

4

Downstream Processing of Fc-Fusion Proteins

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4.1

Introduction and Overview of Fc-Fusion Proteins

Fc-fusion proteins are created by joining the coding sequence for the Fc region of a monoclonal antibody (mAb) to the coding sequence for another protein [1]. Translation produces a single polypeptide with functional properties derived from both fusion partners. Many different fusion partners are used for the expression of recombinant proteins in the laboratory, including the His6 tag, the FLAG epitope, and the glutathione-S-transferase (GST) tag [2]. However, Fc-fusion proteins are the only ones to be used thus far in approved biopharmaceutical products.

The Fc region offers several advantages as a fusion partner. Many biologically active peptides and proteins have a short serum half-life, reflecting their rapid clearance through the kidneys. The Fc region can bind to the neonatal Fc receptor (FcRn) to extend the half-life of antibodies, and the same benefits are conferred on fusion partners [3]. The Fc region is also readily soluble in aqueous solutions and can improve the solubility of more sparingly soluble proteins such as membrane proteins [4]. The Fc region is recognized by staphylococcal protein A, which means Fc-fusion proteins can be recovered easily even at low concentrations by protein A affinity chromatography. Fc-fusion constructs are widely used during fundamental drug discovery research [5]. The Fc fusion also lends itself to other creative therapeutic platforms such as polymeric structures with hexameric protein units [6].

Seven Fc-fusion proteins are commercially available as approved biopharmaceuticals and these are listed in Table 4.1 [1,7]. At least two of them (Enbrel[®] and Orencia[®]) have already achieved blockbuster status with sales of over US\$1 billion per year, and Etanercept (Enbrel) is one of the most successful drugs ever released, with annual sales of more than US\$7 billion [1]. Etanercept was initially indicated for rheumatoid arthritis but also has approvals for ankylosing spondylitis, psoriatic arthritis, and psoriasis. It combines the IgG1 Fc region with tumor necrosis factor (TNF) receptor 2, and works by binding excess TNF α , a key proinflammatory cytokine that triggers a variety of autoimmune conditions [8]. Abatacept (Orencia)

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Table 4.1 Commercial therapeutic Fc-fusion proteins.

Trade name	Company	Fusion partner 1 ^{a)}	Fusion partner 2 ^{a)}	Clinical indication	Year of launch
Enbrel	Amgen	IgG1 Fc	TNFR	Rheumatoid arthritis	1998
Amevive	Biogen-Idec	IgG1 Fc	LFA-2	Psoriasis	2003 ^{b)}
Orencia	Bristol-Myers Squibb	IgG1 Fc	CTLA4	Rheumatoid arthritis	2005
Nplate	Amgen	IgG1 Fc	TPO receptor binding peptide	Chronic idiopathic thrombocytic purpura (ITP)	2008
Arcalyst	Regeneron	IgG1 Fc	IL-1R	Cryopyrin-associated periodic syndrome (CAPS)	2008
Nulojix	Bristol-Myers Squibb	IgG1 Fc	CTLA4 (engineered)	Organ transplant rejection	2011
Eylea	Regeneron	IgG1 Fc	VEGFR-1 and VEGFR-2	Age-related macular degeneration	2011

a) All human sequences.

b) Voluntarily withdrawn in 2011, no safety concerns.

combines the IgG1 Fc region with the extracellular domain of CTLA4 (cytotoxic T lymphocyte antigen 4), which is found on the surface of T cells. The interaction between CD86 on the surface of antigen-presenting cells and CD28 on the surface of T cells results in the activation of T cells and is a key step in the immune cascade, but this can be interrupted by CTLA4 because it has a higher affinity for CD86 and can outcompete the binding of CD28. Abatacept is an effective alternative treatment for patients that do not respond to TNF α inhibitors such as etanercept [9].

Rilonacept (Arcalyst[®], IL-1 Trap) is a dimeric fusion of the ligand-binding regions of the IL-1 receptors (IL-1R1 and IL-1R accessory protein) linked to the IgG1 Fc region [10]. It is approved for the treatment of cryopyrin-associated periodic syndrome (CAPS). Alefacept (Amevive[®]), which combines the LFA-3 antigen with the IgG1Fc region, is an antagonist of T-cell activation approved for the treatment of psoriasis [11]. Belatacept (Nulojix[®]) is, like abatacept, a fusion between CTLA4 and the IgG1 Fc region, but it has two amino acid substitutions that result in slower dissociation from CD86 and thus increased potency. The immunosuppressive properties of this drug resulted in an approval for use to extend graft survival for renal transplant patients [12]. Romiplostim (Nplate[®]), which stimulates thrombopoiesis, has been approved for the treatment of chronic idiopathic thrombocytopenic purpura (ITP) [13]. Finally, aflibercept (VEGF Trap-eye, approved as Eylea[®]) is an inhibitor of vascular endothelial growth factor (VEGF) that combines the IgG1 Fc region with the second binding domain of VEGFR-1 and the third binding domain of VEGFR-2 [14].

A steady growth in the clinical applications of Fc-fusion proteins and other antibody-based therapeutics is anticipated, with several products already in the later stages of clinical development [1,7,15,16].

4.2 Biochemistry of Fc-Fusion Proteins

A typical construct for an Fc-fusion protein places the Fc region (hinge- C_{H2} - C_{H3} domains) at the C-terminus of the fusion partner (Figure 4.1). The hinge region can form intermolecular disulfide bonds, allowing Fc-fusion proteins to be expressed as covalently bound dimers. The hinge region can be omitted so that the Fc-fusion proteins are expressed as monomers; however, in some cases, additional cysteine residues can be placed in the body of the Fc region to allow the formation of dimers without the hinge.

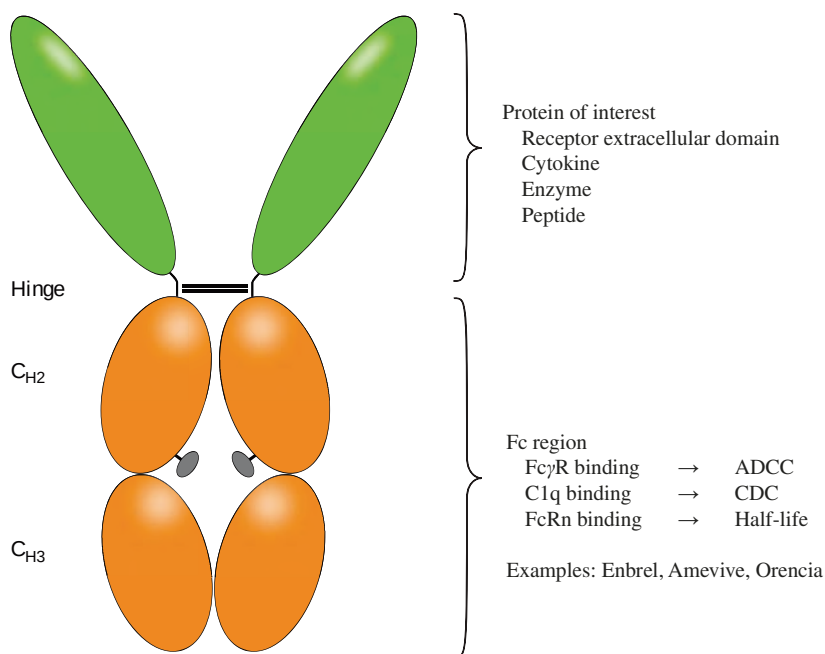


Figure 4.1 Structure of an Fc-fusion protein. The protein of interest can be any of a variety of functional types, including receptor extracellular domains, cytokines, enzymes, and

peptides. The immunoglobulin Fc-region (hinge, C_{H2} , C_{H3}) imparts *in vivo* stability via FcRn binding, among other potential effector functions.

The Fc region confers several antibody-like properties upon Fc-fusion proteins, including a similar general conformation in the form of a Y-shaped structure, with the Fc region driving the formation of the dimer. In terms of purification, the Fc region is responsible for specific interactions with the protein A ligand. The Fc region also contains a single N-linked glycosylation site at Asn 297.

The fusion partner contributes to the overall properties of the fusion protein, often reducing the isoelectric point (pI) to an acidic or neutral value in contrast to the typically basic pI of mAbs. In contrast to a majority of mAbs which have a basic pI, Fc-fusion proteins often have isoelectric points in the neutral or acidic range. Several of these proteins are heavily glycosylated, which further results in heterogeneity in isoelectric points. As a result, isoelectric focusing (IEF) or capillary IEF (cIEF) analysis yields a range of species that constitute the product. Etanercept (TNFR-Fc) has been shown to have isoforms from pH 4.4 to 5.5 and abatacept (CTLA4-Ig) from pH 4.5 to 5.7.

The fusion partner often has greater conformational flexibility than the Fab region of an mAb. This has been demonstrated by consistently higher apparent molecular weights for Fc-fusion proteins on size-exclusion chromatography as compared to monoclonal antibodies of comparable or higher molecular weights [17]. The fusion partner may also contribute additional acceptor sites for N-linked and/or O-linked glycosylation (e.g., etanercept has three N-linked glycosylation sites and a number of O-linked glycosylation sites). Fc-fusion proteins also tend to contain glycans with more terminal sialic acid residues than antibodies [18]. All these differences have a significant impact on the strategies adopted for downstream processing.

4.3

Purification of Fc-Fusion Proteins from Mammalian Cells

4.3.1

Platform Approaches for Downstream Purification

Most approved Fc-fusion proteins are expressed in mammalian cells, with the exception of romiplostim, which is produced in *Escherichia coli*. Several high-yielding expression systems have been designed for the production of Fc-fusion proteins [4,19], and the secretion of soluble Fc-fusion proteins has also been reported in the yeast *Pichia pastoris* [20].

Mammalian cells are usually harvested by centrifugation followed by depth filtration and membrane filtration steps, resulting in a clarified supernatant suitable for chromatography [21]. When purifying Fc-fusion proteins from clarified cell culture supernatant, platform approaches similar to those used for mAbs have been introduced successfully [22–26]. The presence of the Fc region allows the use of protein A chromatography as a capture step, providing a high degree of selectivity and a purity >98% in terms of process-related contaminants [27,28].

Following capture, orthogonal polishing steps are used to remove any remaining process-related impurities such as host cell proteins (HCPs) and DNA, as well as product-related impurities such as high-molecular-weight (HMW) aggregates. Several different strategies have been used as summarized in Figure 4.2. One common approach is cation-exchange (CEX) chromatography as the first polishing step followed by a second step determined by the unique properties of the product, for example, anion-exchange (AEX) chromatography in flow-through mode if HCP and HMW aggregates have already been removed to satisfactory levels and the only remaining significant impurity is host cell DNA. Use of this strategy clearly depends on the protein pI and is usually successful for proteins with a basic pI if the CEX step is operated in a bind-and-elute mode. Membrane-based AEX chromatography offers the advantage of a single-use format operated under high flow rates, resulting in excellent process throughput; however, AEX packed-bed chromatography has also been employed in a weak partitioning mode to partially retain the product on the column to improve the clearance of HCP and HMW aggregates [25]. Other alternatives for the second polishing step include hydrophobic interaction chromatography (HIC) and mixed-mode chromatography. HIC is often selected when the level of HMW aggregates is significant; it exploits hydrophobic interactions between the aggregates and the resin driven by high concentrations of salt (e.g., ammonium sulfate or sodium citrate). However, the conditions must be optimized carefully for each product because high salt concentrations can also induce aggregation, resulting in significant yield losses. HIC resins typically have a low binding capacity and are therefore operated in flow-through mode at moderate salt concentrations to ensure that HMW aggregates are retained more strongly because of their larger size and greater propensity for “salting out.” Flow-through mode also ensures efficient HCP clearance. Higher column loading is possible because only the aggregates take up the binding capacity of the resin, which also favors the use of smaller, disposable devices with convective media such as membranes and monoliths.

Mixed-mode resins [29] typically combine several different types of interactions to improve selectivity, such as electrostatic (AEX or CEX) and hydrophobic or in the case of hydroxyapatite chromatography, AEX and CEX along with metal chelate interactions. This can help to remove HCP and leached protein A ligand. Elution from mixed-mode resins typically involves higher salt concentrations and more extreme pH conditions than standard resins (e.g., Capto adhere resin combines AEX with hydrophobic interactions and is eluted under low pH conditions, often in combination with elevated salt concentrations). Hydroxyapatite resin is composed of calcium phosphate. It has poor packing properties and is unstable under pH 6 and must often be discarded after several operational cycles. This makes it an unfavorable choice for large-scale operations, but it is often used as a polishing step when other chromatography modes do not offer comparable selectivity for the removal of HCP, DNA, or HMW aggregates.

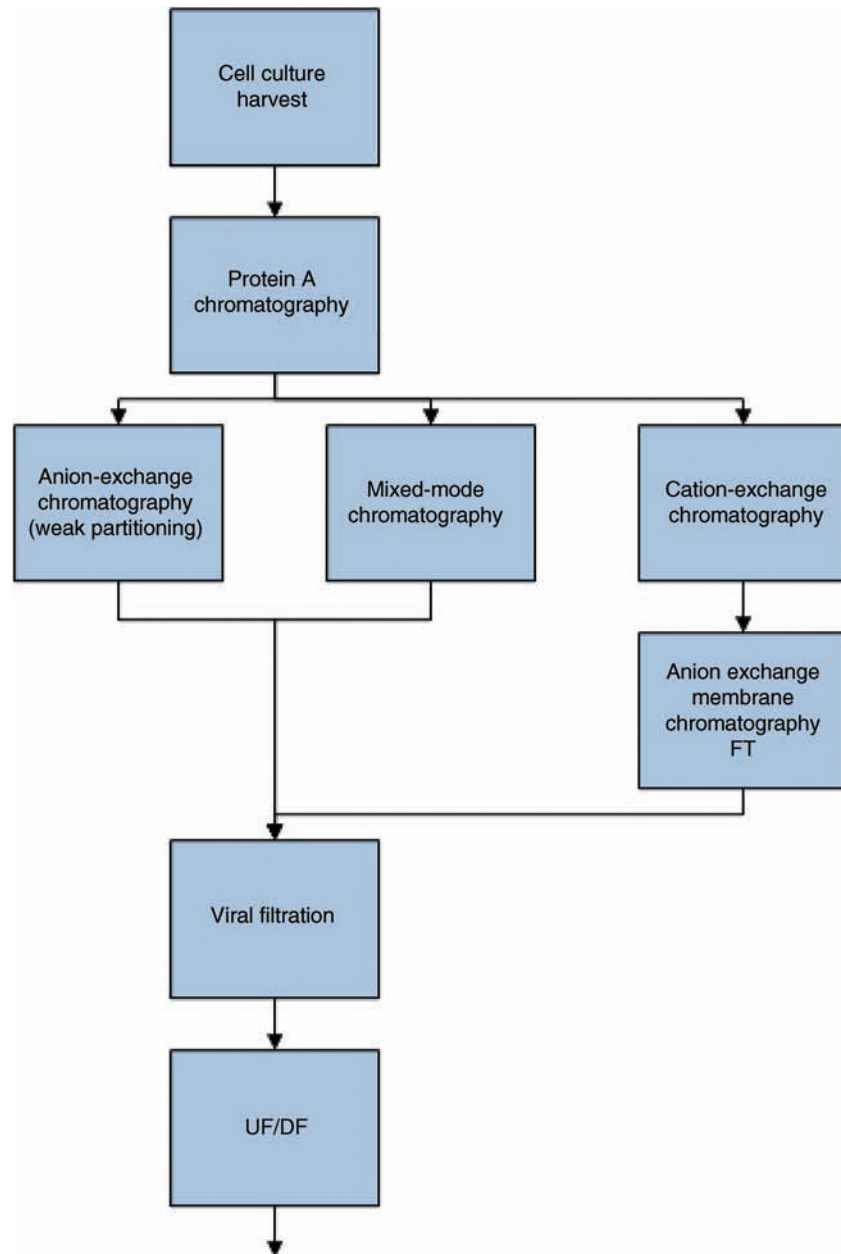


Figure 4.2 Platform approach for the purification of Fc-fusion proteins.

4.3.2

Comparison of Protein A Chromatography, Viral Inactivation, and Polishing Steps

Fc-fusion proteins can be purified using platform approaches similar to those adopted for mAbs, but because Fc-fusion proteins are by their nature more diverse than mAbs because of the biochemical properties of the fusion partners (Table 4.2), there are several differences between the two product classes that are important in the context of downstream processing.

The first issue that often stands out during the purification of Fc-fusion proteins is the increased pH sensitivity compared to mAbs [23,30]. Protein A capture is followed by elution in a low-pH buffer, but with Fc-fusion proteins this can adversely impact biological activity and/or induce the formation of HMW aggregates. The highest pH capable of inducing efficient elution from the resin must therefore be used to release the product (in practice, the effective pH range for efficient elution of IgG from protein A is 2.7–4.0). Even this is often insufficient to prevent the formation of significant levels of aggregates, so various strategies have been explored to reduce aggregation during elution as summarized in Figure 4.3 [31]. It is important to determine whether a potential precipitation event in the elution pool involves the product alone or includes an impurity species; in the latter case, precipitation can be avoided by the appropriate selection and sizing of harvest depth filters to remove those species prior to protein A chromatography. However, if the product itself is aggregating in the elution pool, the protein A operating conditions need to be altered, for example, by including a stabilizing additive in the elution buffer or by inducing elution through the use of chaotropic salts such as 1–2 M MgCl_2 [32]. Commonly used excipients that can preserve protein structure and hence reduce aggregation include salts, amino acids such as glycine and arginine [33], and moderate concentrations of urea [23]. Another strategy is to run the protein A column at cold temperatures (2–8 °C) although this may cause additional problems with bed stability due to outgassing from the mobile phase when the column reaches room temperature. A simpler approach is the careful selection of wash and elution buffers to avoid sudden changes in pH during elution. Citrate buffers can sometimes induce higher levels of aggregation because they efficiently neutralize charges on the protein surface. The use of acetate buffers, or a transition from an acetate wash buffer to a glycine elution buffer, can sometimes reduce aggregation quite significantly by ensuring a gradual pH transition and reducing the duration of product exposure to the lowest pH conditions during elution. The product may also become partially unfolded when bound to the resin, thus increasing the risk of aggregation during elution [23]. The risk of aggregation can therefore be reduced by collecting the protein A eluate directly into a high-pH buffer.

A second key difference is that Fc-fusion proteins have a lower binding capacity on protein A resins compared to mAbs because of the greater impact of intermolecule steric effects [17]. Binding stoichiometries of 2.4–2.6 were observed for Fc-fusion proteins, with a protein A ligand containing five binding regions; in contrast, mAbs had a higher binding stoichiometry of close to three mAbs per

Table 4.2 Typical differences observed between Fc-fusion proteins and mAbs during downstream processing.

Observed differences between Fc-fusion proteins and mAbs	Process impact/solution
Reduced binding capacity during protein A chromatography	Resins that offer enhanced capacity for mAbs do not always provide the same benefit for Fc-fusion proteins. Resin binding capacity requires the optimization of process throughput by balancing flow rate and column loading.
Products often susceptible to significant (up to 20%) soluble HMW aggregate formation under low-pH conditions	Use the highest pH compatible with protein A elution. Include stabilizing additives in the elution buffer (e.g., salts, glycerol, low concentrations of urea). Collect column eluate into a high-pH neutralizing solution to minimize exposure to low-pH conditions. It may not be possible to operate the protein A step under the highest column loading conditions due to product aggregation. In extreme cases of pH susceptibility, a non-protein A capture step (e.g., mixed-mode chromatography) may have to be used. Solvent/detergent or detergent often used instead of low pH for viral inactivation.
High level of post-protein A HMW aggregates	This product-related impurity often drives the selection of polishing steps for an Fc-fusion protein. HIC in bind-and-elute or flow-through mode is often employed if the level of HMW aggregates is high. Since Fc-fusion proteins generally have a lower pI than mAbs, CEX chromatography is not an automatic choice; either AEX or CEX may be used depending on the pI. In some cases, mixed-mode or hydroxyapatite chromatography offer better results.
High levels of product sialylation but with significant heterogeneity	A high degree of product sialylation is sometimes beneficial in the clinic. Enrichment for these glycoforms is a difficult process step to develop and control, and AEX is often used for this purpose.
Low flux on parvoviral-grade viral filters	Planova viral filters (low-flux but low-fouling) are often used. Higher-flux viral filters can often be susceptible to fouling and need to be carefully developed and sized. Surface-modified (hydrophilized) virus-retentive membranes provide another alternative.
Limited product concentration in final formulation	This is driven by the susceptibility of Fc-fusion proteins to aggregate. In liquid formulations, low to moderate protein concentrations are often selected in contrast to mAbs that can have a concentration >100 mg/ml

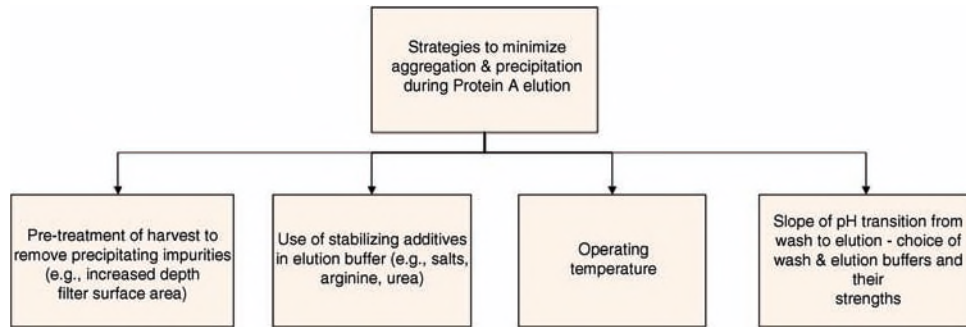


Figure 4.3 Strategies to reduce aggregation during protein A chromatography.

protein A ligand [17]. This reflects the more “floppy” conformation of the fusion partner compared with the Fab portion of a mAb as shown in Figure 4.4. The increased risk of aggregation discussed above also makes it impractical to achieve column loading close to the dynamic binding capacities for Fc-fusion proteins on protein A resins. It is therefore even more important to optimize the throughput and cost of protein A chromatography for Fc-fusion proteins than it is for mAbs. Such aspects can be evaluated using productivity calculations defined as the mass of product purified per unit time per unit resin volume [34]. A ramped flow-rate strategy can also help achieve higher loading capacities during protein A chromatography [35].

The risks associated with low-pH environments generally mean that Fc-fusion proteins may not be stable to an acid hold step, which is used as an effective virus inactivation strategy during the manufacture of mAbs. Therefore, virus inactivation must be achieved using solvent/detergent or detergent [36] alone either directly

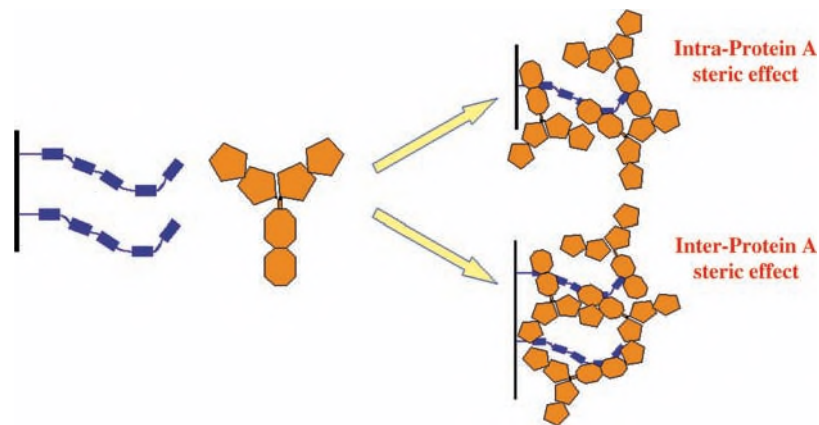


Figure 4.4 Steric properties of Fc-fusion proteins that can limit the binding capacity on protein A resins.

after elution from the protein A column or sometimes in the cell culture broth hold tank so that the protein A column also clears the detergents from the process flow. Both Tween 80 and Triton X-100 are commonly used as detergents for this purpose.

Polishing steps following protein A capture are usually geared toward the clearance of HMW aggregates, which can exceed 5% despite the precautions listed above. As discussed earlier, HIC in the flow-through or bind-and-elute modes is often used for aggregate clearance, although mixed-mode and hydroxyapatite resins may be used for certain products if they offer better clearance of impurities.

Another common polishing step is AEX, but whereas the flow-through mode is usually selected for mAbs, bind-and-elute mode may be more appropriate for Fc-fusion proteins. This is because the pI of most Fc-fusion proteins is less than pH 7 whereas mAbs tend to be strongly basic. Fc-fusion proteins also tend to be more extensively sialylated than mAbs depending on the fusion partner [18]. If a shallow AEX gradient is used, highly sialylated glycoforms tend to bind more strongly and elute at the tail of the peak whereas asialylated or sparsely/moderately sialylated glycoforms tend to elute at the front. In certain cases, there is a desire to achieve a higher average degree of sialylation for Fc-fusion proteins since the sialic acid molar ratio is linked to *in vivo* half-life for many proteins in circulation [18]. In these cases, the only downstream process step that can accomplish sialic acid enrichment is AEX. Preparative glycoform separations are highly challenging, and the use of such a downstream processing step must be considered carefully. A low-salt wash during AEX can remove some of the less sialylated forms of the product, and the careful definition of peak pooling criteria for elution can also result in further enrichment. However, these steps are highly sensitive to column loading as well as the conductivities of the wash and elution buffers. The initial glycoform heterogeneity must also be considered when determining column operating conditions. The modification of operating conditions on a run-to-run basis using real-time analytical data for each batch to ensure that product quality is maintained within a narrow window is a good example of process analytical technology (PAT) as applied to biopharmaceutical manufacturing [37,38]. A potential PAT strategy that could improve the control of this process step would be to alter elution peak collection criteria based on the glycoform profile of the column load, the amount of loaded protein, and the exact values of conductivity for the wash and elution buffers for each batch. Such a “measure-to-control” PAT strategy could potentially reduce product heterogeneity for Fc-fusion proteins by controlling the glycoform profile for these complex glycoproteins.

Mixed-mode chromatography, hydroxyapatite chromatography, and even metal chelate chromatography [39] are often used for the purification of Fc-fusion proteins to increase selectivity under favorable elution conditions. In addition to HCP, DNA, and leached protein A, these resins also clear HMW aggregates. These methods can be used for product capture when the product is too pH-sensitive for successful protein A chromatography, particularly resins such as Capto adhere (hydrophobic plus AEX) and Capto MMC (hydrophobic plus CEX).

Fluxes on parvoviral-grade viral filters (<20 nm pore size) are particularly low for Fc-fusion proteins [30], which may reflect the more hydrophobic nature of these

products as well as the point at which this step is introduced (e.g., fluxes may be lower after ultrafiltration/diafiltration [UF/DF] because this step can generate more particles because of the shear stress on the protein solution during repeated recirculation). Fc-fusion proteins may also cause viral filtration issues because of their greater conformational freedom, which contributes to their intrinsic propensity to aggregate [40]. The enhanced sensitivity of Fc-fusion proteins to this reduces the ability to include UF/DF operations aiming to achieve the high protein concentrations that are often achieved for mAbs (>100 mg/ml). Instead, lyophilized final formulations of Fc-fusion proteins are often used. The filtration issue can also be addressed by using low-flux/low-fouling filters (e.g., Planova 15 N or 20 N [Asahi]) instead of the newer viral filters developed for mAb processing (Viresolve Vpro [Millipore], Virosart CPV [Sartorius]). Next-generation concepts for virus-retentive membranes are required to improve the productivity and process economy of this unit operation, but more effort is also required to screen and optimize current viral filters and their placement in the downstream process for Fc-fusion proteins.

Although this section has highlighted some of the differences between mAbs and Fc-fusion proteins that can affect downstream processing strategies, it should be recognized that platform approaches still work well for Fc-fusion proteins and the overall process flow used for mAbs can be retained as long as the unique biochemical properties of Fc-fusion proteins are noted and taken into consideration when optimizing the relevant steps.

4.4

Purification of Fc-Fusion Protein from Microbial Systems

Fc-fusion proteins can be produced in microbial systems such as *E. coli* if glycosylation is not required for the biological activity of the fusion partner. FcRn-binding activity may be reduced by the lack of N-linked glycans in the Fc region, but peptibodies (Fc-peptide fusion proteins) have been produced with half-lives of 6–8 days (compared to 10–15 days for mAbs). The first approved peptibody produced in *E. coli* is now marketed under the brand name Nplate [41]. Several other peptibodies are in late-stage clinical development, suggesting that the market share of this class of Fc-fusion proteins will grow in the future. The platform is versatile because it involves the screening of large peptide libraries to identify candidates that bind specific targets with high affinity, and the linking of such peptides to the Fc region to overcome the traditional challenge with peptide-based drugs, that is, their short half-lives [42]. Additionally, *E. coli* has traditionally been viewed as a high productivity system with high product titers achieved in a short fermentation time frame.

Figure 4.5 shows a schematic for the downstream processing of Fc-fusion proteins from microbial sources. Peptibodies have been expressed as inclusion bodies in *E. coli* [41], but the high-level expression of soluble peptibodies could potentially be achieved by targeting them to the periplasmic space [43]. Following

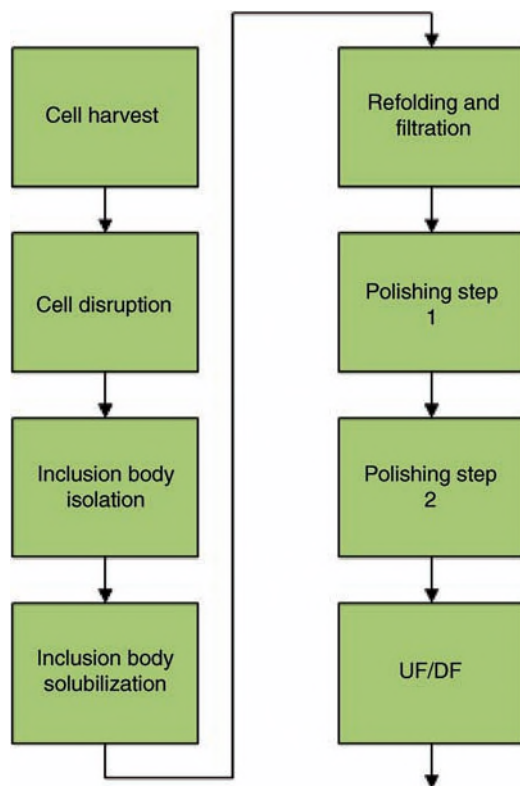


Figure 4.5 Downstream processing of a peptibody.

cell homogenization, inclusion bodies are recovered using a series of washing and centrifugation steps. They tend to contain the denatured protein in a relatively pure form, which circumvents the need for a highly selective capture step such as protein A chromatography. The refolding of inclusion bodies is the most intensive phase of this process because it relies on empirical testing rather than a standardized procedure [44–46]. The inclusion bodies are typically solubilized in high-pH buffers containing high concentrations of chaotropes such as urea, along with reducing agents to break the scrambled disulfide bonds. The inclusion bodies are then refolded by dilution in a refolding buffer that includes a lower concentration of chaotropes, stabilizing agents that can prevent the formation of aggregates at high protein concentrations and a redox couple (e.g., cysteine/cystine) that facilitates the correct formation of disulfide bonds. The refolding reaction is often carried out at cold temperatures. The oxidation events needed for disulfide bond formation are either catalyzed by air oxidation or by the inclusion of metal ions such as Cu^{2+} in the refolding buffer. The refolded solution is often turbid and is therefore passed through appropriate depth and membrane filters to obtain a clarified solution that can be purified by chromatography.

The chromatographic polishing steps are selected to focus on the clearance of misfolded forms of the product as well as HMW aggregates and HCP. HIC is frequently used because it can resolve these species efficiently, although AEX, CEX and/or mixed-mode chromatography may be used, in addition to or instead of, if these provide better clearance. A dedicated virus clearance/inactivation step is unnecessary because *E. coli* does not support mammalian pathogens. The final step is therefore buffer exchange using UF/DF prior to formulation.

4.5

Future Innovations in Fc-Fusion Protein Downstream Processing

A number of innovations in the downstream processing of biopharmaceuticals have the potential to influence the future directions that Fc-fusion protein downstream processing might take [47].

Protein A chromatography has traditionally been a high-cost but highly selective mode of chromatography that is widely used for the purification of Fc-fusion proteins. There has been a consistent emphasis on the optimization of throughput for this unit operation [34,35]. With the expiry of the original patent on recombinant protein A in 2009, several new vendors have entered the protein A chromatography market offering high-capacity resins. In addition, base-stable protein A ligands have been developed that allow the use of sodium hydroxide for cleaning-in-place (CIP) procedures. Some of the newer protein A resins include MAbSelect Xtra, MAbSelect SuRe Ultra (both from GE Healthcare), CaptivA (Repligen), POROS protein A (Applied Biosystems), and a prototype resin from JSR Micro. Some of these new resins are being offered at lower costs per unit volume and/or have higher binding capacities than traditional resins, which can help improve the economics of protein A chromatography for both mAbs and Fc-fusion proteins.

The bottlenecks in downstream processing typically reside in the chromatography steps, and this is leading the industry toward a more serious investigation of alternative, nonchromatographic separations [48]. Fc-fusion proteins can often require large quantities of the product to be produced (both Enbrel and Orencia have commercial production of several hundred kilograms per year). Handling the large quantities of product further emphasize the bottleneck posed by chromatographic separation techniques. Although a number of nonchromatographic techniques such as precipitation and aqueous two-phase separations had fallen out of favor, interest has been rekindled owing to the high titers now achieved in cell cultures and the limited throughput of chromatography [49]. One example is the use of polyelectrolytes to precipitate the impurities in cell culture harvest broths [50]. Many of these applications have been developed for monoclonal antibodies but could be extended to Fc-fusion proteins in the future.

Improvements in separation throughput have also been achieved by the use of high-flow-rate devices such as membrane chromatography cassettes operated in flow-through mode under high loading conditions [51]. Membrane chromatography

involves the use of synthetic porous membranes containing the same functional groups as packed resins, which is advantageous for polishing steps because there is much less mass transfer resistance and therefore more efficient hydrodynamic behavior, which means the membranes can be operated at higher flow rates with a lower overall buffer consumption and a much shorter processing cycle [52]. Disposable membranes are therefore becoming established as a platform for the removal of host cell DNA by AEX and can be performed with a membrane bed height of 4 mm at flow rates of more than 600 cm/h [53,54].

As stated above, viral filtration is a necessary step for the purification of products derived from mammalian cell cultures, but Fc-fusion proteins often experience lower fluxes than mAbs. Viral filtration can be an expensive unit operation because the filters are single-use. A variety of next-generation viral filters have been launched on the market, including the Viresolve Vpro, Virosart CPV, Planova 20N, and Ultipor DV20 [55,56]. With suitable pretreatment, several of these filters could help to increase the performance of the viral filtration step for Fc-fusion proteins while reducing costs, as long as due consideration is given to their appropriate placement in the downstream process.

Single-use technologies are gaining a strong foothold in biopharmaceutical manufacturing, and they offer significant advantages for the manufacture of Fc-fusion proteins [57,58]. There is a de facto upper limit to the volume of single-use bioreactors (currently 2000 l) that dictates the capacity of the downstream process chain and the scale of the corresponding components, but this is due to market factors rather than intrinsic technological limitations. Single-use bioreactors are currently ideal for the production of biopharmaceuticals in early-stage clinical development, whereas process-scale manufacturing requires larger-scale stainless steel bioreactors. However, the increase in titers achieved over the past 5–10 years suggests that process-scale manufacturing should become possible using 5000 l bioreactors in the near future, which means that one step further in terms of scalability will allow the implementation of fully disposable manufacturing trains for biopharmaceutical products on a more routine basis, including mAbs and Fc-fusion proteins.

4.6 Conclusions

Fc-fusion proteins are versatile mAb derivatives that are becoming increasingly successful in the clinic. Seven Fc-fusion proteins have been approved for therapeutic use, and more are emerging from the late-stage clinical pipeline. The biochemistry of Fc-fusion proteins shows that their properties are derived both from the Fc region (dimerized structure similar to mAbs, affinity for protein A) and the fusion partner (rich and diverse N-linked and/or O-linked glycosylation, terminal sialylation of the oligosaccharides).

Fc-fusion proteins produced in mammalian cells are glycosylated. Downstream processing focuses on these glycan structures and robust purification strategies can

be developed using a more liberal interpretation of the mAb platform process. It is important to consider the pH sensitivity of Fc-fusion proteins compared to mAbs and their tendency to form aggregates under low-pH conditions.

Fc-fusion proteins expressed in *E. coli* are not glycosylated and therefore accumulate within inclusion bodies. Downstream processing therefore focuses on the solubilization and refolding of the products and the clearance of HMW aggregates and misfolded polypeptides.

The strategies outlined in this chapter provide some general guidelines reflecting our experience with the manufacture of several Fc-fusion proteins. Clearly, these proteins are much more heterogeneous than mAbs and have diverse properties that are not suitable for a one-size-fits-all downstream processing approach, but as more Fc-fusion proteins enter the clinic and we gain experience from a broader spectrum of products it may be possible to extract some further common principles that will facilitate the early steps in process development in the years to come.

References

- 1 Beck, A. and Reichert, J.M. (2011) Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies. *MAbs*, **3** (5), 415–416.
- 2 Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M., and Nygren, P.A. (1997) Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Expr. Purif.*, **11** (1), 1–16.
- 3 Roopenian, D.C. and Akilesh, S. (2007) FcRn: the neonatal Fc receptor comes of age. *Nat. Rev. Immunol.*, **7** (9), 715–725.
- 4 Lo, K.M., Sudo, Y., Chen, J., Li, Y., Lan, Y., Kong, S.M., Chen, L., An, Q., and Gillies, S.D. (1998) High level expression and secretion of Fc-X fusion proteins in mammalian cells. *Protein Eng.*, **11** (6), 495–500.
- 5 Schneider, P. (2000) Production of recombinant TRAIL and TRAIL receptor: Fc chimeric proteins. *Methods Enzymol.*, **322**, 325–345.
- 6 Mekhaie, D.N., Czajkowsky, D.M., Andersen, J.T., Shi, J., El-Faham, M., Doenhoff, M., McIntosh, R.S., Sandlie, I., He, J., Hu, J., Shao, Z., and Pleass, R.J. (2011) Polymeric human Fc-fusion proteins with modified effector functions. *Sci. Rep.*, **1**, 124.
- 7 Chamow, S. (2011) Fc-fusion proteins: a growing class of therapeutics. At ESACT Meeting; Vienna, Austria.
- 8 Moreland, L.W., Baumgartner, S.W., Schiff, M.H., Tindall, E.A., Fleischmann, R.M., Weaver, A.L., Ettlinger, R.E., Cohen, S., Koopman, W.J., Mohler, K., Widmer, M.B., and Bloch, C.M. (1997) Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc-fusion protein. *N. Engl. J. Med.*, **337** (3), 141–147.
- 9 Genovese, M.C., Becker, J.C., Schiff, M., Luggen, M., Sherrer, Y., Kremer, J., Birbara, C., Box, J., Natarajan, K., Nuamah, I., Li, T., Aranda, R., Hagerty, D.T., and Dougados, M. (2005) Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N. Engl. J. Med.*, **353** (11), 1114–1123.
- 10 Kapur, S. and Bonk, M.E. (2009) Rilonacept (Arcalyst), an interleukin-1 trap for the treatment of cryopyrin-associated periodic syndromes. *P T*, **34** (3), 138–141.
- 11 Chamian, F., Lowes, M.A., Lin, S.L., Lee, E., Kikuchi, T., Gilleaudeau, P., Sullivan-Whalen, M., Cardinale, I., Khatcherian, A., Novitskaya, I., Wittkowski, K.M., and Krueger, J.G. (2005) Alefacept reduces infiltrating T cells, activated dendritic cells, and inflammatory genes in psoriasis vulgaris. *Proc. Natl. Acad. Sci. USA*, **102** (6), 2075–2080.
- 12 Larsen, C.P., Pearson, T.C., Adams, A.B., Tso, P., Shirasugi, N., Strobert, E.,

- Anderson, D., Cowan, S., Price, K., Naemura, J., Emswiler, J., Greene, J., Turk, L.A., Bajorath, J., Townsend, R., Hagerty, D., Linsley, P.S., and Peach, R.J. (2005) Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am. J. Transplant*, **5** (3), 443–453.
- 13 Kuter, D.J., Bussell, J.B., Lyons, R.M., Pullarkat, V., Gernsheimer, T.B., Senecal, F.M., Aledort, L.M., George, J.N., Kessler, C.M., Sanz, M.A., Liebman, H.A., Slovick, F.T., de Wolf, J.T.M., Bourgeois, E., Guthrie Jr, T.H., Newland, A., Wasser, J.S., Hamburg, S.I., Grande, C., Lefrere, F., Lichtin, A.E., Tarantino, M.D., Terebelo, H.R., Viallard, J.-F., Cuevas, F.J., Go, R.S., Henry, D.H., Redner, R.L., Rice, L., Schipperus, M.R., Guo, D.M., and Nichol, J.L. (2008) Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. *Lancet*, **371** (9610), 395–403.
- 14 Stewart, M.W. (2012) Aflibercept (VEGF Trap-eye): the newest anti-VEGF drug. *Br. J. Ophthalmol.*, **96** (9), 1157–1158.
- 15 Piggee, C. (2008) Therapeutic antibodies coming through the pipeline. *Anal. Chem.*, **80** (7), 2305–2310.
- 16 Beck, A., Wurch, T., Bailly, C., and Corvaia, N. (2010) Strategies and challenges for the next generation of therapeutic antibodies. *Nat. Rev. Immunol.*, **10** (5), 345–352.
- 17 Ghose, S., Hubbard, B., and Cramer, S.M. (2007) Binding capacity differences for antibodies and Fc-fusion proteins on protein A chromatographic materials. *Biotechnol. Bioeng.*, **96** (4), 768–779.
- 18 Beck, A., Wagner-Rousset, E., Bussat, M.C., Lokteff, M., Klinguer-Hamour, C., Haeuw, J.F., Goetsch, L., Wurch, T., Van Dorsselaer, A., and Corvaia, N. (2008) Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins. *Curr. Pharm. Biotechnol.*, **9** (6), 482–501.
- 19 Rouiller, Y., Perilleux, A., Marsaut, M., Stettler, M., Vesin, M.N., and Broly, H. (2012) Effect of hydrocortisone on the production and glycosylation of an Fc-fusion protein in CHO cell cultures. *Biotechnol. Prog.*, **28** (3), 803–813.
- 20 Lin, H., Kim, T., Xiong, F., and Yang, X. (2007) Enhancing the production of Fc-fusion protein in fed-batch fermentation of *Pichia pastoris* by design of experiments. *Biotechnol. Prog.*, **23** (3), 621–625.
- 21 Shukla, A. and Kandula, J. (2008) Harvest and recovery of monoclonal antibodies from large-scale mammalian cell culture. *BioPharm. Int.*, **21** (5), 34–45.
- 22 Sommerfeld, S. and Strube, J. (2005) Challenges in biotechnology production—generic processes and process optimization for mAbs. *Chem. Eng. Process*, **44**, 1123–1137.
- 23 Shukla, A.A., Gupta, P., and Han, X. (2007) Protein aggregation kinetics during Protein A chromatography. Case study for an Fc-fusion protein. *J. Chromatogr. A*, **1171** (1–2), 22–28.
- 24 Shukla, A.A. and Thommes, J. (2010) Recent advances in large-scale production of monoclonal antibodies and related proteins. *Trends Biotechnol.*, **28** (5), 253–261.
- 25 Kelley, B., Blank, G., and Lee, A. (2008) Downstream processing of monoclonal antibodies: Current practices and future opportunities, in *Process Scale Purification of Antibodies*, John Wiley & Sons, Inc., Hoboken, pp. 1–23.
- 26 Kelley, B. (2009) Industrialization of mAb production technology: the bioprocessing industry at a crossroads. *MAbs*, **1** (5), 443–452.
- 27 Huse, K., Bohme, H.J., and Scholz, G.H. (2002) Purification of antibodies by affinity chromatography. *J. Biochem. Biophys. Methods*, **51** (3), 217–231.
- 28 Shukla, A.A., Hubbard, B., Tressel, T., Guhan, S., and Low, D. (2007) Downstream processing of monoclonal antibodies—Application of platform approaches. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **848** (1), 28–39.
- 29 Zhao, G., Dong, X.Y., and Sun, Y. (2009) Ligands for mixed-mode protein chromatography: Principles, characteristics and design. *J. Biotechnol.*, **144** (1), 3–11.
- 30 Rea, D., Ultee, M., Chen, S., and Loisel, T. (2008) Solutions for the purification of Fc-fusion proteins. *BioPharm. Int. Supplement*, 20–25.
- 31 Shukla, A., Hinckley, P., Gupta, P., Yigzaw, Y., and Hubbard, B. (2005) Strategies to

- address aggregation during Protein A chromatography. *BioProcess Int.*, **3** (5), 36–45.
- 32 Gagnon, P. (1996) *Purification Tools for Monoclonal Antibodies*, Validated Biosystems, Inc., Tucson, AZ, p. 254.
 - 33 Arakawa, T., Philo, J.S., Tsumoto, K., Yumioka, R., and Ejima, D. (2004) Elution of antibodies from a Protein-A column by aqueous arginine solutions. *Protein Expr. Purif.*, **36** (2), 244–248.
 - 34 Tugcu, N., Roush, D.J., and Goklen, K.E. (2008) Maximizing productivity of chromatography steps for purification of monoclonal antibodies. *Biotechnol. Bioeng.*, **99** (3), 599–613.
 - 35 Ghose, S., Nagrath, D., Hubbard, B., Brooks, C., and Cramer, S.M. (2004) Use and optimization of a dual-flowrate loading strategy to maximize throughput in protein-a affinity chromatography. *Biotechnol. Prog.*, **20** (3), 830–840.
 - 36 Horowitz, B., Lazo, A., Grossberg, H., Page, G., Lippin, A., and Swan, G. (1998) Virus inactivation by solvent/detergent treatment and the manufacture of SD-plasma. *Vox. Sang.*, **74** (Suppl. 1), 203–206.
 - 37 Hinz, D.C. (2006) Process analytical technologies in the pharmaceutical industry: the FDA's PAT initiative. *Anal. Bioanal. Chem.*, **384** (5), 1036–1042.
 - 38 Hou, Y., Jiang, C., Shukla, A.A., and Cramer, S.M. (2011) Improved process analytical technology for protein A chromatography using predictive principal component analysis tools. *Biotechnol. Bioeng.*, **108** (1), 59–68.
 - 39 Shukla, A.A., Sorge, L., Boldman, J., and Waugh, S. (2001) Process characterization for metal-affinity chromatography of an Fc-fusion protein: a design-of-experiments approach. *Biotechnol. Appl. Biochem.*, **34** (Pt 2), 71–80.
 - 40 Fast, J.L., Cordes, A.A., Carpenter, J.F., and Randolph, T.W. (2009) Physical instability of a therapeutic Fc-fusion protein: domain contributions to conformational and colloidal stability. *Biochemistry*, **48** (49), 11724–11736.
 - 41 Shimamoto, G., Gegg, C., Boone, T., and Queva, C. (2012) Peptibodies: a flexible alternative format to antibodies. *MAbs*, **4** (5), 586–591.
 - 42 Sato, A.K., Viswanathan, M., Kent, R.B., and Wood, C.R. (2006) Therapeutic peptides: technological advances driving peptides into development. *Curr. Opin. Biotechnol.*, **17** (6), 638–642.
 - 43 Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.*, **10** (5), 411–421.
 - 44 Clark, E.D. (2001) Protein refolding for industrial processes. *Curr. Opin. Biotechnol.*, **12** (2), 202–207.
 - 45 Lilie, H., Schwarz, E., and Rudolph, R. (1998) Advances in refolding of proteins produced in *E. coli*. *Curr. Opin. Biotechnol.*, **9** (5), 497–501.
 - 46 Singh, S.M. and Panda, A.K. (2005) Solubilization and refolding of bacterial inclusion body proteins. *J. Biosci. Bioeng.*, **99** (4), 303–310.
 - 47 Gottschalk, U., Brorson, K., and Shukla, A.A. (2012) The need for innovation in biomanufacturing. *Nat. Biotechnol.*, **30** (6), 489–492.
 - 48 Thömmes, J. and Etzel, M. (2007) Alternatives to chromatographic separations. *Biotechnol. Prog.*, **23** (1), 42–45.
 - 49 Przybycien, T.M., Pujar, N.S., and Steele, L.M. (2004) Alternative bioseparation operations: life beyond packed-bed chromatography. *Curr. Opin. Biotechnol.*, **15** (5), 469–478.
 - 50 McDonald, P., Victa, C., Carter-Franklin, J.N., and Fahrner, R. (2009) Selective antibody precipitation using polyelectrolytes: a novel approach to the purification of monoclonal antibodies. *Biotechnol. Bioeng.*, **102** (4), 1141–1151.
 - 51 Zhou, J.X., Tressel, T., Gottschalk, U., Solamo, F., Pastor, A., Dermawan, S., Hong, T., Reif, O., Mora, J., Hutchison, F., and Murphy, M. (2006) New Q membrane scale-down model for process-scale antibody purification. *J. Chromatogr. A*, **1134** (1–2), 66–73.
 - 52 Walter, J.K. (1998) Strategies and considerations for advanced economy in downstream processing of biopharmaceutical proteins, in *Bioseparation and Bioprocessing: Processing, Quality and Characterization, Economics, Safety and Hygiene* (ed. G. Subramanian), Wiley-VCH Verlag GmbH, New York, pp. 447–460.

- 53 Gottschalk, U. (2006) The renaissance of protein purification. *BioPharm. Int.*, **19** (Suppl), 8–9.
- 54 Gottschalk, U. (2005) New and unknown challenges facing biomanufacturing. *BioPharm. Int.*, **18**, 24–28.
- 55 Brown, A., Bechtel, C., Bill, J., Liu, H., Liu, J., McDonald, D., Pai, S., Radhamohan, A., Renslow, R., Thayer, B., Yohe, S., and Dowd, C. (2010) Increasing parvovirus filter throughput of monoclonal antibodies using ion exchange membrane adsorptive pre-filtration. *Biotechnol. Bioeng.*, **106** (4), 627–637.
- 56 Lute, S., Bailey, M., Combs, J., Sukumar, M., and Brorson, K. (2007) Phage passage after extended processing in small-virus-retentive filters. *Biotechnol. Appl. Biochem.*, **47** (Pt 3), 141–151.
- 57 Shukla, A., Mostafa, S., Wilson, M., and Lange, D. (2012) Vertical integration of disposables in biopharmaceutical drug substance manufacturing. *BioProcess Int.*, **10** (6), 34–47.
- 58 Shukla, A.A. and Gottschalk, U. (2013) Single-use disposable technologies for biopharmaceutical manufacturing. *Trends Biotechnol.*, **31** (3), 147–154.