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Using precipitation by polyamines as an alternative to chromatographic separation in antibody purification processes

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ABSTRACT

Polyamine precipitation conditions for removing host cell protein impurities from the cell culture fluid containing monoclonal antibody were studied. We examined the impact of polyamine concentration, size, structure, cell culture fluid pH and ionic strength. A 96-well microtiter plate based high throughput screening method was developed and used for evaluating different polyamines. Polyallylamine, polyvinylamine, branched polyethyleneimine and poly(dimethylamine-co-epichlorohydrin-ethylenediamine) were identified as efficient precipitants in removing host cell protein impurities. Leveraging from the screening results, we incorporated a polyamine precipitation step into a monoclonal antibody purification process to replace the Protein A chromatography step. The optimization of the overall purification process was performed by taking the mechanisms of both precipitation and chromatographic separation into account. The precipitation-containing process removed a similar amount of process-related impurities, including host cell proteins, DNA, insulin and gentamicin and maintained similar product quality in respect of size and charge variants to chromatography based purification. Overall recovery yield was comparable to the typical Protein A affinity chromatography based antibody purification process.

1. Introduction

Column chromatographic separation is considered as the workhorse of the recombinant protein purification process since it can provide high resolution to meet the high purity requirement [1]. However, chromatography operations have some limitations, such as batch operation and long processing time. Consequently, alternatives to chromatography are attractive even if only to reduce the number of column chromatography steps [2,3]. Protein precipitation has become an attractive alternative due to its simple and inexpensive operations [4] and the requirement of low concentration precipitants [5]. Using the protein precipitation technique, it is possible to exploit the functional chemistry of chromatographic techniques to a similar extent for protein purification in a solution mode [4,6,7].

Precipitation using polyelectrolytes can be implemented to selectively recover target proteins [7,8] or to remove impurities while leaving the protein product in the solution [9]. Polyelectrolytes are effective in precipitating proteins because of the interaction between the charges on the proteins and on the polyelectrolytes, creating insoluble complexes, and subsequent bridging of insoluble complexes either by residual charge interaction or through hydrophobic patches on the complexes to form larger clusters [5]. On the other hand, the reverse process can occur upon further addition of polyelectrolytes whereby redissolution of proteins occurs, undermining the performance of protein purification [10]. Optimization of the precipitation technique in terms of efficiency and selectivity depends on the understanding of the factors that govern the binding affinity between proteins and polyelectrolytes. In general, the precipitation efficiency of proteins depends on pH, ionic strength, polyelectrolyte concentration (i.e., ratio of protein versus polyelectrolyte) and polyelectrolyte structure and molecular weight [11].

Polyamines are cationic polyelectrolytes with multiple repeating amine functional groups. Due to the basic nature of these amine functional groups, they are protonated under a wide pH range and therefore are positively charged. Therefore, polyamines can be used to precipitate negatively charged proteins in solution. In this paper, the use of polyamine precipitation in the monoclonal antibody purification process was studied.

The primary considerations during monoclonal antibody purification process development are purity and recovery. The purification process is normally designed to employ orthogonal or complementary separation mechanisms to achieve the purity requirements of the monoclonal antibody therapeutics. The purification process targets removal of product-related impurities such

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Table 1
List of polyamines.

Polyamine	Abbreviation	Vendor	Average molecular weight	Structure (1°, 2°, 3°, 4° amines)
Polyallylamine	PAA	Sigma–Aldrich	65 kDa	1°
Polyvinylamine	PVA	Polysciences	25 kDa	1°
Poly(dimethylamine-co-epichlorohydrin-ethylenediamine)	PDMAECHED	Sigma–Aldrich	75 kDa	1°, 2°, 4°
Polyethyleneimine	PEI (branched)	Serva	Unknown	1°, 2°, 3°
Polyethyleneimine	PEI (branched)	MP	Unknown	1°, 2°, 3°
Polyethyleneimine	PEI (branched)	MP	50–100 kDa	1°, 2°, 3°
Polyethyleneimine	PEI (branched)	Acros	60 kDa	1°, 2°, 3°
Polyethyleneimine	PEI (branched)	Polysciences	1.2 kDa	1°, 2°, 3°
Polyethyleneimine	PEI (branched)	Polysciences	10 kDa	1°, 2°, 3°
Polyethyleneimine	PEI (branched)	Polysciences	70 kDa	1°, 2°, 3°
Polyethyleneimine	PEI (branched)	Polysciences	50–100 kDa	1°, 2°, 3°
Polyethyleneimine	PEI (linear)	Polysciences	2.5 kDa	2°
Polyethyleneimine	PEI (linear)	Polysciences	25 kDa	2°
Polyethyleneimine	PEI (linear)	Polysciences	250 kDa	2°
Poly(N-methyl vinyl amine)	PMVA	Polysciences	500 kDa	2°
Poly(4-vinylpyridine)	P4VP	Sigma–Aldrich	60 kDa	2°
Poly(4-vinylpyridine)	P4VP	Sigma–Aldrich	160 kDa	2°
Spermine	Spermine	MP	348 Da	1°, 2°

as size and charge variants, and process-related impurities, including host cell proteins, host cell DNA, cell culture additives (such as insulin and gentamicin), endotoxin, viral particles and leached Protein A if Protein A chromatography is used [12,13]. Among process-related impurities to be cleared during purification processes, host cell proteins are of significant interest due to their potential antigenicity and the diversity of physicochemical properties they represent.

We evaluated using polyamines to efficiently precipitate Chinese hamster ovary (CHO) host cell proteins (CHOP) from harvested cell culture fluid (HCCF). Adding polyamines into HCCF can result in electrostatic interaction between acidic (i.e., negatively charged) CHOP and the positively charged polyamines, leading to precipitation of CHOP. If precipitation is carried out at the appropriate pH, at which the more basic monoclonal antibody of interest has net positive charges, polyamines selectively precipitate and remove the negatively charged CHOP impurities, leaving the monoclonal antibody intact and soluble in HCCF. This precipitation mechanism exploits the similar principle to an anion exchange chromatography operated in a flow-through mode.

In this study, a 96-well microtiter plate based high throughput screening (HTS) method was developed to evaluate polyamines with different structures (e.g., linear and branched) and sizes in precipitating CHOP impurities. The HTS method was also used to examine the effect of pH, ionic strength and polyamine concentration on CHOP removal efficiency by precipitation. In addition, the incorporation of polyamine precipitation into a monoclonal antibody purification process was studied. Replacement of the Protein A chromatography step as a capture step by polyamine precipitation was evaluated. The overall purification process was designed to evaluate whether employment of orthogonal separation mechanisms, including both precipitation and chromatography steps, could remove impurities more efficiently than simple replacement of certain chromatography steps with precipitation. The aspects of how trace amounts of residual polyamines affect the cation exchange chromatography step yield and how to remove residual polyamines through the cation exchange chromatography step were also explored. Several purification processes were compared with respect to the yield of the monoclonal antibody, the levels of CHOP, DNA, insulin, gentamicin as well as monoclonal antibody product quality, such as size and charge variants.

2. Materials and methods

2.1. Material

Chinese hamster ovary cell culture fluid containing recombinant humanized monoclonal IgG1 antibody was obtained from Genentech (Oceanside, CA). The media used during the cell culture process was serum free and contained animal peptones. Cell culture fluid, containing monoclonal antibody at a titer of approximately 1.5 g/L, was processed by continuous centrifuge and depth filtration to remove cellular debris and finally 0.22 μm filtered.

The polyamines used in this study are listed in Table 1 with the corresponding structures shown in Fig. 1. In most precipitation experiments, 5% weight/volume (w/v) polyamine stock solutions were used to spike into the HCCF to reach the final desired polyamine concentrations. The 5% (w/v) solutions were prepared in deionized water and adjusted to either pH 7 or pH 8 with 1.5 M Tris base or 2 M acetic acid.

SP Sepharose Fast Flow, SP Sepharose XL, Q Sepharose Fast Flow and Capto adhere chromatography resins were obtained from GE Healthcare. ProSep-vA High Capacity chromatography resin was obtained from Millipore.

2.2. Precipitation in 96-well microtiter plates

A 96-well microtiter plate (Catalog #7701-5200, Whatman) with a well volume of 2 mL was chosen as the precipitation plate. Conditioned HCCF (1.5 mL) was added into the wells of the precipitation plate. An appropriate amount of the 5% (w/v) polyamine stock solution was then added into each well to initiate precipitation. The plate was shaken on an orbital shaker (Lab-Line Instrument) with the mixing rate set at 7 for 15 min. The plate was centrifuged (Eppendorf Centrifuge 5804R) at 3000 rpm for 5 min to separate precipitate from supernatant. The supernatant in each well was transferred and filtered through the filter plate (Catalog #7700-7206, Whatman) with 0.45 μm hydrophilic polyvinylidene fluoride (PVDF) filter to further remove any fine precipitate. The filtration was conducted by stacking the filter plate onto the collection plate and then centrifuging to collect the filtrate into the collection plate. Precipitation in the well plate format was used to screen different polyamines and precipitation conditions.

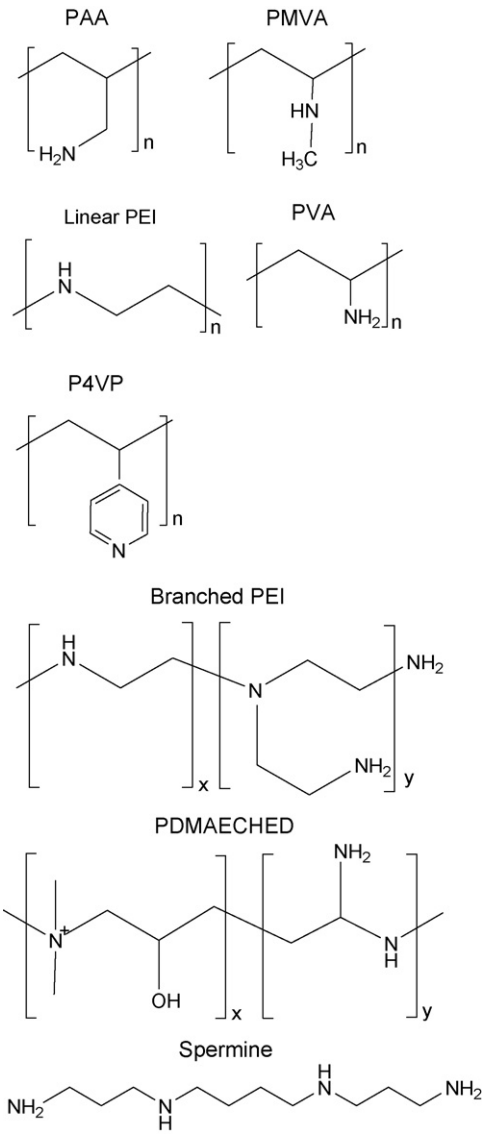

Fig. 1. Structures of polyamines.

2.3. Precipitation at preparative scale

In addition to the well plate format, precipitation was conducted in a beaker to generate loading materials for downstream chromatographic steps. The conditioned HCCF and polyamines were mixed at 60 rpm on a stir plate with a magnetic stir bar for 15 min. The mixture was then centrifuged (Beckman Allegra X-22) at 8000 rpm for 10 min prior to filtration with 0.22 μm membrane. Alternatively, the mixture was held at 4–8 °C overnight after 15 min of mixing to allow the precipitates to settle by gravity before filtration.

2.4. Chromatography steps

A typical monoclonal antibody purification process consists of the Protein A chromatography (ProSep-vA) as the capture step followed by the two ion exchange chromatography steps as polishing steps, SP Sepharose Fast Flow in a bind-elute mode and Q Sepharose Fast Flow in a flow-through mode.

In the precipitation-containing purification processes, polyallylamine (PAA) was used as the model polyamine for precipitation. The second step was the cation exchange chromatography (SP Sepharose Fast Flow or SP Sepharose XL), followed by either the anion exchange chromatography (Q Sepharose Fast Flow) or the multi-modal chromatography (Capto adhere).

The chromatography steps were performed at room temperature using an AKTA explorer 100 chromatography unit (GE Healthcare).

The Protein A chromatography step used ProSep-vA resin (Millipore) to purify the monoclonal antibody present in the HCCF. The column was operated at a flow rate of 420 cm/h. The column bed height was 14 cm. The ProSep-vA was loaded to approximately 14 g/L. After equilibration in 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1, HCCF was loaded onto the column. The column was then washed with equilibration buffer, 0.4 M potassium phosphate, pH 7.0 and then equilibration buffer. After three washes, a 100 mM acetic acid, pH 2.9 elution buffer was applied to the column. Pool collection was initiated based on absorbance at 280 nm (0.5 optical density (OD)) and terminated after 3.0 column volumes. The elution pool was adjusted to pH 5.0 using 1.5 M Tris base solution. The column was regenerated with 100 mM phosphoric acid and stored in 100 mM sodium acetate, 2% benzyl alcohol, pH 5.0.

The cation exchange (CEX) chromatography step used SP Sepharose Fast Flow resin (GE Healthcare) or SP Sepharose XL (GE Healthcare) in a bind-elute mode. The cation exchange column was operated at a flow rate of 150 cm/h. The column bed height was 30 cm. Following equilibration in 50 mM sodium acetate, pH 5.5, the column was loaded at pH 5.0 and a conductivity of ≤6.0 mS/cm to a loading density of 30 g/L. The column was washed with 25 mM MOPS, pH 7.1 and then with equilibration buffer or diluted equilibration buffer (12.5 mM sodium acetate, pH 5.5). After the two washes, a gradient elution was applied from 80 mM sodium acetate to 320 mM sodium acetate, pH 5.5 over 15 column volumes. Pool collection was initiated and terminated based on the absorbance at 280 nm (0.5 OD to 0.5 OD or 2.5 OD to 2.5 OD). The column was regenerated and sanitized with 0.5N sodium hydroxide and stored in 0.1N sodium hydroxide.

The anion exchange (AEX) chromatography step used Q Sepharose Fast Flow resin (GE Healthcare) in a flow-through mode. The anion exchange column was operated at a flow rate of 150 cm/h. The column bed height was 20 cm. The column was equilibrated with 50 mM Tris, 50 mM sodium acetate, pH 8.0. The column was loaded at pH 8.0 and a conductivity of 6.5 mS/cm to a loading density of 40 g/L. The monoclonal antibody flowed through the column. After loading, the column was further washed with the equilibration buffer. Pool collection was initiated and terminated based on absorbance at 280 nm (0.5 OD to 0.5 OD). The column was regenerated and sanitized with 0.5N sodium hydroxide and stored in 0.1N sodium hydroxide.

The multi-modal chromatography step used Capto adhere (GE Healthcare) in a bind-elute mode. The Capto adhere column was operated at a flow rate of 150 cm/h. The column bed height was 20 cm. The column was equilibrated with 50 mM Tris, 50 mM sodium acetate, pH 8.0. The column was loaded at pH 8.0 and a conductivity of 6.5 mS/cm to a loading density of 30 g/L. The column was then washed with equilibration buffer, 0.4 M potassium phosphate, pH 7.0, and then 350 mM sodium acetate, pH 5.5. After the three washes, 25 mM sodium acetate, pH 5.5 was used to elute the monoclonal antibody off the column. Pool collection was initiated and terminated based on absorbance at 280 nm (0.5 OD to 0.5 OD). The column was regenerated with 0.1 M phosphoric acid, pH 2.9, sanitized with 0.5N sodium hydroxide and stored in 0.1N sodium hydroxide.

2.5. Analytical methods

Monoclonal antibody concentration in HCCF was determined using a Poros A 20 μm column (2.1 mm × 30 mm, Applied Biosys-

tems). The column was operated at a flow rate of 2.0 mL/min. It was equilibrated in 100 mM sodium phosphate, 250 mM sodium chloride, pH 6.3 and eluted with 100 mM glycine, 2% acetic acid, pH 2.5. Absorbance was monitored at 280 nm and the elution peak area was used to quantify monoclonal antibody concentration from a standard curve.

Monoclonal antibody concentration in column-purified samples was determined by absorbance at 280 nm with absorbance at 320 nm subtracted to correct for light scattering. The absorbance was measured using an 8453 spectrophotometer (Agilent) with a 10 mm path length cuvette. Antibody concentration was calculated using the equation of $(A_{280} - A_{320}) \times$ dilution factor $\div$ extinction coefficient.

Size exclusion chromatography (SEC) was used to monitor the size heterogeneity of the monoclonal antibody. The assay employed a TSK-GEL G3000SWXL column (7.8 mm × 300 mm, Tosoh) to separate high molecular weight species (HMW), monomer and low molecular weight species (LMW). The column was operated at a flow rate of 0.5 mL/min using a 200 mM potassium phosphate, 250 mM potassium chloride, pH 6.2 running buffer. The column was operated at ambient temperature. Samples were diluted in the running buffer and 50 μg of antibody was injected for each sample. Absorbance at 280 nm was used to monitor levels of HMW, monomer and LMW.

Ion exchange chromatography (IEC) was used to monitor the charge heterogeneity of the monoclonal antibody. The assay employed a ProPac WCX-10 column (4 mm × 250 mm, Dionex) to separate acidic, main and basic species. The column was operated at a flow rate of 0.5 mL/min at 40 °C. Samples were diluted to 1 mg/mL in equilibration buffer (20 mM potassium phosphate, pH 6.5 with 10% acetonitrile) and treated with carboxypeptidase B (CpB) prior to injection. 25 μg of antibody was injected onto the equilibrated column. A linear gradient was performed from 0 to 250 mM potassium chloride in the equilibration buffer. Absorbance at 280 nm was used to monitor levels of acidic, main and basic species.

The analytical methods used to determine CHOP, leached Protein A, insulin, gentamicin and CHO DNA concentrations are described previously [8].

3. Results and discussion

3.1. CHOP precipitation

The extent of protein precipitation is influenced by pH, ionic strength or conductivity, polyamine concentration, size and structure. In this study, these parameters were evaluated for precipitation of CHOP from HCCF containing the monoclonal antibody product. The goal of precipitation was to selectively remove CHOP impurities without co-precipitating the monoclonal antibody product to retain high product yield.

3.1.1. Ionic strength

Polyamine induced precipitation is governed by the interaction between positively charged polyamines and negatively charged CHOP. Since the interaction is electrostatic in nature, ionic strength or conductivity is an important factor in influencing precipitation efficiency.

To examine CHOP precipitation under varying ionic strengths, HCCF was diluted to 3 and 6 mS/cm. The unadjusted HCCF had a conductivity of approximately 12 mS/cm. Both unadjusted HCCF and diluted HCCF were subjected to precipitation with PAA. CHOP removal profiles with different PAA concentrations are shown in Fig. 2. In this study, CHOP removal, expressed in percentage, is defined as a ratio of the difference between the pre-and post-precipitation CHOP concentration in solution to the pre-

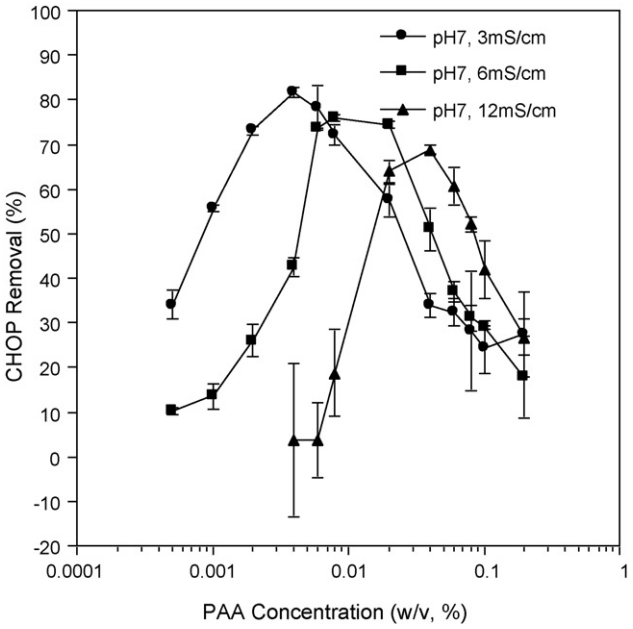

Fig. 2. Chinese hamster ovary host cell proteins (CHOP) removal by precipitation with different concentrations of polyallylamine (PAA) at three different harvested cell culture fluid (HCCF) ionic strength.

precipitation CHOP concentration in solution. At optimal PAA concentrations, precipitation removed up to 70%, 75% and 80% of CHOP at 12, 6, and 3 mS/cm, respectively. As expected, higher CHOP removal was seen at lower conductivity because the electrostatic interaction between CHOP and PAA is not shielded by the ions present in the HCCF at lower conductivity.

3.1.2. pH

Changes in pH can result in changes in the net and surface charges of proteins. At high pH, proteins are more negatively charged or less positively charged. Since polyamines precipitate CHOP by binding to negatively charged patches on protein molecules, precipitation would be favored under high pH conditions. However, a pH higher than pH 8.0 could result in potential deamidation of monoclonal antibodies. Therefore, only pH 7 and pH 8 were examined in this study. Fig. 3 shows CHOP removal profiles by PAA precipitation at pH 7 and pH 8 with a conductivity of 3 mS/cm. Both pH conditions resulted in similar CHOP removal profiles and maximum CHOP removal efficiency. Therefore, compared to conductivity, pH is less important in the range studied to evaluate polyamine precipitation efficiency.

3.1.3. Polyamine concentration

As demonstrated in Figs. 2 and 3, in addition to pH and ionic strength, polyamine concentration is another important factor in determining CHOP precipitation efficiency. At low polyamine concentration, the precipitation efficiency increased with an increase in polyamine concentration; however, as the polyamine concentration was increased further, the precipitation efficiency decreased. There was an optimal polyamine concentration at which maximum CHOP precipitation efficiency was reached. In addition, as shown in Fig. 2, the optimal polyamine concentration increased with the increase of the ionic strength. For example, for PAA precipitation, the optimal PAA concentration was 0.004% at 3 mS/cm and 0.04% at 12 mS/cm.

At low polyamine concentration, CHOP precipitation is not efficient due to the limited amount of polyamines present in the solution. Precipitation can be improved by adding more polyamines to the solution since the formation of bridging between pro-

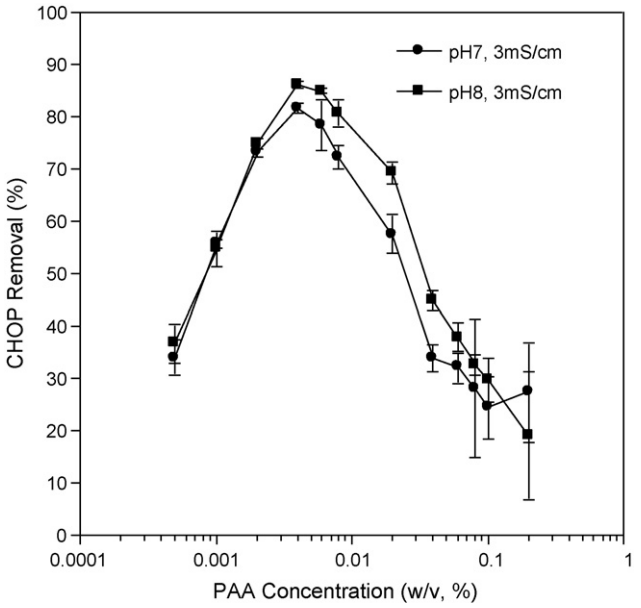

Fig. 3. Chinese hamster ovary host cell proteins (CHOP) removal by precipitation with different concentrations of polyallylamine (PAA) at two different harvested cell culture fluid (HCCF) pH.

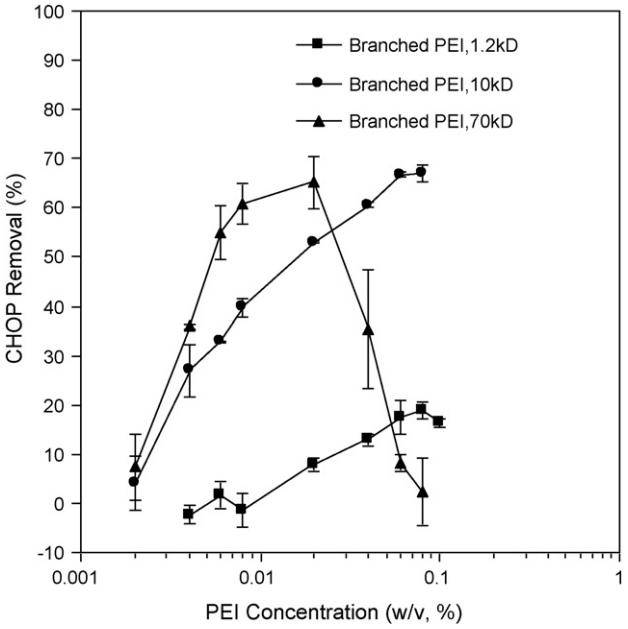

Fig. 4. Chinese hamster ovary host cell proteins (CHOP) precipitation profiles using branched polyethyleneimine (PEI) with three different molecular weight, 1.2, 10 and 70 kDa.

tein molecules through polyamines leads to precipitation [14,15]. However, when the polyamine concentration is further increased, CHOP removal through precipitation decreases. At high polyamine concentration, a polyamine layer is formed around the protein molecules, sterically interfering with precipitation. The positively charged polyamine layers repel each other, preventing the formation of bridging between protein molecules, i.e., redissolution [14–16]. Based on the observations from Figs. 2 and 3, there normally exists an optimal polyamine concentration resulting in maximum CHOP precipitation efficiency. In addition, high conductivity (i.e., high ionic strength) can result in great electrostatic shielding thus preventing the formation of bridging between protein molecules through polyamines. Therefore, the optimal polyamine concentration increased with increasing conductivity to compensate for the electrostatic shielding effect.

3.1.4. Polyamine size

The effect of polyamine size on precipitation was studied using polyethyleneimine (PEI). CHOP precipitation efficiency was compared among three branched PEI with molecular weight of 1.2, 10 and 70 kDa. The HCCF at pH 8 and 6 mS/cm was used in this study.

The smallest PEI (1.2 kDa) was not able to precipitate CHOP as efficiently as the other two larger PEI (10 and 70 kDa), as shown in Fig. 4. The maximum CHOP removal efficiency was approximately 65% for both 10 kDa PEI and 70 kDa PEI. However, the optimal PEI concentration was 0.08% for 10 kDa PEI and 0.02% for 70 kDa PEI. Thus, the smaller PEI needs to be at a higher concentration in order to reach comparable efficiency in CHOP removal. The results indicate that there may exist a critical polyamine size below which CHOP precipitation becomes inefficient. If the bridging theory is applied, the formation of either bridging or steric polyamine layers needs to have a critical polyamine size. Polyamines with shorter chains are less efficient for the formation of bridging, resulting in less precipitation [16]. Simultaneously, the polyamines with shorter chains are less efficient for the formation of steric polyamine layers around the protein molecules, resulting in less redissolution [16].

3.1.5. Polyamine structure

Two similar polyamines in structure, polyvinylamine (PVA) and poly-N-methylvinylamine (PMVA), were studied for their CHOP precipitation efficiency. In comparison to PVA, PMVA has an extra methyl group linked to the amine nitrogen, making it a secondary amine, as shown in Fig. 1. PVA and PMVA were used to evaluate whether primary amine or secondary amine containing polyelectrolyte is more efficient for CHOP precipitation. Fig. 5 shows CHOP removal using different concentrations of the two polyamines in the HCCF at pH 8 and 6 mS/cm. Clearly, PVA with primary amines was more efficient for CHOP precipitation than PMVA with secondary amines. The methyl groups on PMVA could sterically hinder

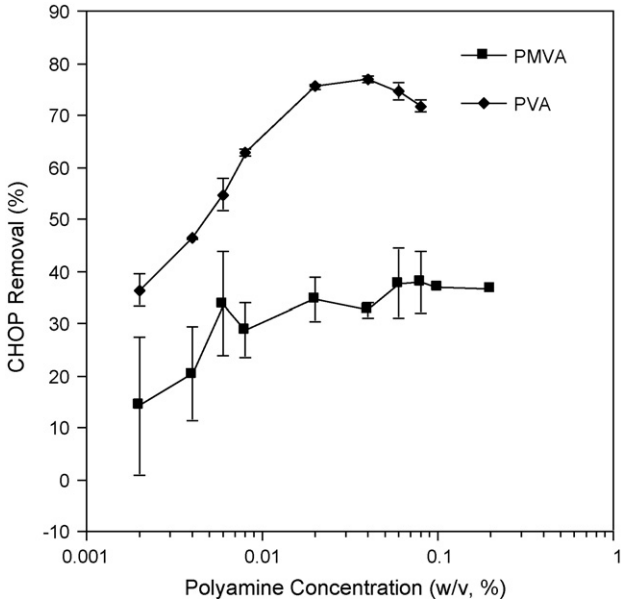

Fig. 5. Comparison of Chinese hamster ovary host cell proteins (CHOP) precipitation profiles between polyvinylamine (PVA) and poly-N-methylvinylamine (PMVA).

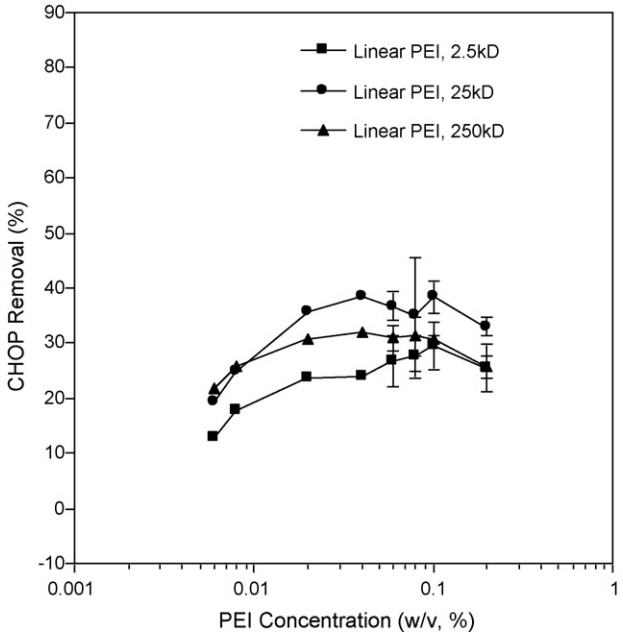

Fig. 6. Chinese hamster ovary host cell proteins (CHOP) precipitation profiles using linear polyethyleneimine (PEI) with three different molecular weight, 2.5, 25 and 250 kDa.

the electrostatic interaction between the positively charged amine and the negatively charged CHOP.

In addition, linear PEI contains secondary amines, while branched PEI contains primary amines in addition to secondary and tertiary amines, as shown in Fig. 1. The linear PEI precipitated CHOP to a lesser degree than the branched PEI, regardless of size (2.5, 25 and 250 kDa) and concentration tested, as demonstrated in Fig. 6. In this study, the HCCF was at pH 8 and 6 mS/cm. Since the primary amine could bind electrostatically to the negatively charged CHOP with less steric hindrance, the primary amine containing branched PEI is able to precipitate CHOP from the HCCF more efficiently as long as the polyamine size is large enough, as discussed in Section 3.1.4.

3.1.6. Screening for efficient polyamines

In addition to investigating how solution properties such as pH and ionic strength and polyamine properties such as concentration, structure and size influence precipitation, we also investigated the ability of different polyamines to precipitate CHOP. All the polyamines listed in Table 1 were tested for CHOP removal from the HCCF at pH 8 and 6 mS/cm. The optimal CHOP removal efficiency for each polyamine is plotted in Fig. 7. PAA, PVA, poly(dimethylamine-co-epichlorohydrin-ethylenediamine) (PDMAECHED) and multiple branched PEI were identified as efficient polyamines due to their abilities to remove CHOP by 60% or more. Interestingly, all efficient polyamines contain primary amines, suggesting that primary amines play an important role in precipitation. In addition, the precipitation efficiency was also confirmed using a second monoclonal antibody for four polyamines identified above (data are not included in this paper). The results indicated that the identified polyamines can potentially provide platform precipitation processes.

3.2. Monoclonal antibody product recovery during precipitation

In addition to the selection of efficient precipitation conditions and efficient polyamines, another important aspect of precipitation processes is product recovery. The goal of the precipitation process was to remove process-related impurities while maintaining satisfactory product recovery. Under the optimal CHOP removal conditions for several efficient polyamines, including PAA, PVA, PDMAECHED and multiple branched PEI discussed in Fig. 7, the monoclonal antibody product recovery was also evaluated. As summarized in Fig. 8, the monoclonal antibody recovery yield was above 95%. This indicates that the optimal CHOP removal conditions not only provide efficient CHOP removal but also maintain satisfactory monoclonal antibody yield.

3.3. Incorporation of precipitation into monoclonal antibody purification processes

Once efficient polyamines were identified for CHOP precipitation, the next step was to incorporate precipitation into the monoclonal antibody purification process and evaluate replacing

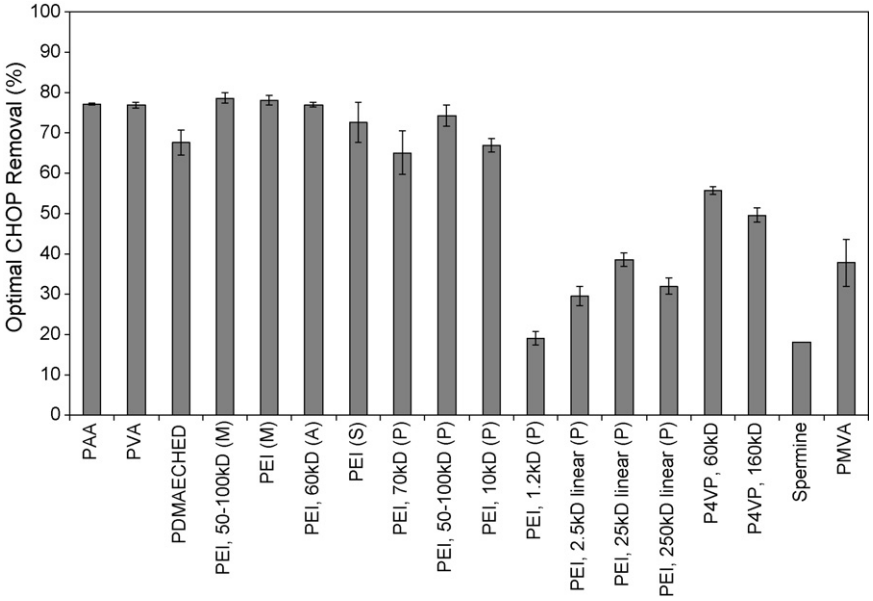

Fig. 7. Comparison of optimal Chinese hamster ovary host cell proteins (CHOP) removal for different polyamines. The vendor names are abbreviated as M for MP, A for Acros, S for Serva and P for Polysciences.

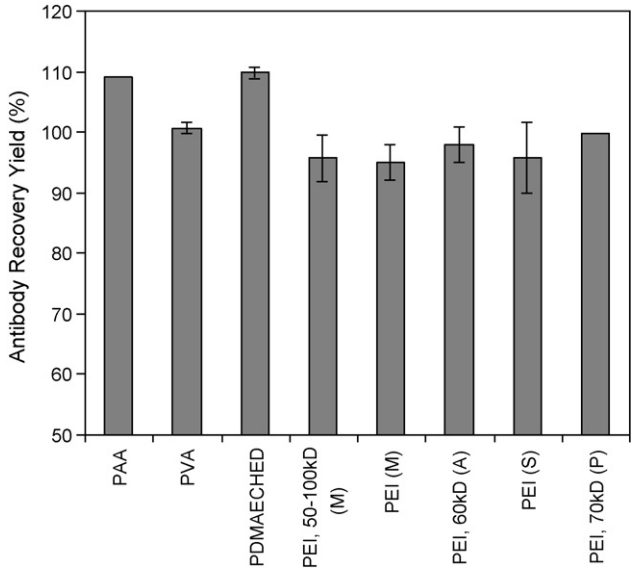

Fig. 8. Comparison of monoclonal antibody yield under the optimal CHOP precipitation conditions for different polyamines. The vendor names are abbreviated as M for MP, A for Acros, S for Serva and P for Polysciences.

the Protein A chromatography step with precipitation without compromising product purity, quality and recovery yield. In this section, PAA was used as a model polyamine in the precipitation step, which was conducted in the preparative scale as described in Section 2.3. The profiles of process-related impurities, including CHOP, insulin, gentamicin, DNA and leached Protein A if Protein A resin was used in the process, were compared for three different purification processes with and without precipitation. In addition, product-related impurities, including size and charge variants, were evaluated as well.

It should be noted that a comparability study in terms of CHOP removal and monoclonal antibody recovery between precipitation in the well plate format and preparative scale was carried out to show that CHOP removal and monoclonal antibody recovery are comparable between two scales (data not shown).

3.3.1. *Typical process with Protein A chromatography step*

A typical monoclonal antibody purification process consists of the ProSep-vA Protein A affinity chromatography followed by the SP Sepharose Fast Flow (SPSFF) cation exchange chromatography and the Q Sepharose Fast Flow (QSFF) anion exchange chromatography. This purification process serves as a control for the processes incorporating the PAA precipitation.

As summarized in Table 2, step yields were ≥90% for all three chromatography steps. CHOP was progressively removed across three steps, resulting in 2 ng/mg in the final QSFF pool. Insulin was cleared down to less than the assay detection limit through the ProSep-vA step. Both the ProSep-vA and SPSFF steps demonstrated efficient removal of gentamicin and DNA, and both impurities were cleared below the assay detection limits after the SPSFF step.

3.3.2. *PAA precipitation-CEX-AEX process*

In this purification process, the ProSep-vA step in the typical purification process was replaced with PAA precipitation while keeping the CEX and AEX steps.

During precipitation, an excess of PAA is present in HCCF solution. The majority of PAA likely interacts with CHOP and DNA, precipitating from solution. However, some PAA likely remains in solution. We found that this residual PAA can interfere with subsequent chromatography steps. In the SPSFF step subsequent to PAA precipitation, during the wash 2 with 50 mM sodium acetate, pH 5.5, antibody loss was significant, resulting in a very low step yield of 49%. The antibody loss during wash 2 may result from weaker interaction between the antibody and the SPSFF resin in the presence of PAA. Both PAA and antibody are positively charged at pH 5.5. With PAA bound on the SPSFF resin, less binding sites are available on the resin so the interaction between the SPSFF resin and the antibody is weakened. To minimize antibody loss during wash 2, the 12.5 mM sodium acetate, pH 5.5 buffer was evaluated and the step yield was improved to 94%. However, due to the weak buffering capacity, approximately 10 column volumes (CVs) of the 12.5 mM sodium acetate, pH 5.5 were required before the column effluent pH reached 5.5 to be ready for elution. The 12.5 mM sodium acetate buffer was chosen as the wash 2 buffer for further study.

We were able to empirically determine that the SPSFF step can remove PAA by observing the chromatographic behaviors of antibody pools on this step. The PAA precipitated HCCF was processed through the SPSFF column using 12.5 mM sodium acetate, pH 5.5 as the wash 2 buffer (~10 CVs). When the elution pool from this experiment was reprocessed on the SPSFF column with the typical wash 2 buffer of 50 mM sodium acetate, pH 5.5 (3 CVs), no product loss was observed during wash 2. This indicated that the SPSFF step removed residual PAA from the precipitation step. Further studies will be needed to quantitate the degree of PAA removal through subsequent chromatography steps.

In addition to minimizing the antibody loss during wash 2, we evaluated alternative cation exchange resin and optimization of the elution pooling. The SP Sepharose XL (SPXL) chromatography resin is similar to SPSFF except that the functional group is linked to the agarose matrix via a long dextran chain. The SPXL resin has higher loading capacity [17] than SPSFF and can tolerate crude load material such as HCCF. The SPXL resin provided more CHOP clearance compared than SPSFF (data are not included in this paper) and thus was selected as the cation exchange resin for further study. Further optimization of the SPXL step showed that the elution pooling of 2.5 OD to 2.5 OD can remove more CHOP without compromising the step yield than the elution pooling of 0.5 OD to 0.5 OD.

As summarized in Table 3 for the purification process including PAA precipitation, SPXL and QSFF, CHOP was cleared to 161 ng/mg in the QSFF pool. Although the main focus of precipitation in this study was to remove CHOP from HCCF, precipitation was also confirmed to be very efficient in removing DNA. The precipitation step itself reduced DNA from 404,658 pg/mg in HCCF to lower than the assay detection limit. However, precipitation was not as efficient in clearing gentamicin as ProSep-vA step in the typical purification process. Gentamicin in the final QSFF pool was 26 ng/mg. Insulin was below the assay detection limit after the SPXL step. The SPXL step yield was 92%, comparable to the SPSFF step yield of 91% in the typical purification process.

3.3.3. *PAA precipitation-SPXL-Capto adhere process*

Capto adhere is a multi-modal chromatography resin with N-benzyl-N-methyl ethanolamine as the ligand. Its multi-functionalities include anionic interaction, hydrophobic interaction and hydrogen bonding. In this study, the Capto adhere resin was evaluated to improve CHOP clearance.

In this purification process, the PAA precipitation and SPXL chromatography steps were used as the initial step and the intermediate polishing step, respectively. The SPXL pool was then adjusted to the same load condition (pH 8.0, 6.5 mS/cm) as the QSFF column for the Capto adhere column. For the model monoclonal antibody used in this study, Capto adhere demonstrated predominantly hydrophobic interaction instead of anionic interaction. Instead of the flow-through mode for the QSFF column, under the same load condition, a bind-elute mode was demonstrated for the Capto adhere column.

Table 2
Step yield of monoclonal antibody and clearance of impurities for typical purification process.

Step	Step yield (%)	CHOP (ng/mg)	Protein A (ng/mg)	Insulin (ng/mg)	Gentamicin (ng/mg)	DNA (pg/mg)
HCCF	NA[a]	350,661	NA[a]	0.29	21,411	231,240
ProSep-vA	97	9374	<2	<0.04	23.4	785
SPSFF	91	1202	<2	<0.03	<0.3	<0.1
QSFF	94	2	<2	<0.06	<0.1	<0.3

[a] NA: not applicable.

Table 3
Step yield of monoclonal antibody and clearance of impurities for PAA precipitation-SPXL-QSFF and PAA precipitation-SPXL-Capto adhere processes.

Step	Step yield (%)	CHOP (ng/mg)	Insulin (ng/mg)	Gentamicin (ng/mg)	DNA (pg/mg)
HCCF (pH 7, 6 mS/cm)	NA[a]	328,475	0.30	16,495	404,658
PAA precipitation	ND[b]	70,608	0.38	16,715	<1.3
SPXL	92%	1674	<0.02	28.4	<0.1
QSFF (as third step)	92%	161	<0.04	26	<0.2
Capto adhere (as third step)	90%	12	<0.02	0.2	<0.1

[a] NA: not applicable.
[b] ND: not determined.

Table 4
Product qualities for PAA precipitation-SPXL-Capto adhere process.

Process Step	LMW (%)	Monomer (%)	HMW (%)	Acidic (%)	Main (%)	Basic (%)
SPXL pool	0.2	99.4	0.4	17.7	62.3	20.0
Capto adhere pool	0.1	99.4	0.5	17.7	64.2	18.1
Reference[a]	0.1	98.9	1.0	14.3	66.7	19.0

[a] The reference is purified through the typical purification process.

Three different wash phases were used in the Capto adhere step. During the wash 2 with 0.4 M potassium phosphate, pH 7.0, there was an A_{280} peak and further analysis indicated that a significant amount of CHOP was washed off during this wash phase. The wash 3 with 350 mM sodium acetate, pH 5.5 was added to allow the column to reach pH 5.5 before the elution phase was initiated with 25 mM sodium acetate, pH 5.5. It would take more elution buffer to allow the pH to reach 5.5 if the wash 3 phase is not included and thus a large elution pool would be generated.

As summarized in Table 3, the CHOP level in the final Capto adhere pool was 12 ng/mg while the gentamicin level was 0.2 ng/mg. In addition, both insulin and DNA were also cleared down to lower than the assay detection limits in the final Capto adhere pool. Furthermore, satisfactory step yields were achieved for both SPXL step (92%) and Capto adhere step (90%). Compared to the purification process with the QSFF step as the final step, removal of CHOP and gentamicin was improved by replacing the QSFF step with the Capto adhere step.

In this purification process, from PAA precipitation to the SPXL and Capto adhere chromatography steps, three orthogonal separation mechanisms were applied: anion exchange in the flow-through like mode for PAA precipitation, cation exchange in the bind-elute mode for SPXL and hydrophobic interaction in the bind-elute mode for Capto adhere. However, in the purification process with the QSFF step as the last step, there was a redundant separation mechanism since both PAA precipitation and QSFF used anion exchange in the flow-through mode. Based on the impurity clearance results summarized in Table 3, the PAA precipitation-SPXL-Capto adhere process is more robust and efficient than the PAA precipitation-SPXL-QSFF process.

In addition, the product qualities, including size and charge variants, were monitored for this purification process, as summarized in Table 4. Both the size variants measured by the SEC assay and the charge variants measured by the IEC assay demonstrated similar product qualities to the typical purification process with Protein A affinity chromatography.

As mentioned earlier in this paper, the major goal of this study was to evaluate whether the Protein A affinity chromatography step can be replaced with precipitation while delivering similar product qualities and purities. As demonstrated in Tables 3 and 4, the PAA precipitation-SPXL-Capto adhere process can deliver similar product qualities, purities and overall recovery yields to a typical Protein A affinity chromatography based purification process.

4. Conclusions

Polyamine precipitation conditions were explored broadly in terms of polyamine structure, size and concentration as well as solution properties such as ionic strength and pH. The use of large polyelectrolytes containing primary amines was confirmed to improve precipitation efficiency of removing CHOP and other process-related impurities. We found there exist optimal polyamine concentrations for CHOP removal that promote sufficient bridging and precipitation of proteins but avoid redissolution. As confirmed, precipitation demonstrated higher efficiency at lower ionic strength which avoids shielding the electrostatic interaction between polyamines and target proteins.

By incorporating polyamine precipitation into a monoclonal antibody purification process, we demonstrated that it is feasible to eliminate the use of the Protein A affinity chromatography step. Such a process is capable of providing acceptable recovery yields and clearance of impurities and can potentially reduce the cost and processing time of the monoclonal antibody purification process. In addition, product quality was also examined to confirm that the introduction of polyamines into purification processes does not negatively affect product quality characterized by the SEC and IEC assay methods. However, the design of the overall purification process must take the orthogonal separation mechanisms into account to fully use each process step to remove process-related impurities, such as CHOP, gentamicin, insulin and DNA.

For full implementation of polyamine precipitation in a monoclonal antibody purification process, several issues still need to be addressed. The first is the impact on viral clearance. Protein A

chromatography and the subsequent low pH hold currently provide viral clearance/inactivation in a monoclonal antibody purification process. The ability of polyamine precipitation to remove viruses remains to be evaluated. Secondly, clearance of the residual precipitant through subsequent chromatography steps to below the level of toxicity needs to be demonstrated and proven to be robust. Lastly, demonstrating that the precipitation process can deal with antibodies and feedstreams of varying properties will be important to show that the process can serve as a platform purification process.

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