Monoclonal Antibody Purification Using Cationic Polyelectrolytes: An Alternative to Column Chromatography

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The potential of cationic polyelectrolytes to precipitate host cell and process related impurities was investigated, to replace one or more chromatography steps in monoclonal antibody purification. The impact of antibody isoelectric point, solution properties (pH and ionic strength), and polyelectrolyte properties (structure, molecular weight and pK_a) on the degree of precipitation was studied. At neutral pH, increasing solution ionic strength impeded the ionic interaction between the polyelectrolyte and impurities, reducing impurity precipitation. Increasing polyelectrolyte molecular weight and pK_a enabled precipitation of impurities at higher ionic strength. PoIy(arginine) was selected as the preferred polyelectrolyte in unconditioned cell culture fluid. PoIy(arginine) precipitation achieved consistent host cell protein clearance and antibody recovery for multiple antibodies across a wider range of polyelectrolyte concentrations. Poly(arginine) precipitation was evaluated as a flocculant and as a functional replacement for anion exchange chromatography in an antibody purification process. Upstream treatment of cell culture fluid with poly(arginine) resulted in flocculation of solids (cells and cell debris), and antibody recovery and impurity clearance (host cell proteins, DNA and insulin) comparable to the downstream anion exchange chromatography step. © 2010 American Institute of Chemical Engineers *Biotechnol. Prog.,* 26: 1322–1331, 2010
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Introduction

During the purification of therapeutic antibodies, impurities including host cell proteins (HCP), antibody variants, DNA, small molecules, and potential contaminants including endotoxin and viral particles must be removed.[1] Current purification techniques typically involve multiple column chromatographic separations to achieve the high purity level required for therapeutic antibodies.[2] The growing demand for therapeutic monoclonal antibodies necessitates cost effective high throughput purification processes to increase productivity. Throughput may be increased in column chromatography by increasing column dimensions, running multiple cycles, or by using high capacity resins. Process throughput may also be increased by reducing the number of purification unit operations or by using alternative techniques that are scalable and achieve similar purification to column chromatography.[3]

Using precipitation techniques, it may be possible to exploit the functional chemistry of chromatographic techniques to achieve a similar level of protein purification in solution.[4] Several precipitants described in the literature are used to selectively purify products of interest, such as proteins and DNA in aqueous solutions. Antibody purification techniques range from salting out with ammonium sulfate to affinity precipitation with smart polymers or affinity macroligands.[5–8] The polymers used for affinity precipitation vary from copolymers, which precipitate on changing pH to polyelectrolyte complexes, which precipitate on changing ionic strength or temperature. The use of affinity precipitation in the purification of monoclonal antibodies is limited by the cost associated with using antibody binding proteins, the lack of histidine tags on these antibodies to facilitate metal affinity purification and the requirement to customize the macroligand with each target antigen.[9]

A more general approach to purify monoclonal antibodies without the need for customization may be to use cationic or anionic polyelectrolytes. Varying solution properties, such as pH and ionic strength, and polyelectrolyte properties, such as functional group, molecular weight, and concentration can lead to the formation of protein-polyelectrolyte complexes that will eventually coacervate and/or precipitate.[10–13] The intermolecular associations in protein-polyelectrolyte complexes are because of electrostatic interactions, hydrogen bonds, and hydrophobic forces.[14] Purification may occur through selective partitioning of the protein of interest and impurities into the solid or liquid phases. Previous studies of polyelectrolyte precipitation include separation of artificial mixtures of proteins, nucleic acids, the recovery of whey proteins, isolation of serum glycoproteins, and viral proteins.[15] Polyamines, such as spermine and spermidine have been used for DNA precipitation.[16,17] Cationic polyelectrolytes, such as poly(ethyleneimine) were effective in precipitating both proteins and nucleic acids.[18–20] Recently, anionic polyelectrolytes have been shown to selectively precipitate monoclonal

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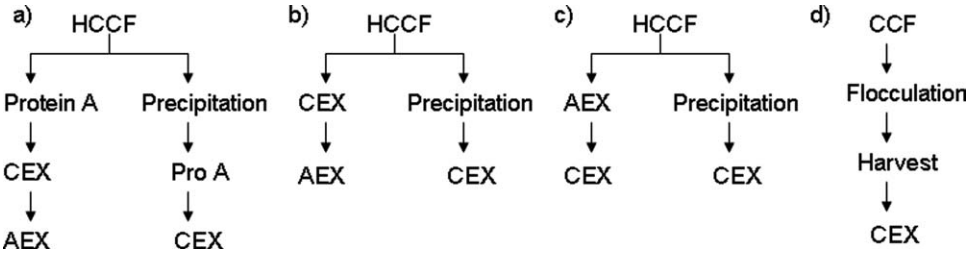


Figure 1. Process flow for cationic polyelectrolyte precipitation in (a) a typical three-step purification with Protein A chromatography as the capture step (b) and (c) a two-step ion exchange-based purification without Protein A chromatography and (d) a two-step ion exchange-based purification with CCF flocculation prior to the harvest step.

antibodies, which have unique properties compared with other impurities in the mammalian cell culture fluid (CCF).[9]

In this article, an alternative approach to monoclonal antibody precipitation is discussed: using cationic polyelectrolytes to precipitate mammalian CCF impurities. At neutral pH, purification occurs when the polycation precipitation agent complexes with the negatively charged components, such as HCP, DNA, and cell culture debris while the antibody remains soluble. Precipitation conditions were identified by investigating the impact of polyelectrolyte and solution properties on HCP precipitation and antibody recovery. Using the identified conditions, cationic polyelectrolyte precipitation was evaluated as a replacement for anion exchange chromatography, in particular, Q-Sepharose Fast Flow chromatography, in both affinity- and nonaffinity-based purification schemes (Figure 1). Q sepharose fast flow resin consists of quaternary amine ligands.[21] This allowed for a comparison of precipitation to chromatography purification, based on amine functional chemistry. Precipitation was integrated into the purification scheme as an initial purification step to (i) reduce the load on downstream chromatography steps for impurity reduction and (ii) allow for the removal of any residual polyelectrolyte in the antibody pools by the subsequent steps. Incorporation of precipitation into an affinity-based purification process allowed for an evaluation in a typical three step purification process. Additionally, a nonaffinity process was evaluated because of the cost advantage associated with the elimination of the Protein A step. Different purification processes were compared based on antibody recovery, percent monomer (by size exclusion chromatography) and clearance of impurities: HCP, DNA, leached protein A, and insulin.

Cationic polyelectrolyte precipitation was evaluated for purification in both CCF and harvested CCF (HCCF). Operating the precipitation step in CCF may reduce the number of purification unit operations by (i) integrating the precipitate removal step with a nonchromatography purification step such as centrifuge harvest and (ii) eliminating the need for multiple downstream chromatography steps for impurity reduction.[2] In addition, studies have demonstrated that the filtration membrane area required for the clarification of the turbid supernatant obtained from conventional cell separation techniques decreased, by using flocculating agents, such as calcium chloride and potassium phosphate in CCF.[22] All the above factors may also result in cost reduction, if an inexpensive polymer is used for precipitation.

Experimental

Purification techniques

Chinese hamster ovary CCF containing a recombinant humanized monoclonal IgG1 antibody was obtained from Genentech (South San Francisco, CA). Four IgG1 monoclonal antibodies were used for screening studies: mAb 1 (pI 9.2, 150 kDa), mAb 2 (pI 9.0, 150 kDa), mAb 3 (pI 8.8, 150 kDa), mAb 4 (pI 7.2, 150 kDa).

Precipitation. Several cationic polyelectrolytes were evaluated (Table 1): Poly(arginine) (Sigma Aldrich), poly (lysine) (Sigma Aldrich), 25 kDa poly(vinylamine) (Polysciences), 340 kDa poly(vinylamine) (BASF), poly(*N*-methylvinylamine) (Polysciences), poly(ethyleneimine) (Polysciences), and poly(diallyldimethyl ammonium chloride) (Polysciences).

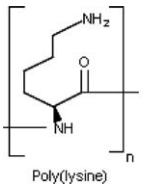
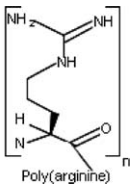
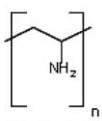
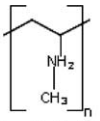
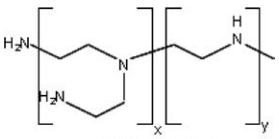
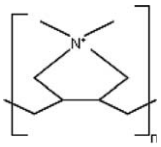
Precipitation was performed either in CCF or HCCF containing antibody at a titer of 1–2 g/L. In addition to soluble antibody and impurities, mammalian CCF typically has about 5% solids comprised of cells and cell debris, which is removed during the harvest step. For precipitation in HCCF, CCF was processed by a continuous centrifuge to remove cell debris before polyelectrolyte addition. The pH of CCF and HCCF was pH 7.0–7.5 with a conductivity of 15 mS/cm. Precipitation was performed at room temperature.

Conditions under which cationic polyelectrolytes precipitated HCP were identified by generating solubility curves. The concentration of soluble HCP and antibody remaining in solution following precipitation was determined. HCP concentration was expressed in terms of ng/mL of solution, ng/mg of the antibody or as a percentage of the starting mass of HCP. Polyelectrolyte concentration was expressed in terms of weight of the polyelectrolyte to the volume of the solution as a percentage. Antibody yield was expressed as a percentage of the starting mass. Solubility curves were generated for antibodies in the pI range 7.2–9.2. Precipitation was conducted in the pH range 5–9 and conductivity range 3–15 mS/cm at polyelectrolyte concentrations 0–0.2% (w/v). If conditioning was required before precipitation, CCF or HCCF was pH adjusted with either acetic acid or Tris base and diluted with purified water to achieve the target conductivity.

To generate solubility curves, 5% (w/v) stock solutions of each polyelectrolyte were added to 25 mL of CCF or HCCF to reach the target polyelectrolyte concentration. The precipitated pools were incubated at 2–8°C overnight before centrifugation to maximize HCP precipitation. The precipitated pools were centrifuged at 5000*g* for 30 min. The supernatant was removed and HCP, antibody concentration and turbidity (for CCF precipitation) were determined. Centrifugation was performed using a Sorvall RTH750 or Sorvall R3CB centrifuge. Turbidity was measured without dilution of the sample using a turbidity meter (model 2100N, HACH).

At a preparative scale, precipitation of CCF and HCCF was performed in a 2-L bioreactor with conditions identified from solubility curves. Precipitation was performed with 110 kDa poly(arginine) in unconditioned CCF or HCCF and with

Table 1. Molecular Weights, Functional Groups, and Structures of the Cationic Polylelectrolytes Evaluated

Polymer	Molecular weight[a] (kDa)	Functional group	Structures
Poly-L-Lysine	2.5–225	$-NH_2R_1$	 Poly(lysine)
Poly-L-Arginine	42–110	Guanidinium cation $-NH_2R_1$, $-NHR_2$	 Poly(arginine)
Poly(vinylamine)	25–340	$-NH_2R_1$	 Poly(vinylamine)
Poly(*N*-methylvinylamine)	500	$-NHR_2$	 Poly(n-methylvinylamine)
Poly(ethyleneimine)	70	$-NH_2R_1$, $-NHR_2$, $-NR3$	 Poly(ethyleneimine)
Poly(diallyldimethyl ammonium chloride)	240	$-N^+R_4$	 PDADMAC

[a] Molecular weights of the polyelectrolytes were obtained from the manufacturer.

340 kDa poly(vinylamine) in CCF conditioned to a target conductivity of 6 mS/cm. The 5% (w/v) stock solution of cationic polyelectrolyte was added to the bioreactor to reach a target concentration: 0.1% (w/v) for 110 kDa poly(arginine) and 0.02% (w/v) for 340 kDa poly(vinylamine). Polyelectrolyte was added over a period of 5 min by a syringe pump at a constant speed with mixing at 50 rpm. After 60 min of mixing, the precipitated pool was stored at 2–8°C overnight before centrifugation. The precipitated pools were centrifuged at 10000*g* for 30 min. The supernatant was analyzed for antibody yield, turbidity (for CCF precipitation), and clearance of HCP, DNA, and insulin. The post precipitation antibody pools were 0.22 μm filtered before downstream chromatography steps and remained visually clear in 2–8°C storage for at least 2 weeks.

At 100-L scale, precipitation of CCF was performed in a 400-L tank. CCF from 100-L fermentation was diluted to achieve a conductivity of 6 mS/cm (two-fold dilution). A 5% (w/v) stock solution of 340 kDa poly(vinylamine) was added to reach a final concentration of 0.02% (w/v) and mixed for 2 h. The flocculated CCF was processed through a continuous disc stack centrifuge (Alfa-Laval BTPX 205) followed by a filtration train consisting of a 12-inch EKSP depth filter (Pall), a 10-inch Durapore guard filter (Millipore) and a Durapore 10-inch sterile filter (Millipore). Centrifugation was operated at a flow rate of 7.5 L/min and 7500 rpm bowl speed. The turbidity of supernatant before filtration was measured. The post filtration antibody pool was assessed for antibody yield and clearance of HCP and DNA.

Chromatography. Chromatography was monitored at 280 nm and performed at room temperature using an AKTA 100 chromatography unit from GE Healthcare. The chromatography pools were analyzed for antibody yield, percent monomer (by size exclusion chromatography) and clearance of HCP, DNA, and insulin.

Protein A chromatography, using Prosep vA resin (Millipore) was used to purify the monoclonal antibody from HCCF, with or without an upstream cationic polyelectrolyte precipitation step. The column diameter was 2.5cm and the bed height was 14 cm. The column was operated at a flow rate of 40 CV/h. The Protein A column was loaded to 14 g/L of resin and run in multiple cycles when operated as the first purification step (four cycles). After equilibration in 25 mM TRIS, 25 mM NaCl, 5 mM EDTA pH 7.1, HCCF was loaded to the column, and the antibody was retained on the column. The column was then washed with equilibration buffer followed by 0.4 M potassium phosphate pH 7.0 and then again by equilibration buffer. The antibody was eluted with 100 mM acetic acid. Pooling was initiated based on absorbance at 280 nm (0.5 OD) and terminated after 2 column volumes. The column was regenerated in 100 mM phosphoric acid and stored in 100 mM acetate, 2% benzyl alcohol pH 5.0. Dynamic binding capacity of Protein A resin was determined with control HCCF and post-precipitation antibody pool as the load. A 0.66 cm i.d. column was used and was loaded to 60 g/L of resin. After equilibration, the column effluent was fractionated during the load phase and the antibody concentration was determined for each fraction. Binding capacity of the resin was calculated as the load density at 5% antibody breakthrough.

For the cation exchange column, SP-Sepharose Fast Flow resin (GE Healthcare) was used in bind and elute mode. The cation exchange column was operated at a flow rate of 150 cm/h. In all cases the column bed height was 30 cm and the column was loaded to 30 g/L of resin. When processing

HCCF, a 1.5 cm diameter column was used. When processing Protein A and anion exchange chromatography pools, a 0.66 cm i.d. column was used. The cation exchange column was equilibrated in 50 mM acetate pH 5.5. The load was conditioned to pH 5.0 and a conductivity of $\leq$6 mS/cm. Following the load, the column was washed with equilibration buffer followed by a wash with 50 mM MOPS pH 7.0 and then again with equilibration buffer. The antibody was eluted using a gradient of 80–320 mM acetate pH 5.5 over 15 column volumes. Pooling was initiated and terminated based on absorbance at 280 nm (0.5 OD). The column was regenerated and sanitized with 0.5 M sodium hydroxide and stored in 0.1 M sodium hydroxide. Dynamic binding capacity of cation exchange resin was determined with control HCCF and post-precipitation antibody pool, as well as with partially purified antibody in Protein A chromatography pool, with and without an upstream poly(arginine) precipitation step, as the load. A 0.66 cm i.d. column was used and was loaded to 70 g/L of resin. HCCF and post precipitation antibody pool was conditioned to pH 5.0 and 5.5 mS/cm and Protein A chromatography pool was conditioned to pH 5.0 and 3.5 mS/cm before loading on cation exchange columns. After equilibration, the column effluent was fractionated during the load phase and the antibody concentration was determined for each fraction. Binding capacity of the resin was calculated as the load density at 5% antibody breakthrough.

For the anion exchange column, Q-Sepharose Fast Flow resin (GE Healthcare) was used in flow-through mode. The anion exchange column was operated at a flow rate of 150 cm/h. In all cases, the bed height was 20 cm. When processing HCCF, a 2.2 cm diameter column was used and the column was loaded to 15 g/L of resin. When processing intermediate purification pools, a 0.66 cm i.d. column was used and the column was loaded to 40 g/L of resin. The column was equilibrated with 50 mM TRIS, 50 mM acetate pH 8.0 and the load was conditioned to pH 8.0 and a conductivity of $\leq$6 mS/cm. Following column loading, the column was washed with equilibration buffer to recover the antibody. Pooling was initiated and terminated based on absorbance at 280 nm (0.5 OD). The column was regenerated and sanitized with 0.5 M sodium hydroxide and stored in 0.1 M sodium hydroxide.

Analytical techniques

Size-exclusion chromatography was used to monitor the size heterogeneity of the antibody under native conditions. HCP, leached protein A, insulin, DNA, and size heterogeneity were measured using assays described previously.[9]

Antibody concentration in HCCF was quantified by a high-performance liquid chromatography assay using a Poros Protein A affinity column (2.1 $\times$ 30 mm, Applied Biosystems). The column was operated at 2.0 mL/min at ambient temperature for duration of 6 min. The column was equilibrated with PBS, pH 7.2. HCCF was injected and eluted with PBS, pH 2.0 (adjusted with 6 N HCl). Absorbance was monitored at 280 nm and elution peak area was quantified. The concentration of the antibody was then calculated using a standard curve. After an entire sequence, the column was regenerated using 0.1 M phosphoric acid and stored in 0.1 M sodium acetate/2% benzyl alcohol, pH 5.0.

Antibody concentration in the downstream mAb1 pools was determined by absorbance at 280 nm (with absorbance at 320 nm subtracted to correct for light scattering), using an Agilent Model 8453 spectrophotometer with a 10 mm path length flow cell. An extinction coefficient of 1.75 mL/(mg cm) was used. Antibody concentration was calculated using the following equation:

$$\text{mg/mL} = \frac{A_{280} - A_{320}}{1.75} \times \text{dilution factor}$$

We evaluated the interference of polyelectrolytes in the HCP ELISA and the antibody titer assays, using purified antibody spiked with different concentrations of polyelectrolytes. With the exception of poly(diallyldimethyl ammonium chloride), the polyelectrolytes did not interfere with the assays at concentrations less than 0.2% (w/v).

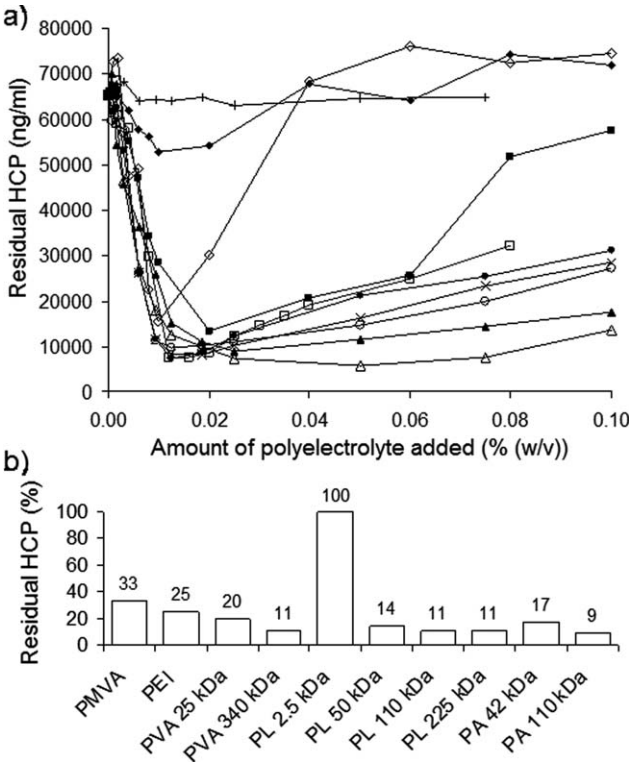


Figure 2. Comparison of HCP precipitation by cationic polyelectrolytes.

Precipitation was performed in mAb 1 HCCF at pH 7.0 and a conductivity of 6 mS/cm with PL 2.5 kDa (+), PMVA (◆), PEI (◇), PVA 25 kDa (■), PVA 340 kDa (□), PL 50 kDa (●), PL 110 kDa (×), PL 225 kDa (○), PA 42 kDa (▲), PA 110 kDa (△). Abbreviations: PL = Poly(lysine); PMVA = Poly(*N*-methylvinylamine); PEI = Poly(ethyleneimine); PVA = Poly(vinylamine); PA = Poly(arginine). Maximum HCP precipitation occurred in the polyelectrolyte concentration range 0.01–0.05% (w/v).

Results and Discussion

Identification of precipitation conditions

For determining precipitation conditions, HCP was chosen as the main target for cationic polyelectrolyte precipitation because it is reduced across each chromatography step in a typical monoclonal antibody purification process and is easy to measure.

Impact of Polyelectrolyte Properties on Precipitation. HCP precipitation was dependent on the polyelectrolyte concentration (Figure 2). HCP precipitation increased with increasing polyelectrolyte concentration until maximum

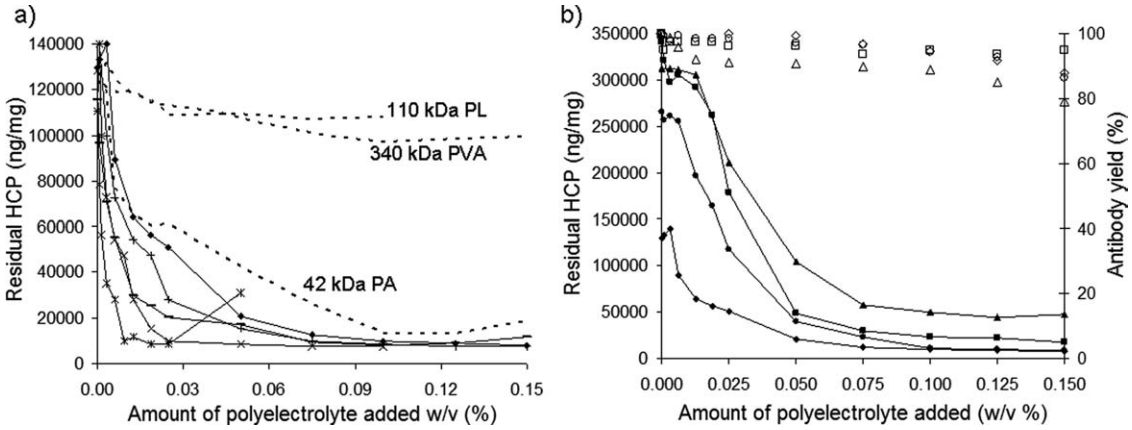


Figure 3. Impact of solution conductivity on HCP precipitation; (a) precipitation was performed with mAb 1 HCCF at pH 7.0 with PVA 340 kDa, PL 110 kDa, PA 42 kDa, and PA 110 kDa (◆) at 15 mS/cm and with PA 110 kDa at 12 mS/cm (+), 9 mS/cm (−), 6 mS/cm (×) and 3 mS/cm (✳). Comparison of HCP precipitation and antibody yield for four antibodies; (b) precipitation was performed with 110 kDa poly(arginine) in unconditioned HCCF at pH 7.0 and conductivity 15 mS/cm for mAb 1 [HCP (◆), Yield (◇)], mAb 2 (HCP (■), Yield (□)], mAb 3 [HCP (●), Yield (○)] and mAb 4 [HCP (▲), Yield (△)]. Abbreviations: PL = Poly(lysine); PVA = Poly(vinylamine); PA = Poly(arginine).

precipitation occurred. Upon further addition of the polyelectrolyte, HCP precipitation decreased. This increase in HCP solubility is likely due to resolubilization of HCP by electrostatic repulsion between an excess of polyelectrolyte in solution and polyelectrolyte bound to the HCP, thereby preventing formation of the insoluble HCP-polyelectrolyte complex.[23] Similar effects of polyelectrolyte concentration on protein recovery have been described previously and polyelectrolyte dosage was found to be dependent on molecular weight as well.[15]

For each polyelectrolyte tested, higher molecular weight polyelectrolytes precipitated more HCP than those with lower molecular weights (Figure 2). The molecular weights of the polyelectrolytes tested ranged from 2.5 to 500 kDa. HCP precipitation with 110 kDa poly(arginine) was 8% higher than with 42 kDa poly (arginine). Similarly, HCP precipitation with 340 kDa poly(vinylamine) was 9% higher than with 25 kDa poly(vinylamine). Polyelectrolyte precipitation of HCP may be caused by multiple mechanisms: polyelectrolyte bridging, charge patch neutralization and electrostatic interactions.[23] Larger polyelectrolytes may bridge the surrounding oppositely charged proteins leading them to precipitate. With poly(arginine), as we increased the molecular weight, the moles of polyelectrolyte required for maximum precipitation decreased. This suggests that higher molecular weight polyelectrolytes bind multiple HCP. However, once polyelectrolyte bridging occurs, there is no benefit to increasing polyelectrolyte molecular weight for additional HCP clearance. The evidence is that both 110 and 225 kDa poly(lysine) precipitated the same level of HCP (89% HCP reduction). Similar results have been reported previously, stating that molecular weight effects on precipitation diminish for larger polyelectrolytes (>100 kDa).[12] Smaller polyelectrolytes may be less effective at bridging multiple proteins: they may bind to regions of large proteins, which may interact with oppositely charged regions of the neighboring proteins, leading them to precipitate. However, for very small polyelectrolytes, the polyelectrolyte charge may be shielded by the surrounding ionic species in solution, preventing the polyelectrolyte-protein interaction. There may be a minimum molecular weight for polyelectrolytes to precipitate HCP, as 2.5 kDa poly(lysine) did not precipitate HCP. However, it would be useful to replicate this lack of precipitation with multiple low molecular weight polyelectrolytes.

Polyelectrolytes with a primary amine functional group precipitated more HCP than those carrying secondary and tertiary amine functional groups (Figure 2). Poly(lysine) and poly(vinylamine) precipitated higher levels of HCP than poly (ethyleneimine) and poly(*N*-methylvinylamine). Polyelectrolyte-HCP interactions may be inhibited by the substituted groups on the nitrogen atom in an amine, as in secondary, tertiary, and quaternary amines. This is supported by the fact that HCP precipitation was lower with poly(*N*-methylvinylamine) in comparison with poly(vinylamine), irrespective of the molecular weights. In this case, the methyl group on the secondary amine may restrict the accessibility of positively charged groups to the negatively charged HCP, which is necessary for the formation of an insoluble polyelectrolyte-HCP complex. Zadow and Hill have also demonstrated that protein precipitation was affected by the different degrees of substitution on the polymer.[24] An exception to this trend is poly(arginine); it has a resonant guanidinium cation with both primary and secondary amine character. Poly(arginine) precipitated HCP levels comparable with polyelectrolytes with primary amine functional groups at 6 mS/cm, thereby exhibiting a dominant primary amine character. HCP clearance with a quaternary amine, poly(diallyldimethyl ammonium chloride), could not be measured due to assay interference. However, we did not visually observe any HCP precipitation at polyelectrolyte concentrations of 0–0.2 w/v %.

Impact of Solution Properties on Precipitation. Polyelectrolytes with higher pK_a values precipitated HCP at higher ionic strengths (Figure 3a). Poly(arginine) precipitated HCP in unconditioned HCCF at 15 mS/cm and achieved HCP reductions similar to those obtained at 3–12 mS/cm. High molecular weight poly(lysine) and poly(vinylamine) precipitations resulted in less than 25% HCP reduction when ionic strength was increased to 15 mS/cm. However, both 42 and 110 kDa poly(arginine) precipitations were able to achieve greater than 90% HCP reduction at 15 mS/cm. This may be explained by the fact that poly(arginine) has the functional group with the highest pK_a value of 12.5, among the cationic polyelectrolytes screened. Primary, secondary, and tertiary

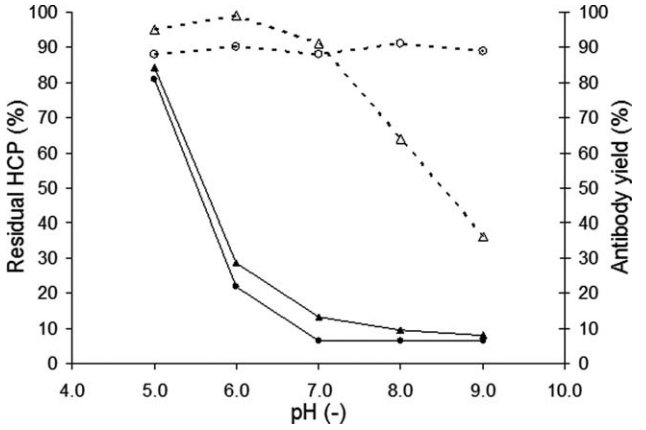


Figure 4. Impact of solution pH and antibody pI on HCP precipitation and antibody yield.

Precipitation was performed using 110 kDa poly(arginine) in HCCF at a conductivity of 15 mS/cm in the pH range 5 to 9 for mAb 1 with pI 9.2 [HCP (●), Yield (○)] and mAb 4 with pI 7.2 [HCP (▲), Yield (△)].

amines have lower pK_a values of 10.6, 10.7, and 9.8, respectively.[25] At neutral pH, this functional group was able to overcome the interference from surrounding ions in solution and interact with the negatively charged HCP thereby achieving precipitation. It has previously been demonstrated that increasing the ionic strength leads to a decrease in separation factor and higher polyelectrolyte dosage requirements.[15] Furthermore, this effect of ionic strength on precipitation was dependent on the polyelectrolyte used to effect precipitation.[24]

For antibodies with basic pI values (>7), optimization of solution pH and ionic strength was not required to achieve high HCP reduction while maintaining high antibody yield (Figures 3b and 4). The majority of HCP expressed in Chinese hamster ovary CCF tend to have acidic pI values.[26] For the antibody with pI 9.2 (mAb 1), HCP reduction (93%) and antibody yield (>85%) remained consistent in the pH range 7 to 9 in unconditioned HCCF at 15 mS/cm. In this pH range, the positively charged antibody was separated from the HCP, which was mostly negatively charged. However, for the antibody with pI 7.2 (mAb 4), we see precipitation of antibody at basic pH (>7). This is likely because of the interaction between the polyelectrolyte and the antibody with its reduced positive charge. At basic pH, both antibody and most HCP are negatively charged and may bind to the polyelectrolyte forming insoluble HCP-antibody-polyelectrolyte complexes. This indicates that for antibodies with acidic pI values, it may be difficult to selectively precipitate HCP and not antibody, because of their similar charge characteristics; further optimization of precipitation conditions may be required to achieve separation.

Impact of Temperature on Precipitation. HCP precipitation was accelerated when temperature was reduced from room temperature (25°C) to 2–8°C (Figure 5). Maximum precipitation was achieved in 15 min at 2–8°C, whereas 60 min was required at room temperature. Additional HCP precipitation of the room temperature sample was observed, when it was held at 2–8°C; HCP reduction was two-fold higher when the temperature was decreased from room temperature to 2–8°C. However, HCP reduction in samples held at 2–8°C after precipitation at room temperature was similar to that obtained when precipitation was performed at 2–8°C. One hypothesis is that temperature reduction may alter the

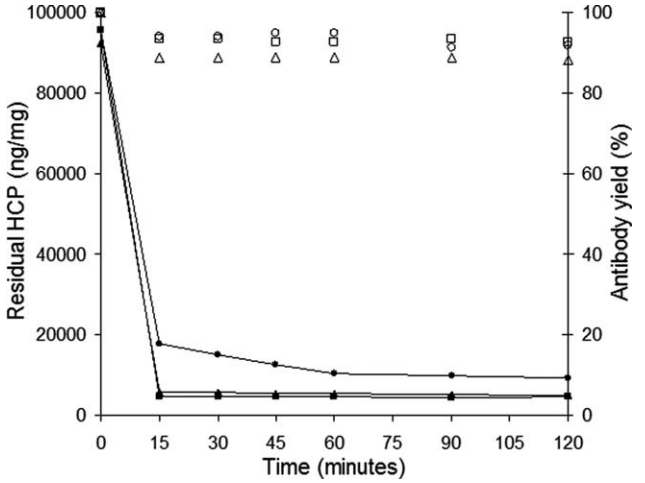


Figure 5. Impact of temperature on precipitation reaction.

Comparison of HCP precipitation and antibody yield with 0.1% (w/v) 110 kDa poly(arginine), in unconditioned mAb 1 HCCF at pH 7.0 and conductivity 15 mS/cm for three precipitation conditions: precipitation performed at (i) room temperature (25°C) [HCP (●), Yield (○)] (ii) room temperature, followed by an overnight 2–8°C hold [HCP (■), Yield (□)] and (iii) 2–8°C [HCP (▲), Yield (△)]. Samples were taken during the 2 hour time course and diluted 100 fold to stop the HCP precipitation.

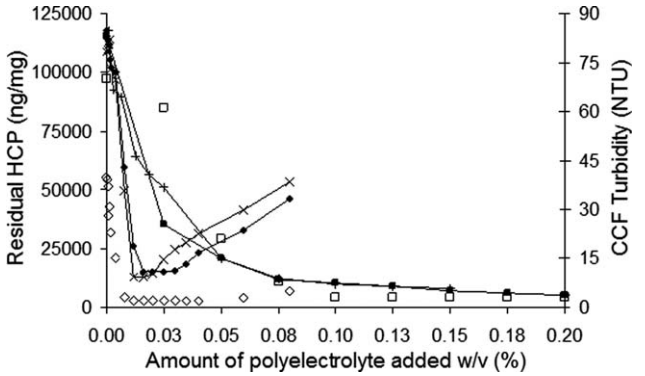


Figure 6. Comparison of HCP precipitation in HCCF and CCF.

HCP precipitation was performed with mAb 1 feedstock with poly(arginine) at pH 7.0 and a conductivity 15 mS/cm in HCCF (+) and CCF (■), and with poly(vinylamine) at 6 mS/cm in HCCF (×) and CCF (◆). Turbidity of the supernatant was measured after centrifugation of the CCF flocculated with poly(arginine) (□) and poly(vinylamine) (◊). Antibody yields after precipitation at all polyelectrolyte concentrations tested were above 80%.

HCP or polyelectrolyte solubility or its confirmation in solution, which may aid the additional HCP precipitation observed. To minimize manipulations during the precipitation unit operation and reduce the risk of additional precipitation during storage or downstream processing, a one step precipitation performed at single temperature (2–8°C) would likely be preferred.

Evaluation of Polyelectrolytes as Flocculants in CCF. Poly(arginine) flocculated solids and precipitated HCP in unconditioned CCF before harvest (Figure 6). A 91% HCP reduction was achieved, comparable with the 92% HCP reduction obtained in HCCF, at a poly(arginine) concentration of 0.1% (w/v). Following the removal of the flocculated solids, turbidity of the CCF decreased with

Table 2. Incorporation of Poly(arginine) Precipitation into a Affinity-Based Purification Process

	Yield (%)	HCP (ng/mg)	Monomer (%)	Leached protein A (ng/mg)	DNA (pg/mg)	Insulin (ng/mg)
HCCF	NA	86,300	NA	NA	373,250	2
Three step purification process						
Initial purification						
Protein A	99	1,690	98.1	9	50	<0.01
Poly(arginine) precipitation	91	4,840	NA	NA	<0.3	0.7
Intermediate purification						
Protein A-CEX	100	300	98.4	<2	<0.2	<0.02
Poly(arginine)-Protein A	103	1,450	97.5	16	<0.3	0.2
Final purification						
Protein A-CEX-AEX	87	<2.8	97.9	<2	<0.2	<0.05
Poly(arginine)-Protein A-CEX	92	3	98.5	<2	<0.1	<0.02
Impact of poly(arginine) on AEX						
Protein A-AEX	98	50	97.2	6	<0.2	<0.05
Poly(arginine)-Protein A-AEX	100	580	97	7	<0.2	<0.03

For the precipitation step, 110 kDa poly(arginine) was added to a final concentration of 0.1 % (w/v) to unconditioned mAb 1 HCCF at pH 7.3 and 15 mS/cm.

All purification steps and analytics were performed according to the procedures outlined in the methods section.

Abbreviations: NA = not applicable; CEX = cation exchange; AEX = anion exchange.

increasing polyelectrolyte concentration, from 70 NTU to 3 NTU.

An alternative polyelectrolyte, poly(vinylamine) also flocculated solids and cleared DNA in unconditioned CCF but was unable to precipitate HCP. However, an 89% HCP reduction was achieved when the ionic strength was decreased to 6 mS/cm, comparable with the 86% HCP reduction obtained in HCCF, at a poly(vinylamine) concentration of 0.02% (w/v). Following the removal of the flocculated solids, turbidity of the CCF decreased from 40 NTU to 2 NTU. The HCP solubility curve and antibody yields for flocculation in CCF were similar to those obtained with precipitation in HCCF. For the feedstocks tested, solids in CCF did not significantly impact the HCP precipitation.

Incorporation of precipitation into monoclonal antibody purification

From the HCP and antibody solubility curves, we identified conditions under which polyelectrolytes precipitated maximum HCP while maintaining high antibody yield in CCF and HCCF.

For precipitation in unconditioned CCF or HCCF, poly (arginine) was selected as the preferred polyelectrolyte. Irrespective of the starting HCP concentrations, we obtained consistent HCP reduction across multiple antibodies with basic pI values, at a polyelectrolyte concentration of 0.1 w/v% (Figure 3b). Although there was a slight increase in HCP reduction at higher poly(arginine) concentrations, 0.1 w/v % was chosen as the optimal concentration to keep excess polyelectrolyte and antibody yield loss to a minimum. Poly (vinylamine) was selected as an alternative polyelectrolyte, as HCP precipitation with poly(vinylamine) was comparable with poly(arginine) when the ionic strength of CCF was decreased to 6 mS/cm. For this polyelectrolyte, 0.02 w/v % was chosen as the optimal concentration.

Using the above conditions, poly(arginine) precipitation was evaluated as a replacement for the anion exchange step in the purification schemes outlined in Figure 1. In this article, HCP and DNA are considered as target impurities to demonstrate comparability; poly(arginine) precipitation should clear target impurities to the same levels achieved by anion exchange chromatography, when integrated with the chromatographic purification.

Incorporation of Precipitation into Three Step Affinity-Based Monoclonal Antibody Purification. The poly(arginine) precipitation-Protein A-cation exchange process resulted in an antibody yields and impurity clearance similar to a typical three step chromatography process with Protein A chromatography as the capture step (Figure 1a, Table 2). Poly(arginine) precipitation resulted in 94% HCP reduction, 50% insulin reduction and DNA clearance to acceptable levels. As initial purification steps, both Protein A chromatography and poly(arginine) precipitation resulted in antibody yields above 90%. Protein A chromatography was more effective than poly(arginine) precipitation at reducing HCP and insulin. By contrast, poly(arginine) precipitation was more effective than Protein A chromatography at reducing DNA. Following initial purification, both intermediate steps resulted in similar antibody yields, above 85%. The poly(arginine) process resulted in higher levels of HCP and leached protein A in the intermediate pools, but similar levels of DNA, insulin and percent monomer. However, after final purification, both processes achieved similar and acceptable levels of impurities and percent monomer.

Incorporation of Precipitation into Two Step Ion Exchange-Based Monoclonal Antibody Purification. The poly(arginine) precipitation-cation exchange process resulted in antibody yields and impurity clearance similar to the ion exchange-based chromatography process with cation exchange chromatography as the capture step (Figure 1b, Table 3). As initial purification steps, both cation exchange chromatography and poly(arginine) precipitation resulted in antibody yields above 90% and cleared DNA to acceptable levels. Cation exchange chromatography was more effective than poly(arginine) precipitation at reducing insulin. By contrast, poly(arginine) precipitation was more effective than cation exchange chromatography at reducing HCP, where cation exchange chromatography pools had 1.5-fold higher HCP. However, after the final purification step, both processes achieved similar and acceptable levels of DNA, insulin and percent monomer. HCP levels were similar (<100 ng/mg) in the final chromatography pools, but not reduced to acceptable levels by either process, in the absence of Protein A chromatography. Furthermore, using

Table 3. Incorporation of Poly(arginine) Precipitation into an Ion Exchange-Based Purification Process

	Yield (%)	HCP (ng/mg)	Monomer (%)	DNA (pg/mg)	Insulin (ng/mg)
Precipitation in HCCF					
HCCF	NA	86,300	NA	373,250	2
Initial purification					
CEX	99	6,730	97.6	0.7	<0.02
Poly(arginine) precipitation	91	4,840	NA	<0.3	0.7
AEX	92	7,850	ND	1.4	<0.07
Final purification					
CEX-AEX	93	80	97.6	<0.2	<0.06
Poly(arginine)-CEX	88	60	98.8	<0.2	<0.02
AEX-CEX	92	400	98.2	<0.2	<0.06
Poly(arginine)-CEX-AEX	99	50	98.3	<0.2	<0.05

For precipitation in HCCF, 110 kDa poly(arginine) was added to a final concentration of 0.1 % (w/v) to unconditioned mAb 1 HCCF at pH 7.3 and 15 mS/cm.

All purification steps and analytics were performed according to the procedures outlined in the methods section.

Abbreviations: NA = not applicable; ND = not determined; CEX = cation exchange; AEX = anion exchange.

Table 4. Comparison of Poly(arginine) and Poly(vinylamine) Precipitation in CCF

	Yield (%)	HCP (ng/mg)	Monomer (%)	DNA (pg/mg)	Turbidity (NTU)
Precipitation in CCF-2 L					
HCCF	NA	101,400	NA	290,200	90(50)[a]
Initial purification					
Poly(vinylamine) precipitation	97	11,640	NA	<0.8	2
Poly(arginine) precipitation	92	6,300	NA	<0.2	2
Final purification					
Poly(vinylamine)-CEX	91	500	99.1	<0.2	NA
Poly(arginine)-CEX	89	80	98.7	<0.2	NA
Precipitation in CCF-100 L					
HCCF	NA	92,000	NA	250,100	40
Poly(vinylamine) precipitation	95	14,700	NA	<0.7	3

[a] unconditioned HCCF was 90 NTU and conditioned HCCF was 50 NTU

For poly(vinylamine) precipitation at 2-L and 100-L scale, 340 kDa poly(vinylamine) was added to a final concentration of 0.02% (w/v) to conditioned mAb 1 CCF at pH 7.3 and 6 mS/cm. For poly(arginine) precipitation at 2 L, 110 kDa poly(arginine) was added to a final concentration of 0.1 % (w/v) to unconditioned mAb 1 HCCF at pH 7.3 and 15 mS/cm.

All purification steps and analytics were performed according to the procedures outlined in the methods section.

Abbreviations: NA = not applicable; CEX = cation exchange.

anion exchange chromatography after poly(arginine) precipitation did not provide any additional target impurity clearance indicating that they may be functionally similar purification steps.

A direct comparison of poly(arginine) precipitation with anion exchange chromatography, as initial purification steps resulted in similar target impurity reduction (Figure 1c, Table 3). Both anion exchange chromatography and poly(arginine) precipitation resulted in antibody yields above 90% and cleared DNA to acceptable levels. Poly(arginine) precipitation was more effective than anion exchange chromatography at reducing HCP, where anion exchange pools had 1.6-fold higher HCP. By contrast, anion exchange chromatography was more effective at clearing insulin to acceptable levels. Insulin is a peptide hormone with a pI of 5.3.[27] At neutral pH, insulin should be negatively charged and form complexes with the positively charged polyelectrolyte along with HCP and DNA. Insulin may not be completely cleared because of the precipitation being operated at high ionic strength of 15 mS/cm. However, after the cation exchange step, the final pools had similar levels of DNA, insulin and percent monomer. HCP reduction was six-fold higher in the poly(arginine) precipitation-cation exchange process.

Combining the Precipitation and Harvest Unit Operations. Poly(arginine) flocculation in CCF was evaluated in the purification process outlined in Figure 1d. Poly (arginine) flocculated the solids and reduced the turbidity of CCF to 2 NTU following harvest, significantly less than the turbidity of control HCCF (90 NTU). Poly(arginine) flocculation was high yielding (>90%) and impurity clearance was similar to poly(arginine) precipitation in HCCF (Table 3 and 4). In addition, CCF flocculation-cation exchange process resulted in antibody yields and impurity clearance similar to the HCCF precipitation-cation exchange process.

Additionally, CCF flocculation (at 6 mS/cm) was evaluated with an alternative polyelectrolyte, poly(vinylamine); Poly(vinylamine) flocculation resulted in antibody yields, percent monomer, turbidity and DNA clearance comparable to poly(arginine) flocculation (Table 4). However, HCP reduction was two-fold lower with poly(vinylamine) flocculation. Similarly, poly(vinylamine) flocculation-cation exchange pools had higher HCP (500 ng/mg) than poly(arginine) flocculation-cation exchange pools (80 ng/mg). However, HCP levels in poly(vinylamine) flocculation-cation exchange pools were comparable with anion exchange-cation exchange pools (400 ng/mg). This demonstrated that poly (vinylamine) may also be a suitable alternative to anion exchange chromatography.

Poly(arginine) precipitation was able to reduce target impurities to the same levels achieved by anion exchange chromatography in all the purification schemes evaluated. Furthermore, aggregate or fragment profile in the antibody pools remained unaffected because of impurity precipitation

by polyelectrolytes as measured by size exclusion chromatography (data not shown). In addition to HCP and DNA reduction, anion exchange chromatography is primarily used to reduce virus and endotoxin.[28] Cationic polyacrylamide has been used as a flocculant in CHO CCF for virus clearance.[29] It has been determined that virus removal by anion exchange resin is strongly disrupted by the presence of high salt concentrations indicating that binding of the virus to the resin is primarily because of electrostatic forces.[22] Hence, to achieve viral and endotoxin clearance using ionic interactions, it may be necessary to operate precipitation at lower ionic strengths similar to the operating conditions of anion exchange chromatography in flow through mode. For cationic polyelectrolyte precipitation to be an effective alternative, future work should demonstrate endotoxin and viral clearance capabilities. With a precipitation step in the purification process, we also need to consider how the residual polyelectrolyte will affect subsequent column chromatography steps and adequately test for its removal in the final bulks.

Impact of Precipitation on Downstream Chromatography Processes. Although polyelectrolyte precipitated with the impurities, residual polyelectrolyte may be present in solution along with the protein of interest.[30] It has been reported that the addition of a poly(arginine) tag to a model protein increased the positive charge of the protein and thus strengthened its interaction with cation-exchange resins.[31] Similarly, when the post precipitation antibody pool is processed downstream, the residual polyelectrolyte may potentially bind to Protein A or cation exchange chromatography resin, displacing the bound antibody or impurities. Alternatively, the residual polyelectrolyte may form a soluble complex with the antibody or impurities affecting their interactions with the resins.

Dynamic antibody binding capacities of Protein A and cation exchange chromatography resins with poly(arginine) precipitated antibody pool were not significantly affected. For Protein A chromatography, the antibody binding capacity was slightly lower (18 mg of antibody/ml of resin) with poly (arginine) precipitated antibody pool, compared with the control HCCF (21 mg of antibody/ml of resin). For cation exchange chromatography, the antibody binding capacities with either poly(arginine) precipitated antibody pool or poly(arginine) precipitation-Protein A pool, were similar to the controls (60 mg of antibody/ml of resin).

HCP and insulin reduction decreased across Protein A chromatography, in the presence of an upstream poly(arginine) precipitation step (Table 2). This indicates that residual poly(arginine) may be present in the post precipitation antibody pool. Furthermore, when the Protein A pool was processed over anion exchange chromatography, we obtained 11-fold higher HCP in the pool, in the presence of an upstream poly(arginine) precipitation step (Table 2). This indicates that residual poly(arginine) may be present in the Protein A chromatography pool that affects the HCP interaction with the anion exchange resin.

By contrast, HCP reduction was enhanced across cation exchange chromatography in the presence of an upstream poly(arginine) precipitation step (Table 3). The enhanced HCP reduction may be due to the preferentially binding of poly(arginine) to the resin, leading to the reduced availability of negatively charged ligands for HCP binding. This is supported by findings that PEI was removed from the protein mixture by adsorption on to a cation exchange resin.[30] Additionally, a concentration of NaCl exceeding 1 M was required for the elution of protein with a polyarginine tag from a SP column, indicating that poly(arginine) binds tightly to the resin.[31] This suggests that cationic polyelectrolytes may be cleared from antibody pools by cation exchange chromatography. However, further work is needed to quantitatively demonstrate clearance of cationic polyelectrolytes. Additionally, alternative techniques may be necessary to ensure adequate polyelectrolyte clearance.

Scale up of precipitation

Another consideration in using precipitation as an alternative to chromatography is the scale up of precipitation, in particular the capture of the precipitate. Because of the high cost of poly(arginine), poly(vinylamine), an inexpensive alternative, was used for scale up of precipitation.

Poly(vinylamine) precipitation in CCF was successfully scaled up using a 100-L fermentation. At 100-L scale, poly (vinylamine) precipitation resulted in 95% antibody yield, 84% HCP reduction, DNA clearance and turbidity reduction from 41 NTU to 3 NTU (Table 4). This was comparable with 2-L scale precipitation, which resulted in 97% antibody yield, 88% HCP reduction, DNA clearance and turbidity reduction from 50 NTU to 2 NTU. However, residual solids tend to stick to the walls of the centrifuge and further optimization is required to improve the pellet consistency to ensure simple and efficient centrifuge cleaning. Alternatively, depth filtration may possibly be used for the capture of the flocculated cell debris and precipitated impurities.

The two step ion exchange process, poly(vinylamine) or poly(arginine) flocculation in CCF followed by cation exchange chromatography did not achieve purity levels comparable with the three-step affinity-based chromatography process, using Protein A chromatography as the capture step. It may be possible to achieve the desired purity levels, through a two step purification process by using alternative cation exchange resins or multimodal resins in the second step. In addition to ionic interactions, multimodal resins use several other types of interactions, including hydrogen bonding and hydrophobic interaction. These mechanisms may provide the additional HCP clearance in the second purification step. Alternatively, a third step may be added to provide additional impurity clearance. Nevertheless, early removal of significant amounts of HCP and DNA in the harvest step with an inexpensive polyelectrolyte such as poly(vinylamine) may result in a cost effective purification process with fewer chromatography steps (without Protein A chromatography).

Summary

Precipitation of impurities is dependent on solution properties (pH and ionic strength), polyelectrolyte properties (structure, molecular weight, and pK_a) and antibody pI. We identified effective polyelectrolytes by studying these factors that affected the separation of antibody from HCP. With increasing solution conductivity, HCP precipitation decreased. However, higher molecular weight polyelectrolytes with higher pK_a were able to precipitate HCP at higher conductivities. Antibody pI is a key factor and optimization of precipitation conditions is not required to achieve acceptable purification levels for antibodies with basic pI values (>7). Antibody titer was not specifically addressed as a

variable in this study. However, antibody titer did vary from 1–2 g/L depending on the feedstock used. Future work should expand on this titer range, especially with a general trend of increasing cell culture titers.

In the purification of monoclonal antibodies, we evaluated poly(arginine) precipitation as the initial purification step in pre-harvest and post-harvest CCF. We successfully integrated the precipitation step with chromatography-based purification processes. Poly(arginine) precipitation reduced host cell impurities, such as HCP and DNA and process impurities, such as insulin. Poly(arginine) precipitation achieved purification similar to anion exchange chromatography, with respect to HCP and DNA clearance. Residual poly(arginine) in post-precipitation pools did not have a significant impact on the Protein A and cation exchange antibody binding capacities, but did affect the HCP clearance achieved by these chromatography steps.

There is the potential that the precipitation step could give the same purification as the anion exchange chromatography step without the throughput constraints dictated by resin load density. Additionally, throughput may be increased by integrating the precipitate removal with the harvest step, which can potentially reduce the number of purification steps. To replace the anion exchange chromatography step, future work should address the ability of cationic polyelectrolytes to precipitate viruses or virus-like particles. Purification by polyelectrolyte precipitation using an inexpensive commercially available polyelectrolyte such as poly(vinylamine) to flocculate cell debris and precipitate impurities may be a cost effective method of applying this purification technique. Purification by poly(vinylamine) precipitation at pilot scale was comparable with lab scale studies. However, for successful large scale implementation, further optimization is required to achieve precipitate consistency suitable for removal by centrifugation.

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