Recent advances in large-scale production of monoclonal antibodies and related proteins

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The rapid development of high-yielding and robust manufacturing processes for monoclonal antibodies is an area of significant focus in the biopharmaceutical landscape. Advances in mammalian cell culture have taken titers to beyond the 5 g/l mark. Platform approaches to downstream process development have become widely established. Continuous evolution of these platforms is occurring as experience with a wider range of products is accrued. The increased cell culture productivity has shifted the attention of bioprocess development to operations downstream of the production bioreactor. This has rejuvenated interest in the use of non-chromatographic separation processes. Here, we review the current state-of-the-art industrial production processes, focusing on downstream technologies, for antibodies and antibody-related products and discuss future avenues for evolution.

Large-scale production of monoclonal antibodies

Monoclonal antibodies (mAbs) and related proteins have been shown to successfully target a wide range of extracellular targets with high specificity. This has led to their introduction to a variety of disease therapies, including several forms of cancer, multiple sclerosis and immunological disorders such as rheumatoid arthritis and psoriasis (Table 1). Significant advances have been made over the years in the design of mAbs as therapeutics that have improved bioavailability, optimized affinity, improved binding specificity, and human antibody sequences to reduce any immunogenic side effects [1]. Further advances in protein engineering are improving the therapeutic profiles of mAbs [2–5]. Several cases for which mAb therapeutics have been approved serve a large patient population and involve chronic therapy with high doses. This has led to the need for production processes that can be developed rapidly to produce consistently and reproducibly large quantities of pharmaceutical-grade mAbs at moderate costs. Process platforms in both upstream cell culture and downstream purification have become widely established in industry to meet this need.

Large-scale production of mAbs utilizes mammalian production systems followed by cell removal and purification through sequential chromatographic and membrane filtration steps, to consistently reduce product- (e.g. protein variants) and non-product-related (e.g. host cell proteins) impurities to acceptable levels. Batch chromatography, which is traditionally used as the core purification method in industrial bioprocesses, has long been labeled as a slow process that requires large volumes of mobile phase and results in very large pool volumes. Recent advances in the design of chromatographic stationary phases available for purification of biomolecules have provided some solutions to the throughput dilemma, and have facilitated reasonably productive platforms for industrial protein purification. As multiple biopharmaceutical companies have become increasingly involved with the development of mAb products, platform approaches to their production have become well established in industry. These platforms leverage similarities in the biochemical properties and chromatographic behavior of this product class as well as experience gained from the first mAbs and Fc fusion proteins that came on the market. The platforms have enabled significant efficiencies.

Glossary

Capture step: the first downstream processing step that captures the product from the harvested cell broth, concentrates the product, and achieves separation from impurities that are most unlike the product (e.g. cells, cell debris, DNA, most proteins).

Cell specific productivity (Qp): productivity of a cell culture expressed in terms of amount of protein produced per cell per unit time. A conventional unit for mammalian cell culture is pg/cell/day.

Downstream process: process steps associated with the purification of a recombinant protein and removal of impurities.

Fed-batch cell culture: a production process based upon feeding a growth limiting nutrient to the culture. This allows the achievement of a high cell density in the production bioreactor and facilitates metabolic control of the cells to avoid generation of side products.

Follow-on biologics (also known as ‘biosimilars’): biopharmaceuticals that are deemed comparable in quality, safety and efficacy to a reference product from an innovator company.

NGNA: N-glycolylneuraminic acid that can form the terminal saccharide unit for an N-linked oligosaccharide structure. Presence of NGNA has been linked to possible immunogenicity. The usual terminal sugar unit is typically NANA (N-acetylneuraminic acid).

Polishing steps: steps that occur after the initial capture step that are aimed at removal of smaller levels of impurities left in the product stream that have more similarity to the product (e.g. aggregated forms of the product, protein structural and sequence variants).

Sanitization: procedures that destroy contaminating microorganisms on a piece of equipment used for bioprocessing. For chromatographic columns, mild alkaline solutions or high concentrations of chaotropes (e.g. urea) are used for sanitization to achieve bioburden control while preserving the resin.

Transient transfection: process of introducing DNA into a host cell by non-viral means such that the genetic material is not inserted into the nuclear genome. As a result, the genetic material is lost over time through mitosis.

Upstream process: process steps associated with the production of a recombinant protein by culture and propagation of the host cells.
in process development from time and resource perspectives. Allied areas in biopharmaceutical enterprises, such as quality and manufacturing, have also benefited through templating their documents and procedures. A few publications have provided details on the process platforms in use at various companies [6–8], and further details have been shared at conferences. The sections below provide details on current platforms and review recent developments that are leading to their evolution. The use of these production platforms has enabled the rapid and efficient introduction of large numbers of mAbs into clinical trials. The process platform continues to evolve as further experience is gained with a wider set of mAbs.

**Downstream process platform for mAb production**

Upstream process platforms in terms of cell line, clone selection methodologies, fed-batch cell culture operating conditions and media have evolved significantly in the last decade. While these two sections focus on downstream process platforms, Box 1 provides details about process operating conditions for large-scale cell culture production of mAbs.

The downstream purification process has evolved toward a common framework across industry in recent years [6–8,28,29]. The platform approach does not imply a templated process; indeed, this has proven difficult even within the same company because of differences in properties and purification behavior between various mAbs [6]. What the platform achieves is the creation of a common philosophy and alignment over the types of unit operations to include in the downstream process. Figure 2 shows some of the downstream process platform schemes adopted at biopharmaceutical companies involved in mAb process development. The schematic diagram for a typical downstream process, including other filtration and viral-clearance-oriented process steps, is depicted in Figure 3.

All of these schemes rely on the use of Protein A affinity chromatography as the capture step in the process. This mode of affinity chromatography is based on the specific binding affinity between the Fc region of mAbs and the Protein A ligand. This specificity allows host cell proteins, DNA and other impurities from the cell culture process to flow through while the product binds to the stationary phase, providing >98% purity in a single step. Elution from Protein A affinity adsorbents is effective under conditions of low pH. In many ways, the high specificity of the Protein A step has facilitated the adoption of a platform approach for downstream purification of mAbs. The polishing steps in the downstream process aim to reduce process- and product-related impurities, particularly host cell

### Table 1. Monoclonal antibodies and related proteins on the market

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Indication</th>
<th>Company</th>
<th>Year</th>
<th>Antibody type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthoclone OKT3</td>
<td>CD3</td>
<td>Acute kidney transplant rejection</td>
<td>Ortho Biotech</td>
<td>1986</td>
<td>Murine</td>
</tr>
<tr>
<td>ReoPro</td>
<td>Platelet GP Iib/IIIa</td>
<td>Prevention of blood clot</td>
<td>Centocor</td>
<td>1994</td>
<td>Murine</td>
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<tr>
<td>Rituxan</td>
<td>CD20</td>
<td>Non-Hodgkin’s Lymphoma</td>
<td>Genentech/ Biogen-Idec</td>
<td>1997</td>
<td>Chimeric</td>
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<tr>
<td>Panorex</td>
<td>17A-1</td>
<td>Colorectal cancer</td>
<td>GlaxoSmithKline</td>
<td>1995</td>
<td>Murine</td>
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<tr>
<td>Zenapax</td>
<td>IL2Ra (CD25)</td>
<td>Acute kidney transplant rejection</td>
<td>Hoffman-LaRoche</td>
<td>1997</td>
<td>Humanized</td>
</tr>
<tr>
<td>Simulect</td>
<td>IL2R</td>
<td>Prolymphaxis of acute organ rejection</td>
<td>Novartis</td>
<td>1998</td>
<td>Chimeric</td>
</tr>
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<td>Synagis</td>
<td>RSV</td>
<td>Respiratory Syncial Virus</td>
<td>Medimmune</td>
<td>1998</td>
<td>Humanized</td>
</tr>
<tr>
<td>Remicade</td>
<td>TNFα</td>
<td>Rheumatoid arthritis</td>
<td>Centocor</td>
<td>1998</td>
<td>Chimeric</td>
</tr>
<tr>
<td>Herceptin</td>
<td>Her2</td>
<td>Metastatic breast cancer</td>
<td>Genentech</td>
<td>1998</td>
<td>Humanized</td>
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<tr>
<td>Mylotarg</td>
<td>CD33</td>
<td>Acute myelogenous lymphoma</td>
<td>Wyeth-Ayerst</td>
<td>2000</td>
<td>Humanized</td>
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<tr>
<td>Campath</td>
<td>CD52</td>
<td>B cell chronic lymphocytic leukemia</td>
<td>Takeda</td>
<td>2001</td>
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<td>Zevalin</td>
<td>CD20</td>
<td>Non-Hodgkins Lymphoma</td>
<td>Biogen-Idec</td>
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<td>Murine</td>
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<td>Humira</td>
<td>TNFα</td>
<td>Rheumatoid arthritis</td>
<td>Abbott</td>
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<td>Human</td>
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<td>Bexxar</td>
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<td>Non-Hodgkins Lymphoma</td>
<td>Corixa/GSK</td>
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<td>Murine</td>
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<tr>
<td>Xolair</td>
<td>IgE</td>
<td>Allergy</td>
<td>Genentech/Novartis</td>
<td>2003</td>
<td>Humanized</td>
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<td>Erbitux</td>
<td>EGFR/Her1</td>
<td>Colorectal cancer</td>
<td>Bristol-Myers</td>
<td>2004</td>
<td>Humanized</td>
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<td>Avastin</td>
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<td>Genentech</td>
<td>2004</td>
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<td>Raptiva</td>
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<td>Genentech/Xoma</td>
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<td>Humanized</td>
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<td>Tysabri</td>
<td>A4 integrin</td>
<td>Multiple sclerosis</td>
<td>Biogen-Idec/Elan</td>
<td>2004</td>
<td>Humanized</td>
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<tr>
<td>Vectibix</td>
<td>EGFR</td>
<td>Colorectal cancer</td>
<td>Amgen</td>
<td>2006</td>
<td>Human</td>
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<tr>
<td>Soliris</td>
<td>C5 complement</td>
<td>PNH – paroxysmal nocturnal hemoglobinuria</td>
<td>Alexion</td>
<td>2007</td>
<td>Humanized</td>
</tr>
<tr>
<td>Stelara</td>
<td>IL12 and IL23</td>
<td>Psoriasis</td>
<td>Centocor</td>
<td>2008</td>
<td>Human</td>
</tr>
<tr>
<td>Simponi</td>
<td>TNFα</td>
<td>Rheumatoid arthritis</td>
<td>Centocor</td>
<td>2008</td>
<td>Human</td>
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<tr>
<td>Actemra</td>
<td>IL-6</td>
<td>Rheumatoid arthritis</td>
<td>Roche</td>
<td>2009</td>
<td>Humanized</td>
</tr>
<tr>
<td>Lucentis</td>
<td>VEGF-A</td>
<td>Age related macular degeneration</td>
<td>Genentech</td>
<td>2006</td>
<td>Fab’</td>
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<tr>
<td>Cimzia</td>
<td>TNFα</td>
<td>Crohn’s disease</td>
<td>UCB</td>
<td>2008</td>
<td>Pegylated Fab fragment</td>
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<tr>
<td>Enbrel</td>
<td>TNFα</td>
<td>Rheumatoid arthritis, psoriasis, ankylosing spondilitis</td>
<td>Amgen</td>
<td>1998</td>
<td>Soluble TNFα receptor fused to IgG1 Fc</td>
</tr>
<tr>
<td>Amevive</td>
<td>CD 2</td>
<td>Psoriasis</td>
<td>Biogen-Idec</td>
<td>2003</td>
<td>LFA3 fused to IgG1 Fc</td>
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<tr>
<td>Orenchia</td>
<td>CD80/86</td>
<td>Rheumatoid arthritis</td>
<td>Bristol-Myers Squibb</td>
<td>2005</td>
<td>CTLA4 fused to IgG1 Fc</td>
</tr>
<tr>
<td>Arcalyst</td>
<td>IL-1</td>
<td>CAPS – Cryopyrin Associated Periodic Syndrome</td>
<td>Regeneron</td>
<td>2007</td>
<td>IL-1 receptor fused to Fc</td>
</tr>
</tbody>
</table>
Box 1. Upstream process platform

The productivity of fed-batch mammalian cell culture processes for mAbs has increased significantly in recent years. Two key factors have underpinned the increase: (i) highly productive cell lines that have both the right growth characteristics and a high specific productivity (Qp); and (ii) improved understanding of chemically defined media and feeding strategies to achieve high cell density and sustained viability over the course of the bioreactor run. Figure 1 is a schematic diagram for an upstream production process that is often used for the production of mAbs and other glycoproteins.

The creation of a rapid-growth and high-specific-productivity (Qp) production cell line sets the right foundation for developing the cell culture production process. Additional requirements include acceptable cell line stability and the ability to grow in chemically defined media [10]. The use of effective expression systems [9,10] is a prerequisite to creating a high-growing cell line. Commonly utilized selection markers within the stable cell line development vectors include those based on genes that encode dihydroxy folic reductase and glutamine synthetase. These expression vectors also typically utilize strong gene promoters that drive product mRNA transcription, such as cytomegalovirus or EF-1α, whereas expression of the gene that encodes the selectable marker is often driven through a weak promoter to maximize selection stringency. A high level of product synthesis in Chinese hamster ovary (CHO) cells has been linked to increased gene copy number and effective transcription [11]. Loss in stability of the cell line in the absence of selective pressure has been linked to decreased transcription rather than loss of copy number, which is often indicated for instability [10]. Although the complete molecular mechanisms are incompletely understood, transgene silencing has been implicated in the decreased productivity. Cell lines used for antibody expression include the most commonly used CHO cell lines, murine lymphoid cell lines (e.g. NS0 and SP2/0), human PER.C6, and often murine hybridoma cell lines [12,13]. All of these cell lines have been shown to produce product glycosylation patterns that are compatible with the human immune system, although there have been reports of high NGNA levels in NS0-derived expression systems [14]. Cells have also been engineered to increase product expression through coexpression of important proteins in the secretory pathway [15]. A number of coexpression strategies have been researched including coexpression of chaperones, protein degradation inhibitors and apoptosis inhibitors. Obtaining more complete knowledge of the CHO proteome will certainly aid coexpression efforts to obtain a more productive cell line.

Production cell lines are screened during their development to select for those with a high specific productivity (Qp). However, not all cell lines with a high Qp (generally 20–90 pg/cell/day [16]) may perform well in a production process. As a result of this and other factors, including stability profiles and desirable product quality metrics, large numbers of clones need to be generated and screened to arrive at the best combination of properties that are most suitable for biopharmaceutical production. Several automated systems for cell line screening are in use today to facilitate the identification of the most suitable production line. Fluorescence-activated cell sorting is one of the techniques used to screen cell lines with high levels of antibody production from within a heterogeneous population during selection. A clone screening technique that utilizes relative mRNA transcript levels to screen predictively for high expressers, using the ratio of light to heavy chain mRNA levels as a predictor of aggregated product levels, has been reported to help identify clones most suitable for antibody production [17]. To monitor clone growth characteristics, some level of screening is typically carried out in shake flasks or even small bioreactors, in addition to early high-throughput screening in 96-well plates [17]. In addition to material generation through the development of stable production cell lines, the use of transient transfectant pools for production is frequently carried out during pre-clinical developmental stages, to produce rapidly significant quantities of the product [18,19], but is currently rarely employed at scales beyond a few hundred liters of cell culture volume. It is greatly preferred to lock in the most desirable subset of clones for biopharmaceutical production early in the clinical development process for a product. This avoids potential changes in glycosylation profile later in development, which can create comparability hurdles if a switch to an alternative cell line is undertaken.

In large-scale cell culture processes, the early stages of inoculum growth viaial thaw are conducted in shake flasks or spinner flasks, which progressively increase in size and/or volume. Wave bioreactors in disposable bags are used extensively at this stage of the process. The cell mass is then scaled up through several bioreactor stages prior to transfer to the production bioreactor. Fed-batch production is the most prevalent and involves the addition of small volumes of feed to supplement the nutrients present in the bioreactor as cell growth and product production progresses. Dissolved oxygen, pH, temperature and mass transfer of oxygen and CO₂ are controlled in the production bioreactor. Perfusion cell culture, in which the cells are retained in the bioreactor and new media fed continuously is employed in some processes, but is less widely adopted because of challenges with maintaining sterility over long time periods. A key development in the achievement of high cell culture titers has been an improved understanding of nutrient limitations during cell culture and the design of chemically defined, serum-free media that can support cell growth and product secretion. Hydrolysates from yeast or plant sources are often a component of cell culture media and have effectively replaced the use of serum in large-scale processes. Amino acids and trace media constituents such as metals have been shown to influence product yields in cell culture [20,21]. Bioreactor processes also have been developed to optimize gas exchange, to supply sufficient oxygen to sustain cell growth and productivity, and to remove CO₂. Maintaining the efficiency of gas exchange is a very important criterion for ensuring the success of scaling up cell culture platforms [22].

mAbs are glycosylated products typically with a single N-linked site on each half of the Fc region. Even though the N-linked glycosylation heterogeneity does not significantly influence pharmacokinetics or half-life of mAbs, because that is predominantly dictated by binding to the neonatal Fc receptor (FcRn binding [23]), N-glycosylation does play a role in mediating antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. Additionally, it is important to prevent the occurrence of glycosylation structures that can trigger a human immune response such as galactose α(1,3)-galactose and NGNA structures [24]. As cell cultures under suboptimal conditions have been shown to produce aberrant oligosaccharide structures [20,21,25], it is important to link media and bioreactor condition optimization to evaluation of glycosylation quality during cell culture design.

A typical harvest procedure for mAb cell culture utilizes centrifugation followed by depth and membrane filters to remove cells and cell debris prior to the purification steps in the downstream process [26]. Depth filters consist of a fibrous bed that can trap particles within their volume in contrast to membrane filters that reject particulates. Recently, depth filters have been shown to possess adsorptive properties for soluble impurities such as host cell proteins and DNA, in addition to their filtration role in removing particulates [27]. As a result, the type of depth filter and the choice of operating conditions need to be studied in an integrative fashion with the downstream purification process.
without the use of an affinity step [31,32]. However, these approaches are more difficult to template. In the past, significant attention has focused on the development of small-molecule ligands that could offer similar selectivity to Protein A. However, comprehensive evaluation of hydrophobic charge induction chromatography (HCIC) [33] and protein A mimetic ligands [34], using multiple antibody process streams, has indicated markedly lower selectivity than the Protein A ligand. Although the success of non-proteinaceous ligands in replacing Protein A chromatography has been low, these alternative modes of chromatography are being employed as polishing steps in antibody purification. Alternatively, other affinity ligands such as single-chain antibodies, against the Fc region, derived from camelid species have been shown to offer promising selectivity for mAbs [35,36]. Improvements in the Protein A ligand itself have also occurred, with one of the recent improvements being the development of a base-stable ligand that can resist alkaline conditions, which are often preferred for sanitization of large-scale chromatography columns. This ligand also has improved the homogeneity of antibody interactions with Protein A, thus facilitating the use of a generic set of operating buffers for the Protein A step [37].

**Recent advances in Protein A capture chromatography**

Given the key significance of Protein A chromatography, recent efforts have focused on improving its understanding and mitigating some of the limitations it poses for large-scale mAb production. Process throughput is constrained by the maximum loading possible on Protein A; therefore, it is important to understand why binding is limited. Using a panel of mAbs and Fc fusion proteins, it has been determined that multiple product molecules can bind to the Protein A ligand. Inter- and intra-ligand steric effects have been shown to limit the amount of mAb that can bind per unit volume of the resin [38]. Dynamic binding capacity on the Protein A affinity step is significantly influenced by flow rate and residence time during loading [39]. The capture step, which involves the loading of relatively dilute cell culture harvest on the resin, is generally throughput-limited. To address this

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**Box 2. Non-chromatographic technologies aimed at improving downstream process throughput**

Renewed interest in non-chromatographic separation has been sparked by some of the recent discussions about process throughput limitations faced by batch chromatography. A number of such techniques have been investigated ever since the advent of protein production on a large scale, but have largely not been adopted for therapeutic protein production. Aqueous two-phase separations using polymer–salt [poly(ethylene glycol)] (PEG) phosphate and PEG citrate) systems have been applied successfully for purification of mAbs from cell culture harvest streams [65]. Expanded bed chromatography has been left behind during the past decade, despite promise as an integrative technology between cell culture and purification. Recently, interest has returned through the introduction of improved resins and flow distribution systems that allow large-scale implementation [66]. Precipitation of proteins using salts has long been a mainstay of laboratory-level protein isolation. It has been realized that application to mAb production will require improvement in the selectivity of this technique [67–69]. Efforts focus on precipitating the product using negatively charged polyelectrolytes such as caprylic acid or poly-ether sulfone. Some efforts have focused on removing impurities by flocculation or precipitation. Promising avenues for future research include the use of selective polymers for product precipitation. Modified PEG molecules have been used for selective capture of mAbs from CHO cell supernatants. Crystallization can result in highly pure proteins and is often used for smaller proteins and peptides. Although this area elicited some early interest for mAb production, it is now realized that the phase behavior of mAbs makes this a less robust process, with relatively narrow operating ranges [70]. Charged UF membranes can provide separation based on size (hydrodynamic radius is strongly influenced by pH and ionic strength) and charge [71,72]. Membrane adsorbers are already a reality in the large-scale downstream processing of some antibodies [73]. Anion exchange membrane adsorbers provide high capacity and throughput but are effective for minimal polishing only (DNA removal, some host cell protein clearance, and for providing additional viral clearance capability). Ability to bind to large quantities of aggregates or host cell protein is minimal.
issue, when ample binding sites are available, a modulated flow rate with rapid initial flow, followed by a progressive decline in flow rate, has been developed [40].

Another area of concern for large-scale Protein A chromatography is the lifetime of the resin, with re-use over multiple cycles in operation. The Protein A ligand is known to be sensitive to extreme conditions of pH and can lose its ability to bind to products. The base-stable ligand mentioned earlier is still not in widespread use because of its higher cost. A progressive decline in binding capacity has been shown to occur upon exposure to the mild alkaline conditions often used for regenerating agarose-based Protein A resins [41]. Protective additives in 0.1 N NaOH [41] or use of a combination solution of phosphoric acid, acetic acid and benzyl alcohol [42] have been found to be effective sanitization solutions that maintain ligand functionality. Overall, adsorbents based on Protein A have been shown to be remarkably stable over many hundred cycles of use, thus making Protein A chromatography a very reliable tool in the antibody purification platform [43].

Host cell proteins constitute an important class of process-related contaminants that need to be reduced to low levels to assure product safety. While the Protein A capture step is highly selective for mAbs over soluble host cell protein impurities, the levels of host cell protein that often persist after Protein A purification can still pose a clearance challenge for non-affinity polishing steps. It has been shown that host cell protein species that persist through...
the Protein A step are often associated with the product species [44]. These protein–protein interactions have been found to be disrupted by chaotrope combinations at high pH at which mAb–Protein A interactions are still strong. This has allowed the development of selective wash conditions that can reduce the host cell protein levels after Protein A chromatography.

In addition to a better mechanistic understanding of host cell protein clearance during mAb purification, better methodologies have been developed to monitor host cell protein (HCP) clearance and to validate the capability of the downstream process to clear them. 2D difference gel electrophoresis has been used to create a map of host cell proteins to monitor their clearance through the downstream process [45]. Multiple strategies, including worst-case studies, bypass and spiking, have been employed to demonstrate robust clearance of host cell proteins through a mAb downstream process [46]. The low-pH conditions used for Protein A elution often result in precipitation issues for the product species. These have been shown to be mitigated by judicious buffer selection or by selecting stabilizing additives for the elution buffer [47]. In some cases, the turbidity has been shown to be caused by the precipitation of host cell protein contaminants, rather than the product. This can still be undesirable from a process reproducibility and filtration perspective [48] and has been shown to be prevented by appropriate sizing and design of the harvest depth filtration step [27].

**Recent advances in unit operations following Protein A capture**

As shown in Figure 2, some platforms adopt a completely templated approach to the polishing steps (predominantly through the use of anion (AEX) and cation (CEX) exchange chromatography), whereas others have a molecule-specific approach based on the particular impurity clearance challenge. Hydrophobic interaction chromatography (HIC) is often selected for its particular ability to clear high-molecular-weight aggregates [6], and mixed-mode ion-exchange chromatography has found recent application as a result of its unique selectivity [49,50]. Several new mixed-mode resins have been launched (e.g. Captoadhere® and CaptoMMC® from GE Healthcare) and are finding increasing application as polishing steps for host cell protein and aggregate clearance. Cation-exchange chromatography column loading is often carried out at low salt concentrations. mAbs have a basic pI, and the initial molecules that adsorb on the resin are often at the entrance to pores in the resin structure, thus repelling more protein molecules and restricting column load capacities [51]. The anion-exchange column is usually operated under flow-through conditions at fairly high load capacities. As described later, this step can sometimes be replaced with a membrane adsorber step. If impurity resolution, particularly HCP and aggregate is needed, the anion-exchange chromatographic step can be operated under a weak partitioning mode as opposed to true flow-through conditions, which results in retention of the impurities [52].

A majority of recently developed mAb production processes employ parvoviral grade viral filters (~20 nm retention size) in contrast to the retroviral grade filters (~50 nm retention size) that were employed in the past [53]. Although the smaller pore size rating provides added viral retention, these filters can be clogged by small levels of particulates that are otherwise not detectable by analytical size exclusion chromatography (SEC) or particle size measurements in the product stream [54,55]. Viral filters are typically single-use filters and can be among the most expensive consumables in the process based on their cost per gram of product. In addition to appropriate placement of these filters in the downstream process, pre-filters can be used to protect the viral filters from flux decay arising from a high particulate load [56]. Viral filters from multiple vendors are typically screened to arrive at the right selection for a process, and significant innovation has taken place in recent years in the development of low-fouling viral filters that can still provide excellent viral clearance [57]. These filters are made of hydrophilic materials that reduce the tendency for proteins to unfold at the surface, thus minimizing nonspecific fouling of the pores. Additional improvements have been made in pore morphology, with a reduction in polydispersity of pore sizes. These have led to progressive improvement in the maintenance of high fluxes with mAb process streams.

The large production quantities of mAbs create another challenge for the handling of the large volumes of drug substance produced. Several ultra-high-concentration antibody formulations with protein concentrations exceeding 100 mg/ml are in use for mAbs, particularly for subcutaneous delivery. The generation of such high protein concentrations requires specialized attention to the design of the UF/DF step that is typically used for buffer exchange into the formulation buffer [58]. Air–water interfaces created during pumping of such highly concentrated protein solutions can cause product aggregation during large-scale production [59]. The flowpath in the UF system is designed to minimize system hold-up volumes and to decrease the incidence of zones where air entrainment might occur. For high-concentration formulations, the concentration at which DF is conducted is typically selected to minimize the number of pump passes to which an average protein molecule might be exposed during the step. Even exposure to stainless-steel surfaces for significant lengths of time can result in aggregation [60]. If the drug substance requires frozen conditions during long-term storage, design of the freezing and thawing steps needs to be carefully approached to avoid product quality changes caused by cryo-concentration and exclusion effects caused by slow freezing rates in large volumes, and product denaturation on high-surface-area ice crystals caused by rapid freezing rates [61].

The adoption of a platform approach has created greater efficiencies in process development and manufacturing for mAbs. Evolution of the platform through experiences accumulated while dealing with challenges faced with atypical antibody processes is an equally essential part of creating a robust and widely applicable platform.

**Non-chromatographic downstream processing**

Chromatographic steps have formed the mainstay of modern-day biopharmaceutical purification processes because of their high selectivity and resolution. However, throughput for conventional preparative chromatography is inherently
limited as a result of the batch nature of the process and the limitations in fluid velocity for medium-sized particle beads (40–120 μm) that are used in these columns. With increasing production amounts per cell culture batch in commercial scale operations (up to 25,000 l working volumes), the achievement of >50 kg drug substance batches per run are close to realization. Significant debate has been sparked by these changes over whether conventional packed bed chromatography can continue to meet downstream process demands in the future. Persuasive arguments have been made for two-column step processes with high product loads on Protein A capture, and a single polishing step operated in the flow-through mode, which indicate that chromatographic steps with adequate cycling can continue to meet these demands into the future [7,8]. It has also been argued that very few mAb products actually require these large production scales because of limitations on market demand. Others have argued that innovative unit operations are worthwhile investigating as alternative means of dealing with ever-increasing bioreactor output [29,62,63]. Unit operations such as aqueous two-phase separation, selective precipitation with polyelectrolytes or polymers, membrane chromatography, and high-resolution UF and crystallization have been cited as possible replacements for chromatography.

In reality, the best non-chromatographic processing steps will likely resemble a combination of these two seemingly divergent scenarios. It is clear that the biopharmaceutical industry is slow to adopt novel processing technologies that require significant capital investments or modifications to existing facilities. Globally, biopharmaceutical manufacturers have made significant investments in current processing equipment, particularly centrifuges and large-scale tanks. By contrast, the industry has readily adopted innovations that fit in within the current facility paradigm, such as novel adsorbents and membrane chromatography.

Understanding some of the reasons behind cost pressures is key in predicting what changes could occur in the future. A pressure that has been cited as a reason for significant change is the coming advent of follow-on biologics [64]. However, follow-on biologics (also known as ‘biosimilars’) will be required to face regulatory requirements for comparability, which is a significantly bigger hurdle for biopharmaceuticals than for small-molecule pharmaceuticals. The burden in demonstrating comparability for these complex molecules often will extend beyond biochemical and biophysical comparability to include safety and efficacy clinical trials. This is likely to diminish the potential for biosimilars to gain a significant foothold in the marketplace. As a result, we feel it is unlikely that the cost pressures from biosimilars will serve as a major driver for innovation in biologic manufacturing processes. By contrast, biopharmaceutical companies already face significant internal pressures to minimize extensive investments in new facilities and optimize the usage of existing facilities. A large part of this pressure is due to the slowing rate of new drug approvals and increased competition among pharmaceutical and biotechnology companies in indications for which new therapeutics are developed. Effective utilization of existing manufacturing facilities then becomes the major driver for process innovation.

Innovative technologies that can operate within the current plant framework are likely to be the first movers in a changing processing landscape. Box 2 lists some of the non-chromatographic technologies that are being investigated to improve process throughput for mAb downstream processing.

**Production of antibody-related products**

In addition to intact mAbs, of which >20 have been approved for therapeutic use, there is growing interest in antibody-related proteins as the next wave of biotherapeutics. These include Fc fusion proteins, which consist of a fusion between the Fc region of mAbs and a protein partner that is often the ligand for a target protein [74]. Four Fc fusion proteins are currently approved for therapeutic use. Chemical conjugates with antibodies are advancing in clinical trials, particularly in oncology [75]. Three conjugated antibodies are commercially approved (Zevalin, Bexxar and Mylotarg). Although they possess several advantages, intact mAbs often have limited bioavailability because of their large size. Antibody fragments are being developed to circumvent this limitation, while retaining the binding characteristics of mAbs [76]. A new generation of products that utilize scaffolds, and are similar to mAbs, have also been developed. These include adnectins that are based on the fibronectin III protein and can be engineered to bind to a wide range of targets in similar fashion to mAbs [77,78]. In addition to improved bioavailability to tissues, these engineered proteins can potentially access clefts or grooves within target antigens that bigger mAbs may not.

The mAb process platform can serve the needs for some of these types of products, including Fc fusion proteins, bispecific antibodies or conjugated antibodies. Antibody fragments are smaller units that are not glycosylated, which allows high productivity that is inherent in bacterial expression systems [79]. Many of these types of products are expressed as intracellular inclusion bodies that require refolding to recover the intact product. Refolding of the inclusion bodies is carried out using chaotropes, such as urea, at low protein concentrations to reduce product aggregation as the denaturant concentration is decreased. The polishing steps for these products need to achieve separation of the misfolded forms in addition to separated-out host cell proteins and aggregates. Dedicated viral clearance steps are not a regulatory requirement for microbially expressed proteins. Refolding can often be a low-yielding unit operation, and, as mammalian cell culture titers improve, there could be occasion to revisit the expression system of choice for antibody fragments. A defined process platform has yet to become widely established for antibody fragment production but will be a key future development as these products mature in the development pipeline.

**Challenges and future trends**

The upstream process platform for mAb production has evolved significantly, both on the cell line creation and production bioreactor process fronts, which leads to the
achievement of product titers that have exceeded all previous experience with cell culture. The downstream process platform for mAb production still includes several chromatographic steps. Improved understanding of process-scale purification of mAbs has arisen as a result of experience with an ever-increasing number of mAbs in development and large-scale manufacturing. Future developments will take place on two fronts: technology that will enable incremental advances in the current platforms, and developments that will cause more radical change in the technology employed for mAb production.

Although the current process platform might appear to be safely ensconced in practice, we feel that several drivers will lead to significant evolution over the next decade. With the high titers now achievable in cell culture, the need to construct new facilities with very large bioreactors will diminish, although current facilities will continue to be in use. Lower production volumes will increase the need for facility flexibility and faster turnaround that will lead to growth in the use of disposables in manufacturing. On the process front, the productivity driver will remain even with the use of smaller bioreactors. This will continue to drive innovation in the development of non-chromatographic downstream steps. However, it has to be noted that, while such technologies have been investigated for a very long time already, implementation into a biopharmaceutical process platform has not yet been demonstrated. We feel that the alternative technologies that will be the first to find application will be those that fit within the current biomanufacturing facility designs, rather than requiring major investment on the facilities front.

Finally, another key development will be the maturation of antibody fragments and novel scaffolds within the clinical development pipelines. We anticipate the birth of a new generation of platform processes that address specific manufacturing issues with these therapeutic modalities, using a combination of traditional and non-traditional methods.

References

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