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Review

Future of antibody purification☆

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Abstract

Antibody purification seems to be safely ensconced in a platform, now well-established by way of multiple commercialized antibody processes. However, natural evolution compels us to peer into the future. This is driven not only by a large, projected increase in the number of antibody therapies, but also by dramatic improvements in upstream productivity, and process economics. Although disruptive technologies have yet escaped downstream processes, evolution of the so-called platform is already evident in antibody processes in late-stage development. Here we perform a wide survey of technologies that are competing to be part of that platform, and provide our [inherently dangerous] assessment of those that have the most promise.

Keywords: Monoclonal; Antibody; Purification; Protein A; Alternatives to protein A; Large scale; Industrial; Protein precipitation; Depth filtration; Membrane filtration; Chromatography; Membrane chromatography; Viral clearance; Future; Centrifugation; Aqueous two-phase separation; Protein crystallization; Manufacturing facilities; PAT; Disposable technology

Contents

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1. Introduction

Despite there being a wide range of approaches that could be taken for the recovery and purification of monoclonal antibodies, most of the processes used in practice show a high degree of commonality, driven largely by the number of antibodies in development and the emphasis on speed to market. The advantages of using a template or platform approach include savings in time and effort, and harmonization of practices and information across different functions and sites within a biotech organization [1]. Given these advantages, why then is there still considerable interest in the industry for alternative recovery and purification processes? Antibody-based therapies will be a major source of new therapies for at least the next 10 years. The large (>200) *number* of antibody products in development certainly supports the case for a standardized approach, but the large (multi-hundred kilograms to tonne) *quantities* in which some of them will be required puts considerable economic pressure on both the current processes and the facilities required (see Fig. 1).

Predicting annual production outputs for speculative medicines, whose efficacy, dosage and indications are unknown, is at best directional; nevertheless, the quantities and growth rates are significant and have given rise to an extended discussion about the so-called "capacity crunch".

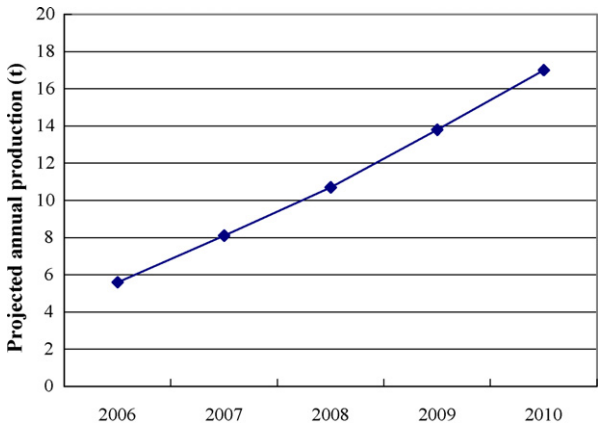

Fig. 1. Projected annual production of monoclonal antibodies. Numbers are in metric tonnes and are a composite from market data supplied by H. Levine and P. Latham.

The industry has responded in several ways, beyond simply building or contracting for more capacity. Molecular engineering approaches can increase and improve the potency and half-life of candidate molecules in the body, meaning that dosages can be decreased [2,3]. Alternatives to cell culture such as transgenics and microbial expression can either decrease the requirements for stainless steel, or improve upstream productivity. Perhaps most significantly, established manufacturers have made considerable improvements in mammalian cell culture titers, which have increased from levels in the low milligrams to multi-gram concentrations per liter [4], which has the benefit of leveraging their existing investment in cell culture capacity, bringing the prospect of >100 kg batch sizes within foreseeable reach.

Improvements in titer bring economies of scale to upstream production, as productivity per unit volume is increased. Unfortunately, these economies of scale do not translate directly into similar benefits downstream, particularly for purification resins, as their usage is determined by the mass of product to be purified, rather than the volume. This impacts both cost of goods, and more significantly, facility costs, as the space and volumes required for downstream operations and the buffers, cleaning and sanitization solutions increase in proportion to the mass of product to be purified. Ultimately there are limits imposed by the technology, equipment and/or facilities that are available, and hence a proportional response by downstream processes within the existing framework of processes and platforms can run into difficulties.

As a result, manufacturers (and suppliers of downstream technology) are exploring multiple ways of streamlining product recovery and purification processes. Strategies include decreasing the number of steps, avoiding complex steps and reducing raw materials costs. In addition, alternative formats for recovery and purification unit operations from the nutraceutical and industrial enzyme industries are being reconsidered. These include expanded and simulated moving beds, membrane chromatography and non-chromatographic methods such as flocculation, precipitation, crystallization and aqueous two-phase systems. Although many of these approaches lack the platform benefits of harmonization and speed to clinic (and potentially the market), they are considered because they are less expensive and may offer economies of scale or benefits in capital avoidance, which make them worth considering for large-scale applications.

In this article, we shall review these efforts and offer our views as to which ones offer the greatest promise. In the long run, however, the decision to adopt any technology is complex, and is ultimately the consequence of highly individual sets of circumstances and timing. It should be noted that our focus is a review of technologies, with only a peripheral discussion on process economics. We recognize the close relationship between the two and its impact on the future of antibody purification, but we refer the readers to another article in the current issue for a detailed review on process economics.

Antibodies and antibody-based alternatives such as fusion proteins and antibody fragments can be produced in a range of hosts, however, the majority of monoclonal antibody recovery and purification processes originate with cell culture, with CHO cells having been preferred to date. Although many variations exist, the vast majority of antibody purification processes seem to follow a process flow outlined in block form in Fig. 2 [5–7].

Particulates are removed by a combination of centrifugation and filtration processes, followed by adsorption of the monoclonal to a resin (almost invariably a Protein A affinity column) followed by two further chromatography steps (to remove host cell protein, DNA, any leached Protein A and aggregates, and to provide an adequate level of overall viral clearance). The process will also typically include a viral inactivation step (low pH hold) and a viral filtration step. We shall use this process outline as the starting point against which to compare alternative technologies.

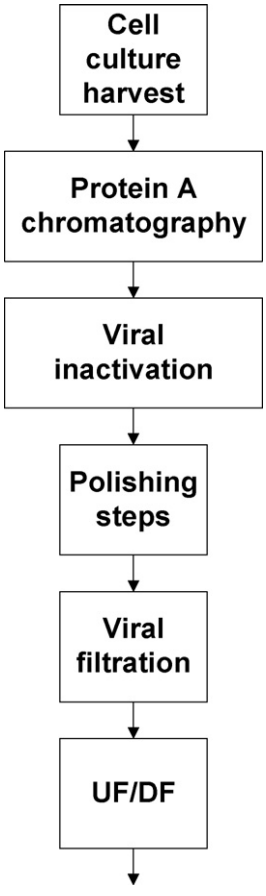

Fig. 2. Platform downstream process for Mabs.

2. Primary recovery

2.1. Centrifugation and microfiltration

Early efforts on primary recovery were typically centered on microfiltration [8] when centrifugation technology was less well developed. When compared with centrifugation alone, microfiltration results in better clarification [9], and has the advantage of requiring less capital. However, it suffers from the inability to be a "platform" unit operation, one that can be used without much optimization in a wide range of feed conditions [10]. With the advent of high cell densities and low shear centrifuges, a hybrid centrifugation–filtration system has become the industry standard for primary recovery [11]. Microfiltration can still be a valuable tool when low capital cost is important and/or when processing is limited to small scales.

Disk-stack centrifugation has emerged as the most widely used form of centrifugation [12–16]. With increasing cell mass coming from the fermentor, modeling the performance of the centrifuge has become increasingly important with a view to minimizing the downstream depth filter requirements, thereby realizing a significant savings [17]. Other efforts to reduce or eliminate the need for depth filtration downstream of the centrifuge include the use of flocculating agents in the cell culture fluid. These act to increase the amount of cellular debris being removed by the centrifuge, thereby improving the clarity of the harvested cell culture fluid, and requiring little or no depth filtration prior to the capture step [18]. Improvements in centrifugation technology, including the use of hermetic inlets to reduce cell shear, have been crucial for widespread adoption of this unit operation. One drawback of disk-stack centrifuges can be their cleanability, especially when compared to tubular bowl centrifuges, which have a much simpler fluid path. Newer models in the latter category [19] help continue the advances in centrifugation equipment technology. As cell densities increase, centrifugation is expected to continue to gain ground over microfiltration, with the filtration focus shifting to downstream polishing of the centrate effluent.

2.2. Depth filtration

Despite low shear centrifuge designs, the centrate effluent may still contain appreciable amounts of smaller sized contaminants (e.g. aggregates and colloids). Depth filtration [11] is most commonly used as a secondary clarification to remove this debris and to prevent plugging of downstream processes, such as chromatography. Charged depth filters are the preferred method for clarification and debris removal downstream of the primary harvest step, due to their ability to retain a large amount of contaminants using both size exclusion and adsorption [20].

Innovations in depth filtration have included newer filter material, pore structure, robust and convenient configurations of the filter. Various types of charged media, sometimes in combination with a tighter polishing filter in a sandwich configuration

have provided good options for designing a robust filtration step. The latter, multi-media, multi-layer design can minimize the number of filtration unit operations as well as hold-up. Filter robustness has also been enhanced by supporting the filter media from both sides, and when combined with shock-resistant durable construction that eliminates contaminant bypass, the filter is capable of reverse flow for backflushing. Some of these filter media can also be steam sterilized multiple times. Another important advance has been the introduction of the encapsulated module technology. This technology offers many advantages over the long-standing lenticular stack configuration, including no contact of process fluids by operators, no housing with product contact (which decreases cleaning and cleaning validation requirements), decreased risk of media damage, and reduced liquid hold-up, which greatly facilitate much-needed high throughput processing requirement for Mabs. All of these advances positions depth filtration not only as an effective post-centrifugation polishing step, but also as a primary clarification step (i.e. without centrifugation) in some cases, e.g. at small scale and/or in cases when the cell density is low.

Additionally, a unit operation used in microbial recovery operations, flocculation, has been used to reduce or eliminate the need for depth filtration downstream of the centrifuge [18]. These act to increase the amount of cellular debris being removed by the centrifuge, thereby improving the clarity of the harvested cell culture fluid, and requiring little or no depth filtration prior to the capture step.

2.3. Expanded beds

Expanded beds offered a great deal of initial promise, but that promise has been somewhat unfulfilled, at least in the realm of antibody purification. The main benefit of expanded beds would be to reduce the number of steps required for product recovery by allowing direct capture of product from the cell suspension, but the size and density of the particles used have resulted in a fairly narrow range of flow rates for acceptable values of bed expansion [21]. The technique has also suffered from problems with the fouling of the adsorbent due to binding of cells and cell debris [21], a problem which is not improving as cell mass and titers increase. A range of column designs has been evaluated in the search for a suitable design. Columns have to be absolutely vertical during operation and the flow rate may need to be adjusted to compensate for differences in the properties of the feedstream [22]. Recently, beads with significantly higher density (permitting much higher flow rates) have become available and are finding applications in the dairy industry for the recovery of lactoferrin and IgG from cheese whey [23]. Other variations on the use of adsorbents include the use of resins with a neutral coat of agarose [24] to afford a physical shield protecting the active groups, or for the formation of supermacroporous matrices, which permit the passage of whole cells [25]. Such matrices have been produced as cryogels and have been used for the isolation of secreted antibody fragments from bacteria, but because of their very high internal volumes, surface area is limited and capacities are inevitably low. Despite all these improvements, the major antibody producers have, as yet, to embrace expanded beds. The strategy appears to be one of keeping the individual unit operations of clarification and capture decoupled, while refining each one to make them highly efficient in meeting the individual step objectives.

3. Protein A chromatography

In the past 15 years, Protein A has been adopted as the capture step of choice by most antibody manufacturers despite initial misgivings about its economic value, reusability, and concerns over the clearance of leached ligand. It has been optimized for throughput, cleaning and re-use and has become the gold standard for antibody manufacturing. Originally the Protein A was isolated from *S. aureus*, and purified over a human IgG column. Since then recombinant forms of the protein have become available and the purification is no longer performed using any human or animal-derived products. Despite this evolution, the expected rise in cell culture titers, combined with the needs for larger masses of antibodies, signal a need for further improvements in the current range of Protein A resins available to the industry, far beyond the incremental improvements over the past 5 years.

When reviewing the currently available options for Protein A chromatography, one is immediately struck by the limitation of choices available. A series by Jungbauer and co-workers [1,26,27] takes an extensive look at all of the Protein A sorbents currently available. The study includes mass transfer characteristics and selectivity for 15 Protein A adsorbents. What is immediately apparent is that the field of Protein A resins divides quickly into those suitable for preparative scale and the rest. Using dynamic binding capacity (DBC) as a metric, the top three resins with the highest DBC were MabSelect Xtra™, MabSelect SuRe™ and ProSep®-vA Ultra. All had capacities greater than 30 mg/mL at linear flow velocities of 200 cm/h (residence time of 3 min). While this throughput is an improvement over the <20 mg/mL capacities in the original Sepharose™ FF Protein A and the ProSep®A, it is still not as high as it needs to be. With expression levels targeted to hit 8–10 g/L in the next 5 years, the processing times would be extremely long with these capacities. There is a ready market for the company that can produce a Protein A resin with a DBC of >50 g/L or an even lower residence time. How can this be achieved? Some of the options that are currently being investigated include higher ligand densities on their resins, ligand orientation/accessibility, particle size, pore size and distribution, and more stable support matrices with increased mass transfer [28]. The theoretical maximum DBC of a Protein A agarose resin has been suggested to be in the region of 70 g/L based on theoretical calculations [28]. Although it is unlikely that this number could be achieved in practice, since the calculation makes several simplifying assumptions, such as that of pores of uniform diameter, there does appears to be some room for improvement from the current status of ∼30 g/L.

Within the limitations of existing binding capacity, a strategy to achieve a higher throughput was proposed by Cramer and co-workers [29] using a dual flow rate approach. In the early phase of loading, the flow rate is kept high, since all of the binding sites are readily available for IgG binding. As these

binding sites become less accessible, the flow rate can be slowed to allow the IgG more time to diffuse into all the pores and bind to the less accessible sites. They developed a mass transfer model to optimize the loading flow rates to the column, thereby optimizing throughput.

In addition to increased capacity and throughput, the other major area of concern for Protein A resins is their stability to cleaning agents. A new Protein A resin is now available, where the asparagines residues in Protein A have been engineered out, thus reducing deamidation under alkaline conditions, while maintaining the ability to bind IgG [30]. This and the development of more efficient protocols for regeneration of resins, have increased the resins stability to base sanitizing solutions. Cleaning regimes consist of alternating cycles of low concentrations of NaOH and salt, resulting in the resin maintaining good yield out to 300 cycles [31], a significant improvement over the earlier Protein A Sepharose FF [32] where the yield of IgG dropped to 50% after 300 cycles. This increased lifetime has a direct impact on the process economics of the manufacturing process. However, since the evaluation of this new resin is still in early stages (e.g. the low DBC conditions utilized in the cited study, the potential need for bacteriocidal studies), further work is required to determine if these advantages translate to a real industrial setting.

4. Alternatives to Protein A capture

Given the wide range of choices for Protein A as a general purpose ligand for purification, what drives the search for alternatives? There are several reasons for this, one of which is that the growth in antibody applications makes for a very attractive market opportunity (>$100 million, growing in excess of 30% for the capture step) for suppliers of purification and recovery technology. Add to this the significant expense of Protein A resins and it is easy to see that even if the current suppliers are well established, they are vulnerable to entry from more competitively priced alternatives.

For manufacturers, the most pressing argument for replacing Protein A is based on process economics (both cost of goods and capacity). Titers have increased steadily over the last decade, from levels in the low milligrams to multi-gram concentrations per liter [4], thus shifting the cost from cell culture to downstream operations. Among the downstream operations, Protein A forms a major cost center, and although Protein A resin lifetimes can be extended to greater than 300 cycles [33,34], with well-defined cleaning cycles, the expense and disposal of these agents makes an alternative further desirable.

Other purported benefits of eliminating Protein A have been based on arguments for safety. Concerns over the safety of Protein A have been based on leakage of Protein A itself, and on the possibility (however remote) of contamination from process impurities stemming from the Protein A manufacturing process, which has historically utilized plasma-derived IgG for purification. These latter concerns that have been addressed by suppliers, who have replaced IgG capture of Protein A with conventional (IEX, HIC) purification steps, and have established that the products produced are equivalent [35–37].

Protein A does play a significant role in immunomodulation [38]. It is interesting to note that immobilized Protein A has been used in approved devices (Prosorba® column, marketed by Fresenius Hemocare Inc., Redmond, WA) for treating a variety of inflammatory diseases, in these applications plasma exposed to immobilized Protein A is returned directly to the body. Quantities as high as 200 μg can be released into the body by the activity of plasma proteases, and may in fact elicit a beneficial response in those patients [38]. Since antibody processes include several steps to ensure the removal of leached Protein A, we would suggest that concerns over leached Protein A should be kept in perspective and not exaggerated.

Finally, the development in antibody engineering capabilities have resulted in significant diversity in antibody-based therapeutics [39,40], allowing for fusion molecules, immunoadhesins, Fab and F(ab)2 structures which may or may not contain Fc portions, so that Protein A no longer provides a universal or platform solution (although this places unreasonable expectations on a platform approach). Furthermore, Protein A shows some variability in binding to antibodies, which appears to be due to interactions with the variable region [41,42], some of which has been explained by the presence of specific sequences in the heavy chain [43].

4.1. Alternative ligands

Extensive effort has been spent on examining alternative ligands of varying selectivity and complexity. Generally, the simpler the ligand, the more stable it is to harsh chemical procedures for cleaning, but with simplicity comes a lower degree of selectivity.

4.1.1. Bioaffinity ligands

There are a number of naturally occurring immunoglobulin-binding proteins that have been described (Table 1) [44,45].

Of these, the most significant are Protein G and Protein L. Protein G ligands bind the same region of IgG-Fc and offer minor benefits over Protein A, the chief of which is a broader sub-class and species specificity, which is relevant in research applications, but less so for commercial production of monoclonal antibodies where the sub-class and species are pre-defined. The drawbacks of Protein G are that the native molecule binds albumin, α2 macroglobulin and kinogen, along with non-specific IgG [46], although recombinant versions are available where the additional binding sites have been removed. Other limitations are that harsh elution conditions are required, and binding

Table 1
IgG-binding proteins from bacteria (from [44])

Protein	Source	Binding
Protein A	*Staphylococcus aureus*	Strong, Fc region
Protein G	Groups C and G streptococci	Strong, Fc region
Protein L	*Peptococcus magnus*	Binds to κ light chains
Protein P	*Clostridium perfringens*	Binds to κ light chains
Protein D	*Branhamella catarrhalis*	IgD. Binds small amounts of IgG
Protein P	Group A streptococci	IgA. Binds weakly to IgG

capacities in general are significantly less than those for Protein A. There are only isolated examples of the use of Protein G at commercial scale. Protein L is the only other bacterial ligand of significance, in part because of its affinity for the Fab portion of antibodies, specifically κ light chains [47]. This would make it appropriate for the purification of engineered antibody fragments. Hybrid combinations of Protein G with Protein A and Protein L have been proposed as general purpose ligands for both conventional chromatography and more novel approaches (membrane chromatography, temperature triggered precipitation, see below) [48–51].

Other protein ligands such as lectins raise more issues than they resolve, as they would result in the preferential selection of certain carbohydrate classes. The naturally occurring plasma carrier, FcRn, appears never to have been considered as a candidate.

Immunoaffinity, has, of course, the potential to resolve very specific separation problems, but the concept of purifying an antibody, with the necessary validation and production economy issues, to purify another antibody, would seem to be inherently prohibitive. However, there could be applications for the purification of antibodies produced by transgenic and/or transchromosomic methods, where separation of fully human antibodies from host species and/or chimeric molecules would be required. Protein A itself is capable of a degree of species specificity.

Recently, antibodies derived from single domain camelid antibodies have become available. These are basically a single variable heavy chain fragment known as VHH, to distinguish it from the traditional VH fragment [52]. These molecules are very stable, low molecular weight (12 kDa) sequences. Antibodies are raised in camels or llamas and the VHH genes are then cloned and expressed in yeast. The VHH fragments contain three CDRs, which allows for both high selectivity and customization for specific requirements (e.g. species selectivity). The resulting molecules show good stability in caustic (0.1N NaOH), both in free solution and when immobilized to agarose. Because of their small size they are able to penetrate deeply into molecules and recognize unique conformational epitopes, although this capability may be limited when immobilized on a chromatography resin [53–55]. Unlike Protein A however, VHH fragments are monomeric and do not have the capability to bind multiple IgG molecules, which may impose a limitation on capacity.

4.1.2. Synthetic ligands

Several approaches have been taken in the quest for a simple, or at least simpler, general purpose ligand as an alternative to Protein A. Ideal candidates would offer most, if not all, of the selectivity at lower cost, but with improvements in capacity and/or chemical stability. Strategies with a primary focus on selectivity prefer more complex structures, and ligand libraries can be built by a variety of techniques including combinatorial chemistry, phage display or on mimetic dye scaffolds. For a recent review, see [40]. Commercial suppliers seeking to offer the same broad usefulness of Protein A generally direct these ligands against the Fc region, but it is perfectly possible to target other regions (for antibody fragments), by mimicking the epitope [56] or by non-mimetic targeting of the paratope itself [57]. Although this does not appeal to a broad market it does have the benefit of distinguishing between active and inactive antibody, useful in standardized immunoassay preparation. Peptidic ligands can offer good selectivity and capacities but can be susceptible to hydrolysis by chemicals and proteolytic enzymes.

Chlorazine dyes have been used as general purpose scaffolds for ligand generation [40] and IgG-binding ligands are commercially available which offer good selectivity and high binding capacities as well as excellent chemical stability. These have been used to purify polyclonal IgG from ethanol precipitates from plasma [58]. Unfortunately, they bind additives such as phenol red and Pluronic® from cell culture fluids and as a result are not recommended for primary recovery. This does not render them ineffective, but does diminish their usefulness in the eyes of many users.

A number of relatively simple, low molecular weight compounds have also been evaluated. These include amines such as histidine and histamine [59,60], thiophilic compounds [61–63], mixed mode adsorbents and peptide and chemical libraries [64–74]. These ligands offer a more complex separation modality than classical ion exchange or hydrophobic interaction chromatography but generally lack the selectivity of Protein A and show some degree of non-specific adsorption. Furthermore, in at least one example there can be differences from one antibody to another, resulting in the need for operational variations making them less suitable as a platform technique [70]. The performance of some of these different ligand types in terms of selectivity and suitability for a platform approach is shown in the following section.

4.1.3. Screening ligands

The critical attributes essential for an alternative to Protein A are capacity and throughput, economy, selectivity and amenability to use as a platform technique. Fig. 3 compares the elution pH of two different antibodies and a fusion protein with a non-antibody marker (α-chymotrypsinogen A) on six different

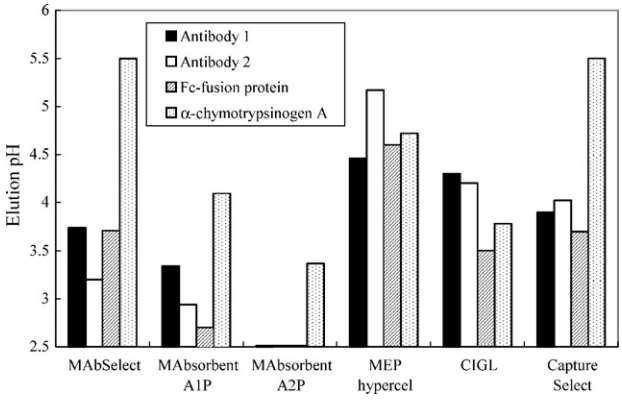

Fig. 3. Elution pH for different antibodies and a fusion protein on different types of antibody-binding ligands. α-Chymotrypsinogen was used as a marker, levels above pH 5.5 indicate lack of binding. *Conditions*: A pH gradient was run from pH 5.5 to 2.5 over 15 column volumes in 25 mM citrate buffer. Sample loading was approximately 5 mg in 1 mL buffer. Column i.d. 1.1 cm; bed height 10 cm; flow rate 100 cm/h. Resins are MabSelect™ (GE Healthcare), Mabsorbent® A1P and A2P (Prometic Biosciences), MEP Hypercel® (Biosepra), CIGL agarose (Millipore, prototype), CaptureSelect®-agarose (BAC).

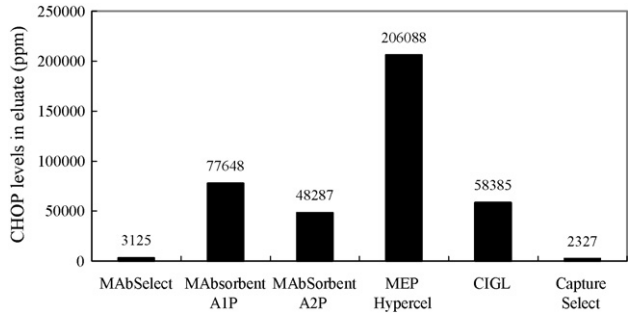

Fig. 4. Host cell protein levels in the eluate of antibody feeds passed over different ligand types. Host cell proteins were assayed using an in-house assay. Other conditions were the same as in Fig. 1.

antibody-binding ligands, respectively, Protein A (MabSelect®, GE Healthcare), synthetic ligands based on mimetic dyes (Mabsorbent® A1P and A2P, Prometic Biosciences), multimodal ligands (MEP Hypercel®, Pall), a prototype ligand (Millipore) and a single domain camelid antibody to IgG (CaptureSelect®, BAC) [40]. The elution pH varied by as much as one pH unit for the different samples, with the higher pH elution conditions generally considered less harsh. The non-proteinaceous ligands showed a tendency to bind the marker.

The eluates were also assayed for host cell protein as a further measure of selectivity, these levels are shown in Fig. 4.

The only ligand to offer similar selectivity to Protein A was the camelid antibody, presumably due to its greater complexity. It is scarcely surprising that increased complexity should offer greater selectivity, rules of thumb (the rule of five) have been described for active drug substances [75] and a similar pattern could be expected to apply for a ligand used to interact with a protein receptor.

A further highly desirable feature, not evaluated here, is the ability to contribute to viral clearance [32]. Choosing a ligand substitute for Protein A that did not demonstrate a similar level of viral clearance would place a greater burden on the downstream steps and may result in the need to include an additional unit operation to reach the required levels of clearance.

4.2. Tags

Rather than attempt to find a highly specific ligand to the antibody, it is possible instead to modify the molecule to add the desirable characteristics and use a highly selective affinity tag instead. This approach has been used for the preparation of recombinant proteins tagged with biotin [76], histidine [77,78] and glutathione *S* transferase [79]. These strategies require that the tag be cleaved from the purified sample after elution by a site-specific protease, which has limited their usefulness due to concerns over non-specific cleavage and the need to remove the proteases. A slightly different approach has been used to purify polyclonal antibodies using biotinylated antigens bound to a column of immobilized avidin [80], which gives flexibility for a research method, but for manufacture one may as well immobilize the antigen directly. Novel systems have been developed which simplify the cleavage by using tags derived from a protein splicing element (intein) [81,82] which have been applied to antibodies [83]. In general these strategies are more useful for research applications and are over-elaborate for manufacturing use.

4.3. Ion exchange and other capture steps

Affinity steps have held the limelight in antibody purification for all the reasons stated earlier, but it is possible to purify monoclonals to the desired level of purity using various combinations of non-affinity purification techniques such as ion exchange, HIC, hydroxyapatite and so on, and by varying selectivity from step to step [44,84]. The sequence of steps is determined by practical considerations such as sample concentration, pH and conductivity [85] but changes in the sequence can impact performance [86].

Ion exchange is the preferred chromatographic technique for the commercial recovery and purification of IgG from plasma. Affinity techniques have mainly been confined to the recovery of coagulation factors from select fractions, perhaps because the ever-present risk of viral contamination has led to manufacturers relying on the use of strong chemical agents for cleaning and sanitization. Ion exchange is certainly amenable to large-scale purification of monoclonal antibodies and is used as the primary capture step in at least one commercial process (Humira®) [87]. Capacities are higher, and cleaning and sanitization steps are simpler, the chief trade-off is a greater burden on the subsequent steps for purification and clearance of host cell protein and potential virus contamination. In a comparison between a Protein A process and a three step non-affinity processes, similar levels of host cell protein clearance could be achieved by both approaches, however the sequence of steps was seen to have a significant impact on host cell protein clearance and yield. Generally yields were best for the Protein A processes [86], however capacities are certainly higher with a non-affinity process. It has recently been argued that a 1.8 m diameter column would be capable of processing batches in excess of 100 kg assuming a capacity in excess of 100 g/L [88], although careful consideration would have to be given to viscosity and solubility effects at such high protein concentrations.

Recent developments in ion exchange focus on improving rigidity, and developing porosities and other characteristics (such as charge density) which have been optimized for the steps used in antibody purification, whether used in bind and elute or flow-through mode [89]. Mixed mode ion exchangers are also being promoted as offering additional selectivity compared to standard modes. However, they lack the general purpose usefulness of Protein A as a platform technique for first in human development, but they could well have a role in a commercial process for an antibody required in large quantities based on their significantly lower costs and higher capacity. Membrane adsorbers are being considered as an alternative for flow-through columns [90,91] because of their excellent kinetic properties; this is discussed in greater detail below [65]. As these steps are downstream from the initial capture step they add convenience but have less of an impact on process economics.

In summary, upon considering all the alternatives to Protein A chromatography, it is clear that there are tradeoffs in costs, selec-

tivity, and capacity. Chemical ligands may offer lower direct (resin) and indirect (cleaning) costs, and excellent capacities, but lack the selectivity of Protein A. Protein A alternatives, of which VHH offers the greatest promise, may deliver the same selectivity as well as cost benefits, but may be limited in capacity. Conventional chromatography (e.g. CEX) approaches will address cost and capacity best of all, but may require the greatest amount of optimization.

Because Protein A is firmly embedded as the resin of choice in platform approaches, and is perfectly adequate for delivering materials at the scales used for clinical trials, we expect that for at least the next 5 years, it will hold its own as the starting point for full-scale manufacturing processes, and productivity of individual chromatography steps can be optimized as discussed in the next section. Beyond the 5 years horizon, other techniques or formats may become more attractive to handle the mass of protein from cell culture. The alternatives are discussed in the subsequent sections.

5. Maximizing productivity and column utilization

The size of the initial capture column will depend on the size of the fermentation vessel and the titer, and a certain over capacity (15–25%) to account for variability in titers. Because of the expense of Protein A resins, the step is designed such that the column is cycled multiple times (typically 4–6) to harvest all the material in a working shift [6,92–94]. This makes it important to use resins with good productivity as discussed previously.

Within the current paradigm of fixed-bed column chromatography, the following conventional approaches can be taken in large-scale applications with higher titers. Scale-up in bioprocess chromatography has traditionally been achieved by increasing column diameter at constant bed height [95], in order to avoid exposing resins susceptible to compression to excessive pressures. Successful scale up requires that performance is predictable, and is maintained from one scale to the next. It is clearly harder to ensure even flow distribution across the entire surface of a large diameter column than if the volume is increased by increasing length, and various designs have been proposed to solve this. One approach has been to use multiple entry and collection ports, but whereas this offers good performance, the design aspects are impractical. Other designs use a single, central port (often with a branching flow distribution system), which offers satisfactory performance up to 1.2–1.4 m diameter, but performance can be resin dependent.

In addition to being able to provide predictable performance from one scale to the next, columns are considered pressure vessels and need to be designed to specific safety standards based on volume and pressure ratings. Modern resins may typically be run at operating pressures up to 3 kPa which in large (800 mm diameter and above) columns translate to pressures of several tonnes being exerted on end plates. As a result end plates have to be either very thick or externally reinforced, to avoid distortion.

Lastly, columns must allow for cleaning and sanitization. Construction materials need to be compatible with proteins, buffers and the cleaning procedures commonly used in sanitary processing, which can include both high and low pH, denaturing solutions and in some cases, steaming-in-place for connective piping and valves. All parts of the column should be swept continuously with eluents as “dead spots” can be places where microbial growth can occur. Larger scale columns generally include valving features which allow for introducing and packing resins, and for emptying them after use, since these very heavy end pieces make other forms of packing impractical and dangerous. These valves may introduce areas of very low flow into the column and should be minimized where practical.

Another, perhaps simpler way around the configuration problem is radial flow, and at least one supplier has developed columns designed for this purpose. In this design the packed bed functions like a hollow cylinder, with flow moving from the outside to the center. The design allows for greater support to the resin and subsequently higher flow rates. In general, however, axial flow columns have been preferred by the biotechnology industry, presumably because of concerns over flow distribution, but there does not appear to be any engineering reason for the lack of adoption of this technology.

More recent generations of chromatography resins have greater resistance to pressure, and as a result engineers have somewhat more flexibility in scale-up. Rather than increasing diameter at constant bed height and flow rate, engineers can opt to hold the ratio between bed height and flow rate constant, so that scale-up occurs at constant residence time in the column. This has the benefit of keeping the process footprint smaller.

Finally, it is worth mentioning a previously mentioned approach to maximize throughput. Since binding capacity declines at higher flow rates, one could load the column at a higher flow rate initially and then reduce it as maximum capacity is reached [29,96].

In designing a recovery and purification train for a very large-scale application, a further approach engineers should consider to maximize productivity and resin utilization is to using two (or more) columns for the initial capture step. Recognizing that this implies greater initial capital investment, this allows for a more effective use of processing time as once one column is close to saturation (typically the most time consuming stage), the stream can be switched to a second column to continue loading while the first column goes through wash and elution stages [97–99]. The logical extension of this would be simulated moving bed (SMB) chromatography. SMB is used in the petrochemical industry and is growing in popularity for separations of enantiomers and binary mixtures [100–105]. The benefits realized are a decrease in the amount of buffer solutions required, as well as a lower resin bed volume. SMB systems can fall into two basic designs—static or carousel type systems; in either case, the movement of the bed in the direction opposite to eluent flow is simulated by sophisticated valve systems. These systems can be very large, up to 100,000 L in volume, and have been used in applications for the purification of antibiotics and the recovery of lysine. The most important use for proteins is in the recovery of lactoferrin from milk whey by ion exchange. The potential of SMB for antibody purification has been recognized and demonstrated at small scale [106]. Case studies for the recovery of albumin and for antibodies have been presented several times [54,107–109]. In general savings in the region of 20%

Table 2
A comparison of fixed and simulated moving bed chromatography

SMB	Fixed bed
Multiple small identical columns	One large column
Single startup/shutdown	Multiple start up/shutdown
Smaller total resin volume	Larger total resin volume
Complex equipment and automation requirements	Simpler equipment and automation
Multiple monitoring points (UV/pH/C)	One monitoring point
Control programmed in space	Programmed in time
Less waste	More waste

for resin expenses and 50–55% for buffer costs are reported. The key features of SMB operation are summarized in Table 2.

SMB has yet to be used for biopharmaceutical applications. Bioreactors are typically operated in batch or fed-batch mode, whereas SMB is most advantageous when used over an extended period. It would, however, be beneficial if a perfusion system was being considered for production. Even here, the savings can be incremental compared to the complexity of the system and the difficulties that could be expected in validation and in maintaining aseptic operation. Furthermore, there is no benefit in using SMB in applications which focus on removing contaminants by binding them and allowing product to flow through the column. However, there could be benefits for bind and elute separations where high loadings and frequent operation are required. A situation where SMB has clearer benefits is in size exclusion chromatography (SEC) for removal of aggregates or other high molecular weight materials. Although SEC is not a preferred industrial unit operation, it may be needed in some specialized applications. In this case, switching to an SMB mode has a relatively greater impact on productivity than with other chromatographic modes because of the low volumetric loadings used in the traditional mode (other than desalting applications). Furthermore, for fractionation applications, long columns are required; and resins which have appropriate porosity for biomolecules can only tolerate low flow rates. Switching to the SMB mode in SEC makes much more effective use of the total column volume available and offers considerable improvements in productivity.

6. Alternative formats—anything but chromatography?

Chromatography is frequently portrayed as a necessary evil in downstream processing because of the costs, batch operation, throughput and complexity of scale up, and its use is limited for non-therapeutic proteins such as industrial enzymes and food additives. Indeed, ethanol precipitation is still widely used for plasma proteins. Where chromatography is used it may be heavily engineered so as to permit semi-continuous processing such as with simulated moving beds as described above. Non-chromatographic separation techniques for antibody purification are a further step away from non-Protein A processes, but it is a worthwhile speculation considering the high cost of antibody manufacturing and need for high throughput unit operations. Alternative operations include precipitation, liquid–liquid extraction systems, crystallization, membrane chromatography and filtration [110]; some of the recent work has focused on making these techniques easier to use and more selective.

6.1. Affinity alternatives

The unique affinity interaction between IgG and Protein A has been used in a variety of alternative recovery operations. For example, affinity ultrafiltration combines the selectivity of affinity approaches with the ability to run filtration in a continuous process [111]. Polymers sensitive to pH or temperature simplify phase separations, and selectivity can be enhanced using ligands and tags [50,112–116]. Any combination of polymer and ligand can be considered. Affinity precipitation has been suggested to be the technique with the greatest large-scale potential and carriers as diverse as Eudragit, poly-*N*-isopolyacrylamide and elastin-like polypeptide have been proposed [113,114,117]. Affinity sinking has been proposed for accomplishing affinity purification with non-immobilized modified ligand [118,119].

A novel system has been developed using Protein A expressed transgenically on oleosin molecules on the surface of oilbodies produced by safflower [120]. Oilbodies can be mixed with cell culture supernatant and separated by centrifugation and the antibody recovered by standard elution methods. Purity is comparable with Protein A chromatography, but capacities are lower [55,121], however this is offset by the low cost of transgenically produced Protein A. A comparison of the antibody peaks from both methods is shown in Fig. 5.

6.2. Aqueous two-phase systems (ATPS)

The unit operation of ATPS has been studied extensively, especially in the recovery of industrial enzymes [122]. However, despite early interest [123] it has not had successful adoption in bioprocesses [124,125]. Interest in its use for Mab recovery has been no exception, and been limited to few research studies [126]. However, this unit operation has certain advantages of scalability, the ability for continuous operation, and high capacity, some of which align quite well with the process needs of low cost and high throughput for antibodies. There have been efforts integrating ATPS with other unit operations, primarily affinity-separation, and utilizing temperature or solute-sensitive polymers (both discussed previously). For example, Kamihira et al. [127] have added the polymer carrier Eudragit, whose pH sensitivity to phase stability can be utilized to facilitate recovery of the extracted protein. Nevertheless, difficulties remain regarding use of this as a platform step due to issues related to complex interactions of the multiple components involved (as the poor understanding thereof) as well as potential sensitivities to feed stream variability.

6.3. Magnetic separations

A further approach with potential is high gradient magnetic separation technology. This has been adapted from chemical and mineral processing industries for protein separations [128–130]. Small (sub-micron) superparamagnetic particles are formed from magnetite crystals and are coated with polyglutarldehyde,

Sample	Host Cell Protein (ppm)
Protein A Chromatography	Approx 750
Oilbodies	4684

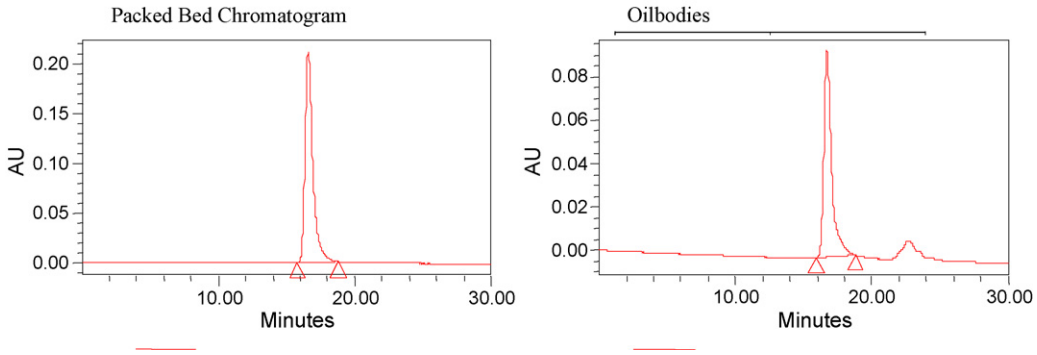

Fig. 5. SEC of eluate from packed bed chromatography and oilbodies.

which can then be derivatized to provide different selectivities. These particles have been used for the purification of enzymes and inclusion bodies. A novel magnetic adsorbent has been used for IgG purification in a magnetically stabilized fluidized bed [131]. In another case of process integration, IgG-immobilized magnetic particles have been used to facilitate ATPS in Protein A purification [132]. Furthermore, certain bacteria also have the ability to produce magnetic particles. These can be engineered to display Protein A on their surface, and could be an economically viable alternative to magnetite if the capacities for antibodies are sufficiently high [133].

6.4. Crystallization

Another approach used in low cost industrial enzymes is crystallization, although its use as a unit operation for therapeutic protein purification has been limited [134–136], primarily due to the difficulty of crystallization from impure process streams and scalability. Crystallization in protein formulation can be more readily envisioned [137], and though still limited in its application, has provided the impetus for development of large-scale crystallization processes. For monoclonal antibodies, crystallization poses a greater challenge even at small scales, due to their large size, glycosylation, and a high degree of segmental flexibility. Nevertheless, this technology has had a new revival at the process scale [138] with the consideration of antibody crystals as a novel delivery vehicle [139–141].

6.5. Membrane adsorbers

Of the many non-chromatographic techniques, membrane adsorbers (also called membrane chromatography) are the closest in terms of implementation in antibody processes. Zhou and Tressel [91] have reviewed the recent literature on the use of membrane chromatography in industrial antibody purification processes. Flow-through anion exchange chromatography has been the most attractive step for contemplating this technology. This was described in a thorough study by Knudsen et al. [142], who evaluated membrane chromatography as an alternative to packed bed chromatography in both flow-through anion exchange and bind-elute cation exchange chromatography, and concluded the attractiveness of membrane technology in the former case. Zhou and Tressel [143] provide additional analysis with a similar conclusion. We envision greater implementation of this technique as further improvements on modules are made. These kind of pre-packed resin configurations greatly facilitate realization of 100 kg batches now within the realm of the near future.

6.6. Charge interactions in traditional membrane separations

Further to membrane chromatography, there is now a growing body of literature where it has been shown that electrostatic interactions [144–148] can be used to enhance traditional size-based membrane-based processes (sometimes termed HPTFF, high performance tangential-flow filtration). This has even led to the proposal of a non-affinity process [149]. A variation of this approach with charged membranes [150,151] may be closer to industrial-scale implementation. Charged membranes could be used to enhance both protein–protein and protein–small solute separations. It is the latter that is more easily imagined in the future industrial processes, once these kinds of membranes are readily available from membrane manufacturers.

7. Viral clearance

A major requirement of antibody purification is the need for adequate clearance of endogenous and adventitious viruses. This

is governed by the guidance ICH Q5A [152] for viral safety of biotech products derived from human or animal cell origin. There is an expectation of multiple orthogonal steps (at least two) for virus inactivation/removal, with at least one step for clearance of non-enveloped viruses. Ultimately, the entire purification process must be capable of inactivating and/or removing substantially more virus that is expected to be present in a single dose of harvested cell culture fluid. In a typical antibody purification process, low pH treatment and viral filtration are specific steps designed for viral inactivation and removal, respectively, with the chromatographic steps providing additional orthogonal clearance.

7.1. Viral inactivation

Currently, low pH inactivation seems to be the preferred and most robust method for achieving retrovirus virus inactivation. Most processes elute with a low pH buffer from the Protein A step, so it is an obvious extension of that step to hold the product pool for a certain period of time to achieve the desired time of inactivation. This method has been used extensively and has been applied in several FDA submissions using a modular approach [153]. Hence, as long as a Protein A capture step is used, this step rides along as a bonus.

Although solvent/detergent treatment used extensively in the plasma processing industry for virus inactivation, it has not been adopted by the monoclonal antibody industry. Other non-invasive inactivation methodologies such as gamma irradiation [154–156] or high temperature short time (HTST) [157] may gain more relevance if and when non-Protein A or non-chromatographic processes become realistic.

7.2. Virus retentive filters

There is an increasing expectation of small (e.g. parvo) virus removal by filtration, a step that challenges both throughput and the ability to handle more concentrated solutions. Therefore, the next generation of virus retentive nano-filters is need to have protein productivities in excess of 2500 g/m^2/h with an ability to process 10 kg of purified MAb in a reasonable timeframe (e.g. a single shift). In order to be able to process MAbs eluting off high capacity chromatography media, these filters should have the ability to process proteins at concentrations of 20 g/L or higher. In addition, these filters will be expected to provide >4log$_{10}$ clearance of 18–24 nm parvoviruses and maintain >6log$_{10}$ clearance of 80–110 nm retroviruses. Furthermore, even though this is an area that is yet uncharted, stability to cleaning agents may become a necessary attribute for these virus retentive filters in order to be able to achieve short turnaround times, and reduce the cost of these high value filters.

Currently, with most parvovirus retentive filters, the filtration performance obtained with the virus is different from the filtration performance of the protein alone. This difference, to a large extent, is attributed to the quality of the preparation of virus. The performance difference may cause one to overestimate the virus retentive ability of the filter and/or may result in oversizing of the filter. Approaches that can be taken to minimize the difference include the use of purified virus preparations, the use alternative virus spiking methodologies such as the "run-spike" method [158] or virus surrogates for size-based filter retention such as bacteriophage ΦX-174 [159]. ΦX-174 has several advantages over small mammalian viruses in that it can be grown to very high titers and purified easily, it can be quantified very easily, it is easy to handle and it has filtration properties that are similar to mammalian viruses.

8. Antibodies from alternate feedstocks

This review on the future of antibody purification would be incomplete without some comments on purification of antibodies from alternate feedstocks. With increasing titers in mammalian cell culture technology, and advances in corresponding downstream operations, the impetus has been low for alternate hosts. However, there have been advances with other hosts that they may also begin to offer valuable alternatives. There are a number of reviews available on the current state of this technology [160–170].

Nikolov and Woodard [171] recently reviewed the purification operations of proteins from animal and plant transgenic sources. The downstream processes of these proteins compared to those from mammalian cell culture differ primarily in the recovery operations. The large variety of the starting feedstocks, is reflected in the diversity of the corresponding recovery operations. Furthermore, methods that have been out of favor in conventional monoclonal antibody processes may experience a resurgence due to this different feedstock (examples include microfiltration [172] and precipitation [161]).

One challenge in the case of transgenic animal sources is for the downstream processes to demonstrate adequate removal of any contaminating viruses and prions, although there is significant data available from conventional purification processes. Nevertheless, plant-based sources have emerged as the alternative, especially for aglycosylated antibodies. There are a number of reviews of purification of plant-derived proteins [173–175]. Soil-based plant production systems face the disadvantage of requiring environmental containment; this has resulted in development of contained, aquatic production systems such as duckweed-based LEX System^{TM} [168]. Since the protein is secreted, the recovery of the protein from these systems are also simpler compared to the soil-based production systems.

An alternate host that may be amenable to quicker adoption for antibodies is the eukaryotic system, *P. pastoris* [170], simply due to the existence of processes for other proteins from these kinds of hosts. Recovery operations for *P. pastoris* and other yeast strains have been in large-scale industrial use for quite sometime [176].

Although it is currently unclear if any of these alternate systems will actually take any bite out of mammalian cell culture, downstream operations for these feedstocks will get increasing attention. In addition to the more promising hosts for therapeutic antibodies discussed above, another possibly quicker application of alternate hosts is for their use in immunoaffinity purification of other therapeutic proteins of interest [177], which would be

free from the post-translational modification limitations if used as a therapeutic itself.

9. Future of antibody purification facilities

9.1. Conventional facilities

Significant capital investment has been the hallmark of current or emerging antibody manufacturing facilities [178]. This leads to inertia for major overhaul in process technologies, however as discussed elsewhere in this manuscript, the universal objective is to maximize productivity of existing facilities, and is achieved by increases in cell culture titers and downstream throughput. Increased upstream productivity by higher titers does not require any additional capital. However, since downstream operations are typically scaled based on the amount of product, higher upstream productivity implies additional capital investment in downstream operations. This challenge of needing higher downstream productivity can be partially addressed without additional capital investment with process improvements already discussed, as well as with innovations in operations such as the use of in-line dilutions and avoiding product pooling between steps. When additional hardware is unavoidable, capital spending can be mitigated by enhanced use of disposable technologies especially at the clinical scale. This is discussed in more detail below.

Furthermore, increasing higher productivities implies that market requirements could be satisfied with fewer batches, resulting in the need to handle multiple products within a single manufacturing facility. This need for flexibility is an argument made elsewhere as well [179]. Another emerging area is the implementation of process analytical technology (PAT) approaches, which is also discussed in detailed below. This enhances the use of on-line monitoring and decision tools, which can be implemented within an existing infrastructure with some effort.

9.2. Disposable technology

Disposable technology is becoming an integral tool for bioprocesses, and is especially relevant for the production of clinical candidates. Although the industry has long used disposable buffer bags, sampling bags and connectors, at multiple scales, what has changed is the discussion about a completely disposable factory!

The main driver for the use of disposables is economics—reduction in capital expenditure, ease and speed of installation, validation and implementation, maintenance, elimination of CIP and SIP procedures. There are also advantages for a reduced risk of bioburden contamination during sampling or handling. Disposable technologies can significantly increase a plant's capacity with little capital expenditure. Currently disposables are being used in several areas—cell culture, purification, liquid bulk storage, bulk transport and sampling. For storage and transport of high value bulk, bag integrity and low gas permeability are key, especially for freeze/thaw situations. The latter can be difficult to control in plastic containers compared to stainless steel or Hastelloy®. Another area for further development in this area is disposable sensors and probes, especially for bioreactors.

In the future, a continued increase in disposable technology use especially for smaller (e.g. pilot) scale and clinical applications is anticipated. There appears to be a general agreement that use of disposable bioreactors and bags at scales greater than 2000 L may not be as cost effective or practical compared to traditional stainless steel equipment. Although stainless steel is the norm for commercial antibody manufacturing, there are significant opportunities for disposables in fully marketed process for niche products, produced at smaller scales.

10. Process analytical technology

An emerging area for all pharmaceutical manufacturing is PAT, which will inevitably have an impact on antibody manufacturing as well. The FDA defines PAT as "a system for designing, analyzing and controlling manufacturing through timely measurements (i.e. during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality" [180]. Its intent is to encourage manufacturers to innovate and implement the most recent scientific advances into manufacturing processes. Benefits arising from this approach are listed as a decrease in cycle times, less waste, improved automation and a decrease in human error, real-time product release and facilitating continuous processing. Other benefits could be a decreased regulatory burden and a shift away from traditional three-batch process validation. Critical to this approach is that the process be well understood. It is also accepted that additional knowledge may surface with extended manufacturing experience even after extensive process characterization. PAT approaches allow for improvements based on these gains in understanding to be incorporated into the process through continuous improvement, without the necessity for revalidation, in a risk-based quality assessment. Table 3 summarizes current and corresponding anticipated practices in the future due to the introduction of PAT.

Control of biological processes begins with control of the fermentation step, and the condition of the harvest material has an impact throughout the process, as the separation is designed to cope with a certain level of bioburden; in addition to pH, temperature and conductivity, the level of nutrients and/or accumulation of products, byproducts and cell mass can be followed directly or by chemometric approaches [181–183]. PAT approaches can be applied to both the process and to the equipment used in the process to indicate and predict continued fitness for use. Typical steps used in biotechnology with an appropriate control strategy are listed in Table 4.

Filtration steps can be controlled through the use of transmembrane pressure and/or surrogates, the packing of chromatography columns can be monitored using transition analysis as a predictor of column health, and peak collection can be managed by forward control [184], or for applications like gel filtration, where flow rates are slower and column design permits removal of materials between sections, by monitoring fractions using at line HPLC [185,186]. Conductivity can be used as an

Table 3
A comparison of current and future practices with the introduction of PAT

	Current	Future
Process	Static, recipe driven	Dynamic, science and knowledge based
Control strategy	Measure and reject	Real-time monitoring and response to deviations
Product/process characteristics	Empirically derived from performance of test batches	Scientifically designed to meet specific objectives
Testing	End product testing	Continuous quality assurance, real-time release
Validation	Validated process	Validated controls
Comparability	Multiple lots	Understanding of how process factors impact quality
CMC Review	Potential for multiple NDA review cycles	Single cycle NDA review
Improvement	Incremental, dependent on approval	Continuous improvement within quality system

indicator of when to start and stop peak collection in gradient elution. The key element is to be able to gather information from the process and elicit a timely response.

PAT is suitable for implementation on existing products (where there is a considerable amount of historical data) as well as in new processes. In the case of existing processes, it may be that many of the steps are already being operated according to PAT principles, however if the process is not defined in PAT terms (i.e. in process end points, as opposed to set recipes), it may be harder to implement improvements within the manufacturer's own quality system, decreasing the regulatory requirement to file submissions. The FDA recognizes that it may be necessary to add new monitors and analyzers to existing processes in order to develop improvements, and has stated that it will not inspect research data gathered for the purpose of evaluating a new tool [180].

Chief concerns over the implementation of PAT are based on a fear of the unknown—fears that extensive monitoring will bring issues to light that have to date remained unnoticed, and fears about the regulatory approach to take. The first concern is readily addressed as most manufacturers, on reflection, will realize that if a process parameter was drifting they would want to know about it and address it. The second concern should be allayed through constructive discussions with regulatory authorities and professional societies such as ASTM and ISPE. Ultimately the benefits from PAT are business based, allowing for superior production technology and lower costs which can ultimately reach the patient.

Table 4
Unit operations and possible control strategies

Operation	Control strategy
Raw materials	Acceptance controls, identity
Mixing, blending	Mixing end points
Fermentation	Fermenter conditions, feed (pH, temperature, gases, cell mass, titer) Process trajectory (NIR, CE, HPLC) Fermenter harvest (cell mass, titer)
Filtration	End points, integrity (TMP, feed/permeate)
Chromatography	Equilibration (pH, C) Gradient elution (C, pH) Peak cutting (in-line, feed forward) Repacking, replacement (transition analysis)
Conjugation, refolding reactions	Reagent concentrations, reaction end points
Lyophilization	End points (humidity, NIR)

11. Concluding remarks

Given the timelines to establish clinical efficacy and safety for biotherapeutics, the "standard" approach for recovery and purification is not likely to change much over the next 5 years. However, as titers increase, and new indications for launched products increase demand, the bottleneck will become downstream operations. The industry is unlikely to respond rapidly as there are several barriers to change; the chief of which are the challenge of "unknown" new technology from a technical, operational and regulatory perspective, and the considerable pre-existing investment in current technologies and the facilities to support them. As a result we will continue to see little other than incremental improvements in downstream processes for some time to come. For example, exploitation of charge interactions in membrane filtration or the use of membrane absorbers falls in this category. Nevertheless, where market demand merits it, manufacturers will realize their options are between continued build out or alternative approaches. What then are likely to be the preferred technologies?

A recent presentation by van Reis et al. [88] suggested similarly that the near- to mid-term is unlikely to see unit operations that are different than those used today. The possibility of a 100 kg batch in existing facilities was presented, by stretching process limits such as a 100 g/L chromatography loading. Even though such a limit may run into viscosity and aggregation issues when extended widely, it is exemplary of extreme possibilities with currently available technology. They further presented a non-chromatographic process consisting of cation and anion membrane chromatography, heat inactivation, virus filtration, low pH inactivation, followed by high performance tangential-flow filtration that met current specifications for yield, host cell protein and DNA removal, viral clearance, and aggregation levels. Although any talk of non-chromatographic processes in this industry is radical, it underscores the great improvements in antibody purification technology already made to date. In another recent presentation, Kelley [187] presented a conceptual 10 t/year Mab process. They showed that their current antibody platform consisting of two chromatography steps (Protein A and anion exchange) is quite capable of handling such a large-scale

production, and hence made the case for sustaining well-proven platforms in the future.

In our (bolder) view, an idealized process would begin with a single, highly selective, concentration step. If this step was insensitive to biomass, so much the better, but the true bottleneck in recovery processes is the first adsorptive column rather than clarification operations such as filtration and centrifugation. Methods such as crystallization or precipitation have the ability to concentrate to the highest degree, that of a solid. Crystallization is a classic criteria for purity, but may be difficult to implement with antibodies at large scale. Precipitation methods can vary in selectivity, either due to entrapment and difficulties in washing the precipitate, or due to co-precipitation of contaminants, but overall offers more options for truly high capacity operations. This is particularly true if contaminants have been engineered out of the host strain. The final objection to precipitation would be loss of yield if the product denatures during precipitation, or problems in removing the precipitating agent. Further in this idealized view, the orthogonal possibility of precipitation of impurities also fares just as well. Such initial precipitation of product or impurities could reduce the burden on expensive adsorption and viral filtration steps downstream.

Once the protein is resolubilized, subsequent steps would ideally be flow-through techniques scaled for the level of contaminants, rather than for the mass of product, as would be the case for bind and elute chromatography steps. Again these would best be run using adsorptive membranes, which are optimized for high volume throughput rather than their ability to adsorb large masses. In certain cases a bind and elute step may be unavoidable to remove a difficult contaminant. This should be arranged to be as late in the process as possible as it would simplify modeling the separation and adapting it to a high productivity technique to optimize the use of resins and buffers. In this case, a simpler three-column system may be preferred to SMB systems, which are more complex to design, validate and operate. Finally the process would need to include adequate measures for viral clearance and inactivation. This would most likely include a viral filtration step in addition to either low pH or UV inactivation, or possibly both (especially if this could decrease the requirements for filtration).

Such a process would provide high productivity, and would be relatively insensitive to further increases in scale, but it requires a certain leap of faith to imagine a completely chromatography free process. The industrial enzymes and nutriceutical industries have deemed chromatography as too expensive for years, preferring to remove their contaminants by eliminating them from hosts in the first place. Where chromatography has been used, it is been engineered to continuous high throughput processing (SMB) modes where possible.

Based on these considerations we believe that the majority of improvements over the next 3–5 years will be incremental, the technology will remain basically the same. This view is exemplified in presentations cited previously [88,187] by existing Mab manufacturers. However, as we see the scale of certain products increase, and the pressures to push ever more product through existing facilities also increasing, the motivation to change will build. Ultimately these decisions will be based on a combination of overall economics and individual circumstances. The possible long-term decline in the dependence on chromatography may seem to be more like “forward to the past” than “back to the future”, but in the end, as Charles Darwin reminds us, “it is not the strongest species that survive, nor the most intelligent, but the ones most responsive to change”.

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