

PART I

INTRODUCTION

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FUSION PROTEINS: APPLICATIONS AND CHALLENGES

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1.1 HISTORY

Proteins as drugs have a long history. In the beginning, natural proteins were extracted from animal, human sources, or in some rare cases even from plants. Large-scale processing of human plasma became a primary source for the isolation of many proteins [1]. For instance, blood factors became available as a therapy against the different forms of hemophilia or the lack of functional (α -1 antitrypsin. The major serum component, albumin, has now been used for more than 50 years as a treatment for shock, trauma, or burns. Immune globulins isolated from human sources are also used successfully in various immunodeficiency diseases. However, despite the great success of plasma products, contaminations with the HIV or hepatitis virus in the 1970–1980s triggered more intensified efforts to prepare virus-free recombinant therapeutic proteins.

Since its identification in the 1920s until the 1980s, insulin from animals was the only treatment for diabetes patients. Particularly, the porcine insulin was widely used since there is only a single amino acid variation from the human form.

Chemical processes were developed to obtain the fully human variant from the pig isoform [2]. Finally, the first recombinant human insulin was manufactured by Eli Lilly & Co in partnership with Genentech, approved in 1982 by the FDA and marketed under the name Humulin[®] [3]. This was also the first therapeutic recombinant protein for human use.

Since that time, the number of recombinant products and approved biopharmaceuticals has increased considerably. Initially, recombinant copies of proteins were made that replaced the natural protein, which until then was harvested from animal or human sources. With the exception of factor VIII against hemophilia, all these proteins such as human growth hormone (hGH) or follicle stimulating hormone (FSH) belonged to the class of hormones. They were soon accompanied by a growing number of first generation therapeutics that could only be obtained recombinantly such as erythropoietin (EPO), interferon (IFN) or tissue plasminogen activator (tPA), just to name a few. After this first enthusiasm and the success with reproducing natural proteins by recombinant DNA technology, researchers started to consider the *de novo* design of therapeutic proteins that do not occur in nature. There is one specific class that can be seen as intermediate link between natural and designed proteins, monoclonal antibodies (mABs).

Antibodies, being a major part of the organism's immune defense, are large proteins that exist in all higher animals. In 1975, a method was developed to generate murine cell lines producing antibody molecules of a single specificity, the so-called monoclonal antibodies (mABs) [4]. The first therapeutic monoclonal antibody, orthoclone OKT3, was of murine origin and approved in 1986. From then, this concept was further refined with the help of modern recombinant DNA technology to obtain the first fully human antibody against tumor necrosis factor- α (TNF- α), marketed under

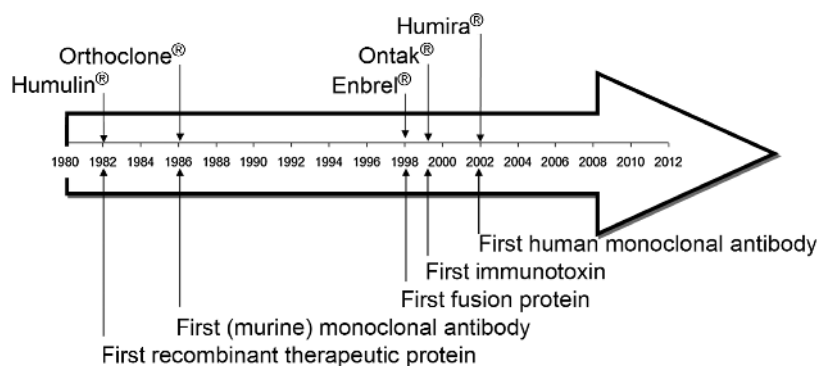


FIGURE 1.1 Milestones of recombinant therapeutic proteins.

the name Humira® in 2002 [5]. The milestones for recombinant therapeutic proteins can be seen in Figure 1.1.

Interestingly, preventing the activity of TNF- α was also the goal for the first fusion protein, Etanercept. It consists of the TNF- α receptor attached to a sequence encoding the Fc portion and hinge region of an IgG1 heavy chain. This drug has been marketed under the name Enbrel® since 1998 and is the best selling fusion protein till date [6]. The two extraordinarily successful drugs Humira and Enbrel can serve as prototype for their molecule classes, exemplifying the different ways to address the same target and present a typical case of competition between antibodies and fusion proteins in the market.

1.2 DEFINITIONS AND CATEGORIES

This book focuses on fusion proteins that are generated by joining two or more genes by genetic engineering that originally code for separate proteins. The result is a single polypeptide with functional properties of both parent proteins. These recombinant proteins are combinations of unrelated domains not occurring in nature. Excluded from the content of this book are multiepitope recombinant vaccines [7], chemical conjugates [8] naturally occurring fusion proteins resulting from chromosomal rearrangements that can be observed in many cancer cells [9] or fusion tags for affinity purification [10].

Bi or multispecific antibodies are special case that do not always represent a single polypeptide chain but usually consist of the combination of heavy and light chains. The Part IIIb of this book discusses some non-natural versions that have more than a single specificity.

The most straightforward classification of these novel proteins can be based on the functions of their incorporated domains. Typically, one part serves molecular recognition or binding, whereas the other part adds certain functionalities such as extending half-life or stability, cytotoxicity, or novel targeting or delivery routes [11].

Recently, a review classified therapeutic proteins according to their pharmacologic activity to (a) replace a deficient or abnormal protein, (b) augment an existing pathway, (c) provide a novel function or activity, (d) interfere with a molecule or organism, or (e) deliver a payload such as a radionuclide, cytotoxic drug, or protein effector [12].

However, this classification is not fully suitable for the scope of this book. Most fusion proteins serve three major purposes that can be summarized under the triple T (T3) paradigm: (a) $t_{1/2}$ (half-life), (b) targeting (or binding), or (c) toxicity (cell killing). Of these three elements, at least two are simultaneously present in fusion proteins (Figure 1.2). Antibodies as natural molecules combine all three aspects in a single molecule. However, antibody derivatives, fragments, or domains have also been used extensively as building blocks for fusion proteins, hence constituting a large part of the portfolio of proteins discussed here, and thus deserving their own category. The main functionality of antibodies, the binding with high affinity and selectivity to a specific epitope, has been reproduced in a number of nonantibody scaffolds that can either be used as single module or by combining two units with different specificity [13]. These molecules together with other bi- or multifunctional therapeutics that do not fit to the T3 categories are classified into the group of novel artificial molecules that is discussed in Part IIIa of this book.

A very practical classification is proposed by the authors of Chapter 3 about “Structural Aspects of Fusion Proteins Determining the Level of Commercial Success” in this book. They suggest sorting fusion proteins based on one of three different functional groups: activity, targeting, or half-life, of which the latter two can be summarized under delivery agents, thus being able to define a two-dimensional landscape of fusion proteins. This is quite similar to a previous scheme using the combination of an effector fragment together with a molecular recognition part as building blocks for fusion proteins [14].

But why should we deal at all with fusion proteins? Several advantages make them very attractive: the combination of two functionalities in a single molecular entity

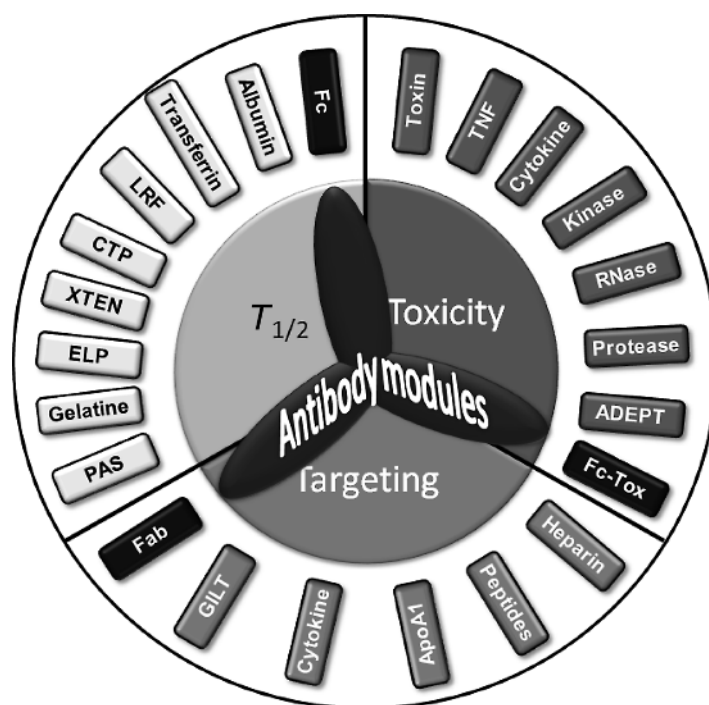


FIGURE 1.2 The triple T paradigm of fusion proteins. Most fusion proteins are composed of mixtures of these modules to combine two functionalities. The multifunctional natural prototype is an antibody combining the constant Fc part that can contribute to half-life extension and toxicity. The Fab part is contained in many fusion proteins for targeting and binding purposes. LRF, ligand receptor fusion; CTP, C-terminal peptide; XTEN, XTENylation; ELP, elastin-like peptide; PAS, PASylation; GILT, glycosylation independent lysosomal targeting; ApoA1, apolipoprotein A1; ADEPT, antibody-directed enzyme prodrug therapy; TNF, tumor necrosis factor family.

simplifies manufacture and drug delivery. Two molecules combined into one will automatically have identical bio-distribution profiles instead of two separate molecules that might have a very different distribution. Furthermore, new functionalities can be created that are lacking in natural or separate proteins. This includes the modification of half-life or targeting specificity. Even economic opportunities such as life cycle extension of products with expired patents are possible. This includes also the generation of novel intellectual property for new and non-natural combinations of proteins. Therapeutic benefits derived from reduced side effects or longer dosing intervals and improved activity are strong drivers to promote the generation of fusion proteins.

But besides all these important advantages, there are also a number of challenges. The combination of unrelated proteins might prove difficult to manufacture because in some cases, the fusion partners have noncompatible properties. This can cause aggregation or misfolding of one domain while the conditions might be perfect for the other domain. Despite the fact that some modules of fusion proteins are elements of other well-proven molecules such as antibodies, the established platform processes might not be applicable because other features shield the required property. This can

go so far that formulation is not possible due to conflicting stability requirements. Furthermore, it will be difficult to control and tune the relative amounts of each component thus complicating dosing for optimal efficacy and safety. Probably most important challenge is the high potential for immunogenicity due to the formation of novel epitopes at the junction between the fusion partners even if only fully human proteins are connected.

1.3 PATENTING

The first generation biologics that represented a true copy of human proteins used for therapeutic applications have already lost or are about to lose their patent protection [15]. In many cases second generation molecules, for example, with improved half-life, are taking their place. A number of them are fusion proteins that are patented as well.

To be able to file a patent for an invention, three characteristics must be achieved: novelty, nonobviousness, and utility or enablement [16]. In the postgenome era, the discovery of novel proteins, at least of human origin, will be difficult. This challenges the first critical parameter on the way to a patent,

the novelty. If we focus on the scope of this book, the fusion proteins, novelty still seems to be easy to reach. As described in the paragraph about definitions, “joining two or more genes by genetic engineering that originally code for separate proteins,” so the generated fusion protein will be novel if nobody did the same earlier. Therefore, novelty is given on one hand through the composition of matter (the new construct, e.g., long-acting human growth hormone [hGH], consisting of hGH fused to human serum albumin [HSA]) or on the other hand by the use that results from combined features of the new molecule (e.g., to treat dwarfism with longer administration intervals). This is in contrast to a natural polypeptide with multiple functions [17].

Taking the example of antibodies as molecules, their individual characteristics, for example, target or captured epitope, affinity, half-life, or sequence of the variable part should be sufficient to enable patenting based on novelty [18]. Surprisingly, exactly these descriptive features are missing in many antibody patents, which is an effect of patenting human genes that often include hypothetical antibodies to that target in the claims [19]. It might be easier trying to patent antibodies based on unexpected advantages, that is, having lower cross-reactivity since higher affinity could be regarded as obvious [20]. This should be taken into account for patenting antibody derived fusion proteins. Particularly, tumor-specific antibodies have had only limited success so far. This led to new intellectual property of improved molecules by coupling antibodies to toxic proteins, thus combining target specificity with nonspecific cytotoxicity [21].

However, if we continue with the second parameter, the nonobviousness, it starts to get more difficult. In the context of fusion proteins in many cases, the existence or functionality of the potential fusion partners will be well known; thus representing prior art. Interestingly, when combining several “prior arts” into a new concept, this patent will only be rejected as obvious if there existed at the time of invention a known problem for which there was an obvious solution encompassed by the patent’s claims [22]. The best argument to get a combination patent is to demonstrate that prior art is not providing a motivation or suggestion to prepare this combination. Even when sufficient suggestions for combinations can be found in prior art, patenting can still be possible if enabling in form of a production method can be claimed additionally. Therefore, when defining patent claims, it can be very beneficial to include methods how to manufacture the protein of interest and what formulations are useful [23].

The third parameter to demonstrate utility is relatively straightforward since the fusion protein was designed with a specific application in mind. Here, both typical aspects of utility, recognition of a benefit and the motivation to make a change to current practice, come into play. Overall it has to be described how the invention can be put into practice, which is ideally done in the form of examples.

In the past, some patent disputes ranked around protected fusion protein technology. For instance, Zymogenetics accused Bristol-Myers Squibb (BMS) to infringe their Fc-fusion technology with Orencia[®]. Initially, the case was settled by a lump sum payment, but finally BMS acquired Zymogenetics in 2010 together with the rights for Fc fusions [24].

1.4 DESIGN AND ENGINEERING

In the design of a novel fusion protein a number of parameters have to be taken into account. Following questions need to be addressed: Will the proteins be functional on either the N-terminus or the C-terminus? In which orientation will the individual proteins be connected? What linker length and sequence should be used? Is there a need for a specific oligomerization? Are mutations or truncations required to enhance or eliminate certain features? In many cases rational design will guide the generation of innovative protein therapeutics [25]. One of the aims of protein design is certainly to improve the functionality of biological drug (Figure 1.3). A key question in this context is: Will the protein reach the target in a significant quantity? Taking the example of a solid tumor it becomes clear that in order to reach the tumor the protein has to be sufficiently small to penetrate the many cell layers. But on the other hand it should not be too small to be excreted too fast. This requires a delicate balancing of the molecule size and has been demonstrated experimentally with a number of different antibody derivatives [26]. Not only size but also valency of antibodies can be modified. Further details on bi-specific and multifunctional antibodies can be found in Part IIIb of this book.

1.4.1 Orientation of Fusion Proteins

Looking at the largest group, the Fc-fusion proteins, it is well known that naturally the Fc part is positioned at the C-terminus of an antibody. But in artificial fusions it can also form the N-terminal part. Several studies have evaluated cytokine mono- or tandem fusions to full antibodies or Fc parts. Here no dependency on the selection of the respective terminus could be observed [27]. However, comparing N- or C-terminal Fc fusions of peptides blocking angiopoietin-2 (Ang-2), it was found that the N-terminal fusion had shorter half-life and weaker binding but better selectivity [28].

Generally, when combining two proteins, there are two orientations in which the fusion partners can be arranged, either at the amino or at the carboxy terminus of the first protein. In many cases the position is without influence on the functional properties, for instance albumin (HSA) can be fused to either end [29]. But during the manufacture of Albuferon[®], a combination of interferon- α 2b (IFN- α 2b) with albumin, it was observed that due to incomplete

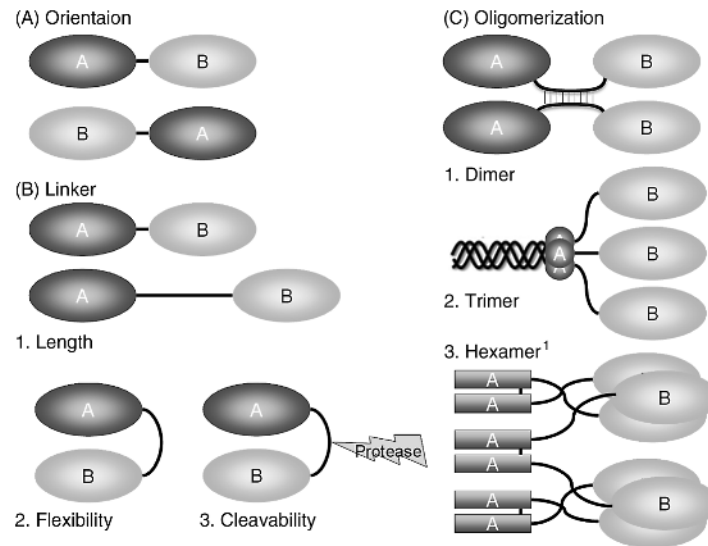


FIGURE 1.3 Fusion protein design. The typical variations of fusion proteins comprise orientation, linkers, and oligomerization. Sometimes proteins require a free N- or C-terminus for their activity. The linker can vary in length, flexibility, and susceptibility to protease cleavage. Dimeric forms of fusion proteins can be generated by including leucine zippers in the linker region. Two examples of higher oligomerization are depicted as well; first, a trimer built from the trimerization domain of human endostatin and second, a hexamer constructed through the combination of three Fc dimers connected via an ILZ coiled coil to two trimers (¹see Morris et al. [51]).

disulfide bridge formation, aggregates formed that drastically reduced the recovery. Interestingly, this phenomenon could be completely abolished by positioning the IFN- α 2b on the N-terminus instead [30]. In another case the order of two angiostatic proteins, human angiostatin (hAS) and endostatin (hES), combined in a fusion protein had to be in a specific orientation to obtain maximum activity. The fusion hES-hAS was 28% more potent than hAS-hES or 7% better than hAS and hES when administered separately [31]. Sometimes a free N-terminus is required. The second partner is then connected at the C-terminus as in the case of receptor traps such as Etanercept. Here, the extracellular receptor domain is combined with an Fc part at the C-terminus to maintain the natural conformation [6]. Another striking example for the positioning effect of fusion partners is the case of elastin-like peptides (ELP). It was observed that C-terminal fusions of ELP resulted in a higher expression level, better yield, and bioactivity. The underlying reason could be increased misfolding, induced by ELPs at the N-terminus; thus reducing the amount of active proteins and increasing their susceptibility to proteolysis [32]. Orientation has a high impact on functionality particularly when fusion proteins contain enzymes that require either a free N- or C-terminus. This has been demonstrated with the Immunoreceptor consisting of angiogenin (ANG) and a single-chain variable domain (scFv) against CD22. Only constructs in the scFv-ANG orientation did not aggregate and were fully functional [33].

1.4.2 Linker Engineering

Again starting with Fc fusions as example, the hinge region fulfills the function of a linker, allowing some spatial flexibility [34]. Besides this exception where a part of a fusion protein ends with a flexible peptide chain, in most cases specific linkers between protein molecules have to be artificially introduced. The multiple aspects of linker design have recently been reviewed [35]. Many researchers use a simple glycine and serine (G_4S)-containing linkers as proposed by a large study of natural domain separating linkers [36]. Spacer peptides that connect both modules of a fusion protein in a spatial conformation are frequently needed to maintain functionality. For instance, the highest potency could be observed when a spacer was introduced between a single chain variable domain (scFv) and ANG [37]. Since fusion proteins ideally consist only of a single polypeptide chain, a reformatting of Fab fragments to scFv is required. This is done with the help of a linker sequence that frequently consists of repeats of glycine and serine, as for example the popular $(G_4S)_3$ linker. The basis of linker design is the rational engineering of both length and conformation. A controlled distance between domains can be achieved by defined repeats of α -helical peptides $A(EAAAK)_nA$ that maintains the separation of domains in contrast to flexible linkers [38].

When evaluating the optimal linker between IFN- α 2b and HSA it could be demonstrated that five amino acids (aa)

are already sufficient. However, maximum activity was obtained with a helical 12 aa linker which was 1.7 times or 2.9 times better than a short rigid or the standard five aa long G₄S linker [39]. The knowledge about linkers was continuously expanded [40]. Recently, a database collecting natural linker sequences and their properties could be established.¹ Inter domain linkers have to be differentiated from intradomain loops based on their function, but also because of their amino acid preference. Interestingly proline is the most frequent amino acid in both cases. Proline destroys α -helix and β -sheet structures as well and cannot form hydrogen bonds [41].

But linker peptides can also be engineered to contain additional functionalities. To optimise the pharmacokinetic profile of a granulocyte-colony-stimulating factor (G-CSF) fused to transferrin, a linker with an intramolecular disulfide bridge was introduced. The peptide sequence was thrombin sensitive, which allowed an *in vitro* cleavage by adding this protease, leaving the two domains only connect through the disulfide bridge. This labile bond could then easily be cleaved *in vivo*, releasing free G-CSF that was more active than the fused molecule [42]. Previously, a similar molecule was engineered to improve oral efficacy. Here, a long rigid helical linker composed of 50 aa between G-CSF and transferrin led to a 10 times lower EC₅₀ than the initial fusion protein with a two aa spacer [43]. This particular linker also increased the expression level more than 10-fold compared to the direct fusion with only two aa in between. However, this time the construct was in an opposite orientation having transferrin at the N- and G-CSF at the C-terminus. Interestingly an unstructured 50 aa linker inhibited expression totally [44].

The orientation of domains and the impact of spacers were also studied when a scFv against human transferrin receptor was combined with the fungal ribonucleolytic toxin restrictocin. Independent of the orientation in both cases, the introduction of a protease-sensitive linker drastically improved the EC₅₀ 2- to 30-fold [45]. The influence of linkers on fusion proteins is discussed in Chapter 4.

1.4.3 Oligomerization of Fusion Proteins

The next level of design involves the correct oligomerization of the protein. In many cases, proteins are dependent on multimerization or have a higher bioactivity as a multimer. One example is the combination of stem cell factor (SCF) and macrophage-colony-stimulating factor (M-CSF) with a 12 aa flexible peptide linker. The fusion protein forms dimers that have a 10- to 20-fold higher potency than the individual monomeric proteins, also benefitting from a synergistic effect [46]. A higher cytotoxicity could also be demonstrated for divalent antibody toxin fusions. Combining a Fab fragment

with two molecules of a truncated *Pseudomonas* exotoxin A resulted in an almost 40-fold more active fusion protein than with only one toxin molecule [47]. Oligomerization can also help to improve valency of recombinant proteins. Sometimes it is required to induce cross-linking of receptors to execute specific functions such as activation or internalization. It has been demonstrated that by the right choice of linkers between V_H and V_L in scFv, aggregates can spontaneously form having a higher avidity [48].

Using an Fc part automatically delivers dimerization, however there are also different approaches possible to enforce dimers. Frequently leucine zippers are used for that purpose. An early example is the generation of bivalent scFv antibodies with Fos or Jun leucine zippers. A covalent bond could be introduced by positioning cysteine in proximity to the zipper. These molecules spontaneously formed dimers when secreted to *Escherichia coli* periplasm [49]. The leucine zipper GCN4 of *Saccharomyces cerevisiae* has been used to dimerize the soluble insulin receptor. This resulted in an improved binding constant, very similar to the original membrane bound native receptor [50].

As in the case of OX40 ligand (OX40L) even a trimerization can be required for full functionality. Here an active construct in the right conformation could be obtained by fusing OX40L to a GCN4 zipper domain connected to an Fc part. The final hexameric molecule consisted of three Fc-induced disulfide linked dimers that allowed the formation of two trimers [51]. Another molecule having a trimer as active natural conformation is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). As before, the GCN4 zipper-induced self-assembled trimers of TRAIL based on three-stranded coiled coils [52]. However, TRAIL can also be trimerized as linear fusion in a head to tail configuration interrupted by short flexible peptide loops. To improve targeting, even a scFv could be included at the N-terminus [53]. Trimerization through tandem repeats in a single chain molecule could also be shown with TNF [54]. CD95 ligand (CD95L) as another member of the TNF homology domain (THD) family could be forced into covalent trimerization by fusion to a tenascin-C (TNC) oligomerization domain, again resulting in improved bioactivity [55]. Higher oligomerization of multiple trimers was tested with the CD40 ligand (CD40L). First a di-trimer conformation was achieved by incorporating the N-terminal part of adiponectin (Acrp30). A tetra-trimer construct could be obtained by combining the N-terminal part of mouse surfactant protein-D (SP-D) with the extracellular part of CD40L. Additional leucine zippers were not required because both Acrp30 and SP-D spontaneously self-assemble into the desired multimers. These constructs have been successfully used as adjuvant in DNA vaccines [56]. A similar approach was also initiated with soluble Fas ligand (FasL). The bioactivity of a single FasL trimer was much lower than di-trimer (hexameric) constructs that either relied on the oligomerization effects of

¹ <http://www.ibi.vu.nl/programs/linkerdbwww/>

Acrcp30 or Fc [57]. Other naturally occurring trimer inducing molecules are the carboxyl-terminal noncollagenous domains (NC1 domains) of collagens XV and XVIII coding for endostatin. This was utilized to generate a multi-functional anti-angiogenic compound consisting of a scFv and a NC1 domain. Cleavage of the NC1 domain by tumor-associated proteases released endostatin from the scFv trimers, thus multiplying the anti-angiogenic effect [58]. Collagen trimers were also used to increase the binding strength of scFv 20- to 1000-fold through multimerization. It was shown that this collagen-like scaffold (Gly-Pro-Pro)₁₀ could either be fused to the N- or C-terminus. Depending on the addition of cysteins flanking the scaffold even a hexameric configuration could be obtained [59]. The trivalent human plasma protein tranectin builds the core of a novel scaffold, the Atrimer™. So far Atrimers have not been used as basis for fusion proteins, but their multiple loops have been engineered for diverse binding applications [60].

1.4.4 Immunogenicity

Rational design of fusion proteins must also be used in order to minimize immunogenicity of the new construct. Even if using only human proteins as starting point for a modular assembly, still the region between both molecules represent a novel epitope that could elicit an immune response. Therefore, it is recommended to use at least *in silico* analysis to predict T- and B-cell epitopes. A lot of work on the removal of T-cell epitopes or de-immunization of fusion proteins has been conducted on immune toxins. In one recent example, a truncated version of Pseudomonas exotoxin A (PE38) fused to an anti CD22 scFv was scanned for the presence of B-cell epitopes with a huge set of antibodies. The identified major epitopes were neutralized by specific mutations [61]. Furthermore, two lysosomal protease-sensitive areas were eliminated from PE38. The resulting mutant had a drastically reduced immunogenicity and an even improved cytotoxicity [62].

A very special case is the antibody-directed enzyme prodrug therapy (ADEPT) that utilizes nonhuman enzymes to metabolize inactive prodrugs into highly potent toxins. One of the promising enzymes, a beta-lactamase had to be mutagenized to remove CD4⁺T-cell epitopes. Site-directed mutagenesis replacing individual aa lowered the T-cell response fivefold [63].

In general, Fc-fusion proteins should also display lower immunogenicity. This effect can be based on the presence of inhibitory Fc receptors on B lymphocytes, the FcγRIIb. This hypothesis was proven with the injection of DNA coding for a Exendin-4 Fc-fusion protein that did not result in the generation of neutralizing antibodies, which was the case for Exendin-4 alone [64].

A number of factors determine the immunogenicity potential of proteins in general. For example, protein

aggregates might cross-link B-cell receptors, or increase protein internalization of antigen-presenting cells (APC), thus initiating an immune response. However, endocytosis can also happen if the target of a fusion protein is up taken. Furthermore, nonhuman modification (e.g., glycosylation) will also induce an immune reaction. Many of these reactions can be predicted *in silico* or analyzed *in vitro* with a wide range of assays [65]. Since both B- and T-cell epitopes contribute to immunogenicity, it is very advisable to identify and remove these epitopes. Due to the high polymorphism of the major histocompatibility complex (MHC) it can be difficult to remove all T-cell epitopes. B-cell epitopes are not restricted to MHC molecules; therefore, it might be easier to eliminate B-cell epitopes [66]. More details on the immunogenicity of fusion proteins can be seen in Chapter 5 of this book.

1.4.5 Mutagenesis for Molecule Optimization

A lot of work on the optimization of fusion proteins by side-directed mutagenesis is focused toward improving parameters beyond immunogenicity. An important factor is the resistance against proteases, on one hand during manufacture and on the other hand while circulating through the patient's organism. In both cases, it must be distinguished between exo- and endopeptidases. For exopeptidases that can cleave their target from either end, usually a modification of the terminal aa abolishes the degradation. For instance, the extension of a glucagon-like peptide-1 (GLP-1) fusion protein by only a single amino acid at the N-terminal prevented cleavage by dipeptidyl peptidase-IV (DPP-IV) [2]. The circulation half-life of an immunotoxin could be doubled when replacing an endoprotease-sensitive arginine residue by serine or lysine [67]. However, proteolytic processing during the endosome/lysosome trafficking is an important step in the mechanism of action of immunotoxins. Therefore, engineering of cleavage recognition sequences must be done very carefully. Recently, it was demonstrated that a deletion of a protease-sensitive region of PE38 abolishes lysosomal degradation while maintaining efficacy and increasing tolerance of high doses [68]. A combination of increased half-life and improved activity induced by single amino acid changes could also be observed with an interleukin-2 (IL-2) immunocytokine. Here, the linker peptide between the C-terminus of the antibody heavy chain and the N-terminus of IL-2 was modified, primarily with the aim to remove protease cleavage sites [69]. The nonselective toxicity of immunotoxins can be avoided by generating in-frame fusions with ubiquitin that triggers rapid degradation. However, the insertion of a cancer protease-specific cleavage sequence can stabilize the immunotoxin by removal of the ubiquitin moiety. For instance, a saporin-based immunotoxin containing a prostate-specific antigen (PSA) recognition sequence had a

10-fold higher activity in the presence of PSA-producing prostate cancer cells [70].

Other mutagenesis approaches are directed to the presence of disulfide bridges. Cysteins can be added, removed, or repositioned. In many cases, the introduction of disulfide bonds improves stability. Heterodimers between V_H and V_L of antibody fragments are relatively unstable but can be stabilized by adding a cysteine in each of the fragments to generate a disulfide bridge. Although there are two possible positions, only one locus retained the full binding activity [71]. Even the rearrangement of existing disulfide bridges is possible as demonstrated with erythropoietin (EPO) where a cysteine was moved from position 33 to 88. This modified EPO in fusion to an Fc part exhibited superior dimerization capabilities, better glycosylation stability and improved pharmacokinetic properties [72]. Stabilization by introducing disulfide bridges can also suppress immunogenicity as shown with an immunotoxin that was mutagenized in the domain III of *Pseudomonas* exotoxin A [73]. However in some cases the presence of an unpaired cysteine can cause aggregation problems. A fusion protein between IFN- α 2b and HSA (IFN- α 2b-HSA) aggregated and caused immunogenicity issues. Only replacing the free cysteine by serine abolished the effect, leading to a more stable and less immunogenic protein [74]. Sometimes disulfide-induced dimerization has multiple effects. The Fab-PE38 dimer with PE38 fused to the light chain and linked through a disulfide bridge in the hinge region had a 16-fold higher refolding yield and 2.5-fold better activity than the initial monomer [75].

Not only free cysteines can cause aggregation, much more frequent is the exposure of hydrophobic residues at protein surfaces that leads to aggregation. In a systematic study, these critical positions and their respective ideal aa of scFvs were identified. Two positions on the V_H (82, 85) and also on the V_L (36, 60) were found, whose replacement with more ideal aa resulted in a significant improvement of stability and yield [76]. A similar approach with another scFv revealed three other heavy chain and one light chain residue that contained not conserved aa. Triple V_H mutants resulted in eightfold higher yields that could be increased to 20-fold, when the orientation was reversed. Interestingly, the mutations did not have a negative effect on binding affinity, but improved plasma stability [77]. Aggregation can also occur by the lack of glycosylation when proteins are expressed in *E. coli*. In the case of erythropoietin (EPO) this was counteracted by replacing asparagine residues with lysine. Due to the increased isoelectrical point (pI) the protein became positively charged under physiological conditions thus eliminating aggregation [78].

Mutagenesis can also have an effect on the activity profile of proteins. For instance, the replacement of the plasminogen activator inhibitor-1 binding site in Tenecteplase[®] by exchanging four aa resulted in a significantly longer activity, because the inhibitor could no longer bind [79].

1.5 MANUFACTURING

The first recombinant protein product, insulin, was initially produced as two separate chains that were conjugated chemically. But soon thereafter the commercial process utilized expression in *E. coli* and subsequent enzymatic maturation to generate insulin from proinsulin [80]. Since that time more than 120 therapeutic proteins, including a number of fusion proteins, have been manufactured for human use in bacteria, yeasts, or animal cells [81]. From a manufacturing perspective in most cases it is not necessary to differentiate between fusion proteins or regular singular therapeutic proteins. The production typically covers three complex steps: upstream processing (molecular biology and fermentation), downstream processing (capture and purification), and finally formulation (transforming the protein into a storable and administrable form). One of the many advantages of fusion proteins is the uninterrupted manufacturing process of a single protein molecule with several functions.

1.5.1 Upstream Process

The choice of the expression system depends heavily on the properties of the desired protein product such as glycosylation, disulfide bridges or other post-translational modifications that can only be obtained from eukaryotic cells [82]. Also protein size plays a role; usually, proteins larger than 100 kDa are by default produced in eukaryotic cells, whereas proteins below 30 kDa are expressed in bacteria. A recent analysis revealed that 39% of recombinant proteins were expressed in *E. coli*, 35% by Chinese hamster ovary (CHO) cells, 15% by yeasts, and 10% by other mammalian cells, but only 1% by other systems [83]. Interestingly, 17 of the 58 approved therapeutic proteins between 2006 and 2010 were manufactured in *E. coli* [84]. The following paragraph focuses on the two major host organisms, *E. coli* and CHO cells, while *Pichia pastoris* has been primarily used for albumin and transferrin fusion proteins [85]. Secreted Fc-fusion proteins in *P. pastoris* require individual optimization of upstream conditions [86].

The modern upstream process consists of three elements: the expression construct, the host cell, and the cultivation conditions. The expression construct in microbial expression is mostly a plasmid vector coding for a resistance gene as selection marker, the gene for the protein to be expressed, both accompanied by the respective promoter sequences, and a replication origin to maintain the plasmid during cell division [87]. It is generally recommended to optimize the codon usage according to the selection of the host organism. Usually, microbial cells are transfected and kept as clonal glycerol stock to serve as master cell bank. Alternatively, the expression cassette can also be integrated into the host genome to create a stable transfected cell. This is the

preferred option for mammalian cell culture. The integration can occur at multiple sites leading to gene duplication and hence higher expression levels [88]. In microbial cells, a similar effect can be achieved with high copy number plasmids. Molecular biology strategies to optimize recombinant protein expression can target transcriptional and translational regulation. On transcriptional level, promoters and terminators play key roles. For the translation reaction, the binding of ribosomes to mRNA during the initiation and their release at termination are very important as well as the stability of the mRNA in the cytoplasm. Folding of the nascent polypeptide chain can be enhanced by co-expression of chaperones [89]. Further optimization can focus at the selective knock out of proteases that might otherwise destroy the protein product [87].

When working with *E. coli*, we should not forget that post-translational modifications are missing and in some cases the amino terminus might contain an extra methionine residue that is not properly removed during translation [90].

The fermentation of *E. coli* cells depends on media composition (e.g., carbon source, antibiotic selection) and cultivation conditions (temperature and oxygen content). One drawback of high-density culture is the accumulation of acetate that limits proliferation. This problem can be solved by a number of different approaches such as introducing the foreign enzyme acetolactate synthase into *E. coli* that generates a less toxic by-product [91]. Alternatively, glucose uptake can be limited by knocking out the *ptsG* gene or the whole phosphotransferase system (PTS) [92].

The attractiveness of *E. coli* is based on the fast, cheap, and simple expression of proteins that can be targeted to the cytoplasm, the periplasm or the cell culture supernatant by secretion. But often intracellular expression of eukaryotic proteins in bacteria results in inclusion bodies (IB) that require refolding approaches during downstream processing. The formation of IBs is mostly caused by an overload of the folding machinery in the bacterial cell that leads to exposure of hydrophobic residues triggering aggregation. In addition, *E. coli* lacks the ability to form disulfide bridges in its reducing environment in the cytoplasm [93].

Particularly, when combining protein toxins and a targeting moiety, the expression level might be significantly lowered due to the translational inhibition by the toxin. One approach to circumvent this difficulty is to express the toxin fusion in form of IB that cannot harm the host cell because of its unfolded, nonactive state [94]. Also secretion to the culture supernatant to keep the toxin away from its target, the ribosomes, has been accomplished [95]. A third variant of producing toxin fusions is the expression in the presence of an inhibitor that is removed during the downstream processing [96]. Another possibility is to express both parts of a fusion protein separately, then fusing them in a later trans-splicing step with the help of split-inteins [97]. Recently, it was shown that an immunotoxin can be

successfully manufactured with high density bacterial fermentation under GMP conditions at a yield of 40% and 97% purity [98].

CHO cells came into the focus of manufacturing after Genentech's market approval of Activase[®], the recombinant tissue plasminogen activator (tPA), in 1987. Currently, CHO cells are the industry standard for glycosylated complex proteins and benefit from three accomplishments: serum-free production is possible and easy, cell engineering achieves high titers, and high-density large-scale fed-batch cultivation is well established. Cell engineering addresses the parameters expression level, duplication time and stability of the cell, control of proliferation and viability, reduction of toxic metabolites, increasing secretion capacity, and modulation of post-translational modifications [99].

The process starts with expression vector design. Strong viral promoters, the elimination of cryptic splice sites and the increase of G/C content by codon optimization are tools to improve expression levels. A further trick is the insertion of an intron to enhance mRNA export and stability by splicing. The gene silencing effect at the genomic insertion site can be avoided by flanking the coding sequence with DNA elements that can block the formation of heterochromatin [100].

Usually, DNA constructs are integrated randomly into the genome by homologous recombination. Besides the gene of interest, the vector also transfers a selection markers; either dihydrofolate reductase (DHFR) or glutamine synthesis (GS). Cultivating the cells in a medium without the respective metabolites lets only transformed cells survive. Furthermore, genes become amplified when cells are exposed to increasing concentrations of the inhibitor methotrexate (MTX) and methionine sulphoximide (MSX) for DHFR and GS, respectively. High producers with good duplication rates are cloned and a master cell bank is created [101].

The growth and productivity of cells is mainly dependent on the metabolism. Therefore, attempts have been undertaken to reduce waste metabolites, thus making the carbon metabolism more efficient. Other strategies involve extending the cell's lifespan by activating anti-apoptosis genes or engineering cell cycle control. Further improvements were achieved by addressing the secretion apparatus and over expressing chaperones [102]. Mutant CHO cell lines can also be useful for the expression of toxic compounds. Usually, immunotoxins are produced as IB in *E. coli* to prevent cell death. Good secretion levels of a scFv-diphtheria toxin fusion could be obtained in an ADP-ribosylation insensitive CHO mutant. However, the glycosylation decreased toxicity. Eliminating the glycosylation sites by mutagenesis improved toxicity 12-fold over the identical molecule prepared from *E. coli* periplasm [103].

In serum-free media nonanimal protein (Sericin) and nonprotein (phosphatidic acid) substitutes enable cultivation. Previously, the most important serum components were

albumin, transferrin, and insulin [104]. Proteins can aggregate even when secreted from CHO cells. This should be minimized already in the upstream process to reduce losses in the downstream and to improve formulation. Adding dexamethasone at nanomolar concentrations increases glutathione reductase levels, but not protein disulfide-isomerase. Thus, a balanced redox condition is established in the cells, effectively reducing aggregate formation as demonstrated on the example of an IgG-fusion protein [105].

In addition to cell engineering, the drastic 100-fold increase of productivity within 20 years can mainly be attributed to better understanding of cultivation conditions. This includes media composition, nutrient content, pH, temperature, the addition of histone deacetylase inhibitors such as sodium butyrate or valproic acid, a better dissipation of dissolved oxygen by reactor design, and metabolite triggered feed strategies [106].

1.5.2 Downstream Process

The downstream process starts with harvesting the product of interest. For secreted proteins that are mainly produced by eukaryotic cells (animal cells or yeast), there are two alternative procedures; either centrifugation to sediment the cell mass, or filtration to obtain a cell-free supernatant. Secreted expression is preferable because in state-of-the-art serum-free medium only few contaminants besides the protein of interest are present. A combined unit operation such as expanded bed absorption (EBA) connects cell removal and first product capture in a single step. The protein of interest retained in the cell-free solution is then separated from other contaminants such as other proteins, DNA, or viruses by a series of chromatographic polishing steps. Finally, the isolated protein is pure and can be transferred to the subsequent formulation step [107].

If the protein is not secreted, a product release step, where the microbial cell is homogenized by physical or chemical means, must be included. Most of the employed methods suffer from a rather high unspecific homogenization. This means the product of interest is released together with many contaminants that have to be removed in laborious downstream procedures. A selective product release step minimizes contaminants, increases the adsorption capacity of chromatography and reduces viscosity. Important parameters to consider for the optimal disruption process are particle size and density, recovery rate, viscosity, processing time, and scalability. In general, mechanical (e.g., high pressure, cavitation), chemical (e.g., osmotic shock, buffer conditions), and genetic (e.g., induced cell lysis) methods can be distinguished. Ideally, the product is accumulated in a subcellular compartment or the periplasmic space that simplifies specific release [108].

A special case for intracellular accumulation is the formation of IB. In many cases, IBs can represent

10–50% of the total cell protein containing up to 95% of a single protein species [109]. Due to their high density IBs can easily be isolated by sedimentation and often allow an enrichment of approximately 90%. Functional proteins from IB require two steps; solubilization under denaturing and reducing conditions, then refolding by removal of denaturants and reformation of disulfide bridges [110].

In some cases, the fusion partner helps by enabling an elegant downstream processing route. One striking example is the utilization of elastin-like peptides (ELP) that facilitate the purification by temperature dependent aggregation that allows simple capturing by sedimentation [111]. A typical antibody-based production, standardized for Fc-based purification, achieves an expression level of 2–5 g/L and an overall yield of 70–80%. Unfortunately, nonantibody Fc fusions do not always achieve such high titers and often they bind less efficiently to Protein A resins [112]. But despite of these deviations, most of the platform solutions derived from antibody processing are also applicable for Fc-fusion proteins. Protein A and its derivatives are the most frequently used capture ligand for Fc-based proteins on chromatography columns. The elution at low pH also inactivates potential viral contaminations if it is below pH 3.7. But an acidic pH might destabilize some Fc-fusion proteins and cause aggregation. This aggregation can be overcome by simultaneously adding a chaotropic agent during elution [113]. Furthermore, ion exchange chromatography step(s) and a final virus filtration complete the standard Fc-dependent purification protocol [114].

If Fab fragments or scFv are fusion partners, also dedicated affinity matrices such as the kappa light chain specific Protein L [115], FabSorbent[®] [116] or other novel synthetic molecules [117] can be used. Protein L was also successfully applied to purify trispecific antibodies lacking an Fc part [118]. Even for albumin fusions a platform technology, AlbuPure[™] was created [119]. Especially for unconventional fusion proteins such as Amediplase[™] customized affinity matrices have been designed that enable a scalable manufacturing process [120].

One of the challenges of contemporary downstream processes is the constantly increasing protein titer resulting from upstream improvements. Sometimes the column capacity in affinity chromatography is not sufficient to capture these high protein concentrations. Therefore, ion exchange chromatography has been used as alternative. This might also be generally applicable for fusion proteins lacking domains for which platform technologies exist.

As alternative to classical chromatographic procedures, aqueous two phase systems (ATPS) have been used recently for the manufacturing of biopharmaceuticals. The phenomenon of ATPS is based on the incompatibility of two components. The typical pairs are either polymers or salts. By forcing the (soluble) protein of interest into the upper phase, simultaneously aggregates can be removed as well.

Since more parameters (concentration, pH, ionic strength, molecule type, and weight) contribute to the separation, this technique has a high resolution power. ATPS is easily scalable, has a high capacity, and can be used in a continuous operation. ATPS could prove quite useful also for the separation of fusion proteins if predictability and economical sustainability is verified [121].

1.5.3 Formulation

Formulation covers the steps that are necessary to create a drug product out of the drug substance. This means the active pharmaceutical ingredient (API), the therapeutic (fusion) protein, is combined with additives or excipients. The proper selection of excipients is a challenging task because only a limited range of molecules is available [122].

The goal of formulation is to prevent protein degradation during storage and to optimize its delivery. To achieve that, a number of buffer parameters such as pH, ionic strength, and composition must be carefully assessed [123]. This can be very demanding for a fusion protein that could be composed of two proteins with different buffer preferences or different sensitivity to degradation. Proteins can be degraded through physical (denaturation, adsorption, aggregation, precipitation) and chemical (hydrolysis, deamidation, oxidation, isomerization, and disulfide exchange) instability. The protein can also be exposed to harmful conditions during handling such as temperature, physical interfaces, and shear forces [124].

Particularly, non-native aggregation of liquid dosage forms can diminish the concentration of the active drug. To limit the amount of aggregates formed, it is necessary to understand the aggregation routes and to predict its formation rate [125].

From a safety point of view, the drug product has to be sterile, free of aggregates that could cause immunogenicity or reduce the concentration of the active substance. The final product must be free of product or process related impurities within a specified tolerance [126].

It was shown that a change of formulation of a well-tolerated product can cause serious safety issues. For example, a change of formulation and ingredients of epoetin- α caused severe pure red cell aplasia [127]. The final step of formulation, fill to finish, has to be done into vessels that maintain sterility, but also do not contain unacceptable extractables and leachables or cause immunogenicity [128]. Both, leachables and extractables can be set free during the filling procedures or later during storage; therefore these conditions must be defined carefully [129]. Stability can be tested in an accelerated approach, where long-term effects are simulated by elevated stress in form of intense temperature, light exposure, or pH.

Beyond physicochemical parameters also patient compliance must be taken into account. This includes the

administration as well. The protein itself can be available either lyophilized (as dry substance) or as liquid formulation, ready for injection. A self-administered therapeutic such as insulin is usually available as prefilled syringe. Noninvasive routes such as oral, pulmonary, nasal, or transdermal delivery would be preferable but suffer from low bioavailability [130]. Recently, a fusion protein approach using transferrin as partner for granulocyte monocyte-colony-stimulating factor (GM-CSF) was successfully applied orally in mice [43]. Pulmonary delivery could be achieved by using monomeric Fc fusions. Here, aerosolized fusion proteins are carried across lung epithelial cells utilizing the neonatal Fc receptor [131]. Part IIc of this book is dedicated to different targeting and delivery approaches with fusion proteins.

Due to the limited stability in the digestive tract therapeutic proteins are usually administered by injection. This can be done through three different ways: intravenous (IV), intramuscular (IM), or subcutaneously (SC). The administration route has an impact on the concentration of the protein. IV injections usually are in the concentration range around 1 mg/mL, IM injections are acceptable up to 100 mg/mL, but only SC injections can have concentrations up to 150 mg/mL. It is very challenging to deliver a soluble, aggregate-free formulation at these very high, viscous concentrations [132]. The concentration and dosage are obviously dependent on the potency and the clearance of the drug. For example, highly active hormone or cytokine drugs are administered at relatively low doses despite their rapid clearance. Antibodies as large molecules on the other side can be required at very high concentrations despite their long half-life. This issue of manufacturability has to be considered from the start [133].

A lot of work has been done to diminish the aggregation. Working with fusion proteins that consist of several unrelated domains that did not evolve together is very challenging. One technique is the selective domain stabilization, where buffer conditions are evaluated that stabilize the least stable domain, because from there aggregation is initiated. Testing an hGH-HSA fusion, best results were obtained when repulsion of protein-protein interaction was increased [134].

1.5.4 Process Economies

As in all other manufacturing processes, cost of goods (COG), processing time, and capital investment are major determinants of fusion protein production. Another important parameter is the usually high dose of therapeutic proteins leading to high demands on capacity. In the last years, a trend to improved processes delivering more output from the same capacity and second generation products with decreased doses and longer half-lives will change biopharmaceutical manufacturing [135].

Many microbial processes generate IBs that need refolding. For instance, the granulocyte macrophage-colony-stimulating factor (GM-CSF) has been produced in *E. coli*. This required a series of washing steps to purify the IBs that are subsequently refolded at low concentration. When combining chemical extraction of IBs with EBA labor costs were fivefold lower and overall costs 50% less [136]. Interestingly, the costs for soluble expression of Heparinase in *E. coli* were twice the expenses of insoluble expression. The difference was primarily dependent on the expression levels that could be achieved in both approaches. Overall it was observed that fermentation related costs represent only a minor fraction, and yield optimization in downstream procedures has the highest impact on cost savings [137].

When comparing the production costs of tissue plasminogen activator stimulating factor (tPA) in *E. coli* and mammalian cells it was found out that refolding concentrations of more than 4 mg and refolding yields above 20% would make the process economically attractive. But it has to be taken into account that the cost of the competing mammalian expression is dominated by the cost for serum in the cultivation medium [138]. This extra cost can nowadays be neglected since most large-scale mammalian cultivations are serum free. Particularly, the absence of animal derived components, minimizes concerns of virus or prion contaminations, and improves the attractiveness of microbial fermentation besides its cost advantage and the short production cycles.

The technological progress of mammalian cell culture with increasing titers for antibodies currently causes bioreactor overcapacity issues. It is anticipated that in 2013 available bioreactor volume will reach 4 million liters compared to 2.4 million liters in 2007. Overall COG projections for antibodies and Fc-fusion proteins currently range from 50 to 100\$/g but achieve a median sales price of 8000\$/g. Therefore, royalty payments have a huge impact on the total COG. A 10-fold increase in titer would decrease the COG for the drug substance by 85%. For other recombinant (fusion) proteins requiring large doses the product titer is still a major determinant of COG [139]. At an antibody production below 10 kg/year and a titer around 0.1 g/L the ratio between upstream and downstream costs is around 50:50. Increasing the output to 100 kg/year at a titer of 0.5 g/L, upstream costs only represent 20%, indicating that at high levels, savings by the expression system plays only a minor role [140].

The aforementioned overcapacity of mammalian cell culture has been observed for many years and increased from 23.6% in 2003 to 36.7% in 2007. This is a result of constantly increasing product titers and simultaneously expanding the capacity by building new factories [141]. New production installations seem to move away from large single product units to more flexible multiproduct plants that nowadays also include disposable equipment. But

disposables can also be used in microbial processes. In a case study, the conventional production of an antibody fragment in *E. coli* was compared to a process with single use equipment. This more than 10-year-old study could demonstrate a 25% advantage of net present value for the conventional manufacture [142]. However, this gap is constantly narrowing, since particularly in downstream processing membrane steps can efficiently replace previous chromatographic column steps. This substitution saves cost in form of 25% less required volume of the stationary phase. Consequently 40% less aqueous waste is generated. Furthermore, labor hours are reduced by 40% because of obsolete cleaning and validation tasks. Overall the antibody purification process based on disposable membranes was 50% faster and 23% cheaper [143]. However, it should be taken into account that smaller and more frequent batches have higher QC and QA costs.

Obviously, the downstream process is a major cost driver suffering from low economy of scale effects. Typical chromatography steps are volume dependent directly impacting space requirements. Further complications are the intermediate cleaning cycles causing downtimes and cost [144].

1.5.5 Glycosylation

Besides production cost also product quality has to be taken into account when selecting an expression host. One element contributing to product quality is glycosylation, and interestingly about 70% of marketed recombinant proteins are glycoproteins. Huge variations and deviations from a typical human glycan pattern can be observed in economically favorable organisms such as bacteria, yeasts, fungi, insects, plants, or nonhuman mammalian species [145].

Glycosylation has a huge impact on the therapeutic effect by improving pharmacokinetics, pharmacodistribution and the selectivity of binding to receptors. This requires a careful selection of upstream process parameters including the host organism [146]. Even the cultivation conditions can affect the glycosylation pattern. For example, *N*-glycolylneuraminic acid (Neu5Gc) does not exist in humans, but is added when glycoproteins are expressed in Chinese hamster ovary (CHO) cells. The addition of Neu5Gc is dependent on a number of cultivation conditions. Neu5Gc content can be significantly lowered in presence of sodium butyrate, a decrease of temperature after exponential growth, high carbon dioxide concentrations and the utilization of sodium hydroxide for pH control [147]. From a downstream perspective glycosylation makes purification more difficult since often heterogeneity is observed. Since it is not easy to separate glyco-isoforms by preparative chromatography all efforts are taken to suppress heterogeneity already during upstream processes. Nevertheless, to guarantee a reproducible, homogeneous drug substance, high resolution analytical tools must be implemented [148].

Glycosylation is a key parameter of fusion proteins, determining immunogenicity, solubility, and stability. Particularly, the terminal sugars of glycans in the CH₂ domain of antibodies are responsible for influencing antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activity [149]. ADCC can be significantly enhanced by the lack of core-fucose on the Fc part as demonstrated with scFv-Fc fusion proteins [150]. An interesting example for the importance of glycosylation is the half-life extension technology of Prolor that relies on a carboxy-terminal peptide, originally derived from the chorionic gonadotropin (CG) β -subunit, bearing four serine-linked oligosaccharides. This short peptide can be fused to the N- or C-terminus of other proteins, expanding the hydrodynamic radius by extensive O-glycosylation, thus preventing kidney filtration [151]. Obviously, glycosylation in that case requires secreted expression in mammalian cells to obtain the proper oligosaccharide pattern. Glycoengineering was also used to optimize half-life and potency of an erythropoietin (EPO) variant called Aranesp[®]. This novel EPO has two additional N-linked carbohydrate chains, resulting in threefold longer serum half-life [152].

Besides half-life extension, glycosylation also contributes to protein stability. The biggest effects are seen in preventing proteolysis by shielding susceptible amino acid sequences, inhibiting aggregation by the hydrophilic nature of oligosaccharides and by protection of the proteins against all kinds of physical denaturation, including freezing [153].

The workhorses of antibody production, CHO cells, deliver a slightly different glycosylation than original human cells. Therefore, CHO cells were engineered to express human glycosyltransferases to improve the ADCC and CDC effects of antibodies [154].

Since cultivation of mammalian cells is rather costly when compared to microbial cells, also attempts were undertaken to obtain human glycoforms in yeasts. As suitable starting point for humanization of yeast the α -1,6-mannose extension must be eliminated. This was followed by the introduction of a α -1,2-mannosidase for mannose trimming. Finally, mannosidase II and N-acetylglucosaminyl transferase II was added to release uniform human-like glycans from yeast [155]. As last step terminal sialylation had to be implemented; this required the introduction of 14 foreign genes into *P. pastoris* [156].

The biggest obstacle of yeast N-glycosylation is the high mannose content that is potentially immunogenic and leads to drastically reduced half lives by the removal of therapeutic glycoproteins through mannose receptors on macrophages, thus reducing the efficacy [157]. But in some cases mannosylation can be beneficial, for example, when an antibody-enzyme fusion protein has to be quickly removed from normal tissue to enable a highly specific antibody-directed enzyme prodrug therapy (ADEPT) approach [158].

1.6 REGULATORY CHALLENGES

Despite the demonstrated success, fusion proteins consisting of two molecules with different functions, still raises concerns. Addressing these issues is of huge interest in a time where biosimilars and biobetters enter clinical development [159]. Several variants of approved drugs can be found within the category of fusion proteins, having improved half-life, potency, stability, or route of administration [11]. In general, three aspects must be clarified satisfactorily: quality, safety, and efficacy. The necessary information is gathered through animal pharmacology and toxicology studies. Here, the pharmacodynamics and pharmacokinetics that cover clearance and absorption, distribution, metabolism, and excretion (ADME), is assessed. The tightly regulated release of proteins in the organisms has to be mimicked by adapting dosing intervals or even activity of the protein [160]. This is much more difficult for non-natural proteins such as fusion proteins.

A separate document contains the chemistry, manufacturing and control (CMC) data. The manufacture must be done under good manufacturing practice (GMP) conditions and demonstrate the ability to consistently and reproducibly supply active batches of the drug. All that must be included in an investigational new drug (IND) application or a clinical trial authorization/exemption (CTA/CTX) for the regulatory authorities in the United States or Europe, respectively.

Since protein therapeutics are produced in living organisms, they could contain intrinsic infectious agents and other process- or product-related impurities. As biological they could have a heterogeneous composition and require extensive analysis. Expected and controllable parameters are size, charge, activity, folding, but on the other hand unexpected and undesired characteristics such as aggregation, amino acid modification or proteolysis could occur as well [161]. Usually, viral clearance with two orthogonal independent steps must be demonstrated. Before release the drug substance's identity, purity, potency, and stability must be verified according to predefined acceptance criteria in a number of validated assays [162].

The key product attributes that determine efficacy, in other words potency and activity, under the conditions of the intended use must be characterized. This includes nonclinical pharmacokinetic, toxicology, and safety studies [163]. Manufacturing conditions have to be chosen that can generate the desired attributes, or product quality, in a reproducible and cost-efficient manner. In the preclinical studies often imperfect replicas of human disease conditions in animal models are used [164]. A further hurdle for human therapeutic (fusion) proteins is that sometimes nonclinical assays must be performed with the respective animal homolog because the human version is nonfunctional in the selected animal species [165].

A fusion protein specific complication is the increased potential of immunogenicity. For instance, immunotoxins contain frequently nonhuman proteins that elicit immune responses and neutralizing antibodies. Also the conjunction site between the two fusion partners forms a novel epitope even when combining only human proteins. In both cases, a detailed study of the potential risk has to be done in form of specialized assays. Chapter 5 of this book explains the current approaches.

Typical challenges for bi-specific molecules are the difficulty to have similar potency/affinity against both targets, the lack of individual dosing or pharmacokinetic and the potential of overlapping and cross-linked toxicity. Dosing and potency can be addressed by combining two molecules with suitable affinities or altering them accordingly. In the case of transferin fusions, the simultaneous or sequential binding of both fusion partners to their respective cell surface receptors has a huge impact on recycling, degradation, and biological activity [166]. Toxicity issues can be predicted to some degree in simulation models. A number of adverse side effects of antibodies that are primarily dependent on the Fc part have been described in the past [167]. It is important to take the multiple functions of Fc domains into account when designing Fc-fusion proteins. Surprisingly, the majority of Fc fusions contain IgG1 that can trigger ADCC or CDC reactions. Therefore, it is advisable to select the Fc part of IgG2 or 4 as fusion partner. A number of novel scaffolds also serve as building blocks for fusion proteins. It is recommended to adapt the preclinical safety testing to the individual molecules and their mode of action [168].

Recently, some fusion proteins failed in clinical trials for different reasons. In the case of ZalbinTM, an interferon- α 2b-HSA fusion, the risk benefit ratio was not sufficiently favorable, which let Novartis and Human Genome Science withdraw their biologics license application (BLA) in 2010.² The glucagon-like peptide-1 (GLP-1)-Transferrin fusion of Pfizer and BioRexis was discontinued in Phase I because a reversible increase in heart rate was observed.³ Surprisingly and without disclosing specific reasons, Amevive[®] (leukocyte function antigen-3 (LFA-3) linked to Fc of IgG1) that was never approved for European markets and was withdrawn from the U.S. market by Astellas Pharma in 2011.⁴

1.7 COMPETITION AND MARKET

Fusion proteins are relative recent entrants to the market of therapeutic proteins, appearing first with Enbrel in 1998. In

the years thereafter only a few were approved by the FDA to reach patients, the majority being FC-fusion proteins [169]. The only exception within the eight marketed fusion proteins in 2011 is an immunotoxin, Ontak[®]. Despite that fact that Ontak does not capture a huge market, many other immunotoxins are in the clinical pipeline, probably expanding the number of approved fusion proteins soon.

In contrast, a total of 25 mABs were accepted by regulatory authorities since 1998. This triple number of products is also reflected in sales. In 2010, the six so far approved fusion proteins collected \$8.3 billion in revenues, whereas mABs had sales of \$40.8 billion globally.⁵ This indicates that the competition of antibodies is really strong, and only Enbrel and Orencia have blockbuster status. On the example of Enbrel, the strong competition and evolution of antibodies in the market segment of TNF- α blockers can be demonstrated (Table 1.1).

However, the big opportunity for fusion proteins can be found in applications where they do not have to face the fierce competition of antibodies. Many arising concepts focus on novel scaffolds that can replace antibodies and represent the binding moiety of fusion proteins [13]. Frequently, these small scaffolds can reach different targets than antibodies, but suffering from pharmacokinetic limitations. Most of them require plasma half-life extension strategies as explained in Part IIa of this book. But due to their small size they are ideal candidates for generating bi-functional molecules by combining two of them.

With regard to bi-specific antibodies, a subtopic of this book, this market is just emerging, with the tri-functional Removab[®] as the first approved multispecific antibody in 2009 [170]. The rising interest in this class of molecules is also reflected in increased merger and acquisition (M&A) activities such as Amgen buying Micromet for \$1.2 billion in 2012.⁶

As described previously, the design of fusion proteins is based on genetic engineering; many of the concepts are patent protected and have been used to build companies. In times when large pharmaceutical corporations are facing expiring patents, these companies became targets for acquisitions to bolster emptying pipelines. In Table 1.2, the M & A deals of the last decade affecting companies with fusion protein technology or drugs in various development stages are collected.

Overall a fast growth for protein therapeutics can be predicted [171]. The top 30 biologicals gathered global sales worth more than \$107 billion in 2010, with Enbrel being the most successful drug.⁷ Overall the global pharmaceutical market grew by 4.1%, but interestingly sales of

² <http://www.hgsi.com/latest/human-genome-sciences-announces-withdrawal-of-european-marketing-authorization-application-for-joulferon-zalbin-for-the-treatment-of-chronic-hepati-7.html>

³ <http://clinicaltrials.gov/ct2/show/NCT00637338?term=PF-04603629&rank=1>

⁴ <http://www.amevive.com/Patient%20letter.pdf>

⁵ Datamonitor: PharmaVita: Monoclonal Antibodies: 2011 – Market Size Update. October 2011.

⁶ http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1653062

⁷ La Merie S.L., Travesia Balmins, 7, Bajos, 2a, 08870 Sitges, Spain: Top 30 Biologics 2010, 04 March 2011.

TABLE 1.1 Competition Between Antibodies and Fusion Proteins that Capture TNF- α

Drug	Approval	Components	Manufacturing	Dosing	Sales 2009 ^a	Sales 2010 ^a	Sales 2011 ^a
Remicade [®]	08/1998	Chimeric mAb	Continuous perfusion in CHO	IV 2–4 weekly	5.9	7.3	8.5
Enbrel [®]	11/1998	Receptor Fc fusion	Fed-batch in CHO	SC 1–2 weekly	6.3	7.4	7.9
Humira [®]	01/2003	Fully human mAb	Extended fed-batch in CHO	SC bi-weekly	5.5	6.7	7.9
Cimzia [®]	04/2008	PEGylated humanized Fab	Fed-batch <i>E. coli</i>	SC bi-weekly	0.11	0.26	0.31
Simponi [®]	04/2009	Fully human mAb	Perfusion in Sp2/0	SC monthly	0.11	0.32	0.67

^aGlobal sales in billion \$.

biological drugs increased by 17% in 2010. Splitting the sales up, it can be seen that the growth rate of proteins was 15%, whereas antibody sales expanded by 21%. This means that of the \$15.91 billion growth antibodies contributed 55% and protein 45%. Another important observation is the higher approval success rate of 32% for biological compared to 13% for small molecules between 1993 and 2004 [172]. In Figure 1.4, the annual sales of important therapeutic protein classes in the United States are presented.

Although the growth of fusion protein sales has been relatively modest in comparison to mABs or hormones, there is a huge potential for this molecule class as can be seen in a much deeper analysis in Chapter 2.

1.8 CONCLUSION AND FUTURE PERSPECTIVE

During the last decades, human imagination has assembled a plethora of new protein combinations from the vast array

TABLE 1.2 Mergers and Acquisitions Involving Companies with Fusion Protein Technology

Buyer	Target	Date	Value (\$ Million)	Technology	Fusion Protein	Indication	Phase (at Acquis.)
Alexion	Enobia	07/02/2012	610	Bone targeting	ENB-0040	Hypophosphatasia	II
Alexion	Taligen	31/01/2011	111	C3d targeting	TT30	Autoimmune	Preclin.
Insys	Neopharm	29/10/2010	135	Immunotoxins	NK-408	Oncology	III
Bristol-Myers Squibb	Zymogenetics	07/09/2010	885	Fc fusion	Atacicept	Autoimmune	II
Biomarin	Zystor	07/09/2010	22	GILT	ZC-701	Pompe's disease	I
Emergent	Trubion	18/08/2010	97	SMIP and Scorpion	SBI-087 TRU-016	Autoimmune Oncology	II
Teva	CoGenesys	12/08/2010	400	Albumin fusion	Neugranin Cardeva	Febrile neutropenia Congestive heart failure	II II
Pfizer	CovX	22/01/2008	n.a.	CovX body (peptide-mAb fusion)	CVX-045 CVX-060 CVX-096	Oncology Oncology Type 2 diabetes	I II
Topotarget	Apoxis	18/12/2007	19	Mega Ligand	APO010	Oncology	I
Pfizer	Biorexis	11/04/2007	n.a.	Transferrin fusion	BRX-0585	Type 2 diabetes	Preclin.
Biogen Idec	Syntonix	01/02/2007	40	Monomeric Fc fusion	FIX-Fc	Hemophilia B	IND
Amgen	Avidia	29/09/2006	290	Avimer (modular binding domains)	IL-6 inhibitor	Inflammation, autoimmune	I
Medigene	Avidex	31/08/2006	62	TCR technology	EsoDex	Oncology	Preclin.

GILT, glycosylation independent lysosomal targeting; SMIP, small modular immunopharmaceutical; TCR, T-cell receptor.

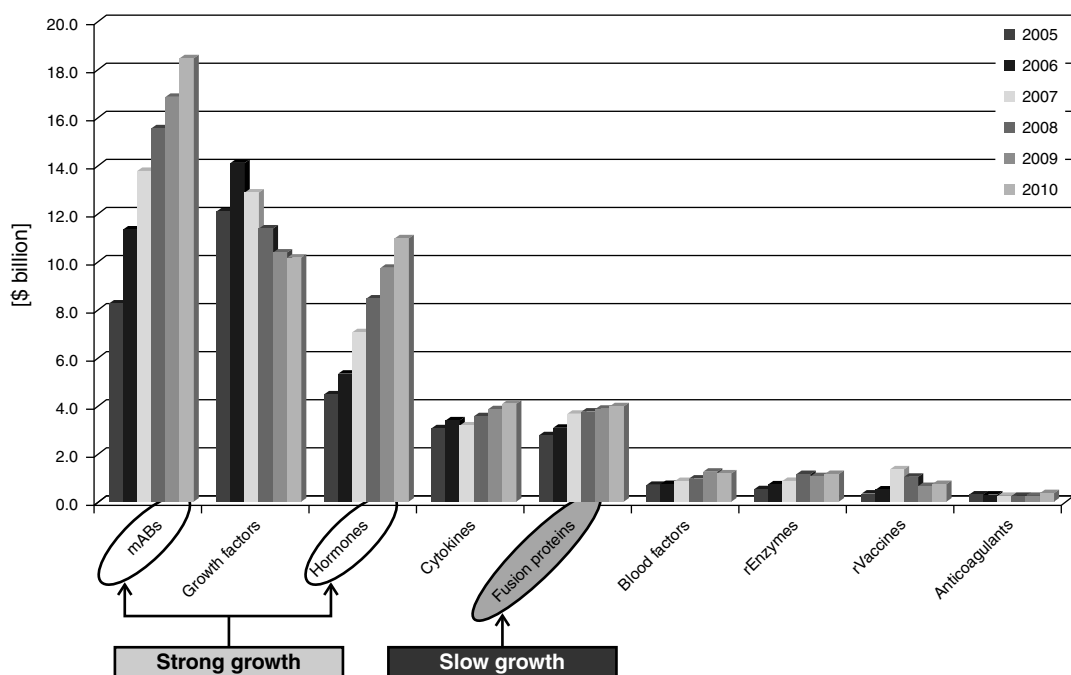


FIGURE 1.4 Annual sales of protein drugs in the United States. The two classes of proteins with a high annual growth rate are monoclonal antibodies (mAB) and hormones. Over the last 4 years, the growth of sales for fusion proteins was minimal, whereas the sales for growth factors even declined.

of nature's building blocks. Many concepts have been heavily influenced by the "magic bullet paradigm" that has been described more than a century ago, but took until now to realize it with artificial recombinations. These novel fusion proteins combine hitherto unrelated functionalities into a single molecule. Several have passed approval from regulatory authorities and many of those currently being in clinical trials will reach the market soon. So far fusion proteins have proven as valuable additions to the arsenal of therapeutic molecules. Still there are many opportunities where innovative fusion proteins can make a significant improvement. For instance, many first generation biopharmaceuticals can benefit from prolonged circulation times. Cancer patients will receive better targeted and more specific drugs with less systemic toxicity based on fusion proteins. So far relapsing tumors can be treated with novel protein drugs that hit two targets simultaneously, so overcoming resistance mechanisms. Novel fusion proteins will make therapies more affordable by lowering manufacture cost and improve quality of life for many patients who benefit from longer administration intervals. However, to fulfill the promises of fusion protein technology still a number of challenges have to be resolved.

A major obstacle is the immunogenicity potential that is always present even in fully human recombination, because at

joint between two molecules will always create a new epitope which can provoke immune reactions. Reduction of immunogenicity and understanding the underlying factors is, therefore, a key element to guarantee future success of fusion proteins. Another point to consider is the potential incompatibility between fusion partners that limit manufacturability. Both challenges will require intense efforts of protein engineering.

This book aims to cover the state of the art of fusion proteins. It presents an overview on the multitude of possibilities to design novel protein drugs while balancing between proven concepts and new ideas that have not reached the clinic yet. The book is structured into three larger parts. First general issues and concepts are discussed before in the second part examples on the three categories ($t_{1/2}$, toxicity, and targeting) are presented. Finally novel concepts and the rising class of multispecific antibodies are described. I hope this book will inspire the reader and create enthusiasm for the exciting topic of fusion proteins.

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