutical needs. Most of these compounds are secondary metabolites, which are present only in low amounts from natural sources. Most medicinal plants are not cultivated, but are collected from the wild and some of them are slow growing. Because of intensive collection from the wild, the current extinction rate of medicinally used plants is estimated

to be 100–1000 times higher than for other plants. As many as 15 000 out of 50 000–70 000 medicinal plant species are now threatened with extinction [19]. Currently between 4000 and 10 000 medicinal plants are on the endangered species list and this number is expected to increase in the future [20].

There are a number of limitations to obtaining plant-derived compounds. They may be restricted to one species or genus, and might be formed only during a particular stage of growth or development or under specific seasonal, stress or nutrient availability conditions [21, 22]. Chemists have also been challenged to synthesize plant-derived compounds via organic chemistry. This is often hampered by the chemical complexity, specific stereochemistry, and the economic feasibility.

Metabolic engineering may offer prospects to overcome the lack of availability of such compounds, through the advancement of molecular biology techniques, including cloning, recombinant DNA, and knowledge of the plant biosynthetic pathways. In this chapter we discuss the major strategies in plant metabolic engineering and their principle approaches and its prospects and limitations for the production of drugs and fine chemicals. Case studies are used as illustrations.

19.2
The Plant as a Source of Natural Products

19.2.1
Plant Cell Cultures

In principle, whole plants, plant organs, and even single cells can be used for the production of natural products. Plant cell culturing was initiated in the 1930s [23] and could offer alternatives to improving the production of the secondary metabolites, as natural harvesting is sometimes bulky and not feasible from an economic point of view. The main advantages of plant cell culturing are easy up-scaling, simple purification schemes due to product secretion, environmental friendliness, and amenability to strict control with regards to meeting FDA manufacturing standards [24]. Plant cell cultures are also not subject to changes in environmental conditions, thus the production of the desired compounds could take place at any location and in any season [25].

In 1959, plant cells were first cultured in a 10 l glass or steel bioreactor [26] and later, in 1977, the first larger scale 20 l stirred tank bioreactor of *N. tabacum* cells was reported [27]. Today, undifferentiated plant cell suspension cultures can easily be scaled up for commercial production purposes, but the productivity is often hampered by the fact that the compounds of interest are not produced in the undifferentiated cells. Currently 14 plant cell cultures have been commercialized for secondary metabolites production for pharmaceutical, food, and cosmetic purposes [10, 28]. Examples are scopolamine from *Dubosia* sp. (Sumitomo Chemical Industries, Japan), ginsenosides from *Panax ginseng* (Nitti Denko, Japan), and paclitaxel from *Taxus* sp. (Phyton Biotech, USA and Samyang Genex, Korea) [10].

One success story of a plant cell culture produced drug is paclitaxel (Taxol® Bristol Myers Squibb). While the plant itself, *Taxus brefolia*, only produces paclitaxel at approximately 0.01% of the dry weight of the bark [29], the plant cell suspension culture has been shown to produce steadily in the region of 140–295 mg/l, reaching 295 mg/l at a maximum under two-stage culture with the elicitation of methyl jasmonate and high density conditions [11].

The main constraints of using plant cell cultures for the production of secondary metabolites include slow growth of plant cells in comparison with microorganisms, no accumulation of the desired metabolites in undifferentiated cultures, compartmentalization of the production of secondary metabolites, low and variable yields, and the decrease of metabolite accumulation as the cell line ages [24, 30–32]. Differentiated cells produce the same product as the plant itself, but in large-scale production, when aiming at an economically attractive route, the yield remains a bottleneck, especially for slow-growing plants. A variety of approaches, such as the growth of differentiated cells (root and shoot culture) and the induction of pathways by elicitors have had limited success so far [22]. The plant production of secondary metabolites is controlled in a tissue-specific manner, thus the dedifferentiation results in loss of production capacity [22] and undifferentiated cell cultures that are genetically unstable, and often lose, partially or totally, their ability to produce secondary products [33, 34].

For example, artimisinin, a potent antimalarial drug, was not found in cell suspension cultures of *Artemisia annua*, while considerable amounts were detected in shoot cultures [35, 36]. Deoxypodophylltoxin, the main lignan in *Anthriscus sylvestris* was also detected in trace amounts in callus and cell suspension cultures [37].

19.2.2 Transgenic Plants

In 1907, Smith and Towsend reported on the cause of crown gall disease of paris daisy (*Chysanthemum frustescens*) by *Bacterium tumefaciens*. Later on the bacterium was classified as *Phytomonas tumefaciens*, and finally as *Agrobacterium tumefaciens*, a gram-negative soil dwelling bacterium [38]. At the end of the 1970s, it was reported that the T-DNA of this microorganism was covalently integrated into the plant nuclear genome in tobacco teratoma cell lines [39, 40]. This has led to many studies to date. Since 1994, transgenic technology has been used and commercialized to produce new crop products with herbicide tolerance, insect resistance, virus resistance, and improved post-harvest quality [41]. Transgenic approaches can be applied to target a rate-limiting step through manipulation of the expression of individual structural genes [5].

There are two transformation approaches commonly used to produce recombinant pharmaceuticals in plants. The first is to subject plants to *Agrobacterium*-mediated transformation, particle bombardment, electroporation, and then secondly to infect plants with recombinant viruses that express transgenes during their replication in the host [42–45]. Genetic transformation of medicinal plants

is usually carried out using *Agrobacterium rhizogenes* to obtain hairy root cultures, or using *Agrobacterium tumefaciens* to produce transformed cells that can be maintained in cell cultures or can be regenerated as whole plants [2].

In principle, the wounded plant tissues, caused by insect or mechanical damage, produce phenolic compounds, which attract *Agrobacterium* by chemotaxis to infect the plant cell on the wounded site and allow the transfer of T-DNA from *Agrobacterium* into the plant nuclear chromosome. The T-DNA contains genes that encode enzymes directing the plant cells to produce peculiar amino acids called opines, and express genes to direct the plant cells to produce plant hormones such as cytokinin and auxin. Opines are used as primary sources of carbon and nitrogen by the cohabiting bacteria, and cytokinins and auxin promote cell division and tumor formation, providing a steadily increasing supply of nutrients for the bacteria [46]. The infection from *A. rhizogenes* in the wounded site will cause a number of small roots to protrude as fine hair growth and proliferate rapidly, causing hairy roots [47]. T-DNA carries the *rol* and *aux* genes. The *rol* genes are responsible for the phenotype of hairy roots and the *aux* genes are involved in root induction by directing auxin synthesis [48].

The major drawbacks of this approach are the unstable gene expression, instability of cell lines that often lose their capacity to produce target molecules over time, and high cost of bioreactors [2]. For example, the alkaloid accumulation in transgenic *Catharanthus roseus* cell cultures quickly returns to the level of the non-transgenic ones [49].

The capacity to regenerate whole plants from single cells without changing the genetic features of the cells and the gene transfer mechanism via *Agrobacterium tumefaciens* facilitates efforts to engineer secondary metabolic pathways [2, 50]. The constraint is the subsequent regeneration of transgenic plants, which remains problematic and time consuming. Unwanted somaclonal variation may be introduced through the tissue culture regeneration system in some cases [51].

Plants have been and are still used as hosts to produce genuine and recombinant proteins and enzymes of industrial and pharmacological value [52]. More than 200 novel antibody-based potential products are in clinical trials worldwide, and the market demand will constrain the capabilities of existing production systems [53]. One would expect the biopharmaceuticals from transgenic plants to be safer and less expensive than those from animal-based sources, which have the potential for contamination with human pathogens [42].

Enhanced productivity of valuable secondary plant metabolites can also be achieved via hairy root cultures [25]. Hairy root cultures can be obtained from transformed root cultures using *Agrobacterium rhizogenes*, a gram-negative soil dwelling bacteria. The term "hairy root" was introduced in 1900 [54] and the first transformation of higher plants using *A. rhizogenes* was achieved in 1973 [55].

Hairy root cultures are genetically stable, capable of unlimited growth without additional hormones, and have an increased capacity for secondary metabolites formation and accumulation [24]. Genetically transformed root cultures have been shown to produce levels of secondary metabolites comparable to those of an intact plant. It has further been shown that hairy root cultures can accumulate

secondary metabolites that normally occur only in the aerial part of the plant. An example is artemisinin in *Artemisia annua* [56]. The transformation of *Artemisia annua* using *A. rhizogenes* carrying the cDNA encoding FDS (farnesyl diphosphate synthase) under a 35S CaMV (cauliflower mosaic virus) promoter yielded fourfold higher artemisinin accumulation compared with untreated control plants [57]. The transformation of *Atropa belladonna* with H6H (hyoscyamine 6β-hydroxylase) from *Hyoscyamus niger*, under the control of the 35S CaMV promoter in a binary plasmid via *A. rhizogenes* mediated transformation, resulted in an accumulation of scopolamine up to fivefold higher compared with untreated control plants [58]. These examples indeed show that hairy root cultures are able to produce the same compounds as in the plant itself. Other examples of hairy root cultures producing secondary metabolites can be found in the work of Srivasta and Srivasta [25].

Another advantage is that transformed roots are able to regenerate whole viable plants, maintain their genetic regeneration, and in addition produce secondary metabolites that are not present in the parent plant [59]. Furthermore, they show fast auxin-independent growth and are suitable for adaptation to bioreactor systems [25]. In addition to production of secondary metabolites, hairy roots are also used to produce human therapeutic proteins, vaccines, and diagnostic monoclonal antibodies. For example, hairy root cultures of potato carrying pBSHER containing the gene for hepatitis B surface antigen (HBsAg) expressed higher levels of HBsAg compared with control cultures [58].

Despite the potentials discussed, challenges during large-scale cultivation, such as unusual rheological properties of hairy root cultures, have to be addressed. Non-optimal fermentation made it necessary to investigate novel approaches to apply hairy root cultures to fermentor [25] and process design [24].

In conclusion, the use of hairy roots as factories for the production of novel plant-based bioactive compounds, vaccines, antibodies, and other therapeutic proteins offers good prospects for feasibility of commercial production.

19.3 Optimizing Biochemical Pathways

19.3.1 Strategies and Goals of Metabolic Engineering

Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to produce new compounds in an organism, to improve the production of existing compounds, or to mediate the degradation of compounds [60]. Metabolic engineering of plants offers interesting perspectives to improve the productivity of the plant as a cell factory. This approach may create new opportunities in agriculture, environmental applications, production of chemicals, and medicines [22, 51]. The main goal of metabolic engineering in general is to produce the desired natural products in a sustainable and economically attractive

Table 19.2 Important goals of metabolic engineering in general.

Physiological understanding	
Novel compounds	To yield a novel compound in a plant and other precursors by introducing the appropriate heterologous genes [2] To give a new trait (color, taste, smell) to food, flowers or ornamental plants [22]
To increase	To improve production of a desired compound or enzyme in a cell culture and also in the plant itself To achieve production in a related plant species or even in microorganisms To improve agronomic traits, such as resistance of a plant to various stresses, pests, diseases, and to increase the seed yield of a crop plant through the expression of certain metabolites [22, 61]
To decrease	To decrease levels of noxious or antinutritional factors in food and feed crops [2]
Regulatory understanding	
To improve our understanding of pathways regulation and flux when some of the intermediate pathways increase in abundance beyond their usual concentration range [61]	

way [24]. Several more specific goals of metabolic engineering are listed in Table 19.2.

The production level of a compound of interest that is present in trace amounts can be enhanced through the following: by increasing the flux of precursors; by blocking a competitive (parallel) pathway using the same precursor or intermediate compound; by introducing new routes of metabolism; by overcoming rate-limiting steps; by reducing flux through enhancing competing pathways; by over-expressing regulatory genes or transcription factors that induce the pathway; by inhibiting or limiting catabolism of the molecule; or by increasing the number of specialized cells producing the compound [2, 62].

Some of the scientific challenges comprise a better understanding of the partly known secondary metabolite biosynthetic pathways on a genetic level, the generation of heterologous organisms with desirable biosynthetic characteristics, and optimized tools for pathway manipulation such as vectors, synthetic genes, and regulating elements [62]. Moreover, biosynthetic pathways are often species-specific. Features such as cell compartmentalization, tissue differentiation, and multi-enzyme complexes, will make the outcomes unpredictable.

19.3.2 Metabolic Pathways of Interest

A comprehensive understanding of different metabolic pathways and their genetic control is essential for the application of a genomics approach to the improvement of medicinal plants [5]. In this chapter we confine the discussion to the metabolic

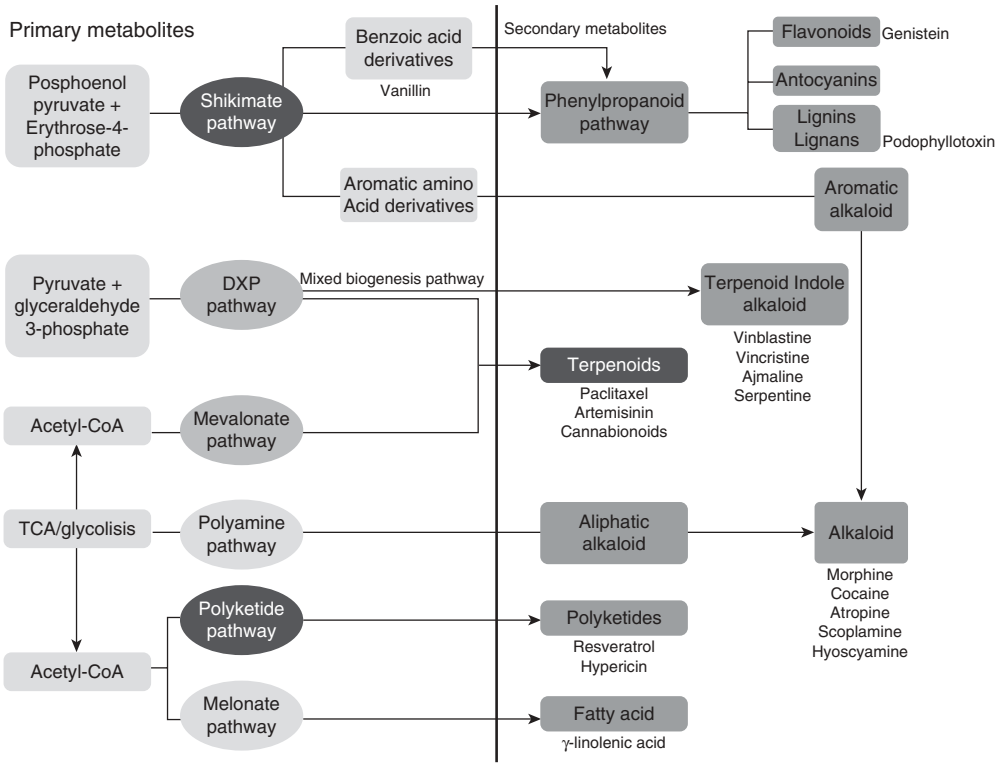

Figure 19.2 Simplified biosynthetic pathways of primary and secondary metabolism in plants (adapted from reference [5]).

pathways of medicinal compounds, among other pathways of interest (Figure 19.2).

Most secondary metabolites are derived from the shikimate, terpenoid, and polyketide pathways. The shikimate pathway is the major source of phenylpropanoids and aromatic compounds [63, 64], such as flavonoids, coumarins, isoquinoline and indole alkaloids, lignans, lignins, and anthocyanins.

The terpenoid pathway leads to more than one-third of all known secondary metabolites, including mono-, seseui-, di-, tri-, and tetraterpenes. It is also the source of the C5-building block (isoprene) in many skeletons from other biosynthetic origins, such as anthraquinones, naphthoquinones, cannabinoids, furanocoumarines, and terpenoid indole alkaloids [65].

The polyketide pathway is a rich source of bioactive molecules such as anthranoids. It is attractive as a model for metabolic engineering studies because the complex structure results from simple C2 units combined in different ways and the modular construction of enzymatic catalysts allows control of enzyme structure [62].

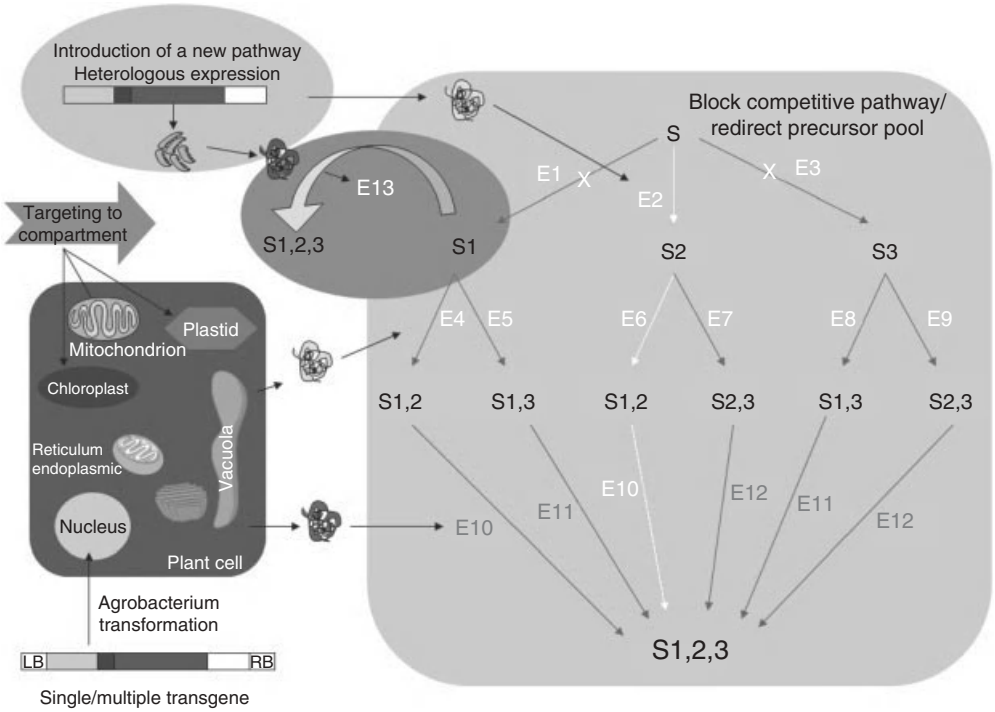

Figure 19.3 Schematic biosynthetic network: S, basic skeleton to which functional group 1, 2, and 3 are added; E1–E13, enzymes that catalyze biosynthetic steps (adapted from reference [66]).

The complexity of a metabolic pathway and some strategies to engineer the pathway are illustrated in Figure 19.3. It is supposed that a basic skeleton S (substrate) is present in which three functional groups can be introduced. If highly specific enzymes would catalyze all steps, 12 enzymes (E) (E1 to E12) could be involved in the formation of three different products with one functional group, three different products with two functional groups, and one final product with all three functional groups. If the specificity of the substrate is broad, it is likely that three different enzymes will be adequate. Heterologous genes (E13) can also be introduced into the plant metabolic pathway, which could catalyze all three functions of the substrate (S1) into product "S1,2,3."

19.3.3
Synthetic Biology

Synthetic biology is a rapidly growing multidisciplinary field among biologists, chemists, physicists, engineers, and mathematicians [67]. It is defined as the design and construction or engineering driven building of new or artificial

biological components or increasingly complex biological entities, such as enzymes, genetic circuits, and cells, or the redesign of existing biological systems for novel applications [68, 69]. The goals are to build complex systems into specific hosts [69], to engineer synthetic organisms [69, 70], to improve understanding of biological systems, and to produce bio-orthogonal systems with new functions [67].

The distinguishing element that differentiates synthetic biology from traditional metabolic engineering is the focus on the design and technological construction of core components (the enzyme, genetic circuit, metabolic pathway, etc., parts), which can be modeled, understood, and tuned to meet specific criteria. The assembly of these components into integrated systems, which enables a systematic forward-engineering of (or parts of) biological systems for improved and novel applications, is a second key issue of synthetic biology [68, 69].

Synthetic biology is categorized into two broad classes. One uses non-natural molecules to reproduce emergent behaviors from natural biology, with the goal of creating artificial life. The other seeks interchangeable parts from natural biology to assemble into systems that function unnaturally [71].

The knowledge of these tools and methods may enable synthetic biologists to design, fabricate, integrate, test, and construct artificial biological systems that originate from the insights discovered by experimental biologists and their holistic perspectives [70].

Although synthetic biology offers promising applications for novel compounds and novel approaches, the success so far is rather limited as it is quite a young science. It is further hindered by the fact that the production processes of the most effective biological components (promoters, gene, plasmids, etc.) have been patented. Royalty payments increase the costs, which make it economically no longer attractive [68]. Another drawback is that living systems are highly complex. Currently, biologists lack information about how the integration of living systems works [70]. The success of synthetic biology depends on its capacity to surpass traditional engineering. It should blend the best features of natural systems with artificial designs that are extensible, comprehensible, user-friendly, and implement stated specifications to fulfill user goals [70].

19.4 Metabolic Engineering Strategies and Techniques in Medicinal Plant Biotechnology

The major metabolic engineering strategies and techniques applied in medicinal plant biotechnology are discussed in detail in this section (see also Figure 19.1). They include up- or down-regulating of pathways, redirecting common precursors, targeting metabolites to specific cellular compartments, and creating storage of overproducing secondary metabolites. Examples of techniques used and their application are given in Table 19.3.

Table 19.3 Techniques used in metabolic engineering and their applications.

Technique	Genes/enzymes	Plant species	Target compounds/ goals	Ref.
Single transgene (biotransformation)	Codeinone reductase (COR)	*Papaver somniferum*	Increase of morphine	[72]
	CYP80B3	*Papaver somniferum*	Increase of morphine alkaloid	[73]
Multiple transgene insertions	10 genes	*Oryza sativa*	Resistant to multiple pathogens, insects, and herbicides, and antibiotic marker free	[74]
Polycistronic vectors (artificial chromosomes)	Cholera toxin β-subunit (*CTB gene*)	*Nicotiana tabacum*	Production of cholera toxin β-subunit	[75, 76]
Transcription factor	ORCA	*Catharanthus roseus*	Increase of terpenoid indole alkaloid	[77]
Sense/antisense suppression	Antisense *CYP80B3*	*Papaver somniferum*	Decrease of benzylisoquinoline alkaloids up to 84% of total alkaloid	[73]
Virus inducing gene silencing (VIGS)	Phytoene desaturase (PapsPDS)	*Papaver somniferum*	Reduction of transcript level of endogenous PapsPDS and photobleach phenotype; and assessing gene function	[78]
	Putrescine *N*-methyltransferase (PMT)	*Nicotiana tabacum*	Reduction of nicotine	[79]
Repressor silencing	Hydrolases, polygalacturonase and pectinesterase	*Solanum lycopersicum*	Altering fruit ripening	[80]
RNAi	Berberine bridge enzyme (BBE)	*Eschscholzia californica*	Accumulation of (s)-reticuline	[81]
	Salutaridinol 7-*O*-acetyltransferase (SalAT)	*Papaver somniferum*	Accumulation of salutaridinol	[82]
	δ-Cadiene synthase	*Gossypium hirsutum*	Reduction of gossypol	[83]

19.4.1 Upregulating of Pathways (Overexpression)

Transcription factors (in multi-enzyme pathways) are regulatory proteins that can be used to regulate multiple steps or even to modulate an entire pathway in order to produce a significant yield of a desired product through sequence-specific DNA binding and protein–protein interactions [61, 84]. They can act as activators or repressors of gene expression, which mediate, respectively, an increase or a decrease in the accumulation of messenger RNA [85]. They are also able to regulate steps for which the enzymes are unknown [84]. Using this approach it is often necessary to increase precursor availability and to understand the coordination of multiple branches or sections of the metabolic pathway. The use of transcription factors requires integrated information from genomics, transcriptomics, proteomics, and metabolomics [24].

Several transcription factors have now been identified. A relevant example is transcription factor MYB12, a flavonol-specific regulator of the phenylpropanoid biosynthesis in developing seedlings. Total flavanol content of the seed was increased when MYB12 was expressed in developing *Arabidopsis thaliana* seedlings. The expression of the genes encoding the four flavonoid biosynthetic enzymes was upregulated, increasing the flux through the flavanone pathway [86]. Three transcription factors – ORCA1, ORCA2, and ORCA3 (octadecanoid-responsive *Catharanthus* AP2-domain) – have been identified in the medicinal plant *Catharanthus roseus* and are involved in biosynthesis of terpenoid indole alkaloids. They belong to the AP2/ERF transcription factor family. The overexpression of ORCA3 in *C. roseus* cultured cells increased the expression of the terpenoid indole alkaloid biosynthesis genes TDC (tryptophan decarboxylase), STR, CPR (cytochrome P450 reductase), D4H (desacetoxyvindoline 4-hydroxylase) [87] and SLS (secologanin synthase) [88]. Moreover, ORCA3 also regulated two genes encoding enzymes (ASα, α-subunit of anthranilate synthase; and DXS, D-1-deoxyxylulose 5-phosphate synthase) in the primary metabolism leading to terpenoid indole alkaloid precursor formation [77].

Next to frequently studied pathways, such as the phenylpropanoid biosynthesis, and the well-characterized MYB transcription factor family, finding a transcription factor that acts on specific pathway genes is very challenging [61]. Transcription factors are difficult to identify in non-model species [84]. An alternative is to design synthetic transcription factors, which target one or more genes of choice [89]. As an example, we mention the design of a synthetic zinc finger protein transcription factor (ZFP-TF) targeted to a methylphytylbenzoquinol methyltransferase (VTE3). The expression of the ZFP-TF increased the activity of native γ-methyltransferase (VTE4) and the α-tocopherol content in *Arabidopsis thaliana* seeds [90]. Transcription factors, natural or synthetic, are used only if the pathway is endogenous to the plant [61].

19.4.2
Redirecting Common Precursors

Many branching points are found in a biosynthetic pathway where enzymes compete for a common precursor. Increasing and redirecting the precursor pool towards the biosynthesis of the target compounds can theoretically increase their production. This can be achieved by blocking the competitive pathway or by inducing overexpression of genes in the precursor pathway [65].

For example, overexpressing the gene that encodes taxadiene synthase in the tomato, a precursor in the carotenoid pathway, increased the production of taxadiene in a tomato mutant. The production was 660–20 000 times higher than in *Arabidopsis thaliana* [91]. By overexpressing genes in precursor pathways in both peppermint [92] and lavender [93] an increase in the monoterpene fraction of the essential oils was found.

However, owing to tight regulation of metabolite accumulation, this approach may also have a limited impact on target products. The increase of intermediate precursor resulted in a limited accumulation of alkaloid target product in *C. roseus* [94]. In this case, the direct overexpression of related genes in the alkaloid pathway was shown to be more effective at increasing the alkaloid accumulation in *C. roseus* [95]. The effect seems to be temporary. It might be due to a result of the same factors, which induce variability in non-transgenic plants [24].

19.4.3
Targeting Metabolites to Specific Plant Cell Compartments

Targeting gene expression to a specific cellular compartment or organelle that contains the precursors could increase the level of the target compounds. Plants are able to express the transgene with organelle targeting signals from the nuclear DNA and the resulting recombinant proteins will be targeted to the appropriate organelles. Specific amino acid sequences required for targeting of proteins to particular organelles and for retention of proteins in organelles have been identified [51]. Thus targeting the enzymes to the compartment of the substrate seems feasible. However, the products formed may cause toxicity problems in a compartment other than the usual one [65].

Using this approach, overexpression of a target gene, either in plastids or the cytosol, allows transport of a sufficient pool of common precursors in the right direction. This leads to a more than 1000-fold increase in concentration of the sesquiterpenes patchouli alcohol and amorpha-4,10-diene, and a 10–30-fold increase of the monoterpene limonene in transgenic tobacco plants compared with untreated control plants [96].

19.4.4
Creation of Storage of Overproduced Secondary Metabolites

A plant may have the capacity to produce secondary metabolites but sometimes it lacks a proper subcellular compartment to store them [97]. Modifications to metabolic storage of products or secondary metabolic pathways have been generally more successful than manipulations of primary and intermediary metabolism [98, 99]. The genes controlling the formation of subcellular compartments have been isolated and characterized in plants [100].

For example, expressing of the *Or* gene encoding a DnaJ cysteine-rich domain-containing protein led to the formation of large membranous chromoplasts in cauliflower curd cells [100]. The expression of the same gene in transgenic potato under the control of a potato granule-bound starch synthase promoter increased the total carotenoid up to sixfold compared with the original, non-transgenic plants [100].

19.4.5
Downregulating of Pathways (Silencing)

The production of a certain compound can be reduced by decreasing the flux towards that product by reducing the level of enzyme in the pathway, increasing catabolism, and increasing flux into competitive pathways [2, 66].

A particular step in the pathway that leads to undesirable compounds can be blocked by suppressing genes that upregulate the pathway or by increasing their catabolism [2]. Antisense, co-supression, and RNA interference (RNAi) methods are used to block, to reduce or to eliminate levels of undesirable compounds. This so called silencing can be targeted to specific plant tissues and organs with minimal interference of the normal plant life cycle, by using tissue or organ-specific RNAi vectors. Mutants with the RNAi effect have been shown to be stable for at least 20 generations [101].

19.5
Challenges in Plant Metabolic Engineering

19.5.1
Unexplored Regulation of Secondary Metabolism

The lack of complete understanding of the regulation of secondary metabolism, especially in the complex alkaloid biosynthesis, hinders the determination of an effective metabolic engineering strategy to achieve a specific production phenotype. The complexities comprise a pathway compartmentalization, the existence of multiple alkaloid biosynthetic pathways and the regulatory control mechanisms [102]. To date, only four biosynthetic routes of alkaloid subclasses have been partially characterized, in particular the benzylisoquinoline, monoterpenoid indole,

purine, and tropane alkaloids [102]. This could be ascribed to the limited genome/cDNA sequence information of medicinal plants [5].

19.5.2 Pathways Are Often Species Specific

A number of genes encoding enzymes, which control key steps of secondary metabolic pathways, have been cloned from a number of a medicinal plant species using classical and modern genomics approaches [5]. However, this represents a small fraction of a total of about 1000 plant genes known to function in secondary metabolism [103]. The progress in isolating genes involved in secondary metabolism is limited due to species specificity, the difficulty in producing large numbers of mutants, their intermediate precursor availability, their analysis, and to the instability of secondary metabolites caused by environmental factors [104]. The major bottleneck for secondary metabolism will remain, as per definition, species specific. Only early parts of the pathways are common to most plants, for example in the flavonoid and terpenoid biosynthetic pathways, thus homology between genes can be used for strategies to clone genes from other plants [104]. The genes encoding enzymes involved in the more specific "decoration" of the basic skeletons can only be studied at the level of the producing plant [105].

19.5.3 Cell Compartmentalization and Tissue Differentiation

Plant cells have a complicated intercellular organization with metabolite flow between compartments highly regulated and orchestrated depending on the biosynthetic needs of the plants [106]. They have numerous organelles of which some are not found in mammalian or yeast cells [51]. The highly compartmentalized nature of enzymes, substrate precursors, and metabolic intermediates also contributes to the complexity of secondary metabolites production, which is regulated at a different level [2].

Plants also have numerous specialized and differentiated organs in which physiological processes and gene expression may differ substantially. Next to organelles, the compartmentalization of secondary metabolite pathways also occurs at the subcellular level [107]. Furthermore, temporal and developmental processes can profoundly influence whether and when a transgene is active. Thus, the issues of compartmentalization complicate the targeting gene strategy. Moreover, if the engineered plants are going to be propagated as crops, environmental effects may add to the level of variability and unpredictability, which is not encountered in a fermentor based system [51].

There is increasing evidence that intra- and intercellular translocation of enzymes are key elements in secondary metabolite production. Localization of enzymes to diverse cellular compartments showed the importance of protein targeting in the assembly of the alkaloid pathway [2]. Alkaloids are generally stored in specific types of compartments due to their cytotoxicity and probable role in

plant defense responses. The subcellular compartmentalization of alkaloid pathway enzymes is extremely diverse and complex because of the cell type-specific localization of the gene transcripts, enzymes, and metabolites [108].

Other examples are phenylpropanoid derivatives. Their biosynthesis occurs in the cytoplasm, but the precursors are derived from metabolism in other organelles, including the chloroplasts and mitochondria [106].

19.5.4
Unpredicted or Unexpected Outcome

The use of metabolic engineering approaches in medicinal plant species to improve the yield of pharmaceutical products has been, and still is, a challenge. There are several limitations such as gene silencing, unpredictable results due to complex network genes, and no increase in concentration of desirable metabolites up to the level of commercialization [5]. Techniques used to introduce new genes into plants also do not allow a prediction about the site of integration and the level of gene expression, even when a strong promoter is used [65].

Single-enzyme perturbations of alkaloid pathways resulted in unexpected metabolic consequences, suggesting the existence of key rate-limiting steps, potential multi-enzyme complexes, or unsuspected compartmentalization [108]. Overexpression of COR1 (codeinone reductase), the final enzyme in morphine biosynthesis, increased the morphine and codeine contents in transgenic poppy [109]. However, thebaine, an upstream metabolite in the 23 branch pathways, was also unexpectedly significantly increased. The knock down of COR1 with RNAi technology would expect to suppress 23 upstream biosynthetic steps and the accumulation of codeinone and morphinone, the immediate precursor of COR. The amount of morphinan alkaloids decreased, while the biosynthesis of (*S*)-reticuline, an early upstream metabolite in the pathway, was increased instead of the target compounds codeinone and morphinone [110]. The complexity and redundancy of many biosynthetic pathways coupled with incomplete knowledge of their regulation could lead to an unpredictable outcome from a targeted metabolic engineering strategy [24].

Selected case studies using different approaches and strategies in metabolic engineering are discussed in the next paragraph.

19.6
Metabolic Engineering Applications in Medicinal Plant Biotechnology

19.6.1
Case Study: Podophyllotoxin Production in *Anthriscus sylvestris*

Anthriscus sylvestris (L.) Hoffm. (Apiaceae) is a common wild plant in Northwest Europe that accumulates considerable amounts of lignans. Deoxypodophyllotoxin, an aryltetralin-lignan is the main attractive constituent that is much more

abundant in the plant kingdom than podophyllotoxin, can be used as a precursor for the production of podophyllotoxin. Podophyllotoxin is used as a precursor for the semi-synthesis anticancer drugs: Etoposide phosphate and Teniposide [111]. To date, podophyllotoxin has been obtained by isolation from *Podophyllum* species. In the future, the availability of podophyllotoxin from this source is likely to become a major bottleneck. *Podophyllum* species are on the endangered species list, proving that the increasing demand of podophyllotoxin is a serious threat to the plant [112]. An alternative source of podophyllotoxin may be obtained by (biotechnological) hydroxylation of deoxypodophyllotoxin at the C7 position (see Figure 19.1). Human cytochrome P450 3A4 in *E. coli* DH5α selectively hydroxylates deoxypodophyllotoxin at the C7 position yielding podophyllotoxin [113]. Studies to transform *A. sylvestris* with this cytochrome are in progress.

19.6.2
Case Study: Scopolamine Biosynthesis in *Nicotiana tabacum*

Scopolamine and hyoscyamine are tropane alkaloids. They form an important class of plant derived anticholinergic compounds occurring in several genera of the Solanaceae, such as *Hysoscyamus*, *Atropa*, *Duboisia*, *Scopolia*, and *Datura* [12, 108]. Scopolamine has a higher commercial market value than hyoscyamine but has a lower yield from plants than hyoscyamine [12]. The world demand for scopolamine is estimated to be about ten times higher than hysocyamine and its racemic form atropine. The main sources of raw material worldwide are *Duboisia* leaves containing 2–4% of total alkaloids, with more than 60% scopolamine and 30% hyoscyamine [15]. Up to 6% of scopolamine has been achieved by conventional cultivation of selected varieties in Australia, Equador, and Brazil, producing 1 t/ha of plant material for industrial alkaloid extraction [15].

The heterologous expression of PMT (putrescine *N*-methyltranferase) from *Nicotiana tabacum* in *Scopolia parviflora* yielded an 8-fold increase in scopolamine and a 4.2-fold increase in hyoscyamine production [114]. A similar effect has been achieved in *Hyoscyamus muticus* and *Datura metel* [115]. Surprisingly, this PMT expression has no effect on alkaloid production when it is expressed in other tropane alkaloid producing hairy root cultures of *Hyoscyamine niger*, *Atropa belladonna*, and *Duboisiana hybrid* [16, 116, 117]. It was suggested that PMT expression in the roots was insufficient to boost the tropane alkaloid synthesis of these plants. Overexpression seems to be species related due to a different, specific post-translational regulation of the endogenous enzyme with respect to the foreign one [117].

The constitutive expression of H6H (hyoscyamine 6β-hydroxylase) from *Hyoscyamus niger* in *Atropa belladonna*, a plant that normally accumulates hyoscyamine, converts hyoscyamine into scopolamine up to 1.2% dry weight [118]. The alkaloid composition of aerial parts of mature plants changed from over 90% hyoscyamine in controls and wild type plants, to almost exclusively scopolamine in transgenics [118]. In transgenic hairy roots of *Atropa belladonna*, up to a 5-fold scopolamine

increase was observed [119]. In *Hyoscyamus muticus* hairy root, expressing the H6H gene, up to a 100-fold increase of scopolamine was found, while the hyoscyamine content remained unaltered [120].

Transgenic tobacco plants expressing constitutively H6H were fed with hyoscyamine and 6β-hydroxyhyoscyamine. These precursors were converted into scopolamine in the leaves of the plants [118]. The hairy root cultures of *Nicotina tabacum*, which do not produce hyoscyamine, were used to express the H6H gene from *Hyoscymanus niger*. The cultures successfully converted added hyoscyamine into scopolamine. They showed efficient uptake of hyoscyamine (average of 95%) from the culture medium and a higher rate of bioconversion of hyoscyamine into scopolamine (10–45%). Up to 85% of the total scopolamine was released into the culture medium [121]. This was in contrast to the normal metabolic behavior of tropane alkaloid-producing hairy roots in which the scopolamine remained accumulated in the root tissues [122]. Feeding exogenous hyoscyamine to cell suspension cultures, which were obtained from the hairy root, showed considerable capacity to convert hysocyamine into scopolamine and the product was secreted into the culture medium [123]. The scaling up of the transgenic cells grown in a 5l turbine stirred tank reactor in a batch mode yielded scopolamine up to a 1.6-fold higher than the small-scale cultures. Almost 18% of the hyoscyamine added to the medium was transformed into scopolamine, which showed a 65% increase with respect to the same alkaloid obtained by bioconversion in shakes flasks [15].

The constitutive co-expression of genes encoding the rate-limiting upstream enzyme PMT and the downstream enzyme H6H of scopolamine biosynthesis yielded only a modest increase in alkaloid accumulation when it was expressed alone, but exhibited a synergistic effect on alkaloid levels when expressed together [16]. It resulted in the highest production of scopolamine in hairy root culture reported of 411 mg/l. It is a 10-fold increase over control cultures and a 2–3-fold increase over cultures that expressed only H6H [16].

19.6.3
Case Study: Genistein Production in Transgenic *Arabidopsis*, Tobacco, Lettuce, Corn, Petunia, and Tomato

Genistein is a common precursor of the isoflavonoid biosynthesis, occurring in particular in the subfamily Papilionoideae of the Fabaceae [124]. Isoflanoids are interesting because of their pharmaceutical and nutraceutical activity that attract considerable interest with the prospect of introducing them into vegetables, grains, and fruits for dietary disease prevention [125].

Genistein production in nonlegume plants has been performed but as yet with unsatisfactory yields. This might be due to the competitive use of narigenin between isoflavon syntase (IFS) and the endogenous flavonoid pathway [125–127].

Soy products are the major dietary sources of isoflavonoids (genistein) for humans. The IFS isolated from soybean has been introduced into *Arabidopsis*

thaliana, corn (*Zea mays*), and tobacco (*Nicotiana tabacum*) [127]. There was no accumulation of free genistein in *Arabidopsis*, but genistein was glycosylated with glucose-rhamnose-genistein and rhamnose-genistein [125].

The overexpression of soybean IFS in tobacco, petunia (*Petunia hybrida* Vilm), and lettuce (*Lactuca sativa* L.) resulted in genistein accumulation in transgenic plants [128]. Another approach was the introduction of a heterogeneous phenylalanine ammonia-lyase (PAL) and IFS into genetically manipulated plants. This increased the genistein content in tobacco petals (1.80-fold) and lettuce leaves (1.5-fold) [128]. The overexpression of IFS soybean in tomato (*Solanum lycopersicum* L) resulted in the presence of genistein 7-*O*-glucoside as the major isoflavone metabolite in the transgenic plants [129].

19.6.4
Case Study: Expression of Spearmint Limone Synthase in Lavender

Essential oil quantity and quality can be regulated by metabolic engineering [92]. In principle, it is possible to engineer the biosynthesis of monoterpenes in order to increase or to modify the essential oil profiles in the target plant [130]. For example, the expression of a sense of the 1-deoxy-D-xylulose-5-phosphate (DXP) reductoisomerase cDNA, and with an antisense of menthofuran synthase cDNA under the control of CaMV 35S promoter, resulted in up to 50% more essential oil in *Mentha* × *piperita* L. without changing the composition of the monoterpenes as compared with the wild-type [92]. Meanwhile the expression of DXP synthase in *Lavandula latifolia* increased the essential oil up to 3.5-fold in leaves and up to 7-fold in flowers as compared with the control, without obvious deleterious effects on plant development and fitness [93].

Until now, studies on the expression of monoterpene synthase in transgenic aromatic plants have been scarce and have only been focused on mint species transformed with limonene synthase (LS). LS catalyzes the stereo-specific cyclization of geranyl diphosphate to yield the monocyclic monoterpene limonene [130].

Spike lavender (*Lavandula laftifolia* Med.) is an aromatic shrub that is cultivated worldwide for oil production, which has limonene as a minor constituent (0.5–2%). Overexpression of the LS gene from spearmint (*Mentha spicata*) in spike lavender under the regulation of the CaMV35S constitutive promoter showed more than 450% increase of limonene content in developing leaves as compared with the control [130].

19.6.5
Case Study: Artemisinin Biosynthesis in *Artemisia annua*

In the early 1980s, efforts began to establish *Artemisia annua* L. cultures that produced artemisinin [131]. A range of variable but always low artimisinin levels were found in callus, shoot, and root cultures but no artemisinin in cell suspension cultures, suggesting that some degree of differentiation is required for the production [132]. Transformation of *Artemisia annua* with *Agrobacterium*

rhizogenes resulted in hairy root cultures that produced artemisinin, and currently many efforts are directed toward optimizing production in hairy root cultures [133].

Several key genes involved in the biosynthesis of artemisinin have been introduced in *A. annua*. Approaches with genetic engineering have been focused on the overexpression of cloned key enzymes involved in the biosynthesis, such as farnesyl diphosphate synthase (FDS) [134] and amorpha-4,11-diene synthase (AMS) [135].

Genetic transformation and regeneration of *A. annua* has been established to introduce genes of interest via *A. tumefaciens* [136]. *A. annua* expressing FPS from *Gossypium arboreum* accumulated higher levels of artemisinin compared with *A. annua* expressing the FPS from *A. annua*. *A. annua* expressing FPS accumulated up to 10.08 mg/g DW artemisinin [136–138].

Hairy root cultures of *A. annua* were established by transforming it via *A. rhizogenes* carrying the farnesyl diphosphate synthase (FDS) gene. The artemisinin content in the transgenic plants, which were regenerated from the hairy root cultures, was significantly higher than in the control plant [96].

Despite all the genetic engineering attempts, the mean of production of artemisinin is still mainly from the plant itself. Recently, the FDA has approved Coartem® (Novartis) as the first artemisinin-based combination treatment (ACT) for malaria in the United States [139]. Novartis has stimulated the cultivation of *Artemisia annua* in more than 1000 hectares in Kenya, Tanzania, and Uganda. In addition, it has also cultivated in China, where, in total, it reaches up to 10 000 hectares [140].

19.6.6
Case Study: Morphine Biosynthesis in *Papaver somniferum*

Papaver somniferum remains the sole source of morphine. The commercial chemical synthesis of morphine, codeine, and other benzylisoquinoline alkaloids is not economically feasible due to the complexity of the molecule and multiple chiral centers [12].

Reticuline is an essential precursor leading to the biosynthesis of benzylisoquinoline alkaloids such as codeine, berberine, and morphine. One of the strategies to increase the flux into morphinan alkaloid is by blocking the BBE (berberine bridge enzyme). Blocking the BBE will increase the (*S*)-reticuline concentration. The expression of an antisense-BBE construct in transgenic opium poppy plants indeed showed increased flux into the morphinan and the tetrahydrobenzylisoquinoline branch pathway [141].

Another strategy is to increase the precursor pool leading to the formation of (*S*)-reticuline, which is (*S*)-*N*-methylcoclaurine 3′-hydrolase. The overexpression of cytochrome P450 monooxygenase (*S*)-*N*-methylcoclaurine 3′-hydrolase (CYP80B3) resulted in an up to 450% increase of total morphinan alkaloids [73]. The suppression of this gene by an antisense construct led to a reduced total alkaloid content in the transgenic poppy [73].

The existence of multi-enzyme complexes has been proposed for flavonoid [142–144] and polyamine metabolism [145]. The occurrence of multi-enzyme complexes also seems to exist in the morphine biosynthesis [108], therefore the metabolic engineering strategies have to be developed carefully. However, not all enzymes of the morphinan branch are necessarily involved in such a macromolecular complex [146].

A further approach is to increase salutaridinol, a precursor of thebaine, by overexpressing salutaridinol 7-*O*-acetyltransferase (SaIAT) and salutaridine reductase (SaIR). RNAi-silenced SalAT in opium poppy plants showed an accumulation of salutaridine instead of salutaridinol, which is normally not abundant in the plants [82]. Salutaridine may be channeled to thebaine through an enzyme complex that includes SaIR and SaIAT. Recent results showed that there is an interaction between SaIR and SaIAT [146]. Morphine, codeine, and thebaine levels were increased in both SaIAT overexpressing and SaIAT RNAi plants [82].

The codeinone reductase (COR) converts codeinone into codeine. Hypothetically, the morphine production can be increased by blocking this enzyme using the RNAi technique. On the contrary, there was an accumulation of (*S*)-reticuline instead of morphine, codeine, oripavine, and thebain [110]. The reasons are unknown, but there were some speculations. It was suggested that there was a feedback mechanism preventing intermediates from the general benzylisoquinoline synthesis entering the morphine-specific branch [2]. The impairment of a required metabolic channel composed of morphinan branch pathway enzymes resulted in accumulation of alkaloid intermediates produced by enzymes that were not part of the same complex [108]. The COR could be part of a multienzyme complex, which cannot function if one of the enzymes is removed [12]. It might be that the side effect of silencing COR was the suppression of 1,2-dehydroreticuline reductase [108]. The potential homology between the two reductases could lead to cosilencing [108]. The COR seems to be an important target for metabolic engineering. The overexpression of COR in *Papaver somniferum* yielded a 15% increase of benzylisoquinoline alkaloids as compared with the high-yielding control genotypes and a 30% increased as compared with the non-transgenic control [72].

19.6.7
Case Study: Gossypol Reduction in Cottonseeds by Blocking δ-Cadinene Synthase

Cotton (*Gossypium hirsutum* L.) could become, apart from its existing production, a nutritionally important crop, not only in developed countries but also in many developing countries where malnutrition and starvation are widespread and it is mainly used for fiber production. The plant produces approximately 1.65 kg of seed for every 1 kg of fiber [83].

After fiber extraction, the cottonseed could be used extensively as a source of proteins and calories, but it is hampered by the presence of the toxic gossypol. Gossypol is a cardiotoxic and hepatotoxic terpenoid and is unsafe for human and

monogastric animal consumption [147]. Gossypol and related terpenoids are present in the glands of foliage, floral organs, and bolls, as well as in the roots [83]. It protects the plant from both insects and pathogens [148, 149].

Gossypol and other sesquiterpenoids are derived from (+)-δ-cadinene. An RNAi-silencing approach on δ-cadinene synthase, coupled with a highly seed-specific α-globulin B gene promoter from cotton, showed significant and selective reduction of gossypol content from cottonseed, without diminishing its content and related defensive terpenoids in non-seed tissues of the plant usually attacked by insects [83]. If the gossypol level in the seeds can be reduced under the safety limit set by United Nations, Food and Agricultural Organization, and World Health Organization, it might be safe for human consumption [83]. Their limit of free gossypol in edible cottonseed products is less than 0.6 g/mg (600 ppm) [150].

19.7 Crossing Borders – Heterologous Production of Plant Compounds in Microorganisms

19.7.1 Artemisinic Acid

One of the success stories of using the synthetic biology approach is related to artemisinic acid. It is a naturally occurring precursor of artemisinin, used as an antimalarial drug. Malaria causes nearly a million deaths each year, mostly of children below 5 years old. The World Health Organization (WHO) estimated 247 million malaria cases among 3.3 billion people who were at risk in 2006 [151]. This leads to a demand to supply artimisinin in an economically attractive and environmental friendly way. The relatively low yield (0.01–0.6%) of artemisinin from *Artemisia annua* is unable to supply the world demand [136]. The total chemical synthesis of artemisinin is difficult and costly [152]. However, the semi-synthesis of artemisinin or any derivatives from microbial sourced artemisinic acid and its immediate precursor gives an alternative for availability and economic feasibility [153]. Using the synthetic biology approach with the use of appropriate promoters and an expression vector resulted in the production of artemisinic acid of up to 300 mg/l in the yeast *Saccharomyces cerevisiae* [68, 154].

19.7.2 Stilbenes

Stilbenes are polyketides, produced by plants. Resveratrol is a representative of stilbenes and is known as a constituent of red wine, which has possible interesting biological activities as an anti-cancer agent [155], inhibitor of inflammation, tumor promotion, angiogenesis and metastasis, and regulation of cell cycle progression [156].

The biosynthetic pathway and the enzymes have been characterized and metabolic engineering has been achieved in plants, microbes, and animals [157]. *E. coli* cells carrying PAL (phenylalanine ammonia-lyase), 4CL (4-coumarate:CoA ligase), STS (stilbene synthase), and ACC (acetyl CoA carboxylase) produced 40 mg/l resveratrol (1 h) from tyrosine. The PAL is from the yeast *Rhodotorula rubra*, 4CL is from actinomycete *S. coelicolor* A3 [2], and STS is from *Arachis hypogaea* [158, 159]. Resveratrol yields were >100 mg/l in *E. coli* expressing 4CL and STS [160].

Stilbenes are rapidly absorbed and metabolized when given orally. The modification of the resveratrol scaffold by hydroxylation and methylation enhanced its bioactivities. The recombinant *E. coli* carrying PAL, 4CL, STS, ACC, and OsPMT (pinosylvin methyltransferase in rice) with the addition of tyrosine resulted in the production of 18 mg/l pinostilbene and 6 mg/l pterostilbene. Addition of phenylalanine resulted in production of pinosylvin monomethyl ether and pinosylvin dimethyl ether almost in the same yield of 27 mg/l [161].

19.7.3 Curcuminoids

Curcumin, bisdemethoxycurcumin, and dicinnamoylmethane are known as curcuminoids [162]. Curcumin is the active ingredient of turmeric (*Curcuma longa*), which has a surprisingly wide range of beneficial claims, but not yet clinically proven. Its use is related to traditional medicine as an anti-inflammatory, antioxidant, anti-HIV, chemopreventive, and chemotherapeutic agent. These actions are partly supported by preclinical pharmacology [163, 164].

Horinouchi [161] discovered a type III polyketides synthase (PKS) in *Oryza sativa* (rice) that can synthesize curcuminods via *p*-courmaroyl-CoA. This PKS, named CUS (curcuminoid synthase), is part of an artificial biosynthetic pathway for production of curcuminoids in *E. coli* [165]. The *E. coli* expressing PAL, 4CL, CUS, and ACC with the additional supply of 1 mM each of the phenylpropanoid acid (*p*-coumaric acid, cinnamic acid or ferulic acid) yielded about 100 mg/l of curcumin or dicinnamoylmethane, and bisdemethoxycurcumin, respectively [161]. *E. coli* carrying 4CL, ACC, and CUS with the addition of ferulic acid isolated from 1 g of rice bran pitch yielded 60 mg of curcumin. Rice bran pitch is a dark and viscous oil, which is a waste from the production of rice edible oil from rice bran. Rice bran pitch (1 g) contains about 22 mg of ferulic acid [162].

19.7.4 Flavonoids

For the first time the complete flavonoid pathway from a plant has been successfully transferred into a microorganism [161]. Genes from various organisms were assembled in *E. coli* on a single pET plasmid for the production of flavanones. They are PAL from the yeast *Rhodotorula rubra*, 4CL or ScCCL from the

actinomycete *S. coelicolor A3* [2], CHS from the plant *Glycyrrhiza echinata*, and CHI from the plant *Pueraria lobata* [166, 167]. The construction proved to be optimal using isopropyl β-D-thiogalactopyranoside (IPTG) inducible T7-promoter and a synthetic ribosome-binding sequence in front of each of the four genes in a *recA*-host [167]. The yield of pinocembrin from 3 mM phenylalanine exogenously added, and naringenin from 3 mM tyrosine were both 60 mg/l [158]. Flavanone-3β-hydroxylase (F3H), flavonol synthase (FLS) and flavone synthase (FNS) were introduced into *E. coli* to modify flavanones into flavonols (kaempferol and galangin). The expression of the genes led to the production of kaempferol (15.1 mg/l) from 3 mM tyrosine and galangin (1.1 mg/l) from 3 mM phenylalanine [168]. Cloning of an FNS gene from *Petroselinum crispum* into pACYC in the *E. coli* host led to production of flavones: apigenin (13 mg/l) from tyrosine and chrysin (9.4 mg/l) from phenylalanine [168].

19.7.5 Vanillin

An example of "white biotechnology" is the production of vanillin as one of the most important aromatic flavor compounds used in foods, beverages, perfumes, and pharmaceuticals. The production scale is more than 10 000 tons per year by chemical synthesis [13]. The increasing demand of customers for natural flavors has shifted the interest of the flavor industry to produce vanillin from natural sources by biotransformation instead of organic synthesis [13]. The aim of the biotransformation of vanillin is to avoid toxic and mutagenic solvents such as phenol and dimethyl sulfate and to avoid corrosive compounds such as hydrogen peroxide, which are used for the organic synthesis [169].

Many different possibilities have been investigated for the biotechnological production of vanillin using different types of bacteria and fungi and different precursors [13]. The transformed *E. coli* BL21(DE3) cells carrying the isoeugenol monooxygenase gene of *Pseudomonas putida* IE27 produced up to 28.3 g/l of vanillin from 230 mM isoeugenol, with a molar conversion yield of 81% at 20 °C after 6 h [170]. The growing knowledge regarding enzymes involved in biosynthetic pathways as well as the identification and characterization of the corresponding genes offers new opportunities for metabolic engineering and for the construction of genetically engineered production strains [13].

19.8 Conclusion and Future Prospects

Plants definitely play an essential role in modern pharmacy and medicine. Efforts to obtain the desired natural compounds to be used as drugs in an efficient way are ongoing and include various approaches.

Metabolic engineering has been applied to both plants and plant cell cultures. Plant cell cultures have been shown to be feasible for industrial production only

to a limited extent, as shown for paclitaxel. Understanding secondary metabolism within cells and cell cultures is essential to use them as a means to supply natural products. The characteristics and metabolic capacities of plant cell/tissue and microbial systems are inherently different; therefore they can serve as complementary unit operations in order to solve the long-standing problem of robust secondary metabolites production [102].

The lack of complete information about the genomes of most medicinal plants is still an immense challenge for applying the appropriate metabolic engineering strategy. To date, only a few plant genomes (e.g., *Oryza sativa*, *Zea mays*, and *Arabidopsis thaliana*), but none of the medicinal plants, have been fully sequenced. The challenges of unravelling the unknown biosynthetic pathways, the encoding genes, and the transcription factors are still there. However, with the progress of sequencing techniques, it will likely be feasible to fully sequence medicinal plants in a shorter time. The only main constraint will, however, be the funding.

Conventional breeding of medicinal plants is another way to enhance the concentration of the desired compounds. Breeding and genetic engineering essentially go hand in hand and are necessary to ensure the availability of the desired compounds.

Until now, it seems that there has been limited success in engineering medicinal plants in which the product could be commercialized based on the economic feasibility. However, genetic engineering strategies have been applied to crop plants such as rice, maize, soybean, and cotton with great and significant success. The genetically modified crop plants with Bt (*Bacillus thuringiensis*) toxin for pest resistance have been grown commercially in approximately 42 million hectares worldwide [171]. In addition, Bt transgenic rice varieties are in field tests and are close to approval for commercialization [172].

The advance of technology holds great promise for the future of plant metabolic engineering. Genomics approaches may lead to the identification of regulatory genes and proteomic approaches may explain why the expression level of some biosynthetic genes do not correlate with the metabolites profile [108].

Finding alternative ways to produce originally plant-derived compounds are still continuing. Microorganisms such as endophytes may serve as an alternative host for production of bioactive substances as reviewed in reference [173]. The success of transferring the biosynthetic pathway from plants into microorganisms or other hosts for the production of artemisinic acid and flavonoids showed that it is feasible to engineer the entire pathway into microorganisms.

The latest promising approach is through synthetic biology for optimizing the biotechnological production of the plant-derived compounds. However, well-characterized biological components, such as the knowledge of the biosynthetic pathways, the genes involved, the promoters, and the precursors, are essential to build the system. An integrated approach to synthetic biology and metabolic engineering will be necessary in the near future. For successful engineering to enhance and optimize the production of the desired metabolites, crossing borders of different disciplines will be needed.

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20
Metabolomics as a Bioanalytical Tool for Characterization of Medicinal Plants and Their Phytomedical Preparations

Nizar Happyana, Remco Muntendam, and Oliver Kayser

20.1
Introduction

For many centuries, humankind has used medicinal plants directly or extracts thereof for their basic healthcare needs. Medicinal plants contain a complex mixture of secondary natural products and show synergistic effects against a broad variety of diseases. According to the World Health Organization (WHO), more than 80% of the world population use medicinal plants for everyday healthcare [1]. At present there are some 50 000 different medicinal plant species used for medication, and in Europe alone there are around 1300 medicinal plants in commercial use [2]. In the United Kingdom more than 25% of the population uses medicinal plants regularly [2], and in Germany approximately 30% of all over the counter (OTC) drugs are herbal medicinal products (HMPs).

Commercial medicinal plants in the world are mostly harvested from the wild. It is not only the so-called low-income countries that have to face this situation, but also in Europe 90% of medicinal plants used for extraction are collected from the wild [2]. The use of HMPs is becoming more and more popular and the demographic development in Europe, the United States, and subsequently in Asia [3] will increase demand in the future significantly. With this background, plant collection from the wild should be replaced by controlled cultivation to ensure sustainability of HMPs. Moreover, emerging of new illnesses, and an increase of resistance to current drugs have also emerged as challenges for medicinal plant sciences.

HMPs are made of complex biological matrices. To ensure patient safety and a high level of quality with regard to composition and activity, sophisticated analytical methods have to be developed and applied. Production and quality management is regulated by Good Manufacturing Practice (GMP) regulations and predominantly GC, HPLC, NMR, GC-MS, and LC-MS methods of analysis have been introduced.

Significant developments in analytical chemistry, bioinformatics, and computers have speeded up the procedures and a new strategy has been developed to provide huge amounts of information from some simple testing, namely metabolomics. This is a breakthrough in the approach to accelerating and streamlining

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Table 20.1 Some definitions related to metabolomics.

Metabolomics	• Holistic, simultaneous and systematic qualitative and quantitative determination of metabolites over time after stimulus
Metabolom	• Dynamic situation
	• Total number of all low molecular metabolites
Metabolite	• Intermediates and products of metabolism
	• Primary and secondary natural metabolites (e.g., lipids, sugars, alkaloids, flavonoids)
	• Low molecular compounds (MW <1000)
Metabolic profiling	• Analyzes a selected group of compounds or set of metabolites in a specific biochemical pathway
	• Targeted metabolites analysis
Metabolic fingerprinting	• Global screening approach
	• Classify samples based on metabolite patterns or "fingerprints"
	• Detect discriminating metabolites without identifying all of the compounds present

the analytical process of medicinal plant research. Metabolomics allows quick and efficient identification and quantification of the secondary metabolites within plants and is easily coupled to high throughput bioactivity screening. Furthermore metabolomics is well known as the youngest of the "omics" methods. A summary of some definitions related to metabolomics are provided in Table 20.1.

In this chapter we review bioanalytical tools often used in the metabolomics of medicinal plant research, such as NMR, DIMS (direct injection mass spectrometry), LC-MS (liquid chromatography–mass spectrometry), and GC-MS (gas chromatography–mass spectrometry) with a particular focus on quality control and metabolic profiling for herbal medicinal products. We highlight how bioanalytical tools are used and may be applied in routine industrial work. Furthermore, we summarize the important roles of metabolomics for medicinal phytochemistry by explaining applications of metabolomics in medicinal plants.

20.2 Bioanalytical Tools

The main purpose of metabolomics is to analyze all metabolites both qualitatively and quantitatively in medicinal plant samples. Basically, metabolomics analysis consists of three steps: sample preparation including the extraction process; metabolite measurements using bioanalytical tools, such as NMR, LC-MS, GC-MS;

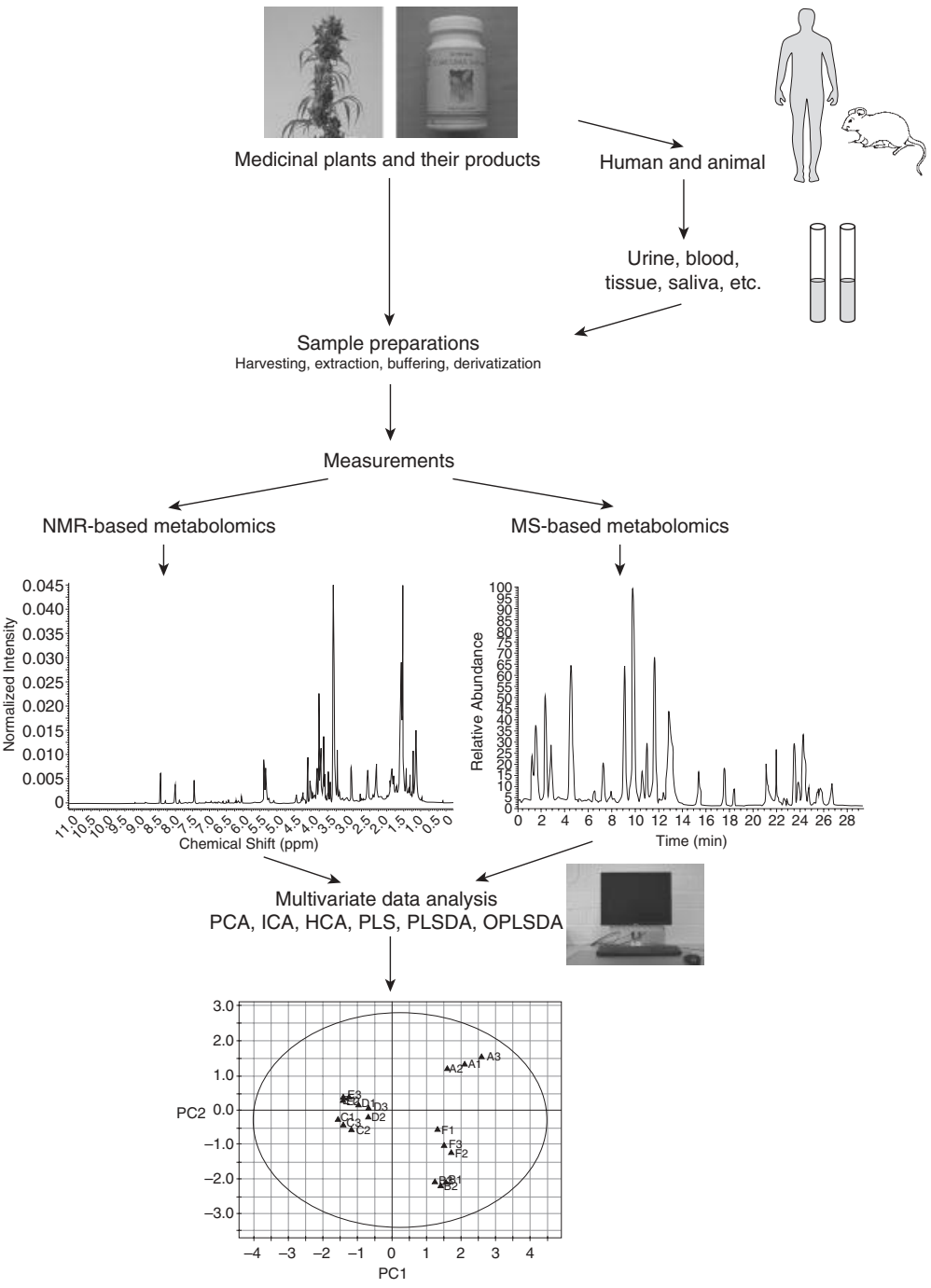

Figure 20.1 Workflow of metabolomics analysis in medicinal plant research.

and mining the raw complex data using chemometric software. An illustration of the steps required in a metabolomic analysis of medicinal plants is explained in Figure 20.1.

20.2.1 Sample Preparation

Sample preparation is considered as one of the most important steps in metabolomics analysis. The procedure must be standardized; starting from the harvesting, extraction, storage, and applying validated analytical parameters. Standardization and validation procedures are essential to obtaining reproducible results over time.

In harvesting, it is critical that metabolism processes in the plants should be stopped immediately. Damage due to cutting the plants could release active enzymes (e.g., glycosidases) that can change the metabolite profile significantly. Typical degradation reactions after harvesting are oxidation, hydrolysis, and decarboxylation. To prevent these, harvesting should be done rapidly and the harvested material should be dried to stop further metabolism. Alternatively lab samples can be frozen immediately (–20 or –80 °C) or submerged in a liquid nitrogen tank, followed by mechanical disruption to release metabolites from the cells [4]. However, for commercial preparation this technique is difficult to implement.

The first step in the extraction of medicinal plants can be classified as a solid–liquid extraction. This means that metabolites from a plant sample in the solid phase transfer into the liquid extraction medium. In general the solvents used for extraction can be divided into four classes: polar solvents (e.g., methanol, ethanol, and water), medium-polar solvents (e.g., chloroform, dichloromethane, diethyl ether), non-polar solvents (e.g., n-hexane), and a combination of such solvents [4]. Each solvent will give different profiles of the metabolites extracted. Metabolites that have high polarity are mostly extracted in polar solvents, while those that have low polarity are mostly extracted in medium-polar solvents. We have to consider that for commercial HMPs, ethanol, with or without water, is the standard solvent for extraction. It seems that by using one unique solvent, metabolomic analysis is becoming simpler, as various water : ethanol ratios and different extraction processes varying in time and temperature give a high diversity of extracts, which are reflected by the high number of different HMPs on the market.

The goal of metabolomics is to identify and quantify metabolites in biological samples that consist of a complex spectrum of different natural product classes. Currently there is no single solvent that can be used to extract all compounds directly. However, solvents that can dissolve the most diverse group of compounds must be chosen, but due to the limitations discussed, several extractions with different solvents must be conducted to give a total overview of the metabolites [5]. In the case of metabolic profiling studies, with the purpose of quantifying a selected group of metabolites in medicinal plants, the selection of solvents should be based on the physicochemical properties of the target metabolites to be analyzed.

For NMR-based metabolomics, polar solvents such as methanol and combined solvents such as water–methanol are often used for the extractions. A two-phase

solvent system, composed of a mixture of chloroform, methanol, and water (2 : 1 : 1, v/v), has also been used successfully for the extractions in NMR-based metabolomics [6–8]. Moreover, the use of deuterated NMR solvents for the extraction has also been reported [9–11]. This method avoids the need to evaporate the original extraction solvent and to redissolve the sample in the NMR solvents [5]. In LC-MS-based metabolomics, samples must be dissolved in solvents preferably similar to the eluent of the HPLC system. Regarding GC-MS-based metabolomics, compounds must be volatile in order to be measured, thus for non-volatile compounds derivatization prioir to measurement is needed.

20.2.2 Nuclear Magnetic Resonance

NMR spectroscopy is based on magnetic nuclei resonance in a strong magnetic field to determine physical and chemical properties of molecules. Basically it consists of a magnet, radio frequency (RF) transmitter or oscillator, and a suitable RF detector [12]. If an organic compound or an extract is placed in a magnetic field, interactions between NMR active nuclei, such as ^{1}H and ^{13}C, and the electromagnetic radiation will produce resonance signals to be collected by the detector. The resonance frequency of the NMR active nuclei is dependent on the chemical environment. Different chemical environments will give different resonances, thus each compound will posses specific NMR spectra.

NMR is widely used as a bioanalytical tool for the analysis of organic molecules and is considered as one of the most promising metabolomic tools [13]. It is well known as a powerful technique for elucidation of compound structures, including stereochemistry details. NMR is non-destructive and can be used for structural analysis of metabolites in crude extracts, cell suspensions, intact tissues or whole plants [14, 15]. Moreover, NMR allows the exploration of metabolic pathways, leading to qualitative information on the link between labeled precursors and their products and quantitative information on metabolic fluxes [16–19].

In NMR, ^{1}H NMR is the most popular technique used for qualitative and quantitative metabolomics analysis. It is very fast in its measurements, typically less than 5 min for one measurement (depending on the concentrations and the resonance frequency), facilitates high-throughput analysis, and usually has simple sample preparation. Moreover, quantitative analysis using ^{1}H NMR has no need for a calibration curve, because the molar concentration of a compound is directly represented by the intensity of a proton signal [4]. By adding an internal standard to the sample, we can compare proton signals of the internal standard with those of the sample and thus quantify compounds in the sample. For ^{1}H NMR, the concentration threshold for a routine detection of a metabolite in an extract using a modern high field spectrometer is probably 10 μM, corresponding to an amount of 5 nmol in the typical sample volume of 500 μl [17]. Furthermore, metabolomics based on ^{1}H NMR approaches is highly reproducible, which means that NMR-metabolomics data are valid for ever, provided the same extraction procedures and the same NMR solvents are used [20].

Although the sensitivity of ^{1}H NMR is low, this weakness can be solved through various approaches. Today, NMR microprobes are available that can be used for measuring small samples. With such probes, the small samples are dissolved in a small volume of NMR solvent, thus the samples are more concentrated and produce better quality spectra. High-resolution NMR is also available. This has high sensitivity and can give a substantial improvement to the detection of the signals, thus it also produces high quality NMR spectra. Finally, there are also cryogenic NMR probes. These probes are small (3 or 5 mm in diameter) but have the capability of improving sensitivity and reducing noise through cooling the receiver coil and the preamplifiers down to cryogenic temperatures. They are powerful probes that can be used for measuring low samples, as has been demonstrated by Schneider and Holscher [21].

Another problem of using ^{1}H NMR in metabolomics studies for medicinal plants is signal overlap that can obstruct the identification and quantification of metabolites. However, 2D NMR techniques can be used to solve this problem. These techniques give better signal resolution and reduce signal overlap by distribution of the resonances in a second dimension. Although 2D NMR has all advantages of ^{1}H NMR it requires a longer recording time. ^{1}H J-resolved NMR (JRES) is a 2D NMR technique that is mostly used in metabolomics studies. JRES has the capability to split the effects of chemical shift and J-coupling into two independent dimensions. The use of JRES in metabolomics has been reviewed in detail by Ludwig and Viant [22]. Beside JRES, other 2D NMR techniques such as HSQC (heteronuclear single quantum coherence), COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), HMBC (heteronuclear multiple quantum coherence), and NOESY (nuclear Overhauser effect spectroscopy) have been applied in plant metabolomics studies [15, 23, 24].

20.2.3 Mass Spectrometry

A mass spectrometer is an analytical instrument measuring the mass-to-charge (m/z) ratio of ions. MS instruments consist of three main parts, namely: an ionization chamber where the molecules are being ionized; a mass analyzer that separates ions according to their m/z by applying electromagnetic fields; and a detector to record m/z. The common techniques used as ionization sources are electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI). In addition, mass analyzers such as single quadrupole, triple quadrupole, ion trap, and time-of-flight (TOF) analyzers are usually applied in metabolomics analysis.

Besides NMR, MS is also well known as an analytical tool for metabolomic analysis, particularly in metabolic profiling and metabolic fingerprinting. In metabolomics, MS separates metabolites based on m/z ratios of their ions. Furthermore, MS also provides both sensitive detection and metabolite identification through mass spectrum interpretation and comparison or molecular formula

determination via accurate mass measurements [13]. Moreover, MS is considered as the most sensitive method for metabolomics analysis [4], because it can identify ionized components at very low levels. A major disadvantage of using MS is its reproducibility. MS measurements are dependent on the type of instrument, operating parameters, and matrix effects on the ionization, making it difficult to produce similar results across various laboratories [4].

20.2.3.1 Direct Injection Mass Spectrometry

A direct injection mass spectrometer analyzes metabolites through the injection of a sample directly into the ionization chamber without prior chromatographic separation, and provides fast and high throughput measurements. DIMS mostly use ESI and APCI as the ionization sources. Both are known as soft ionizations, which provide minimal fragmentation of molecular ions and a less complex mass spectrum. Therefore, through interpreting the molecular ions, metabolites can be identified without chromatographic separations [25].

Time-of-flight is usually used as the mass analyzer in DIMS methods. TOF separates ions based on the time the ions take to reach the detector. All ions in TOF are accelerated by an electric field that ensures they all have the same kinetic energy. Thus the velocity of the ions just depends on their m/z, hence heavier ions will reach the detector later compared with lighter ions. Depending on the flight-tube geometry and instrument tuning, TOF-MS instruments provide masses of 6000–17 000, with a mass accuracy in the range of 3–5 amu [26]. In addition, to improve the performance of TOF-MS, a quadrupole has been attached as a scanning device or a mass filter [27]. The quadrupole has four rods at high voltage to create a quadrupole field to select ions according to their m/z (only ions with m/z values within a certain range can pass the rods). Therefore the quadrupole enhances the capability of TOF-MS in the separation of metabolites.

Different metabolites with the same molecular weights cannot be separated with the previous techniques. However, this problem can be addressed by using tandem MS/MS. This technique provides great selectivity through the specific fragmentation of each metabolite. After ions (precursors) pass through the first MS, they are activated by collision with an inert gas such as nitrogen or argon to produce ion fragments. The newly created fragment ions can subsequently be analyzed by the second MS. MS/MS is usually coupled with a quadrupole as the scanning device and TOF as the mass analyzer. The quadrupole-TOF-MS/MS instrument can clarify the fragmentation process by distinguishing the m/z of precursor and fragment ions. Therefore it allows good interpretation of spectra [26]. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) is a powerful MS technique. It is provided with high resolution to separate and distinguish very complex mixtures and has high mass accuracy, allowing calculation of elemental compositions to aid in structural differentiation and characterization [28]. Nevertheless, it cannot be used to separate structural isomers. Furthermore, FTICR-MS is an expensive instrument, thus its utilization is not widespread.

20.2.3.2 Gas Chromatography–Mass Spectrometry

Gas chromatography–mass spectrometry is well known as one of the popular techniques for global metabolic profiling [29]. It provides fast analysis at a relatively cheaper cost compared with other mass spectrometry techniques, while retaining the capability for specific metabolite detection and quantification. Furthermore, GC-MS can be used to separate large numbers of compounds in a single measurement and identify unknown metabolites as well.

GC-MS is a combined technique of gas chromatography (GC) and mass spectrometry (MS) used to analyze different metabolites within a measurement. In this technique, the GC separates the metabolites while MS functions as the metabolite detection tool. The GC is equipped with a capillary column as the stationary phase, a carrier gas as a mobile phase (He, N_2, H_2), and a sample injector. Electron impact (EI), known as hard ionization, is mostly used for ionization in GC-MS-based metabolomics. In EI, electrons are produced through thermionic emission interacting with the molecules in the gas phase to form molecular ions and fragment ions. TOF provides fast metabolite detection and can be used as mass detection, meanwhile single quadrupole and ion trap have also been used as mass analyzers in GC-MS.

Metabolites identification in GC-MS analysis is conducted by comparing the retention time or retention index of sample metabolites with the retention time or retention index of pure reference metabolites or spectral library databases [30]. Quantitative metabolomics using GC-MS requires preparing calibration curves for each metabolite, because the sensitivity of GC-MS varies for all metabolites [4].

Only GC-MS can be used for analyzing volatile metabolites with thermal stability. Derivatization can be used to measure non-volatile and polar metabolites, to increase volatility, thermal stability, and reduce the polarities of the functional groups of the metabolites. Silylation is a derivatization procedure mostly applied by replacing active hydrogen with a trimethylsilyl (TMS) group. The silylation reagent is moisture sensitive and reacts readily with water, which decreases the efficiency. This all samples should be fully dry and solvents should be as pure as possible, to avoid inefficient derivatization. Alternatively, alkylation and acylation can be used for derivatization of functional groups, such as –COOH, –OH, –NH, and –SH.

20.2.3.3 Liquid Chromatography–Mass Spectrometry

Liquid chromatography–mass spectrometry is the other widely used technique for targeted or non-targeted metabolomics analysis and offers high selectivity and good sensitivity analyses. A large number of metabolites can be separated with LC-MS, as overlapping peaks of different metabolites on the chromatogram can be identified as separate compounds in the mass analyzer of the LC-MS instrument. However, LC-MS has disadvantages in the reproducibility of separations that can be caused by matrix effects of the complex sample extract and different parameters in the various LC-MS systems [4].

LC-MS instruments consist of two main parts, namely, liquid chromatography for metabolites separation and MS for detection. ESI is commonly used for ioniza-

tion of molecules in LC-MS, although APCI also has been used to ionize more difficult metabolites. In ESI, the sample, in a suitable solvent at atmospheric pressure, is ionized by application of a high electric charge to the sample needle [31]. Moreover, ESI can cover a broad range of metabolites, as it operates ionization in negative and positive modes. LC-MS commonly applies TOF and single quadrupole as the mass analyzer, and a combination of both has also been used. In LC-MS analysis each metabolite has a different sensitivity, therefore calibration curves of each metabolite are definitely needed for quantitative analysis.

Reversed-phase columns are mostly employed in metabolomics analysis using LC-MS, as they are easy to use and can be applied for the separation of the majority of metabolites. The characteristics of LC columns, such as column internal diameter and packing particle size of the column, influence the level of LC-MS resolution. Improving chromatographic resolution can be achieved by reducing the diameter particle of the column packing material, as has been applied in ultrahigh pressure liquid chromatography–mass spectrometry (UPLC-MS) system [32]. This technique can reduce ionization suppression significantly and decrease co-elution of metabolites. Highly polar metabolites are usually difficult to separate by reversed-phase columns. Alternatively, hydrophilic interaction chromatography (HILIC) columns can be used for separation of highly polar metabolites. Moreover, HILIC-MS also has been used for the analysis of highly polar compounds in *Curcubita maxima* leaves [33].

20.2.4
Data Processing

The data obtained from medicinal plant metabolomics experiments are very large numbers and complex, thus are very difficult to interpret by eye. However, after the development of computer technologies and the emergence of analytical software, the large amounts of data sets can be automatically visualized and interpreted. The common method for data processing in metabolomics is multivariate data analysis. It is a chemometric method that can visualize large numbers of compounds resulting from metabolomics experiments, and data mine information about the relationships between the levels of different metabolites [34]. To provide appropriate data for multivariate data analysis, the spectra from metabolomics experiments must be extracted.

To date, three methods have been developed for the raw data extraction, namely, binning, peakpicking, and deconvolution [35]. Binning or bucketing is the method most often for data extraction in metabolomics [36]. With this method, spectra are subdivided into several regions, called “bins” or “buckets” and the total area within each bucket is used as a representation of the original spectra [37]. In NMR based plant metabolomics, binning can reduce 16k data points to 250 data points [38], while in MS based plant metabolomics the number of bins is usually below 2000 at a bin size of 1 amu [26]. However, binning in crowded spectra has the potential for significant loss of information, for example by including peaks belonging to multiple compounds within a single bin [39].

The peakpicking method, as an alternative to bucketing, basically consists of peak finding, baseline subtraction, and alignment steps [35]. An investigation has been conducted to compare peakpicking with bucketing on the data extraction, and the results showed that the peakpicking approach was more interpretable than the bucketing [40].

In deconvolution, defined as targeted profiling, the data are integrated by an algorithm from pure compound spectra and interrogated for identifying and quantifying the metabolites in the mixture [41]. In NMR-based metabolomics, this method provides NMR signal vectors and quantitative metabolite data [35], whereas in MS-based metabolomics, deconvolution reduces the complexity of the data [26].

Normalization is the next step after raw data extraction. In NMR-based metabolomics, integral normalization is a standard method for normalizing and is required to control possible variations in sample concentrations and variable sample dilutions [37]. Meanwhile in MS-based metabolomics, normalization coupled with transformation is introduced to minimize the impact of the variability of high-intensity peaks [26].

After normalization, the extracted data are further analyzed by multivariate data analysis. Basically, multivariate data analysis can be distinguished into two general types, namely, unsupervised and supervised approaches. Unsupervised approaches are often known as clustering techniques, which do not use independent variables, provide a simplified description of the data with general information, and visualize the relationship between the dependent variables [34]. Moreover, unsupervised approaches are powerful methods for sample classification. Meanwhile, supervised approaches describe the subset of variation in the dependent variables. These approaches do not describe the irrelevant variations to the experimental questions, thus providing simplified information [34]. Supervised approaches are appropriate for metabolomics analysis that aims to discover characteristic compounds and where the sample identity is often known [26].

One of the most popular multivariate data analysis methods for unsupervised approaches is principal component analysis (PCA) [42]. It is basically a data reduction technique and represents multivariate data in a low-dimensional space. Furthermore, PCA has capabilities for finding relationships and variances in the data, making a model of how the chemical system behaves, and separating any underlying systematic data from noise [43]. Figure 20.2 describes a graphical overview of the matrices and vectors used in PCA. The extracted data are represented by a set of new variables known as principal components (PCs). Similarities and variances of samples according to metabolomic data are shown in a score plot and the influence of each metabolite signal is visualized by the loading plot of PCA [4].

The other common methods that have been used for unsupervised approaches are independent component analysis (ICA) and hierarchical cluster analysis (HCA). ICA apparently is an improvement of PCA, because in the beginning step it needs PCA for reduction of the high dimension of the dataset, and the quality of the ICA is also determined by the number of principal components

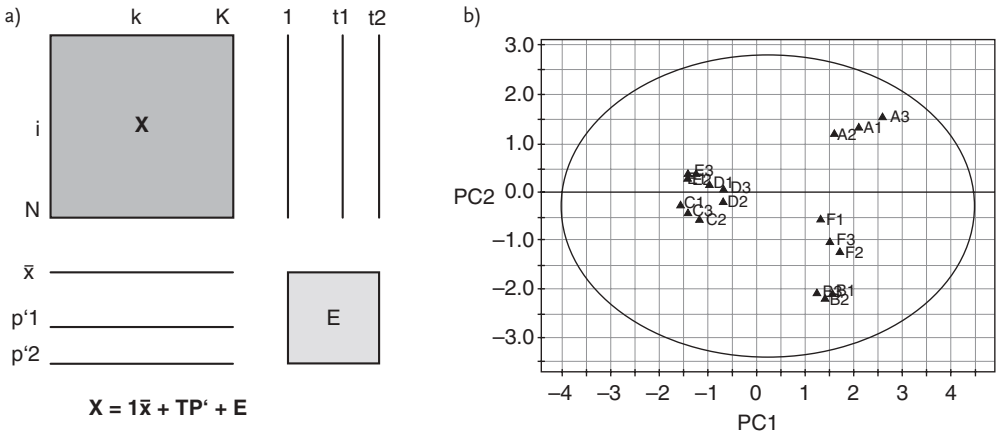

Figure 20.2 A, a graphic of a data matrix X with its first two principal components. Index i is used for objects (rows) and index k for variables (columns). There are N objects and K variables. The matrix E contains the residuals, the part of data not "explained" by the PC models [43]. B, an example of a score plot.

[27]. In ICA, a new set of components known as independent components (ICs) are calculated to detect more meaningful components, and different ICs represent different non-overlapping information [27]. ICA has shown good results in metabolic fingerprinting for a small number of high-dimensional samples when PCA failed to do so [27]. HCA classifies samples in a dataset based on their similarity. It creates a hierarchy of clusters that are commonly visualized in a tree structure called a dendogram, where the root consists of a single cluster containing all observations and the leaves correspond to individual observations. Therefore, HCA easily draws a description of the similarities of the samples within datasets [28].

Partial least squares (PLS) regression is one of the common methods used for supervised approaches. PLS combines features from PCA and multiple regressions. It can be used for discrimination along with creating a linear regression model by projecting the predicted and the observable variables to a new dimension. PLS has been improved in several variants that also can be used for supervised approaches, such as partial least squares discriminant analysis (PLSDA), and orthogonal partial least squares discriminant analysis (OPLSDA). PLSDA can discriminate samples in a dataset through identification of variables that significantly show relevant variations in the dataset. OPLSDA is an improvement of PLSDA and removes irrelevant information and aligns the projections precisely with the aspect of interest, thus gives a better interpretation than PLSDA. This technique discriminates between two or more groups in a dataset [44] in which the regression model is calculated between the multivariate data and a response variable that only contains class information [45].

20.3
Metabolomics Applications in Medicinal Plants

Metabolomics can be considerd to represent the phenotype on a metabolic level and is the most informative technique for understanding biological systems [28]. Moreover, it is a beneficial technique that quickly provides required information in the study of medicinal plants. In addition, metabolomics has been applied in various areas of research into medicinal plants, such as classification of medicinal plants, characterization of plant cell cultures, and transgenic medicinal plants, quality control, and proof for efficacy of medicinal plants from urine or blood samples.

20.3.1
Discrimination for Classification of Medicinal Plants

Many factors influence the profiles of metabolites biosynthesized by medicinal plants. Different environmental conditions such as soil, fertilizers, climate, pest control, and insects for plant cultivation, can create a high, and not necessarily constant, diversity of the biochemical composition. Factors affecting metabolite production in medicinal plants are described in Figure 20.3. Because of the commercial utility, some medicinal plants, such as *Rhodiola rosea*, a folk medicine in Scandinavia and Russia [46], are now cultivated at many locations with different geographies. These cultivations show variations in the metabolite profiles, which are affected by geographic and soil conditions. Furthermore, morphological diversity, representing varieties of the plants, also leads to variations in the profiles of the metabolites. As a consequence, the medicinal value and pharmacological

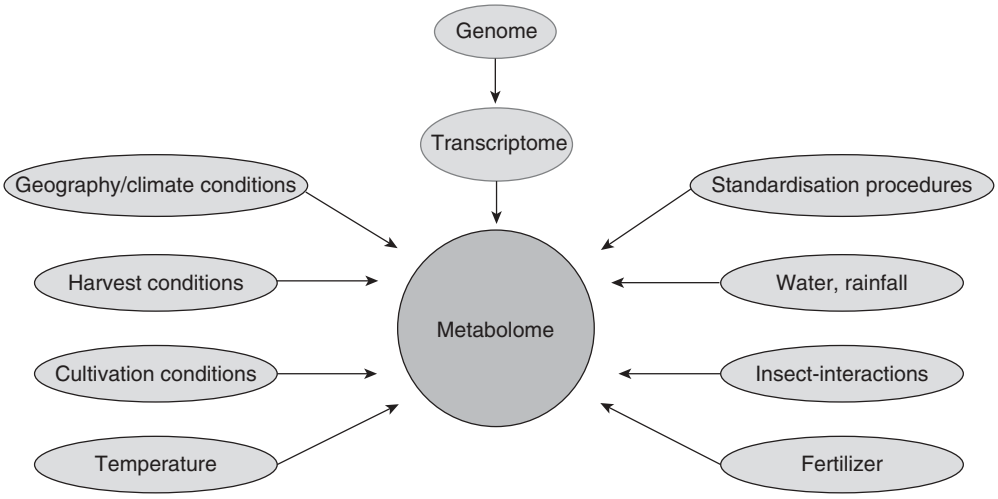

Figure 20.3 Factors affecting the metabolome of a medicinal plant.

activity of an herbal medicinal product strongly depends on the desired metabolite composition, and there is great interest by the pharmaceutical companies in having the cultivation and production process under as much as control as possible. A smart, reliable, and cost efficient analytical method is of significant interest, in order for the in-process control to characterize and classify medicinal plants consistently.

Metabolomics meet these requirements for metabolite analysis in herbal medicinal products, as it covers the complete spectrum of all natural products affected by cultivation conditions (temperature, rain, and soil conditions) and location. All relevant data for internal use or for declaration to the authorities can be systematically compared and stored. As an example, using ^{1}H NMR coupled with PCA, an unsupervised metabolic profiling has been conducted to discriminate nine rhizomes of *R. rosea* collected from Switzerland, Russia, and Finland [46]. According to PCA models, volatile compounds characterizing the essential oil spectra have been identified as the discriminating factor of rhizomes of *R. rosea* [46]. Similarly, an approach was addressed to the rhizomes and roots of *Notopterygium incisum* Ting ex H.T. chang (Qianghuo), which is known in Traditional Chinese Medicine (TCM). Comprehensive GC/GC and GC/GC-MS combined with PCA analysis have been applied to analyze 15 samples of volatile oils of Qianghuo from Sichuan, Ningxia, Inner Mongolia, Hunan, and Gansu. The results indicated that monoterpenes and oxygenated sesquiterpenes were responsible for the differentiation of the Qianghuo [47].

Metabolomics also has been used to discriminate medicinal plants that were cultivated under the same environmental conditions but were from different varieties. Metabolomic analysis using ^{1}H NMR coupled with PCA has been applied to discriminate 12 *Cannabis sativa* cultivars cultivated under the same standardized conditions. In the chloroform fraction THCA and CBDA were identified as important metabolites to discriminate the cultivars from each other, whereas in the water fraction, sucrose, glucose, asparagine, and glutamic acid were identified as major discriminating metabolites [7]. Discrimination of 11 varieties of *C. sativa* has also been conducted by quantification of the concentration of monoterpenoids, sesquiterpenoids, and cannabinoids using GC coupled with PCA [48].

Besides discrimination of medicinal plants from the same species, metabolomics can also be applied to distinguish closely related species with similar metabolic profiles. Three species of the genus *Panax*, namely *P. ginseng*, *P. notoginseng*, and *P. japonicus*, have different pharmacological activities and highly similar morphology, making it hard to differentiate between them. By using UPLC-MS combined with PCA, the three species of *Panax* have been discriminated [49]. PCA showed that chikusetsusaponin IVa, ginsenoside R0, ginsenoside RC, ginsenoside Rb1, ginsenoside Rb2, and ginsenoside Rg2 were the critical markers for discrimination of each species [49]. The same problem, similarity in morphology, has been addressed for the roots from *Astragalus membranceus* Fisch. and *Hedysarum polybotrys* Hand. The identification of nine active compounds using HPLC-DAD, LC-MS coupled with HCA showed significant

differences in the metabolome and allowed exact and definitive identification of both roots [50].

In another case, the discrimination of *Ephedra sinica*, *Ephedra intermedia*, and *Ephedra distachya* var. *Distachya*, known to be old Chinese medicinal herbs, has been conducted using ^{1}H NMR coupled with PCA [51]. Benzoic acid analogs in the water fraction and ephedrine-type alkaloids in the organic fraction were identified to be the discriminating chemical markers in *Ephedra* species [51]. A method that combines ^{1}H NMR with PCA has also been applied to discriminate the three most common species of *Strychnos* with different pharmacological activities. Brucine, loganin, fatty acids, icajine, and sungucine were identified as the key compounds for discrimination of *S. nux-vomica*, *S. ignatii*, and *S. icaja* [52].

20.3.2
Characterization of Metabolites in Plant Cultures and Transgenic Medicinal Plants

Plant cultures may offer an attractive solution for solving the limitations of extracting active metabolites from natural medicinal plants. Some attempts have been made through introducing elicitors or molecular biological approaches to the cultures for improving metabolite production and studying metabolite biosynthesis. Moreover, metabolomics, as a powerful method, has been used as a promising method for identification of metabolites in medicinal plant cultures.

Metabolomics has been applied for monitoring the elicitation process in medicinal plant cell cultures. By using ^{1}H NMR coupled with PCA, a metabolomic profiling approach has been conducted for identifying the effects of elicitation of silver nitrate in cell suspension cultures of *Vitis vinifera*. It was found that the metabolite profile in an elicited suspension culture is different from the control and that lactate, alanine, acetic acid, choline, fructose, glucose, and sucrose were the key compounds for differentiation of elicited cultures from the control [53].

In addition, metabolomics has been used to study the effects of elicitation in the metabolite biosynthetic pathway of cell cultures made from medicinal plants. A metabolomic profile using ^{1}H NMR combined with PCA has been taken for monitoring the elicitation effects of yeast extract and yeast extract–methyl jasmonate in suspension cultures of *Silybum marianum*. The results revealed that elicitation influenced the phenylpropanoid pathway [54]. Furthermore, elicitation of yeast extract and methyl jasmonate caused a metabolic reprogramming that affect amino acid and carbohydrate metabolism, whereas yeast extract alone affected threonine and valine metabolism [54]. GC-MS coupled with PCA has been carried out to investigate the response of *Medicago trunctula* cell cultures towards elicitations of methyl jasmonate, yeast extract, and ultraviolet light. The results indicated that methyl jasmonate increased production of β-amyrin and saponins, while yeast extract induced accumulation of shikimic acid, an early precursor in the prenylpropanoid pathway, and ultraviolet light gave insignificant effects [55]. However not all elicitations can stimulate targeted metabolite production, such

as elicitation in cell suspension cultures of *C. sativa* L., which did not fulfill the expectation of a stimulation of cannabinoids production [56].

Metabolomics is also a powerful tool for identifying metabolites in hairy root cultures. A metabolomic profiling approach has been applied to discriminate two hairy root clones of *Psoralea corylifolia* L. and an untransformed control. GC-MS and LC-MS analysis showed that the isoflavanoid formononetin and its glycoside were present only in the hairy root clones, and suggested that clones are dissimilar in their secondary metabolism [57]. Beside this, metabolomics has also been used to validate a cryopreservation protocol for suspension cell cultures of *Tabernaemontana divaricata* [58]. The validation was based on metabolic profiling of a fresh control and cryopreserved *T. divaricata*. A comparison of the obtained data clearly indicated that the level of the main alkaloid precursor, tryptamine, did not change and differences in the metabolome was only detectable at the level of several amino acids, carbohydrates, and fumaric acid [58].

In medicinal plant biotechnology, metabolomics has not only been applied in plant cell cultures, but also for transgenic plants. Metabolomics has been used to identify the effects of different genetic modification approaches in metabolite production. For example, two lines of the transgenic *Artemisia annua* L. where amorpha-4,11-diene synthase (ADS) were overexpressed and suppressed and were analyzed by a metabolic profiling approach using GC/GC-MS combined with PLSDA. According to the data analysis, overexpression of the ADS influenced the whole terpenoid metabolic pathway, especially the artemisinin biosynthesis, whereas suppression of ADS showed insignificant effects in artemisinin biosynthesis [59]. Beside ADS, the effects of overexpression of farnesyl diphosphate synthase at five developmental stages of *A. annua* L. have been analyzed by metabolomic profiling. The results showed that there are clear differences during all flowering stages [60].

20.3.3
Quality Control of Medicinal Plants

Many factors can cause variations in the metabolite profile of an HMP, such as harvesting treatment, storage process, extraction technique, preparation method, and packaging (Figure 20.3). Alterations of the metabolite profile change the pharmacological activities of the products. Moreover, these lead to different quality grades, alter the efficacy of the products, and also gives ambiguous results in clinical trials using medicinal plant products. In some cases, several additional components could be illegally mixed into HMPs, such as aminophylline and prednisone acetate, as reported for TCM products [61]. Thus, the composition of metabolites in the HMPs is very complex and difficult to analyze. Therefore, good analytical methods are required to guarantee the quality of medicinal plant products at a high standard. Furthermore, metabolomics with different approaches have been used as smart analytical methods for quality control of medicinal plant products, as they offer comprehensive, efficient quantitative and qualitative analysis of metabolites.

Commercial medicinal plant products mostly contain complex mixtures of compounds, plus the correlation between the composition of compounds in the mixtures and the pharmacological action is often unclear. Moreover, many pharmaceutical companies sell identical herbal products and claim the same pharmacological activity and efficacy. A closer look clearly shows that the extraction processes are different, the extraction media are not the same, sometimes the species used are different, and the formulations (tablet, liquids, and sachets) cannot be compared. Typical examples for this phytochemical dilemma are *Gingko biloba*, *Echinacea purpurea*, St. John's wort HMPs, and many more. However, metabolomics has been successfully applied to monitor complex compound mixtures and to discriminate commercial herbal products. A simple method using ^{1}H NMR coupled with PCA has been used to characterize ten samples of commercial St. John's wort from some suppliers. This method not only discriminated the samples from different suppliers but also showed differences between samples from the same suppliers [62]. Moreover, 14 commercially available *Tanacentrum parthenium* L. samples have also been discriminated using ^{1}H NMR combined with PCA [63]. These examples thus show that ^{1}H NMR coupled with PCA is an effective method in the quality control of medicinal plant products.

Metabolomics in combination with ^{1}H NMR and PCA has also been used to investigate commercial capsules that claim to be from *A. annua* and contain a high artemisinin content, an active compound for malaria medication. The results have clearly shown that the capsules contain low concentrations of artemisinin and are indeed not *A. annua* but *A. afra* [64]. Therefore, it can be seen that this approach also provides a simple method for investigating fake commercial medicinal plants.

In some cases, metabolomics combining ^{1}H NMR and PCA is not sufficient for identification of each compound in very complex mixtures of medicinal plant products. However, ^{1}H J-resolved NMR (JRES) coupled with PCA offers an alternative solution to solve this problem. This has been demonstrated in the quality control of commercial ginseng preparations. This method can discriminate between the preparations, based on accumulation of ginsenosides, alanine, arginine, choline, fumaric acid, inositol, and sucrose [11].

Improvements to multivariate data analysis in metabolomics, such as the combination of PCA and PLSDA, have been applied to the quality control of medicinal plant products. Combination of PCA and PLSDA in data processing can reduce the dimensionality of multivariate data while preserving most of the data information, maximizing separation between classes, and minimizing the distance between intragroup clustering [65]. This technique has been demonstrated in the quality control of *Curcuma*. Using GC-MS coupled with PCA and PLSDA, the key compounds for the discrimination of *C. phaeocaulis*, *C. kwangsiensis*, and *C. wenyujin* have been identified, namely curzerenone, germacrone, curdione, and epicurzerenone [65]. Furthermore, this combination has also been used to assess the quality control of *Pericarpium Citri reticulatae* and *Pericarpium citri reticulatae viride* [46].

Besides the combination of PCA and PLSDA, an orthogonal projection (OP) technique has also been proposed for quality control. This technique was demonstrated in the metabolic fingerprinting of *Houttuynia cordata* Thunb. and its results were compared with those from other techniques, such as PCA. According to this study, OP shows better results in the discrimination and identification of fingerprints than the other methods, and thus it might be a promising tool in evaluating and discriminating the quality of medicinal plants products in the future [66].

Besides improvements to the data processing, combination of LC-MS and GC-MS analysis can also be another alternative technique useful for quality control. This technique coupled with HCA has been used to identify ten samples of *Caulophyllum robustum*. According to HCA, it can discriminate all samples and can also distinguish unacceptable samples for quality control purposes [67]. As a consequence, this technique offers a meaningful method for quality control of medicinal plant products.

20.3.4
Identification of Medicinal Plant Bioactivity

Identification of the components that are responsible for bioactivity of medicinal plants can be conducted using metabolomics coupled with bioactivity assays. This approach has been demonstrated for identification of the components of xiaoyaosan that are responsible for antidepression. Xiaoyaosan is a TCM product that is composed of several medicinal plants [68]. Different fractions were administrated to stressed rats and subsequently the urine was analyzed using NMR and the data were mined using PLSDA. The results showed that the metabolite profile of the rat treated with a petroleum ether fraction was more similar to that of the positive control. These workers concluded that the fraction containing mainly lipophilic compounds had the highest antidepression activity [68].

Metabolomics has also been used to investigate therapeutic and synergistic effects of three major ingredients > (T, S, and G) of Compound Danshen Formula (CDF), namely tanshinone IIA (T), salvianolic acid B (S), and ginsenoside Rb1 (G). CDF is a TCM formula derived from the herbs of *Radix salviae miltiorrhizae* (Danshen in Chinese), *Radix notoginseng* (Sanqi), and *Borneolum syntheticum* (Bingpian) [69] and is used to improve coronary and cerebral circulation [70]. Myocardial ischemia rats were administrated with T, S, G, TSG, and CDF. The blood plasma was subsequently analyzed by LC-MS and PLSDA, which revealed that the activity of TSG was closer to the activity of CDF, whereas the activities T, S, and G were low. Therefore, it was confirmed that T, S, and G have a synergistic effect when they are used together [69].

In other case, metabolomics also can be used to predict biological activity. This method has been demonstrated in the prediction of anti- plasmodial activity of *A. annua* extracts. Some extracts of *A. annua* obtained from different locations have been discriminated by ^{1}H NMR combined with PCA, and it was found that artemisinin is the main discriminating factor for clustering. PLS and PLSDA

were then used to create the models for prediction of anti-plasmodial activity. The results showed that the predictive activities were very similar to the experimental activities [71].

20.3.5 Study Efficacy of Medicinal Plants

Efficacy and toxicity (see Section 20.3.6) of HMPs have to be assessed by *in vivo* studies (phase I) and later by clinical studies (phase II and III). *In vivo* studies are carried out in animal models, such as rats, and later clinical studies are carried out in humans, with samples of blood and urine being taken for further analysis. An illustration of the hierarchy levels for quality assurance in HMPs is explained in Figure 20.4. Besides approving the clinical efficacy, bioequivalence and phytoequivalence of HMPs are important questions. In contrast to phytoequivalence, defining bioequivalence is rather simple as it is a term for describing biological and pharmacological equivalence of two proprietary preparations of an HMP. In principal, phytoequivalence can be considered as a physiological equivalence between the effects of two herbal extracts, the efficacy of one of which has been clinically demonstrated, but according to drug regulating authorities, this definition does not include the condition of two phytochemically identical extracts.

To achieve phytoequivalent extracts both preparations must have the same metabolic profile, qualitatively and quantitatively. Owing to different extraction procedures, which are usually disclosed by the company that is the marketing authorization holder, for a generic company it is then nearly impossible to get two

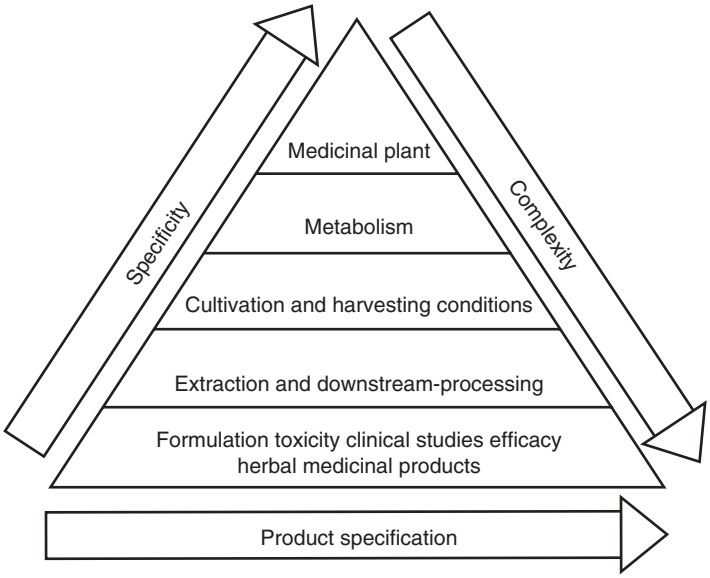

Figure 20.4 Hierarchy levels for quality assurance in HMPs.

completely equal HMPs based on the original production process. To solve this dispute, it would be in the interests of the patient and consumer to define phytoequivalence according to existing regulations in the biotech field for so called "biosimilars." Here, for recombinant therapeutic proteins, the basic amino acid structure must be identical to the innovator product, but other decorations, such as glycolisation, may differ if both have the same or comparable biological effect. To follow up on this idea, HMPs are phytoequivalent if the main pharmacologically relevant compounds or biomarkers are present and both have similar pharmacological and pharmacokinetic profiles. To meet the criteria of similar phytochemical profiles for pharmacologically active components, metabolomic is an important tool for identifying relevant compounds in a complex mixture.

UPLC-MS and PCA coupled analysis have been used to study efficacy of *Epimedium brevicornum* Maxim, a TCM, in kidney deficiency syndromes. A high dose of hydrocortisone was injected into rats to induce a pathological condition similar to kidney deficiency syndrome. These rats were later treated with an extract of *E. brevicornum* Maxim and the urine and blood were analyzed. Based on PCA, the metabolite profiles of treated rats were shifted closer to that of pre-hydrocortisone administration. Therefore, the efficacy of *E. brevicornum* for kidney deficiency syndromes was proven [72]. Also, the efficacy of antidepressive HMP of xiaoyaosan has been investigated using GC-MS and three multivariate data analysis techniques, PCA, PLSDA, and HCA. Thirteen metabolites relating to antidepression were identified in a urine sample from the rats. Moreover, the multivariate data analysis revealed that the metabolite profile of the rats treated with a high dose of xiaoyaosan was much closer to the control, and gave clear evidence to the efficacy of xiaoyaosan [73].

In another case, ^{1}H NMR and PCA were utilized to demonstrate the anti-aging efficacy of the total flavones in *Epimedium* (TFE), considered to be the major active components of *Epimedium koreanum Naka*. Data showed 26 characteristic resonances related to the age of the rats and successfully identified ten of them, namely, creatinine, lactate, alanine, acetate, acetone, succinate, allantonin, methylamine, dimethylamine, and trimethylamine-*N*-oxide [74]. Furthermore, according to the PCA, the metabolite profile of 24 month old rats treated with TFE was similar to that of 18 month old rats without TFE. It thus showed the anti-aging efficacy of TFE [74]. ^{1}H NMR coupled with PCA has also been used to study the efficacy of *Ginkgo biloba*. Stressed rats were treated with *Ginkgo*, and subsequently their urines were analyzed with ^{1}H NMR. Results showed the metabolite profile of the treated rats had changed to close to that of the control rats, which suggested that *Ginkgo* was responsible for this action [75].

20.3.6
Investigation of Medicinal Plant Toxicity

Toxicity is one of the obstacles to the use of medicinal plants, and can also be investigated with metabolomics. This method is based on the fact that organ damage could be associated with the corresponding changes in metabolite

profiles. This has been documented in a metabolomics study of the toxicity of Hei-Shun-Pian, a TCM derived from the roots of *Aconitum Carmichaelii* Debx., and known for its analgesic, antipyretic, anti-rheumatoid arthritis, and anti-inflammation effects [76]. Urine samples and plasma from the rats that had been administered orally with the decoction of Hei-Shun-Pian were measured with ^{1}H NMR and the data analyzed by PCA. In the early stages of the dosing period, urinary excretion of trimethylamine-*N*-oxide and taurine, which is closely related to the cardiovascular system [77], were decreased, while the concentration of citrate, 2-oxoglutarate, succinate, and hippurate were increased. These results revealed that the extract of Hei-Shun-Pian has cardiac toxicity and causes disturbances to the heart and liver.

In addition to studying HMPs, metabolomics has also been applied to investigate the toxicity of single compounds. A metabolomic approach has been conducted to study the toxicity of aristolochic acid, widely present in *Aristolochia*, *Bragantia*, and *Asarum* species [78]. Urine samples from treated rats and control rats were analyzed with GC-MS and LC-MS combinations and data mined by PCA and OPLSDA. The results revealed that metabolite profiles of treated rats were different from that of the control rats. Furthermore, the concentrations of homocysteine and serine, accepted levels for diagnosis of kidney morbidity, were lower than those of the control. This thus showed that aristolochic acid caused disruption to the kidney [78].

Toxicity of aristolochic acid has also been studied in comparison with the urinary profiles from rats treated with aristolochic acid and other known toxicants, such as sodium chromate ($NaCrO_4$) and mercury(II) chloride ($HgCl_2$), as controls at various time intervals. The results also revealed that aristolochic acid induced renal toxicity [79]. From this report we learn that the combination of metabolomics and histopathological experiments gives a new perspective to the potential of these new bioanalytical tools (LC-MS coupled with PCA and linear discriminate analysis) [80]. In a more recent case on aristolochic acid toxicity, a metabolomic approach using LC-MS/MS coupled with PCA has been used to investigate renal toxicity, but in addition these workers also identified potential biomarkers related to kidney disease, namely kynurenic acid and hippuric acid [81].

20.4 Conclusions

Many factors influence the profiles of metabolites biosynthesized by medicinal plants. Different environmental conditions, such as soil, fertilizers, climate, pest control, and insects for plant cultivation, can create a high but not constant diversity of the biochemical composition. However, metabolomics has been proven as a powerful method to discriminate and classify the same medicinal plants cultivated at different geographies, different varieties of plants, and different plants but having the same morphology, by identifying the key compounds for discrimination. Moreover, metabolomics has been used for monitoring the

elicitation process in medicinal plant cell cultures and for identifying the effects of different genetic modification approaches in the metabolite production of transgenic plants.

Harvesting treatment, storage conditions, extraction technique, preparation method, and packaging all affect the quality of medicinal plant products significantly. Therefore, good analytical methods are required to guarantee a high level of quality, from production to the pharmacy shelf. However, metabolomics through various approaches has been successfully applied as a smart analytical method for the quality control of medicinal plant products, as it offers comprehensive, efficient quantitative and qualitative analysis of metabolites. Metabolomics has also been coupled with bioactivity assays to identify the components responsible for bioactivity, to investigate synergistic effects, and even to predict bioactivity of a medicinal plant product. Moreover, metabolomics has also been applied to study the efficacy of medicinal plant products through measuring urine, blood or other biofluids of the addressed object (human or rats) and comparing it with the control. The fact that organ damage could be associated with the corresponding changes in metabolite profiles leads to application of metabolomics in toxicity investigations of medicinal plant products.

All of these have shown that the emergence of metabolomics has opened up new opportunities to answer the challenges in the area of medicinal plants. Metabolomics has been proven to be a breakthrough method to accelerate and streamline the analytical process of medicinal plant research, by allowing quick, efficient identification and quantification of the metabolites within the samples.

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21
Integration of Biotechnologies for the Development of Personalized Medicine

Kewal K. Jain

21.1
Introduction

Personalized medicine, also referred to as individualized therapy, simply means the prescription of specific treatments and therapeutics best suited for an individual, taking into consideration both genetic and environmental factors that influence response to therapy [1]. Most of the current drugs are approved and developed based on their performance in a large population of people, but medicine of the future is developing as personalized solutions for a particular patient's needs. The conventional "one-drug-fits-all" approach involves trial and error before an appropriate treatment is found for a disease. Clinical trial data for a new drug merely shows the average response of a study group. There is, however, considerable individual variation; some patients show no response whereas others show a dramatic response. It is obvious that a more individualized approach is needed. Although individualization of certain treatments had been carried out in the pre-biotechnology era, the concept of personalized medicine as described in this chapter follows progress in the study of human diseases at the molecular level, advances in molecular diagnostics and drug development based on genomics, proteomics, metabolomics, and biomarkers. Personalized treatment of various diseases has been described in a textbook on this subject [1].

The aim of the personalized medicine is to match the right drug to the right patient and, in some cases, even to design the treatment for a patient according to genotype as well as other individual characteristics. A broader term is integrated healthcare, which includes development of genomics based personalized medicines, predisposition testing, preventive medicine, combination of diagnostics with therapeutics, and monitoring of therapy. This fits in with the concept of systems biology as applied to healthcare. Personalized medicine is considered to be the best way to translate emerging biotechnologies and integrate them into healthcare. The relationship of personalized medicine to various biotechnologies and drug development is shown in Figure 21.1.

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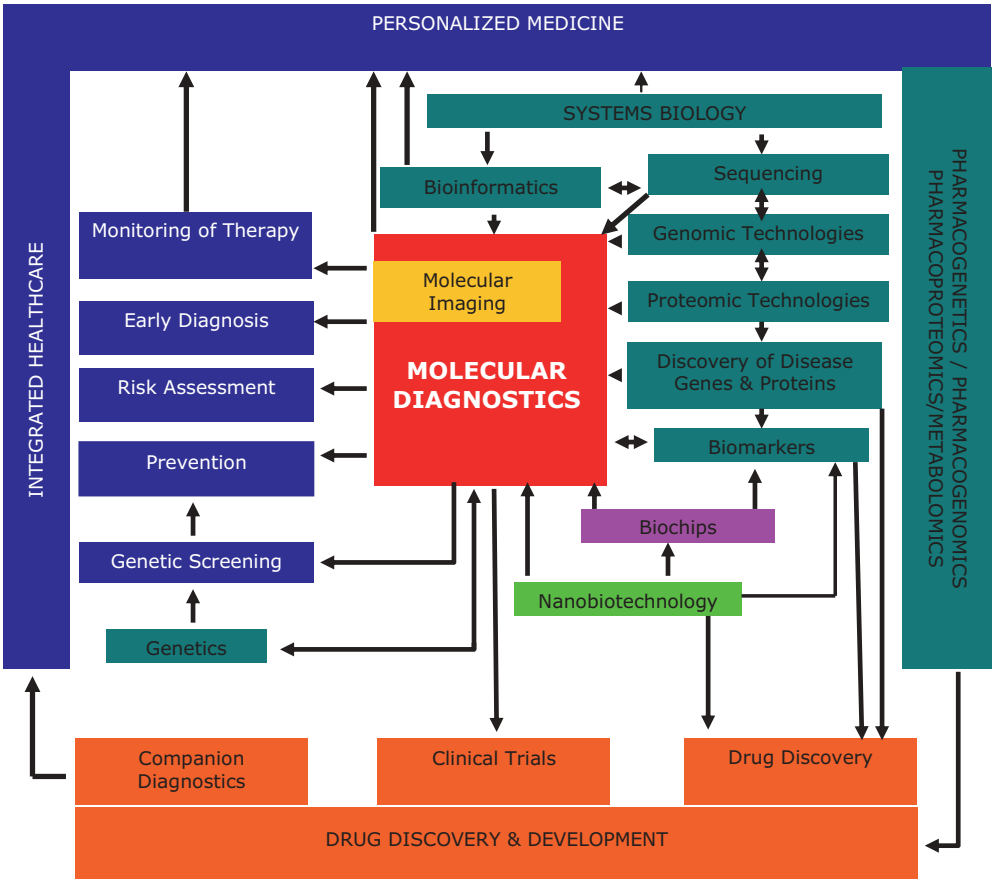

Figure 21.1 The role of molecular diagnostics in personalized medicine. Among various technologies that are linked and that interact, molecular diagnostics has a central place. In an integrated healthcare system, the role of molecular diagnostics does not stop at initial detection of a disease but continues on to monitoring of the therapy. Molecular diagnostic technologies are used in drug discovery and development to develop personalized medicines and to act as companion diagnostics. There is a two-way relationship with biomarkers, as not only are molecular diagnostic technologies used for detection of biomarkers, but also some biomarkers form the basis of novel diagnostics. © Jain PharmaBiotech.

21.2
Genetic Variations in the Human Genome

Human genome rearrangements can occur by several mechanisms, which include both recombination- and replication-based mechanisms. The latter can result in complex genomic rearrangements. Although many studies have been conducted to identify SNPs (single nucleotide polymorphisms) in humans, few studies have

Table 21.1 Genetic variations in the human genome. © Jain PharmaBiotech.

Variation	Features
Complex chromosomal rearrangements (CCRs)	CCRs account for a large fraction of non-recurrent rearrangements at a given locus
Copy number variation (CNV)	DNA segments >1 kb in length, whose copy number varies with respect to a reference genome. ~12% of human genes vary in DNA sequences they contain
Insertions and deletions (INDELs) in the human genome	INDELS are an alternative form of natural genetic variation that differs from SNPs
Interspersed repeated elements	Long and short interspersed nuclear elements are a significant portion of human genome
Large-scale variation in human genome	Large portions of DNA can be repeated or missing for no known reason in healthy persons
Segmental duplication	Duplicons have >90% sequence homology to another region in the genome
Single nucleotide polymorphisms (SNP)	SNPs are sequence variations at single base pair level with a population frequency of >1%
Structural variations (SVs)	SVs involve kilobase- to megabase-sized deletions, duplications, insertions, inversions, and complex combinations of rearrangements
Tandem repeats	Tandem sequences repetitions represent ~10% of the genome

been conducted to identify alternative forms of natural genetic variation. Significant genetic variations in the human genome are shown in Table 21.1.

21.2.1 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms, which comprise some 80% of all known genetic polymorphisms, are small stretches of DNA that differ in only one base. The number of SNPs in public databases exceeds 15 million, as of May 2011. Identification of SNPs is important as it helps in understanding the genetic basis of common human diseases. Some SNPs have been linked to disease susceptibility, or disease status, while others have been linked to drug response. SNPs can be used as biomarkers to segregate individuals with different levels of response to treatment, both beneficial and adverse, in clinical settings.

SNP markers can also be utilized in drug development for prediction of adverse effects or response. In the absence of functional information about which polymorphisms are biologically significant, it is desirable to test the potential effect of all polymorphisms on drug response. Of the millions of SNPs identified in public

databases using a large number of methods, only a small fraction are well characterized and validated [2].

High-resolution genome-wide association studies using panels of 300 000 to 1 million SNPs aim to define genetic risk profiles of common diseases. These studies provide the opportunity to explore the pathomechanism of human diseases in a manner that is unbiased by previous hypotheses or assumptions about the nature of genes that influence complex diseases. Many genetic variants identified as risk factors for diseases by such studies have been localized to previously unsuspected pathways, to genes without a known function. Although identification of SNPs as risk factors may be clinically useful, in many cases SNP genotyping for complex diseases may be insufficient to predict whether a person is at risk for a particular disease.

21.2.2
Complex Chromosomal Rearrangements

Complex chromosomal rearrangements (CCRs) have been known for some time but their mechanisms have remained elusive. Recent advances in technology and high-resolution human genome analyses have revealed that complex genomic rearrangements can account for a large fraction of nonrecurrent rearrangements at a given locus [3]. Various mechanisms, most of which are DNA-replication based, for example, fork stalling and template switching, have been proposed for generating such complex genomic rearrangements and are probably responsible for CCR.

21.2.3
Insertions and Deletions in the Human Genome

Whereas SNPs are differences in single chemical bases in the genome sequence, insertions and deletions (INDELs) result from the insertion and deletion of small pieces of DNA of varying sizes and types. If the human genome is viewed as a genetic instruction book, then SNPs are analogous to single letter changes in the book, whereas INDELs are equivalent to inserting and deleting words or paragraphs. INDELs were discovered using a computational approach to re-examine DNA sequences that were originally generated for SNP discovery projects. INDELs are distributed throughout the human genome with an average density of one INDEL per 7.2 kb of DNA. Variation hotspots were identified with up to 48-fold regional increases in INDEL and/or SNP variation compared with the chromosomal averages for the same chromosomes. A map is available of 415 436 INDELs in the human genome [4]. The scientists expect to expand the map to between 1 and 2 million by continuing their efforts with additional human sequences.

INDELs are already known to cause human diseases. For example, cystic fibrosis is frequently caused by a three base pair deletion in the CFTR gene, and DNA insertions, known as triplet repeat expansions, are implicated in fragile X syn-

drome and Huntington's disease. Transposon insertions have been identified in hemophilia, muscular dystrophy, and cancer. INDEL maps will be used together with SNP maps to create one big unified map of variations that can identify specific patterns of genetic variation to help predict the future health of an individual. The next phase of this work is to figure out which changes correspond to changes in human health and develop personalized health treatments.

21.2.4 Large-Scale Variation in the Human Genome

Large-scale disparities in the DNA of healthy people have been revealed, which challenge the previous findings, and reveal a largely ignored source of genome variation. Several loci have been identified across the human genome that contain genomic imbalances among unrelated individuals; half of these regions overlap with genes, and many coincide with segmental duplications or gaps in the human genome assembly. Thus healthy persons can have large portions of DNA that are repeated or large portions that are missing for no known reason. This previously unappreciated heterogeneity may underlie certain human phenotypic variations and susceptibility to disease and suggest arguments for a more dynamic human genome structure.

21.2.5 Variation in Copy Number in the Human Genome

Copy number variation (CNV) is a source of genetic diversity in humans. Numerous CNVs are being identified with various genome analysis platforms, including array comparative genomic hybridization (aCGH), SNP genotyping platforms, and next-generation sequencing. CNV formation occurs by both recombination-based and replication-based mechanisms and *de novo* locus-specific mutation rates appear much higher for CNVs than for SNPs. CNVs can cause Mendelian or sporadic traits, or be associated with complex diseases through various molecular mechanisms, including gene dosage, gene disruption, gene fusion, position effects, and so on. However, CNV can also represent benign polymorphic variants. CNVs, especially gene duplication and exon shuffling, can be a predominant mechanism driving gene and genome evolution. CNVs form at a faster rate than other types of mutations.

The CNV of DNA sequences is functionally significant but has yet to be fully ascertained. A study has shown that ~12% of human genes vary in the CNV of DNA sequences they contain – a finding that contradicts previous assumptions that the DNA of any two humans is 99.9% similar [5]. The discovery indicates that copy number variation could play a larger role in genetic disease than previously thought, with broad implications in disease association studies, genetic diagnostic testing, and cancer research. CNVRs regions (CNVRs), which can encompass overlapping or adjacent gains or losses, contain hundreds of genes, disease loci, functional elements, and segmental duplications. Notably, the CNVRs encompass

more nucleotide content per genome than SNPs, underscoring the importance of CNV in genetic diversity. The data obtained delineate linkage disequilibrium patterns for many CNVs, and reveal marked variation in copy number among populations. They also demonstrated the utility of this resource for genetic disease studies. Several CNVs are already known to be associated with diseases, including AIDS, inflammatory bowel disease, lupus, cataracts, arterial disease, and schizophrenia. The findings could change the direction of future genetic disease research, which has primarily focused on SNPs. Some diseases are caused by CNV rather than SNPs.

Structural variations of DNA greater than 1 kilobase (kb) in size account for most bases that vary among human genomes, but are still relatively under-ascertained. Tiling oligonucleotide microarrays were used to generate a comprehensive map of CNVs, of which most have been validated independently [6]. By correlation with known trait-associated SNPs, several loci with CNVs were identified that are candidates for influencing disease susceptibility. The results show that any two genomes differ by more than 1000 CNVs, or approximately 0.8% of a person's genome sequence. Most of these CNVs are deletions, with a minority being duplications. This map complements the cataloging of SNPs delineated in the HapMap Project. In spite of the power of this map, the heritability void left by genome-wide association studies will not be accounted for by common CNVs in cases of complex diseases, such as diabetes or heart disease.

Sequence-based strategies, particularly with the advent of new sequencing tools, have facilitated the identification of CNVs. Although these techniques are expensive and best suited to small sample sizes, they offer distinct advantages over array-based tools [7]: (i) they are the best approach for detection of small CNVs (<10 kb); and (ii) sequence information allows the detection of not only the extra or missing copies of DNA but also information as to their genomic arrangement and structures, such as inversions and SNPs.

21.2.6
Structural Variations in the Human Genome

Structural changes are extremely common in human populations. Genetic variation among individual humans occurs on many different scales, ranging from gross alterations in the human karyotype to SNPs. More bases are involved in structural changes in the genome than are involved in single base pair changes.

Although the original human genome sequencing effort was comprehensive, it left regions that were poorly analyzed. Later investigations revealed that, even in healthy individuals, many regions in the genome show structural variations (SVs), which involve kilobase- to megabase-sized deletions, duplications, insertions, inversions, and complex combinations of rearrangements.

One study has offered a new view of what causes the greatest genetic variability among individuals, suggesting that it is due less to single-point mutations than to the presence of structural changes that cause extended segments of the human genome to be missing, rearranged or present in extra copies [8]. This study was

designed to fill in the gaps in the genome sequence and to create a technology to rapidly identify SVs between genomes at very high resolution over extended regions. A novel DNA-based method known as Paired-End Mapping was used for this study. Researchers broke up the genome DNA into manageable-sized pieces about 3000 bases long; tagged and rescued the paired ends of the fragments; and then analyzed their sequence with a high-throughput, rapid-sequencing method developed by 454 Life Sciences. This method of sequencing enables identification of "hot spots," that is, regions with a lot of variation, which are often regions associated with genetic disorder and disease. These results will have an impact on how genetic effects in diseases are studied. It was previously assumed that "landmarks," such as the SNPs, were fairly evenly spread out in the genomes of different people. Now, one has to take into account that the SVs can distort the map and differ between individual patients. Even in healthy persons, there are variants in which part of a gene is deleted, or sequences from two genes are fused together without destroying the cellular activity with which they are associated. These findings show that the parts list of the human genome may be more variable and flexible than previously considered. The discovery of CNVs in human genomes has dramatically changed the perspective on DNA structural variation and disease.

21.3
Role of Biomarkers in the Development of Personalized Medicine

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention. Classical biomarkers are measurable alterations in blood pressure, blood lactate levels following exercise, and blood glucose in diabetes mellitus. Any specific molecular alteration of a cell on the DNA, RNA, metabolite or protein level can be referred to as a molecular biomarker. In the era of molecular biology, biomarkers usually mean molecular biomarkers and can be divided into three broad categories [9]:

1) those that track disease progression over time and correlate with known clinical measures;
2) those that detect the effect of a drug;
3) those that serve as surrogate endpoints in clinical trials.

Development of personalized medicine is closely linked to biomarkers, which may serve as the basis for diagnosis, drug discovery, and monitoring of diseases. Molecular diagnostic technologies are used for detecting biomarkers and some biomarkers may form the basis of diagnostic tests. They can be used for following the progression or regression of disease as well as the effect of treatment.

The economics and uncertainties of drug development demand that pharmaceutical companies quickly demonstrate the safety and efficacy of their

compounds. Using biomarkers to make key development decisions early can reduce development timelines and increase the likelihood of regulatory and clinical success, thereby maximizing market positioning. Biomarkers are becoming more critical in the process of discovering and developing new drugs and in personalizing the use of established drugs.

21.4 Technologies Used for the Development of Personalized Medicine

Several technologies have facilitated the development of personalized medicine. These can be categorized as follows:

- **Technologies used for molecular diagnostics**.
- **Technologies for detection of biomarkers**, for example, proteomics, genomics, and metabolomics.
- **DNA sequencing**.
- **Bioinformatics** for evaluation and use of data from various technologies.
- **Pharmacogenomics** is the application of genomics (variations of DNA as well as RNA) to drug discovery and development. It involves the study of the mechanism of action of the drugs on the cells as revealed by gene expression patterns.
- **Pharmacogenetics** is a term recognized in pharmacology in the pre-genomic era and concerns the study of the influence of genetic factors on the response to drugs. With advances in genomics, the role of gene polymorphisms on the action of drugs has been added to this.
- **Pharmacoproteomics** is the application of proteomics to drug discovery and development. Discovery of protein biomarkers may serve as a common basis of diagnostics and therapeutics. Subtyping patients on the basis of protein analysis may help to match a particular target-based therapy to a particular biomarker in a subgroup of patients.
- **Pharmacometabolomics** is the application of metabolomics for the study of diseases as well as diagnostics and therapeutics.

21.5 Molecular Diagnosis as a Basis for Personalized Medicine

The use of molecular diagnostics to understand the molecular mechanisms of disease affecting an individual will be pivotal in the delivery of safe and effective therapy for many diseases in the future. Molecular diagnostic technologies have been described in a detailed report on this topic [10]. Technologies that are

particularly relevant to personalized clinical laboratory diagnostics are reviewed elsewhere [11]. Molecular diagnostics has three important roles in personalized medicine:

1) **Early detection** and selection of appropriate treatment determined to be safe and effective on the basis of molecular diagnostics.
2) **Integration** of molecular diagnostics with therapeutics.
3) **Monitoring therapy** as well as determining prognosis.

In parallel with two important components of personalized medicine, pharmacogenetics and pharmacogenomics, there are two types of tests relevant to personalized medicine:

1) **A pharmacogenomic test** is an assay intended to study inter-individual variations in whole genome SNP maps, haplotype markers or alterations in gene expression, or inactivation that may be correlated with pharmacological function and therapeutic response. In some cases the pattern or profile of the change rather than the individual biomarker is relevant to diagnosis.

2) **A pharmacogenetic test** is an assay intended to study inter-individual variations in the DNA sequence related to drug absorption and disposition (pharmacokinetics), including polymorphic variations in genes that encode the functions of transporters, metabolizing enzymes, receptors, and other proteins.

Molecular diagnostic technologies relevant to personalized medicine are shown in Table 21.2. Important technologies relevant to personalized medicine include real-time and quantitative polymerase chain reaction (PCR), single nucleotide polymorphism (SNP), copy number variations, DNA sequencing, genotyping, and microarray/biochips.

21.5.1 Real-Time PCR and Personalized Medicine

Real-time PCR systems offer many general technical advantages over standard PCR, including reduced probabilities of variability and contamination, as well as online monitoring and the lack of need for post-reaction analyses. An example of the usefulness of real-time PCR is for the identification of pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) as well as methicillin-sensitive *S. aureus* (MSSA), and the accurate determination of antimicrobial sensitivities for prompt optimal patient therapy. Real-time quantitative PCR (Q-PCR) is useful in detection and staging of lung cancer as well as construction and validation of prognostic and predictive gene expression signatures. It can be applied to the analysis of clinical samples to help stratification of patients in a personalized medicine approach. It enables the measurement of gene expression or DNA copy number in specific cell types that are available only in a small amount. Limitations of real-time PCR include that PCR product increases exponentially and variation

Table 21.2 Molecular diagnostic technologies used for personalized medicine. © Jain PharmaBiotech.

Polymerase chain reaction (PCR) based methods
Cold-PCR
Digital PCR
Quantitative fluorescent PCR
Real-time PCR
Reverse transcriptase (RT) PCR
Scorpions™ (DxS Ltd): closed-tube platform for the efficient homogeneous detection of PCR amplicons
Non-PCR methods
Enzyme mutation detection
Fluorescense resonance energy transfer (FRET) based assays: Invader assay
Locked nucleic acid (LNA) technology
Peptide nucleic acid (PNA) technology
Transcription-mediated amplification
Gene chip and microfluidic microarrays
Nanodiagnostics
Nanoparticle-based integration of diagnostics with therapeutics
Nanotechnology-based diagnostics for pharmacogenetics/pharmacogenomics
Toxicogenomics
Single nucleotide polymorphism genotyping
Copy number variations (CNVs)
DNA methylation studies
Gene expression based tests
DNA sequencing
Multiplex DNA sequencing
Sequencing in microfabricated high-density picoliter reactors
Whole genome sequencing
Cytogenetics
Comparative genomic hybridization (CGH)
Fluorescent *in situ* hybridization (FISH)
Proteomic-based methods
Proteomic-based methods
Fluorescent *in situ* protein detection
Protein biochip/microarray technology for identification of biomarkers in body fluids
Toxicoproteomics
MicroRNA-based diagnstics
Molecular imaging
Functional MRI with nanoparticle contrast material
FDG-PET
Imaging combined with therapy of cancer
Optical imaging
Point-of-care diagnostics

increases with cycle number. There is an increased variation after transformation to linear values and increased risk of false negative results.

PCR-based methods are time-consuming and are used mostly in laboratory settings. For the practical development of personalized medicine, point-of-care testing is more suitable and rapid results are required, such as in the office or

out-patient management of infection, where identification of the microorganism is required before suitable treatment is prescribed and the patient leaves the office.

21.5.2
Analysis of Single Biological Molecules for Personalized Medicine

Direct analysis of single biological molecules is the key to the next generation of revolutionary technologies, which can detect individual molecules of DNA, RNA, and proteins. These methods include real-time PCR, digital PCR, emulsion PCR, rolling circle amplification technology, single-molecule imaging, and nanobiotechnology-based diagnostics. With the accuracy and sensitivity of direct measurements, they can replace traditional molecular biology techniques, which are hampered by the need for amplification. Additional advantages of these technologies include:

- sensitivity in the femtomolar range;
- no need for thermal cycling or enzymatic procedures, eliminating a major source of cost and bias;
- small sample material requirements;
- flexibility across sample types (e.g., DNA, RNA, and protein) and assays.

21.5.3
Molecular Imaging and Personalized Medicine

Positron emission tomography (PET) is the most sensitive and specific technique for imaging molecular pathways *in vivo* in humans. PET uses positron emitting radionuclides to label molecules, which can then be imaged *in vivo*. The inherent sensitivity and specificity of PET is the major strength of this technique. Indeed, PET can image molecular interactions and pathways, providing quantitative kinetic information down to sub-picomolar levels. Generally, the isotopes used are short-lived. Once the molecule has been labeled, it is injected into the patient. The positrons that are emitted from the isotopes then interact locally with negatively charged electrons and emit what is called annihilating radiation. This radiation is detected by an external ring of detectors. It is the timing and position of the detection that indicates the position of the molecule in time and space. Images can then be constructed tomographically, and regional time activities can be derived. The kinetic data produced provide information about the biological activity of the molecule.

Molecular imaging provides *in vivo* information in contrast to the *in vitro* diagnostics. Moreover, it provides a direct method for the study of the effect of a drug in the human body. The ability to image biological and pathological processes at a molecular level using PET imaging offers an unparalleled opportunity to radically reform the manner in which diseases are diagnosed and managed. The translation of this molecular imaging into clinical practice will impact upon

personalized medicine, particularly for the management of cancer and neurological disorders such as Alzheimer's disease.

21.5.4
Point-of-Care Diagnostics and Personalized Medicine

Point-of-care (POC) or near-patient testing involves analytical patient testing activities provided within the healthcare system, but performed outside the physical facilities of the clinical laboratories. POC does not require permanent dedicated space, but instead includes kits and instruments, which are either hand carried or transported to the vicinity of the patient for immediate testing at that site. Sites where POC may be performed include the doctor's office, the bedside in the case of hospitalized patients, the emergency room or a disaster or war zone. POC may be performed in the field for several other indications, including screening of populations for genetic disorders and cancer. The patients may even conduct the tests themselves at home. The most important application of molecular diagnostics is estimated to be at the POC and rapid results are required.

POC diagnosis is important for the development of personalized medicine. An example is management of infections requiring use of antibiotics in the emergency room or at the physician's office. For selection of the appropriate antibiotic to start the treatment, rapid tests are required that could provide the results within half an hour. Culture and sensitivity from the specimen obtained and even conventional PCR is not a practical option due to waiting time of a few hours or a few days. Another example of POC testing is in coronary care units of hospital emergency departments, where new cardiac biomarker tests can provide rapid results that physicians can use to make critical patient management decisions.

Hand-held diagnostic devices, biochips, and electrochemical devices for the detection of DNA are particularly suited to POC diagnostics. Protein biochips, particularly microfluidic immunoassays, appear to be likely to get to the POC first as several technical problems associated with use of nucleic acid biochips outside the laboratory are being worked out. Biochip and microfluidic technologies are also used for miniaturizing other laboratory tests such as cell count and automated immunoassays. Continued improvements in biosensor technology and miniaturization will increase the ability to test for many analytes at the POC.

21.6
Sequencing and Personalized Medicine

The term DNA sequencing refers to methods for determining the exact order of the 3 billion nucleotide bases – adenine, guanine, cytosine, and thymine – that make up the DNA of the 24 different human chromosomes. Following the development of dye based sequencing methods with automated analysis, DNA sequencing has become easier and faster. The rapid speed of sequencing attained with

modern DNA sequencing technology has been instrumental in the sequencing of the human genome.

Personalized genome sequencing is expected become an integral part of personalized medicine as the cost comes down. Sequencing will also lead to the development of many diagnostic assays that will contribute to personalized medicine. Simple-to-operate and affordable small sequencers can be integrated in point-of-care diagnostics for personalized medicine.

21.6.1 Sequencing of Variations in Human Genomes

In various parts of the human genome, some people have segments of DNA sequence that other people do not have. Large genetic regions may be flipped in one person compared with another and these differences can influence a person's susceptibility to various diseases. These data provide a standard for genotyping platforms and a prelude to future individual genome sequencing projects. The results also indicate that the human genome sequence is still incomplete, that sequencing of additional genomes will be required to fill the remaining gaps. The eight people studied are part of a much larger group whose genomes will be sequenced as part of the 1000 Genomes Project, an international effort to sequences the genomes of people from around the world.

In order to understand structural variation, it is also essential to develop new technologies designed to detect genetic differences among people. For example, SNP biochips, whether used in research or in clinical applications, need to reflect this SV to find links between particular gene variants and diseases. Currently available biochips would miss an association for nearly half of these sites. Besides their potential applications, the results provide a wealth of data from eight reference human genomes to explore new hypotheses and make discoveries.

21.6.2 Study of Rare Variants in Pinpointing Disease-Causing Genes

Genome-wide association studies (GWAS) use gene chips in automated systems that analyze sites where SNPs tend to occur. Use of these SNP chips over the past decade for comparing DNA samples of healthy subjects and patients has revealed SNPs that associate with common complex diseases. However, SNPs investigated by the gene chips do not themselves cause a disease, but instead serve as a marker linked to the actual causal mutations that may reside in a nearby region. After a GWAS finds SNPs linked to a disease, a "fine-mapping" study is needed by additional genotyping, that is, sequencing of the gene regions near the SNP signal, to uncover an altered gene that harbors a mutation responsible for the disease.

GWAS have been successful in identifying disease susceptibility loci, but pinpointing of the causal variants in subsequent fine-mapping studies remains a challenge. A conventional fine-mapping effort starts by sequencing dozens of

randomly selected samples at susceptibility loci to discover candidate variants, which are then placed on custom arrays, and algorithms are used to find the causal variants. A study challenges the prevailing view that common diseases are usually caused by common gene variants (mutations); rather the culprits may be numerous rare variants, located in DNA sequences farther away from the original "hot spots" that are usually examined [12]. Thus causal variants may not be easily unveiled by conventional efforts, as one or several rare or low-frequency causal variants can hitchhike the same common tag SNP. The authors of this study demonstrated that sequencing of DNA in subset of patients most likely to carry causative mutations leads to identification of more actual mutations. This refined technique may identify individuals more likely to have mutations in causal genes. This approach will facilitate personalized medicine, in which treatment will be tailored to an individual's genetic profile. Identifying causal variants in disease genes provides an opportunity to develop drugs to rectify the biological consequences of these mutated genes.

21.7
Role of Biochips/Microarrays in the Development of Personalized Medicine

Biochip is a broad term indicating the use of microchip technology in molecular biology and can be defined as arrays of selected biomolecules immobilized on a surface. This technology has been described in more detail elsewhere [10]. The DNA microarray is a rapid method of sequencing and analyzing genes. An array is an orderly arrangement of samples. The sample spot sizes in the microarray are usually less than 200 microns in diameter. It is comprised of DNA probes formatted on a microscale (biochips) plus the instruments needed to handle samples (automated robotics), read the reporter molecules (scanners), and analyze the data (bioinformatic tools).

Microarrays enable one to view subtle changes in many genes at one time. They provide a snapshot of what genes are expressed or active, in normal and diseased cells. Patterns of gene expression can emerge when normal cells or tissues are compared with those known to be diseased, enabling classification of the severity of the disease and identification of genes that can be targeted for therapy. Thus microarrays can be used to develop personalized medical treatments.

Protein biochips/microarrays are well-established tools for research and some products for *in vitro* diagnostics are available commercially. Profiling proteins on biochips will be useful for distinguishing the proteins of normal cells from early-stage cancer cells, and from malignant metastatic cancer cells. In comparison with the DNA microarrays, the protein microarrays offer the possibility of developing a rapid global analysis of the entire proteome leading to protein-based diagnostics and therapeutics. Protein microarrays are reliable tools for multiple biomarkers with only a minimal amount of sample and have enormous potential in applications for personalized medicine [13]. Applications of biochips/microarrays in personalized medicine are listed in Table 21.3.

Table 21.3 Applications of biochip technology relevant to personalized medicine. © Jain PharmaBiotech.

Rapid DNA sequencing
Drug discovery and development
High-throughput drug screening
Design and stratification of clinical trials
Drug safety: applications in pharmacogenetics
Toxicogenomics
Clinical drug safety
Molecular diagnostics
Genetic screening
Detection of mutations
Inherited disorders
Identification of pathogens and resistance in infections
Molecular oncology
Cancer prognosis
Cancer diagnosis
Pharmacogenomics
Gene identification
Genetic mapping
Gene expression profiling
Detection of single nucleotide polymorphisms
For storage of the patient's genomic information
Integration of diagnosis and therapeutics

21.8 Role of Cytogenetics in the Development of Personalized Medicine

The term "cytogenetics" has been traditionally used for studies of the cellular aspects of heredity. It has been used mainly to describe the chromosome structure and identify abnormalities related to disease. Because of its emerging role in diagnosing disease at the molecular level, cytogenetics is now an important part of molecular diagnostics and can be referred to as molecular cytogenetics. The limitations of conventional banding analysis in the accurate diagnosis and interpretation of certain chromosome abnormalities have largely been overcome by these new technologies, which include fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and multicolor FISH. CGH reveals chromosomal locations of CNVs in the DNA segments of the study genome and has a role in clinical cytogenetics for detection and identification of unbalanced chromosomal abnormalities. Array CGH (aCGH) has a 1000-fold better resolution than chromosome karyotyping, which is limited to 5–10 million bases, and brings it down to the level of hundreds of bases. aCGH measures CNVs at multiple loci simultaneously, providing an important tool for studying cancer and developmental disorders and for developing diagnostic and therapeutic targets.

Clinical applications include diagnosis of microdeletion and microduplication syndromes, detection of subtelomeric rearrangements in idiopathic mental

retardation, identification of marker and derivative chromosomes, prenatal diagnosis of trisomy syndromes, and gene rearrangements as well as gene amplification in tumors. Molecular cytogenetic methods have expanded the possibilities for precise genetic diagnoses, which are extremely important for personalized management of patients.

Cytogenetics is a part of the broader field of "cytomics," which means that the structural and functional information is obtained by molecular cell phenotype analysis of tissues, organs, and organisms at the single cell level. This is done through image or flow cytometry in combination with bioinformatic knowledge extraction concerning the nucleic acids, proteins, and metabolites (cellular genomics, proteomics, and metabolomics) as well as cell function parameters, such as intracellular pH, transmembrane potentials or ion gradients.

Cytomics at the single-cell level, in conjunction with data-pattern analysis, high-content screening by image analysis or flow cytometry of clinical cell- or tissue-section samples, provides differential molecular profiles for the personalized prediction of therapy-dependent disease progression in patients. The molecular reverse-engineering of these molecular profiles, which is the exploration of molecular pathways, backwards, to the origin of the observed molecular differentials, by systems biology has the potential to detect new drug targets in knowledge spaces, typically inaccessible to traditional hypotheses [14]. Furthermore, predictive medicine, by cytomics in stratified patient groups, opens a new route not only to personalized medicine but also for the early detection of adverse drug reactions in patients.

21.9
Role of "Omics" in Personalized Medicine

There are several "omics" that play a role in the development of personalized medicine. Only the most important of these will be briefly described: pharmacogenetics, pharmacogenomics, pharmacoproteomics, and pharmacometabolomics.

21.9.1
Role of Pharmacogenetics

Pharmacogenetics, a term recognized in pharmacology in the pre-genomic era, as the study of the influence of genetic factors on the action of drugs as opposed to genetic causes of disease. Now it is the study of the linkage between the individual's genotype and the individual's ability to metabolize a foreign compound. However, variability in drug response among patients is multifactorial, including environmental, genetic, and disease determinants that affect the disposition of the drug. Individual variation in response to drugs is a substantial clinical problem. Such variations include failure to respond to a drug, adverse drug reactions (ADRs) and drug–drug interactions when several drugs are taken concomitantly.

The term molecular toxicology covers the use of molecular diagnostic methods for studying the toxic effects of drugs. Toxicology studies are an important part of the drug development process. During preclinical testing, pharmacogenetics methods can be applied to determine drug toxicity at the molecular level during animal studies or to provide an alternative to *in vitro*/*in vivo* assays. A number of assays have been developed to assess toxicity, carcinogenicity, and other genetic responses that arise when living cells are exposed to various chemical compounds. Two important categories of molecular toxicology are: toxicogenomics (use of genomic technologies for the study of toxicology) and toxicoproteomics. The object of these studies is to detect suitable drug candidates at an early stage of the discovery process and to reduce the number of failures in later stages of drug development.

Predicted clinical developments from application of pharmacogenetics are:

- **Establishment of prescribing guidelines**, based on clinical studies, for drugs that are subject to substantial polymorphic metabolism.
- **Prescribing advice** will relate dose to genotype and will highlight the possibility of drug interactions when multiple drugs are prescribed concomitantly.
- **Establishment and recording of individual patient genotypes**, that is, "personal pharmacogenetic profiles."
- **Pharmacogenetic testing** will substantially reduce the need for hospitalization, and its associated costs, because of adverse drug reactions.
- **Development of new drugs for patients with specific genotypes**, that is, "drug stratification."

The number of polymorphisms identified in genes encoding drug metabolizing enzymes, drug transporters, and receptors is rapidly increasing. In many cases, these genetic factors have a major impact on the pharmacokinetics and pharmacodynamics of a particular drug and thereby influence the sensitivity to such drug in an individual patient with a certain genotype. The highest impact is seen for drugs with a narrow therapeutic index, with important examples emerging from treatment with antidepressants, oral anticoagulants, and cytostatics, which are metabolized by CYP4502D6, CYP2C9, and TPMT, respectively. Many of the genes examined in early studies were linked to single-gene traits, but future advances hinge on the more difficult challenge of elucidating multigene determinants of drug response. In order to apply the increasing amount of pharmacogenetic knowledge to clinical practice, specific dosage recommendations based on genotypes will have to be developed to guide the clinician, and these recommendations will have to be evaluated in prospective clinical studies. Such development will lead to personalized medicines, which would be more efficient and will result in fewer adverse drug reactions.

21.9.2 Role of Pharmacogenomics

Pharmacogenomics implies the use of genetic sequence and genomics information in patient management to enable therapy decisions. The genetic sequence

and genomics information can be that of the host (normal or diseased) or of the pathogen. Pharmacogenomics will have an impact on all phases of drug development – from drug discovery to clinical trials. It will also apply to a wide range of therapeutic products including bioengineered proteins, cell therapy, antisense therapy, and gene therapy. These treatments are also subject to constraints and complexities engendered by individual variability.

Pharmacogenomics promises to enable the development of safer and more effective drugs by helping to design clinical trials such that non-responders would be eliminated from the patient population and take the guesswork out of prescribing medications. It will also ensure that the right drug is given to the right person from the start. In clinical practice, doctors could test patients for specific SNPs known to be associated with non-therapeutic drug effects before prescribing in order to determine which drug regimen best fits their genetic makeup. Pharmacogenomic studies are rapidly elucidating the inherited nature of these differences in drug disposition and effects, thereby enhancing drug discovery and providing a stronger scientific basis for optimizing drug therapy on the basis of each patient's genetic constitution.

21.9.3
Role of Pharmacoproteomics

The termed "pharmacoproteomics" was coined to describe the role of proteomics in drug development can [15]. There is an increasing interest in proteomics technologies now because DNA sequence information provides only a static snapshot of the various ways in which the cell might use its proteins, whereas the life of the cell is a dynamic process. Proteomic technologies are described in detail elsewhere [16]. Proteomics-based characterization of multifactorial diseases may help to match a particular target-based therapy to a particular marker in a subgroup of patients. Individualized therapy may be based on differential protein expression rather than a genetic polymorphism. Advantages of application of pharmacoproteomics in personalized medicine are:

- Pharmacoproteomics is a more functional representation of patient-to-patient variation than that provided by genotyping.
- Because it includes the effects of post-translational modification, pharmacoproteomics connects the genotype with the phenotype.
- By classifying patients as responders and non-responders, this approach may accelerate the drug development process.

21.9.4
Role of Pharmacometabolomics

The human metabolome is best understood by analogy to the human genome, that is, where the human genome is the set of all genes in a human, the human metabolome is the set of all metabolites in a human. In a systems biology approach,

metabolomics provides a functional readout of changes determined by genetic blueprint, regulation, protein abundance and modification, and environmental influence. Metabolomics is the study of the small molecules, or metabolites, contained in a human cell, tissue or organ (including fluids) and involved in primary and intermediary metabolism.

In view of the chemical and physical diversity of small biological molecules, the challenge remains of developing protocols to gather the whole "metabolome."

No single technique is suitable for the analysis of different types of molecules, which is why a mixture of techniques has to be used. The technologies used in metabolomics include nuclear magnetic resonance, direct infusion mass spectrometry (MS), and/or infrared spectroscopy. Gas chromatography–MS and liquid chromatography–MS technologies achieve a lower sample throughput but provide definite identification and quantification of individual compounds in complex samples.

Metabolomic technologies enable analysis of large arrays of metabolites for extracting biochemical information that reflects true functional end-points of biological events, whereas functional genomics technologies and proteomics merely indicate the potential cause for phenotypic response. Therefore, they cannot necessarily predict drug effects, toxicological response or disease states at the phenotype level unless functional validation is added. Metabolomics bridges this information gap by depicting in particular such functional information, as metabolite differences in biological fluids and tissues provide the closest link to the various phenotypic responses. Such changes in the biochemical phenotype are of direct interest to the biopharmaceutical industry and hence the justification for the term "pharmacometabolomics," which covers the use of metabolomic technologies to identify biomarkers for disease as well as off-target side effects in marketed drugs and new chemical entities in development.

Compared with 25 000 genes and approximately a million proteins, there are only 2500 metabolites. Their limited number enables an easier, more quantitative method of analysis. Examination of a sample using multiple mass spectrometry based technologies, integration of the data, and analysis by proprietary software and algorithms enables faster and more accurate understanding of a disease than previously possible. Plasma samples obtained from patients can be analyzed for signatures of neurodegenerative disorders by measuring the spectrum of biochemical changes and mapping these changes to metabolic pathways. The advantages of metabolomics technologies are:

- **Ability to analyze all bodily fluids** such as blood, CSF, and urine as well as cultured or isolated cells and biopsy material.
- **High throughput capability** enabling simultaneous monitoring of biological samples.
- **Analysis of multiple pathways and arrays** of metabolites simultaneously from microliter sample amounts.

A major factor underlying inter-individual variation in drug effects is variation in metabolic phenotype, which is influenced not only by genotype but also by

environmental factors such as nutritional status, the gut microbiota, age, disease, and the co- or pre-administration of other drugs. Thus, although genetic variation is clearly important, it seems unlikely that personalized drug therapy will be enabled for a wide range of major diseases using genomic knowledge alone. Metabolite patterns that are characteristic of the individual can be used to diagnose diseases, predict an individual's future illnesses, and their responses to treatments. A clear connection has been demonstrated between an individual's metabolic phenotype, in the form of a pre-dose urinary metabolite profile, and the metabolic fate of a standard dose of the widely used analgesic acetaminophen [17]. Pre-dose and post-dose urinary metabolite profiles were determined by NMR spectroscopy. It is hoped that metabolomic profiling would be included in personalized medicine.

21.10
Role of Nanobiotechnology for the Development of Personalized Medicine

Nanotechnology is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer-length scale, that is, at the level of atoms, molecules, and supramolecular structures. It is the popular term for the construction and utilization of functional structures with at least one characteristic dimension measured in nanometers (10^{-9} m). Application of nanobiotechnology in molecular diagnostics is termed "nanodiagnostics" [18]. Because DNA, RNA, proteins, and their functional subcellular scaffolds and compartments, are in the nanometer scale, the potential of a single-molecule analysis approach would not be fully realized without the help of nanobiotechnology. Advances in nanotechnology are providing nanofabricated devices that are small, sensitive, and inexpensive enough to facilitate direct observation, manipulation, and analysis of a single biological molecule from a single cell. This presents new opportunities and provides powerful tools for nanomedicine, which includes various applications of nanobiotechnology in healthcare, including nanodiagnostics, nanopharmaceuticals, and nanosurgery [19]. Nanobiotechnology has refined many of the technologies used in healthcare. Advances in nanobiotechnology will facilitate the development of personalized medicine by achieving the following [20]:

- Improving the sensitivity and extending the present limits of molecular diagnostics/molecular imaging.
- Integration of information from nanotechnology-based detection of biomarkers, point-of-care devices, nanochips, and nanobiosensors.
- Enabling targeted drug delivery by use of nanoparticles.
- Integration of diagnosis and therapy.

Some pharmacogenetic assays based on the nanotechnology have been approved by the FDA. Two examples are: (i) Verigene® (Nanosphere Inc), a nucleic acid test to detect variants of CYP2C9 and VKORC1 genes, responsible for sensitivity

to the anticoagulant warfarin; and (ii) Verigene® F5/F2/MTHFR (methylenetetrahydrofolate reductase) nucleic acid test, which detects disease-associated gene mutations that can contribute to blood coagulation disorders and difficulty in metabolizing folate.

Nanotechnology-based devices that provide site-specific drug delivery and efficient monitoring of drug effects may contribute to the development of personalized medicine. Examples have been given in various therapeutic areas in preceding sections. Personalization of cancer therapies is based on a better understanding of the disease at the molecular level and nanotechnology will play an important role in this area. Biomarkers, discovered by application of nanobiotechnology, are used to diagnose and treat cancer based on the molecular profiles of individual patients. The most important feature of personalization of cancer therapy is use of the same nanoparticles for diagnosis as well as therapy.

The future of cardiovascular diagnosis is already being impacted by nanosystems that can both diagnose pathology and treat it with targeted delivery systems. The potential dual use of nanoparticles for both imaging and site-targeted delivery of therapeutic agents to cardiovascular disease offers great promise for individualizing therapeutics. Image-based therapeutics with site-selective agents should enable verification that the drug is reaching the intended target and a molecular effect is occurring.

21.11
Systems Biology and Personalized Medicine

Systems biology is defined as the biology of dynamic interacting networks. It is also referred to as pathway, network, or integrative biology. The US National Institute of General Medical Sciences has defined systems biology as "an integrated experimental, informational, and computational science" that has "benefited from advances in genomics, proteomics, metabolomics, and other high-throughput technologies and is driven by innovations in computational analysis and simulation." Systems biology attempts to study biological systems in a holistic rather than an atomistic manner [21]. Ideally, this involves gathering dynamic and global datasets as well as phenotypic data from different levels of the biological information hierarchy, integrating them, and modeling them graphically and/or mathematically to generate mechanistic explanations for the emergent systems properties. This requires that the biological frontiers drive the development of new measurement and visualization technologies and the pioneering of new computational and mathematical tools – all of which requires a cross-disciplinary environment composed of biologists, chemists, computer scientists, engineers, mathematicians, physicists, and physicians speaking common discipline languages.

The combination of high-throughput methods of molecular biology with advanced mathematical and computational techniques has made it possible to screen and analyze the expression of entire genomes, to simultaneously assess

large numbers of proteins and their prevalence, and to characterize in detail the metabolic state of a cell population. Complementing large-scale assessments, there are more subtle analyses that rationalize the design and functioning of biological modules in exquisite detail. This intricate side of systems biology aims at identifying the specific roles of processes and signals in smaller, fully regulated systems by computing what would happen if these signals were lacking or organized in a different fashion.

An analysis of the structure and dynamics of the network of interacting elements provides insights that are not obvious from analysis of the isolated components of the system. Systems biology can facilitate the development of personalized medicine by identification of the biological networks in which SNPs associated with the response to therapy exert their influence. It may help in determining how SNPs modify key biological processes such as cell differentiation, apoptosis, and cell communication. Identification of the role of multiple SNPs in modifying the function of signaling pathways, which are implicated in complex disease pathogenesis, may enable development of interventions that are required to change from the non-responder to the responder status of a patient.

Systems biology will have a major role in creating a predictive, personalized, preventive, and participatory (P4) approach to medicine [22]. It will also facilitate the transfer of technologies relevant to personalized medicine from the preclinical to clinical phase. The systems biology concept has been applied to other sciences relevant to personalized medicine: systems pathophysiology of diseases and systems pharmacology. Systems pharmacology seeks to develop a global understanding of the interactions between pathophysiology and drug action [23]. It will enable an understanding of adverse effects of drugs by considering targets in the context of the biological networks in which they exist. Experimental and computational approaches allow systems pharmacology to obtain holistic, mechanistic information on disease networks and drug responses, and to identify new drug targets and specific drug combinations. Systems pharmacology will integrate pharmacogenetics, pharmacogenomics, and pharmacoproteomics. The concept of systems biology is applied to systems medicine and is relevant to personalized medicine. Systems approaches are starting to provide deeper insights into the mechanisms of human diseases, and to facilitate the development of better diagnostic and prognostic biomarkers for cancer and many other diseases.

21.12
Personalized Biological Therapies

Historically, blood transfusion and organ transplantation were the first personalized therapies as they were matched to the individuals. Some cell therapies that use patient's own cells are considered to be personalized medicines, particularly vaccines prepared from the individual patient's tumor cells. More recently recombinant human proteins might provide individualization of therapy.

21.12.1
Recombinant Human Proteins

There are a large number of therapeutic proteins approved for clinical use and many more are undergoing preclinical studies and clinical trials in humans. Most of them are human or "humanized" recombinant molecules. Virtually all therapeutic proteins elicit some level of antibody response, which can lead to potentially serious side effects in some cases. Therefore, immunogenicity of therapeutic proteins is a concern for clinicians, manufacturers, and regulatory agencies. Immune response to therapeutic proteins in conventional animal models has not been, except in rare cases, predictive of the response in humans. It is expected that computer driven prediction followed by *in vitro* and/or *in vivo* testing of any potentially immunogenic epitopes will help in avoiding, or at least minimizing, immune responses to therapeutic proteins. It is possible to develop recombinant proteins in combination with diagnostic tests to limit their use to patients where they are least likely to induce immune reactions.

Another approach to protein therapy is *in vivo* production of proteins by genetically engineered cells where the delivery of proteins can be matched to the needs of the patient and *in vivo* production and controlled delivery might reduce adverse effects.

21.12.2
Therapeutic Monoclonal Antibodies

Compared with small-molecule drugs, antibodies are less likely to cause toxicity based on factors other than the mechanism of action. They can be designed to be very specific with high affinity for the target and can combine diagnosis with therapy. Genetic engineering of antibodies to produce humanized monoclonal antibodies (hmAbs) has greatly advanced their utility in molecular targeting therapies. Many clinical trials of mAbs as a single agent, or in a combination protocol with current standard chemotherapy or immunoconjugates have shown promise in the treatment of specific diseases, particularly cancer. The accumulating results from many basic, clinical, and translational studies may lead to more individualized therapeutic strategies using these agents directed at specific genetic and immunologic targets.

21.12.3
Cell Therapy

Cell therapy is the prevention or treatment of human disease by the administration of cells that have been selected, multiplied, and pharmacologically treated or altered outside the body (*ex vivo*). The aim of cell therapy is to replace, repair or enhance the function of damaged tissues or organs. The cells used can originate from the patient or from a donor or from another species. Other sources include cell lines and cells from patients' tumors to make cancer

vaccines. Cells can be encapsulated in selectively permeable membranes that block entry of immune mediators but allow outward diffusion of active molecules produced by the cells. Genetic engineering of cells is part of *ex vivo* gene therapy. The cells may be introduced by various routes into the body and selectively implanted at the site of action. Problems of rejection of grafted cells can be solved by using the patient's own (autologous) cells and encapsulating cells from other sources.

Increasing potential of embryonic stem cells is now being realized. Adult stem cells of the individual patient are more suitable for personalized therapy [24]. Availability of technologies to derive induced pluripotent stem cells from adult somatic cells would enhance the potential of personalized cell-based therapy.

With the ability to isolate, expand, and study mesenchymal stem cells (MSCs) *in vitro*, individual patient's MSCs can be tested for their sensitivity to various drugs. For example, selection of individual dosing regimens can be based on the *in vitro* responsiveness in a simple assay performed using a patient's own MSCs.

21.12.4 Gene Therapy

Gene therapy is usually defined as the transfer of defined genetic material to specific target cells of a patient for the ultimate purpose of preventing or altering a particular disease state. However, the broad scope of gene therapy includes cells that may be genetically modified to secrete therapeutic substances, such as neurotrophic factors. *Ex vivo* gene therapy involves the genetic modification of the patient's cells *in vitro*, mostly by use of viral vectors, prior to re-implanting these cells into the tissues of the patient's body. This is a form of personalized therapy.

There are several opportunities for developing personalized cancer gene therapies based on knowledge of gene expression patterns of tumors, cancer pathways, and biomarkers.

21.12.5 RNA Interference

Gene silencing by RNA interference (RNAi) involves the use of a double-stranded RNA, which enters the cell and is processed into short, 21–23 nucleotide double stranded RNAs, termed small interfering RNAs (siRNAs) that are used in a sequence-specific manner to recognize and destroy complementary RNAs. The RNAi approach using oligonucleotide-based drugs may provide the required selectivity for allele-specific inhibition (ASI), where cancer cells are attacked at the site of loss of heterozygosity (LOH). siRNAs can not only be used as tools to study gene function, but might also be used as genotype-specific drugs to mediate ASI. siRNA has been shown to produce genotype-specific inhibition of tumor growth *in vivo*, by targeting an SNP in POLR2A (gene of the large subunit of RNA polymer-

ase II located in close proximity to the tumor suppressor gene p53, which frequently shows LOH in cancer cells [25]). Thus RNAi may play an important role in personalized medicine.

21.13 Personalized Vaccines

The next era in vaccinology will be ushered in by the new science of vaccinomics, which will enable the development of personalized vaccines, based on our increasing understanding of immune response phenotype/genotype information by applications of "omics" technologies. Two important areas for application of personalized vaccines are viral infections and cancer.

21.13.1 Personalized Vaccines for Viral Diseases

Many factors can contribute to the heterogeneity of vaccine-induced immune responses, including polymorphisms of immune response genes. Identification of genes involved directly or indirectly in the generation of the immune response to vaccines is important. Associations between SNPs in HLA class I and class II genes, cytokine, cell surface receptor, and Toll-like receptor genes and variations in immune responses to measles vaccine have been reported [26]. Such information may provide further understanding of genetic variations that influence the generation of protective immune responses to vaccines, and eventually the development of new vaccines. Rapid advances in developing personalized vaccines are already occurring for hepatitis B, influenza, measles, mumps, rubella, anthrax, and smallpox vaccines [27]. In addition, newly available data suggest that some vaccine-related adverse events may also be genetically determined and, therefore, predictable.

21.13.2 Personalized Cancer Vaccines

Cancer vaccines attempt to harness the specificity and resistance potentials of the human immune system. The aim of cancer vaccines is to stimulate the immune system to recognize, attack, and destroy tumor cells. In contrast to vaccines for prophylaxis of infectious diseases, cancer vaccines are therapeutic. There are several types of cancer vaccines, which include nucleic acid based, mAb-based and cell-based vaccines. Various types of cells are used including tumor cells and dendritic cells. Combination of different methods and genetic modification of cells are also used. Many of these vaccines can be personalized and the topic is discussed in more detail elsewhere [28]. A few examples are given here.

Autologous cancer vaccines are made from patients' own tumor tissues. This approach is based on the fact that antigenic fingerprint of each person's cancer

is unique and can never be duplicated in another person's cancer. Although cancers may arise by common mechanisms, that is, through mutations in genes implicated in cell transformation, they undergo additional random mutations in other genes. Several such vaccines are in development and differ in the technologies used for preparation. Melacine melanoma vaccine, which consists of lysed cells from two human melanoma cell lines combined with an adjuvant, is approved in Canada but not in the United States. It is the first cancer vaccine to be considered solely for patients with certain gene types, a sort of personalized vaccine. However, a true personalized vaccine will be one in which patient's own cells are used.

Development of active immunotherapy begins with the selection and modification of a tumor antigen specific to the cancer (prostatic acid phosphatase in prostate cancer), which is produced using recombinant DNA technology. The modified antigen is combined with antigen presenting cells that are isolated from a cancer patient and the activated cells are then re-administered to the patient to stimulate T-cells to recognize and attack cancer cells that carry the target antigen. The lead product in this category is sipuleucel-T (Provenge™), which targets the prostatic acid phosphatase. It is approved by the FDA for the treatment of patients with early-stage and advanced prostate cancer.

Multipeptide cancer vaccines offers many advantages, such as the possibility of bypassing tumor heterogeneity and selection of antigen-negative clones escaping peptide-specific immune responses, thus eliciting both CD4- and CD8-mediated immune recognition. Advances in antigen discovery technologies have enabled further optimization of peptide selection. With the ultimate goal of combining peptide selection with a patient-specific immunogenic profile, peptide based anticancer vaccines remain a promising personalized treatment for cancer patients, as shown by preclinical and clinical studies.

Patient-specific cancer vaccines involve generation of an antigen-specific response even when the tumor antigens are not known. A cell therapy product is created using a technique that fuses the patient's own tumor cells with powerful, immune-stimulating dendritic cells (DCs). The fusion product is then injected back into the patient with the goal of sparking a specific immune response against the cancer. This individualized cell therapy presents the full complement of antigens specific to the patient's tumor. This is the most promising approach. A personalized melanoma vaccine that fuses the patients' own melanoma cells with their own DCs to help the immune system to recognize and eradicate cancer cells is in clinical trials for metastatic melanoma.

21.14
Concluding Remarks and Future Prospects of Personalized Medicine

Considerable evidence has accumulated in the past decade to support the feasibility of personalized medicine, that is, the selection of the best treatment for an individual. Considerable contribution has been made by sequencing of the human

genome and development of molecular diagnostics. Several other "omics" technologies and other advances in new technologies, particularly nanobiotechnology, are making significant contributions to the development of personalized medicine, which is already being practiced at some institutions. The concept of personalized medicine is the best way to integrate and translate new technologies for improving healthcare.

Availability of low-cost genomic sequencing will expand the use of genomic information in the practice of medicine. Drugs will be targeted better to diseases in particular patients based on genotype information. Toxicity will be predictable in most cases prior to drug administration. By the end of the second decade of the 21st century, it is anticipated that the general population will have the opportunity to carry a chip card, like a credit card, with all the genetic information of the person coded on it. Such a database can be constructed by taking a blood sample of the individual, resequencing the functional DNA, and identifying the genetic variations in functional genes.

It is expected that within the next decade, we will have a better understanding of how to coat or chemically alter nanoparticles to reduce their toxicity to the body, which will allow us to broaden their use for disease diagnosis and for drug delivery. Nanodevices will be available to examine tissue in minute detail for better understanding of disease mechanisms in order to administer more effective therapies. Nanobiotechnology will enable therapeutic interventions at the cell level to correct the cause of disease. Construction of nanoscale computer-controlled molecular tools that are much smaller than a human cell will be possible and they will be built with the precision of drug molecules. Finally, nanobiotechnology will provide many tools for the implementation of personalized medicine in the second decade of the 21st century.

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22
Xenotransplantation in Pharmaceutical Biotechnology

Gregory J. Brunn and Jeffrey L. Platt

22.1
Introduction

For nearly a century, xenotransplantation, the transplantation of animal cells, tissues and organs into humans, has been seen as a potential way to replace the function of diseased or damaged organs. Application of xenotransplantation for this purpose has been blocked for the most part because xenografts confront a severe and destructive immune response. Genetic engineering of large animals has allowed the ability to add and remove genes from lines of animals, and other new technologies could address this barrier and physiologic and infectious barriers to xenotransplantation. These technologies might also expand potential applications of xenotransplantation.

22.1.1
Alternative Sources of Transplantable Tissues

No field of medicine has generated more excitement and more controversy than transplantation. Transplantation of foreign cells, including stem cells, has long been envisioned as a treatment for failure of the heart, kidney, liver, and lungs, for diabetes and for diseases of the blood and other tissues. The main limitation to applying transplantation for the treatment of disease is a shortage of human donors. This shortage limits the clinical application of organ transplantation to approximately 5% of the number of transplants that would be performed were the supply of organs unlimited [1, 2] and precludes widespread use of tissue transplants.

Possible solutions to the shortage of donor organs have garnered considerable interest and include use of artificial organs, "engineered tissues," stem cell transplants, and xenotransplants. Although some newer technologies have excited interest, xenotransplants of the heart, lung, kidney, and pancreatic islets function well enough to sustain life, and if feasible could offer the least-expensive approach. Enthusiasm for xenotransplantation also stems from the possibility that animal tissues and organs might be less susceptible to disease

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recurrence compared with allotransplants. Advances in cellular and molecular biology and in genetics open up possibilities for use of cells, tissues, and organs to address the complications of disease, not only by replacement of abnormal cells and tissues, but by use of transplanted tissues to impart novel physiological functions. In this regard and for some purposes, xenografts may be an ideal vehicle for introducing a novel gene or biochemical process that could be of value to the transplant recipient.

If interest in xenotransplantation is substantial, the hurdles to application are equally so. For the past three decades, the first and preeminent obstacle to transplanting organs and tissues between species has been the immune reaction of the host against the graft. A second, and still theoretical, hurdle is the possibility that beyond the immune barrier, there might be physiological limitations to the survival or function of a xenograft and the possibility that a xenotransplant might engender medical complications for the xenogeneic host. A third hurdle is the possibility that a xenograft might transfer infections to the host and that from the host such agents might spread to other members of society. This chapter will consider the current state of efforts to overcome the hurdles to xenotransplantation and will evaluate how genetic engineering might be applied to this end.

22.1.2 The Pig as a Source of Tissues and Organs for Clinical Xenotransplantation

Although it might be intuitive that nonhuman primates would be the best source of xenogeneic tissues for clinical transplantation, most research today focuses on the pig. The reasons for favoring the pig as a xenotransplant source include the availability of pigs in large numbers, the ease with which the pig can be bred, the limited risk of zoonotic disease engendered by the use of pigs, and the possibility of introducing new genes into the germline of the pig.

Recently, lines of pigs that might be favorable for transplantation have been generated by breeding through adding anti-inflammatory genes and by targeting genes that generate a key antigen [3–5]. Newer technologies will improve the efficacy of genetic engineering and provide other ways to break down barriers to xenotransplantation. Accordingly, these barriers are considered in detail.

One of the major obstacles to testing genetic manipulation of pigs as an approach to xenotransplantation has been the difficulty in generating mutant pigs. Recent advances may overcome this limitation [6] using sperm-mediated gene transfer to incorporate the human decay accelerating factor (hDAF) gene into pigs, obtaining a high efficiency of transgenesis (80% of pigs incorporated hDAF into the genome) and hDAF expression (43% of the transgenic pigs). This method in theory could be used to introduce multiple genes at once, or a tailor-made set of human genes that may be useful for transplant-mediated genetic therapies, which has been discussed elsewhere [7] (Table 22.1).

Table 22.1 Genetic engineering in xenotransplantation: conventional gene therapy versus transgenic therapy versus cloning.

	Conventional gene therapy	Conventional transgenic techniques	Cloning
Delivery	Vector or vehicle required	Injection of genetic material directly into pro-nuclei of fertilized egg	Transfection of cultured somatic cells
Expression	Dependent on ability of each cell to take up genetic material Requires treatment for every transplant or recipient	Genetic material introduced into the germline, leading to expression in a line of animals	Genetic material introduced into the germline, leading to expression in a line of animals
	May require repeated treatment	One manipulation	One manipulation
Immunogenicity	Delivery vehicle or transgene may be immunogenic	The transgene may elicit immune response	The transgene may elicit immune response
Target of genetic manipulation	The recipient and the graft may be transduced	Genetic manipulation of the donor only	Genetic manipulation of the donor only
Genetic manipulation	Gene addition Dominant negative	Gene addition Dominant negative	Gene addition Dominant negative Gene knockout

22.2 Biological Barriers to Xenotransplantation

22.2.1 Graft Vascularization

All xenografts elicit an immune response. This response includes antibodies, cell-mediated immunity, natural killer cells, and inflammation [8]. However, the fate of xenografts confronted by immune responses is dictated in large part by the way in which the graft receives its vascular supply (Figure 22.1). Isolated cells, such as hepatocytes, and "free" tissues, such as pancreatic islets and skin, derive their vascular supply through the in-growth of host blood vessels. The process of neovascularization, as such, might be impaired in a xenograft by incompatibility of donor growth factors with the host microvasculature, and this impairment might cause a cell or tissue xenograft to fail. Once the vascular supply is established, however, a xenogeneic cell or tissue graft is susceptible mainly to cellular immunity, leading to cellular rejection, but is protected by the blood vessels from the recipient's antibodies. Whole-organ grafts in contrast have their own "foreign" vascular tree. These foreign blood vessels can be attacked by cellular immunity and also by antibodies. Indeed, in organ xenografts, rejection caused by antibodies is the most difficult barrier to surmount.

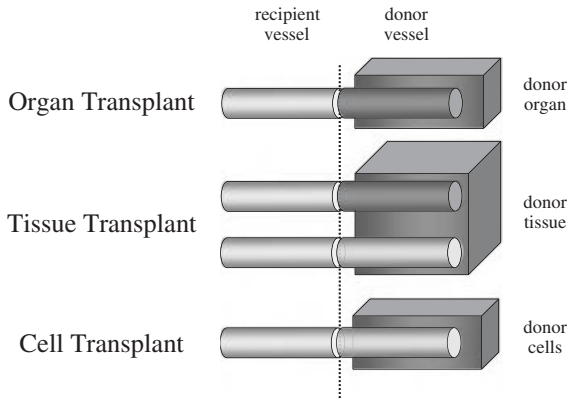

Figure 22.1 Mechanisms of xenograft vascularization. Organ xenografts receive recipient blood exclusively through the donor blood vessels (top). Free tissue xenografts (e.g., pancreatic islets and skin) are vascularized partly by the in-growth of recipient blood vessels and partly by spontaneous anastomosis of donor and recipient capillaries (middle). Cellular xenografts (e.g., hepatocytes and bone marrow cells) are vascularized by the in-growth of recipient blood vessels (bottom).

22.2.2 Hyperacute Rejection

Hyperacute rejection is caused by anti-graft antibodies existing in the recipient at the time of transplant. This type of rejection begins immediately as the blood of the recipient perfuses the graft. Hyperacute rejection destroys the graft within minutes to a few hours and is characterized histologically by interstitial hemorrhage and thrombosis, the thrombi consisting mainly of platelets [9].

Research over the past decade has clarified the molecular basis for the hyperacute rejection of pig organs by primates [10, 11], and this knowledge has led to the development of new and incisive therapeutic approaches to averting this problem. Hyperacute rejection of porcine organ xenografts by primates is initiated by the binding of xenoreactive natural antibodies to the graft [9, 12–14]. Xenoreactive natural antibodies are present in the circulation without a known history of sensitization [15]. Contrary to expectations, xenoreactive antibodies are predominantly directed against only one antigen, a saccharide consisting of terminal Galα1,3Gal [16–19]. The importance of Galα1,3Gal as the primary antigenic barrier to xenotransplantation was demonstrated first by experiments in which specific depletion of anti-Galα1,3Gal antibodies using immunoaffinity columns [20] prevented immediate rejection of porcine hearts.

Although the identification of the relevant antigen for pig-to-primate xenotransplantation allows specific depletion of the offending antibodies, more enduring and less intrusive forms of therapy would be preferred. The most obvious approach to developing animals with organs resistant to host antibodies would be to genetically target or "knock out" the enzyme α1,3-galactosyltransferase, which catalyzes

synthesis of Galα1,3 [21]. This gene has been knocked out in mice [22] and in pigs [3], demonstrating that absence of the enzyme is not lethal. Prather and colleagues [23] and the group working with Ayares [24] have used similar strategies to disrupt one allele of the α1,3GT gene (GGTA1) in pigs by first targeting the gene for disruption in fetal porcine fibroblasts. Selected clones were used as nuclear donors for enucleated pig oocytes, the resulting embryos implanted into surrogate gilts. Recently, cloned pigs harboring a functional knockout of both alleles of α1,3GT were generated [3]. These pigs are miniature swine with homozygous deletion of the α1,3GT gene ($\alpha1,3GT^{-/-}$), do not express Galα1,3Gal epitopes on any tissue and, as might be expected, make anti-Galα1,3Gal reactive natural antibodies that are cytotoxic to cells from $\alpha1,3GT^{+/+}$ pigs. Organs from these pigs do not undergo hyperacute rejection after transplant into nonhuman primates [25, 26].

22.2.3 Complement Activation

A second step in the development of hyperacute rejection is activation of the complement system of the recipient on donor blood vessels [13]. Complement activation is triggered by the binding of complement fixing xenoreactive antibodies, such as natural antibodies directed against Galα1,3Gal, to graft endothelium, and, to a smaller extent perhaps, by reperfusion injury. Regardless of the mechanism leading to complement activation, a xenograft is extraordinarily sensitive to complement-mediated injury because of multiple defects in the regulation of complement (Figure 22.2) [27–29]. Under normal circumstances, the complement

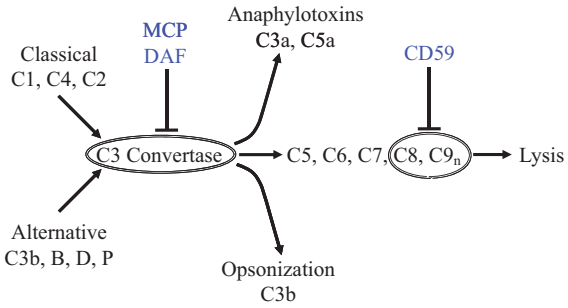

Figure 22.2 Regulation of the complement system. The complement cascade, which can be activated via the classical or alternative pathway, is regulated under normal circumstances by various proteins in the plasma and on the cell surface. Three of the cell-surface complement regulatory proteins are shown here. Decay-accelerating factor (DAF) and membrane cofactor protein (MCP) regulate complement activation by dissociating or promoting the degradation of C3 convertase. CD59, also known as protectin, prevents the functions of terminal complement complexes by inhibiting C8 and C9. An organ graft transplanted into a xenogeneic recipient is especially sensitive to complement-mediated injury because decay-accelerating factor, membrane cofactor protein, and CD59 expressed on the xenograft endothelium cannot effectively regulate the complement system of the recipient.

cascade is regulated or inhibited by various proteins in the plasma and on the surface of cells. These proteins protect normal cells from suffering inadvertent injury during the activation of complement. The proteins that regulate the complement cascade function in a species-restricted fashion; that is, complement regulatory proteins inhibit homologous complement far more effectively than heterologous complement [28, 30]. Accordingly, the complement regulatory proteins expressed in a xenograft are ineffective at controlling the complement cascade of the recipient, and the graft is subject to severe complement-mediated injury [27].

To address this problem, pigs transgenic for human complement regulatory proteins have been generated as potential sources of xenografts (Figure 22.2) [28, 31, 32]. Animals transgenic for human decay accelerating factor, which regulates complement at the level of C3, together with CD59, which regulates complement at the level of C8 and C9 [32] or CD46, which controls complement activation at the level of C3 and C4 [33], demonstrate that the expression of even low levels of human decay accelerating factor and CD59 or CD46 in porcine-to-primate xenografts prevents hyperacute rejection [4, 34]. These results, and the dramatic prolongation of xenograft survival achieved by expressing higher levels of human decay accelerating factor [35], underscore the importance of complement regulation as a determinant of xenograft outcomes.

22.2.4
Acute Vascular Rejection

If hyperacute rejection of an organ xenograft is averted, acute vascular or antibody mediated rejection, so named because of its resemblance to antibody mediated rejection of allografts [36, 37], may ensue. Acute vascular rejection (sometimes also called delayed xenograft rejection) can begin within 24 h of reperfusion and usually leads to graft destruction over the following days and weeks [36, 38, 39]. Acute vascular rejection is triggered by the binding of xenoreactive antibodies to the graft. Removal of antidonor antibodies from the circulation of recipients can prevent acute vascular rejection [40]. Delivery of antidonor antibodies to the recipients of a functioning graft induces acute vascular rejection [41]. When organs of swine and other non-primates are transplanted into higher primates, acute vascular rejection is caused by antibodies directed against Galα1,3Gal [42, 43], at least initially.

Regardless of the cause, however, acute vascular rejection is associated with intravascular coagulation caused by the activation of endothelial cells in the transplant [36, 44, 45]. Activated endothelial cells express procoagulant molecules, such as tissue factor, and proinflammatory molecules, such as E-selectin and cytokines [46, 47]. The pathogenesis of acute vascular rejection is summarized in Figure 22.3.

Acute vascular rejection poses a difficult therapeutic challenge. The various possible approaches for combating acute vascular rejection are listed in Table 22.2. Acute vascular rejection is sometimes treated effectively by plasmapheresis. Such treatment in conjugation with immune depletion of antibodies can prevent acute vascular rejection of xenografts [40]. Although useful experimentally, depletion of

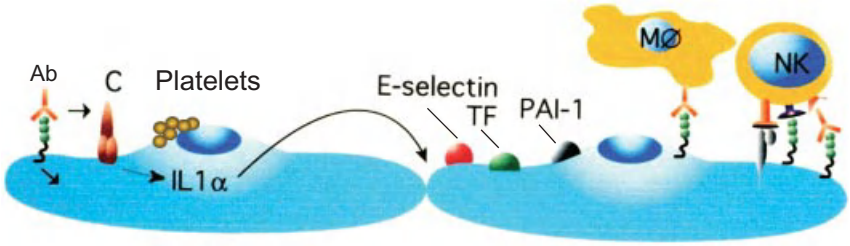

Figure 22.3 Pathogenesis of acute vascular rejection. Activation of endothelium by xenoreactive antibodies (Ab), complement (C), platelets, and perhaps by inflammatory cells (natural killer (NK) cells and macrophages (MØ)) leads to the expression of new pathophysiologic properties. These new properties, such as the synthesis of tissue factor (TF) and plasminogen activator inhibitor type 1 (PAI-1), promote coagulation; the synthesis of E-selectin and cytokines such as IL-1α promote inflammation. These changes in turn cause thrombosis, ischemia, and endothelial injury, the hallmarks of acute vascular rejection. (Adapted from *Nature* (1998), **392** (Suppl.), 11–17, with permission.)

Table 22.2 Therapeutic strategies for acute vascular xenograft rejection.

Possible mechanism targeted	Manipulation of	
	Recipient	**Donor**
Antibody–antigen interaction	Specific depletion of xenoreactive antibodies	Generating transgenic pigs with low levels of antigen
	Prevention of xenoreactive antibody synthesis (e.g., cyclophosphamide, leflunomide)	Generation of pig clones lacking antigen
Complement activation	Systemic anticomplement therapy (e.g., CVF, sCR1, gamma globulin)	Generation of donor pigs transgenic for human complement regulatory proteins
Endothelial cell activation	Administration of anti-inflammatory agents	Inhibition of NFκB function Introduction of protective genes
Molecular incompatibilities	Administration of inhibitors (e.g., inhibitors of complement or coagulation)	Introduction of compatible molecules

antibodies might fail because binding of a small amount of antibody from a graft can initiate acute vascular rejection [48]. Although antibodies directed against Galα1,3Gal can induce acute vascular rejection [49] the organs of α1,3-galactosyltransferase-deficient pigs are still susceptible to this condition [50].

Expression of human complement regulatory proteins may help prevent acute vascular rejection. Preliminary studies suggest that interfering with the antigen–

antibody reaction and controlling the complement cascade may be sufficient to prevent acute vascular rejection for at least some period of time [40]. These goals were accomplished by using animals transgenic for human decay accelerating factor and CD59 as a source of organs and baboons depleted of immunoglobulin as recipients. Cozzi *et al.* [35] achieved prolonged survival of xenografts, presumably preventing acute vascular rejection, by using transgenic pigs expressing high levels of decay accelerating factor and cynomolgus monkeys treated with very high doses of cyclophosphamide. The immunosuppression perhaps prevented the synthesis of antidonor antibodies. McGregor *et al.* [51]. have achieved excellent results using hearts expressing CD46.

Work on rodents points to the potential involvement of natural killer (NK) cells and macrophages in mediating acute vascular rejection. However, the ability of immunoglobulin manipulation to prevent acute vascular rejection suggests that the involvement of NK cells and macrophages might be less important than *in vitro* studies and studies on rodents have suggested [31, 45]. On the other hand, NK cells might exacerbate the injury triggered by xenoreactive antibodies, as human NK cells have been shown to activate porcine endothelial cells *in vitro* [52–54].

22.2.5
Accommodation

Fortunately, the presence of antidonor antibodies in the circulation of a graft recipient does not inevitably trigger acute vascular rejection. If antidonor antibodies are temporarily depleted from a recipient, an organ transplant can be established so that rejection does not ensue when the antidonor antibodies are returned to the circulation [55]. This phenomenon is referred to as "accommodation" [28]. Accommodation may reflect a change in the antibodies, in the antigen, or in the susceptibility of the organ to rejection [56]. If accommodation can be established, it may be especially important in xenotransplantation because it would obviate the need for ongoing interventions to inhibit antibody binding to the graft. One potential approach to accommodation may be the use of genetic engineering to reduce the susceptibility of an organ transplant to acute vascular rejection and the endothelial-cell activation associated with it [45]. Unfortunately, successful intervention at the level of such effector mechanisms has yet to be achieved. However, disruption of antibody–antigen interaction has brought about accommodation in human subjects [44, 55].

22.2.6
Cellular Mediated Immune Responses

Organ transplants and cellular and free tissue transplants are subject to cellular rejection. In allotransplantation, cellular rejection is controlled by conventional immunosuppressive therapy, but there is concern that, for several reasons, cellular rejection may be especially severe in xenotransplants. Firstly, the great variety of

antigenic proteins in a xenograft may lead to recruitment of a diverse set of "xenoreactive" T-cells. Secondly, the binding of xenoreactive antibodies and activation of the complement system may amplify elicited immune responses [57, 58]. For example, deposition of complement in a graft may cause activation of antigen-presenting cells, in turn stimulating T-cell responses. Still another factor that might amplify the elicited immune response to a xenotransplant involves "immunoregulation," which ordinarily would circumscribe cellular immune responses, but may fail or be deficient across species. Such failure could reflect limitations in the recognition of xenogeneic cells or incompatibility of relevant growth factors, as but two examples.

Induction of immunologic tolerance has been an erstwhile goal of transplant surgeons and physicians. Especially in the case of xenotransplantation, if the current immunosuppressive regimens are not sufficient, induction of immunologic tolerance may be required. At least three approaches are being pursued: (i) the generation of mixed hematopoietic chimerism, (ii) the establishment of microchimerism by various means, and (iii) thymic transplantation [59–61]. The development of mixed hematopoietic chimerism through the introduction of donor bone marrow [62] has worked very well across rodent species [63, 64], although success may be limited by xenoreactive antibodies and the engraftment impaired by incompatibility of host growth factors or the microenvironment [65]. Fortunately, there is evidence that these problems can be overcome [60]. Various approaches to peripheral tolerance, such as the blockade of costimulation by administration of a fusion protein consisting of a soluble form of the CTLA-4 molecule and immunoglobulin (CTLA-4-Ig), are being pursued.

Still another factor in the cellular response to a xenotransplant involves the action of NK cells. Natural killer cell functions can be amplified by cell-surface receptors that recognize Galα1,3Gal [66]. Natural killer cell functions are down-regulated by receptors that recognize homologous major histocompatibility complex (MHC) class I [67, 68]. Human NK cells may be especially active against xenogeneic cells because of stimulation on the one hand and failure of down-regulation on the other. The possible involvement of NK cells in xenograft rejection might be addressed by generation of transgenic pigs expressing on the cell surface MHC-like molecules that will more effectively recognize corresponding receptors on NK cells and which will down-regulate the function of NK cells.

How a xenogeneic donor could be modified genetically to enhance the development of tolerance or to limit elicited immune responses is still uncertain. Clearly, efforts to control the natural immune barriers to xenotransplantation may contribute to limiting the elicited immune response. To the extent that recipient T-cells recognize donor cells directly, that is, the T-cell receptors of the recipient recognizing native MHC antigens on donor cells, a xenogeneic donor might be engineered in such a way to reduce corecognition (through CD4 and CD8) or costimulation (through CD28 or other T-cell surface molecules), or to express inhibitory molecules, such as CD59 or Fas ligand. These approaches and the

expression of inhibitory molecules, which are being considered as approaches to gene therapy in allotransplantation, may well prove more effective in xenotransplantation because inhibitory genes can be introduced as transgenes and thereby expressed in all relevant cells in the graft. Another useful and perhaps necessary approach will involve genetic modifications to allow the survival and function of donor bone-marrow cells.

22.3 Physiological and Infectious Hurdles to Xenotransplantation

22.3.1 Interspecies Challenges

Progress in addressing some of the immunological obstacles to xenotransplantation has brought into focus the question of the extent to which a xenotransplant would function optimally in a foreign host. A recent demonstration that the porcine kidney and the porcine lung can replace the most important functions of the primate kidney and primate lung are encouraging [69, 70]. Subtle defects in physiology across species may nevertheless exist. Organs such as the liver, which secrete a variety of proteins and which depend on complex enzymatic cascades, may prove incompatible with a primate host. Accordingly, one important application of genetic engineering in xenotransplantation may be the amplification or modulation of xenograft function to allow for more complete establishment of physiological function or to overcome critical defects. For example, Akhter *et al.* [71] and Kypson *et al.* [72] aimed at improving the function of cardiac allografts by manipulation of β-adrenergic signaling, and this technique might be adapted to the xenotransplant to improve cardiac function. On the other hand, most cellular processes and biochemical cascades are intrinsically regulated to meet the overall physiological needs of the whole individual. The key question, then, is which of the many potential defects actually need to be repaired?

Another potential hurdle to the clinical application of xenotransplantation is the possibility that the xenograft may disturb normal metabolic and physiological functions in the recipient. For example, Lawson and coworkers [73, 74] have shown that porcine thrombomodulin fails to interact adequately with human thrombin and protein C to generate activated protein C. This defect could lead to a prothrombotic diathesis because of failure of generation of activated protein C. Robson *et al.* [75] have found aberration control of coagulation in xenografts. Of even greater concern is the possibility that the transplantation of an organ, such as the liver, could add prothrombotic or proinflammatory products into the blood of the recipient. Although perhaps a great many physiological defects can be detected at the molecular level, the critical question will be which of these defects is important at the whole-organ level or with respect to the well-being of the recipient, and which must be repaired by pharmaceutical or genetic means.

22.3.2 Zoonosis

The increasing success of experimental xenotransplants and therapeutic trials bring to the fore the question of zoonosis, that is, infectious disease introduced from the graft into the recipient. The transfer of infectious agents from the graft to the recipient is a well-known complication of allotransplantation. To the extent that infection of the recipient in this way increases the risks of transplantation, that risk can generally be estimated and a decision made based on the risk versus the potential benefits conferred by the transplant. The concern about zoonosis in xenotransplantation is not so much the risk to the recipient of the transplant, but the risk that an infectious agent will be transferred from the recipient to the population at large. Fortunately, all of the microbial agents known to infect the pig can be detected by screening and potentially eliminated from a population of xenotransplant donors. There is concern, however, that the pig may harbor endogenous retroviruses, that are inherited with genomic DNA and which might become activated and transferred to the cells of the recipient.

The possibility that an endogenous retrovirus from swine might pose a hurdle to xenotransplantation was first raised by Patience *et al.* [76]. Reports from several laboratories have raised concerns about transmission of porcine retroviruses. Patience *et al.* [76] found that the porcine endogenous retrovirus (PERV) can be activated in pig cells, leading to the release of particles that can infect human cell lines. Whether this virus or other endogenous viruses can actually infect across species and whether such infection would lead to disease are unknown, but remain a subject of current epidemiologic investigation. The transfer of PERV to humans has been sought but not definitively demonstrated [77]. However, Ogle *et al.* [78] found that human hematopoietic stem cells introduced into swine acquire PERV by fusing with host cells to form human–swine hybrid synkaryons. The hybrid cells appear to have a survival advantage over unfused cells and transmit the virus to uninfected human cells. Because the hybrid cells express swine genes, they might have been overlooked in clinical surveys.

If cross-species infection does prove to be an important issue, genetic therapies might also be used to address this problem. The simplest genetic therapy would involve breeding out the organism, but this approach might fail if the organism was widespread or integrated at multiple loci. Some genetic therapies have been developed to potentially control human immunodeficiency viruses [79]. Although these therapies have generally failed because it has been difficult or impossible to gain expression of the transferred genes in stem cells and at levels sufficient to deal with high viral loads, the application of such therapies might be much easier in xenotransplantation because the therapeutic genes could be delivered through the germline. Ultimately, if elimination of endogenous retroviruses were necessary, it could potentially be accomplished by gene targeting and cloning, as discussed above.

22.4
Scenario for the Clinical Application of Xenotransplantation

Successful application of xenotransplantation in the clinical arena requires insights into not only immunology, but also physiology and infectious disease, all of which have been discussed briefly here in the context of genetic therapy. In recent years, important advances have been made in elucidating the immunological hurdles of pig-to-primate transplantation. Although this scientific progress is important and exciting, xenotransplantation will likely enter the clinical arena through a step-by-step process. A first step, free-tissue xenografting, is in limited clinical trials already [80–82] and preliminary evidence is encouraging as porcine free tissue xenografts appear to endure in a human recipient [82]. One immediate application of free-tissue xenografting would be treatment of cirrhosis caused by the hepatitis virus, using targeted infusion of porcine hepatocytes [8]. The promise of this approach is enhanced because: (i) pig hepatocytes are resistant to viral re-infection, (ii) rat models of cirrhotic liver failure indicate that porcine hepatocyte xenotransplants may endure and function well [83]; and (iii) predicted demand for hepatic transplantation due to hepatitis C-induced cirrhotic liver disease is likely to worsen the already acute shortage of livers available for transplant. Swine hepatocytes can survive for months in nonhuman primates [84, 85]. Another potential extension of free-tissue xenografting is transplantation of xenogeneic islets of Langerhans for the treatment of type I diabetes [86]. Xenogeneic islet transplants may prove to be less liable to destruction by the autoimmune processes that underlies this disease.

Temporary or "bridge" organ transplantation will probably follow free-tissue xenografting. Bridge transplants will not address the problem of the shortage of human organs, but incisive analysis of the outcomes of these transplants will provide important information about the remaining immunologic hurdles and the potential physiological and infectious considerations. With this information, further therapies including genetic engineering may allow the use of porcine organs as permanent replacements. Even then, one can envision ongoing efforts to apply genetic therapies that will optimize graft function and limit the complications of transplantation.

While it may be that the use of pigs as a source of organs and tissues for transplantation is not far off, exciting advances in tissue engineering, stem cell technology, and *in vitro* organogenesis may broaden the use of animals in human medicine. Adult and embryonic stem cell culture has given rise to organ-specific tissues with functional characteristics of the corresponding organs [87]. Although these cultures are unlikely to yield fully developed functional organs for transplantation into humans, pigs or other animals could be used as recipients of these culture-initiated tissues and allow completion of development. These organs, grown and maintained in animals, may then be available for transplantation on an "as needed" basis. Pigs may thus serve as xenograft "recipients" prior to becoming organ "donors."

The recent success in cloning of animals, including pigs, raises the possibility of transferring nuclei from a human patient's cells into enucleated stem cells of

an animal, and then growing the cells in animals to generate differentiated human tissue that is autologous with the patient. The lessons learned from genetic manipulation of animals in the quest to make animal organs suitable for transplantation into humans may find their best application in generating animals suitable for use as biological reactors to grow human organs suitable for transplantation into humans.

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23
Nutraceuticals–Functional Foods for Improving Health and Preventing Disease

Jian Zhao

23.1
Introduction

Sufficient and balanced nutrients from the diet provide vital support for normal functioning of the human body. However, this statement may mean very different and more complicated things to each individual due to diverse health status and the complex relationships between food and health. Advancing scientific research and knowledge of medicine and food nutrition has dramatically changed the concepts of food, medicine, and healthcare, and brought in a revolution with huge changes to people's dietary habits in recent decades.

Functional foods and nutraceuticals (FFNs), and alternative therapies based on FFNs, have emerged as different and overwhelming healing systems. Strong recommendations from nutritional or clinical professionals for consumption of FFNs and their use as therapies mean they have become increasingly popular. It is believed that good FFNs hold great promise to benefit human health, due to their potential to enhance overall health status, to reduce side effects associated with chemotherapy or radiotherapy, to complement current therapies that often fail for treatment of many chronic or degenerative diseases, and to reduce the cost of healthcare [1, 2].

Today, a huge portion of the worldwide population take FFNs or use alternative therapies, and this is increasing. Riding the trends, biotechnology to improve food nutrition values, production of nutraceuticals or even medicines in traditional foods, is blooming. Meanwhile, it has been realized that what people eat or drink has become the major cause of various diseases and health problems, besides infections or epidermal diseases; people now pay detailed attention to what they eat, including foods and medicines, which can largely determine one's health throughout life.

With the fast growth of manufacturing, marketing, services, consumption of medicines, FFNs, and other new types of foods, concerns have also been raised. While legislation on FFNs is not yet complete, the concerns over the use, safety, efficacy, quality control, or standardization of these edible products have become major issues [1]. The information regarding FFNs and their related therapies from

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various aspects of the media, and even the scientific research, is often incomplete, out of date, confusing, or sometimes controversial. This chapter attempts to update and comment on these areas of FFNs and their related therapies, biotechnological developments, and problems, in order to lead to a complete understanding of most aspects of nutraceuticals and functional foods.

23.2
Plant Food, Pharmaceuticals, Nutraceuticals, and Human Health

Humans rely extensively on plant resources for food and their living environments. Plant-derived foods, such as staple grains, fruits, and vegetables, provide humans with the majority of their life-supporting dietary nutrients, including carbohydrates, lipids, proteins, vitamins, minerals, and dietary fibers. Although many of these basic nutrients can also be taken up from animal-derived foods, plant foods provide rich, diverse, and ideal phytonutrient combinations that are unique and more suitable for maintaining human health. Plant foods contain whole sets of vitamins, essential amino acids, essential fatty acids, macro- and micro-minerals, lipids, carbohydrates, and plant-specific nutrients. The presence of other non-healthy or even potentially harmful ingredients, such as high levels of cholesterol, saturated fatty acids, toxic metabolites, and various chemical contaminations in meats, milks, or even epidemic disease infection, makes animal-derived foods less desirable. Even fish, which are popularly regarded as the top omega-3 polyunsaturated fatty acids (PUFA) containing healthy food, are often inevitably contaminated with heavy metals, such as mercury or other toxic chemicals. Consumption of fish has become a common cause of heavy metal toxicity. Today, the increasing incidence of various cancers, heart diseases, diabetes, and obesity has been more or less associated with excessive consumption of meats as a regular part of daily life in the Western world.

With unaffordable medications and unsatisfactory chemotherapy and radiotherapy treatments, preventive FFNs and less expensive alternative healing systems have become overwhelmingly popular. Plant foods, particularly raw organic plant foods, such as whole grains, fresh vegetables, nuts, and unprocessed fruits with high phytonutrient values, are strongly recommended by professionals to be good for human health. Epidemiological and clinical studies have clearly demonstrated the relationship between diet and health status. For example, epidemiological and prospective studies indicate that comprehensive lifestyle changes may modify the progression of prostate cancer [3]. Similarly, dietary habits and changes could significantly affect overall health status and lifespan [4], although the molecular mechanisms are poorly understood. Recent studies have suggested that dietary choices may have an effect on the epigenetics of individual genomes, providing a profound insight into our understanding of the relationships between diet and human health [5].

Human beings rely on plants not only for foods that contain many basic nutrients and for the relief of starvation, but also for the medical functions and health benefits of various plant natural products. From ancient times, dating back at least

5000 years, humans have been using medicinal plants to diagnose and cure various diseases, or to enhance health and increase the longevity of their lives. Three thousand years ago, the well-recognized father of modern medicine, Hippocrates, stated "Let food be thy medicine and medicine be thy food." The truth in this saying is widely recognized: if foods are properly prepared, they can be medicine to promote health, and prevent or treat diseases [1].

Extensive use of plants for healthcare and in the treatment of disease has led to numerous discoveries of important medicinal plants and bioactive natural products, and accumulation of invaluable therapeutic experience. The heritage of folk or traditional medicine has been of benefit to human health for thousands of years and still continues to serve mankind [6, 7]. Many important drugs used in chemotherapy, for example, are obtained or derived from plant natural products. Some examples of plant products are: aspirin, which originates from salicin and salicylic acid, a natural product found in *Salix alba* (white willow) plants; anti-lymphoma and histiocytosis drug vinblastine/vincristine from Madagascar periwinkle (*Catharanthus roseus*); anticancer drug Taxol (paclitaxel) from the pacific yew tree; anti-malaria drugs artemisinin from the Chinese tradition medicinal plant wormwood (*Artemisia annua*); and the anticancer (ovarian and small cell lung cancers) drug topotecan is synthesized from its natural analog podophyllotoxin in Mayapple (*Podophyllum peltatum*) (http://www.rain-tree.com/plantdrugs.htm, [7]).

Modern pharmaceuticals based on the plant natural products, however, have now turned to simple drugs produced through chemical synthesis. The development of chemically synthesized simple drugs is often associated with high costs, even though they are powerful relievers of patient symptoms. However, these unaffordable drugs can also lead to serious and sometimes lethal side-effects for patients. Moreover, these simple drugs often fail to treat many chronic and degenerative diseases, which have been occurring more frequently for human beings living in modern societies.

All these issues can dissatisfy patients and force them to change their lifestyles, and to look for protective functional foods and nutraceuticals, and safer and cheaper alternative medicines and therapies. Plant foods and natural products have thus become frequently recommended; FFNs that have the potential to combine the dual functions of food and medicine are produced for alternative therapies. More than one third of the populations in the developed countries (e.g., about 47% of the population in Japan, 40% in the United States) consume function foods and nutraceuticals, or take alternative therapies, and these numbers are continuing to grow.

23.3
Concepts of Functional Foods, Nutraceuticals, and Other Related Terms

Driven by consumer demand, the profit-pursuing manufactures, professionals' enthusiasm, and advances in scientific research on food nutrition and medicine, FFNs have spread worldwide. Consequently, numerous similar or related products

with different trade names have appeared on the market with a variety of labels and claims. The labels and claims, such as functional foods, medical foods, dietary or nutritional supplements, nutraceuticals, and food supplements, often vary in various areas, nations, or at different stages of development, and from different manufacturers or providers. According to the regulations, food labels are the most important documents to classify, and explaining the food products, mislabeling, or improper claims may cause confusion and misunderstanding, and even serious consequences. Clarifying these concepts is of particular importance to avoid any misunderstanding or misleading of patients, professionals, researchers, or other interested parties.

23.3.1 Functional Foods

Functional foods are a class of foods containing fortified or enhanced essential nutrients specifically designed to meet a consumer's special demands. They generally look like regular foods and are not in the form of a drug. However, beyond the basic nutritional function expected of conventional foods, functional foods emphasize their "functions" by enhancing or fortifying certain essential nutrients that are well-documented to promote health, prevent or treat nutrient deficiency or related diseases: for example, vitamin D and A enhanced milk, omega-3 fatty acid docosahexaenioc acid (DHA) enhanced infant milk or juices. The functional components in functional foods could be any known components or ingredients with specific medical or physiological benefits for preventing or controlling disease, such as vitamins, antioxidants, or bioactive herbal nutraceuticals, such as bioactive flavonoids, terpenoids, and other bioactive natural products.

23.3.2 Nutraceutical

Nutraceutical is a term combining the words "nutrition" and "pharmaceutical," coined by Stephen DeFelice to define a special food product intended to provide health and medical benefits, such as the prevention and treatment of disease [1]. This evolving concept may refer to such food products as isolated or purified nutrient components, vitamins mixed with other bioactive compounds, dietary nutrient supplements, specific diets intended to promote health status for certain patients, plant-derived bioactive products, or herbs, and bioactive natural compounds of varying purity from other resources. Nutraceuticals emphasize their health benefit or medical functions by promoting human health and preventing disease. However, they are not drugs intended to diagnose, treat, or cure any disease, and thus are not subject to the same testing and restricted regulations as pharmaceutical drugs. Nevertheless, many nutraceuticals are sold in medicinal forms such as pills, tablets, powders, and capsules.

23.3.3 Medical Foods

Medical foods refer to the special foods "formulated to be consumed or administered internally under the supervision of a physician, and which are intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, on the basis of recognized scientific principles, are established by medical evaluation" by the US FDA. These foods meet certain nutritional requirements for patients diagnosed with specific illnesses, are administered by professionals, and monitored through medical supervision. Therefore, medical food is more often used as a clinical term. Medical foods are also designed to more effectively manage and deliver pharmacologically active compounds or drugs to patients, in addition to the necessary nutrients.

23.3.4 Novel Foods

As the concept of novel foods appears often and people confuse them with functional and medical foods, it is essential to clarify what they are. In all authorized regulations, novel foods refers to foods or novel food ingredients that have never been used as foods or have no history of significant and safe consumption. What is covered by the term novel foods varies from country to country, but normally includes plant preparations, plant extracts or animal-derived products that have not been extensively examined from the safety aspect. Therefore, they are subject to strict regulation in use. In Canada, genetically modified (GM) crops, animals, and fish with components or traits that have potentially been altered from the natural ones are also considered as novel foods. GM foods could possibly have differences from natural foods with respect to composition, and the safety with respect to human health and natural environments may not have been evaluated. These novel foods made from GM sources are still subject to hot debate and regulation restrictions. Even though many GM bioproducts have various types of benefits, for example, fortified nutrient contents, engineered pharmaceuticals, or oral vaccinations against degenerative and infectious diseases, along with other merits that people expect, most GM products now are not commercialized because of concerns on their potential consequences in either long-term health or agricultural ecosystems. For example, in the case of pro-vitamin A-enforced "Golden Rice," can consumers' health really benefit from a high content of pro-vitamin A? What is the result of its long-term consumption as a staple food? What is the long-term environmental impact of "Golden Rice" cultivation? [1].

Unlike other GM crops aimed at improving agronomic traits or nutrient values, scientists have also recently developed GM crops and livestock for the production of pharmaceutically important proteins for clinical applications [8]. So far numerous therapeutic proteins, including vaccines, antibodies, hormones, and other various bioactive peptides, have been produced in GM crops and livestock.

However, the foods containing homologous extracts of these pharmaceuticals, such as medical antibodies, antibiotics, vaccines, or other therapeutic peptides generated from GM plants, are considered as medical foods. They are specifically designed for particular patients for definite medical purposes.

23.3.5 Food (Dietary) Supplement

A food (dietary) supplement is the term widely used to refer to a product taken by humans that contains a "dietary ingredient" intended to supplement the diet. The dietary supplements should be nutrient factors such as vitamins, essential amino acids, essential fatty acids, and minerals, or some bioactive herbs or botanicals, enzymes, and known natural products. This concept may cover nutraceuticals.

23.3.6 Food Additives

Food additives are the substances added to foods to help process food or improve the physical or chemical qualities. These additives, such as food colors, flavors, food thickener gels, acidity regulators, antioxidants, stabilizers, and preservatives, usually have no nutritional value, but can preserve the flavor or improve the taste and appearance. Because of safety concerns with the use of these chemicals, most nations have legislation to regulate their use, including categories, dosage, and labeling.

23.3.7 Phytonutrients

Phytonutrients refer to plant-derived natural products with particular biological activities for supporting human health. The concept covers the basic food nutrients, such as vitamins and minerals; it also covers plant secondary metabolites with certain biological activity [1, 2, 6]. Unlike phytochemicals, which generally refer to all plant-derived chemicals regardless of their biological activity, or phytomedicines, which only includes plant natural products that show potent biological activity and can potentially be developed into medicines, phytonutrients focus on plant derived bioactive products with health benefits. Therefore, phytonutrients here include phytochemicals with nutritional value, biological activity or a medical function, so not only function-known vitamins, proteins, lipids, and carbon hydrates, and essential minerals, but also unknown health-promoting plant natural products, including many secondary metabolites. Considering the vast amounts of renewable plant resources that remain to be explored, our understanding of plant natural bioactive products is still limited but subject to expansion with advanced scientific research on phytochemistry, nutrition, and pharmacological sciences.

23.3.8
Herbs, Herbal Nutraceuticals, or Botanicals

Herbs, herbal nutraceuticals, or botanicals refer to plants or parts of plants and their extracts with certain biological activities that can be used to promote health, and prevent or treat human disease. The health benefits of plant materials are either evidence-based through a long history of use in folk and traditional medicine, or partly proved by recent scientific research on their bioactive components and biological activity [6, 7]. They belong to a large group of nutraceuticals. However, a herbal nutraceutical can contain multiple bioactive components with either health-beneficial or harmful effects, which makes their overall therapeutic effects more complicated and research on them difficult. These multiple ingredients may show synergistic or different, and even antagonist, effects because of their targets on biochemical pathways or cellular processes.

23.3.9
Alternative Therapies

Alternative therapies refer to therapies other than chemotherapy or radiotherapy that currently dominate clinic therapeutic treatments in modern medicine. These alternative therapies include many different therapeutic strategies.

Nutritional therapy uses functional foods containing enhanced dietary nutrients such as vitamins, essential amino acids, and essential fatty acids, to improve health, and prevent or treat diseases that are normally related to certain nutrient deficiencies [9–11]. Phytotherapy represents another healing system that uses whole, or parts of, medicinal plants, effective herbal extracts, or partly purified fractions (containing bioactive phytonutrients) to improve health, and prevent or treat diseases [6]. Although most phytotherapies have developed from herbal remedies used in folk medicines to promote health and treat diseases, phytotherapy is actually based more on modern scientific research and have usually been tested in various types of clinical trials [7]. The phytonutrients are continuously being improved for their safety, effectiveness, quality and quantity control. Advanced studies on their phytochemical principles and biological activities, and clinical trials on feeding protocols will provide a solid base for patient practice.

Nutraceutical therapy uses nutraceuticals to promote health status, prevent disease, or even treat certain chronic diseases [12]. Because nutraceuticals cover a wide range of bioactive products, including most phytonutrients such as vitamins, minerals, and other bioactive compounds from other sources, for example, omega-3 containing fish oils, nutraceutical therapy is an umbrella concept covering several other therapies, such as nutritional therapy and phytotherapy. Nutraceutical therapy takes effect by promoting overall health status, prevention or treatment of health disorders, degenerative, and chronic diseases [12]. Many nutraceuticals currently on the market, mainly natural products, have been found to have new favorable health benefits from preventing cardiovascular diseases, delaying organ aging, protecting brain- and neuro-function, to preventing digestive diseases and

obesity, as well as diabetes. The targeted health disorders and chronic diseases may range from chronic fatigue, energy loss, insomnia, and osteoarthritis, to backache, skin complaints, and asthma [1, 2].

Therefore, some good nutraceutical therapies may represent more comprehensive therapies. In addition, a comprehensive therapy also tries to employ as many health-affecting factors as possible to improve the overall health conditions of an individual. It will depend not only on balanced dietary nutrients, but also on sufficient exposure to sunlight, fresh air, pure water, proper exercise and rest, emotion and self-control, to build up a healthy human immunity. A comprehensive therapy concept such as this is being paid more attention and is applied in clinical settings as well as other recovery processes for various types of patients [1].

23.4 FFN Principles and Their Potential Health Benefits

Today, there are thousands of FFNs on the market worldwide, these being made from plant-based products, marine products, and animal-based products to serve consumer's demands. However, the bioactive components in these FFNs can be simply classified into several groups according to their chemical nature, bioactivities, or health benefits. Generally, the recognized potential health benefits of FFNs to improve health status or prevent or even treat chronic or degenerative diseases largely depend on these principles.

23.4.1 Dietary Fibers

Dietary fibers, non-starch polysaccharides of plant foods, are the main components and have multiple dietary functions in human health. They not only physically contribute to stomach distension, satiation, and satiety of foods, but also benefit gut microbiota movements and activities, food absorption and defecation, and other aspects. Thus, dietary fibers in FFN products, such as oligofructose, polydextrose, fructo-oligosaccharides, inulin, and galacto-oligosaccharides, have been shown to provide many health benefits. Dietary fibers promote colonic health, stimulating the probiotics *Bifidobacterium* and *Lactobacillus*, reducing cholesterol and triglyceride levels and the risk of cardiovascular diseases, and thus promoting coronary artery health, stimulating glucose metabolism and insulin response for diabetes, and preventing cancers, and so on. Solid data from medical research and clinical trials support that rye- and oat-meals, as excellent sources of dietary fibers such as beta-glucan, have the above mentioned health benefits [13].

A wide range of consumers, including obese and diabetic patients, use FFNs containing plant dietary fibers to maintain a healthy weight [14]. The first approval has now been given by the FDA that the claim about scientifically proven function of soluble fiber from plant foods has many health benefits. Plant foods rich in dietary fibers also include wheat bran (rich in insoluble fiber), psyllium, oat, and barley meals (rich in soluble fiber and beta-glucan). As many insoluble dietary

fibers effectively stimulate growth and activity of probiotics such as *Bifidobacterium* and *Lactobacillus* strains in the human gut, they are also regarded as prebiotics.

23.4.2 Phenolic Products

Chlorogenic acid is marketed under the trademark Svetol™ in Norway and the United Kingdom as a food active ingredient used in coffee, chewing gum, and mints to promote weight reduction. Resveratrol found in nuts and red wine has strong antioxidant, antithrombotic, anti-inflammatory, and anti-carcinogenesis activities. Hydroxytyrosol from olives and olive oil is a potent antioxidant [1]. Curcumin from the Indian spice turmeric shows strong antioxidant, anti-inflammatory, and anticancer activity [15]. Some clinical trials have also shown that natural phenolic compounds show various health benefits [16].

Plant lignin polyphenolic compounds are contained in plant-derived foods at low levels and have multiple biological activities, including weak estrogenic or anti-estrogenic effects, such as antimitotic, antifungal, antioxidant, and antiviral activity, and are prebiotics [17]. The most famous lignan is podophyllotoxin, which has potent anticancer activity and its derivatives have been developed into drugs for chemotherapy against various types of cancers. Secoisolariciresinol and matairesinol were the first plant lignans identified in foods. The main dietary lignans in our daily foods might be pinoresinol and lariciresinol, which contribute about 75% of the total lignan intake, whereas secoisolariciresinol and matairesinol contribute only about 25% [18]. Flaxseed and sesame seed are rich in lignans, such as secoisolariciresinol diglucoside. Other sources of lignans include cereals (rye, wheat, oats, and barley), pumpkin seeds, soybeans, broccoli, beans, and some berries.

Flavonoids, such as flavonones, flavonols, isoflavones, anthocyanins, and flavol-3-ols (proanthocyanidins, commonly known as condensed tannins), are one of the most abundant classes of secondary metabolites in dietary plants. Flavonoids are present in most plant foods. Oligomeric proanthocyanidins are mixtures of oligomers and polymers of flavol-3-ols extracted mainly from grape seeds that have potent antioxidant and anti-aging activity, and therefore are recommended for use in preventing many degenerative diseases. Recent studies have highlighted that the active component in grape-seed extracts, epicatechin or catechin, can delay the development of Alzheimer's disease; the molecular mechanism of the bioactivity is under investigation [19]. Epicatechin from blueberry-leaf extract can inhibit hepatitis C virus proliferation by binding to virus replication enzymes [20]. Proanthocyanins can improve urinary tract health by preventing urinary tract infection and reducing the risk of cardiovascular disease, through their strong antioxidant activity. There is a positive correlation between dietary flavonoid (such as myricetin, quercetin, and isoflavones) intake and decreased mortality from coronary heart disease, partly due to the inhibition of LDL (low-density lipoprotein) oxidation and reduced platelet aggregability by flavonoids [21–25].

Estimates of the dietary intake of flavonoids range between 23 and 200 mg per day, which is mainly through green tea, onions, apples, and red wine. The consumption of 30–50 mg per day of soy isoflavones in the traditional Eastern diet may help

to lower the incidence of breast cancer [1]. The soy isoflavone phytoestrogens, such as genistein and daidzein, and a daidzein metabolite equol, produced by intestinal microflora, have potent antioxidant activity. In particular, equol is an inhibitor of LDL oxidation taking place in the arterial intima [1, 26]. Intake of soy-derived phytoestrogens provides protection against oxidative modification of LDL, and helps to reduce the risk of atherosclerosis, cardiovascular disease, and cancer [26].

A randomized, double-blind, placebo-controlled, cross-over study with 30 healthy postmenopausal women indicated that eight weeks consumption of cereal bars enriched with 50 mg of soy isoflavones per day increased their plasma nitrite and nitrate concentrations and improved endothelium-independent vasodilatation [26]. The numbers of breast cancer patients in Western societies are continuously increasing, and these are higher than those in eastern Asian countries, mainly because Asian women eat more soy and other plant-derived foods such as tofu, vegetables, and raw cereals that contain phytoestrogens, lignans, and fibres [1, 26]. It is now widely recognized that soy isoflavone phytoestrogens benefit human health, particularly women's health, in different ways, although the mechanisms for such convincing effects remain to be completely understood.

FFNs have been extensively studied for their beneficial effects on the improvement of cognitive functions, and the prevention or delay of Alzheimer's disease-related neuropathology [19]. The single or combined FFNs include vitamins, phenolic products (such as curcumin, epicatechin, and resveratrol), essential fatty acids (such as DHA and alpha-lipoic acid), and essential amino acids have been tried for neuroprotection benefits, although more confirmative studies are still needed [27–29].

23.4.3 Terpenoids

Plant terpenoids are an important group of phytonutrients, and include pre-vitamin A carotenoid, zeaxanthin, and vitamin E, coenzyme Q10, and bioactive monoterpene, sesquiterpenes, diterpenoids. Coenzyme Q10 is a lipid-soluble antioxidant and a very popular food supplement. Coenzyme Q10 may help to improve blood pressure and glycemic control in type II diabetes. Epidemiological studies have clearly shown the great benefits of the consumption of tomato to human health due to tomato carotenoids, mainly lycopene, β-carotene, and lutein [30]. Lycopene from tomatoes and other fruits is a potent antioxidant carotenoid, offering protection against prostate and other cancers and inhibiting tumor cell growth in animals [30]. β-Carotene from carrots, fruits, and vegetables is not only a form of pre-vitamin A, but also has potent antioxidant activity through neutralizing free radicals, which are regarded as one of the major causes of aging and various cancers. Humans benefit from eating various plants and vegetables that are rich in dietary caroteniod, containing vitamin E, lycopene, lutein, zeaxanthin, due to their strong antioxidant and anti-aging activity.

Monoterpenes in citrus fruits, cherries, peppermint, and herbs have anticarcinogenic actions in experimental models, as well as cardioprotective effects.

Although most monoterpenes, also known as essential oils, can be used as flavors, fragrances, and medicines, herbs that are rich in monoterpenes are also widely used for the treatment of various diseases, such as, the sesquiterpenes from plants usually have strong antibacterial, antiviral, antifungal, and insecticidal effects, and can be used to treat related diseases. For example, unique monoterpene derivatives, thujaplicins, from trees are widely used as an antifungal medicine in clinical treatments, and they have also shown other multiple potential activities [31]. The most well-known sesquiterpenes, such as artemisinin and their derivatives, are dominant anti-malarial drugs [32]. Many plant diterpenes are also medicines, such as the familiar anticancer drug taxol and its derivatives.

23.4.4 Phytosterols

Another important class of terpenoids are phytosterols, which include plant sterols such as stigmasterol, sitosterol, campesterol, and plant stanols. They are natural components of many plant foods. Because of the similarity of their structures with cholesterols from meats, phytosterols competitively inhibit intestinal cholesterol absorption [33–35]. Reducing blood cholesterol levels has been a major target of medications and FFNs and FFN-based therapies. Phytosterols decrease both dietary and biliary cholesterol absorption by the small intestines, and consequentially increase cholesterol depletion. Today phytosterols purified from plants are used in function foods. Vegetable oils containing plant sterols, such as esters of fatty acids and plant stanol esters, in the form of enriched margarines, have been developed as functional foods [33, 36, 37]. Studies have shown that a daily intake of plant sterols or stanols of 1.6–2 g per day can reduce cholesterol absorption by ~30% and plasma LDL cholesterol levels by 8–10% [33, 36–38]. The FDA have approved the claim "Foods containing at least 0.4 gram per serving of plant sterols, eaten twice a day with meals for a daily total intake of at least 0.8 gram, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease."

23.4.5 Fatty Acids and Lipids

Fat occupies nearly 60% of the human brain and determines the brain's integrity and performance. Dietary essential fatty acids and long-chain PUFAs are important for fetal and infant development. Clinical studies have associated the imbalanced dietary intake of essential fatty acids including alpha-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid) with impaired brain development, performance, and diseases [39, 40]. This is particularly true for omega-3 PUFA, as they are essential for the human body to make many important molecules that affect neurofunction, cellular function, inflammation, mood, and behavior. Inadequacy of long-chain PUFAs may lead to a failure to accomplish a specific component of brain growth and irreversible damage, because both the brain and the retina are rich in arachidonic acid (ARA) and decosahexaenoic acid (DHA) and rely on them

for their normal functions. Both epidemiologic and clinical studies have demonstrated that omega-3 type PUFA decrease the risk of cardiovascular disease [39, 40]. Fish oil, flaxseed, flaxseed oil, walnuts, canola oil, and soybean oil can provide safe, affordable, and renewable plant sources of these essential fatty acids.

Dietary PUFAs, such as arachidonic acid (ARA), A-linolenic acid (ALA), eicosapentaenoic acid (EPA), and DHA, or the natural sources of these fish oils, flaxseeds, and other plant oils, are probably the most widely used and studied FFNs [39–46]. People now take extra omega-3 fatty acids from fish oils, marine foods, flaxseeds, or other omega-3 FFN products, as recommended by scientific research and clinical trials. However, using method dosage and frequency of taking these essential fatty acids for optimal health benefits is still speculative, even though a large number of studies have shown that substitution of dietary saturated fat by oleic acid and/or omega-3 PUFA benefit cardiovascular health, by reducing blood cholesterol, LDL-cholesterol, and triglycerides [39–46].

There is a plenty of evidence to show the *deteriorating effects of* trans fats on human health [47]. Trans fat such as *trans*-10,*cis*-12 conjugated linoleic acid (CLA) can induce insulin resistance and various metabolic syndromes, for example, diabetes, obesity, nonalcoholic fatty liver disease. Despite inconsistencies, most clinical trials and animal studies indicate that fish oil and individual omega-3 PUFA, including ALA and DHA, have significant clinical effects on the prevention and reversal of insulin resistance, which is related to many syndromes and diseases [41–46].

Recent studies have shown that medium-chain fatty acids (MCFAs) or medium-chain triglycerides (MCTs) also have benefits to human health [48]. Unlike long- or very long-chain fatty acids, these fatty acids passively diffuse into the portal system without requiring modification or digestion. Therefore, malnutrition or malabsorption patients are treated with these MCFAs and MCTs. Metabolic syndromes, such as abdominal obesity, dyslipidemia, hypertension, and impaired fasting glucose, contribute to increased cardiovascular morbidity and mortality. In animal and human subjects, medium-chain fatty acids and medium-chain triglycerides suppress fat deposition through enhanced thermogenesis and fat oxidation [49]. Several reports have suggested that MCFAs/MCTs offer the therapeutic advantage of preserving insulin sensitivity in animal models and patients with type II diabetes [49]. Coconut oil is composed of approximately 66% medium-chain triglycerides. Alpha-lipoic acid, a natural fatty acid synthesized in both plant foods and the human body, is regarded as an important nutraceutical for prevention or treatment of weight loss, obesity, or type II diabetes. For its potent antioxidant activity, alpha-lipoic acid can reduce symptoms of diabetic peripheral neuropathy, increase insulin sensitivity, and improve neuropathic deficits in diabetic patients [50]. However, these results from the 1990s and early 20th century still need further investigation.

23.4.6 Essential Amino Acids

Eight essential amino acids are so called because mammals cannot synthesize them by themselves but they have to taken up from plant foods or meat sources.

Taking up adequate essential amino acids are very important for health as they are the building blocks of proteins, which carry the functions of the human body [1, 51].

- **Tryptophan** is used for synthesis of the neurotransmitter serotonin and relief of depression, flaxseeds have high tryptophan levels.
- **Tyrosine** is necessary for dopamine, norepinephrine, and epinephrine synthesis for normal neurosystem activity and enhances positive moods.
- **Isoleucine** is necessary for the synthesis of hemoglobin in red blood cells.
- **Leucine** has beneficial effects for skin, bone, and tissue wound healing, and promotes growth hormone synthesis.
- **Lysine and valine** are essential for muscle proteins, as well as for the synthesis of hormones and L-carathine, which is essential for the functioning of a healthy nervous system.
- **Methionine** is essential for all protein synthesis and helps in the breakdown of fats and in reducing muscle degeneration.

All these essential amino acids can be found in plant foods such as cereals, soybean, flaxseeds, nuts, and peas and in animal-derived foods [52]. In addition, phenylalanine is beneficial for healthy nervous systems and boosts memory and learning. Phenylalanine may be useful in the treatment of depression and suppressing appetites. L-Arginine is a conditional essential amino acid for infants and growing children, as well as for pregnant women [53]. Glutamine is considered to be a conditional essential amino acid in metabolic stress. *N*-Acetyl cysteine is a derivative of cysteine widely used as both a drug and a dietary supplement and as a nutraceutical for multiple medical purposes.

23.4.7 Prebiotics and Probiotics

The gastrointestinal tract of human beings is populated by micro-organisms, which can be either harmful or beneficial to human health [54]. The predominant and most important populations are in the colon, where a true symbiosis with the host exists, which is key to well-being and health. Antibiotic therapy, poor dietary options, and other physiological disorders may cause intestinal imbalance and dysbiosis, which results in overgrowth of bad bacteria and yeast such as *Clostridium difficile*. Dysbiosis has been linked with disorders such as colon problems, irritable bowel syndrome, and rheumatoid arthritis [55]. Prebiotics and probiotics can restore the balance of gut microbiota. Probiotics refers to beneficial gut microbiota that can also be found in various foods [54]. Probiotics, such as common bacteria strains of the *Lactobacillus* and *Bifidobacterium* families, can be taken as nutraceuticals into the intestinal tract to restore the balance of gut microbiota. Human beings have historical traditions of using probiotics in the fermentation of various foods, such as breads, cheese, and milks (yogurts).

Prebiotics are non-digestible foods that can selectively stimulate the growth and/or activity of the health beneficial gut microbiota. Some prebiotics, such as dietary fibers (e.g., fructo-oligosaccharides), provide nourishment for beneficial bacterial growth and function, and thereby confer health benefits to humans. Regarding the human gut intestine microbiota relationships, the management of not only antibiotics, but also prebiotics, probiotics, and symbiotics has become important in current clinical practice to modulate the intestinal bacterial flora in man under healthy and pathologic conditions [56].

From another point of view, metabolic disorders, including obesity and type II diabetes, are pathological conditions resulting from the failure of a human's energetic balance for the equality of intake, metabolism, absorption, storage, and energy distribution [55–57]. Such pathological conditions may be reversed by the intake of FFNs and probiotics and/or prebiotics, and changes to a healthy dietary habit.

The benefits of prebiotics and probiotics on insulin sensitivity, inflammation, postprandial incretions, and glucose tolerance continue to be the subjects of extensive study [56–59]. A community based double-masked, randomized controlled trial on the efficacy of prebiotic oligosaccharide and probiotic *Bifidobacterium lactis* HN019 with children aged between 1 and 3 years showed that consumption of milk added with prebiotics and probiotics resulted in reduction of dysentery, respiratory morbidity, and febrile illness [57]. However, further studies on other aspects of the encouraging results from this and other clinical trials need to be carried out [57]. A recent evaluation by an expert committee concluded that there is insufficient data to recommend the routine use of probiotic- and/or prebiotic-supplemented infant formulae, although the supplementation with probiotics and/or prebiotics is an important area of research [54].

23.4.8 Phytoestrogen

Phytoestrogens are diverse nonsteroid natural products with similar structures to the estrogen hormone estradiol, and thus have the ability to cause estrogenic or/and anti-estrogenic effects. Many phytoestrogens are contained in our daily diets, such as in soybean and cabbage, nuts, and oilseeds, and thus they are also called "dietary estrogens." These phytoestrogens, such as the polyphenolic compounds coumarins, prenylated flavonoids, and isoflavones, can act both as antioxidants and as estrogen agonists and antagonists, with multiple and complicated effects [60].

Because of the significance of human hormones on human health, the production, balances, homeostasis, and metabolism of estrogen are associated with various health problems. The dietary intakes of hormone analogs or hormone biosynthesis- and metabolism-related nutrients, such as phytoestrogens from plant foods, hormones from animal-derived foods, are important.

An optimal "estrogen balance" has implications for cancer prevention and successful aging in both women and men. In particular, dietary intake of phytoestro-

gens is well recognized as a much safer therapy than estrogen replacement therapy with pills for women at the menopause stage of life. They protect against heart disease, anticancer activity against certain cancers such as breast cancer, lower LDL, and total cholesterol [60]. Soybean products and cruciferous vegetables, such as cabbage, cauliflower, and broccoli, possess phytoestrogens, nature's estrogen hormone modulators, which provide estrogenic effects and an anti-estrogenic competitive effect to enhance the beneficial action and safety of estrogen. Several large-scale investigations have found that Asian women have significantly lower incidence rates of breast cancer than women in the West have, and suggested that the likely contributory factor to the dramatic difference may be that Asian women take a vegetarian diet, with a higher intake of legumes and other plant foods containing a variety of lignans, dietary fibers, and isoflavonoid phytoestrogens [60, 61].

23.5 Herbal Nutraceuticals and Multiple Herbal Component Formulations

Another large portion of marketed nutraceuticals are herbal nutraceuticals. Phytotherapy uses herbal nutraceutical combinations to archive the superior efficacy and lesser side effects in comparison with single isolated components of plant extracts. Many of these multi-herbal nutraceuticals have been assessed in clinical trails as well as in pharmacological studies [1, 6, 7]. These nutraceutical formulas have multiple functions, by targeting multiple biochemical pathways and physiological processes, and, therefore, they can generate well-balanced health benefits with much fewer side-effects.

A multi-component herbal nutraceutical, STW5, has been clinically proved to be effective for the treatment of patients with functional dyspepsia and irritable bowel syndrome [62]. As with other Chinese herbal medicines, combinations of various herbs containing different bioactive substances, STW5 is a combination of nine plant extracts with different active constituents [63]. In addition to single-component nutraceuticals, such as glucosamine and chondroitin, many herbal nutraceuticals have been tried for the treatment of osteoarthritis and rheumatoid arthritis diseases [1, 64].

Other degenerative diseases and immunosystem problems, for which current synthetic drugs often fail, have now become the targets of herbs, nutraceuticals, and nutraceutical therapies. Allergic rhinitis is the most frequently occurring immunological disorder. A traditional Chinese formulation Aller-7, comprising seven herbal extracts, was shown to be well tolerated and efficacious in patients with allergic rhinitis without any serious adverse effect [65]. Similarly, another formulation was also studied in clinical trials and appears to offer symptomatic relief and improvement to the quality of life for some patients with seasonal allergic rhinitis [66]. The butterbur leaf extract Ze 339 was also found to be safe and efficacious in the treatment of patients with seasonal allergic rhinitis [67].

All of these studies clearly suggest that multi-component traditional herbals can offer a very efficacious and better therapeutic option to patients with various

diseases. However, a lack of information on the phytochemistry and pharmacology, or on the synergistic effects of phytotherapies, may threaten and damage the customers and the markets [68].

23.6 FFNs and Metabolic Syndrome, Facial Aging, and Cosmetic Surgery

In contrast to nutrient deficiency that commonly occurs in undeveloped countries and threatens millions of peoples' lives today, people in developed countries often take excessive or imbalanced diets or nutrients, which may cause other types of health problems. An increasing prevalence of overweight and obese populations has reached global proportions. Being overweight or obese can generate a major risk of chronic disease, such as type II diabetes, cardiovascular disease, hypertension, stroke, and cancers. These are diet-related health problems; however, symptoms of patients may not be easily reversed by simply reducing diet consumption or changing their dietary habits, perhaps because the obesity has changed many physiological and psychological processes in the patients, some of which are irreversible.

At the same time, diabetes, particularly type II diabetes, which is normally coincident with obesity, is becoming an increasingly prevalent metabolic syndrome worldwide, with increased risks of cardiovascular, renal, and pulmonary morbidity, and mortality. While the serious side-effects of synthetic drugs cannot be ignored, FFNs are being developed for the treatment and prevention of diabetes, overweight problems, and obesity, as well as for diet-control. FFNs and therapies for that include a wide range of products, such as bars made from oats, whole grains, nuts, or other health-beneficial foods with lower calories but higher nutritional values, or formulas containing herbs and bioactive compounds, as well as combinations of these formulas with FFNs and physical exercise [69].

Many plant-based foods are actually lower in saturated fats and calories, have high levels of dietary fibers, and have more balanced minerals and vitamins contents; thus they would be powerful in the fight against many metabolic syndromes, including diabetes and obesity [69, 70]. Moreover, some nutraceuticals derived from plant foods or medicinal plants have potent effects on the prevention and treatment of obesity. For example, clinical studies have demonstrated that plant-derived sterols, phytosterols, can repeatedly lower bad LDL cholesterol in the blood, which is a major risk factor for coronary heart disease [71].

Hydroxycitric acid, extracted from *Garcinia cambogia*, is an analog of citric acid. It inhibits ATP citrate lyase and carbohydrate conversion into triglycerides, suppressing the appetite without stimulating the central nervous system [72–75]. Hydroxycitric acid formulas are widely used to treat overweight problems, glucose dysfunctions, and metabolic syndromes, for example, to reduce cholesterol and triglycerides. Hydroxycitric acid in combination with other nutrients or nutraceuticals has been used for diabetes and obese individuals. Because of the many debates on its effectiveness and safety, more clinical trials are needed [73, 75, 76].

FFNs are also believed to delay facial aging, and to improve facial rejuvenation and facial beauty, because of their rich contents of amino acids, vitamins, antioxidants, and other phytonutrients that possess antibacterial, antifungal, and anti-inflammatory, anti-aging activity, and overall health-promoting effects that are helpful for the skin [77]. There is widespread use of FFNs, in particular, phytonutrients or herbal nutraceuticals, effective cosmetics (many of which also contain phytonutrients), esthetic plastic and cosmetic surgery, or combinations thereof, at various levels for beauty purposes [77, 78].

There are many claims by manufacturers and marketers about certain herbal nutraceuticals or their phytonutrients having potent effects on the reduction of excees weight, obesity, and diabetes, the prevention of facial aging, degenerative diseases, and nutritional deficiencies, or improvements in overall health and beauty [78, 79]. However, there are also many warnings of negative effects of uses of some FFNs and herbal nutraceuticals or natural products on numerous surgeries, clinical processes, and recovery. Particularly, the perioperative use of herbs and phytonutrient supplements with respect to esthetic plastic and cosmetic surgery should be used with caution. These herbal nutraceuticals with unknown phytochemistry or phytonutrients with unclear pharmacological effects may have a significant impact on surgical outcomes and cause complications [80]. Although some phytonutrients have beneficial effects on some esthetic plastic and cosmetic surgeries [77, 80], the raw herbs are more complex due to the multiple components in herbs. Because very limited information is available on their phytochemical components, medical, or toxic effects, using method and dosage, and the clinical research, perioperative taking of herbs by patients who have undergone surgery may have unexpected influences on the surgical outcome.

A descriptive "top-10" list of such herbs and perioperative recommendations has been compiled for the plastic surgeon and includes [81]:

- **chondroitin**, used to treat osteoarthritis in conjunction with glucosamine;
- **ephedra**, *Ephedra sinica*, used to promote weight loss, to treat respiratory tract conditions, but it has been banned by the FDA because of sympathomimetic toxicity of ephedrine alkaloids, active ingredients ephedrine and pseudoephedrine;
- **echinacea**, *Echinacea purpurea*, used for chronic wounds, immune stimulant and arthritis, active component phenolic compounds;
- **Gingko biloba**, improves blood circulation and mental function, active components, ginkgoflavoneglycosides;
- **goldenseal**, *Hydrastis canadensis*, used as a strong antibacterial reagent, active ingredient berberine;
- **milk thistle**, *Silybum marianum*, used for liver problems such as liver cirrhosis, chronic hepatitis, active ingredient silymarin;
- **ginseng**, used to revitalize and boost energy and reduce stress and fatigue, active component ginsenosides;

- **kava**, *Piper methysticum*, promotes relaxation and is an anti-depressant, active components kavalactones;
- **garlic**, used to maintain healthy cholesterol and an anticoagulant, or as an antibiotic reagent, active ingredient is the sulfur compound allicin;
- **black cohosh**, *Cimicifuga racemosa*, estrogenic activity to treat gynecological and other age-related disorders, active components triterpenoid glycosides;
- **valerian**, *Valeriana officinalis*, used as a mild sedative to treat insomnia and anxiety, active ingredients sesquiterpenes and valepotriates;
- **saw palmetto**, *Serenoa repens*, used for improvement of urinary symptoms and benign prostatic hyperplasia, bioactive ingredients fatty acids and phytosterols;
- **arnica Montana**, used as an anti-inflammatory and antibiotic reagent, active ingredient helenalin;
- **St. John's wort**, *Hypericum perforatum*, used for mild and moderate mood disorders or depression, active ingredient hypericin;
- **bromelain**, pineapple stem, anti-inflammatory, antibacterial, and proteolytic activity;
- **thunder god vine**, *Tripterygium wilfordii*, root extracts used to treat rheumatoid arthritis, bioactive component triptolide.

However, some of these herbs may have a certain toxicity, dependent on the dosage, and have negative effects on surgical procedures or recovery, such as bleeding (ginger, ginseng, ginkgo biloba, and garlic), immunosuppression (echinacea), inflammation (garlic, ginkgo), wound healing (echinacea and garlic), blood pressure, and/or heart rate (ephedra, garlic, ginseng, and goldenseal), increased anesthesia effects (kava, St. John's wort, valerian), and unexpected hormone-like effects (saw palmetto). Some of these are in the FDA's public warning list due to their potential toxicity, such as kava for potential liver toxicity, and St John's wort for possible decreasing indinavir plasma concentration [82, 83].

Thus, extensive studies on these herbs, including ones on their biological activities, phytochemical components, clinical trials, and potential toxicity, are needed before firm claims can be made. It is still responsible and helpful for surgeons or professionals to learn the potential effects of herbal nutraceuticals on patients and communicate well with them to incorporate nutritional and supplementation management into their perioperative office for optimizing surgical outcome.

23.7 Absorption and Metabolism of FFNs and Interaction with Drugs

Although we take great benefits from eating plant foods and their bioactive phytonutrients, our understanding of the absorption and metabolism of phytonutrients is limited. It is now generally accepted that the bottleneck for alleviating

nutrient deficiencies may be largely due to the limited absorption of nutrients from diets. Although the exact percent absorption of most phytonutrients has yet to be determined, recent studies suggest that many important phytonutrients, such as vitamins, minerals (Ca^{2+}, Fe^{2+}, and Zn^{2+}), and bioactive plant products, are not fully absorbed in the human body [1]. Absorption of essential minerals such as Ca^{2+}, Fe^{2+}, and Zn^{2+} from the diet can be subject to interference from other factors from both human and dietary nutrients, and thus poor absorption and deficiency of these nutrients occurs widely, from infants to elders.

Even for important and common nutraceuticals such as vitamins that have been known for almost two centuries, their absorption and metabolism from various food sources are still not fully understood. However, unlike essential minerals, the low absorption rates of most water-soluble vitamins, such as vitamin C and vitamin Bs, and fat-soluble vitamins ,such as vitamins A, E, D, and K, can be readily complemented by intakes of more vitamin-rich plant diets and relevant FFNs [1].

The low levels of bioactive ingredients in plant foods often make it difficult to trace their absorption and metabolism in the gut intestine system. Moreover, some phytonutrients may be metabolized by microbial organisms that live in the gut systems [84]. The human gut is populated by an array of bacterial species that develop important metabolic and immune functions and markedly affect the nutritional and health status of the host. FFNs and their metabolic products may also affect the gut microbiota, either positively, such as prebiotics, or negatively, such as antimicrobial agents. These gut bacteria in turn are also able to transform these so-called bioactive principles in FFNs into various products with different bioavailability (absorption rates by intestinal cells) or bioactivity. These reciprocal interactions between the gut microbiota and FFNs influence their effects on human health [84]. The gut microbiota transforms dietary compounds into different bioactive metabolites *in vivo* and, in turn, plant food bioactive compounds might influence the gut microbiota composition and its physiological effects on mammalian tissues [85].

The most extensively studied plant natural products and their absorption and metabolism by mammalian models might be flavonoids. Anthocyanins and proanthocyanidins are the most abundant flavonoids in plant diets. These flavonoids are most often found to be glucuronylated or methylated in blood plasma after ingestion [86]. However, it is not clear where (intestine cells, liver, or other organs) these flavonoids are actually glucuronylated or methylated and how these flavonoids can pass the blood–brain barrier. Studies have been designed to investigate why the majority of the dietary anthocyanins and proanthocyanidins are metabolized into phenolic acids. It is proposed that most likely these glucuronylated or methylated flavonoids circulating in the blood and brains may exert physiological functions [87].

Many drugs are metabolized by the human cytochrome P450 class of enzymes, and one of the well studied targets of nutraceutical– or herb–drug interactions is how nutraceutcials affect P450 enzyme activity. Gurley *et al*. evaluated the effects of several herbal nutraceuticals, including milk thistle, black cohosh, kava,

goldenseal, St. John's wort, and echinacea on P450 CYP2D6 activity *in vivo* and found that only goldenseal shows significant inhibition on drug urinary recovery [88]. By effective searching of the literature on such research, Brazier and Levine [89] identified about 50 possible drug–herb interaction pairs. Among these, 22 drug–herb pairs were supported by randomized clinical trials, case-control studies, cohort studies, case series, or case studies. Warfarin was the most common drug and St. John's wort was the most common herbal product reported in drug–nutraceutical interactions.

Other types of targets of plant natural products in mammalians are transporters in intestine walls or throughout the gastrointestinal tract, liver, and kidney, which are responsible for drug absorption into the body and metabolism in the liver, and release through the urinary system. Dietary flavonoids have been reported to exert mostly inhibitory effects on mammalian ABC transporters, and therefore affect drug absorption, metabolism, and release [90]. A recent survey reported that about 49% of the American elderly community with ages 57 through 85 used dietary supplements on a regular basis, and about 25% of them are at risk for a major drug–drug interactions [91]. In addition, different types of herbal nutraceuticals also interact each other and affect their biological activity and toxicity, which is even more complicated and important, but little is known about this [92].

23.8
Epidermiological Study and Clinical Trials on FFNs

Strictly, any claim about the health benefits of FFNs, or other food supplements should be based on scientific research, or at least based on evidence of long time use, for example, traditional medicines and herbs. Scientific research on FFNs includes identification of bioactive chemical principles in FFNs, biological activity, toxicity, use dosage, as well as their pharmacological behavior, such as absorption and metabolism of the bioactive principles in the human body or valid medical models. Therefore, food chemistry, pharmacological test, clinical trials, and epidemiological studies are required to provide enough supports for the health benefits of an FFN.

Numerous clinical trials and epidemiological studies have been conducted with FFNs for various health problems. These include vitamin nutrient supplements, essential fatty acids, dietary flavonoids, dietary fibers, phytosterols, phytoestragens, and many herbal nutraceuticals. There is plenty of evidence to prove that an appropriate nutritional contribution is related to lower morbidity and mortality, for example, a recent clinical trial has shown the effectiveness of external supplements with glutamine, arginine, and omega-3 PUFA on acute severe pancreatitis [9].

Clinical trials and epidemiological studies on the effects of FFNs are often full of debates, because of controversial results. However, strict and high-quality clinical trials and epidemiological studies, particularly the latter ones, require not only

rationale designs, proper controls, a long period for feeding, observation and physiological measurements, data collection, but also a large population of patients (participants) willing to cooperate with the study, as well as a final systematic analysis. This is largely because the effects of phytonutrient are usually marginal, long-term, and individually differential.

Other factors can also significantly affect the outcome of clinical trials and epidemiological studies, for instance, the quality and quantity of the herbs or phytonutrients, absorption and metabolism of the phytonutrient, and drug–herb or drug–phytonutrient interactions. Therefore, it is not easy to obtain reliable results from clinical trial and epidemiological studies on phytonutrients. The results from clinical trials and epidemiological studies need to be re-evaluated carefully. For example, FFNs, such as glucosamine, chondroitin, collagen hydrolysate, *S*-adenosylmethionine, vitamins, and fish oil, have been widely used for prevention and treatment of widely occurring chronic and degenerative disease arthritis, and numerous clinical trials at various levels have been carried out. However, controversial results are often reported and no firm conclusion can be reached for the efficacy of these FFNs on arthritis [64, 93].

One of the widely commercialized examples of phytotherapy, saw palmetto (*Serenoa repens*), in combination with others, is commonly used for the treatment of lower urinary tract symptoms and benign prostatic hyperplasia. Although the majority of the adverse events of using saw palmetto are mild, the results from many different clinical trials are complicated and controversial [94, 95]. This is partly due to the complex pathogenesis of arthritis, but objective and subjective causes in clinical trials are probably the main reasons.

However, high-quality clinical trials and epidemiological studies on phytonutrients and phytotherapies provide the most close-to-reality and reliable evaluation of the biological effects on human health. Therefore, they are essential and highly necessary to evaluate phytonutrients and phytotherapies for the continuous and healthy development of natural resources and healing systems. The rapidly increasing number of such proof-of-concept studies strongly support the success of some phytonutrients and their phytotherapies on improvements to health or even to solve health problems.

23.9
Biotechnology for Improved Nutritional Value and Creation of Medical Foods

Biotechnology is the technique that utilizes various living organisms to produce desired products for the pharmaceutical, food, agricultural, and chemical industries, or to carry out tasks for human purposes [1]. From the oldest biotechnology, such as fermentation of microorganisms to make breads, wine, and sauces, to new biotechnologies to create novel foods, such as transgenic rice and potato with enhanced pro-vitamin A nutrient [1], scientists are now using genetic manipulation or metabolic engineering to bring another revolution to human foods to the table and to challenge human society with all types of novelties. The list of these

novel foods is continuously getting longer: from GM tomatoes with increased anthocyanins [96, 97], GM pigs producing more omega-3 PUFA [98], GM salmon with fast growth and higher productivity [99], GM cows with lactoferrin for immunity enhancement [100], GM livestock producing human therapeutic proteins [101], to GM crops producing vaccines and pharmaceutical peptides [1, 102].

Since approval was first sought to market a GM tomato with an extended shelf life by Calgene Corporation in 1989, plant biotechnology has created more than 50 GM crops that are on the market. However, there are concerns and debates over these biotechnologies and their products, regarding food safety, food allergies, environmental problems, threats of natural biodiversity, and food, agricultural, and environmental regulations. The US Government was once most open to new biotechnology, but has recently started to pull back on the deregulation of biotechnology products. GM crops and animals, and other GM products are now subject to more strict regulations. Nevertheless, the huge advantages of biotechnology may allow it to continue to develop, because global climate changes, increasing human population and burdens on agriculture, decreasing arable lands, and worsening of some other factors could threaten global food security. Today agricultural biotechnology is believed to hold great promise in meeting global food needs and in the fight against hunger and malnutrition in the developing world. Agricultural biotechnology is expected to be used, under strict regulations from all possible aspects, to increase the production of the main food staples, improve the efficiency of production, reduce the adverse environmental impact of agriculture, and provide access to food for small-scale farmers.

Current biotechnology allows scientists to produce and over accumulate nutrients such as vitamins, minerals, essential fatty acids or amino acids, and other phytonutrients in crops, medicinal plants, or animals, fish. More advanced biotechnology also enables humans to change the natural compositions of organisms into more desirable ones to facilitate human uses. With these breakthroughs and further successes in the production of animal-originated vaccines, antibodies, and other medical proteins in crops, biotechnology can also generate medical foods. For vitamin nutrients, "Golden Rice," a GM rice accumulating higher levels of pro-vitamin A β-carotenoid in its grains has become a excellent example [1]. In addition, engineering the overproduction of vitamin Bs (such as folate) and vitamin Es (such as tocotrienol and tocopherol) in plants has been tried. Dietary flavonoids such as genestein and anthocyanins, carotenoids such as zeaxanthin and lycopene, terpenoids such as peppermint oil and coenzyme Q, essential fatty acids such as DHA and EPA, and other health-promoting plant natural products have also been the targets for the metabolic engineering of crops [1, 103–105]. Studies were also conducted with these GM plants with increased flavonoid production on medical models, which show clear health benefits in the proof-of-concept [96, 97]. In addition, transgenic plants or crops or livestock that can express exogenous proteins with therapeutical roles have also been generated, such as GM plants or livestock producing oral vaccines, antibodies, and therapeutic peptides [102, 106]. These materials can be made into medical foods for the treatment of diseases.

23.10 Future Developments

Current scientific evidence strongly suggests that FFNs hold great potential and promise in promoting human health, by preventing and curing chronic and degenerative diseases. However, more scientific research, epidemiological studies, and clinical trials are needed to test the efficacy, safety, standardization, use of the methods, and other aspects of FFNs. The acceptance and use of FFNs by increasing populations could continue to be encouraged through more food and medical research and clinical studies. From a government point of view, FFNs provide a means to improve the overall health conditions of citizens, to complement the current and defective medicines and therapies, and to reduce the increasing costs of healthcare systems by the continuous protective or therapeutic function of FFNs. Most governments' regulations on FFNs are not complete due to the fast development and specialty of FFNs. The FFNs and their related therapeutic practices are generally subject to similar regulations as foods. Many concerns and compliances have been raised as a result of the loosely regulated FFNs markets, in particular, with regard to herbal nutraceuticals and their clinical efficacy, quality control, safety test, acute, subacute, subchronic, chronic, and long-term toxicity [107]. Governments, health professionals, nutritionists, and regulatory toxicologists are now strategically working together to enhance the regulations on FFNs and their therapeutic applications [108].

Large variations in numerous pharmacological and epidemiological studies and clinical trials of FFNs led to inconclusive and controversial results, and massive confusion for patients or consumers. The lack of standardization of herbal nutraceuticals and phytonutrient products, and other FFNs, with regard to quality and quantity controls, efficacy, and other important parameters, has been one of the causes of subjective and objective symptom improvement beyond a placebo effect. Because the bioactive components and composition of herbs can change over the time of growth, collection, and storage, and the efficacy of many herbal nutraceuticals may depend not only on one phytochemical composition but on multi-component effects, standardization of these FFNs has been a great challenge.

There is growing evidence from clinical trials, epidemiological and basic studies that potential toxicity of some herbal nutraceuticals should not be neglected. Therefore, more regulations need to be worked out, through consultations with expert panels, for these products and practices, on good manufacturing practice compliance, on what is generally recognized as a safe status, and on analytical methods and validation. The safety assessment of FFNs, in particular herbal nutraceuticals and phytonutrients, is complicated and involves chemical identification of the bioactive composition, quantification of the material, quality control, bioactivity and toxicity tests (including acute, subacute, subchronic, chronic, and long-term toxicity studies, and reproductive toxicology). A large portion of the claims on the effectiveness of herbs and phytonutrients need to be verified [109].

In this "omics" era, transcriptomics, proteonomics, metabolomics, and bioinformatics have emerged as powerful and routine tools in nutrition and medical studies. The application of these technologies in FFNs study has great potential, for example:

- **metabolomics** tools for identification and assessment of the chemical components in herbal nutraceuticals or metabolism products of them in the human body [110];
- **proteomics** technology for testing the biochemical pathway or protein targets of FFNs in mammalian models or the human body;
- **transcriptomics**, global gene expression detection by using microarray technology to probe the safety and efficacy of phytonutrients or herbal nutraceuticals, which will greatly hasten the processes such as validation of toxicity, safety, effectiveness, and health-promoting mechanism of FFNs [111].

Integration of these strategies and technologies into studies of the safety, efficacy, toxicity, and use methodology, and other nutritional aspects of FFNs, has emerged as so called functional nutrigenomics, which study how FFNs affect the global gene expression in an individual and how an individual responds to nutrients and bioactive compounds at transcriptomic, proteomic, and metabolomic levels.

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