ARTICLE

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Selective Antibody Precipitation Using Polyelectrolytes: A Novel Approach to the Purification of Monoclonal Antibodies

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ABSTRACT: We evaluated the potential for polyelectrolyte induced precipitation of antibodies to replace traditional chromatography purification. We investigated the impact of solution pH, solution ionic strength and polyelectrolyte molecular weight on the degree of precipitation using the anionic polyelectrolytes polyvinylsulfonic acid (PVS), polyacrylic acid (PAA), and polystyrenesulfonic acid (PSS). As we approached the p*I* of the antibody, charge neutralization of the antibody reduced the antibody–polyelectrolyte interaction, reducing antibody precipitation. At a given pH, increasing solution ionic strength prevented the ionic interaction between the polyelectrolyte and the antibody, reducing antibody precipitation. With increasing pH of precipitation, there was an increase in impurity clearance. Increasing polyelectrolyte molecular weight allowed the precipitation to be performed under conditions of higher ionic strength. PVS was selected as the preferred polyelectrolyte based on step yield following resolubilization, purification performance, as well as the nature of the precipitate. We evaluated PVS precipitation as a replacement for the initial capture step, as well as an intermediate polishing step in the purification of a humanized monoclonal antibody. PVS precipitation separated the antibody from host cell impurities such as host cell proteins (HCP) and DNA, process impurities such as leached protein A, insulin and gentamicin, as well as antibody fragments and aggregates. PVS was subsequently removed from antibody pools to <1 μg/mg using anion exchange chromatography. PVS precipitation did not impact the biological activity of the resolubilized antibody.

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KEYWORDS: polyelectrolytes; monoclonal antibody; precipitation; purification

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 (Fahrner et al., 2001; Shukla et al., 2007). Current purification techniques typically involve multiple chromatographic separations employing orthogonal modes of separation. A typical process includes three chromatography steps: protein A affinity chromatography, cation exchange chromatography and anion exchange chromatography. Increasing demand for therapeutic antibodies and increasing pressure to reduce the cost of goods has placed a greater emphasis on improving manufacturing productivity. Recent increases in cell culture productivity have resulted in cell culture titers of multiple grams per liter (Wurm, 2004). This increase in cell culture productivity must also be met by increased purification throughput to fully realize the benefit of higher cell culture titers. Chromatography throughput can be increased through the use of higher capacity chromatography media, increasing column dimensions or column cycling. Another approach is to evaluate alternative purification technologies that may achieve similar purification to column chromatography but with reduced cost and improved scalability (Low et al., 2007; Thommes and Etzel, 2007).

We are investigating the use of antibody precipitation using polyelectrolytes as an alternative to column chromatography. Several approaches have previously been taken to the purification of antibodies and antibody fragments through precipitation. These range from salting-out the antibody with ammonium sulfate (Venkiteshwaran et al., 2008) to affinity precipitation with smart polymers or affinity macroligands. Affinity macroligands consist of polymers onto which affinity ligand(s) are conjugated (Hilbrig and Freitag, 2003; Mattiasson et al., 1998a, b). The polymer can vary from elastin like polypeptides that

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precipitate with a change in temperature (Kim et al., 2005) to copolymers of methacrylic acid and methyl methacrylate (Eudragit S-100) that precipitate with changing pH (Taipa et al., 1998, 2001). Affinity ligands used in the precipitation of antibodies and antibody fragments include antigens to the target antibody (Dainiak et al., 1999; Powers et al., 1994; Taipa et al., 1998, 2001), antibody binding proteins such as protein G and protein L (Kim et al., 2005) and chelating agents charged with metal ions to facilitate metal affinity precipitation (Kumar et al., 2003). 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.

In contrast, precipitation with anionic polyelectrolytes may be more generally applied to a greater number of monoclonal antibodies without the need for customization. We evaluated the anionic polyelectrolytes polyvinylsulfonic acid (PVS), polyacrylic acid (PAA) and polystyrenesulfonic acid (PSS) over the molecular weight range outlined in Figure 1. Through the manipulation of solution pH and ionic strength, as well as polyelectrolyte properties such as type of polyelectrolyte and polyelectrolyte molecular weight, a protein–polyelectrolyte complex can form resulting in a solid–liquid phase separation (Dubin et al., 1994; Hallberg and Dubin, 1998; Hattori et al., 2000; Kaibara et al., 2000; Mattison et al., 1998). This phase separation can take the form of a protein precipitate or coacervate. In this article, we use the general term of precipitation to describe either precipitation or coacervation. Purification may occur through selective partitioning of the protein of interest and impurities into the solid or liquid phases. Wang et al. (1996) demonstrated the separation of a mixture of model proteins (ribonuclease, β-lactoglobulin, γ-globin, and bovine serum albumin) through coacervation using the cationic polyelectrolyte poly(diallyldimethylammonium chloride). Xia and Dubin (1994) have also compiled a list of examples of protein separation using polyelectrolytes.

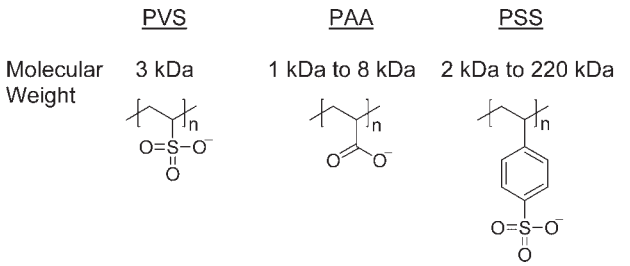

Figure 1. Structure and molecular weights of polyelectrolytes evaluated. The molecular weight of PVS was determined by size exclusion chromatography. Two lots of PVS were tested and found to have molecular weights of 3 kDa with polydispersity (M_w/M_n) of 1.7 and 2.5 respectively. The molecular weights of PAA and PSS were obtained from the manufacturer.

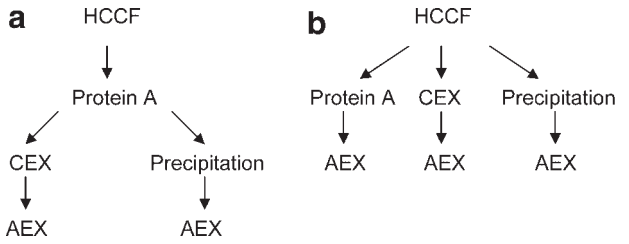

Figure 2. Process flow for (**a**) the comparison of PVS precipitation to cation exchange (CEX) chromatography as an intermediate purification step and (**b**) the comparison of PVS precipitation to protein A and CEX chromatography as an initial purification capture step. In both cases anion exchange (AEX) chromatography was used as a polishing step.

We evaluated the use of anionic polyelectrolytes in the purification of humanized monoclonal antibodies from harvested cell culture fluid (HCCF). We evaluated polyelectrolyte precipitation as an intermediate purification step replacing the cation exchange column in a three chromatography step process and also as a direct capture step for a monoclonal antibody from HCCF (Fig. 2). We used an anion exchange chromatography column operated in flow through mode to remove the negatively charged polyelectrolytes. We compared the different processes based on antibody yield, levels of HCP, DNA, leached protein A, insulin and gentamicin as well as antibody aggregates and fragments.

Experimental

Purification Techniques

Chinese hamster ovary cell culture fluid containing recombinant humanized monoclonal IgG1 antibodies were obtained from Genentech (South San Francisco, CA). The media used during the cell culture process was serum free and contained animal peptones. Cell culture fluid, containing antibody at a titer of about 1 g/L, was processed by continuous centrifuge to remove cellular debris and 0.22 μm filtered before performing the capture step. The protein pools were also 0.22 μm filtered prior to additional purification steps. Chromatography was monitored at 280 nm and performed at room temperature using an AKTA 100 chromatography unit from GE Healthcare (Uppsala, Sweden).

The protein A column used Prosep vA resin (Millipore, Bedford, MA) and was used to purify the antibody present in the HCCF. The column diameter was 2.5 cm and the bed height was 14 cm. The column was operated at a flow rate of 560 cm/h. The Prosep vA column was loaded to approximately 14 g/L_{resin}. After equilibration in 25 mM Tris, 25 mM NaCl, 5 mM EDTA pH 7.1, HCCF was applied to the column. The column was then washed with equilibration buffer followed by 0.4 M potassium phosphate and then again with equilibration buffer. After these washes were

complete, a 100 mM acetic acid elution buffer was applied to the column. 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.

Prior to precipitation, HCCF, partially purified antibody or bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was adjusted to the target pH using dilute hydrochloric acid or sodium hydroxide and diluted with purified water to achieve the target conductivity. Each polyelectrolyte was added to the conditioned pool with mixing. We obtained PVS and PAA from Sigma Aldrich and PSS from Polysciences, Inc. (Warrington, PA). Following precipitation, the precipitated pools were centrifuged for 30 min at 1,500*g* (for solubility curves) to 4,600*g* (for downstream purification) using a Sorvall RTH750 or Sorvall R3CB centrifuge. In order to generate solubility curves, the supernatant was removed and protein concentration was determined and expressed as a percentage of the starting concentration. For downstream purification or while assessing impurity clearance, the precipitate was washed with 25 mM MOPS, pH 7.1. The precipitate was then resolubilized with mixing in 50 mM Tris, 50 mM acetate pH 8.0 and analyzed for yield and impurities. The resolubilized pools were processed across anion exchange chromatography without any additional conditioning.

The cation exchange column used SP-Sepharose Fast Flow resin (GE Healthcare) in bind and gradient elution mode. The cation exchange column was operated at a flow rate of 150 cm/h. In all cases the bed height was 30 cm. When processing HCCF, a 2.2 cm diameter column was used and the column was loaded to 10 g/L_{resin}. When processing intermediate purification pools, a 0.66 cm i.d. column was used and the column was loaded to 40 g/L_{resin}. The column was equilibrated in 50 mM acetate pH 5.5. The load was conditioned to pH 5.0 and a conductivity of $\leq$5.5 mS/cm. Following the load, the column was washed with equilibration buffer followed by a wash with 25 mM MOPS pH 7.1 and then again with equilibration buffer. After these washes were complete, the antibody was eluted in a gradient from 80 mM acetate to 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.

The anion exchange column used Q-Sepharose Fast Flow resin (GE Healthcare) in flow through mode. The anion exchange column was operated at a flow rate of 150 cm/h. The column diameter was 0.66 cm and the bed height was 20 cm. The column was equilibrated with 50 mM Tris, 50 mM acetate pH 8.0. The column was loaded at pH 8.0 to a loading density of 40 g/L_{resin}. The antibody flowed through the column, which was then washed with equilibration buffer. 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

Antibody concentration in HCCF was determined using a Poros protein A HPLC assay (Applied Biosystems, Foster City, CA). The column was operated at a flow rate of 2.0 mL/min where it was equilibrated in PBS and eluted with PBS adjusted to pH 2.0 with hydrochloric acid. Absorbance was monitored at 280 nm and the elution peak area was used to quantify antibody concentration from a standard curve. Antibody concentration in partially purified pools was determined by absorbance at 280 nm (with absorbance at 320 nm subtracted to correct for light scattering), using an 8453 spectrophotometer (Agilent, Santa Clara, CA) with a 10 mm path length flow cell. Antibody concentration was calculated using the following equation:

$$\{[(A_{280\,nm})-(A_{320\,nm})]\times(\text{dilution})\}/\text{Excitation Coefficient}.$$

Size-exclusion chromatography was used to monitor the size heterogeneity of the monoclonal antibodies under native conditions. The assay employed a TSK-GEL G3000SWXL column (7.8 mm $\times$ 300 mm, Tosoh Bioscience, Montgomeryville, PA) to separate aggregates, monomer, and fragments. The column was operated at a flow rate of 0.3 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 running buffer and 20 μg of antibody was injected for each sample. Absorbance at 280 nm was used to monitor levels of aggregates, monomer and fragments.

An enzyme-linked immunosorbent assay (ELISA) using goat anti-HCP antibodies was used to determine HCP concentrations in all of the pools. For the ELISA, affinity purified goat anti-HCP antibodies were immobilized on microtiter plate wells. Dilutions of the pool samples were incubated in the wells, followed by incubation with peroxidase-conjugated goat anti-HCP antibodies. The horseradish peroxidase enzymatic activity was quantified with *o*-phenylenediamine which produces a colorimetric signal. Samples were serially diluted in assay diluent so that the absorbance reading fell within the range of the assay (5–320 ng/mL).

A sandwich Protein A ELISA was used to determine leached protein A concentrations in the pools. Chicken anti-staphylococcal protein A antibodies were immobilized on microtiter plate wells. Samples were diluted to 0.2 mg/mL antibody and applied to the wells. Protein A, if present in the sample, bound to the coat antibody. Bound protein A was detected with horseradish peroxidase conjugated anti-protein A antibodies. Horseradish peroxidase enzymatic activity was quantified with *o*-phenylenediamine.

An ELISA using guinea pig anti-insulin polyclonal antibodies was used to determine insulin concentrations in all of the pools. For the ELISA, affinity purified anti-insulin antibodies were immobilized on microtiter plate wells. Dilutions of the pool samples were incubated in the wells, followed by incubation with horseradish

peroxidase-conjugated guinea pig anti-insulin antibodies. The horseradish peroxidase enzymatic activity was quantified with tetramethyl benzidine. Samples were serially diluted in assay diluent so that the absorbance reading fell within the range of the assay (0.094–3.000 ng/mL).

A competitive ELISA was used to determine gentamicin concentrations in all of the pools. Goat polyclonal antibodies to gentamicin-BSA were immobilized on microtiter plate wells. Gentamicin competed with biotinylated-gentamicin for binding to the antibodies. The amount of bound biotin-labeled gentamicin was detected with the addition of horseradish peroxidase-streptavidin and *o*-phenylenediamine substrate. Samples were serially diluted in assay diluent so that the absorbance reading fell within the range of the assay (0.37–90 ng/mL).

The method for DNA quantification used PCR to amplify Chinese hamster ovary DNA. DNA from samples and controls were extracted using Qiagen's Viral RNA Mini kit. The extracts and standard curve, along with PCR master mix containing primers and probe, were then loaded in a 96-well plate format onto ABI's sequence detection system, where the DNA was quantified using real-time PCR. TaqMan PCR employs primers and probe that are designed specific to the target Chinese hamster ovary DNA sequence. Product amplification was measured using a fluorogenic probe, labeled with a reporter dye at the 5′ terminus, which is suppressed by a quencher dye at the 3′ terminus. Taq polymerase begins amplification of the target DNA and upon reaching the probe, its 5′ nuclease activity displaces the probe, releasing the reporter dye. With the reporter dye no longer in proximity to the quencher dye, the reporter fluoresces, and the increase in emission intensity is measured. Cycle numbers at which DNA has amplified past the threshold are calculated for the standard curve, against which unknown sample concentrations are quantified. Samples were serially diluted in assay diluent so that the fluorescence readings fell within the range of the assay (1–10,000 pg/mL).

Biological activity of the monoclonal antibody was measured using a complement dependent cytotoxicity assay. The assay is based on the ability of the antibody to lyse human B lymphoblastoid (WIL2-S) cells in the presence of human complement. WIL2-S cells were incubated with monoclonal antibody standard, controls, and samples in the presence of a fixed amount of human complement. The plate was then incubated at 37°C in a humidified incubator, during which time the Fab region of the monoclonal antibody bound to the target receptor on the WIL2-S cells, and the Fc region bound to the C1q component of complement. This sequence of events activated complement, leading to cell lysis and death through enzymatic steps. At the end of the incubation, the remaining live cells were quantified by adding alamar blue, a redox dye that fluoresces at an excitation wavelength of 530 nm and an emission wavelength of 590 nm when reduced by live cells. The results, expressed in relative fluorescence units, were plotted versus the log of the antibody concentrations using a 4-parameter curve fitting program, and the antibody concentrations/potency were calculated from the standard curve.

It has been reported that PVS is a potent RNase A inhibitor (Smith et al., 2003). An RNase A inhibition assay was developed by modifying Ambion's RNase Alert Kit to quantify levels of PVS in purification pools. The assay used an RNA analog with a fluorescent label on one end and a quencher on the other. Once the RNA analog was cleaved by RNase A, the fluorescent label was released from the quencher. The released label was quantified by measuring fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The presence of PVS inhibited RNase A activity, limiting fluorescent emission. The amount of PVS was determined by comparing the observed fluorescence from a test sample to a standard curve with a 4-parameter fit.

Results and Discussion

During the purification of therapeutic antibodies, impurities including host cell impurities, process impurities and antibody variants must be removed while maintaining a high antibody yield. Antibody precipitation using polyelectrolytes must meet these criteria and provide adequate throughput in order to replace column chromatography in antibody purification processes. We investigated the impact of polyelectrolyte properties and solution conditions on yield and impurity clearance, integrating a precipitation step into the purification process for a therapeutic antibody.

Identification of Precipitation Conditions

We identified conditions under which polyelectrolytes caused protein precipitation through the generation of solubility curves. In the presence of the polyelectrolytes, we determined protein solubility (C/C_0) from protein concentration remaining in solution following precipitation (C) divided by initial protein concentration (C_0) expressed as a percentage. We expressed the concentration of polyelectrolyte in terms of weight of polyelectrolyte to weight of protein. Protein precipitation decreased as we approached the isoelectric point (p*I*) of the proteins tested. With increasing pH, lower ionic strength was required to achieve complete precipitation. Increasing polyelectrolyte molecular weight allowed the precipitation to be performed at higher solution ionic strength.

We investigated the impact of precipitation pH on protein solubility with bovine serum albumin and three monoclonal antibodies (Fig. 3). Bovine serum albumin with a p*I* of 4.7 was included in the study to broaden the range of protein p*I*s tested. The monoclonal antibodies tested had basic p*I*s between 7.6 and 9.2. At a solution pH more than one pH unit below the protein p*I*, protein–polyelectrolyte interactions led to the formation of an insoluble protein–

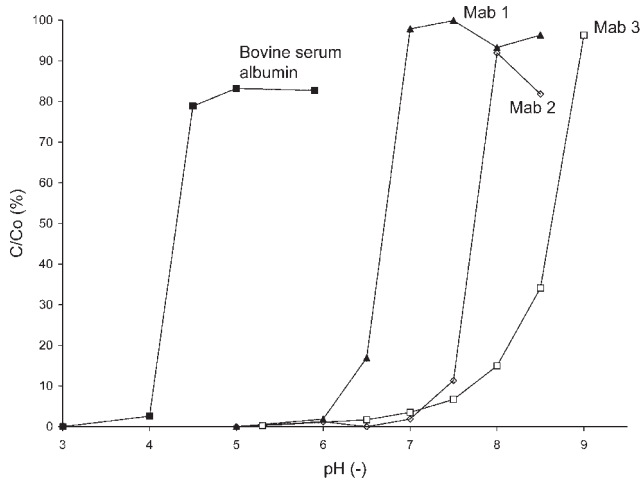

Figure 3. The effect of pH on precipitation for bovine serum albumin (p*I* of 4.7, 66 kDa), Mab 1 (p*I* of 7.6, 150 kDa), Mab 2 (p*I* of 7.9, 150 kDa) and Mab 3 (p*I* of 9.2, 150 kDa) determined at 0.05–0.12 $g_{PVS}/g_{protein}$ at a conductivity of $<$1.5 mS/cm with an initial protein concentration ranging from 17 to 21 g/L.

polyelectrolyte complex. Under these conditions, 0.05–0.12 $g_{PVS}/g_{protein}$ was sufficient to achieve complete precipitation. As solution pH approached the p*I* of either bovine serum albumin or the antibodies, protein solubility increased. Proteins with a lower p*I* were soluble at a lower pH in the presence of PVS. With increasing pH, charge neutralization of the protein likely reduced its net positive charge, reducing the protein–polyelectrolyte interaction and decreasing the amount of precipitation.

Within the pH range of 5–7, we investigated the impact of ionic strength and PVS concentration on antibody solubility (Fig. 4a–c). At each pH, precipitation increased with increasing PVS concentration until antibody solubility reached a minimum. At a solution pH of 5, the antibody fully precipitated from solution under the two ionic strengths evaluated. By contrast, the antibody did not fully precipitate from solution at pH 6 and 7 until the solution ionic strength was reduced to 1.5 mS/cm at pH 6 and 0.7 mS/cm at pH 7. As solution pH increased, the net positive charge on the antibody decreased. Under these conditions of reduced positive charge, the ionic strength of the solution played a more significant role in shielding the remainder of the positive charge on the antibody, preventing the formation of the insoluble antibody–PVS complex. Reducing solution ionic strength was therefore required to achieve complete precipitation. We further investigated the impact of solution ionic strength using two molecular weights of PAA, 1 and 8 kDa at pH 7 (Fig. 4d). At pH 7, the 1 kDa PAA was not as effective at precipitating the antibody as the 3 kDa PVS polyelectrolyte. At both 1.5 and 3 mS/cm, antibody solubility was lower in the presence of PVS. By increasing the PAA molecular weight to 8 kDa, we achieved complete precipitation at 1.5 mS/cm. At 3 mS/cm, the larger

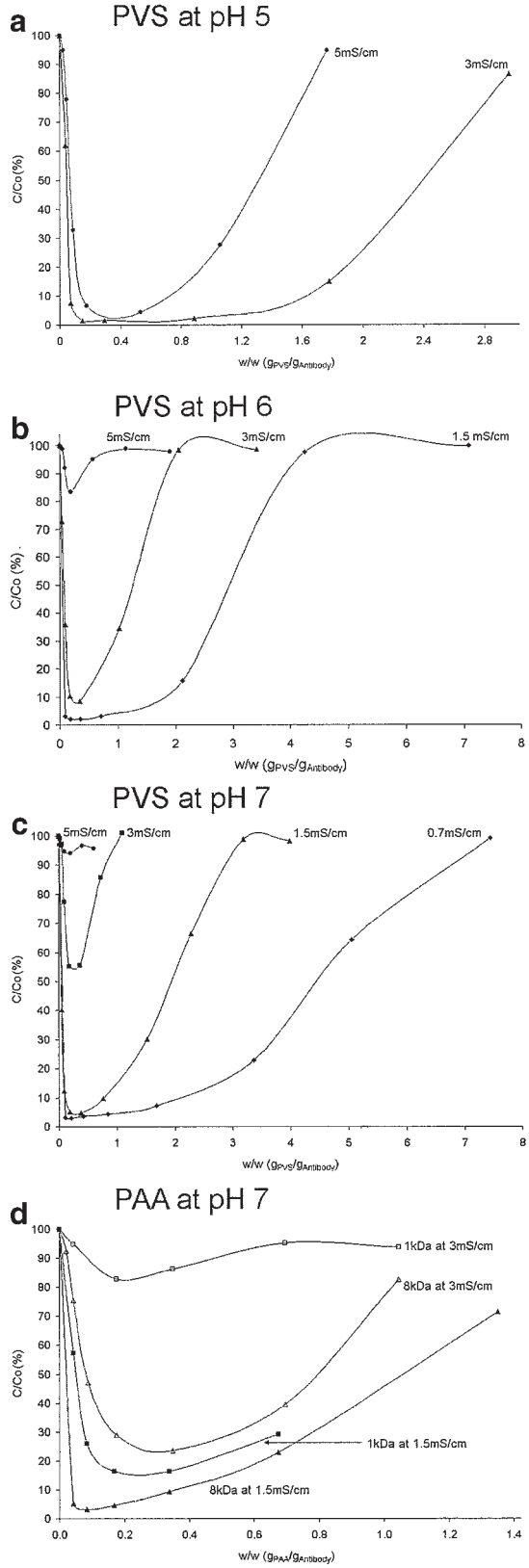

Figure 4. Solubility curves for Mab 4 (p*I* of 9.0) with PVS at (**a**) pH 5, (**b**) pH 6, (**c**) pH 7, and (**d**) with PAA at pH 7, using protein A purified antibody within a conductivity range of 5 to 0.7 mS/cm.

molecular weight PAA was more effective at precipitating the antibody. However we still did not achieve complete precipitation with the 8 kDa PAA. The PAA–antibody precipitate tended to be gel-like in nature, in contrast to the more powder-like PVS–antibody precipitate.

For both PVS and PAA, polyelectrolyte concentration also influenced antibody solubility over the range of solution pH and ionic strength tested (Fig. 4a–d). The formation and resolubilization of the insoluble antibody–polyelectrolyte complex is likely an equilibrium reaction driven by the addition of polyelectrolyte. At each pH and ionic strength tested, increasing polyelectrolyte concentration increased antibody solubility once minimum antibody solubility had previously occurred. This increase in antibody solubility was likely due to electrostatic repulsion between an excess of polyelectrolyte in solution and polyelectrolyte bound to the antibody. This electrostatic repulsion prevented the formation of the insoluble antibody–polyelectrolyte complex. This increase in antibody solubility through the addition of excess polyelectrolyte was predicted by Carlsson et al. (2003) using Monte–Carlo simulation to predict polyelectrolyte–lysozyme interactions. The disruption of the antibody–polyelectrolyte complex through increasing solution ionic strength or through increasing polyelectrolyte concentration could be exploited during the resolubilization process.

We further evaluated the impact of polyelectrolyte molecular weight on antibody solubility using PSS under high ionic strength conditions at pH 7. PSS has the same sulfonic functional group as PVS while also having an aromatic ring on each subunit (Fig. 1). Both the sulfonic acid functional group and the aromatic ring likely play a role in forming the antibody–polyelectrolyte complex. We had previously observed that PAA and PVS with molecular weights of 1–3 kDa respectively required a solution conductivity of $\leq$1.5 mS/cm to achieve significant antibody precipitation at pH 7 (Fig. 4c and d). At a solution ionic strength of 12 mS/cm, PSS with molecular weights of 2 and 5 kDa did not precipitate the antibody even out to 0.35 $g_{PSS}/g_{Antibody}$ (Fig. 5). However, with increasing PSS molecular weight, precipitation increased until complete precipitation occurred with a 220 kDa polyelectrolyte at 0.30 $g_{PSS}/g_{Antibody}$. As a molar ratio, the moles of 220 kDa PSS per moles of antibody required to achieve complete precipitation was 0.2. This suggests that multiple antibodies were complexing with a single polyelectrolyte chain. As the molecular weight of the PSS increased, the precipitate became gel-like in appearance.

Solubility curves identified solution conditions and polyelectrolyte concentrations under which the antibody precipitated for the three polyelectrolytes. For PVS and PAA, the solubility curves also indicated several options for the resolubilization of the antibody: increasing solution pH, increasing solution ionic strength and the addition of excess polyelectrolyte. The addition of excess polyelectrolyte was not pursued as we would need to remove the excess polyelectrolyte on subsequent purification steps. We selected a resolubilization buffer at pH 8.0 with a conductivity of approximately 7 mS/cm (50 mM Tris, 50 mM acetate). This buffer system allowed us to directly load the resolubilized pool onto an anion exchange chromatography step that was used to remove residual polyelectrolyte. Resolubilization with this buffer allowed us to achieve resolubilized antibody pools with antibody concentrations up to 35 g/L. The resolubilization of the antibody between 4 and 35 g/L did not have a negative impact on percent purity by SEC (Table I). Due to the high salt tolerance of the PSS–antibody complex, additional development would be required to identify resolubilization conditions that did not involve the addition of excess PSS. Based on the gel-like nature of the PSS–antibody precipitate and the need for additional development of a resolubilization buffer, PSS was not evaluated further.

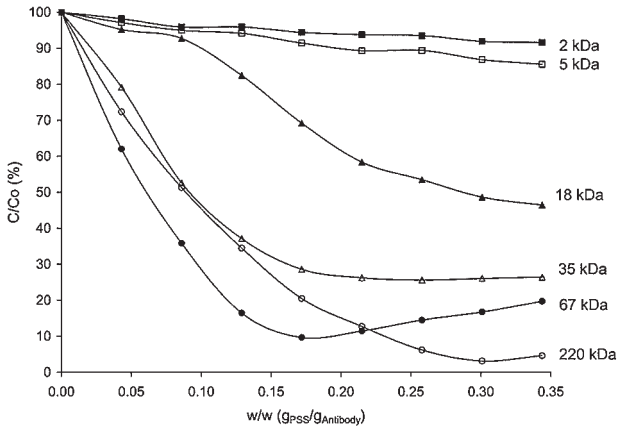

Figure 5. The impact of PSS molecular weight on antibody precipitation using protein A purified antibody (Mab 3) at pH 7 and a conductivity of 12 mS/cm.

Initial Evaluation of Impurity Clearance

Solubility curves identified conditions under which the antibodies precipitated. Using those conditions, we evaluated the impact of precipitation pH as well as polyelectrolyte type and molecular weight on impurity clearance. With increasing pH, antibody purity improved. By contrast, with increasing polyelectrolyte molecular weight, antibody purity decreased. Based on these results, we incorporated a PVS precipitation step at pH 7 into the purification scheme

Table I. The impact of antibody resolubilization concentration on percent purity by SEC for Mab 4.

Resolubilized concentration (mg/mL)	Monomer by SEC (%)
4	98.9
6	98.9
12	99.0
30	99.1
35	99.2

for a therapeutic antibody where it reduced host cell impurities, process impurities and antibody variants such as antibody aggregates and fragments.

Using precipitation conditions identified from the solubility curves, we evaluated the ability of PVS and the two molecular weights of PAA to precipitate and purify a partially purified antibody (Table II). The PVS precipitation step was high yielding. By contrast, we observed a reduced yield for PAA precipitation with both molecular weights tested. Like the PSS–antibody complex, the PAA–antibody complex was gel-like in nature. The difficulty in manipulating this gel, in particular due to its sticky nature, likely contributed to the reduced yield. Precipitating the antibody with either PVS or PAA did not increase antibody aggregates in the resolubilized pools as measured by SEC. The three polyelectrolytes tested reduced HCP and leached protein A. There was a 4.5-fold decrease in HCP with PVS precipitation using a 3 kDa PVS polyelectrolyte and a 6.7-fold decrease in HCP with a 1 kDa PAA polyelectrolyte. Increasing PAA molecular weight from 1 to 8 kDa reduced the fold clearance of HCP from 6.7- to 2.8-fold in the resolubilized pool. Increasing polyelectrolyte weight may increase the incorporation of HCP into the insoluble antibody–polyelectrolyte complex. The increase in HCP with increasing molecular weight of PAA suggests there may be a trade off between operating the precipitation step at higher ionic strengths with larger polyelectrolytes and reduced impurity clearance with those larger polyelectrolytes. Since the low molecular weight PAA and PVS displayed similar impurity clearance, we moved forward with PVS based on the higher resolubilization yield and the more desirable precipitate characteristics; that is a powder-like precipitate rather than a gel-like precipitate, which may be easier to separate during downstream processing.

Table II. The impact of polyelectrolyte molecular weight on yield and impurity clearance for Mab 4 at pH 7.

	Protein A pool	Resolubilized pool (3 kDa)
PVS		
Yield (%)	NA	100
HCP (ng/mg)	1,105	240
Protein A (ng/mg)	23	4
Monomer (%)	98.9	99.2

		Resolubilized pool	
	Protein A pool	1 kDa	8 kDa
PAA			
Yield (%)	NA	80	90
HCP (ng/mg)	1,098	160	390
Protein A (ng/mg)	5	<2	<2
Monomer (%)	99.1	99.2	99.1

All precipitations and analytics were performed according to the procedures outlined in the Experimental Section. The precipitations were performed at conditions to achieve complete antibody precipitation. PVS precipitation was performed at 0.19 $g_{PVS}/g_{Antibody}$ at 0.7 mS/cm. For PAA, the precipitations were performed at 0.19 $g_{PAA}/g_{Antibody}$ at 0.8 mS/cm for the 1 kDa PAA and 0.10 $g_{PAA}/g_{Antibody}$ at 1.5 mS/cm for the 8 kDa PAA.

With PVS, we investigated the impact of precipitation pH on impurity clearance between pH 5 and 7 using both protein A purified antibody and antibody in HCCF (Table III). At each pH, yield after resolubilization was greater than 90%. With increasing pH, the level of HCP decreased in the resolubilized pools for the three antibodies tested. We observed this decrease with increasing precipitation pH whether the starting material was HCCF or partially purified antibody. The majority of HCP expressed in Chinese hamster ovary cell culture fluid tend to have acidic p*I*s (Champion et al., 1999). As we increased pH, we crossed or approached the p*I* of many HCP. Under these conditions, some populations of HCP were negatively charged. These HCP should not interact with the negatively charged PVS. Other HCP populations likely had a reduced positive charge. In this case, the ionic strength of the solution may have shielded the remainder of the charge on these HCP, preventing their integration into the insoluble complex. Although solution conditions may have prevented the formation of a complex with PVS, some HCP may have become non-specifically incorporated during the precipitation event. In addition, other HCP likely retained sufficient

Table III. The impact of precipitation pH on yield and impurity clearance for PVS precipitation of three antibodies.

	Starting pool (protein A pool)	Resolubilized pool		
		pH 5.5	pH 6.0	pH 7.0
Mab 2				
Yield (%)	NA	95	94	91
HCP (ng/mg)	507	220	210	120
Monomer (%)	ND	ND	ND	ND

	Starting pool (protein A pool)	Resolubilized pool		
		pH 5.0	pH 6.0	pH 7.0
Mab 4				
Yield (%)	NA	100	93	99
HCP (ng/mg)	2,344	1,440	520	60
Monomer (%)	97.2	98.4	99.0	99.0

	Starting pool (HCCF)	Resolubilized pool		
		pH 5.0	pH 6.0	pH 7.0
Mab 3				
Yield (%)	NA	99	92	93
HCP (ng/mg)	120,600	14,950	4,260	2,610
Monomer (%)	NA	97.3	98.9	99.2

All precipitations and analytics were performed according to the procedures outlined in the Experimental Section. The precipitations were performed at conditions to achieve complete antibody precipitation. For Mab 2, protein A pool was conditioned to 5 mS/cm, 0.21 $g_{PVS}/g_{Antibody}$ at pH 5.5; 3 mS/cm, 0.42 $g_{PVS}/g_{Antibody}$ at pH 6; 0.8 mS/cm, 2.13 $g_{PVS}/g_{Antibody}$ at pH 7. For Mab 4, protein A pool was conditioned to 3 mS/cm, 0.18 $g_{PVS}/g_{Antibody}$ at pH 5; 1.5 mS/cm, 0.18 $g_{PVS}/g_{Antibody}$ at pH 6; 0.7 mS/cm, 0.18 $g_{PVS}/g_{Antibody}$ at pH 7. For Mab 3, HCCF was conditioned to 3.5 mS/cm, 0.25 $g_{PVS}/g_{Antibody}$ at pH 5; 2 mS/cm, 0.51 $g_{PVS}/g_{Antibody}$ at pH 6; 1.5 mS/cm, 0.57 $g_{PVS}/g_{Antibody}$ at pH 7.

positive charge to complex with PVS forming an insoluble complex. As we increased the precipitation pH, percent purity by SEC also increased for Mab 3 and Mab 4 (Table III). The increase in percent purity was due to the clearance of antibody aggregates and fragments. We speculate that these antibody variants were cleared due to differences from the antibody in p*I* or in surface charge. Based on impurity clearance, we further evaluated PVS precipitation at pH 7 in the purification schemes outlined in Figure 2, where it was directly compared to chromatography based purification schemes.

Comparison of Precipitation to Chromatography

During antibody purification, we use cation exchange chromatography as an intermediate purification step to reduce HCP, leached protein A, DNA, gentamicin as well as antibody aggregates (Fahrner et al., 2001). We evaluated PVS precipitation as a replacement for the cation exchange step, in particular as a replacement for SP-Sepharose Fast Flow chromatography (Table IV). This allowed us to directly compare sulfonic acid based purification through precipitation to sulfonic acid based purification on a chromatography resin. Both the cation exchange step and the precipitation step were high yielding ($\geq$95%). Leached protein A and gentamicin were reduced across the precipitation step and cation exchange step to similar levels. PVS precipitation removed antibody fragments that were not removed by the cation exchange step as shown by SEC. DNA clearance could not be assessed across the precipitation step because residual PVS interfered with the PCR-based assay used to detect DNA. Insulin was at a low level in the protein A pool so its clearance could not be assessed.

Following the intermediate purification step, we purified the resolubilized precipitation pool and the cation exchange chromatography pool across an anion exchange chromatography step in flow through mode. During antibody purification, we use anion exchange chromatography as a polishing step where the anion exchanger binds trace levels of impurities such as HCP and DNA while allowing the antibody to flow through (Fahrner et al., 2001). Prior to loading onto the anion exchange column, the cation exchange pool required a pH adjustment as well as a dilution to reduce load conductivity. By contrast, with the precipitation step, we loaded the resolubilized pool in 50 mM Tris, 50 mM acetate pH 8.0 directly onto the anion exchange column. The elimination of these adjustment steps allowed easy integration of the precipitation step with the subsequent anion exchange step.

With its cationic functional chemistry, the anion exchange step can also remove residual PVS in the resolubilized antibody pool. Smith et al. (2003) previously demonstrated the removal of PVS (called oligovinylsulfonic acid) from contaminated ethanesulfonic acid buffers using anion exchange chromatography. As measured by the RNase inhibition assay, PVS was reduced to <1 μg/mg across the anion exchange step. Comparing the anion exchange pools for the PVS precipitation step and the cation exchange chromatography step, both pools had similar levels of HCP, leached protein A, insulin and DNA as well as antibody aggregates and fragments. The anion exchange pool from the PVS precipitation arm of the study has slightly higher levels of gentamicin. The biological activity of the antibody was not impacted by the inclusion of a precipitation step.

Based on purification performance, the PVS precipitation step was an effective replacement for the cation exchange chromatography step tested. The successful implementation of a precipitation step at larger scales requires a scalable method for capturing, washing and resolubilizing the precipitate. However, there is the potential that a precipitation step could give the same purification as

Table IV. Comparing PVS precipitation to cation exchange chromatography as an intermediate purification step for Mab 4.

		Intermediate pools		AEX pools	
	Protein A pool	CEX	PVS pool	Protein A-CEX–AEX	Protein A-PVS–AEX
Yield (%)	95	95	100	96	92
Impurities					
HCP (ng/mg)	1,100	200	240	<0.7	1.0
Protein A (ng/mg)	23	<2	4	<2	2
Insulin (ng/mg)	0.002	<0.03	<0.06	<0.06	<0.08
DNA (pg/mg)	200	<0.2	ND	<0.3	<0.4
Gentamicin (ng/mg)	9	0.3	1.3	0.2	1.3
Size exclusion					
Aggregate (%)	0.8	0.8	0.7	0.7	0.7
Monomer (%)	98.9	98.9	99.3	99.3	99.3
Fragment (%)	0.3	0.2	0.0	0.0	0.0
PVS (μg/mg)	NA	NA	291	NA	<1
Biological activity (%)	ND	ND	ND	99 ± 2	99 ± 2

All purifications and analytics were performed according to the procedures outlined in the Experimental Section. For the precipitation step, the protein A pool was adjusted to pH 7, 0.7 mS/cm. PVS was added to a final concentration of 0.19 $g_{PVS}/g_{Antibody}$.

NA, not applicable; ND, not determined; CEX, cation exchange; AEX, anion exchange.

intermediate chromatography steps without the throughput constraints of particular chromatography resins that are dictated by maximum resin load density.

We further evaluated PVS precipitation as an antibody capture step from HCCF (Table V). With higher levels of host cell impurities (HCP and DNA), process impurities (insulin and gentamicin) than the partially purified protein A pool, HCCF was a more challenging feedstock on which to test the precipitation step. We compared PVS precipitation to both an affinity capture step using protein A chromatography and a cation exchange chromatography capture step.

HCP were reduced across the precipitation step but not to the same level as either the protein A step or the cation exchange step. HCP may have been incorporated into the precipitate through ionic interactions with PVS. Alternatively, HCP may be incorporated into the precipitate in a non-specific manner during the precipitation event. With this more challenging feedstock, more stringent washing of the precipitate may reduce HCP in the resolubilized pool. Even at a low load density of 10 g/L_{resin}, the cation exchange capture step was not as effective at removing HCP as the protein A step. In both cases, non-affinity purification of the antibody distributed the removal of HCP across all the purification steps in the process. This is in contrast to the protein A based purification where the majority of HCP clearance occurred across that step. In this scenario, the subsequent chromatography steps typically remove low levels of host cell impurities as well as antibody related impurities such as aggregates or process impurities such as leached protein A.

The cation exchange capture step was more effective than protein A chromatography at removing host cell DNA. Although DNA clearance could not be assessed across the precipitation step, there were similar levels of DNA in all anion exchange pools.

Both the cation exchange capture and PVS capture steps reduced insulin to similar levels as the protein A step. The p*I* of insulin is 5.3. At a precipitation pH of 7, insulin was negatively charged and remained in solution while PVS formed an insoluble complex with the antibody and other impurities. On the cation exchange step, insulin was likely removed during the wash phases at either pH 5.5 or 7.

Gentamicin was reduced across the precipitation step but not to the same level as either the protein A step or the cation exchange step. There was a 13-fold reduction in gentamicin across the precipitation step. The question remains as to why gentamicin was not completely removed by the precipitation step. Gentamicin sulfate (LEK Pharmaceuticals, Wilmington, NC) used in the cell culture process consists of four forms: C1 ($C_{21}H_{43}N_5O_7$), C2 ($C_{20}H_{41}N_5O_7$), C1a ($C_{19}H_{39}N_5O_7$), and C2a ($C_{20}H_{41}N_5O_7$). Using the Grid Molecular Interaction Fields method (Milletti et al., 2007), we calculated the pK_a of these four forms. Gentamicin sulfate has a total of eight pK_a varying from pH 4.96 to 14.12. Of these eight pK_a, only two vary between the four forms of gentamicin sulfate. The first and most significant variation is the nitrogen directly attached to the chiral center where the pK_a varied from 8.27 to 8.74. The second variation was at the NH_2 in position 28 (attached to the same ring as the chiral center but directly opposite) where the pK_a varied from 7.32 to 7.47. We speculate that certain forms of gentamicin with higher pK_a are more difficult to clear using ion exchange based purification such as PVS precipitation. This theory is supported by the fact that gentamicin was also significantly reduced but not removed across the cation exchange chromatography capture step. The precipitation step and cation exchange step may be selecting for gentamicin with a higher pK_a and greater net positive charge. This is further supported by the lack of gentamicin clearance on the anion

Table V. Comparing PVS precipitation to affinity and cation exchange chromatography capture steps for Mab 4.

		Capture Step			AEX Pools			
	HCCF	Protein A	CEX	PVS	Protein A–AEX	CEX–AEX	PVS–AEX	PVS–AEX–CEX
Yield (%)	NA	95	82	82	92	100	95	102
Impurities								
HCP (ng/mg)	234,000	1,100	4,250	27,400	4	71	360	18
Protein A (ng/mg)	NA	23	NA	N/A	23	NA	NA	NA
Insulin (ng/mg)	2.41	0.002	<0.09	0.01	<0.05	<0.15	<0.19	<0.03
DNA (pg/mg)	3,000,000	200	0.55	ND	<0.3	1.4	4.4	<0.18
Gentamicin (ng/mg)	25,500	9	146	2,000	5	170	1,200	4
Size exclusion								
Aggregate (%)	ND	0.8	0.3	3.6	0.6	0.3	1.2	1.0
Monomer (%)	ND	98.9	90.4	95.9	99.1	91.0	98.8	98.8
Fragment (%)	ND	0.3	9.2	0.5	0.3	8.7	0.0	0.2
PVS (μg/mg)	NA	NA	NA	255	NA	NA	<1	ND
Biological activity (%)	ND	ND	ND	ND	102 ± 2	87 ± 10	N/D	95 ± 0

All purifications and analytics were performed according to the procedures outlined in the Experimental Section. For the precipitation step, HCCF was adjusted to pH 7, 0.7 mS/cm. PVS was added to a final concentration of 0.76 $g_{PVS}/g_{Antibody}$. The PVS–AEX pool was purified through an additional CEX step to achieve further purification.

NA, not applicable; ND, not determined; CEX, cation exchange; AEX, anion exchange.

exchange step from the cation exchange capture step and the partial reduction across the anion exchange step from the precipitation step. At pH 8, gentamicin with a pK_a of 8.27 may bind to the anion exchange column, whereas gentamicin with a pK_a of 8.74 (similar to the pI of the antibody) would likely flow through with the antibody.

The process impurity leached protein A was not removed across the two step protein A-anion exchange process. By contrast, residual PVS was removed across the two step PVS-anion exchange process. To meet a similar standard of purity as the protein A capture process, we incorporated an additional purification step (cation exchange chromatography) to further reduce HCP and gentamicin in the precipitation capture portion of this study. The final cation exchange pool in the precipitation capture portion of the study had similar biological activity to the protein A based chromatography processes (either protein A-anion exchange or protein A-cation exchange–anion exchange from Table IV).

Antibody in the protein A pool had a high percent purity of 98.9% as measured by SEC. By contrast, the cation exchange capture step had 90.4% purity due to the presence of 9.2% fragments. These fragments were not removed through the subsequent anion exchange step. These fragments may arise from the co-purification of a host cell protease on the cation exchange step. This high fragment level was reflected in a reduced biological activity of 87%. This data confirmed that a decrease in product quality could result in reduced biological activity. The PVS precipitated pool had 3.6% aggregates that were removed through the subsequent anion exchange step. Anion exchange chromatography operated in flow through mode typically does not remove antibody aggregates except under conditions of weak partitioning not used in this case (Kelley et al., 2008). These antibody aggregates may have been associated with residual PVS that bound to the anion exchanger while the antibody monomer flowed through.

As expected, the ion exchange based precipitation was not as effective as the affinity based protein A chromatography as a capture step. Nevertheless, a three step purification process including a precipitation capture step did have similar purification performance to the three step protein A-cation exchange–anion exchange process. It should be noted that the protein A capture arm of the study also required three purification steps, in particular a cation exchange step to remove leached protein A. Since an equivalent number of purification steps were required to achieve similar levels of all the impurities tested, there may be a potential cost advantage to replacing protein A chromatography with a precipitation step, especially when using inexpensive polyelectrolytes such as PVS. A comparison of the total cost of purification through precipitation versus chromatography should be performed once a scalable method for performing solid–liquid separation has been identified. Continuous centrifugation and filtration are both feasible approaches for the large scale capture and washing of antibody precipitates. Venkiteshwaran et al. (2008) recently demonstrated the use of crossflow membrane microfiltration as a method of capturing and resolubilizing antibody precipitates. Ge et al. (2006) also used microfiltration for the capture of precipitated elastin-like fusion protein.

Summary

Through the generation of solubility curves, we identified conditions of pH and ionic strength under which monoclonal antibodies precipitated in the presence of polyelectrolytes. With increasing precipitation pH, solution ionic strength inhibited precipitation through disruption of the antibody–polyelectrolyte interaction. Increasing polyelectrolyte molecular weight allowed the precipitation step to be operated at higher ionic strengths. However, using larger polyelectrolytes reduced HCP clearance and increased the potential for the formation of gel-like precipitates. As precipitation pH increased, HCP decreased and percent antibody purity by SEC increased. There is likely a compromise between precipitation pH and polyelectrolyte molecular weight that will allow us to perform the precipitation at higher ionic strengths while still achieving an acceptable level of purification.

We evaluated PVS precipitation as an intermediate purification step and as a capture step for the purification of therapeutic antibodies. PVS precipitation reduced host cell impurities such as HCP and DNA, process impurities such as leached protein A, insulin and gentamicin as well as product variants such as antibody aggregates and fragments. Precipitation did not have a negative impact on the biological activity of the antibody tested. Anion exchange chromatography removed residual PVS. We successfully integrated the precipitation step with other purification steps meeting purity levels similar to current chromatography based processes. The successful implementation of a precipitation step at production scale requires a scalable method for capturing, washing, and resolubilizing the precipitate.

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