Continuous Precipitation for Monoclonal Antibody Capture Using Counter-current Washing by Microfiltration

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Submitted to

Biotechnology Progress

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Abstract

There is renewed interest in the possibility of using precipitation for initial capture of high value therapeutic proteins as part of an integrated continuous downstream process. Precipitation is greatly facilitated by the high product titers now achieved in most cell culture processes, in sharp contrast to chromatographic processes whose performance is reduced at high titers. The current study used a combination of reversible cross-linking (zinc chloride, $ZnCl_2$) and volume exclusion (polyethylene glycol, PEG) agents to precipitate a monoclonal antibody product directly from harvested cell culture fluid (HCCF) using a continuous tubular precipitation reactor. The precipitates were then dewatered and continuously washed using tangential flow filtration, with a counter-current staged configuration used to reduce the amount of wash buffer required and increase host cell protein (HCP) removal. Long term operation was achieved by operating the membrane modules below the critical filtrate flux to avoid fouling. Experimental results demonstrate the feasibility of this fully continuous integrated precipitation process at bench-scale, with design calculations used to explore the key factors affecting the performance of this system for initial antibody capture.

Keywords: Precipitation; continuous processing; antibody; microfiltration; staging

Introduction

Precipitation is one of the earliest developed techniques used for large-scale protein purification due to its low-cost and the flexibility to fine-tune the separation based on the choice of precipitation methods. Plasma proteins are still purified using precipitation, now in combination with chromatographic polishing steps, with the different fractions (products) obtained by proper choice of ethanol concentration, solution pH, temperature, and salt concentration.[1,2] This is facilitated by the high concentrations of several key plasma proteins including albumin, IgGs, fibrinogen, alpha-1-antitrypsin, and apoliprotein.[3] However, the very low titer of early recombinant proteins (<0.1 g/L) made target protein precipitation impractical if not impossible. Instead, the initial capture was performed using bind-and-elute chromatography, e.g., Protein A affinity chromatography is currently used for the initial capture of essentially all monoclonal antibody (mAb) products.[4,5]

The significant increase in product titer over the past two decades, with concentrations above 5 g/L now fairly routine for mAbs[6], has led to a renewed interest in the potential use of precipitation for antibody capture. Previous studies have examined the use of cold ethanol and $CaCl_2$[7], polyethylene glycol (PEG) and $CaCl_2$[8], PEG and low pH[9], and $ZnCl_2$ and PEG[10] for antibody precipitation. More recently, Burgstaller et al.[11] achieved high recovery of a monoclonal antibody product from harvested cell culture fluid (HCCF) by continuous precipitation using a combination of 2 mM $ZnCl_2$ with 7% 6 kDa PEG. The precipitate was then concentrated and washed using tangential flow filtration with 0.2 μm pore size hollow fiber membranes. The mAb was recovered in the washed product stream at a yield of 95% with 97% purity, with a significant reduction in high molecular weight impurities (HMWI).

The objective of this study was to examine some of the key design variables in a fully integrated continuous precipitation process for the purification of a mAb using the $ZnCl_2$ – PEG system. However, in contrast to Burgstaller et al.,[11] the $ZnCl_2$ and PEG were added sequentially (instead of at the same time) to better control the yield and morphology of the precipitated mAb. The precipitates were then directly and continuously dewatered using a hollow fiber membrane module without any retentate vessel (hold tank) as used by Burgstaller et al.[11] In addition, the washing step was performed using a 2-stage countercurrent configuration without any $ZnCl_2$ or PEG to significantly reduce the buffer requirements / costs while enhancing removal of host cell proteins (HCPs). We also employed an inline re-solubilization step using a low pH glycine buffer, providing a final product stream that would be suitable for subsequent purification as part of a fully integrated continuous downstream process. Model calculations were used to obtain additional insights into the overall system performance.

Materials and Methods

Cell Culture

Experiments were performed with a monoclonal antibody produced in Chinese hamster ovary (CHO) cells in a 2 L perfusion bioreactor. Cell culture fluid was harvested around day 6 using tangential flow filtration with 0.2 μm pore size hydrophilized polyethersulfone (PES) hollow fiber membranes in a MiniKros® module with 470 cm^2 membrane area (Repligen Corporation, Rancho Dominguez, CA). The mAb titer was around 4.6 g/L as determined by a Cedex Bio HT Analyzer (Roche CustomBiotech, Indianapolis, IN).

Precipitation Conditions

Small-scale batch precipitation experiments were performed to identify appropriate conditions for mAb purification. Data were obtained over a range of $ZnCl_2$ (Sigma-Aldrich, St. Louis, MO, from 2.5 to 20 mM) and PEG with number average molecular weight of 3350 g/mol (Sigma-Aldrich, St. Louis, MO, from 2 to 15 weight percent) concentrations, with the $ZnCl_2$ added first followed approximately 30 s later by the PEG; note that these concentrations are based on the final solution volume after addition of both precipitants. The precipitates were centrifuged and the mAb concentration in the supernatant determined by analytical Protein A chromatography. The mAb yield was calculated by simple mass balance. A more detailed analysis of the effects of $ZnCl_2$ and PEG on the effectiveness of the precipitation will be provided in a future publication.

Continuous Precipitation

The continuous precipitation – dewatering – washing – re-solubilization was conducted using the experimental system shown schematically in Figure 1 consisting of a series of static mixers and hollow fiber membrane modules. The tubular precipitation reactor was constructed from silicone tubing with inner diameter of 0.48 cm = 3/16 inch (Masterflex L/S Platinum-Cured Silicone Tubing, model 96416-15, Cole-Parmer, Vernon Hills, IL) fitted with a static-mixer (Koflo Corporation, Cary, IL). The HCCF was continuously fed to the static mixer through a Y-shaped connector with barbed hose fittings using a peristaltic pump (Watson-Marlow Fluid Technology Group, Wilmington, MA). $ZnCl_2$ was mixed with the HCCF at the Y to initiate precipitate nucleation, with PEG added as a volume exclusion agent between the first and second static mixers to facilitate the growth of the precipitates.

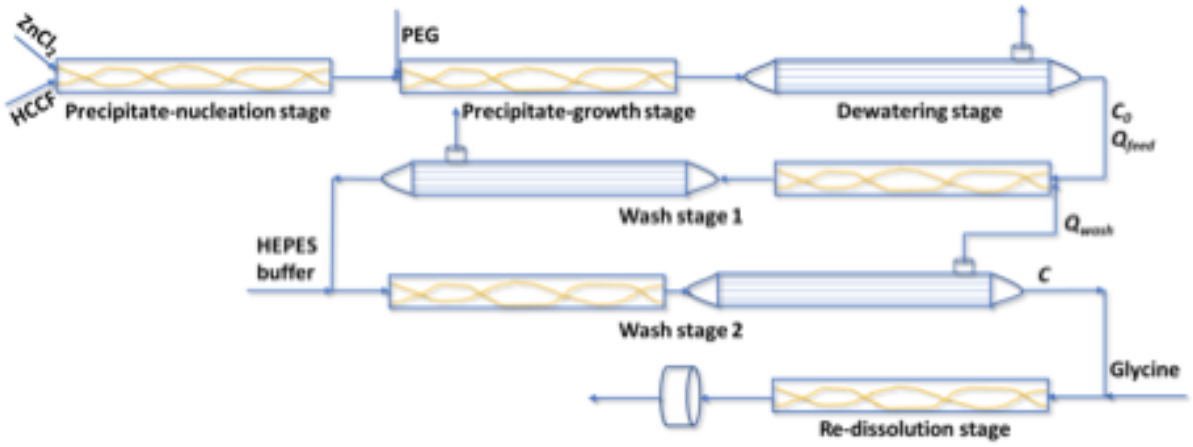

Figure 1. Schematic of continuous precipitation system with 2-stage countercurrent washin

Most continuous precipitation experiments were performed by pumping HCCF into the precipitation reactor at a flow rate of 5 mL/min, where it was combined with 1 mL/min of a 0.1 M $ZnCl_2$ solution. A 17.5 weight percent PEG solution was added between the two static mixers to achieve final concentrations of about 2.3 g/L for the mAb, 10 mM for the $ZnCl_2$, and 7 weight percent for the PEG. The total residence time in the tubular precipitation reactor was only 30 s, which is much smaller than the 2.5 to 22.5 min used by Burgstaller et al.[11]. The more efficient precipitation in this work is likely due to the sequential addition of the $ZnCl_2$ and PEG (these were added simultaneously by Burgstaller et al.); both studies were performed with approximately the same mAb concentration (between 2 and 3 g/L).

The precipitates were concentrated with 0.2 µm hydrophilized polyethersulfone (PES) hollow fiber membranes in a MidiKros® module with 140 cm^2 membrane area (Repligen Corporation, Rancho Dominguez, CA). Soluble impurities were further removed by washing using 50 mM HEPES buffer at pH 7.0 without any added $ZnCl_2$ or PEG. HEPES was used as the buffering species, rather than phosphate or TRIS, as it has minimal interactions with zinc

ions.[12,13] Figure 1 shows the 2-stage counter-current washing format used, with the HEPES buffer added to the second stage and recycled back to the first stage; small static mixers were used between the hollow fiber modules to insure complete mixing of the wash buffer and precipitate. Experiments were also performed with just a single washing stage.

The precipitated protein was re-solubilized by inline addition of 2 M glycine at pH 3.2; the low pH disrupted the crosslinking by the Zn^{2+} causing nearly complete dissolution of the precipitated protein. The re-solubilized mAb was then collected through a double layer Sartoclear® depth filter (DL60, 8 μm/0.8 μm, Sartorius Corporation, Bohemia, NY) to remove any insoluble material.

Critical Flux Experiments

The critical flux for the protein precipitate in the hollow fiber module was evaluated using the flux-stepping procedure described previously by Li and Zydney[14]. The precipitated protein was generated continuously using the tubular precipitation reactor shown in Figure 1, with the critical flux for the precipitation stage evaluated by removing the wash and re-dissolution modules (with the retentate outflow simply discarded). A peristaltic pump (Watson-Marlow Fluid Technology Group, Wilmington, MA) was placed on the permeate exit line from the hollow fiber module to control the filtrate flux, while the transmembrane pressure (TMP) was monitored by placing SciLog pressure sensors (Parker Hannifin Corporation, Cleveland, OH) on both the feed and permeate lines.

Analytics

The mAb concentration and purity were evaluated by size exclusion chromatography (SEC) and a CHOP ELISA. The product purity was calculated from the SEC chromatograms as the ratio of the monomer peak area to the sum of all peak areas. Note that the $ZnCl_2$ appeared to have some interaction with the column, resulting in an unexpected peak tailing as reported previously by Burgstaller et al.[11]

Results and Discussion

Precipitation

High mAb yields (> 97%) were obtained at $ZnCl_2$ concentrations above 15 mM (even in the absence of PEG). The $ZnCl_2$ concentration could be reduced to 10 mM when using 7 weight percent PEG. All subsequent precipitation experiments were performed using these latter conditions.

During continuous precipitation, the solution became turbid immediately after mixing HCCF with the $ZnCl_2$, i.e., just after the entrance to the first static mixer, reflecting the rapid kinetics of precipitation using $ZnCl_2$ as a cross-linking agent. The overall mAb yield for the precipitation step was approximately 97%, with the precipitates remaining stable for at least 24 hr during batch holding (which was the maximum holding time examined). The collected precipitate looked fluffy when observed in a graduated cylinder; it settled to approximately one-half the original volume after 24 hr. The average size of precipitates was on the order of 1 μm based on the initial settling velocity.

Critical flux

In order to insure stable operation of the dewatering and washing steps over extended periods of time, it is desirable to operate the hollow fiber membrane modules below the critical flux, which is defined as the value of the filtrate flux at which membrane fouling first becomes significant, in this case defined as an increase in TMP of more than 50 kPa in 24 hr (i.e., >2 kPa/hr). The critical flux in tangential flow microfiltration is governed by the mass transfer characteristics of the membrane module, possibly in combination with any long-range interactions (e.g., electrostatic repulsion) between the precipitate and the membrane surface[10].

Typical data from the critical flux experiments with the precipitate formed with 10 mM $ZnCl_2$ and 7% PEG in a MidiKros® hollow fiber membrane module containing 0.2 μm hydrophilized polyethersulfone (PES) hollow fiber membranes with 140 cm^2 membrane area are presented in Figure 2. The TMP is shown as a function of time, with each interval corresponding to a different (constant) value of the filtrate flux (labeled directly above the pressure data). The TMP was below 6.9 kPa (1.0 psi) throughout the entire experiment, reflecting the high permeability of the 0.2 μm hollow fiber membrane (around 70 $L/m^2/h/kPa$). The TMP for the first 2 intervals remained essentially constant over each 40-min filtration (variation less than 0.5 kPa/hr); the greater TMP for the periods with higher filtrate flux is as expected. However, the TMP at a permeate flux of 30 $L/m^2/h$ (8.3 μm/s) increased by nearly 1.5 kPa over 24 min, corresponding to a rate of more than 3.7 kPa/hr. Thus, the critical flux for this experiment was greater than 26 $L/m^2/h$, the highest flux at which the TMP remained stable, but below 30 $L/m^2/h$, the first flux at which the TMP showed a clear increase in time during the constant flux filtration.

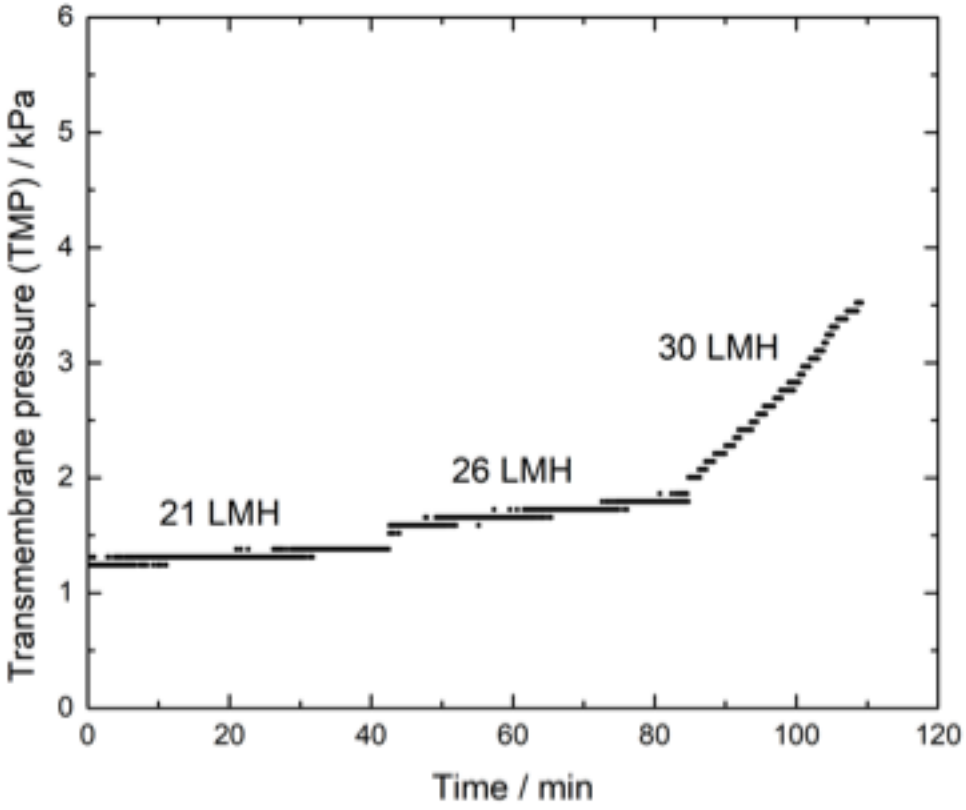

Figure 2. Transmembrane pressure as a function of time during critical flux experiments with precipitate formed from a mixture of 2.3 g/L mAb, 7 weight percent PEG, and 10 mM $ZnCl_2$. The permeate flux values for each interval are labeled above the TMP data.

The critical fluxes for a series of hollow fiber modules, with different number, length, and inner diameter for the hollow fibers, are summarized in Table 1. All of the data were obtained using the same concentrations of mAb, $ZnCl_2$, and PEG as determined previously. In each case, the critical flux was evaluated from the flux-stepping experiments at a feed flow rate of 10 mL/min using the average of the filtrate flux values determined just above and below the critical flux. The critical flux for Module A was 28 $L/m^2/h$. Module B was designed with fewer fibers but with 65 cm fiber length; however, this caused more than a 50% reduction in the critical flux even though Module B had a higher shear rate. This behavior is in contrast to previous

results in which the critical flux increased with increasing shear rate[14], suggesting that the difference in critical flux between Modules A and B was not due to the change in back mass transfer. Instead, the lower critical flux in Module B may be due to the large axial pressure drop in the longer hollow fibers, which was more than 10 times that in Module A (due to the combination of the higher shear rate and the longer fiber length). The large ΔP leads to a high inlet transmembrane pressure, which would in turn cause the local flux near the module inlet to exceed the critical flux for fouling even though the average filtrate flux in Module B was fairly low. Critical flux experiments were thus performed with Module C having fibers with larger ID (1.0 mm) but shorter length. The critical flux in Module C was slightly smaller than that in Module A (24 $L/m^2/h$ vs 28 $L/m^2/h$), which is probably due to the lower shear rate. It was not possible to operate Module C at higher shear rates since this would have required much larger volumetric feed flow rates. Additional studies will be required to fully characterize the critical flux behavior for the precipitated mAb as a function of the module geometry and shear rate.

Table 1. Critical flux of hollow fiber membranes with different fiber geometries. Each module comprised 0.2 μm hydrophilized polyethersulfone (PES) hollow fiber membranes.

Module	Fiber ID (mm)	Surface area (cm^2)	Shear rate (s^{-1})	Number of Fibers	Fiber length (cm)	Critical flux ($L/m^2/h$)
A	0.5	140	300	45	20	28
B	0.5	92	1500	9	65	<13
C	1.0	88	120	14	20	24

Based on the results in Table 1, the dewatering step used Module A with the filtrate flux maintained at a value of 26 L/m^2/h, corresponding to a permeate flow rate of 6.1 ml/min. This provided a 2.5-fold increase in the concentration of the precipitated mAb while removing approximately 60% of the host cell proteins (CHOP) and DNA (assuming no retention of either impurity by the membrane).

Washing

Further reductions in the concentration of soluble impurities (e.g. CHOP, DNA, and excess $ZnCl_2$ and PEG) were achieved by "washing" the precipitated mAb, i.e., by diluting with buffer and then re-concentrating the precipitated mAb using a hollow fiber membrane module. In order to reduce costs, and eliminate the need to remove $ZnCl_2$ and PEG later in the downstream process, the washing was performed using HEPES buffer without any $ZnCl_2$ or PEG. Initial experiments used 6 mL/min of HEPES, providing a 2.5-fold dilution of the dewatered feed (which was at a flow rate of 4 mL/min). The wash buffer and precipitate were mixed using a short static mixer, with a residence time around 20 s, followed by re-concentration (back to the initial precipitate flow rate of 4 mL/min) in a separate hollow fiber module, providing additional reduction in CHOP by approximately 60% (again assuming no retention). The permeate from the washing stage was initially clear, but it did become turbid upon incubation overnight, likely due to further precipitation caused by the $ZnCl_2$ remaining in the permeate solution.

Although it would be possible to increase CHOP removal simply by increasing the amount of added buffer, very high dilution ratios would be needed to achieve the high levels of purification typical for an initial capture step based on affinity chromatography, and the large quantity of added buffer could lead to re-solubilization of the precipitated mAb (prior to the

included re-solubilization step) which would reduce the final product yield. Alternatively, the wash buffer can be effectively "re-used" by performing the washing in a counter-current staged configuration as shown in Figure 1. In this case, fresh wash buffer is added into the second stage of the 2-stage washing process, with the permeate from this stage (containing only a low concentration of CHOP) recycled back to the first stage. This type of counter-current staging has been discussed in some detail by Nambiar et al.[11] in the context of diafiltration for mAb formulation and by Dutta et al.[12] in the context of continuous counter-current tangential chromatography (CCTC).

The extent of impurity removal (R) in this type of counter-current staged washing is given as:

$$R = \frac{C_0}{C} = \frac{\alpha^{N+1}-1}{\alpha-1} \quad (1)$$

with

$$\alpha = \frac{Q_{wash}S}{Q_{feed}} \quad (2)$$

and where C_0 is the concentration of soluble impurities in the feed to the washing step, C is the concentration of soluble impurities in the final retentate leaving the washing step, Q_{feed} is the feed flow rate, Q_{wash} is the buffer flow rate used for washing, S is the sieving coefficient (transmission) for the impurity through the hollow fiber membrane, and N is the number of stages (modules) in the washing step. For example, a flow ratio of 9:1 ($\alpha = 9$) in the wash step would provide a 10-fold reduction in the concentration of a soluble impurity for a single stage (assuming $S = 1$). However, a 2-stage system using the same value of α would provide a 91-fold reduction in impurity level and this would be increased to more than 800-fold using a 3-stage system, all with the same quantity of fresh wash buffer.

Figure 3 shows results for the CHOP removal after the continuous integrated dewatering (2.5-fold concentration) and either a 1- or 2-stage washing step using $\alpha = 1.5$ (wash buffer flow rate of 6 mL/min with a feed flow rate of 4 mL/min and assuming $S = 1$). The fold-removal was defined as the ratio of the CHOP mass flow rate in the feed from the bioreactor divided by the CHOP mass flow rate leaving the wash step, with the CHOP concentrations determined by a CHOP ELISA. The CHOP removal for the single stage washing was only R = 6.6, but this increased to R = 10 when using the two-stage counter-current washing. Note that it was not possible to operate at higher values of α using the available hollow fiber membranes without extensive fouling since these high conversions would have required operation at a filtrate flux above the critical flux. The solid curves in Figure 3 are model calculations accounting for the 2.5-fold concentration in the de-watering step, with the degree of CHOP removal in the washing step given by Equation (1) assuming 100% CHOP transmission through the membrane. The experimental results are in good agreement with the model calculations, providing a framework to design / optimize the washing step to achieve the desired degree of CHOP removal. More than 100-fold CHOP removal could be obtained using a 3-stage washing step with $\alpha = 3$. We would expect similar levels of removal for DNA and other low molecular weight species since the 0.2 μm pore size hollow fiber membranes should have minimal retention of all soluble components.

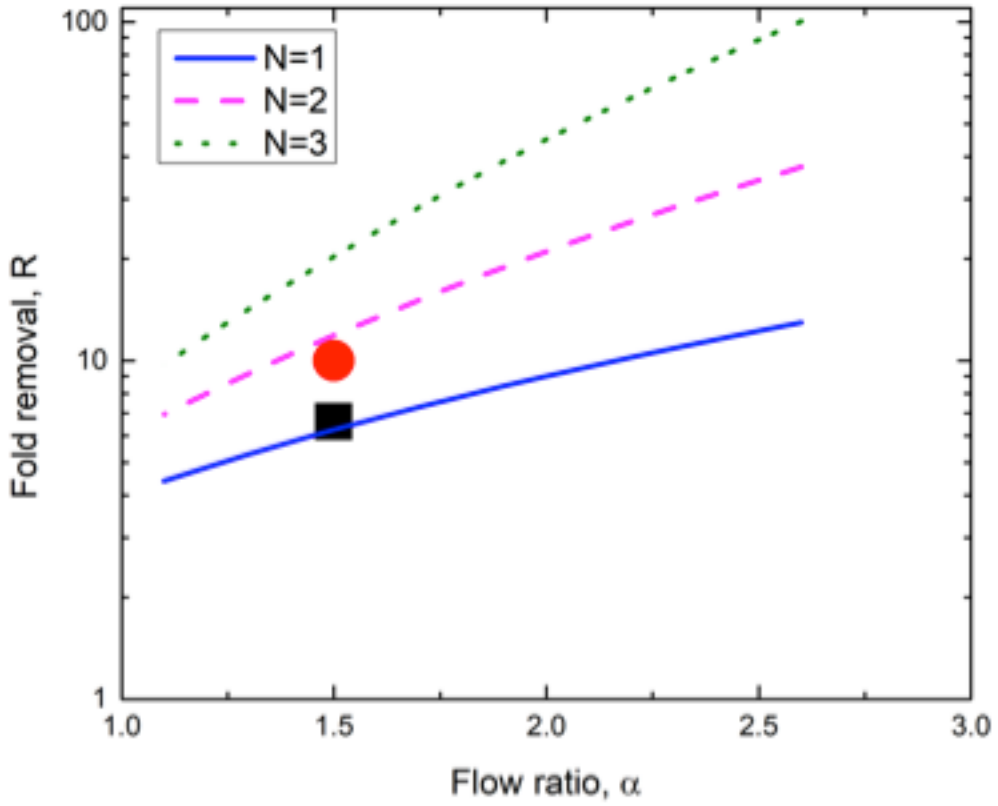

Figure 3. Experimental data and model calculations for CHOP removal after de-watering (2.5-fold concentration) and counter-current washing with 1, 2, or 3 stages.

Re-dissolution

The precipitated mAb was re-solubilized by lowering the pH to disrupt the cross-linking associated with the Zn^{2+} cations. A 2 M glycine buffer at pH 3.2 was mixed inline with the washed precipitate as shown previously in Figure 1. The required amount of re-dissolution buffer was first determined by performing a series of batch titration experiments with results summarized in Figure 4. In this case, the precipitates were collected from the retentate of the dewatering step (without washing), mixed with different amounts of glycine, and the pH and percentage of re-solubilized mAb were evaluated after incubation for 30 minutes. The addition of

higher relative amounts of glycine provided lower pH and in turn greater yield of soluble mAb. More than 90% mAb recovery was achieved when the pH was reduced to below 5.

The continuous re-dissolution was performed using a small static mixer, with length chosen to provide a residence time of around 20 s; this was more than sufficient to obtain a non-turbid solution as determined by visual inspection and then confirmed by dynamic light scattering (DLS). The size distribution of the re-solubilized product, as determined by DLS, was very similar to that seen in the initial HCCF feed. The re-dissolution used a glycine buffer flow rate of 2 mL/min which achieved a pH of 3.5 in the re-solubilized product stream. This low pH would greatly facilitate the implementation of an inline virus inactivation step (immediately after the re-solubilization) using a continuous flow reactor.[13]

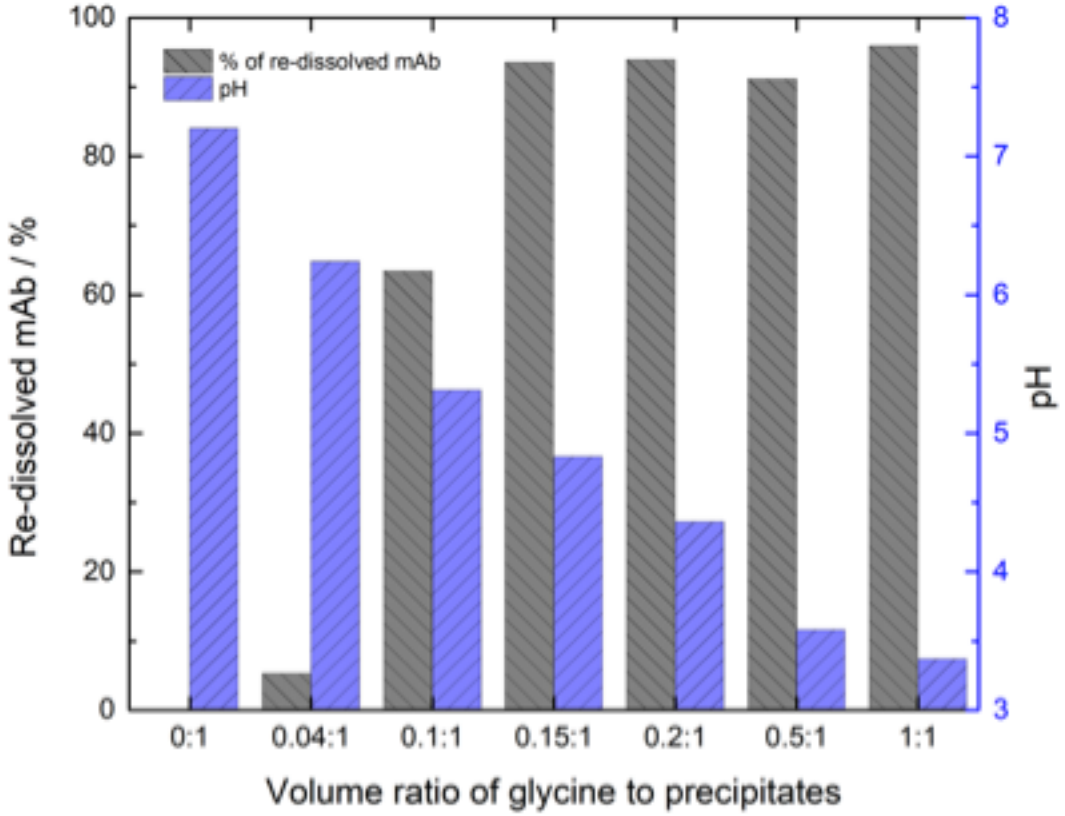

Figure 4. Re-dissolution of precipitated mAb using batch titration with 2 M glycine buffer at pH 3.2

Integrated Continuous Process

The operating conditions for the integrated continuous precipitation – dewatering – washing – re-solubilization process are summarized in Table 2. The washing was conducted using the 2-stage counter-current configuration shown in Figure 1 with $\alpha = 1.5$. The 2 M glycine re-solubilization buffer was added inline at a flow rate of 2 mL/min, providing a re-solubilized mAb at a pH of approximately 3.5, well-suited for a subsequent inline virus inactivation step. The re-solubilized mAb was passed directly through a double layer Sartoclear® depth filter to remove any insoluble material.

Table 2. Operating conditions and CHOP concentrations for the integrated continuous process

Stream	Flow rate (ml/min)	CHOP (ppm)	mAb concentration (g/L)
HCCF	5	130,000	4.6
0.1 M $ZnCl_2$	1	--	--
17.5 wt % PEG	4	--	--
Feed to dewatering step	10	130,000	2.3
HEPES Wash buffer	6	--	--
Re-solubilization buffer	2	--	--
Solubilized mAb	6	14,000	3.1

The integrated process was run continuously for 1 hr; longer operating times were not practical due to the limited amount of feed material available. This 1-hr process yielded more than 1 g of re-solubilized mAb. The entire system was operated without any intermediate feed pumps, with a total pressure drop of only 50 kPa = 7.3 psi (evaluated from the difference between the pressure at the inlet to the tubular precipitation reactor and that at the outlet from the final depth filter). There was no evidence of any membrane fouling, as the transmembrane pressures in the de-watering and wash modules remained stable throughout the process at values less than 2 kPa (<0.3 psi). The CHOP and mAb concentrations were also stable based on off-line assays for samples collected at multiple time points during the process. The final mAb

concentration was 3.1 g/L and the overall mAb yield was 80%, with the majority of the lost mAb appearing in the permeate from the wash step. This mAb loss could be reduced by adding $ZnCl_2$ to the wash buffer as per the work by Burgstaller et al.[8] The final CHOP concentration was 14,000 ppm, corresponding to a 10-fold reduction in CHOP levels relative to the mAb. Monomer purity was 89% as determined by the main peak in SEC.

Conclusions

Although several recent studies have demonstrated the feasibility of using precipitation for the initial recovery / capture of mAb products, the work presented in this paper provides the first demonstration of a fully integrated continuous process for precipitation, dewatering, washing, and re-solubilizing a mAb without any intervening hold steps. The precipitation was conducted in a tubular precipitation reactor with sequential inline addition of $ZnCl_2$ (a cross-linking agent) and PEG (a volume exclusion agent), enabling the use of very short residence times (<30 s) in the precipitation step, significantly reducing the overall system hold-up volume.

The precipitated mAb was de-watered and washed using hollow fiber membrane modules, with the washing conducted using a counter-current 2-stage washing configuration. To the best of our knowledge, this is the first reported use of this type of staged operation as part of a continuous precipitation process. The de-watering and 2-stage wash step were able to remove 90% of the host cell proteins using a wash buffer flow rate of 6 mL/min, which is only 20% more than the HCCF feed flow rate. Much higher levels of CHOP removal could be obtained using higher flow rates of wash buffer and / or more stages as per the model calculations presented in Figure 3. However, this would require the use of hollow fiber modules that could be operated with higher

conversion (ratio of permeate to feed flow rates); additional experimental studies are required to identify opportunities to increase the critical flux with precipitated proteins.

The mAb yield for the fully integrated process was 80%, with most of the mAb loss occurring in the wash step, likely due to premature re-solubilization of the mAb by the wash buffer (which was free of both $ZnCl_2$ and PEG). The final re-solubilized product was depth-filtered to remove any insoluble material and was obtained at pH 3.5, making it directly suitable for processing in a continuous virus inactivation reactor in combination with appropriate polishing operations, e.g., flow-through membrane chromatography. Thus, the precipitation system examined in this study could provide the basis for a low-cost fully integrated continuous process for purification of mAb products. Such a system would be linearly scalable in terms of membrane area of hollow fiber modules by changing the number of fibers. Future studies will be required to demonstrate the full potential of this downstream processing platform.

Acknowledgments

This work was supported in part by an NSF GOALI grant CBET 1705642. The authors would like to acknowledge support from the analytics group at BI and the Process Science Upstream group for providing the HCCF.

Conflict of interest: The authors declare that they have no conflict of interest.

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