Journal of Chromatography B 1230 (2023) 123919

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Short communication

Alleviation of the necessity for supernatant prefiltering in the protein a recovery of Monoclonal antibodies from Chinese hamster ovary cell cultures

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ARTICLE INFO

Keywords:
Monoclonal antibodies (mAbs)
high-performance liquid chromatography (HPLC)
capillary-channeled polymer (C-CP) fibers
protein A

ABSTRACT

Protein A (ProA) chromatography is a mainstay in the analytical and preparative scale isolation/purification of monoclonal antibodies (mAbs). One area of interest is continuous processing or continuous chromatography, where ProA chromatography is used in the large-scale purification of mAbs. However, filtration is required prior to all ProA isolations to remove large particulates in cell culture supernatant, consisting of a mixture of cell debris, host cell contaminants, media components, etc. Currently, in-line filters are used to remove particles in the supernatant, requiring replacement over time due to fouling; regardless of the scale. Here we demonstrate the ProA isolation of *unfiltered* Chinese hamster ovary (CHO) cell media using capillary-channel polymer (C-CP) fiber stationary phases modified with S. aureus Protein A (rSPA). The base polymer of the analytical scale C-CP columns costs ~$5 per 30 cm column, and when modified with ProA, the base cost is ~$25 per 30 cm column, a cost-effective option in comparison to analytical-scale commercial columns. To directly sample unfiltered media, a 5 cm gap was created at the head of the C-CP column, where the large particulates are trapped, while molecular solutes flow through the capillary channels without sacrifice in analytical performance, mAb loading capacity, or backpressure increases. The binding capacity of the gap ProA C-CP column was ~ 2 mg mL^{-1} of IgG per bed volume. The same analytical column could be operated after processing a total of ~ 56 column bed volumes of supernatant (>25 analytical cycles) without the need for caustic clean-in-place processing.

1. Introduction

Monoclonal antibodies (mAbs) comprise a large part of the bioprocessing and pharmaceutical sectors, as mAbs can be used as therapeutics to treat a wide variety of diseases and cancers. Large titers of mAbs, up to 10 g/L of material are desirable and becoming common in bioprocesses [1]. The biopharmaceutical industry is driven by a focus on increasing productivity and efficiency while maintaining essential quality attributes, with sales of mAbs expected to reach ~$200 billion worldwide in 2022 [2,3]. Recent efforts towards higher efficiencies include evolution from bulk to continuous manufacturing approaches. As such, aspects of continuous chromatography of mAbs for the large-scale purification and measurements of productivity are under development [4–6]. Currently, protein A (ProA) chromatography is the gold standard for mAb isolation on both the analytical and preparative scales, due to the high specificity of ProA to the Fc region of mAbs [7–9].

In the biomanufacturing sector, ProA chromatography plays an important role in two areas: 1) monitoring titer and quality attributes during upstream processes and 2) isolation and purification in downstream processes. Very often, analytical ProA chromatography is followed by subsequent chromatographic operations to determine the degree of mAb agglomeration (via size exclusion chromatography), charge variant distributions (via ion exchange chromatography), or mass spectrometric analysis [10–12]. Currently, there are online (automated sampling) and at-line (manual sampling) measurement methods in upstream assessments of the bioreactor productivity [13]. As is common with most forms of biomatrix chromatography, ProA separations require the removal of extraneous sample components such as particles, vesicles, etc., which tend to foul column head frits, bind irreversibly to stationary phase materials and within their pores, and shorten column lifetimes. In general, such species are removed in the case of bioreactor supernatants by centrifugation followed by filtration with a 0.2 μm filter membrane [1,14].

Following the initial ProA isolation for downstream processes, there

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https://doi.org/10.1016/j.jchromb.2023.123919
Received 22 September 2023; Received in revised form 18 October 2023; Accepted 29 October 2023
Available online 31 October 2023

are polishing steps, which consist of anion exchange chromatography, cation exchange chromatography, and hydrophobic interaction chromatography, which are required before the therapeutic product can be ready for packaging [9,15,16]. To this end, process intensification approaches including continuous chromatography, are implemented to create a cost-efficient and high throughput process for the isolation of mAbs [4–6,15,17]. More so than in the case of batch processing, with continuous chromatography in-line filtration (by many means) is essential to protect costly commercial columns from large particles entering the columns [2]. The common modalities of filtration in continuous chromatography include centrifugation [18–20], depth filtration [21–23], and tangential flow filtration [24–26]. For both upstream (analytical) and downstream (preparative) processes, filters by their very nature present the potential for fouling and require replacement. With the potential for filter failure present, this requires intervention and therefore, is not a fully automated process. Cost effective column formats, which are more forgiving towards common debris in cell culture supernatants would have potential benefits on both analytical and preparative scales.

This laboratory has previously described the use of a novel column format, based on capillary-channeled polymer (C-CP) fibers, for the high throughput chromatographic separations of proteins, and more recently extracellular vesicles [27–29]. C-CP fibers form multiple parallel, single micrometer-sized channels, resulting in very high column permeability. A lack of fiber porosity, and thus intraphase diffusion, results in a virtual lack of any solute mass transfer resistance (i.e., no van Deemter C-term limitations) [30,31]. The capillary channels mean high linear velocities can be employed with relatively low system backpressures. Additionally, high levels of specificity can be derived through relatively simple chemical modifications of the fiber surfaces [32–34]. To this end, ProA columns have been made by simply flowing a solution of recombinant S. aureus protein A (rSPA) through polypropylene C-CP fiber columns [35–37], with the ligand robustly affixed through chemical adsorption. Those efforts demonstrated the cost effectiveness, efficiency, and reusability of the columns, with mAb throughput and purity characteristics that are very competitive with commercial analytical-scale columns.

As a route to lower the processing time and potential losses of critical products to filter substrates, we present the analytical-scale ProA isolation of CHO supernatant where no inline filter is required. To alleviate the necessity of a syringe or inline filter, a gap was created at the head of a ProA-modified C-CP fiber column. Particulates are held up in the void volume while molecular-scale solutes pass through the capillary channels. It is not believed that such an approach has been described relative to packed-bed columns as a means of excluding particulates from column passage. The effort looks at the potential effects of unfiltered supernatant on the ProA separation characteristics. Various volumes (10-to-200 μL) of 0.71 mg mL^{-1} IgG unfiltered CHO supernatant were injected onto the gapped rSPA modified C-CP fiber column, itself having a void volume of < 0.1 mL. Over the range of unfiltered supernatant volume loaded, the elution of IgG and backpressure of the separation were unaffected. A linear response was determined for the IgG elution across volumes of 10-to-100 μL (7.1 μg to 71 μg IgG) for filtered and 10-to-75 μL (7.1 μg to 58 μg IgG) for unfiltered with a binding capacity per bed volume of ~ 2 mg mL^{-1}. While the alleviation of the filtration step has immediate consequences for analytical separations, it may also have important implications as the C-CP fiber format is expanded to the preparative scale and continuous chromatography applications.

2. Materials and methods

2.1. Chemicals and reagents

Sodium phosphate, monobasic, monohydrate (EMD Millipore, Merck, Germany), sodium phosphate, dibasic, heptahydrate (EMD Millipore, Merck), citric acid (BDH, Dubai, United Arab Emirates) were used for solvent preparations. Each was dissolved in deionized water (DI-H_2O) obtained from an Elga PURELAB flex water purification system, (18.2 MΩ-cm, Veolia Water Technologies, High Wycombe, England). For C-CP fiber modifications, native recombinant S. aureus Protein A (rSPA) (animal free) (Syd Labs, Hopkinton, MA) was used. The rSPA was diluted with a 1X solution of Gibco phosphate-buffered saline (PBS) 10x pH 7.4 (ThermoScientific, MA, USA). Chinese hamster ovary (CHO) cell supernatant from CHO K1 cell line was provided by the Harcum laboratory (Department of Bioengineering, Clemson University). The supernatant was centrifuged at 1000g and stored at −20 °C before use. Supernatant was thawed prior to use, where both filtered, and unfiltered supernatant was used. A 0.22 μm Polyethersulfone (PES) syringe filter (FroggaBio, NY, USA) was used for the filtered samples.

2.2. Column preparations

Polypropylene (PP) base fibers were manufactured at Clemson University [38]. Fibers were pulled through polyether ether ketone (PEEK) tubing (0.76 mm i.d.) and cut to 30 cm to create the C-CP columns. Two columns were prepared, where 1) the fiber was cut flush to the ends of the PEEK tubing, and 2) where a ~ 5 cm gap was left at the beginning of the column, as depicted in Fig. 1. The gap and no gap columns were packed identically to one another, however, in the case of the gap column, the fibers were pulled further through the PEEK tubing to produce a ~ 5 cm gap at the beginning of the column. It is important to point out that the gap volume generated (~23 μL) represents a substantial fraction (38 %) of the total remaining column void (interfiber) volume (~59 μL). As such, one might anticipate large perturbations in column hydrodynamics. That said, the actual fiber stationary phase surface (loading) area is only reduced by ~ 17 % in creating the gap. After packing, the columns were washed with DI, ACN, then DI to remove any contaminants from the extrusion and packing processes. For fiber modifications, the columns were equilibrated on the HPLC system with 1X PBS, prior to loading the rSPA solution. A 0.5 mg mL^{-1} solution of rSPA was flown through the columns for 5 min to modify the surface with ProA [35].

2.3. Instrumentation

A Dionex Ultimate 3000 HPLC consisting of a quaternary pump and diode array detector (ThermoFisher Scientific, Sunnyvale, CA, USA), controlled with Chromeleon 7 software, were used for all separations. All chromatographic measurements were taken in triplicate at an absorbance of 280 nm. A Hitachi Regulus 8230 was used for all SEM imaging.

2.4. Methods

For ProA isolations, the method was varied based on the loading volume of the CHO supernatant. The solvents included 50 mM sodium phosphate, pH 7 (MP A) and 150 mM citric acid, pH 2.5 (MP B). A stock 0.71 mg mL^{-1} mAb solution of filtered and unfiltered CHO supernatant (MP C) was used for all separations. For loading studies, the separation started with equilibrating the column with MP A (0 – 2 min), followed by sample loading with MP C and column equilibration with MP A, and finally elution of IgG using MP B initiated at t = 7 mins. For each run, the injection period was adjusted based on the injection volume, with the times of the equilibration and elution steps kept constant. The injection volumes were varied from 10 μL to 200 μL of filtered and unfiltered CHO supernatant. Each load/elution sequence was performed in triplicate at flow rates of 1.0 mL min^{-1}. For SEM imaging, cross sections of the C-CP gap in close proximity to the column head were cut then sputter coated with platinum.

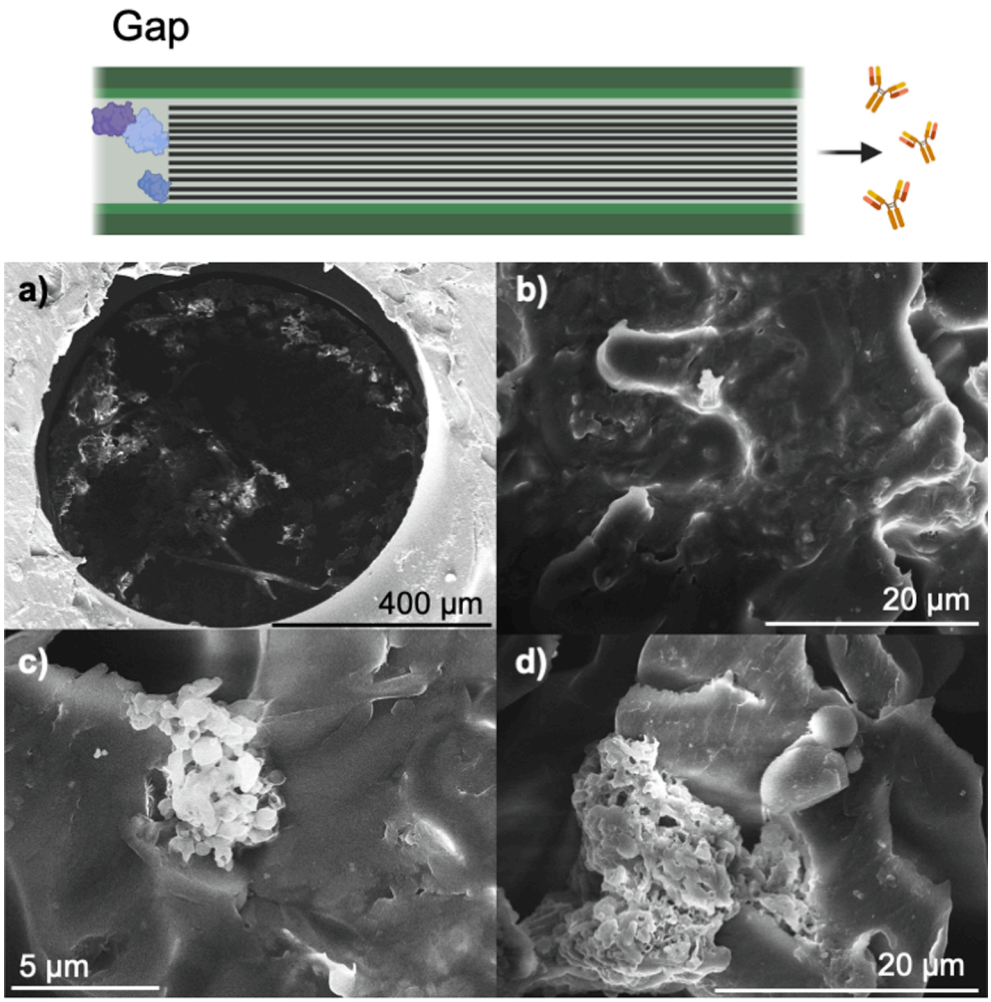

Fig. 1. Depiction of gap at head of ProA C-CP fiber columns. SEM images of ProA C-CP gap column including a) cross section of gap and b-d) particulates trapped at the column head.

3. Results and discussion

3.1. ProA column elution characteristics

Filtering supernatant can be time intensive and expensive as often multiple filters are needed. With the method presented here, isolation of IgG from unfiltered CHO supernatant only requires the standard 1000 g centrifugation for sample preparation. By creating a ~ 5 cm gap at the head of the C-CP fiber column, particulates are held-up in the gap region, while molecular-scale constituents pass through the micrometer-sized channels in fiber column. It is postulated that a level of turbulence is generated in that region, where the particulates remain suspended and molecular solutes continue in the fluid stream. SEM images of the gap present at the fiber column head are shown in Fig. 1. The C-CP column was exposed to unfiltered media prior to imaging. In Fig. 1a, the entire cross-section of the gap column is shown, with the fibers encased in PEEK tubing, represented by the lighter smooth surface in the image. The fibers are inside the casing, at a slightly lower depth. Some charging of the fiber surface (bright spots) as the platinum is not able to fully coat the inner regions. Further removal of the casing down towards the column head allows effective visualization of the fibers. The higher-magnification SEM images (Fig. 1b-d) show large particulates (~5 – 20 µm) captured at the head of the fiber bundle. In Fig. 1b specifically, the interdigitating of the fibers is clearly shown, with the outline of a single fiber highlighted by the lighter region of the image. Clusters of particulates are revealed in Fig. 1c, with the interdigitating fibers seen in Fig. 1d, capturing larger particulates in between the fibers. With the visual confirmation of SEM, the fibers proved to be effective at capturing particulates present in the unfiltered media. That said, it would be easy to envision the use of a simple counter-flow of solution as a means of evacuating particulates from the column void region to waste, increasing the number of processing cycles for a single column.

The general performance of the gap column was tested against a typical (no gap) ProA C-CP fiber column for filtered and unfiltered supernatant injections. Averaged chromatograms for triplicate injections for each case are shown in Fig. 2. The reproducibility of the recoveries for the gap and no gap columns, with filtered and unfiltered media, was excellent, with an overall variability of < 5 %RSD. For the standard (no gap) column, the peak area of the filtered supernatant (green) was ~ 105 mAu min (2.32 %RSD), while the unfiltered supernatant (yellow) was ~ 121 mAu min (4.90 % RSD). The slight decrease in peak area with the filtered sample may be attributed to losses during filtration, while the slight increase in % RSD of unfiltered sample can be attributed to the complex matrix. For the gapped C-CP column, the peak areas of the filtered and unfiltered sample were ~ 102 mAu for each (filtered: 2.12 % RSD, unfiltered: 4.82 % RSD), showing that there was no difference in recovery based on the filtration of the sample. Very importantly, in terms of potential perturbations in the column hydrodynamics, there are no appreciable changes in the elution band characteristics in the two column formats. Overall, the recovery for the gapped column is the same as if having performed the filtering step, with the overall precision still remaining very acceptable. Moving forward, only the gap C-CP columns

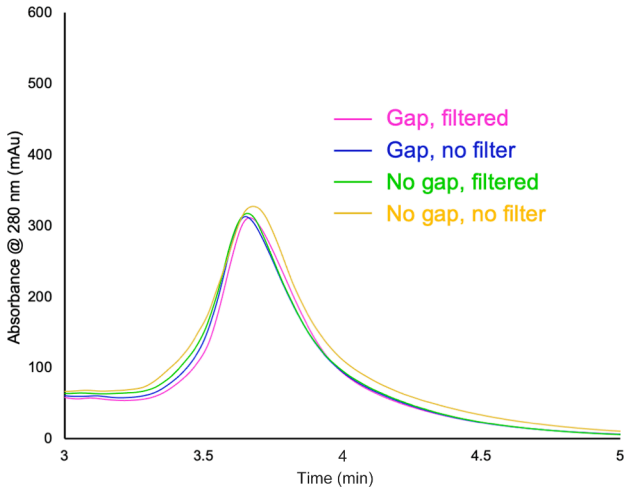

Fig. 2. Comparison of IgG elution profiles derived from injections of filtered and unfiltered CHO cell supernatants employing a standard-format ProA C-CP fiber column and one employing a 5 cm gap at the column head. The column was equilibrated in 50 mM Na_3PO_4 and elution at 2.5 min with 150 mM $C_6H_8O_7$, pH 2.5.

were used for subsequent evaluations of the role of supernatant filtration steps.

3.2. Quantification and binding capacity

Having dismissed any potential ill-effects in the addition of the gap void space at the head of the ProA C-CP fiber columns, potential effects on recoveries, quantification, and loading capacities were evaluated. The isolation and elution profiles are presented in Fig. 3 for the case of the injection of IgG from the unfiltered CHO supernatant. The injection volume was varied from 10 μL to 200 μL for the 0.71 mg mL^{-1} IgG in CHO supernatant. As described previously, all equilibration and elution times were consistent across each injection volume. With increasing the volume of CHO supernatant loaded, there are increases in the elution peak areas and heights, as expected. The same injection volumes were used for filtered supernatant, with the same trends observed, and the peak areas determined for comparison to the unfiltered separations.

The resultant IgG elution peak areas of triplicate filtered and unfiltered supernatant injections across the 10 – 200 μL load volumes are presented in Fig. 4. To be clear, the absorbance values reflect not specifically the mass of IgG adsorbed, but that which is recovered. For injection volumes of 10 - to – 100 μL (equivalent to 7.1 – 71 μg IgG injected on-column), the filtered supernatant represented by green circles, there is excellent linearity in the integrated absorbance responses, with an R^2 of 0.9997. Beyond 100 μL (71 μg IgG) and on, there is a negative deviation from linearity, suggestive of column overload. The recoveries for the unfiltered CHO supernatant are represented by the red triangles. As might be expected, the linear response range for the unfiltered supernatant was slightly smaller than for the filtered media; from 10 to 75 μL supernatant. Beyond that volume, the peak area responses begin to plateau, indicating column saturation. Very significantly, the two response curves are virtually superimposable across their linear regions, reflecting unit recovery efficiencies regardless of whether or not filtration is employed. Ultimately, the unfiltered supernatant does not significantly affect the binding capacity of the column. For both the unfiltered and filtered supernatant samples, slightly larger error bars are observed above 80 μg loadings as the column is becoming saturated at this point. The respective volume (y_v)- and mass (y_m)-based linear regression data are presented in the figure. The respective error bars (±1 sd) are plotted for the triplicate injections at each volume, with the data symbols covering the spreads in most instances. The precision for triplicate injections at each volume is noted on the figure. The overall precision across the data set is 0.84 %RSD and 1.40 %RSD for unfiltered and filtered, respectively, reflecting excellent consistency across the 27 separate, consecutive measurements for the two supernatant conditions (filtered/unfiltered).

In comparison to previous efforts in IgG isolation with the ProA-modified C-CP fiber phases, the binding capacities here for process CHO cell supernatants (~2 mg mL^{-1} fiber bed volume) are very much in line with those obtained for neat IgG solutions (~2.3 mg mL^{-1} fiber bed

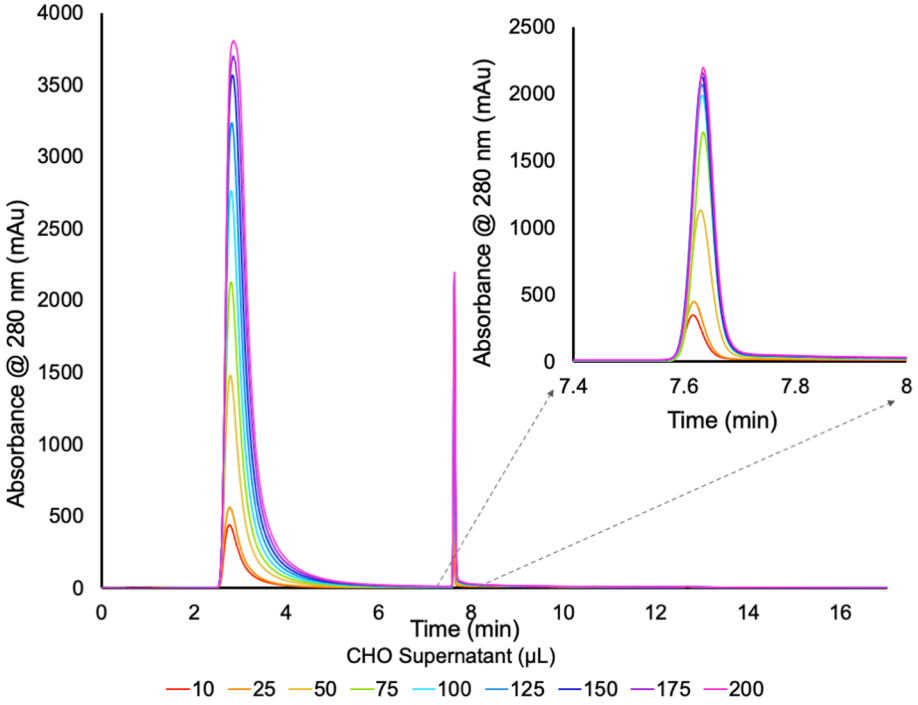

Fig. 3. Chromatograms of increasing volumes (10 – 200 μL) of unfiltered supernatant loaded onto a ProA C-CP fiber column. Column equilibration: 50 mM Na_3PO_4, injection time: 2.1 min, elution solvent: 150 mM $C_6H_8O_7$, pH 2.5.

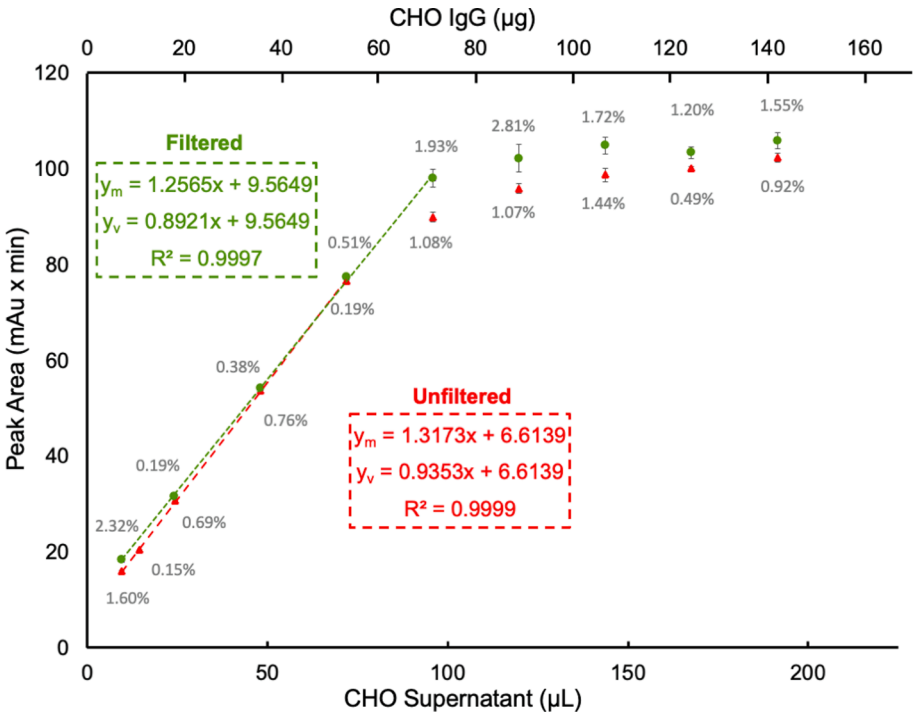

Fig. 4. Recoveries of eluted IgG (absorbance peak area) as a function of the injection volume/solute mass. Filtered supernatant is represented by green symbols, while unfiltered supernatant is represented by red symbols.

volume) [37], reflecting minimal impact from the highly complex supernatant matrix itself. It is believed that the non-porous nature of the fiber surfaces minimizes the potential effects of pore fouling. That said, it is difficult to make comparisons with commercial packed-bed and monolithic analytical-scale columns, where figures of merit are generally reported in regards to neat IgG solutions (i.e., not actual process supernatants) [39,40], where binding capacities of ~ 5 mg mL^{-1} fiber bed volume are observed. Product data sheets typically cite values in the single to low tens of mg mL^{-1}. The somewhat higher binding capacities in those columns are clearly attributable to the high porosity/surface area supports. That said, those attributes work counter to the fouling effects of complex media, which surely would limit the binding capacities and column lifetimes when processing bioreactor samples.

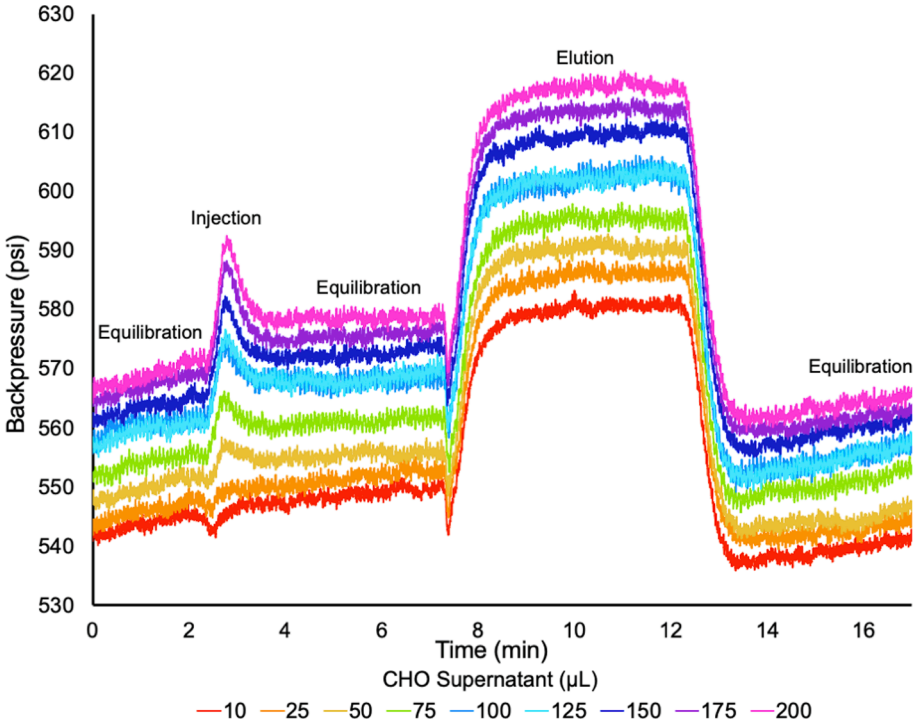

Fig. 5. Temporal responses of system backpressure as a function of supernatant injection volume. Column equilibration: 50 mM Na_3PO_4, elution solvent: 150 mM $C_6H_8O_7$, pH 2.5.

3.3. Backpressure effects of unfiltered media

The backpressure of the isolations was monitored to ascertain any ill-effects of the unfiltered media, reflective of clogging and fouling at either the column head or within the column channels themselves. The system-generated backpressure traces for each of the n = 3 sets of injections making up the response function of Fig. 4 were averaged and are presented in Fig. 5. For each injection of the unfiltered supernatant, the backpressure remains steady to the point of the supernatant injection, where a steady increase is observed as the loading solvent is introduced. Following the completion of the injection, the 7 min equilibration step is initiated, followed by the IgG elution step of 5 min, and a return to the equilibration solvent. What is seen across the traces, which were recorded with increasing sample volumes, is a steady increase in the backpressure, with incremental offsets that are uniform across the complete analytical cycles. While seemingly pronounced, the backpressure in the final re-equilibration following the last 200 μL injection is only ~ 25 psi higher than the beginning of the entire data set across 27 injections; an ~ 6 % change without the use of any clean-in-place procedures. Indeed, the change here corresponds only to the increased resistance affected with the larger sample volumes, as blank injections for a fresh column using the same gradient program were virtually superimposable on those for the infiltered supernatant. Thus, there was no backpressure burden placed on the system by the cell culture matrix. To emphasize the relative immunity towards fouling for the C-CP ProA column, it must be noted that a total of ~ 3 mL of CHO cell supernatant was introduced to the single column; representing a total of ~ 56 column volumes.

4. Conclusions

While ProA separations are simple and efficient, commercial columns are often expensive and require sample filtration prior to chromatographic isolations to preserve separation integrity and column lifetimes. In addition to added processing times and costs, such filtration inevitably leads to losses of valuable product to the filter surfaces. This is true on the analytical or preparative scales. The gapped ProA C-CP fiber column presented here was unaffected by the introduction of unfiltered supernatant in terms of chromatographic integrity, binding capacity, quantification, and back pressure. After loading > 55 bed volumes of unfiltered supernatant, significant backpressure increases were not observed. The quantification was also excellent across concentrations of 7.1 – 71 μg filtered (0.9997) and 7.1 – 54 μg unfiltered (R^2 = 0.9999) injection masses. Additionally, the binding capacity per bed volume of the unfiltered supernatant on the column was ~ 2 mg mL^{-1}, in line with commercial analytical scale ProA columns.

Further benchmarking with regards to commercial column technologies is in order, inclusive of other practical considerations such as processing times, consumables, and column costs. In any case, the fact that supernatant filtration is a requirement in those system's protocols reflects a potential advantage of the approach presented here. In addition, opportunities exist for ProA C-CP column scale-up. Perhaps most intriguing is implementation in continuous chromatography workflows where alleviating the supernatant filtration step would be attractive. Beyond this, the evacuation of particulates from the gap region with a brief counterflow could be easily employed as a standard process step to increase functional lifetimes.

CRediT authorship contribution statement

Sarah K. Wysor: Methodology, Data curation, Visualization, Writing – original draft. **R. Kenneth Marcus:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [R. Kenneth Marcus reports financial support was provided by Clemson University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper].

Data availability

Data will be made available on request.

Acknowledgements

This project was supported in part by National Science Foundation grant CHE-2107882.

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