Pharmaceutical BIOPROCESSING

Industrial application of impurity flocculation to streamline antibody purification processes

Flocculation technologies offer significant benefits to industrial mammalian cell culture processes, including increased clarification efficiency, impurity removal and process simplification. In this paper, the application and performance of flocculation technologies employed in the harvest process of monoclonal antibody Chinese hamster ovary cell culture are reviewed. Attention has been placed on technologies enhancing the removal of cells, cellular debris and reduction in impurities while maintaining the antibody in the product stream. Many flocculants are systematically evaluated with respect to their mechanism of action, impact on downstream processing and product quality, and potential disadvantages. Practical considerations and future directions for application of flocculation in antibody manufacturing are discussed.

Michael Felo[1], Yun Kenneth Kang*[,2], James Hamzik[1], Paul Balderes[2] & Dale L Ludwig[2]

[1]Process Solutions, EMD Millipore, 290 Concord Road, Billerica, MA 01821, USA

[2]Bioprocess Sciences, Eli Lilly & Company, 450 East 29th Street, New York, NY 10016, USA

*Author for correspondence:
Tel.: +1 646 638 5109
Fax: +1 212 213 4785
yun.kang@lilly.com

Flocculation is a process that has been widely implemented in the chemical and food industries, as well as in waste water treatment. To the contrary, it has only been relatively recently that flocculation-based pretreatment of bacterial fermentation and mammalian cell culture has been explored and, in several cases, implemented in order to circumvent both harvest and purification challenges experienced in therapeutic protein production [1–3]. Flocculation has been slow to gain acceptance in the manufacture of monoclonal antibodies (mAbs), where mammalian cell culture processes are typically utilized. Until recently, commercial processes employing cell lines such as CHO, NS0 and SP2/0 have only rarely reached cell densities where removal of solids or cell-related soluble impurities during the harvest step becomes excessively burdensome. Standard unit operations such as centrifugation, depth filtration and microfiltration, in most cases, have efficiently produced harvested cell culture fluid (HCCF) suitable for downstream purification [4].

Through advances in mammalian cell culture over the past 10–15 years, particularly those in fed-batch and product-retaining perfusion processes, the mAb titers at the time of harvest have dramatically increased, though typically with corresponding elevations in cell density, cellular debris and related cellular impurities [5,6]. As a result, the challenges of separating cells and debris now more closely parallel those of microbial fermentation processes. It is therefore not surprising that the evaluation of flocculation to enhance the performance of this step has become an area of intense investigation. Specifically, flocculants such as simple acids, divalent cations, polycationic polymers, caprylic acid and **stimulus-responsive polymers** have been evaluated for their ability to enhance cell culture clarification and reduce the levels of DNA, host cell proteins (HCP) and viruses.

In this paper, the application and performance of various flocculation method in the harvest process of CHO cell culture production of mAbs are reviewed. Focus has been placed on methodsthat enhance the removal of cells, cellular debris and reduce impurity levels while maintaining the antibody within the product stream. Though the process goals are similar for each of these flocculants, the mechanisms of action, impact on product quality and potential toxicity from residual flocculant vary significantly.

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Key terms

Flocculation: Flocculation is the agglomeration of particles (cells, cell debris or colloids in mammalian cell culture) caused by a bridging effect induced by the flocculant.

Stimulus-responsive polymer: Stimulus-responsive polymers are high-performance polymers that change physical properties according to their environment. Such materials can be sensitive to a number of factors, such as temperature, pH, conductivity and specific stimulus reagents.

Although the effectiveness of flocculation has been frequently demonstrated, adoption remains slow in biopharmaceutical industrial applications. Critical issues including platform process fit, flocculant clearance downstream and regulatory concerns over implementation have hindered routine incorporation of the technology. Still, process and industry trends toward high cell density cell cultures, continuous processes, single-use technologies and lower culture production volumes are anticipated to continue to drive the development and implementation of these flocculation technologies into industrial biopharmaceutical manufacturing processes.

Advances in cell culture processes drive development of flocculation for mAb production process

Advances in cell culture processes have significantly increased mAb titers to levels as high as 30 g/l. These improvements have led to more challenging conditions for downstream processing, including cell densities (up to 10^8 cells per ml), packed cell volume (up to 40% v/v) and increased levels of soluble impurities such as HCP and high molecular weight (HMW) species [2,3,7–9]. Advances in mAb downstream processing are required in order to overcome the significant challenges posed by increased cell culture productivity.

Increases in cell mass, cell debris and colloids from high cell density culture, place a greater burden on the clarification train, specifically on continuous disk-stack centrifugation and depth filtration, which continue to be routinely employed in the harvest step [10]. Culture suspensions containing high solid content with a wide particle size distribution and a high percentage of small particles further complicate the harvest unit operation, requiring significantly higher depth filter surface area and, in some cases, exceeding the existing filtration train capacity [11]. During the centrifugation process, high solid content in the cell culture feed leads to increases in pellet ejection interval and decreases in product recovery due to volume losses. Submicron particles are not easily removed by centrifugation and are left to the depth filtration, and perhaps sterilizing-grade filter, for removal. Increases in filtration areacan lead to further product loss and increases in process costs for both filters and housings [10].

In recent years, flocculation-based pretreatment of cell cultures for mAb production has been investigated [2,3,9,12–17]. Many flocculation agents, or flocculants, ranging from simple electrolytes to synthetic polyelectrolytes (or polymers) have been evaluated (Table 1). Flocculation is the agglomeration of particles (cells, cell debris or colloids in mammalian cell culture) caused by a bridging effect induced by the flocculant. Flocculation agents bind to particles largely through electrostatic charge interaction, although additional interactive forces such as hydrophobic interaction or hydrogen bonding may be involved [3,4,18]. Flocculated cell cultures have higher mean particle sizes compared with their untreated counterparts (Figure 1), which has been observed to consistently correlate with improved centrate quality and filterability. Notably, positively charged flocculants, such as polyamines [14], calcium chloride/potassium phosphate [16], chitosan [15], polydiallyldimethyl ammonium chloride (PDADMAC) [2,9,19] and stimulus-responsive polymers [3] have been shown to be successful in inducing flocculation, resulting in improved clarification efficiency, process yield and clearance of impurities during the primary mAb recovery process from mammalian cell culture.

Performance, limitations & application of flocculation methods for mAb production processes

Acid precipitation

Reduction of cell culture pH at the time of harvest is a straightforward approach to induce flocculation and precipitation. This is accomplished through the introduction of concentrated acid solutions including acetic, phosphoric and citric acids. The acid solution typically drives the cell culture fluid from a neutral pH to between pH 5.5 and 4.0, most commonly between pH 5.0 and 4.5. This change in pH forces a transition across the isoelectric points of some HCP impurities, rendering them insoluble through charge neutralization. However, this impurity reduction must be balanced with the stability and quality of the product under tested conditions during the harvest operation.

The shift in particle size caused by acid precipitation/flocculation is generally less pronounced than that induced by polymer-based flocculation methods. Still, particle size increase induced by lower pH has been reported, leading to improved efficiency of continuous disk-stack centrifugation [11] or microfiltration harvest processes [20]. For harvest processes utilizing centrifugation, the larger particulates are more easily removed from the cell culture, and the capacity of the secondary depth filtration step has been increased by

Table 1. Comparison of different flocculants used to streamline cell culture harvest process and enhance the removal of impurities.

Flocculant	Mechanism of action	Additive dosage	Process yield	HCP reduction	DNA reduction	Effectiveness/ robustness	Residual level/ control	Toxicological concern	Other comments	Ref.
Acid precipitation	Precipitation by charge neutralization	pH: 4.5–5.0	>90%	0.2–0.8 LRV	1–3 LRV	Molecule dependent	High/pH adjustment and TFF or chrom	None	Product stability issues at low pH	[11,16, 20–23]
Cationic metal salts	Coprecipitation	2–200 mM metal salt (pH 5–6)	>80%	≤0.5 LRV	≤3 LRV	Insufficient data	High/pH adjustment and TFF or chrom	Very low	Potential for centrifuge damage	[16,21,24]
Caprylic acid	Coagulation and charge neutralization	0.5–1.5% (v/v)	>80%	>1.1 LRV	Variable	Molecule dependent	High/TFF or chrom	Low	Single-use extractable concern, and odor	[13,25–31]
Cationic polymers (PEI, PDADMAC, chitosan)	Charge interaction	0.01–1.0% (w/v)	>80%	<0.3 LRV	5–6 LRV	Effective/lacks in robustness, susceptible to cell culture variability	Medium/ chrom	High	Sourcing if natural product, fouling of chromatography resin, residual polymer detection	[3,9,15,19, 25,32–40]
Stimulus-responsive polymers	Charge, hydrophobic and hydrogen bonding, possibly in combination of acid precipitation; residual polymer precipitation by stimulus reagents	0.1–0.6% (w/v)	>90%	30–70%; <10 ppm post-Protein A	>5 LRV	Very effective and very robust	Low/ stimulus addition, filtration and chrom	High	Fouling of chromatography resin, additional step of adding stimulus, residual polymer detection	[3,41]

Chrom: Chromatography; LRV: Log reduction value; PDADMAC: Polydiallyldimethylammonium chloride; PEI: Polyethylenimine; TFF: Tangential flow filtration.

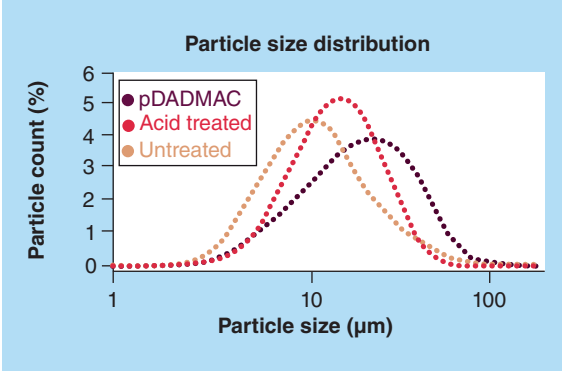

Figure 1. A comparison of particle size distribution for a monoclonal antibody-expressing Chinese hamster ovary cell culture which is untreated (pH 7.0), acid treated (pH 4.8) and treated with polydiallyldimethyl ammonium chloride (0.0375% w/v).

two–four-fold due to the lower particle content in the centrate [11,16,21]. For harvest processes using microfiltration, the higher particulate reduction resulted in greater than twofold increase in capacity in the subsequent depth filter clarification [20].

While results are cell culture and pH-dependent, reductions in DNA concentrations of 1–3 $\log_{10}$ reduction value (LRV) and HCP levels of 0.2–0.8 LRV have been reported [16,20]. Viruses have also been shown to precipitate at acidic pH conditions, resulting in 3–4 LRV murine leukemia virus removal [22,23]. For pH-sensitive molecules, product recovery as low as 40% has been reported, though recovery of greater than 90% is typical [11,20]. Incubation time at low pH should be minimized when product recovery and quality can be impacted.

Acid precipitation increases the mean particle size in the cell culture, thus improving centrifuge efficiency, increasing the capacity of an open grade depth filter, and decreasing the filter area required at manufacturing facilities. Acid precipitation of cell culture improved purity, turbidity and filterability of Protein A pools. Most molecules were stable in acid treated cell culture for the duration of the processing time predicted for manufacturing scale. However, the lower limit of the pH range for an acid precipitation process should be defined by the stability of the molecule.

Calcium phosphate flocculation

The formation of insoluble calcium phosphate *in situ* in CHO cell culture broth has been demonstrated to be an efficient method for impurity and turbidity reduction in mAb processes. In this method, calcium chloride solution is added to the cell culture at the time of harvest. A second soluble salt, potassium phosphate, is then added to the culture [21], producing an insoluble calcium phosphate precipitate. Cells and cell debris, colloids and soluble impurities bind to the precipitant, leading to flocculation and reduction in the turbidity and impurity levels in the cell culture supernatant. In many cases, after the formation of the precipitate, the pH and temperature of the cell culture fluid are lowered to further decrease the solubility of the calcium phosphate. The proposed mechanism of action is similar to that of ceramic hydroxyapatite, with the calcium phosphate binding particulates and impurities through charge and other mixed-mode interactions [24]. While these types of insoluble metal salts can be formed using many metal cations and counteranions, calcium phosphate has been the precipitant most widely studied for mAb production processes.

Calcium phosphate flocculation offers several benefits. The reagents are easily sourced, commonly used in protein purification processes, and generally nontoxic. The method generates a relatively dense precipitate which enhances the efficiency of continuous disk-stack centrifugation, depth filtration and microfiltration [16,21]. The levels of impurities reduction are similar to those achieved through acid precipitation. However, with the introduction of the calcium phosphate precipitate, these levels can be implemented under higher pH conditions. DNA reduction of 3 LRV and HCP reduction of up to 0.4 LRV have been reported [16], along with mAb product recovery of 80–100% depending on salt concentration, final culture pH and processing time [21].

Calcium phosphate flocculation does have some potential drawbacks. Since the salt has low solubility, precipitation can continue to occur post harvest. This can lead to an increase in HCCF turbidity before Protein A capture and product instability due to possible precipitate/product interactions. Although calcium phosphate is generally recognized as safe, the impact of residual levels of calcium phosphate on the long-term stability and solubility of the therapeutic protein should be considered. Another concern is that the rigidity and granular nature of the precipitate may also damage the polish and potentially decrease the lifetime of disk-stack centrifuges, though confirmation of such damage has not been reported.

Caprylic acid treatment

Caprylic acid is an eight-carbon saturated fatty acid, commercially manufactured by the oxidation of octanol or synthesized from 1-heptene. It has been used effectively to precipitate nonimmunoglobulin protein impurities from serum, plasma and ascites fluid [13,25,26]. It was also reported as an effective agent for inactivating enveloped viruses [27–29].

Application of caprylic acid in antibody production processes was investigated recently [30,31] not only as an alternative to Protein A capture or polishing

chromatography step commonly used for mAb purification, but also as an impurity reduction step in cell culture harvest operation. It was postulated that at pH 4.0–6.0, the hydrophobic interactions of caprylic acid with HCP make these soluble impurities precipitate. Antibodies with basic isoelectric point (pI), however, have sufficient charges to counteract the hydrophobic interaction and thus remain in the supernatant.

With carefully optimized pH and caprylic acid concentration, HCP reduction was achieved and greater than 90% process yield was observed. In addition, Protein A purification of caprylic acid-treated HCCF generated a less turbid eluate pool. The clearance efficiency of both enveloped and nonenveloped viruses was not affected by varying the caprylic acid or mAb concentration, suggesting that incorporation of caprylic acid flocculation into mAb harvest or purification process would add a robust and effective orthogonal viral clearance step.

However, inclusion of a novel clarification strategy after this flocculation process may be required as the precipitated impurities float on the surface of the supernatant after centrifugation. Furthermore, due to the possible toxicity of the molecule, caprylic acid must be removed from the final antibody product. This may require an additional separation step and in-process monitoring or clearance study.

Cationic polymer flocculation

Numerous cationic polymers of varying functionality have been studied for use in CHO cell culture flocculation. These polymers are typically added to the cell culture at the time of harvest at a concentration of 0.01–1% (w/v). The pH of the treated cell culture may also be lowered to induce precipitation using concentrated simple acids.

Through the use of polymers, the particle size in cell culture can be dramatically increased from CHO cells at 10–12 nm to agglomerates greater than 1000 nm [32]. These particles or 'flocs' can be easily removed by centrifugation or depth filtration or can be allowed to settle in the bioreactor before transferring the supernatant. Polymer flocculation also greatly reduces the population of submicron particulates, allowing for more efficient removal by adsorptive depth filtration [11]. Due to the high charge density of these polymers, these flocculation steps can result in greater than five LRV of DNA. HCP reduction, however, is largely dependent on the functionality of the polymers. Classical flocculants like polyethylenimine (PEI) operate largely on electrostatic interactions and can only remove HCP having a pI below the solution pH. HCP clearance with these polymers is typically less than 40% (<0.2 LRV) [33], though higher levels of HCP clearance have also been reported [14]. Other positively charged flocculants like PDADMAC exhibit hydrophobic and electrostatic properties and are able to clear a larger amount of HCP with reports of up to 50% clearance [3,34]. Product recovery is generally greater than 80%. More sophisticated, two-polymer systems can be used to achieve higher levels of HCP clearance, though in many cases the selectivity between HCPs and mAbs is undesirable [34]. Industrial application of these more complicated systems also poses a number of serious challenges.

Clearance of the cationic polymer through the downstream process is critical for three reasons. First, the polymers are known to be toxic at levels of 1 ppm or above [3,35,36]. Second, residual polymer present in HCCF after the harvest process may continue to cause precipitation or flocculation. Finally, residual polymer could bind irreversibly to chromatography media causing a reduction in binding capacity or altering the selectivity of the resin. Polymer clearance must be verified as part of the process development and validation cycle.

Polyethylenimine

One of most well-known polymer flocculants in the biotechnology industry is PEI. This polymer has been utilized in some industrial microbial processes [37]. However, there are no publications on utilization of PEI in industrial mammalian cell culture processes. Lack of adoption is likely due to its well-characterized cytotoxicity [38]. Among polymer flocculants, PEI has been shown to have the highest degree of cytotoxicity, which is both structure and molecular weight dependent [36]. Branched chain PEI has shown cytotoxicity in mammalian cells at 1 ppm. Validating clearance of the polymer to this low level can place a significant burden on the biologics manufacturer. For mAb cell culture processes, the benefits of PEI flocculation may not outweigh the burden and risks. This would likely explain the limited commercial adoption of this flocculant.

A potential improvement to PEI flocculation that may avoid toxicity issues has been investigated. Schirmer *et al.* [33] reported on the use of silica beads coupled with PEI for the flocculation of a Per.C6 cell culture. This functionalized bead approach sequestered the polymer from the clarification solution while allowing for rapid settling of the bound cells and cell debris due to the high density of the silica bead. This combined flocculation and settling step may also be applied in single-use manufacturing processes, though the high dosage of silica beads required for effective settling may prove cost prohibitive.

PDADMAC

The use of PDADMAC, a polymer flocculant traditionally used in water treatment has been extensively evaluated in CHO cell culture processes [25]. Through

increase in mean particle size in the cell culture, PDADMAC has demonstrated significant improvement in the efficiency of centrifugation and reduction in secondary depth filter area [32]. A depth filter has been designed specifically for the larger particles induced by PDADMAC, allowing for single-stage clarification [19,39]. Alternatively, combining PDADMAC flocculation with addition of polyethylene glycol (PEG) leads to formation of a significantly denser floc, enhancing the settling speed and reducing the residual flocculant remaining in solution. PEG has been widely studied on its own for both precipitation and aqueous-two-phase extraction. In addition to PEG, numerous polymers and additives have been explored for the precipitation of mAbs. These methods are beyond the scope of this review.

PDADMAC is known to have lower cytotoxicity than PEI [36], allowing for lower clearance validation requirements. Using a standard three-column mAb purification process, residual PDADMAC level was reduced to less than 50 ppb [9].

Chitosan

Chitosan, a natural polymer produced from nonmammalian sources (typically arthropod shells), was used for flocculation of mammalian cell culture [15]. Chitosan was prepared as 1% solution in 1 M acetic acid and added to the cell culture at the time of harvest at concentrations of 0.05–1% (w/v). Similar to other positively charged polymer flocculants, the efficiency of centrifugation and filtration was significantly increased. Increased toxicity of chitosan was observed with increases in either charge density or molecular weight [40]. The natural sourcing of chitosan has also lead to concerns that there could be unacceptable variability between lots of the flocculant as well as the potential for introduction of impurities from environmental sources. Immunogenicity of chitosan in certain patient populations who are sensitive to shellfish has also been expressed as a concern.

Stimulus-responsive polymer flocculation

Stimulus-responsive polymers or smart polymers refer to a class of molecules that change in confirmation and properties in response to a stimulus, such as pH, temperature or ionic strength. A novel stimulus-responsive polymer [3,41], partially benzylated poly(allylamine), was investigated as a flocculant to address the mAb clarification and purification challenges derived from high-titer cell cultures. This novel molecule, termed SmP by the investigators, is a salt-tolerant, cationic polymer substituted with hydrophobic residues that undergoes a soluble-to-insoluble transition when exposed to multivalent anions such as phosphate. Traditional polymeric flocculants typically have a narrow operating window for optimal flocculant dose. Underdosing a traditional polymeric flocculant does not provide enough polymer to effectively bridge particles thus leading to incomplete clarification while overdosing results in restabilization of the suspension leading to elevated levels of residual polymer and higher turbidity. With inherent variation in cell culture, traditional flocculation processes can be difficult to develop and operate at manufacturing scale. Because SmP becomes insoluble with the introduction of a stimulus, there is less concern about polymer overdosing. This allows reasonable control over residuals and turbidity even at dosing levels that result in an excess of polymer for traditional flocculants (Figure 2).

When SmP was used, flocculated cells, cellular debris and impurities were formed and efficiently removed by clarification. The introduction of a stimulus reagent leads to the precipitation of residual SmP. The residual polymer level was reduced to <0.1 ppm after the subsequent Protein A chromatography step. No additional ion exchange chromatography was therefore required for polymer removal [3]. While the toxicity profile of SmP has not yet been fully established, it is reasonable to assume that SmP has toxicity comparable to other substituted cationic polymers.

SmP displayed highly efficient binding to negatively charged and hydrophobic impurities such as HCP and host DNA under typical cell culture conditions while enhancing the removal of cells and cell debris and controlling the level of residual polymer. In a study of four different mAbs, the developed process demonstrated high step recovery, and improved clarification efficiency, as well as efficient reduction of process and product related impurities such as HCP, host DNA and HMW species. HCP levels were reduced 20–50% with 5.2 to >6.4 LRV DNA clearance. HMW species, including aggregated mAb, were reduced to less than 1% in the Protein A eluate in all cases. Significant improvement in filterability was observed during the depth filtration. The developed process can potentially offer a solution in cases where mAbs demonstrate poor purification process performance.

Discussion

Flocculation processes employed in the cell culture harvest step have led to improvements in the efficiency of subsequent separation steps such as centrifugation and depth filtration through the generation of larger particulates and precipitates. Additionally, these processes have demonstrated increased DNA and HCP removal through flocculant-specific mechanisms.

PEI and caprylic acid have been implemented in microbial [37] and plasma processes [12,13,28], respectively. Acid precipitation and PDADMAC are being

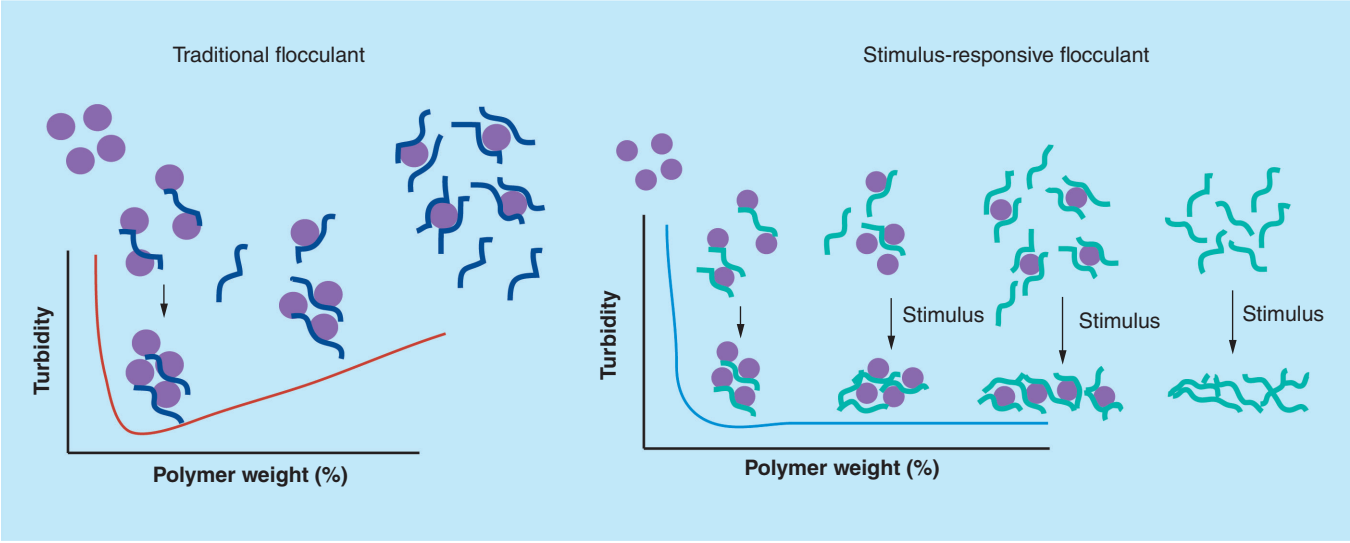

Figure 2. Traditional flocculants can be either overdosed or underdosed, leading to suboptimal clarification. Using a stimulus-responsive flocculant, residual polymer is controlled through the application of a stimulus which renders residual flocculant insoluble and being easily removed.
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implemented in mAb production at large scale. However, other flocculants reviewed in this paper have yet to see implementation in large-scale production. This is not surprising given regulatory concerns around flocculant clearance and potential impact on product quality. Also, it is not clear whether cell culture flocculation is demanded. Historically established downstream platform processes have been able to handle the solids and impurities generated in CHO cell cultures. It is only with the advent of very high titer cell cultures that an additional burden has been placed on downstream platform processes. Some even have questioned whether the benefit of increased cell culture productivity in the bioreactor is worthwhile in view of the resulting changes required to the downstream processes [42].

The implication is that in order to be broadly adopted, a 'new' technology such as flocculation must overcome an unmet process or manufacturing challenge, either by enabling a process to fit into an existing platform process or by providing new capabilities which justify a change to the platform. Acid precipitation would fit into this first category, where the technology has been investigated primarily with the goal of maintaining facility and platform fit [11].

Improvements in cell culture production, for example, the extremely high cell densities and solid loads achieved with the Per.C6 cell line, necessitate the intensification of the harvest process and change of purification platform [33]. While most CHO cell culture processes have not achieved these cell density levels, the use of perfusion technology may soon drive manufacturers in this direction [9]. In addition to the high solid loads, current CHO cell culture processes may also generate increased levels of soluble impurities which are difficult to clear in a current downstream platform process [3]. In these cases, flocculant pretreatment of the cell culture allows for a significant reduction in impurities prior to downstream purification.

Current trends in the biopharmaceutical industry point not only toward increasing product titers but also to smaller patient populations for new indications. With product titers of 5–10 g/l common for current clinical processes, a 2000 l bioreactor could be expected to produce 10–20 kg of mAb product. These elevated expression levels, coupled with lower mass requirements for patient treatment, may push the industry to use smaller bioreactors in multiproduct facilities. At a reduced scale, flocculation matched with settling or depth filtration may become advantageous on an operational and cost basis when compared with traditional disk-stack centrifugation [10]. Settling and depth filtration to remove the flocculated solids and impurities have already been shown to be efficient unit operations, providing a clarified, high-quality product stream ready for capture chromatography [3,9].

Application of flocculation in combination with a new antibody therapeutic landscape in the biopharmaceutical industry has also driven significant changes in the way mAbs are manufactured. There are two important factors shaping the way mAb products are produced. First, biosimilars, follow-on versions of innovative biologics that are at or near patent expiry, offer the opportunity for companies to commercialize products in an aggressive timeframe for established patient populations. Second, the desire of many governments to

have 'in-country, for-country' manufacturing of drugs for their population has created the need for smaller, light-asset production facilities designed to meet the local demand [43]. Taken together, these developments again have driven focus to the 2000 l single-use bioreactor as the center piece of future manufacturing strategies. The productivity of the 2000 l single-use bioreactor may now meet or exceed that of the 10,000 l stainless steel bioreactor in legacy processes, with all of the benefits of a single-use system. The implementation of flocculation in the harvest process of a bioreactor enables fully disposable upstream and downstream processes. The facility for a 2000 l bioreactor can be built, commissioned and qualified in 12–18 months in any geography at capital costs that are a fraction of a traditional facility. At the 2000 l bioreactor scale, flocculation technologies enable the use of filtration technologies, eliminating the need for a disk-stack centrifuge, thus facilitating a fully single-use process.

Furthermore, flocculation technologies may potentially allow for transition from batch processes to continuous production processes [44]. Taking these operations together, one can envision a continuous, perfusion single-use bioreactor process with continuous harvest by flocculation for improved clarification and impurity clearance.

The application of flocculation using a stimulus-responsive polymer demonstrated acceptable process yield and efficient clearance of HCP, host DNA, HMW and residual polymer and may enable a robust alternative cell culture harvest step. More importantly, as a result of flocculation, HCP and host DNA were further removed in the Protein A step to levels that meet the requirements of drug substance and thus reduce the impurities load to subsequent purification steps. With advances on stimulus-responsive polymer technology, the primary goal of the flocculation in mAb processing may be achieved: to develop a separation unit operation alternative to chromatography, with equivalent separation efficiency, high process capacity, good facility fit and sound process economics.

Conclusion & future perspective

There is little doubt that flocculation technologies have the potential to significantly benefit industrial mAb production. Increased clarification efficiency, host cell impurity reduction, aggregate removal and process simplification have all been demonstrated with multiple approaches. However, wide adoption of these technologies by the biopharmaceutical industry remains uncertain at the moment. Several technical issues need to be fully addressed including platform process fit, unit operation cost, and flocculant toxicity and clearance. An additional barrier to adoption is the lack of a strong driver for the introduction of a new step to the standard mAb manufacturing process.

Despite these challenges, industry and market forces are likely to necessitate advances to current mAb manufacturing processes. Increasing cell densities and product titers in the bioreactor, a desire for fully disposable single-use processes, and the need for low capital investment, local manufacturing will continue to drive evaluation and implementation of flocculation technologies. In many ways, these technologies are in their infancy with respect to mammalian cell culture processes. Given time, it is likely that they will be incorporated into commercial processes for mAb manufacturing. Perhaps the key question will not be whether flocculation will be adopted, but at what scale, to what extent and in which cases it will be implemented across the biotechnology industry.

Financial & competing interests disclosure

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Executive summary

Introduction

- Application of flocculation technologies has demonstrated significant benefits to mammalian cell culture clarification.
- However, adoption of flocculation in monoclonal antibody cell culture processes has been slow.
- Benefits and disadvantages for many flocculation techniques are reviewed and discussed in light of current status and future trends to mammalian cell culture processes.

Advances in cell culture processes drive development of flocculation for monoclonal antibody production processes

- Through advances in mammalian cell culture in fed-batch and product-retaining perfusion processes, monoclonal antibody (mAb) titers at the time of harvest have dramatically increased with corresponding elevations in cell density, cellular debris and related impurities.

Executive summary (cont.).

Advances in cell culture processes drive development of flocculation for monoclonal antibody production processes (cont.)

- Increases in solids and impurities place a greater burden on the clarification train, particularly disk-stack centrifuges and depth filters.

Performance, limitations & application of flocculation methods for mAb production processes

- Acid precipitation:
  - Description: reduction of cell culture pH at the time of harvest induces flocculation and precipitation using concentrated acid solutions to adjust solution pH to 5.5 and 4.0;
  - Benefits: while results are cell culture and pH-dependent, reduction in DNA concentrations of 1–3 log reduction value (LRV) and host cell proteins (HCP) levels of 0.2–0.8 LRV have been reported with increases in secondary depth filter capacity of 2–4-fold. Viruses have also been shown to precipitate at acidic pH conditions, resulting in 3–4 LRV for murine leukemia virus;
  - Disadvantages: for pH-sensitive molecules, product recoveries as low as 40% have been reported.
- Calcium phosphate flocculation:
  - Description: calcium chloride solution is added to the cell culture at the time of harvest. A second soluble salt, potassium phosphate, is then added, producing an insoluble calcium phosphate precipitate. Cells and cell debris, colloids and soluble impurities bind to the precipitant, leading to flocculation and reductions in supernatant turbidity and impurity levels;
  - Benefits: reagents for this method are easily sourced, and impurity reduction levels are similar to that of acid precipitation while generating a more dense precipitate that is more efficiently removed during primary clarification;
  - Disadvantages: precipitation can continue to occur after clarification due to the low solubility of calcium phosphate. Antibody product stability and solubility can also be impacted. The rigid precipitate may harm the finish of process equipment, especially disk-stack centrifuges.
- Caprylic acid treatment:
  - Description: caprylic acid is an eight-carbon saturated fatty acid. It has been used effectively to precipitate nonimmunoglobulin protein impurities from serum, plasma and ascites fluid, typically at pH 4–6;
  - Benefits: with carefully optimized pH and caprylic acid concentration, HCP reduction was achieved and greater than 90% process yield was observed. In addition, Protein A purification of caprylic acid-treated harvested cell culture fluid generated a less turbid eluate pool;
  - Disadvantages: with caprylic acid treatment, the precipitated impurities float on the surface of the supernatant after centrifugation. Caprylic acid must be removed from the final antibody product due to toxicity concerns.
- Cationic polymer flocculation:
  - Description: numerous cationic polymers of varying functionality have been studied for use in CHO cell culture flocculation. These polymers are typically added to the cell culture at the time of harvest at a concentration of 0.01–1% (w/v), often at lowered pH conditions;
  - Benefits: these polymers dramatically increase the mean size of particulates in the treated cell culture. DNA clearance is typically greater than five LRV. Significant HCP clearance can be achieved with a well-optimized process. Impurity clearance is correlated to polymer change density and hydrophobicity;
  - Disadvantages: polymer toxicity is the primary concern for implementation of this process step. Impact on downstream purification steps must also be addressed and validated as part of the development process;
  - Polyethylenimine, polydiallyldimethyl ammonium chloride, chitosan and stimulus-responsive polymers are specifically reviewed.

Discussion, conclusions & future directions for flocculation technologies

- Acid precipitation and polydiallyldimethyl ammonium chloride are being implemented in mAb production at large scale. Other flocculants have yet to see implementation in large-scale production, likely due to regulatory concerns around flocculant clearance and impact on product quality.
- Use of flocculation for cell culture clarification fits well with many industry trends, specifically increasing cell densities, lower process volumes with focus on the 2000 l bioreactor scale and a move toward single-use processing in flexible and reduced-capital facilities.
- Flocculation technologies have the potential to significantly benefit industrial mAb production. However, wide adoption of these technologies by the biopharmaceutical industry remains uncertain. Several technical and regulatory issues need to be fully addressed including platform process fit, unit operation cost and flocculant toxicity and clearance.

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