Part One
Concepts and Methods for Recombinant Drug Production

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Pharmaceutical Biotechnology and Industrial Applications – Learning Lessons from Molecular Biology

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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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