Alternatives to Chromatographic Separations

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Alternatives to Chromatographic Separations

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Up to now, the productivity of mammalian cell culture has been perceived as limiting the productivity of the industrial manufacture of therapeutic monoclonal antibodies. Dramatic improvements in cell culture performance have changed this picture, and the throughput of antibody purification processes is gaining increasing attention. Although chromatographic separations currently are the centerpiece of antibody purification, mostly due to their high resolving power, it becomes more and more apparent that there may be limitations at the very large scale. This review will discuss a number of alternatives to chromatographic antibody purification, with a particular emphasis on the ability to increase throughput and overcome traditional drawbacks of column chromatography. Specifically, precipitation, membrane chromatography, high-resolution ultrafiltration, crystallization, and high-pressure refolding will be evaluated as potential large scale unit operations for industrial antibody production.

Introduction

Cell culture titers of proteins, especially monoclonal antibody (mAb) proteins, have increased dramatically over the past decade from tenths of a gram per liter to 10 grams per liter (Low et al., 2006). Cell culture volumes have also increased to where 25,000-L batches are a reality. Imagining the day of 100-kg batches is no longer difficult (Kelley, 2006). Advances in cell culture technology and the expansion of the monoclonal antibody market have shifted the burden to downstream processing in the race for increased manufacturing productivity and decreased cost of goods.

Chromatographic separations have been the workhorse in downstream processing of mAb therapeutics since the beginning of the industry. A minimum of two column chromatography steps is typically employed: first, a recombinant Protein A capture column, and second, an anion exchange flow-through column for impurity removal (Figure 1). In many cases, a third chromatographic step is added to provide additional clearance of impurities and contaminants. A simple calculation using Protein A affinity chromatography can illustrate the problem posed by using chromatographic separations at this scale. Assuming an optimistic loading capacity of 50 g/L, a 2,000-L column is required to capture 100 kg of mAb. If the entire batch is captured in a single load-elute cycle, then the required column diameter is 3.2 m at a 25 cm bed height. Typically, industrial scale columns today are about 2.0 m in diameter and are operated at 10−20 cm bed height. Alternatively, a single 100-kg batch can be purified in several cycles, thus reducing the required amount of stationary phase. While this reduces the initial financial investment, more cycles are required, and there are a finite number of useful cycles for Protein A affinity adsorbents. Furthermore, because chromatographic separations involve equilibration, wash, elution, regeneration, and sanitization steps, substantial liquid volumes are required. Large column volumes to process antibody batches of 100 kg (or more) also result in large volumes of solutions that have to be prepared, stored, and disposed of properly. Use of a Protein A affinity column of 2,000 L, as outlined above, may require buffer volumes of more than 50,000 L, depending on the number of column volumes selected for each process step. Finally, elution of the bound mAb from the Protein A resin is performed at low pH, where many antibodies are known to form aggregates. These are perceived as potentially immunogenic and must be cleared from the product, decreasing yield and increasing cost. It is not hard to imagine why alternatives to chromatographic separations are hot topics.

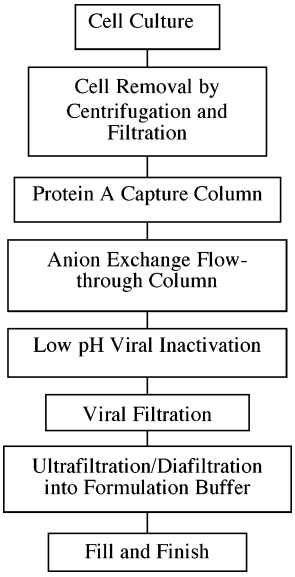

Figure 1. Minimal separation train for a mAb therapeutic.

Several promising alternatives to chromatographic separations will be discussed in this report. As presented by Van Reis et al. (2006b), the biotechnology industry has a large installed capacity in centrifugation, chromatographic, and membrane filtration equipment. This equipment will continue to be used

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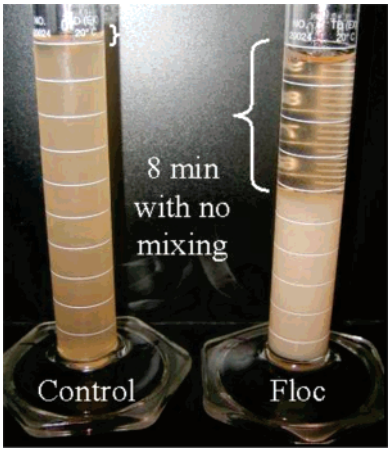

Figure 2. Flocculation of cellular debris by addition of nontoxic salts calcium chloride and potassium phosphate. (Copyright 2006 Wyeth Biopharma. Reproduced with permission.)

in the future. New developments in charged ultrafiltration membranes, membrane chromatography, precipitation prior to centrifugation and microfiltration, and pressure-induced refolding that use this installed equipment are advantageous for this reason. Protein crystallization also does not require substantial investments in new equipment and has been part of the biotechnology industry since the 1980s for recombinant human insulin. Its use for the manufacture of mAb therapeutics has yet to be realized. All of these alternative technologies have one goal in common: achieve a separation power equal to that of column chromatography, while reducing the cost of manufacture, the number of steps in the separation train, and increasing the scale of manufacture.

Precipitation Prior to Centrifugation/Microfiltration

Cellular debris is now more difficult to completely clear because of advances in cell culture technology leading to higher titers, cell densities, and decreased cell viability (Shpritzer et al., 2006). Conventional cell separation techniques such as centrifugation and microfiltration often leave the supernatant highly turbid because of residual particulate material, frequently of submicron size. Polishing of turbid supernatants by further dead-end filtration steps is expensive but necessary before purification by column chromatography steps to prevent plugging and fouling of the bed. Often, polymers such as polyethyleneimine are added to the solution to induce flocculation and enhance removal of cellular debris by centrifugation. Coffman et al. (2006) and Shpritzer et al. (2006) propose addition of nontoxic flocculation agents such as calcium chloride and potassium phosphate to avoid toxicity and removal issues caused by use of polymers. By adding 30 mM calcium chloride and then 20 mM potassium phosphate, a calcium phosphate precipitate forms rapidly that flocculates cellular debris (Figure 2). Within minutes a clear supernatant layer begins to form during gravity sedimentation. Centrifugation for 10 min at 340*g* yields a clear supernatant and about 95% recovery of mAb. Subsequent clarification by filtration required less membrane area. Flocculation lead to a reduction in process-related impurities such as host cell protein and host cell DNA, thus reducing the load challenge to the purification process. Although not an alternative to chromatographic separations by itself, precipitation steps do lessen the load on chromatographic steps and may eliminate the need for chromatographic steps located downstream that remove impurities such as host cell protein and mAb aggregates.

Membrane Chromatography

Chromatographic membranes are emerging as an attractive alternative to traditional column chromatography, especially for removal of large impurities in a flow-through mode (Zhou et al., 2007; Etzel and Riordan, 2006; Zhou and Tressel, 2006; Phillips et al., 2005; Zhou and Tressel, 2005; Knudsen et al., 2001). Anion exchange chromatography columns are used to remove traces of process-related impurities such as host cell protein, DNA, RNA, and endotoxin and also play an important role in the overall viral clearance strategy for antibody purification (Curtis et al., 2003; Norling et al., 2005; Zhou and Tressel, 2006). Many of these impurities are mega-Dalton in size and bind to the external surface only of chromatographic beads, resulting in low loading capacity. Conversely, for adsorptive membranes, loading capacity increases as the size of the impurity increases (Etzel and Riordan, 2006). In essence, the larger size results in more mass bound per unit of surface area for membranes. Problems packing large-scale columns, equipment and adsorbent cleaning, column lifetime limitations, validation, flow rate limitations, buffer usage, and floor space requirements can all be reduced using disposable membrane chromatography systems (Mora et al., 2006).

First generation membrane adsorbents suffered from problems related to both adsorptive properties and device issues. Fluid distribution in early devices was less than optimal, leading to a substantial performance loss during scale-up (Gebauer et al., 1995). Additionally, low loading capacity and membrane fouling was observed (Reichert et al., 2001), limiting the practical use of these devices in routine manufacturing. Next generation membrane chromatography products will likely solve these problems, thus expanding utilization in the biotechnology industry. As utilization for flow-through applications expands, other uses such as for the capture step are more likely to result (Boi et al., 2006).

Charged Ultrafiltration Membranes

Using both size and charge rather than simply size alone is the basis of charged ultrafiltration membrane separation. A positively or negatively charged moiety is covalently attached to the pore structure of the membrane to reject like-charged proteins and pass neutral proteins. The pH and conductivity of the feed solution are adjusted to control the charge on the proteins and attenuate charge shielding from reduction of the double layer. Proteins containing marginally different sizes that cannot be cleanly separated by uncharged ultrafiltration membranes can be separated using both size and charge. The promise is to advance the resolving power of ultrafiltration from separation of proteins differing in molecular mass by at least factor of 10, to separation of proteins having little or no difference in molecular mass.

In one of the earliest reports, Nakao et al. (1988) separated dextrans and model protein mixtures using polyethersulfone ultrafiltration membranes containing either a positive or a negative charge. Cellulose ultrafiltration membranes containing a positive charge were used to separate different mAb therapeutics from Chinese hamster ovary cell protein impurities by van Reis (2006a). Much of the progress on developing a theoretical understanding of charged ultrafiltration membrane separations was developed by Zydney and collaborators (see, e.g., Zeeman and Zydney, 1996; van Reis et al., 1997; van Reis et al., 1999). In the future, charged ultrafiltration membranes may be able to provide some of the protein fractionation now accomplished using column chromatography, for example, by concentration of positively charged mAb therapeutics using

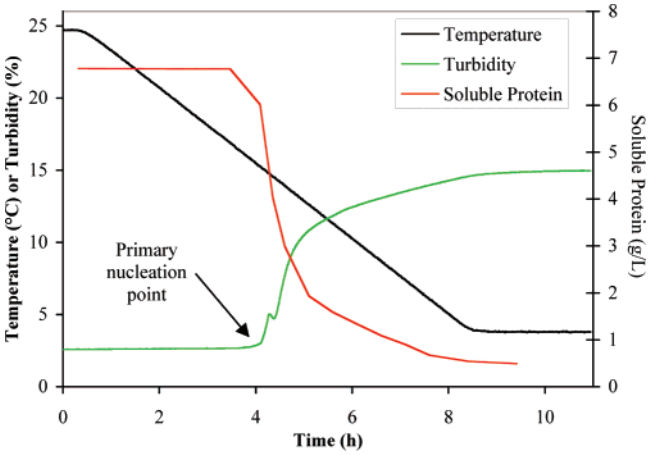

Figure 3. Apo2L crystallization during decreasing temperature ramp. (Copyright 2006 Genentech. Reproduced with permission.)

positively charged membranes that have a pore size large enough to pass process related impurities. Because membrane separations are easier to scale up and operate than chromatographic separations and the cost of installation, cleaning, and media replacement is lower using membrane technology, it may become a lower cost alternative to some chromatographic separations.

Crystallization

Protein crystallization is a powerful separation technology because it simultaneously concentrates, purifies, and stabilizes the product (Etzel, 2006). In 1982, Eli Lilly launched the world's first successful product of modern biotechnology for human healthcare: recombinant human insulin for treatment of diabetes. Its manufacture relies on a crystallization step. Peters et al. (2005) describe using a crystallization step in the manufacture of the recombinant protease inhibitor aprotinin. Genentech used crystallization to manufacture the therapeutic protein Apo2L, a member of the tumor necrosis factor family of cytokines, and thus eliminate a chromatographic step (Matthews, 2006; Flores et al., 2006).

The Genentech process is a classic case study in the development of a protein crystallization process. Crystals were first observed by accident when a solution was left overnight. Solubility screening was used to identify the impact of temperature, pH, and poly(ethylene glycol) (PEG) anti-solvent addition. The metastable region of the solubility diagram was determined. Crystallization was initiated by decreasing the solution temperature to induce primary nucleation (Figure 3). A downward temperature ramp and PEG addition were used to reduce solubility during crystallization and control the crystal growth rate. Large 150-μm crystals of hexagon-tablet habit were produced. Crystals were recovered by filtration.

The next major milestone for protein crystallization for the biopharmaceutical industry will be the development of a crystallization process for a mAb therapeutic. This has been an elusive goal. Jion et al. (2006) grew 100-μm crystals of a mAb in 3 weeks from a solution of 11% PEG 1000, 10.5 g/L mAb, pH 6 at 30 °C. Their mAb had a pI of about 4.7, much lower than most commercial mAb therapeutics, perhaps making their achievement less relevant to industry. Furthermore, their target was crystalline mAb formulations, because of the longer shelf life and stability, and not alternatives to chromatographic separations. Nevertheless, advances in crystallization of non-mAb protein therapeutics for separation and purification, and success in crystallization of mAb therapeutics for formulation purposes may build the foundation needed for the first crystallization process for a mAb therapeutic as an alternative to chromatography.

Pressure-Induced Refolding/Disintegration of Aggregates

Despite the fact that mammalian cells typically secrete properly folded proteins, ineffective polypeptide processing in the endoplasmic reticulum can lead to the formation of high molecular weight aggregates (Seefeldt et al., 2006). Furthermore, aggregates may also be formed during purification, for example, when eluting monoclonal antibodies from Protein A affinity adsorbents at low pH. Since high molecular weight aggregates have immunogenic potential, they must be efficiently removed during antibody purification. High-resolution techniques to remove aggregates will have a negative impact on the overall process yield and thus on process throughput and cost. An alternative method to dealing with high molecular weight aggregates is refolding under high hydrostatic pressure. This technique, initially introduced to unfold and refold recombinant proteins from inclusion bodies, has recently been shown to be quite effective in dealing with aggregated proteins from mammalian cells (Hesterberg, 2006; Seefeld et al., 2004; St. John et al., 2001). The technology is based on the fact that native proteins typically are unfolded under very high pressure of greater than 4000 bar, whereas multimeric proteins dissociate at a more moderate pressure of about 2000 bar. Aggregates behave like multimers and can be dissociated under such pressures. Because the native, i.e., properly folded, state is still thermodynamically favored under the moderate pressure conditions, the aggregates are refolded into monomeric protein. Utilizing this "refolding window", the high-pressure technology can be employed to turn aggregated species into useable monomeric protein, thus increasing the overall process yield. As discussed by Carpenter et al. (2005), adding reducing and oxidizing agents to the refolding buffers may even allow the use of disulfide shuffling to rectify disulfide scrambling. The kinetics of refolding is protein dependent, with refolding times reported between minutes and several hours. Overall process duration is a topic requiring future elucidation and optimization to make this interesting technology ready for industrial bioprocessing.

Conclusions

The rapidly expanding marketplace for mAb therapeutics has been a boon to society and the biotechnology industry. Advances in cell culture technology have shifted the burden downstream to deliver increased amounts of mAb at a lower cost of goods needed for the future. Chromatographic separations are frequently mentioned as a potential bottleneck to expansion of mAb manufacture. Alternatives to chromatographic separations are of high interest for this reason. As mentioned in this report, help is on the way. New technologies such as charged ultrafiltration membranes, protein crystallization, and membrane chromatography promise to become valuable alternatives in place of column chromatography steps in the manufacture of protein therapeutics. Precipitation steps and pressure-induced refolding of mAb aggregates may lessen the load on downstream column chromatography steps. Charged ultrafiltration membranes and membrane chromatography are likely the first technologies to offer a robust alternative to traditional column chromatography, because these technologies are already being used in the biotechnology industry in some pioneer applications. Crystallization of mAb therapeutics is further off. Identification

of anti-solvents to reduce solubility, proper selection of pH, temperature, and mAb concentration, and the use of theoretical principles to augment Edisonian approaches may lead to first time applications in product formation to increase stability and shelf life of mAb therapeutics. Implementation of protein crystallization at the process scale for separation and purification purposes is a reality for industrial proteins and is also occasionally used for non-mAb therapeutics but remains a challenge for mAb therapeutic products. Similarly, high-pressure refolding is still at a very early stage and will require a credible scaling strategy and pilot scale applications to demonstrate a clear path forward to industrial scale applications for antibody therapeutics. As the biotech industry moves to higher and higher productivity, alternatives to chromatography will be increasingly studied. The production of biotherapeutics will benefit tremendously from borrowing from the production of industrial proteins (e.g., enzymes), where high throughput at minimal cost is a governing principle in process design.

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