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Purification of monoclonal antibodies from clarified cell culture fluid using Protein A capture continuous countercurrent tangential chromatography

Amit K. Dutta^{a,d}, Travis Tran^a, Boris Napadensky^a, Achyuta Teella^a, Gary Brookhart^b, Philip A. Ropp^b, Ada W. Zhang^c, Andrew D. Tustian^c, Andrew L. Zydney^d, and Oleg Shinkazh^{a,*}

^aChromatan Corporation, 200 Innovation Blvd., Suite 260B, State College, PA 16803

^bFujifilm Diosynth Biotechnologies, 101 J. Morris Commons Lane, Morrisville, NC 27560

^cRegeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591

^dDepartment of Chemical Engineering, The Pennsylvania State University, University Park, PA 16802

Abstract

Recent studies using simple model systems have demonstrated that Continuous Countercurrent Tangential Chromatography (CCTC) has the potential to overcome many of the limitations of conventional Protein A chromatography using packed columns. The objective of this work was to optimize and implement a CCTC system for monoclonal antibody purification from clarified Chinese Hamster Ovary (CHO) cell culture fluid using a commercial Protein A resin. Several improvements were introduced to the previous CCTC system including the use of retentate pumps to maintain stable resin concentrations in the flowing slurry, the elimination of a slurry holding tank to improve productivity, and the introduction of an “after binder” to the binding step to increase antibody recovery. A kinetic binding model was developed to estimate the required residence times in the multi-stage binding step to optimize yield and productivity. Data were obtained by purifying two commercial antibodies from two different manufactures, one with low titer (~0.67 g/L) and one with high titer (~6.9 g/L), demonstrating the versatility of the CCTC system. Host cell protein removal, antibody yields and purities were similar to that obtained with conventional column chromatography; however, the CCTC system showed much higher productivity. These results clearly demonstrate the capabilities of continuous countercurrent tangential chromatography for the commercial purification of monoclonal antibody products.

*Communication concerning this manuscript should be addressed to: Oleg Shinkazh, Chromatan Corporation, 200 Innovation Blvd., Suite 260B, State College, PA 16803, oleg.shinkazh@chromatan.com, **Phone:** (617) 529-0784.

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Keywords

Continuous; chromatography; monoclonal antibody; Protein A

1. Introduction

Monoclonal antibodies (mAbs) have revolutionized cancer treatments, significantly reducing and, in some cases, eliminating the need for traditional chemotherapy (Oflazoglu et al., 2010). Antibody products have also been developed for the treatment of rheumatoid arthritis, age-related macular degeneration, and a variety of cardiovascular diseases (Drewe et al., 2002). Antibodies are typically produced in CHO cells, with the downstream process designed to remove host cell proteins, DNA, and other impurities to very low levels. The cost of large scale purification of mAb from the cell culture fluid is often the largest component of the overall production cost (Ghosh, 2002).

Many companies have developed platform technologies for mAb purification, with the initial product capture accomplished using Protein A affinity chromatography (Liu et al., 2010). Protein A chromatography is a highly robust technology that provides high mAb recovery and impurity removal directly from clarified cell culture fluid (CCF) without the need for any pretreatment of feed pH or ionic strength. Although packed column Protein A chromatography is used successfully in the production of essentially all commercialized mAb products, this technology has a number of significant drawbacks that include high costs of Protein A resins, low productivity batch operation, necessity for capital intensive large stainless steel columns, and the often problematic and time consuming column packing and cleaning procedures. In addition, significant advances in cell culture technology in the past ten years have resulted in ten-fold increases in mAb titers, shifting the production bottleneck and cost optimization burdens to downstream operations, which have resulted in even a greater necessity for innovation in the mAb capture market.

Furthermore, a strategic shift in bio-manufacturing towards single-use disposable systems and continuous processes is gaining popularity, enabling companies to produce drugs with higher flexibility, lower cost, and continuous and on-demand manufacturing (Masser, 2014). Single-use equipment can also significantly reduce time-to-market for new drug substances. Rader and Langer (Rader et al., 2012) project that more than 50% of new commercial manufacturing facilities will be based on single-use technology, with a market size of more than \$15 billion by 2020. This has created increased interest in the use of membrane chromatography (also known as membrane adsorbers), but these systems currently have much lower binding capacities than traditional resins and have thus been non-competitive for initial mAb capture using Protein A (Ghosh, 2002).

There has thus been considerable interest in the development of continuous chromatographic processes that can be directly integrated with a perfusion bioreactor for continuous bioprocessing. For example, Warikoo et al. (2012) and Godawat et al. (2012) examined the use of periodic counter current (PCC) multi column chromatography for continuous production of mAb from a perfusion bioreactor. This integrated system provided 5× higher volumetric productivity than a fed batch process with comparable yield and purity and

smaller column size (Warikoo et al., 2012). The design and optimization of a PCC based chromatography system has also been studied by Pollock et al. (2013), while Muller-Spath et al. (2010) have examined the use of multi-column countercurrent solvent gradient (MCSG) chromatography with anion and cation exchange resins. Although multi column chromatography has been successfully integrated with perfusion bioreactors, these systems require column packing and complex valve switching, causing them to operate in a cyclic rather than true steady state mode. Other groups have examined alternatives to chromatography that can provide steady-state product capture, including aqueous two-phase extraction (Rosa et al., 2013) and precipitation (Hammerschmidt et al., 2014), although these systems typically cannot provide the robust operation and high purity that can be achieved using Protein A affinity resins.

Recent work by Shinkazh et al. (Shinkazh et al., 2011; Napadensky et al., 2013) has demonstrated that Continuous Countercurrent Tangential Chromatography (CCTC) can overcome many of the key limitations of conventional batch and multi-column chromatography while providing opportunities for truly continuous product capture. The CCTC process utilizes the resin in the form of a slurry which flows through a series of static mixers and hollow fiber membrane modules as shown in Figure 1. The micro-porous hollow fiber membranes retain the large resin particles while letting all dissolved species, including proteins and buffer components, pass through the membrane and into the permeate. The buffers used in the binding, washing, elution, stripping, and equilibration steps flow countercurrent to the resin slurry in a multi-stage configuration, enabling high resolution separations while reducing the amount of buffer needed for protein purification. In addition, the pressure drop in CCTC is very low (< 70 kPa), allowing the use of a completely disposable flow path, providing the type of single-use technology that can enhance manufacturing flexibility, reduce labor and capital costs, and decrease time-to-market for new drug substances. In contrast to other continuous bioprocessing systems, the CCTC system provides the opportunity to run at steady state without having to switch any valves after system start up, while allowing the users to completely abandon all activities related to handling packed beds including packing, cleaning, validation and storage.

The previously published studies of CCTC used model protein systems involving the separation of bovine serum albumin (BSA) and myoglobin using an anion exchange resin (Shinkazh et al., 2011) and the purification of an IgG₄ from a mixture of BSA and myoglobin using a Protein A affinity resin (Napadensky et al., 2013). The objective of this work was to demonstrate the ability of CCTC to purify monoclonal antibodies produced in CHO cells from clarified cell culture fluid. Data were obtained with two different mAbs, with low and high titers, to show the versatility of the CCTC system. In each case, the binding stage was optimized using a kinetic model developed from batch uptake experiments. Several improvements were made to the earlier CCTC system: an additional static mixer was placed after the binding step to improve mAb recovery, flow control was maintained using retentate pumps to simplify operation, and the large slurry tank was eliminated to increase productivity. The purity and yield performance of the resulting CCTC system was comparable or better than Protein A column chromatography, however the CCTC system showed much higher overall productivity.

2. Materials and methods

2.1. Materials

Data were obtained with two monoclonal antibodies: a high titer (6.9 g/L) mAb1 from Regeneron Pharmaceuticals and a low titer (0.67 g/L) mAb2 from Fujifilm Diosynth Biotechnologies. Both mAbs were provided in a clarified cell culture fluid obtained from CHO cell bioreactors. The CCF was stored at -80°C and then thawed prior to use. Most hollow fiber membranes that were used in CCTC experiments were obtained from Spectrum Labs (52 cm², 0.5 μm pore size, 1 mm inner diameter, and 40 cm length, Rancho Dominguez, CA.). High surface area membranes were obtained from and GE Healthcare (225 cm², 0.45 μm pore size, 1 mm inner diameter, and 60 cm length, Pittsburgh, PA). All CCTC experiments were performed with POROS[®] MabCapture Protein A resin with 45 μm particle size obtained from Life Technologies (Norwalk, CT). MabSelect SuRe resin with 85 μm particle size was obtained from GE Healthcare (Piscataway, NJ) for column chromatography runs. Static mixers with 28.6 cm length and 1.0 cm inner diameter were obtained from Koflo Corporation (Cary, IL). Both the high and low titer CCF were sterile filtered (AseptiCap[®], Advanced Microdevices, India) after thawing. All buffers were filtered using Whatman Polycap[®] TF (GE Healthcare Bio-Sciences, Pittsburgh, PA). Packed bed chromatography was performed using an AKTAProcess chromatography skid (GE Healthcare) and a 25 cm inner diameter (I.D.) Vantage VS250 column (EMD-Millipore, Billerica, MA). UPLC analysis leveraged an ACQUITY UPLC system from Waters Corporation (Parsippany, NJ). HPLC analysis was performed using an Agilent 1200 Infinity Series analytical LC system (Agilent Technologies, Santa Clara, CA).

2.2. Equilibrium binding isotherm

The equilibrium (static) binding capacity of the resin was evaluated by mixing 5 mL of 25% resin slurry with CCF at multiple loadings of the mAbs. The resulting mixtures were stirred continuously for 30 min, at which point small samples of the fluid phase were obtained through 0.22 μm syringe filters (PES membrane, Membrane Solutions, Dallas, TX). Antibody concentrations were analyzed by analytical Protein A chromatography, with the concentration of bound mAb determined by mass balance.

2.3. Batch binding kinetics

Binding kinetics were evaluated using a batch system in which a known volume of resin slurry was rapidly mixed with CCF in a 100 mL beaker. Small samples (approximately 1.5 mL) were collected as a function of time through 0.22 μm syringe filters. Each syringe filter was used once and then discarded. mAb concentrations were determined by analytical Protein A chromatography as described above.

2.4. Critical flux experiments

In order to ensure stable operation, the permeate flux in the hollow fiber modules was maintained below the critical flux (Bacchin et al., 2006). The basic procedure for evaluating the critical flux has been described by Wu et al. (1999), with specifics for the CCTC system given elsewhere (Shinkazh et al., 2011; Napadensky et al., 2013). Briefly, the slurry was

circulated through the hollow fiber module in total recycle mode, with the permeate flow rate controlled using a Masterflex[®] peristaltic pump. Pressures at the feed inlet, retentate outlet, and permeate outlet were continuously monitored using pressure transducers (Pendotech, Princeton, NJ) connected to a computer through Omega 400 as hardware and DASyLab as the software interface. Data were obtained at a fixed ratio of the permeate to retentate flow rate (γ) while the total flow rate was incrementally increased every 15–20 minutes. The critical flux was identified as the permeate flux at which the transmembrane pressure (*TMP*) became unstable (increasing with time during the constant flux operation).

2.5. Binding step experiments

The performance of the binding step was examined in both single and double stage (patent pending) systems as shown in Figures 2A and 2B. The concentration of the resin slurry, the appropriate buffer and CCF flow rates, as well as the static mixer volumes were determined from equilibrium binding and batch kinetics experiments. Sufficient membrane area was used so that the filtrate flux was at least 20% below the critical flux.

The entire system was first filled with binding buffer (pH 7.2, 150 mM phosphate buffer), with flow rates adjusted to the desired values using LabVIEW to control the Watson Marlow peristaltic pumps. The system was allowed to stabilize, at which point the resin was pumped into the initial static mixer by switching a three-way valve. The system was then allowed to reach steady-state until the outlet resin concentration was equal to that of the inlet stream. The CCF was then introduced into the system, starting the purification process. Samples were collected from the inlet and outlet streams at various times for off-line determinations of the mAb concentrations using analytical Protein A chromatography.

2.6. CCTC experiment

A schematic of the CCTC system is shown in Figure 1. The CCTC process was comprised of six chromatographic steps: (1) binding, (2) wash-1, (3) wash-2, (4) elution, (5) stripping, and (6) equilibration, with the composition of the different buffers shown in Table 1. All flow rates were controlled by multi-head peristaltic pumps (Watson Marlow, Wilmington, MA). The system was initially filled with buffers from their corresponding steps, with the flow rates set using LabVIEW. The pressures in each stage were monitored continuously. The resin was introduced once the system pressures had stabilized by adjusting the three-way valve on the inlet to the first static mixer. The outflow from the equilibration stage was taken to waste until the resin concentration in that stream was at least 99% of the slurry volume fraction entering the system. At that point, the outflow from the equilibration stage was recycled directly to the inlet of the binding stage by initiating resin tank bypass (Figure 1). The CCF was then introduced, with permeate samples collected from all of the stages for subsequent analysis.

2.7. Packed bed chromatography

Packed bed chromatography for mAb2 was performed to allow comparison between the CCTC system and a typical industrial Protein A chromatography. Chromatography was performed on a 10L (20 cm bed height, 25 cm I.D.) column packed with MabSelect SuRe resin. The Protein A column was equilibrated with two column volumes (CVs) of binding

buffer before load application. Following loading, the column was washed with wash-1 and wash-2 buffers sequentially before a three CV elution step. A constant linear velocity of 230 cm/h was used throughout the process step. Buffer compositions are given in Table 1.

2.8. Particle size analysis

The stability of the resin particles was evaluated from particle size data obtained with a Malvern Mastersizer S (Malvern Instruments Ltd, U.K.). Fresh resin and resin collected after the CCTC experiment were analyzed to evaluate particle size distributions.

2.9. Analytics

2.9.1. SEC-UPLC—Soluble aggregates for both mAbs was determined by SEC-UPLC using an ACQUITY UPLC PrST SEC column, with 200Å pore size, 4.6 × 150 mm column, and a 1.7 µm bead size (Cat#186005225, Waters Corporation). The mobile phase for mAb1 was 150 mM sodium phosphate, 250 mM NaCl, pH 7.2. For mAb2, data were obtained using a 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 mobile phase.

2.9.2. Host cell proteins—CHO host cell protein levels were measured using a commercially available ELISA kit (Cat#, F550, Cygnus Technologies, Wrentham, MA).

2.9.3. Antibody titer—Antibody titers were measured via HPLC using a POROS A 20 µm column 2.1 mm × 30 mm, 0.1mL (cat#2-1001-00, Life Technologies). Peak Detection was by UV at 280 nm.

2.9.4. Leached Protein A—Residual Protein A leachate levels were determined using a commercial ELISA kit capable of quantifying both native Protein A and the MabSelect SuRe ligand (Cat#F400, Cygnus Technologies).

2.9.5. Antibody charge variant analysis by analytical weak cation exchange—Cation-exchange HPLC was performed on a CM-STAT 4.6 × 100 mm cation-exchange column (TOSOH, Minato, Japan) with mobile phases consisting of 10 mM MES, 3 mM NaCl, pH 6.0 (Buffer A) and 20 mM MES, 200 mM NaCl, pH 6.0 (Buffer B). Samples were eluted by employing a linear gradient at ambient temperature of 10% Buffer B to 20% Buffer B over 25 CV. Flow rate was set at 1.0 mL/min. Detection was by UV at 280 nm. The sample injection load was 40–60 g/L.

3. Results and discussion

3.1. Binding characteristics

The equilibrium binding isotherms for mAb1 and mAb2 in their corresponding CCF are shown in Figures 3A and 3B, respectively. In each case, the bound concentration (Q) was evaluated from the measured values in the free solution using a simple mass balance:

$$Q = \frac{(C_0 - C_f)V_s}{V_R \varphi} \quad (1)$$

where C_0 is the initial mAb concentration after mixing with the resin slurry, C_f is the final concentration of mAb in solution, V_S is the total volume of the fluid phase in the final solution, V_R is the volume of the resin slurry, and ϕ is the resin concentration determined from the settled resin volume. The maximum binding capacity for the two mAbs were 26 ± 1 and 21 ± 2 g/L. This difference is likely due to the differences in molecular properties of the mAbs and CCF impurities. In both cases, the equilibrium isotherm is essentially a step function, with resin saturation achieved as soon as there is sufficient mAb to reach Q_{max} .

The operating Q value was chosen based on the adsorption isotherms to insure negligible product loss: 20.5 g/L for mAb1 and 15 g/L for mAb2 corresponding to approximately 80% and 70% saturation, respectively. The required slurry flow rate (or γ value) was determined directly from the target Q and the resin concentration as: $\gamma = q_{CCF}/q_{slurry} = 0.74$ for mAb1 and $\gamma = 3.1$ for mAb2 based on resin volume fractions of 0.25 and 0.14, respectively. The lower resin volume fraction for mAb2 was chosen because of the lower titer.

The required residence times in the static mixers in the binding step were then determined from binding kinetics data. Results for a typical binding experiment using mAb1 are shown in Figure 4, with the solid and dotted curves representing the lumped parameter model fits using piece-wise values of the rate constants as described in the Appendix. The lumped parameter model has been previously employed by Bak et al. (2007) in their analysis of the antibody breakthrough profile in Protein A column chromatography. This model is simple to implement in both the analysis of binding kinetics data and process design. More advanced binding models have been developed, e.g., pore diffusion, surface diffusion, etc., but these models often involve multiple fitted parameters that often depend on the specific binding conditions (Chen et al., 2002). The binding data were obtained by mixing 24.4 mL of the slurry with 18 mL of the CCF, which is the same ratio as that to be used in the CCTC system ($\gamma = 0.74$). The model is in excellent agreement with the data using $k_I = 0.64 \text{ min}^{-1}$ for the initial binding (up to 50% saturation) and $k_I = 0.43 \text{ min}^{-1}$ for the approach to saturation (see Figure 4). The reduction in effective binding constant as the resin approaches saturation likely reflects the presence of binding sites with different affinities in combination with mass transfer limitations, with the initial binding dominated by the more accessible sites near the exterior surface of the porous resin. The k_I value obtained from fitting the whole plot was 0.49 min^{-1} which was used to design a single stage system. The value obtained from “after binder” kinetics experiments (approaching steady-state) was 0.34 min^{-1} , with this value used to size the “after binder”. Similar kinetics experiments were conducted for mAb2 resulting in $k_I = 1.22 \text{ min}^{-1}$ for a single stage; $k_I = 1.49 \text{ min}^{-1}$ for stage 1, $k_I = 0.92$ for stage 2, and $k_I = 0.96 \text{ min}^{-1}$ for a two-stage system with the “after binder”.

The kinetic parameters were then used to optimize the design of the static mixers for the binding step in the CCTC system. Three configurations were examined: single stage binding with one large static mixer, a two-stage system with countercurrent arrangement of the stages, and a two-stage system with an “after binder” (an additional static mixer placed after the second hollow fiber membrane module in the binding step to capture remaining free mAb before the wash steps).

For the low titer mAb2, the total residence time required to achieve a 98% product capture was 10.13 min for a single stage and 10.10 min when using 2 stages with countercurrent contacting of the slurry and CCF. Most of the product loss in both of these systems was due to free mAb in the exit stream from the binding stage (which would be “lost” in the subsequent wash step). Thus, the binding step was re-designed to include an additional static mixer placed after the hollow fiber membrane module to capture residual unbound mAb – this was referred to as the “after binder”. The total residence time for the double stage system with the “after binder” was only 7.15 min, which is 30% less than that required for either the single or double stage system without the after binder. The optimum design had $t_1 = 3.40$ min, $t_2 = 1.80$ min, and $t_{after} = 1.95$ min, corresponding to static mixers with volumes of $V_1 = 100$ mL, $V_2 = 53$ mL, and $V_{after} = 14$ mL.

Corresponding calculations for the high titer mAb1 yielded total residence times for 98% product yield of 5.75 min for the single stage system, 5.70 min for the two stage system, and 4.92 min for the double stage system with “after binder”. In this case, the CCTC process was designed using a single stage binding step ($V = 68$ mL) to minimize the complexity of the overall system; the small increase in residence time compared to that for the two stage system with after binder has a relatively small effect on the overall system productivity.

The performance of the binding step was confirmed by running experiments with the binding step and a single stage wash as shown in Figure 2. In this case, the permeate samples from the binding and the wash 1 steps were both collected for analysis of the mAb concentration. Data for the mAb1 yield, determined from an overall mass balance based on the loss of mAb1 in both the binding and wash permeates, are shown in Figure 5 at four time points. The mAb1 yield was greater than 99% throughout the experiment, which is actually somewhat higher than that predicted based on the original design calculations. This small increase in yield is likely due to additional binding of mAb1 in the static mixer used in the wash step (which functions like an additional “after binder”). This was confirmed based on the very low mAb1 concentrations in the permeate collected from the wash step (<1% loss of mAb1).

3.2. CCTC system design

Critical flux experiments were conducted to determine the size (membrane area) needed for the hollow fiber modules. In contrast to the experiments conducted in Shinkazh et al. (2011) and Napadensky et al. (2013), data were obtained with constant ratios of permeate to feed flow rates, which is consistent with the operational mode of the CCTC system (in which the resin concentration in the exit of the hollow fiber module is set equal to that in the slurry fed to the static mixer in that step). Data were obtained using the flow rate ratio required in the corresponding binding steps for both the mAbs as shown in Figures 6A and 6B. The data give $J_{crit} = 323$ L/m²/h ($= 89.7 \times 10^{-6}$ m/s) for mAb1 and $J_{crit} > 850$ L/m²/h ($= 236 \times 10^{-6}$ m/s) for mAb2. The much higher critical flux for mAb2 is a direct result of the lower resin volume fraction used for mAb2. The hollow fiber modules were designed to operate with at most $J = 0.8J_{crit}$ to provide a margin of safety, with the membrane area calculated as $A = q_p/J$ where q_p is the permeate flow rate through the hollow fiber module.

The yield for the elution step in a CCTC system containing n stages is given by:

$$Y = \frac{\alpha(\alpha^n - 1)}{\alpha^{n+1} - 1} \quad (2)$$

$$\alpha = \gamma S \kappa \quad (3)$$

$$\kappa = \frac{1}{1 - \phi_{ave}(1 - \lambda)} \quad (4)$$

where γ is the ratio of the buffer flow rate to the concentrated slurry flow rate, S is the average sieving coefficient for the species of interest ($S=1$ for a mAbs with the 0.45 μm pore size membrane), κ is a correction factor that accounts for the presence of solids in the slurry, ϕ_{ave} is the average slurry volume fraction in the hollow fiber (evaluated as the mean value of the inlet and outlet values), and λ is the liquid fraction contained in the packed (settled) resin bed. For $\gamma = 3$, $S = 1$, a resin volume fraction of 14% in the concentrated slurry ($\phi_{ave} = 0.0875$), and $\lambda = 0.6$, the product yield given by Equation (2) is 76%, 93%, and 98% for an elution step with one, two, and three stages, respectively. Thus, a three stage system was used for the elution step to minimize product loss. Similar calculations were performed for the washing steps, in this case focusing on impurity removal. The stripping and equilibration steps were designed with two stages, both to facilitate equilibration at the desired pH and to remove any strongly bound impurities that were removed during stripping.

A summary of the key operating parameters for the CCTC system for both monoclonal antibodies is provided in Table 2. The permeate flux used in the binding step was much lower than that used in the other steps due to the greater fouling potential of the CCF processed in this step. As an additional safety factor, the resin concentration was increased by 3% (28% instead of 25%) to ensure higher recovery of mAb1. For mAb2, a different safety factor was used – the CCF flow rate was reduced by 15% vs. the modeled flow rate to also ensure high recovery.

Several significant innovations (patent pending) were implemented in this work compared to that described previously (Shinkazh et al., 2011; Napadensky et al., 2013) [see Figure 7A and 7B]. All permeate pumps were eliminated [7B] and replaced with retentate pumps for each stage [7A]. This change resulted in elimination of level control on the exit streams, thus simplifying the control strategy and decreasing hardware requirements. It also eliminated the pressure gradient across the system, allowing individual steps to be pressurized using their corresponding buffer pumps. Because level control was no longer needed on the exit streams, it was also no longer needed for the resin slurry. Therefore, the system was able to become fully closed after being loaded with slurry (utilizing slurry tank bypass shown in Figure 1). These changes reduced the total hold-up volume in the CCTC system, thereby increasing the overall productivity by at least 20–30%. The net result of these changes is a significant reduction in the equipment footprint and a more robust process operation.

3.3. CCTC performance

Experimental results for the CCTC process for mAb1 are summarized in Figures 8, 9, 10, and 11. The CCTC system was operated for a total of 8 hrs, with the CCF introduced 30 min after the system was first loaded with slurry. The stability of the system was verified by monitoring the A₂₈₀ signal on the permeate stream from the elution step, which should be nearly pure mAb1. The absorbance was essentially constant throughout the process as shown in Figure 8. This is in sharp contrast to the cyclical product concentrations obtained in both simulated moving bed (Rajendran et al., 2009) and multi-column (Holzer et al., 2008) chromatography systems. In addition, the pressure profiles in the hollow fiber membrane modules were all below 28 kPa (Figure 9), which is 5–10× less than typical pressures in packed column chromatography. The pressure in the binding, wash-2, stripping, and equilibration steps remained essentially constant throughout the 8 hr operation. There was a small increase in pressure for both wash 1 and the elution steps, which is probably related to mild membrane fouling by host cell proteins and mAb1, respectively.

Data for the overall yield of mAb1, determined from off-line analysis of samples obtained from the elution step using analytical Protein A chromatography, are shown in Figure 10. The yield is nearly constant throughout the CCTC process at a value slightly above 90%, which is similar to the yield obtained in Protein A process columns. However, the steady state productivity of the CCTC system was 55 g mAb /L resin/hr, which is an increase of 175% over the corresponding process Protein A column. Note that the overall productivity of the CCTC system was somewhat lower (47g mAb/L resin/hr). This is because the overall productivity is reduced due to system ramp up and ramp down time.

The composition of the CCF and all the effluent streams were analyzed using size exclusion chromatography (SEC), with results shown in Figure 11. Most of the impurities were washed away in the binding and subsequent wash steps. The elution stream was quite pure and contained very low levels of both small and large impurities (likely mAb fragments and oligomers).

A more detailed comparison of the CCTC system and a Protein A column fed with the same CCF is shown in Table 3. Whereas the CCTC system used POROS® MabCapture Protein A resin for its smaller particle size (45 µm) and perfusive mass transfer properties (Afeyan et al., 1990; Whitney et al., 1998), the packed bed column was performed using a base stable industry standard resin for Protein A chromatography, MabSelect SuRe (85 µm). The host cell protein concentration in the initial CCF was reduced from more than 50,000 ppm in the CCF to 179 ppm in the CCTC system compared to 604 ppm in the column. The greater HCP removal in the CCTC system may be due to the difference in chromatographic resins, differences in treatment of the CCTC load compared to the packed bed load (freeze/thawed versus fresh respectively), or more efficient washing in CCTC compared to that in a packed bed. In addition, the concentration of soluble aggregates (as determined by SE-UPLC) was only 8.6% in the CCTC system compared to 11% in the column.

Charge variant analysis via analytical cation exchange chromatography indicated that material purified by CCTC was of equal or better quality compared to the packed bed system: the column run had a slight increase in acidic species and a corresponding decrease

in main peak. Examples of protein modifications that result in acidic variants are deamidation and glycation (Du et al., 2012; Vlasak et al., 2008).

In both systems the level of Protein A leaching into the product pool is low enough (< 7 ppm) to be easily removed by standard downstream processing unit operations.

Corresponding results for mAb2 are shown in Table 4. The CCTC system was again run continuously for 7 hr, with the overall yield of 93% corresponding to a steady-state productivity of 26 g mAb/L resin/hr. The lower productivity for mAb2 compared to mAb1 is a direct result of the low feed titer (0.67 g/L versus 6.9 g/L for mAb1) and the slightly lower binding capacity (21 g/L versus 26 g/L for mAb1). The final pool productivity was ~ 22 g/L/hr. The HCP removal was higher in the CTC system by 21%, with the aggregate concentrations in the elution pool comparable to that of the column.

The particle size distribution of the resin was analyzed before and after the CCTC experiment to identify any possible degradation in the resin or generation of fines (see Figure 12). The median particle diameter of the resin before and after the experiment was 38.3 and 39 μm , respectively, demonstrating that circulation of the resin through the pumps and CCTC system caused no detectable damage to the resin particles. The binding capacity of the resin remained almost the same before (26 ± 1 g/L) and after (25 ± 1 g/L) the CCTC experiment.

4. Conclusions

The results presented in this study provide the first demonstration that CCTC can be used for the initial capture and purification of monoclonal antibodies directly from clarified cell culture fluid. Data were obtained with two different antibodies from two different bio-manufacturers (Regeneron Pharmaceuticals and FujiFilm Diosynth Biotechnologies) with very different titers, spanning most of the range currently seen in commercial mAb processes. In both cases, the CCTC system provided the mAb product at high yield and purity, comparable to that obtained in packed Protein A columns, but at much higher productivities. The higher productivity is a direct result of running an efficient continuous process, with the resin being cycled through all of the chromatographic steps much more efficiently than in a batch column.

While the CCTC productivities shown in this study are significantly higher than the “status quo” column chromatography productivities, simple process modeling shows that developing a smaller particle size Protein A resin (10–15 μm) will result in even greater productivity improvements. Because the CCTC system is no longer constrained by column geometry / pressure drops, a specifically designed CCTC small particle size resin will be expected to increase the binding kinetics by an order of magnitude, thus enabling the CCTC system to reach productivities of over 150 g of mAb / L of resin / hr.

The CCTC system was optimized for each mAb based directly on the binding characteristics, using data for both the equilibrium isotherm and the binding kinetics. Several significant changes were introduced compared to previous efforts using CCTC. In particular, an “after binder” was added to the binding step to capture residual free (unbound)

mAb before entering the wash step, significantly reducing the required residence time (and hold-up volume) in the binding step. In addition, retentate pumps were used to control the flow rates and resin concentrations, providing lower pressure operation throughout the CCTC system while eliminating the complexity associated with the flow control system used in the past. This also allowed us to remove the slurry recirculation tank (after filling the system with resin), further reducing the hold-up volume and increasing the overall productivity of the CCTC system.

Interestingly, the CCTC process provided better host cell protein (HCP) removal for both mAb with significantly lower aggregate levels (for mAb1) than that obtained with an analogous Protein A column. This improved performance may be related to the significant reduction in the time during which the mAb remains bound to the resin – the total residence time in the binding, wash, and elution steps in the CCTC system is less than 7–12 min while the Protein A column typically requires greater than one hour for these steps. Alternatively, this could be related to greater exposure of the slurried resin to wash buffer in CCTC as opposed to a packed bed. However, effects arising from the different resin type and load treatment (freeze/thaw versus fresh) cannot be ruled out.

The CCTC system operates at very low pressures (under 70 kPa), allowing the use of inexpensive plastic tubing and hollow fiber membrane modules. The system requires no initial packing (charging the system with resin takes less than 30 min), and the entire flow path is fully disposable, making CCTC ideal for flexible manufacturing designed around single use technology. In addition, CCTC provides truly continuous operation, with the mAb purity and yield remaining completely constant (true steady-state operation) throughout the process. These features will provide unique opportunities for future process developers to easily integrate CCTC with other continuous technologies such as perfusion bioreactors, membrane chromatography, single-pass TFF, and hybrid purifiers to enable fully continuous production of mAbs.

The future work of this group will be focused on further optimization of the CCTC system performance as well as generating the necessary data for cGMP commercialization. This will include evaluations of alternative resin technologies, comprehensive assessment of process economics, integration of the CCTC system with other continuous downstream processing technologies, and performing studies on viral clearance, extractables, and leachables.

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Appendix

The binding kinetics data were analyzed using a simple lumped parameter model assuming irreversible adsorption:

$$\frac{d\theta}{dt} = k_1 C (1 - \theta) \quad (\text{A1})$$

where $\theta = Q/Q_{max}$ is the dimensionless concentration of bound mAb and C is the mAb concentration in free solution.

$$C = C_0 - \beta\theta \quad (\text{A2})$$

where,

$$\beta = \frac{\phi V_R Q_{max}}{V_s} \quad (\text{A3})$$

where, V_R is the volume of slurry, V_s is the total volume of solution, and ϕ is the volume fraction of resin in the slurry.

$$V_s = V_M + V_R(1 - \phi) + 0.3V_R\phi \quad (\text{A4})$$

The last term in Equation (A4) accounts for the fluid volume in the resin packed bed. Differentiating Equation (A2) with respect to time (t) and combining with Equation (A1):

$$\frac{dC}{dt} = -(k_1 C^2 + k_1 \beta C - k_1 C_0 C) \quad (\text{A5})$$

Equation (A5) can be directly integrated to evaluate the free mAb concentration as a function of time in the binding experiment using $C = 0$ at $t = 0$. Calculations to simulate the behavior of a two stage binding step used the same basic approach, but with the initial conditions adjusted to account for the presence of bound mAb in the feed. The static mixers were assumed to have perfect plug flow, i.e., there was a uniform residence time for all mAb in each stage of the binding step.

Although it was possible to fit the kinetic data to a single value of k_I (shown in Figure 4, Panel A), a more accurate description of the binding data could be achieved using different values of the binding rate constant during the early and latter stages of the experiment. The data up to $\theta = 0.5$ were used to evaluate the rate constant for binding to the clean (mAb free) resin, yielding $k_I = 0.64 \text{ min}^{-1}$, while the data at longer times were used to determine the rate constant for the resin near saturation, yielding $k_I = 0.43 \text{ min}^{-1}$. These rate constants were used in the design of the first and second stages in the two-stage binding step (Panel B in Figure 4). The best fit value of the rate constant for the entire data set ($k_I = 0.49 \text{ min}^{-1}$ in Panel A in Figure 4) was used to design the single stage binding step. The appropriate rate constant for the after binder was determined using pure mAb at high levels of saturation to achieve the appropriate resin concentration and mAb loading.

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Highlights

- Successful use of continuous countercurrent tangential chromatography (CCTC)
- First application of CCTC for antibody capture from clarified cell culture fluid
- Significantly greater productivity than packed Protein A column
- Similar yield, host cell protein removal, and purity as in column chromatography
- Several improvements implemented in CCTC including after binder to increase yield

Figure 1. Schematic of the CCTC system with binding, two washes, elution, stripping, and equilibration steps, each consisting of a combination of static mixers and hollow fiber membrane modules (see inset for the elution step). The pumps are not shown to reduce complexity.

Figure 2.
Schematic of the experimental set up used for (A) single stage and (B) double stage binding. The wash step was used to collect resin-free samples to evaluate the concentration of free mAb exiting the binding step.

Figure 3. Adsorption equilibrium isotherms for (A) mAb1 and (B) mAb2, both in CCF, using the POROS® MabCapture Protein A resin.

Figure 4. Batch binding kinetics data for mAb1 in CCF. Model calculations for the binding kinetics data for mAb1 using the rate constants for (A) a single stage and (B) the first and second stages. Model calculations are described in the Appendix.

Figure 5. Yield of mAb1 during binding step experiments. The predicted yield from the binding model was 98%.

Figure 6.

Critical flux data for (A) mAb1 (with $\gamma=0.74$) and (B) mAb2 (with $\gamma=3.1$). The numerical values on the plot are the filtrate flux (in L/m²/h) for that portion of the experiment. The critical flux was defined as the value of the filtrate flux at which the pressure first became unstable.

A

B

Figure 7.
Schematic of the binding step in the (A) new patent pending and (B) old CCTC system.

Figure 8.
 A_{280} signal from elution step during CCTC process for mAb1 purification.

Figure 9.

Pressure profiles during CCTC process for mAb1 showing (A) average pressure for binding and wash-1 steps, (B) average pressure for wash-2 and elution steps, and (C) average pressure for stripping and equilibration steps.

Figure 10.
Yield of mAb1 from elution step during CCTC experiment.

Figure 11.
Size exclusion chromatogram of mAb1 CCF and different effluent streams.

Figure 12.
Particle size distribution of the resin (A) before and (B) after the CCTC experiment.

Table 1

Buffer composition for CCTC.

Buffer	Compositio	pH _{in}	pH _{out}
Binding	20 mM Na ₂ HPO ₄	7.2	7.1
Wash-1	20 mM Na ₂ HPO ₄ , 500mM NaCl	7.2	7.1
Wash-2	20 mM Na ₂ HPO ₄	7.2	7.0
Elution	Acetic acid	2.6	3.1
Stripping	HCl	2.0	2.3
Equilibratio	20 mM Na ₂ HPO ₄	8.0	6.9

pH_{in} refers to the pH of the inlet buffer in the last stage of a particular step. pH_{out} is the pH of the solution coming out from the first stage of the same particular step.

Table 2

Key operating parameters for the CCTC system.

	mAb1	mAb2
Titer (g/L)	6.9	0.67
q_{CCF} (ml/min)	5.0	19.0
q_{slurry} (ml/min)	6.8	7.2
Resin (volume fraction)	0.28	0.14
q_{buffer} (ml/min)	20.3	21.6
J_{bind} (L/m ² /hr)	58	51
$J_{other\ steps}$ (L/m ² /hr)	230	249
Steady state yield (%)	90	93
Steady state productivity (g/L/hr)	55	26

Table 3

Comparison between performances of CCTC and column chromatography formAb1.

Performance	CCTC	Column chromatography
Yield* (%)	90.4	> 90
Final elution pool Productivity** (g/L/hr)	47.0	20.0
Steady state productivity (g/L/hr)	54.8	NA
HCP (ppm)	179	604
HMW*** aggregate (%)	8.6	11.0
Leached protein A (ppm)	< 7	< 7
Charge variants		
Acidic (%)	33.5	35.7
Main peak (%)	53.0	50.1
Basic (%)	13.5	13.4

* Load and eluate concentration was determined by analytical proteinA

** Productivity for column chromatography was calculated assuming a 2kL production using four Protein A cycles, including column CIP and -use storage.

*** High molecular weight

The CCF contained 53960 ppm of HCP and 11.0% aggregate.

The time period used to calculate the productivity for CCTC system was based on starting CCF flow and stopping collection of elution sample.

Table 4

Comparison between performances of CCTC and column chromatography for mAb2.

Performance	CCTC	Column chromatography
Yield* (%)	92.8	~100
Final elution pool productivity** (g/L/hr)	22.2	6.4
Steady state productivity (g/L/hr)	26.2	NA
HCP (ppm)	2195	2787
LMW aggregate (%)	0	0.3
HMW aggregate (%)	3.9	2.1

* Load concentration determined by analytical protein A, eluate concentration determined by A280

** Productivity for column chromatography was measured using 2 cycles, pre-use sanitization through end of elution.

The CCF contained 3,992,900 ppm of HCP.