Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 848 (2007) 28–39
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Downstream processing of monoclonal antibodies—Application of platform approaches☆

Abhinav A. Shukla[a,1], Brian Hubbard[a], Tim Tressel[b], Sam Guhan[b], Duncan Low[b,*]

[a] Process Development, Amgen Inc., 1201 Amgen Court W., Seattle, WA 98119, United States
[b] Process Development, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, United States

Received 31 May 2006; accepted 8 September 2006
Available online 13 October 2006

Abstract

This paper presents an overview of large-scale downstream processing of monoclonal antibodies and Fc fusion proteins (mAbs). This therapeutic modality has become increasingly important with the recent approval of several drugs from this product class for a range of critical illnesses. Taking advantage of the biochemical similarities in this product class, several templated purification schemes have emerged in the literature. In our experience, significant biochemical differences and the variety of challenges to downstream purification make the use of a completely generic downstream process impractical. Here, we describe the key elements of a flexible, generic downstream process platform for mAbs that we have adopted at Amgen. This platform consists of a well-defined sequence of unit operations with most operating parameters being pre-defined and a small subset of parameters requiring development effort. The platform hinges on the successful use of Protein A chromatography as a highly selective capture step for the process. Key elements of each type of unit operation are discussed along with data from 14 mAbs that have undergone process development. Aspects that can be readily templated as well as those that require focused development effort are identified for each unit operation. A brief description of process characterization and validation activities for these molecules is also provided. Finally, future directions in mAb processing are summarized.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Monoclonal antibodies; Fc fusion proteins; Cell culture harvest; Protein A chromatography; Viral inactivation; Viral filtration; Ultrafiltration/diafiltration; Process characterization; Process validation

Contents

1. Monoclonal antibodies and Fc fusion proteins 29
2. Purification of mAbs—literature review and templated purification schemes 29
3. A platform approach to process development 31
4. A flexible, generic platform for mAb downstream processing 31
   4.1. Cell culture harvest operations 32
   4.2. Protein A chromatography 33
   4.3. Low pH viral inactivation 35
   4.4. Polishing chromatographic steps 35
   4.5. Viral filtration 36
   4.6. Ultrafiltration/diafiltration (UF/DF) 36
   4.7. Absolute filtration 37
5. Process characterization and validation activities 37

☆ This paper is a part of a special issue entitled "Polyclonal and Monoclonal Antibody Production, Purification, Process and Product Analytics", guest edited by A.R. Newcombe and K. Watson.

* Corresponding author. Tel.: +1 805 313 1754; fax: +1 805 480 0613.
E-mail address: dlow@amgen.com (D. Low).
[1] Bristol-Myers Squibb, 6000 Thompson Road, East Syracuse, NY 13057, United States.

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.09.026

6. Future directions in mAb purification . . . 38
7. Conclusions . . . 38
References . . . 38

1. Monoclonal antibodies and Fc fusion proteins

Monoclonal antibodies and Fc fusion proteins have emerged as one of the most exciting therapeutic modalities in the biopharmaceutical industry. Nineteen monoclonal antibodies and 3 Fc fusion protein-based therapeutics have been approved for sale in the U.S. and the European Union [1] with combined annual sales already exceeding $9 billion [2]. Several of these molecules serve significant unmet medical needs (Table 1). Nearly a quarter of biologics undergoing clinical trials belong to this class of molecules (PhRMA 2004 survey, www.phrma.org) ensuring that the importance of this product class will continue to increase over the coming years.

Some of the crucial properties of monoclonal antibodies for biological applications include their specificity for in vivo disease targets as well as the near infinite range of targets for which they can be generated. All therapeutic antibodies are IgGs with IgG1 and IgG2 being the most common subclasses [3]. IgGs have a well-defined biochemical structure consisting of two heavy and two light chains held together by intra-molecular disulfide bonds. Each chain consists of constant and variable regions (heavy chain: C_H1, C_H2, C_H3 and V_H; light chain: C_L and V_L). Fig. 1 shows a schematic structure of a monoclonal antibody. Fc fusion proteins, as the name implies, consist of the fusion of the Fc region (C_H2, C_H3 and hinge) of an antibody with a fusion partner (commonly a receptor or a cytokine). In contrast to antibodies, Fc fusion proteins do not possess a common biochemical structure beyond the dimeric Fc region [4]. This construct takes advantage of the interaction of the Fc region with a receptor on endothelial cells called the FcRn receptor that rescues these molecules from intracellular degradation and increases their half-life quite significantly. As will be explained later, the presence of the Fc tag also enables purification of these molecules by Protein A chromatography and facilitates the use of a common platform approach for both classes of molecules.

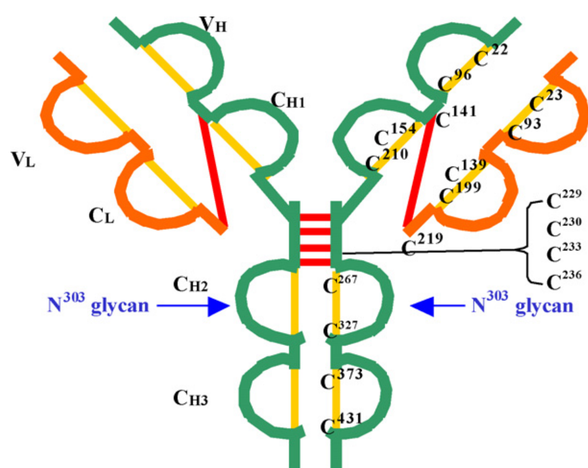

Fig. 1. Structure of a monoclonal antibody. V_H, variable region, heavy chain; C_H, constant domain, heavy chain; V_L, variable region, light chain; C_L, constant domain, light chain.

The advent of monoclonal antibodies and Fc fusion proteins has significantly increased the production scales for biopharmaceuticals. Most biotechnology products that were approved until the mid 1990s (including a variety of vaccines, hormones and growth factors) required very small quantities of purified product. In contrast, due to the high doses and the large patient populations in the indications they have been approved for, monoclonal antibodies and Fc fusion proteins commonly require annual production of several hundred kilograms of bulk drug substance. Thus, the considerations for large-scale production of pharmaceutical grade antibodies and Fc fusion proteins are quite different from their routine laboratory scale purification that have been described elsewhere [5].

Since monoclonal antibodies and Fc fusion proteins are typically large, glycosylated molecules, they are most often produced commercially by deep tank mammalian cell culture [6]. Recent increases in cell culture titers to >2 g/L [7] have enabled this production technology to stave off immediate competition from transgenic sources of production although these production methods might still find broad applicability in the future [8]. This review deals with the recovery and purification of antibodies and Fc fusion proteins (termed mAbs for the rest of this paper) from mammalian cell culture sources.

2. Purification of mAbs—literature review and templated purification schemes

Efficient recovery and purification of mAbs from cell culture media is a critical part of the production process and can dictate a significant proportion of the total manufacturing costs [9]. The primary consideration during downstream process development is purity. Another important consideration is the speed of process development given that process development needs to occur prior to introduction of a therapeutic candidate into clinical trials. Other key considerations include overall yield and process throughput. In addition, the process must meet several manufacturability criteria including robustness, reliability and scalability.

Important product purity attributes include process related contaminants (e.g. host cell protein levels, DNA, endotoxin, leached Protein A and some cell culture media additives) and product related impurities (e.g. high molecular weight aggregate and clipped/low molecular weight species). In addition, the process must be capable of clearing viruses to ensure product safety in the event of an undetected contamination.

A variety of preparative modes of chromatography have been employed for the process-scale purification of mAbs. Most schemes have involved the use of Protein A affinity chromatography exploiting the specific interactions that take place between

Table 1
Approved monoclonal antibodies and Fc fusion proteins

Trade name	Indication	Company	Year of approval
(a) Approved monoclonal antibodies			
Orthoclone OKT3	Acute kidney transplant rejection	Ortho Biotech	1986
ReoPro	Prevention of blood clot	Centocor	1994
Rituxan	Non-Hodgkin's lymphoma	Genentech/Biogen-IDEC	1997
Panorex	Colorectal cancer	GlaxoSmithKline	1995
Zenapax	Acute kidney transplant rejection	Hoffman-LaRoche	1997
Simulect	Prophylaxis of acute organ rejection in allogenic renal transplantation	Novartis	1998
Synagis	Respiratory synctial virus	Medimmune	1998
Remicade	Rheumatoid arthritis	Centocor	1998
Herceptin	Metastatic breast cancer	Genentech	1998
Mylotarg	Acute myelogenous lymphoma	Wyeth-Ayerst	2000
Campath-1H	B-cell chronic lymphocytic leukemia	Millenium/ILEX	2001
Zevalin	Non-Hodgkin's lymphoma	Biogen IDEC	2002
Humira	Rheumatoid arthritis	Abbott	2002
Bexxar	Non-Hodgkin's lymphoma	Corixa/GSK	2003
Xolair	Allergy	Genentech/Novartis	2003
Erbitux	Colon cancer	Imclone/BMS/Merck	2004
Avastin	Metastatic colon cancer	Genentech	2004
Raptiva	Psoriasis	Genentech/Xoma	2004
Tysabri	Multiple sclerosis	Biogen-Idec	2006
Vectibix	Metastatic colorectal cancer	Amgen	2006
(b) Approved Fc fusion proteins			
Enbrel	Rheumatoid arthritis, psoriasis, ankylosing spondylitis	Amgen	1998
Amevive	Psoriasis	Biogen-Idec	2004
Orencia	Rheumatoid arthritis	Bristol Myers Squibb	2005

the Fc region of mAbs and immobilized Protein A which is a cell wall component of *Staphylococcus aureus* [10,11]. Protein A affinity chromatography has been shown to be highly selective for mAbs, resulting in >95% purity in a single step starting from complex cell culture media [12].

Other modes of chromatography have been combined with Protein A chromatography to achieve pharmaceutically acceptable purity levels. These steps are typically chosen to provide orthogonal modes of interaction with the product to enable effective separation from host cell proteins and other contaminants. Direct capture by Protein A chromatography followed by anion-exchange chromatography and size exclusion chromatography has been employed for purifying a monoclonal antibody expressed by hybridoma cell culture [13]. Anion-exchange was selected as the second chromatographic step for DNA and endotoxin clearance, while size-exclusion was employed as the last step for removal of aggregates and degradation products. An ultrafiltration/diafiltration (UF/DF) buffer exchange step was employed prior to size exclusion chromatography to concentrate the product. Other modes of chromatography have also been employed successfully for mAb purification including hydroxyapatite and immobilized metal affinity chromatography (IMAC) [12]. Genentech has adopted the use of Protein A chromatographic capture followed by cation-exchange chromatography (CEX) and anion-exchange chromatography (AEX) operated in the flowthrough mode [14]. The CEX step clears host cell proteins, aggregats leached Protein A while the AEX flowthrough step removes DNA and achieves further reduction in host cell protein impurities. This sequence of steps has been adopted as a generic purification scheme [15] for a number of monoclonal antibody products.

While Protein A chromatography is highly selective for mAbs, the use of an immobilized protein as a ligand also lends its own share of challenges to this mode of chromatography. The ligand is prone to proteolysis and the cleaved domains can adhere to product molecules creating a separation challenge. Conventional Protein A ligands cannot be exposed to alkaline conditions that are commonly employed to sanitize other column modes thus necessitating the use of high concentrations of chaotropes such as urea for column regeneration and sanitization. The use of high concentrations of chaotropes creates a cost issue as well as a disposal challenge. The need to elute the column at a low pH can induce product aggregation for some mAbs. Most significantly, the cost of Protein A resins is nearly an order of magnitude higher than conventional chromatographic resins. Clearly, there is a significant driver for the development of small molecule ligands that can match the selectivity of Protein A for binding to mAbs.

Hydrophobic Charge Induction Chromatography (HCIC) employs a heterocyclic ligand such as 4-mercaptoethanol (MEP) that takes on an inducible positive charge at low pHs. This resin has been reported to be selective for antibody separations [16,17]. However, more recent investigations [18] have found this mode of chromatography to be based on non-specific hydrophobic interactions with electrostatic repulsion at low pH being responsible for product elution. In the capture mode, this resin was nearly an order of magnitude less selective for mAbs over host cell proteins as compared to Protein A chromatog-

raphy but was found to be a potentially useful polishing step. Ligands that can mimic the binding pocket of Protein A for the Fc region of mAbs have been found [19] and developed into Protein mimetic resins marketed as MAbSorbent A1P and A2P [20]. In internal investigations, these resins have also been found to possess lower selectivity than Protein A. Thus, at this point none of the small molecule ligands can universally match the selectivity offered by Protein A chromatography for mAb separations. However, they might be useful additions to the downstream process sequence due to their orthogonal selectivity with conventional modes of chromatography.

Similar to what is done for other proteins, it is conceptually possible to design a downstream process for mAbs without a Protein A affinity step by employing combinations of conventional chromatographic modes. Three-step combinations of cation-exchange, anion-exchange flowthrough, hydrophobic interaction chromatography and mixed mode cation-exchange chromatography were found to deliver adequate clearance of host cell protein contaminants for a CHO derived monoclonal antibody [21]. However, such purification schemes by-and-large have not caught on in commercial downstream operations due to the need to design the purification sequence separately for each mAb. Given the almost universal applicability of Protein A chromatography and the development of workarounds for most of its limitations (described in Section 4.2), it appears that this ligand will continue to be employed for commercial scale mAb purification at least in the foreseeable future.

The Protein A chromatographic step is typically employed for direct capture of the product from cell culture supernatant after harvest operations designed to remove cells and cell debris. In a few cases, the Protein A step is the second step in the process following capture on a conventional mode of chromatography [22]. This was done to protect the expensive Protein A resin from possible fouling through direct exposure to cell culture harvest media. However, the development of effective column regeneration schemes commonly allow Protein A resins to be employed for over 100 cycles with direct load of the cell culture supernatant. This also eliminates the need for concentration or buffer exchange of the harvest prior to chromatography. For the vast majority of commercial mAb processes, Protein A chromatography appears to be firmly ensconced as the primary capture step that also delivers a high purification factor.

3. A platform approach to process development

Process development can often be the rate-limiting step in the introduction of biopharmaceuticals into clinical trials [23]. Given the explosion in the numbers of mAbs entering clinical trials, there is a clear driver for employing a templated approach to process development. Indeed, if it was possible to have a generic process that could be employed for all mAb candidates it would greatly reduce the time and resources needed for process development. This can have a significant impact on the number of clinical candidates who can be introduced into clinical trials, and some kind of a generic approach is increasingly forming the cornerstone of the business strategy of most companies focusing on this therapeutic modality.

A generic process assumes that a pre-defined purification process works for all mAbs. However, in our experience, significant physicochemical differences exist among mAbs making this approach either impractical or resulting in a non-robust process. Fig. 2 shows analytical cation-exchange elution profiles for eight monoclonal antibodies under identical chromatographic conditions. As can be seen from the figure, the eight molecules differ quite significantly from each other in their affinity and elution behavior. Our experience with over 20 mAb candidates indicates that an inflexible generic process, with fully templated operating conditions, is not a desirable approach given these dramatic differences in properties. Even if a single set of operating conditions that work were to be arrived at, the resulting processes would clearly not be optimal or robust for each of the molecules. Accordingly, our approach has been to adopt a platform strategy instead of a fully generic set of operating conditions for all mAbs. The platform serves as a guidance document that defines the overall scheme of downstream processes and brackets the operating conditions for individual unit operations, thus limiting the scope of experimentation required to reach a solution for a given molecule.

A platform approach has several advantages from a business standpoint. Speed to the clinic is often the key determinant of business advantage for biotechnology companies since very often several companies may try and target similar biological pathways. The reduction in time and resources required to carry out process development are usually the primary economic driver for adopting a platform approach. However, once such a strategy is adopted several other advantages also become apparent. Other organizations in the company such as Quality and Manufacturing can better align with Process Development and integrate templated documents into their systems. Since raw materials are now selected from a significantly limited list, better deals can be negotiated with vendors. In addition, efforts can be directed towards multi-sourcing of critical raw materials to better manage operating risk. The platform also lays down a common, aligned philosophy that can be adopted across multiple geographic process development sites of a company (such as Amgen), resulting in a site-independent process that can be transferred to multiple manufacturing sites. The platform can also serve as a planning tool while planning across the entire organization since it lays down a common set of expectations. For instance, while designing manufacturing facilities for multiple future products, the platform can serve as a guide of what a process will look like and thus be a key planning tool.

4. A flexible, generic platform for mAb downstream processing

The previous section described the concept behind the platform process. In this section, we describe the downstream platform for mAbs that we have developed at Amgen and applied for the production of over 20 molecules over a range of scales ranging from clinical production to commercial launch. Fig. 3 shows a schematic for the platform downstream process for mAbs. Each of the unit operations shown in the figure is further described below.

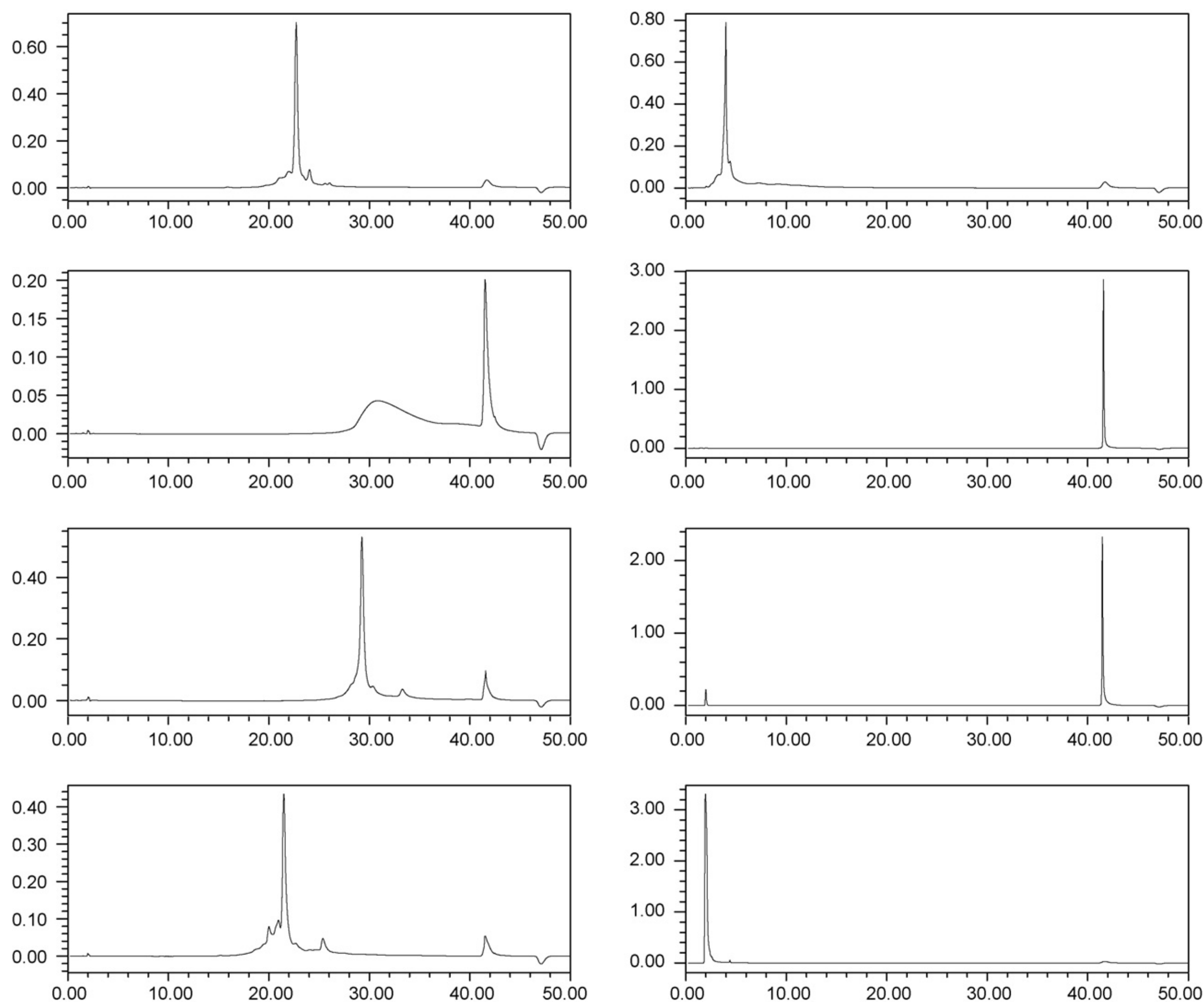

Fig. 2. Analytical cation-exchange linear gradient elution profiles for eight monoclonal antibodies. *x*-axis is time (min), *y*-axis is absorbance units at 280 nm measured by an in-line UV spectrometer.

4.1. Cell culture harvest operations

Since mAbs are secreted into the cell culture medium during mammalian cell culture, the first step in the downstream process is to remove cells and cell debris. At commercial scale this is accomplished by centrifugation using a continuous disk-stack centrifuge. Centrifugation [24] is preferred over other harvesting technologies such as cross-flow microfiltration [25] due to its scalability and economical operation for large volumes (typically 2–15,000 L/batch). Large-scale centrifugation acts as the primary harvesting step but cannot accomplish complete removal of cells and cell debris, which must be removed prior to chromatography.

For this reason, centrifugation is followed by depth filtration step(s) to remove residual cellular debris. Depth filtration refers to the use of a porous medium that is capable of retaining particulates throughout its matrix rather than just on its surface [26]. Depth filters employed in bioprocessing typically consist of a fibrous bed of cellulose or polypropylene fibers along with a filter aid (diatomaceous earth) and binder. The flat sheets are packed into single-use cartridges that can be stacked in a housing and pressurized to drive fluid flow through the system. While conventionally, depth filters have been regarded solely as a particulate removal operation, recent evidence suggests that the adsorptive properties of depth filters can be exploited to remove soluble species as well [27]. In this work, host cell protein contaminants were shown to be effectively removed from cell culture harvest supernatant of a mAb by appropriate selection of depth filter size and flux. Removal of these contaminants was shown to prevent problems with turbidity during elution of the capture Protein A chromatographic column.

Depth filters typically do not come with an absolute pore size rating unless they include a membrane layer at the end of the flow path. The depth filter is followed by a filter with an absolute pore size rating (typically 0.45 μm or 0.2 μm) that ensures the removal of solid particulates (and bacteria in case of the 0.2 μm filter) from the cell culture harvest supernatant.

Operating conditions for the harvest operations can be successfully templated for practically all mAbs. The combination of various harvest operations results in a process that is robust

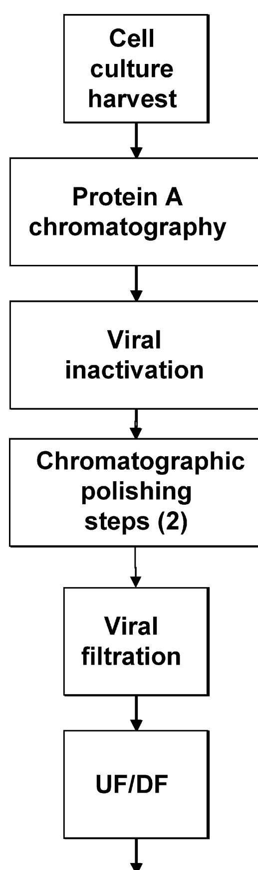

Fig. 3. Platform downstream process for mAbs.

enough to account for variations in cell density at harvest and final cell viability between molecules.

4.2. Protein A chromatography

Protein A chromatography serves as the capture step in the platform process. The Protein A step also serves as the key volume reduction step in the process since the product stream is concentrated from a relatively dilute cell culture supernatant to the eluate, which is typically at a concentration of >10 g/L. This step has proved to be highly selective for mAbs and can in many cases yield >99% purity starting from the cell culture supernatant. The overall scheme of operating a Protein A step also lends itself readily to a platform format. The cell culture supernatant can be directly loaded on the column (at a neutral pH) and the product is eluted from the column at low pHs. A wash step introduced between column load and elution is often at an intermediate pH and removes host cell protein and other contaminants. Finally, the column is stripped (an acidic solution of pH ∼2) and regenerated (high concentrations of chaotropes such as urea or guanidine hydrochloride are employed since conventional Protein A ligands are not alkaline stable). The high purities that are achieved by Protein A chromatography make the concept of a platform process possible for mAbs. The polishing steps have to remove remaining levels of host cell protein contaminants, DNA and product-related species (high molecular weight aggregate and low molecular weight clipped species) as well as provide additional clearance steps that help assure viral safety. The Protein A step also adds another impurity into the process in the form of leached Protein A ligand that is typically cleaved by proteases present in the cell culture supernatant.

Protein A chromatography does suffer from several limitations. The primary disadvantage is the high cost of the resin, which can be up to 10 times as expensive as conventional chromatographic supports. The high cost of the resin often leads to an operating strategy in which a smaller Protein A column is cycled several times while purifying a batch of cell culture supernatant. Protein A column loading is usually the rate-limiting step during this unit operation, since a large volume of cell culture supernatant is loaded on a relatively smaller column. This in turn means that throughput during Protein A chromatography becomes a key consideration. Differences in the dynamic binding capacity at various flow rates [28] and in the pressure-flow characteristics of various Protein A chromatographic media can result in wide variations in throughput [29,30] and were an important consideration during selection of a platform Protein A chromatographic resin. Interestingly, as titers achievable in cell culture increase, the focus will shift from how fast one can load the column to how much one can load (i.e. dynamic binding capacity).

Another key limitation of Protein A chromatography is the need to carry out product elution at low pHs. Exposure to low pH conditions can result in the formation of soluble high molecular weight aggregates (as can be detected by analytical size exclusion chromatography) and/or insoluble precipitate formation during product elution. Table 2 shows the occurrence and qualitative severity of these phenomena with 14 mAbs that underwent process development. As can be seen from the table, these problems occur quite frequently during Protein A chromatography. High molecular weight aggregate formation can lead to a reduction in product yield if a significant level of the product species aggregate. This also places an added burden on the polishing steps to achieve clearance since aggregate species

Table 2
Aggregation and precipitation during Protein A chromatography

Molecule	Soluble high molecular weight aggregate (measured by SEC)	Insoluble precipitate formation (measured byOD410)
1	−	−
2	+	+
3	+	−
4	+	++
5	+	+
6	+	+++
7	+	+++
8	+	−
9	+	−
10	++	−
11	++	−
12	+++	−
13	+++	+++
14	+++	−

Soluble HMW: (+++) >10%; (++) 4–10%; (+) 1–4%; (−) <1%. SEC refers to size-exclusion chromatography. OD410 refers to an optical density measurement at 410 nm as an indication of turbidity.

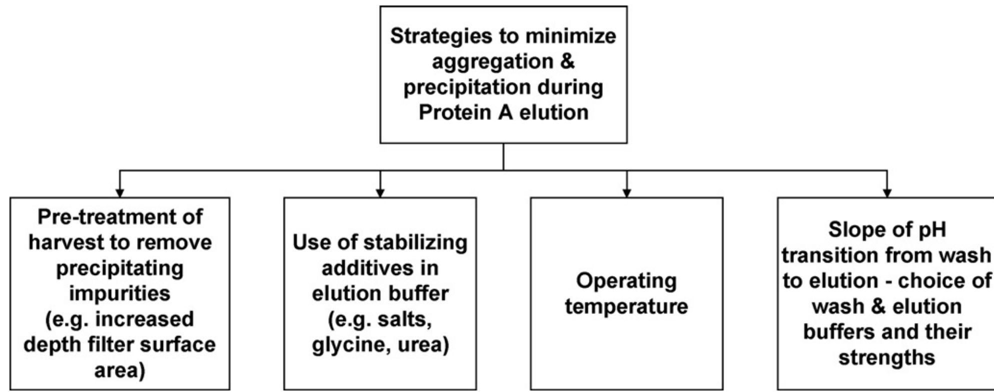

Fig. 4. Strategies adopted for addressing aggregation/precipitation during Protein A chromatography.

can be potentially antigenic. Insoluble aggregate formation can be the result of either the product species or impurities such as host cell proteins precipitating. In either event, there exists the risk of reduction in column lifetime if precipitation occurs during elution. If the product precipitates there might be a threat to product activity.

A variety of strategies have evolved to address the issue of aggregation/precipitation during Protein A elution and are reviewed elsewhere [31]. Fig. 4 shows a schematic of the strategies that have been taken in such situations. The modification of the Protein A elution buffer to make the buffer conditions more conducive to product stability is often the simplest solution. Stabilizers such as arginine have been added to the Protein A elution buffer to reduce aggregation of eluting antibodies [32]. If host cell protein impurities precipitate and prove to be removable during harvest depth filtration, appropriate selection of the depth filter chemistry, flux and loading can form a viable solution [27]. Low temperature operation of the Protein A step can in some cases reduce product aggregation. Yet another strategy can be to influence the slope of the pH transition from wash to elution buffers. Even though Protein A chromatography is operated under step gradient conditions, the mixing of the two buffers during the transition creates a pH transition that can be steep or gradual depending on the choice of wash and elution buffers and their strengths. Clearly, molecule specific solutions have to be developed and a templated set of conditions will not apply in the event of aggregation/precipitation issues.

Given the conventional wisdom that mAbs interact with Protein A through their Fc regions and the fact that >95% sequence homology exists even between the four Fc sub-types, one might expect Protein A elution pH to be readily templated. Fig. 5 plots the elution buffer pH employed in the downstream process for 14 mAbs. As can be seen from the figure, elution pH varies quite substantially from close to pH 3.0 to 4.1, even though the molecules belonged either to the IgG1 or IgG2 subclasses. A recent explanation for this has been provided through the demonstration of monoclonal antibody interactions with Protein A through their variable regions [33]. These interactions were shown to be eliminated on SuRe Protein A media in which the ligand consists solely of the B domain of Protein A which is not implicated in variable region interactions [34]. Thus, Protein A media with this alternative ligand can lend themselves more readily to templated elution buffer pH as shown in Fig. 6.

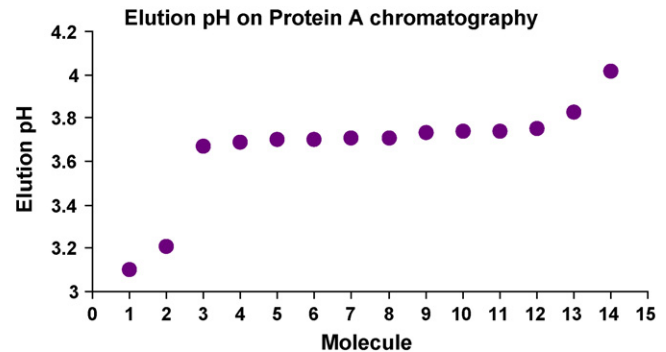

Fig. 5. Elution buffer pH during Protein A chromatography for 14 mAbs.

Binding capacity on Protein A media also vary quite significantly between molecules. Fig. 7 shows the operational loading capacity varying between 10 and 40 g/L resin for 14 molecules. Clearly, loading capacity also requires experimental determination for each molecule. Fig. 7 plots data for the same set of molecules as in Fig. 5 but employs a different numbering scheme to avoid an erroneous correlation of the data in the two figures. The numbering scheme for each of the figures in this text is unique to that particular plot although the same set of molecules are employed through the text.

Column regeneration for Protein A columns is typically carried out with high concentrations of chaotropes although there are recent reports suggesting the use of weakly alkaline conditions [35]. High concentrations of chaotropes (e.g.

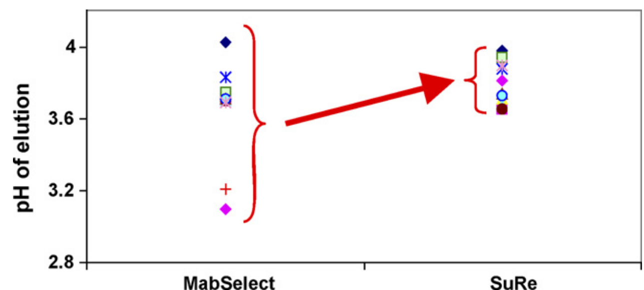

Fig. 6. Comparison of elution pH on MAbSelect® (conventional ligand) and SuRe (engineered ligand) resins for 14 mAbs.

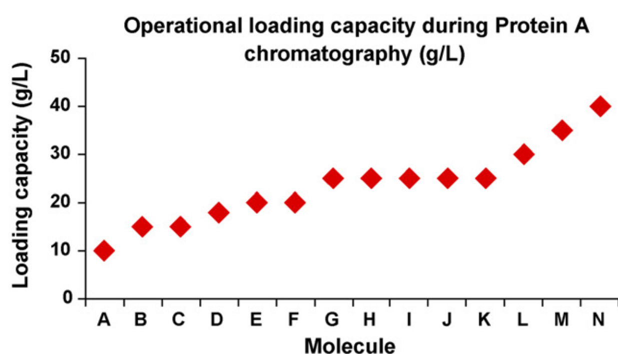

Fig. 7. Maximum operational load capacity during Protein A chromatography.

urea, guanidine hydrochloride) are costly and require special handling during disposal. The MAbSelect SuRe® Protein A resin with the engineered ligand is designed to be alkaline stable and permits the use of sodium hydroxide for column regeneration [36].

Table 3 summarizes the operating parameters for Protein A chromatography and highlights the ones that can be templated and those that require development effort specific to a molecule. The operational load capacity, wash and elution buffers require development although the platform for this step does define a set of possible buffers.

4.3. Low pH viral inactivation

The FDA Q5A guidance document [37] requires the use of two dedicated orthogonal steps for viral reduction in addition to the clearance achieved on chromatographic steps to assure safety of products produced by mammalian cell culture. Since the Protein A column eluate is at low pH and given that most mAbs are stable in solution under low pH conditions, it is relatively easy to include a low pH incubation step to inactivate viruses. Low pH treatment has been shown to successfully inactivate retroviruses for a variety of biotechnology products [38]. The pH of the Protein A elution pool is adjusted to pH ≤3.8 by addition of an acid solution (e.g. 0.5 M phosphoric acid). The use of strong acids such as HCl is avoided despite the advantage of low volume addition due to the risk of product denaturation in the localized region where the solution is added. In addition, storage of high concentrations of halide containing solutions can cause corrosion concerns for stainless steel vessels, especially when these solutions are at a low pH.

Retroviral inactivation kinetics in the specific solution dictate the duration of the incubation step. For molecules that are stable under low pH conditions (true for most monoclonal antibodies), a generic low pH condition that assures complete inactivation of retroviruses can be selected [39]. Following acid inactivation, the solution is neutralized to move the product into a more stable pH range. Once again, the use of strong bases (such as sodium hydroxide solutions) is avoided and the use of higher concentrations of weaker bases (e.g. Tris base solution) is preferred. An important consideration during neutralization is the presence of a buffering species that can help maintain pH around the final target pH. This is important to help prevent pH overshoot. For example, if an acetate buffer is employed for Protein A elution and the target pH following neutralization is pH 7.0 where this species cannot buffer, it might be required to add a buffering salt such as phosphate along with the high pH solution.

Some mAb solutions might exhibit a turbid appearance following neutralization. If the product species is not involved in the precipitation, this might not be as much of a concern as precipitation during Protein A column elution. Filtration of the solution with 0.45 μm/0.2 μm absolute filtration or in the case of excessive turbidity, use of depth filtration followed by absolute filtration might be an expedient process solution.

Table 3
Operational parameters for Protein A chromatography

Parameter	Platform conditions
Resin	Predetermined
Residence time during loading	
Resin load capacity	Development needed
Bed height	Pre-determined
Operating temperature	
Equilibration/post-load wash buffers	
Equilibration buffer Volume	
Post Load Eq. Wash Volume	
Optional: Wash II buffer	Development needed
Optional: Wash II Volume	
Elution buffer	
Elution pH	
Strip	Pre-determined
Strip Volume	
Flush	
Flush Volume	
Regeneration	
Regeneration Residence Time	
Regeneration Volume	
Storage	
Storage Volume	

Parameters in gray are templated across molecules, others require molecule specific development.

4.4. Polishing chromatographic steps

The subsequent chromatographic steps are aimed at reducing host cell protein impurities, high molecular weight aggregates, low molecular weight clipped species, DNA and leached Protein A that remain after the Protein A chromatographic step to acceptably low levels that assure safety of the product. At least two subsequent chromatographic steps are typically employed in mAb downstream processes, with a sufficient level of redundancy between them that assures robust operation of the entire process. These steps are typically referred to as the polishing steps in the downstream process.

In our platform, we typically select the polishing steps from cation-exchange chromatography (CEX), anion-exchange chromatography (AEX), hydrophobic interaction chromatography (HIC) and hydroxyapatite. Typically, one of the two polishing steps is operated in the flowthrough mode (in which the product does not bind to the column whereas impurity species are retained). AEX and HIC steps are often operated in the flowthrough mode for monoclonal antibodies since in general these molecules possess high p*I*s (isoelectric points). Higher column loadings are usually possible in the flowthrough mode

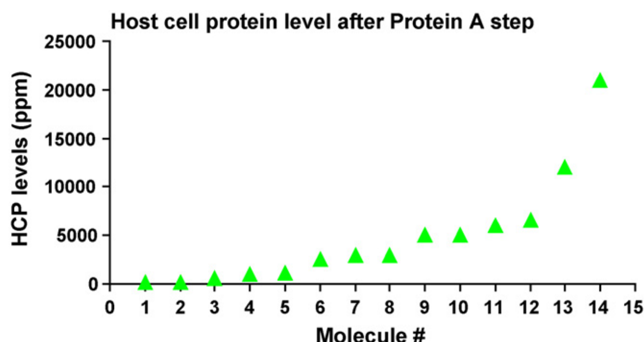

Fig. 8. Host cell protein contaminant levels after Protein A chromatography.

than in the bind and elute mode. In addition, operating in the flowthrough mode can make for a more robust operation. Successful operation of the step depends primarily on accuracy in achieving the correct column load conditions whereas bind and elute operation has a greater number of degrees of freedom in which errors can potentially occur (load, wash and elution conditions).

The choice of which modes of chromatography are employed depend on the nature of impurities that require clearance and how difficult they are to remove. Fig. 8 plots the host cell impurity levels after the Protein A chromatographic step. As can be seen from the figure, host cell protein levels can vary quite widely between molecules. Separate experiments have indicated that these variations are linked to differences in surface chemistry as well as cell culture and harvest conditions between molecules.

The percent high molecular weight aggregate levels after Protein A chromatography are shown in Fig. 9. In this case, the data was obtained from a generic set of operating conditions on Protein A and subsequent optimization along the lines discussed earlier successfully reduced percent aggregate to <10% in all cases. Furthermore, it is quite clear that aggregate levels can also vary between molecules. A large proportion of these aggregates is produced during cell culture (as demonstrated by the use of non-Protein A and neutral pH capture steps). The extent of host cell proteins and percent aggregate after Protein A chromatography is usually indicative of the degree of difficulty in developing appropriate polishing steps for a given molecule. Most molecules require a larger time and resource investment

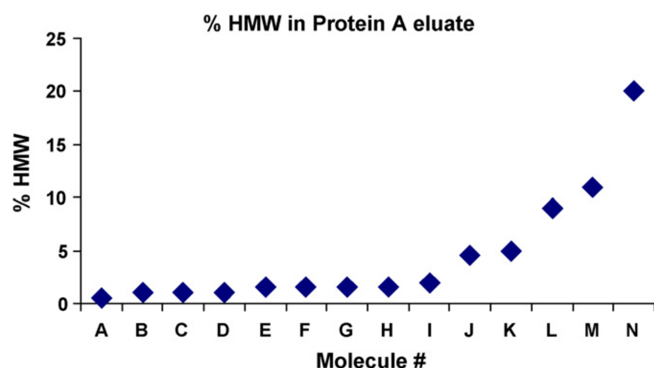

Fig. 9. Percent high molecular weight aggregate levels after Protein A chromatography.

Table 4
Modes of chromatography employed as polishing steps in mAb processes for clearing specific kinds of contaminants

Impurity	Mode of chromatography
High molecular weight aggregate	HIC, CEX
Host cell protein impurities	AEX, HIC, CEX
Leached Protein A	Hydroxyapatite, HIC, CEX
Viral clearance	AEX, CEX, HIC, HA

for removing these impurities as compared with leached Protein A or clipped low molecular weight species. Table 4 lists the modes of chromatography that have typically been successful for removing a particular kind of impurity in our experience. Note that this discussion does not include the requirement for the removal of product variant species that are sometimes required. Since variant removal (typically misfolded or inactive product species) is not a typical occurrence, strategies for their removal fall outside the definition of a platform process. An additional factor in the selection of polishing chromatography steps is the number of logs of viral clearance they can offer. In general, operation in the flowthrough mode can decrease the viral clearance capability except in the case of anion-exchange chromatography. AEX flowthrough steps have been demonstrated to achieve >4 logs of retroviral and parvoviral clearance for monoclonal antibodies [40].

4.5. Viral filtration

Viral filtration is employed in the platform process to complement the low pH viral inactivation step. Viral filters can be classified on the basis of their pore sizes into retroviral (<50 nm) and parvoviral (<20 nm) grade filters [41]. Viral filters are placed in the platform process following either one of the polishing chromatographic steps based on the volume of the intermediate product stream to be filtered or the volume of solution that can be filtered per unit surface area of membrane. Viral filters are typically operated at constant pressure. Due to their relatively small pore sizes, viral filters (especially parvoviral grade filters) can clog relatively quickly in the presence of particulate aggregates. As a result, pressurized tanks are employed to drive the fluid through the filters in preference to the use of pumps which can generate particulates due to shear through their moving parts. While the type of viral filter employed can be standardized across molecules, the placement and size of filters employed typically vary from product to product.

4.6. Ultrafiltration/diafiltration (UF/DF)

Following the completion of downstream purification, the product is buffer exchanged into the formulation buffer [42]. This is best accomplished by the use of an ultrafiltration/diafiltration setup. The type of membrane used, the transmembrane pressure employed, the cross-flow rate and the concentration at which diafiltration is carried out can be templated for all mAbs. With several formulations requiring the use of very high protein concentrations, issues of viscosity

and product aggregation can occur for certain molecules and have to be dealt with on a case-by-case basis.

4.7. Absolute filtration

Filtration through an absolute filter with a microfiltration membrane is often employed during the downstream process to ensure bioburden control or to remove small amounts of particulates. Such filters are often employed as in-line filters to a chromatographic column to help prevent fouling due to particulates. Following buffer exchange, the UF/DF retentate is filtered to generate the bulk drug substance which is often stored prior to the fill and finish operations. The type of the absolute filter can be standardized for the downstream process and validated to demonstrate bioburden clearance. Filter sizing can vary depending on the volume being filtered and the extent of particulates in the feed stream.

5. Process characterization and validation activities

Process characterization is a set of activities conducted to demonstrate robustness of a commercial manufacturing process through studies conducted at a small-scale [43]. The clinical entry (first-in-human) manufacturing process is usually not characterized in detail. However for a biologic entering late-stage clinical trials, process characterization is an essential component of the regulatory filing package. Operational and performance parameters for each unit operation are categorized as non-key, key or critical based on this exercise. This also helps to guide the setting of alert and action limits for these parameters and the setting of acceptance criteria for process validation studies.

Designing successful process characterization studies requires a significant amount of planning, determining which performance and operational parameters need to be studied on the basis of process history and understanding gained during development or formalized risk quantitative risk analysis such as FMEA analysis [44]. Since process characterization is carried out at small-scale, qualification of a scale-down model is essential [45].

For the characterization studies themselves, the characterization range for operational parameters are set to be at least as wide as the normal operating ranges and narrower than the zone of failure. A variety of fractional factorial experimental designs are employed to study the impact of varying various operating parameters within the predetermined range used for the characterization study. A typical study is a two-level, resolution IV, single replicate design with center points to assist in an independent estimation of error. An important outcome of the process characterization is the identification of parameters as non-key, key and critical. Prior to carrying out the characterization study, performance parameters (which include a set of in-process product quality analytical assays as well as parameters which monitor consistency of the run) are classified as shown in Table 5. If an operational parameter when varied within the characterization range causes significant variation in a critical performance parameter, it is classified as key. If on the other hand, it does not impact a critical performance parameter for that step, it is classified as non-key. As shown in Fig. 10, a subset of the key operational parameters will be critical, in that they can cause failure of the process. Clearly, this sub-classification can only be determined from separate experiments called worst case runs in which operating parameters for a step are combined to result in a worst-case scenario with respect to a particular critical performance attribute. The subsequent steps are then operated at their center-points to determine if that particular impurity is cleared to acceptable levels. If not, that operating parameter is deemed to be critical and should be carefully controlled during large-scale manufacturing operation. For such a parameter, the acceptable range might be as narrow as the operating range defined during process development and not wider.

Process validation is defined in the Q7A guidance document from the FDA as "... providing documented evidence that the process, operated within established parameters, can perform effectively and reproducibly to produce an intermediate or API meeting its predetermined specifications and quality attributes". Consequently, process validation is typically carried out at large-scale during a series of runs termed the conformance or validation campaign. These runs are carried out at large-scale following the engineering/practice runs and prior to start of the actual manufacturing campaign to produce and stockpile bulk drug substance in anticipation of commercial launch. Data from these runs are aimed at demonstrating control over the entire process. A written validation master plan is created prior to carrying out process validation activities. Some process qualification activities are carried out at small-scale using a qualified scale-down model of the process. These are employed to com-

Table 5
Definition of non-key, key and critical performance and operational parameters

Critical performance parameter: parameter that is a direct measure of the functionality of a step
Key performance parameter: parameter that measures process consistency and performance
Critical operational parameter: an operational parameter that when varied within the CR will cause process to fail acceptance criteria in one or more critical performance parameters
Key operational parameter: a parameter that if varied within the CR will significantly impact the performance of the process but not cause it to fail in terms of acceptance criteria around critical performance parameters
Non-key operational parameter: a parameter that if varied within CR will have no significant impact on product or process

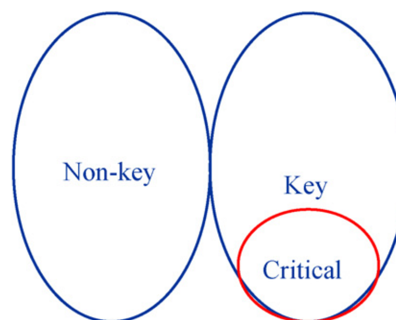

Fig. 10. Classification of performance and operational parameters resulting after process characterization.

plement data from large-scale operation (in the case of extended time/reuse studies) or in situations in which particular kinds of validation data cannot be obtained at large-scale (e.g. contaminant spiking studies including viral clearance validation studies and clearance of certain types of process chemicals). Further details on process validation can be had from several references [46,47].

6. Future directions in mAb purification

Future challenges to the current paradigm in mAb purification will be provided by the scale of production for many of this class of products. Successful increases in cell culture titer are anticipated to continue for the next several years, making the downstream process rate limiting. A large part of the limitations stem from limited tankage for buffers and process intermediates and the inability to increase large-scale column diameter to beyond 2 m without encountering significant issues with flow distribution and packing. Chromatographic operations thus become limited in terms of the throughput they can provide and necessitate extensive cycling to process a single cell culture batch. Innovative facility designs are likely to emerge as the first line of solutions to this challenge. Further in future, non-chromatographic purification techniques such as selective precipitation or liquid–liquid separations employing highly selective ligands are likely to emerge. Alternative ligands to Protein A are also likely to continue to be introduced in the market and their selectivity will improve with time. All of these will result in a gradual evolution of the downstream platform for mAb purification over the next decade. For further information on this area, please consult the review by Low et al. [48] also included in this volume.

7. Conclusions

Platform processes have emerged as a direct result of the need to develop clinical manufacturing processes for a vast pipeline of monoclonal antibody and Fc fusion proteins in the biopharmaceutical industry. This business strategy has resulted in significant savings in time and resources and harmonization of practices and information flow across the process development, operations and quality organizations at Amgen. This review described some of the essential elements of a platform downstream process for large-scale production of mAbs. Common elements in the mAb platform are described for each unit operation as well as aspects that pose a challenge to templated conditions across molecules. A key focus of continued technology development efforts is to template as many parameters as possible for each unit operation. Improved understanding of the fundamentals of each step is essential in making this a reality in the years to come.

References

[1] G. Walsh, Biopharm. Intnl. 17 (12) (2004) 18.
[2] D. Milroy, C. Auchincloss, Monoclonal Antibodies–on the Crest of a Wave. Horizons, Wood Mackenzie, Boston, MA, 2003, p. 6.
[3] M.A. van Dijk, J.G.J. van de Winkel, Curr. Opin. Chem. Biol. 5 (4) (2001) 368.
[4] A. Aruffo, in: S. Chamow, A. Ashkenazi (Eds.), Antibody Fusion Proteins, Wiley-Liss, New York, 1999, p. 221.
[5] R.W. Scott, S.A. Duffy, B.J. Moellering, C. Prior, Biotechnol. Prog. 3 (1987) 49.
[6] H.E. Chadd, S.M. Chamow, Curr. Opin. Biotechnol. 12 (2001) 188.
[7] F. Wurm, Nat. Biotechnol. 22 (2004) 1393.
[8] T.J. Menkhaus, Y. Bai, C. Zhang, Z.L. Nikolov, C.E. Glatz, Biotechnol. Prog. 20 (2004) 1001.
[9] K. Keller, T. Friedmann, A. Boxman, Trends Biotechnol. 19 (11) (2001) 438.
[10] H. Hjelm, K. Hjelm, J. Sjöquist, FEBS Lett. 28 (1972) 73.
[11] G. Kronvall, et al., J. Immunol. 111 (1973) 141.
[12] P. Gagnon, Purification Tools for Monoclonal Antibodies, Validated Biosystems, Tucson, AZ, 1995.
[13] L.S. Hanna, P. Pine, G. Reuzinsky, S. Nigam, D.R. Omstead, Biopharm. Int. (October) (1991) 33.
[14] R.L. Fahrner, H.L. Knudsen, C.D. Basey, W. Galan, D. Feuerhelm, M. Vanderlaan, G.S. Blank, Biotechnol. Gen. Eng. Rev. 18 (2001) 301.
[15] G. Blank, Recovery of Biological Products X Conference, Cancun, Mexico, June, 2001.
[16] L. Guerrier, P. Girot, W. Schwartz, E. Boschetti, Bioseparation 9 (2000) 211.
[17] W. Schwartz, D. Judd, M. Wysocki, L. Guerrier, E. Birck-Wilson, E. Boschetti, J. Chromatogr. A 908 (2001) 251.
[18] S. Ghose, B. Hubbard, S. Cramer, Biotechnol. Prog. 21 (2005) 498.
[19] R. Li, V. Dowd, D. Stewart, S. Burton, C. Lowe, Nat. Biotechnol. 16 (1998) 190.
[20] J. Curling, Genet. Eng. News 21 (20) (2001) 1.
[21] D. Follman, R.L. Fahrner, J. Chromatogr. A 1024 (2004) 79.
[22] G. Follena-Wassermann, Presented at Recovery of Biological Products X, Cancun, Mexico, June, 2001.
[23] G. Pisano, The Development Factory, HBS Press, Boston, MA, 1997.
[24] J.P. Maybury, M. Hoare, P. Dunnill, Biotechnol. Bioeng. 67 (2000) 265.
[25] C.S. Parnham, R.H. Davis, Biotechnol. Bioeng. 47 (1995) 155.
[26] J.V. Fiore, W.P. Olson, S.L. Holst, in: J. Curling (Ed.), Methods of Plasma Protein Fractionation, Academic Press, New York, 1980, p. 239.
[27] Y. Yigzaw, R. Piper, M. Tran, A.A. Shukla, Biotechnol. Prog. 22 (2006) 288.
[28] R. Hahn, R. Schlegel, A. Jungbauer, J. Chromatogr. B 790 (2003) 35.
[29] R.L. Fahrner, D. Whitney, M. Vanderlaan, G. Blank, Biotechnol. Appl. Biochem. 30 (1999) 121.
[30] R.L. Fahrner, H. Iyer, G. Blank, Bioproc. Eng. 21 (1999) 287.
[31] A.A. Shukla, P. Hinckley, P. Gupta, Y. Yigzaw, B. Hubbard, Bioproc. Int. 3 (5) (2005) 36.
[32] T. Arakawa, J. Philo, K. Tsumoto, R. Yumioka, D. Ejima, Protein Express. Purif. 36 (2004) 244.
[33] S. Ghose, M. Allen, B. Hubbard, C. Brooks, S.M. Cramer, Biotechnol. Bioeng. 92 (2005) 665.
[34] M. Starovasnik, M. O'Connell, W. Fairbrother, R. Kelley, Protein Sci. 8 (1999) 1423.
[35] H. Johansson, A. Bergenstable, G. Rodrigo, K. Oberg, The Use of NaOH for CIP of rProtein A Media: a 300 Cycle Study Waterside Conference, Savannah, May 2002. Available at http://www5.amershambiosciences.com/applic/upp00738.nsf/vLookupDoc/250173076-P516/$file/18117764AA.pdf.
[36] R. Hahn, K. Shimahara, F. Steindl, A. Jungbauer, J. Chromatogr. A 1102 (2006) 224.
[37] FDA Q5A Guidance Document: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin, Federal Register, vol. 63(185), 1998, p. 51074. Available at http://www.fda.gov/cder/guidance/Q5A-fnl.PDF.
[38] G. Sofer, Biopharm. Int. 16 (1) (2003) 50.
[39] K. Brorson, S. Krejci, K. Lee, E. Hamilton, K. Stein, Y. Xu, Biotechnol. Bioeng. 82 (2003) 321.
[40] S. Curtis, K. Lee, G. Blank, Y. Xu, Biotechnol. Bioeng. 84 (2) (2003) 179.
[41] T. Ireland, H. Lutz, M. Siwak, G. Bolton, Biopharm. Int. 11 (2004) 38.

[42] R. van Reis, E. Goodrich, C. Yson, L. Frautschy, S. Dzengeleski, H. Lutz, Biotechnol. Bioeng. 55 (1997) 737.
[43] J. Seely, R. Seely, Biopharm. Int. (August) (2003) 24.
[44] R. Seely, J. Haury, in: A. Rathore, G. Sofer (Eds.), Process Validation in Manufacturing of Biopharmaceuticals, Taylor and Francis, Boca Raton, FL, 2005, p. 13.
[45] R. Godavarti, J. Petrone, J. Robinson, R. Wright, B. Kelley, in: A. Rathore, G. Sofer (Eds.), Process Validation in Manufacturing of Biopharmaceuticals, Taylor and Francis, Boca Raton, FL, 2005, p. 69.
[46] L. Conley, J. McPherson, J. Thommes, in: A. Rathore, G. Sofer (Eds.), Process Validation in Manufacturing of Biopharmaceuticals, Taylor and Francis, Boca Raton, FL, 2005, p. 469.
[47] C. Bussineau, R. Seely, G. Lovitt, J. Fernandez, G. Blank, E. Brandreth, D. Conrad, R. Devine, R. Ferris, K. de Heyder, R. Juffras, P. Levy, W. List, M. Munk, B. Neely, S. Notarnicola, R. O'Leary, H. van Deinse, PDA Journal Technical Report #42 59 (2005) 1.
[48] D. Low, R. O'Leary, H. Pujar, J. Chromatogr. (2006) (JCB-06-583R1), submitted for publication.