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Ultrafiltration Behavior of Partially Retained Proteins and Completely Retained Proteins Using Equally-Staged Single Pass Tangential Flow Filtration Membranes

Abhiram Arunkumar
Global Product Development and Supply, Bristol-Myers Squibb and Company, 38 Jackson Road, Devens, MA 01434

Junyan Zhang
Global Product Development and Supply, Bristol-Myers Squibb and Company, 38 Jackson Road, Devens, MA 01434

Nripen Singh
Global Product Development and Supply, Bristol-Myers Squibb and Company, 38 Jackson Road, Devens, MA 01434

Sanchayita Ghose
Global Product Development and Supply, Bristol-Myers Squibb and Company, 38 Jackson Road, Devens, MA 01434

Zheng Jian Li
Global Product Development and Supply, Bristol-Myers Squibb and Company, 38 Jackson Road, Devens, MA 01434

DOI 10.1002/btpr.2671
Published online 00 Month 2018 in Wiley Online Library (wileyonlinelibrary.com)

This work examines the ultrafiltration behavior of partially retained proteins like lysozyme and completely retained proteins like monoclonal antibodies using single pass tangential flow filtration (SPTFF) modules with different screen channels and molecular weight cut-offs. When the staging of the SPTFF used the same membrane area in each stage, there was no impact of the module screened channel or the buffer matrix on the final concentration achieved for completely retained monoclonal antibodies. A hybrid configuration containing 30 kDa membranes and 50 kDa membranes increased the maximum achievable concentration for both the monoclonal antibodies used in this work, at the same time, allowing a two-fold to four-fold increase in normalized feed flow-rate through the system compared to only the 30 kDa or only the 50 kDa membranes. The sieving coefficient of lysozyme measured and calculated using SPTFF was lower than those measured during conventional recirculation TFF indicating a more complicated concentration polarization effect than conventional recirculation TFF. Moreover, the sieving coefficients of lysozyme were the same for the 10 kDa regenerated cellulose and 50 kDa PES membranes while it was higher for the 30 kDa regenerated cellulose membrane. The difference in TFF and SPTFF behavior is important when the product of interest is desired to be permeated. This work presents the first body of data for partially and completely retained solutes together in the SPTFF mode and provides a strategy to increase protein concentration at higher feed flow rates. © 2018 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2018
Keywords: single pass tangential flow filtration, ultrafiltration, continuous processing, sieving coefficient, screen channel, lysozyme, monoclonal antibody

Introduction

Significant advances have been made in biologics process development to increase upstream productivity (titers). As a result, the downstream purification platform is continuously evolving to increase capacity and selectivity to handle the increased biomass.[1] In addition to improved capacity, selectivity, better utilization of capacities and uniformity in product quality, one of the primary benefits of continuous processing is in the cost of the drug substance: Past work has reported that the manufacturing operating cost reduced from $1230 per gram for a batch process to $250 per gram for a continuous process with a three-fold decrease in capital costs.[2,3]

In order to enable continuous processing, perfusion, continuous chromatography, and multicolumn column chromatography need to form the workhorse of the purification process. There have been significant advances in continuous chromatography, with companies like Merck and Sanofi adapting a variant of continuous bioprocessing in their manufacturing pipeline.[4] While perfusion and technologies like alternating tangential flow filtration (ATF) is desired to express the therapeutic protein and harvest them continuously and chromatography is used to perform the purification to acceptable standards, the final step in producing the drug substance involves concentration of the protein and exchanging the protein into the formulation buffer. This unit

Correspondence concerning this article should be addressed to A. Arunkumar at abhiramarunkumar@outlook.com.

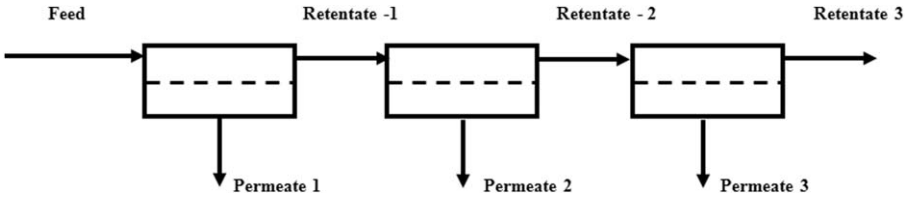

Figure 1. Single Pass Tangential Flow Filtration (SPTFF) flow path. The area of all the stages is the same, but could be different in principle.

operation is traditionally performed in the batch recirculation mode using an ultrafiltration membrane that is retentive to the protein, but permeable to buffer components. The recirculation of the protein solution makes the operation a batch process. Batch TFF has been the subject of extensive research in order to produce highly concentrated monoclonal antibodies and Fc-Fusion proteins.[5–7] The adaptation of continuous processing in the final step to concentrate proteins and exchange them in the formulation buffer has been a slow process.

Single pass tangential flow filtration (SPTFF) is a technology that eliminates the recirculation loop and allows for concentration in a single pump pass. This is achieved by increasing the residence time of the protein solution within the module and increasing the effective length and area simultaneously. Past work discussed the use of commercially available SPTFF modules to concentrate proteins, and the key hydraulic differences between TFF and SPTFF.[8] However, much of the work on SPTFF has been on retentive proteins using retentive membranes that had a molecular weight cut-off of 10 or 30 kDa.[9–11] There is no available literature that compares the performance of partially retained solutes and completely retained solutes using SPTFF, or the effect of membrane molecular weight cut-off on achieving concentrated protein solutions. This is necessary when membrane steps will be used to replace chromatographic polishing steps as Zydney points out[12] or when the purpose of the SPTFF is to isolate the product of interest in the permeate. This is also true for emerging new modalities for biotechnology such as viral vector purification, plasmid DNA, or RNA, when the product of interest appears in the permeate, and single-pass TFF might be the only option to achieve purification objectives. In case of retentive monoclonal antibodies, SPTFF has been examined as an alternative technology to replace TFF to achieve highly concentrated solutions.[8] Several studies have reported on the complex TFF behavior of concentrated monoclonal antibody solutions and the dependence on the inter-molecular interactions in the protein and the buffer composition, and their interaction with the module hydraulics. For example, Binabaji et al. found that the type of the screen channel taken along with the buffer affects the maximum achievable concentration[5,6] and confirmed by Arunkumar et al.[7]

This work provides experimental data on the sieving behavior of a partially retained model protein, lysozyme (Molecular mass = 14.3 kDa) using ultrafiltration membranes of different molecular weight cut-offs in SPTFF, and on the effect of membrane molecular weight cut-off, screen type and the buffer matrix on the behavior of completely retained monoclonal antibodies exclusive to Bristol-Myers Squibb. A hybrid molecular weight cut-off solution was identified as the ideal strategy to obtain high concentrations at higher feed flow-rates. The outcomes of this study provide the first experimental data set to show substantial differences between TFF and SPTFF for partially retained and completely retained proteins, and provides the basis for protein separations using SPTFF for the biotechnology and the food processing industries.

Theory

SPTFF is a membrane technology that has become commercially available in recent times, to enable protein concentration in a single pump-pass through the module feed channels. The single pass concentration is achieved by reducing the feed flow rate into the module and allowing enough contact time of the protein solution within the module to allow high conversion of the feed solution into the permeate. The length of the module is increased, the modules arranged in series, and the area of the membrane is consequently increased to allow higher conversions in a single pump pass (Figure 1). Two different approaches to staging are possible: the unequal area staging (also called the Christmas Tree staging), and the equal area staging. The basic premise to increase the overall membrane area and the module length is the same for both these approaches to device design. This work will focus only on the equal area staging.

In ultrafiltration, the partially and completely retained solutes accumulate at the wall of the membrane and form a polarized boundary layer. This phenomenon is called concentration polarization and the concentration of the solute (protein) at the wall, C_W is related to that at the bulk, C_b by means of Eq. (1).

$$C_W = C_b\left(S_o + (1-S_o)\exp\left(\frac{J}{k}\right)\right) \quad (1)$$

where J is the filtrate flux, k is the boundary layer mass transfer coefficient, and S_o is the observed sieving coefficient given by $S_o = C_p/C_b$, where C_p is the concentration of solute (protein) in the permeate. In a system where the membrane is completely polarized and the polarized boundary layer controls the separation, the wall concentration should have a constant value at a given axial position, z, and it will change (increase) throughout, depending on the filtrate flux and the protein concentration at each z. This means that as long as the feed solution to the membrane module is the same, the wall concentration at each z is constant (pressure-independent ultrafiltration), the filtrate flux and the retentate concentration are constant at each axial point, allowing for constant operating conditions throughout the process.

When the solute is partially retained as some applications for protein fractionation or permeation may require, the sieving coefficient will depend on the wall concentration. It is well known in ultrafiltration that increasing the wall concentration increases the sieving coefficient for pure protein solutions in buffer.[13,14]

Table 1. List of Monoclonal Antibodies Used in This Work Along With Their Physical Properties

Monoclonal antibody	Isoelectric point (pI)	Phosphate buffer composition	Histidine buffer composition	Molecular mass (kDa)	Target concentration (g/L)
mAb1	9.2–9.6	20 mM sodium phosphate pH 7.0–7.4	20 mM histidine 200–260 mM Sucrose pH 5.6–6.2	140–150	200
mAb2	7.2–7.8	20 mM sodium phosphate pH 7.0–7.4	20 mM histidine 200–260 mM sucrose pH 5.6–6.2	140–150	75
Lysozyme	11.4	20 mM sodium phosphate 150 mM sodium chloride pH 7.4	N/A	14.3	N/A

Strictly speaking, the sieving coefficient of a partially retained protein is not constant in SPTFF since the protein concentration changes throughout the length of the module. Furthermore, it is difficult to measure the sieving coefficient in SPTFF because of changing hydraulics at each point in the module. Nonetheless, it is possible to break down the SPTFF system into three-stages, exactly as it is assembled, and an "average" sieving coefficient can be estimated based on the permeate and retentate concentrations at each stage using the equations presented by Arunkumar and Etzel[15] according to Eq. 2:

$$\bar{S_o} = 1 - \ln\left(\frac{C_{R,i}}{C_{R,i-1}}\right) / \ln \mathrm{VCF}_i$$

[2]where $C_{R,i}$ is the retentate coming out of stage i, $C_{R,i\text{-}1}$ is the retentate exiting the (i-1)th stage (thereby becoming the feed to the ith stage), VCF_i is the volume concentration of the ith stage. The overall mass balance of the system is given by Eq. (3):

$$C_F = \hat{Q}_t C_P + \left(1 - \hat{Q}_t\right) C_R \tag{3}$$

$\hat{Q}_t$ is the total conversion of the feed to the permeate (given by $\hat{Q}_t = Q_P/Q_F$), C_P is the overall permeate concentration, and C_R is the overall retentate concentration.

The overall mass balance can be used to calculate an "overall average" sieving coefficient, $\langle S_O \rangle$ using Eqs. 2 and [3] according to Eq. (4) to achieve an overall average VCF_t:

$$\langle S_o \rangle = 1 - \ln\left(\frac{C_R}{C_F}\right) / \ln\left(\mathrm{VCF}_t\right) \tag{4}$$

The monoclonal antibodies (Molecular Mass = 140–150 kDa) used in this work were completely retained by the 10 kDa composite regenerated cellulose (CRC), 30 kDa CRC, and 50 kDa polyethersulfone (PES) membranes. Thus, S_o was set to $S_o = 0$ for the retained antibodies. Lysozyme was partially retained by these membranes ($0 < S_o < 1$). While it is straightforward to understand lysozyme behavior using conventional TFF by measuring the sieving coefficient as a function of polarization conditions (filtrate flux and crossflow rate), the above equations are necessary to estimate the sieving behavior of lysozyme in each section of the SPTFF module.

Experimental

This purpose of this work was to experimentally understand the single-pass ultrafiltration behavior of partially permeable proteins (hen egg white lysozyme, MW = 14.3 kDa) and completely retentive proteins like specific monoclonal antibodies exclusive to Bristol-Myers Squibb (MW = 140–150 kDa). To this end, experiments were performed using membrane modules with molecular weight cut-offs of 10, 30, and 50 kDa, and with different turbulent promoters (feed screens).

Modules with different turbulent promoters based on different differential pressure are commonly used for protein concentration to achieve concentration targets.[5,7]

Materials and methods

The two monoclonal antibodies (mAb1 and mAb2) used in this work were IgG4 monoclonal antibodies and had molecular masses of 140–150 kDa with physical characteristics provided in Table 1. Hen egg white lysozyme was obtained from MilliporeSigma (L-6876) and dissolved in 20 mM sodium phosphate 150 mM sodium chloride pH 7.2 to achieve a protein concentration of 10 mg/mL. This particular buffer composition was chosen because it had a conductivity of 16 mS/cm, and was enough to overcome electrostatic exclusion effects for lysozyme that has been known to impact protein sieving through a 30 kDa ultrafiltration membrane.[16]

The 10, 30, and 50 kDa membranes used were obtained from MilliporeSigma had different screens tabulated in Table 2. The 10 and 30 kDa membranes were made of Ultracel® CRC, while the 50 kDa membrane was made of Biomax® modified polyether sulfone (PES). The 50 kDa PES membrane was used because this was the only commercial option for a cut-off beyond 30 kDa that would completely retain a monoclonal antibodies. A 50 kDa CRC membrane was not available commercially for use in this work.

Pressure sensors were obtained from Pendotech Corporation, Nassau, NJ.

Tangential flow filtration experiments under conditions of total recycle to measure lysozyme sieving coefficients

Conventional TFF in recirculation mode was performed for lysozyme to compare the sieving behavior in the TFF and SPTFF modes. Sieving coefficients were measuring using the approach used previously in literature.[13,17] One membrane module with an area of 0.11 m^2 for the 10 kDa CRC, 30 kDa CRC, and the 50 kDa PES membranes was used for these measurements.

10 mg/mL lysozyme was prepared by dissolving the lysozyme in 20 mM sodium phosphate 150 mM sodium chloride pH 7.2, and, was recirculated through the 10 kDa CRC (C screen, Catalog Number P3C10C01), 30 kDa CRC (D screen, Catalog Number P3C030D01) or 50 kDa PES (A screen, Catalog Number P3B050A01) ultrafiltration membrane under conditions of total recycle, at a membrane area-normalized feed flow-rate (or "feed flux," referred to as normalized feed-flow rate throughout this manuscript) of 100 L/h/m^2. The control valve on the retentate was used to adjust the inlet pressure on the feed to 2.0 bar (30 psig). A pump on the permeate side was used to control the permeate flux

Table 2. Membrane Modules and Their Characteristics Used in This Work

Membrane train	Modular arrangement	Total area (m^2)	Screen channel	ΔP at hydraulic flow-rate of 4 L/min/m^2	Vendor
10 kDa Ultracel (CRC)	3 × 0.11 m^2–10 kDa membranes	0.33	Type C, 515 μm	14	MilliporeSigma
30 kDa-C, Ultracel (CRC)	3 × 0.11 m^2–30 kDa membranes	0.33	Type C, 515 μm	10	MilliporeSigma
30 kDa-D, Ultracel (CRC)	3 × 0.11 m^2–30 kDa membranes	0.33	Type D, 610 μm	2	MilliporeSigma
50 kDa-A Biomax (PES)	3 × 0.11 m^2–50 kDa membranes	0.33	Type A, 420 μm	17	MilliporeSigma
30D-30D-50A	2 × 0.11 m^2–30 kDa D screen membranes followed by 1 × 0.11 m^2 50 kDa A screen membrane	0.33	Hybrid of the D screen and A screen	10	MilliporeSigma

to the desired value and samples were collected from the permeate tubing and retentate tubing at different filtrate fluxes to measure the protein sieving coefficient.

Single pass tangential flow filtration of lysozyme

A Pellicon 3™ Single-Pass TFF system was used with a filtration area of 0.11 m^2 per stage. A diverter plate (Catalog: XXSPTFF01) was placed in a Pellicon-2™ Mini holder (Catalog Number XX42PMINI), with a gasket in between to seal the feed and permeate channels. Then, 0.11 m^2 Pellicon 3™ TFF cassettes were inserted after the first diverter plate, giving a three-in-series system with a total area of 0.33 m^2, with each cassette separated by a diverter plate. The assembly was torqued to 23 Nm using a torque wrench.

Single-pass concentration of lysozyme was performed using two normalized feed flow-rates of 55 and 18 L/h/m^2 using the different ultrafiltration membranes. The retentate pressure was adjusted to provide at least a five times concentration in a single pass. Permeate and retentate were collected from every stage and measured for protein concentration. Based on the protein concentrations and flow-rates, average sieving coefficients were calculated using Eq. 2 for each stage and for the overall module for a given membrane and a given feed flow-rate, using Eq. (4).

SPTFF excursion experiments for monoclonal antibodies

Single pass TFF experiments were performed using the same method as Arunkumar et al.[9] The protein solution was pumped through the membrane module at different flow-rates and a retentate pressure of 10.0–15.0 psig to begin the process, with the value being increased using a control valve as target concentrations increased. A retentate pressure of 10–15 psig was chosen to ensure that all the ultrafiltration experiments were performed in the pressure independent regime of the flux vs. TMP plot, which was generated separately at different normalized feed flow-rates. While reporting the data in this work subsequently, the feed pressure and retentate pressures were not separately reported because the system control used two parameters—the area normalized feed flow rate and the retentate pressure. The manipulation of the flow-rate and retentate pressure set a system feed pressure to the inlet of the SPTFF system. The absolute values of the feed pressure or retentate pressure did not give a trend; however, the feed flow-rate coupled with the differential pressure through the channel was sufficient to provide a trend with the volume concentration factor and describe the system hydraulics completely.

The retentate was connected to a highly sensitive inline protein concentration measurement system based on absorbance at 280 nm (FlowVPE, C Technologies) that gave the continuous output of the protein concentration on the retentate. Each data point corresponding a particular normalized feed flow rate was collected only after equilibrating the system at the given normalized feed flow rate for at least 30 min. The attainment of equilibrium and constant output was determined by the Pendotech pressure trace and the protein concentration trace on the FlowVPE as a function of time. All the data points reported in this work did not show deviations from constant outputs in the 30 min during which the measurement was made and reported.

Any discrepancy in the measured outlet concentration was immediately investigated. The flow-rate, feed pressure, retentate pressure, and the corresponding concentration were noted before proceeding to a different normalized feed flow-rate. Samples were collected from the permeate of each stage separately to analyze for any losses due to protein sieving into the permeate. The procedure was repeated for different modular configurations and different protein solutions in their respective buffer compositions.

Measurement of protein concentrations

Protein concentration of the pool samples was measured using a DropSense 96 well plate system (Trinean, Genbrugge, Belgium). 4 μL of sample was loaded onto a 96-well plate and absorbance at 280 nm was measured. Absorbance was converted to protein concentrations using the empirically determined extinction coefficient, assuming that the Beer-Lambert law was valid. The measurements from the pool samples was used to confirm measurements from the FlowVPE.

Results

This work examined the single-pass ultrafiltration behavior of two types of proteins: a partially retained protein, lysozyme, and two completely retained monoclonal antibodies using 10 kDa CRC, 30 kDa CRC, and 50 kDa PES molecular weight cut-off ultrafiltration membranes. These two types of solute-retention behavior cover a broad range used in bioprocessing, across different modalities and even across different industries (biotechnology, ADCs, food and dairy processing). Some applications may require separation of a small molecule or protein from larger molecules using semipermeable ultrafiltration membranes,[13,15,17,18] and some applications may require concentration of protein solutions using completely retentive ultrafiltration membranes.[5–7,19] With the exploration of single pass TFF as a new technology as an alternative to TFF, it is important to understand the single-pass ultrafiltration behavior of both partially retained and completely retained proteins. This is the first work to provide experimental data demonstrating the behavior of partially retained proteins and completely retained proteins as a function of membrane molecular weight cut-off.

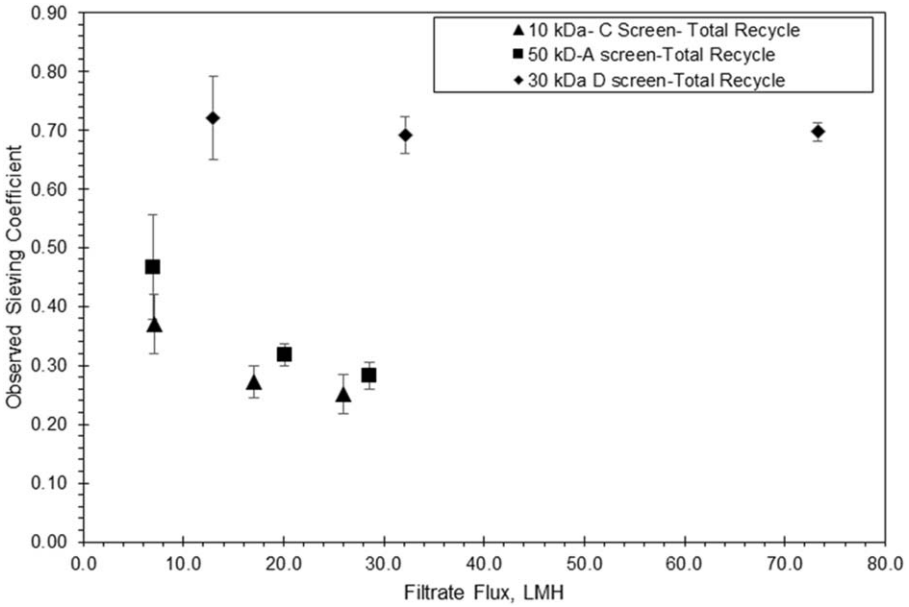

Figure 2. Variation of sieving coefficients of lysozyme with filtrate flux using different membranes in the recirculation TFF mode at a normalized feed flow-rate of 100 L/h/m^2. No concentration was performed, only total-recycle of retentate and permeate into the feed container.

Sieving behavior of partially retained lysozyme using conventional TFF

The purpose of this experiment was to obtain the TFF sieving data for lysozyme as a control to compare with the single-pass TFF lysozyme sieving and study any differences.

Figure 2 shows the variation of lysozyme sieving coefficient as a function of filtrate flux at a normalized feed flow rate (crossflow rate) of 100 L/h/m^2 using different membranes, operated in the total recycle TFF mode. The data shows that the sieving coefficients of lysozyme using the 10 kDa CRC and 50 kDa PES ultrafiltration membranes are identical ($P > 0.05$), with the data for the 10 kDa CRC and 50 kDa PES ultrafiltration membranes essentially lying on top of each other. The sieving coefficient of lysozyme (MW = 14.3 kDa) using the 30 kDa CRC ultrafiltration membrane module was 150% higher than the 10 kDa CRC membrane and 120% higher than the 50 kDa PES membrane ($P < 0.05$) at a comparable filtrate flux of 17 LMH. The sieving coefficients did not change with flux for the 30 kDa membrane, while the sieving coefficients decreased with flux from 7 LMH to 30 LMH for the 10 and 50 kDa membranes and remained constant thereafter. This behavior is explained and discussed in "Comparison of sieving coefficients of lysozyme using TFF and SPTFF" section.

Sieving behavior of lysozyme in single-pass TFF

Single-pass concentration of lysozyme was performed using two normalized feed flow-rates of 55 and 18 L/h/m^2 using the 10 kDa CRC, 30 kDa CRC, and 50 kDa PES ultrafiltration membranes. Based on the protein concentrations and flow-rates, average sieving coefficients were calculated using Eq. 2 for each stage and the overall module using Eq. (4) for a given feed flow rate. The sieving coefficients of lysozyme calculated using SPTFF were lower than those measured during TFF for all the membranes ($P < 0.05$). Furthermore, the calculated sieving coefficients of lysozyme for the 10 kDa CRC membrane were constant through all the stages, and also did not change as a function of the normalized feed-flow rate with $\langle S_o \rangle = 0.17 \pm 12\%$ CV ($P > 0.05$) (Figure 3 and Table 3).

In case of the 50 kDa PES membrane, the stage-wise sieving coefficient of lysozyme increased with decreasing normalized feed flow-rate ($P < 0.05$) for all the stages. The same trend was observed for the 30 kDa CRC membrane ($P < 0.05$). The stage-wise sieving coefficients of lysozyme reported using the 30 kDa CRC membrane were the highest compared to the 10 kDa CRC and the 50 kDa PES membranes.

While the stage-wise sieving coefficient of lysozyme using the 10 kDa CRC membrane did not change with the stages, or the normalized feed-flow rate, the sieving coefficient of the 50 kDa membrane decreased from stage 1 to stage 3, with the sieving data between stage 2 and stage 3 being indifferent at a given normalized feed flow-rate ($P > 0.05$) (Figure 3).

The stage-wise sieving coefficient of lysozyme using the 30 kDa CRC membrane went through a maximum with the values at stage 2 being the highest for a given feed flow-rate. Nonetheless, the differences between stage 1 and stage 2 were small, with the sieving coefficients at stage 3 being 24% lower than stage 1 at 18 L/h/m^2 and 20% lower than stage 1 at 55 L/h/m^2.

The distribution of lysozyme in the permeate followed the exact trend of increasing overall sieving coefficients $\langle S_O \rangle$, with decreasing normalized feed flow rates (Table 3). The $\langle S_O \rangle$ for the 50 kDa PES membrane did not differ from the 10 kDa CRC at 55 L/h/m^2, but was 31% higher than the 10 kDa CRC membrane at 18 L/h/m^2 ($P < 0.05$). The data set for all the membranes reported in Figure 3 and Table 3 were highly reproducible with a coefficient of variation (%CV) of $<10\%$.

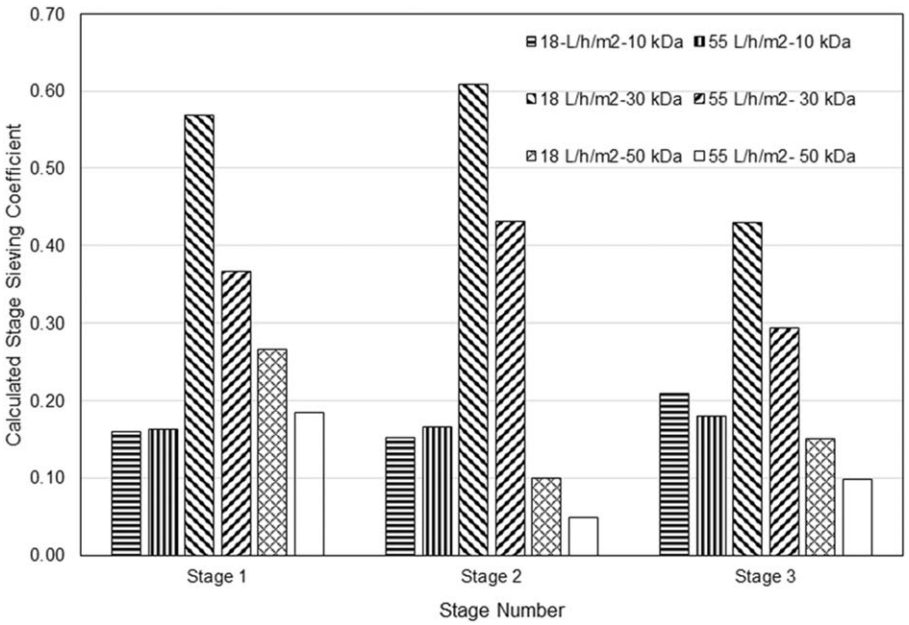

Figure 3. Stage-wise sieving coefficients of lysozyme using different membranes in the SPTFF mode. Data is presented for each stage as a function of feed flow-rate. The TMP was maintained above the critical TMP for all these experiments.

Table 3. Summary of Protein Concentrations, Sieving Coefficients and Distributions in the Permeate and Retentate for the Concentration of Lysozyme Using Different SPTFF Modules

Membrane configuration	Feed flow rate (L/h/m^2)	Overall conversion	Overall sieving coefficient, $\langle S_o \rangle$	% Distribution in permeate	% Distribution in retentate	Retentate concentration (g/L)
10 kDa, (3 × 10 kDa, C)	55	0.81	0.17 ± 0.02	23.5	75.0	41.8
	18	0.94	0.16 ± 0.01	37.2	61.9	117
30 kDa, (3 × 30 kDa, D)	55	0.87	0.37 ± 0.03	49.7	50.2	43.8
	18	0.86	0.55 ± 0.04	61.6	37.4	29.4
50 kDa, (3 × 50 kDa, A)	51	0.86	0.14 ± 0.03	19.7	80.3	48.5
	19	0.91	0.21 ± 0.01	37.1	66.6	62.9

Data is presented as average ± SD.

Ultrafiltration behavior of completely retained monoclonal antibodies using different modular configurations

The monoclonal antibodies mAb1 and mAb2 were completely retained using all the membranes. Since SPTFF is primarily used to concentrate these protein solutions for producing high concentration formulations, the effect of membrane molecular weight cut-off and the type of screen channel was examined. As shown in Figure 4, the normalized feed flow rate vs. protein concentration plots for the 10 kDa CRC membrane and 30 kDa CRC membrane for mAb1 were indistinguishable. The corresponding differential pressures for the 10 kDa CRC and 30 kDa CRC membranes were similar but not the same. Nevertheless, the molecular weight cutoff between the 10 and 30 kDa or the screen type did not affect the performance for mAb1. The 50 kDa PES membrane had a higher differential pressure, presumably because of the tight screen in the 50 kDa PES module.

Furthermore, it was also observed that using a 50 kDa PES membrane in the last stage in a 30-30-50 kDa configuration helped in pushing the maximum concentration further than just using 30 kDa membranes or 50 kDa membranes, and allowed operation at a flow rate that was three-fold higher compared to the 30 kDa membranes or the 50 kDa membrane alone, even though the differential pressures were the same as the 30 kDa membranes (Figure 4).

Similar observations were made for mAb2, where the 30-30-50 kDa configuration significantly pushed the maximum concentration beyond 150 mg/mL, even though the target required to be achieved during processing was only 75 mg/mL (Figure 5). The 50 kDa PES membrane met the target of 75 mg/mL, but the 30-30-50 kDa hybrid system was able to operate at a higher feed flow-rate to achieve the same concentration objectives as the 30 kDa or the 50 kDa membranes. The data in both Figures 4 and 5 were averaged for mAb1 and mAb2 in both phosphate and histidine buffers, indicating that the buffer matrix did not affect the capability of the equal area staging to achieve final concentration targets ($P > 0.05$). Both these figures also indicated that the differential pressures were similar for both the phosphate and histidine buffers with a %CV on the differential pressure being $<5\%$.

Concentration experiments for 1 h were performed for mAb1 and mAb2 using the 30 kDa, 50 kDa and 30-30-50 kDa hybrid system at the lowest normalized feed flow-rate realistically possible (which means the retentate flow-rate was measurable accurately). The permeate flow-rates from

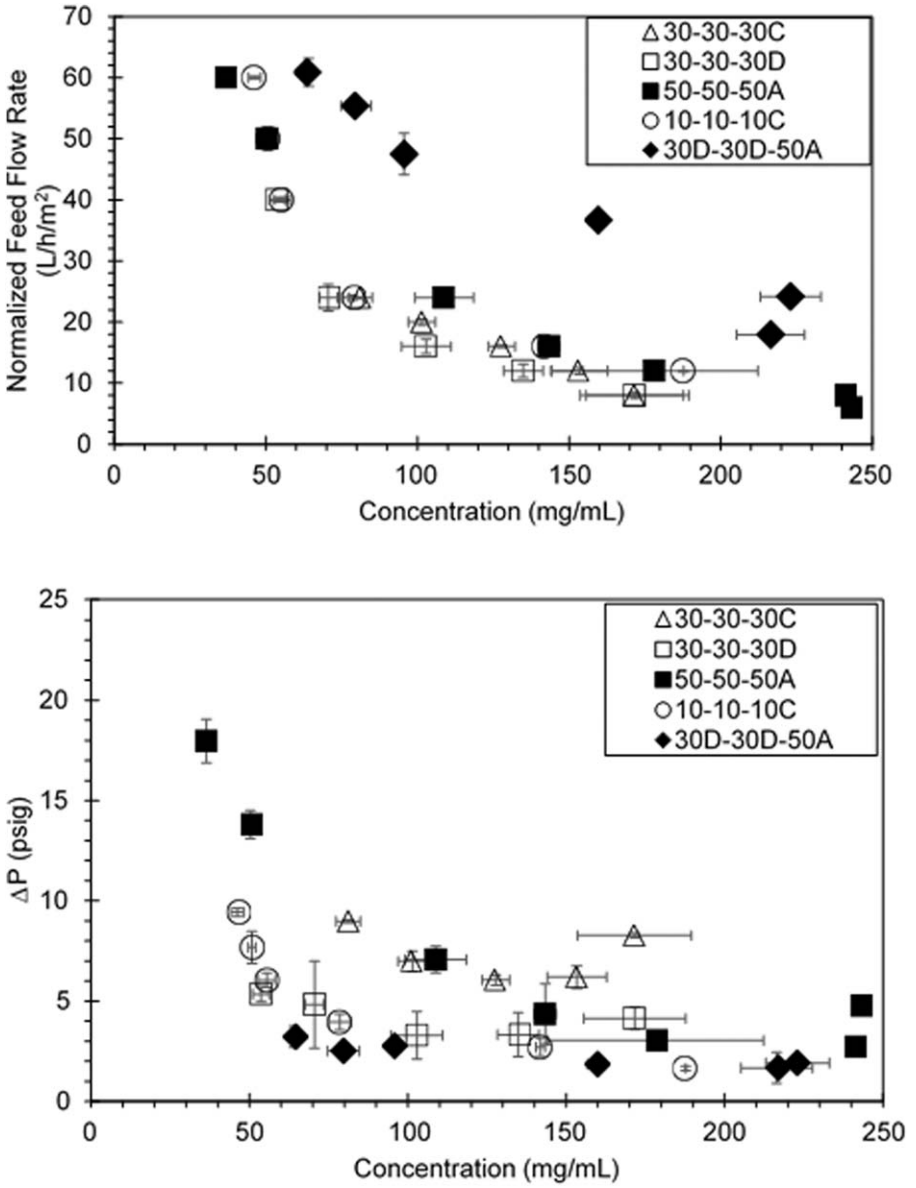

Figure 4. (A) Normalized feed flow rate vs. final retentate concentration using different SPTFF arrangements for mAb1. The feed concentration was 16 ± 3 g/L. (B) Differential pressure vs. final retentate concentration for different SPTFF arrangements for mAb1.

each stage were measured to calculate the contribution of each stage. The stage-wise cumulative volume concentration factor data is presented in Figure 6. The 30-30-50 kDa configuration was capable of achieving significantly higher concentration factors compared to the standard 30-30-30 kDa configuration: 12× for mAb1 and about 80× for mAb2, even though the flow-rates were 183% higher for the 30-30-50 kDa configuration for mAb1 compared to the 30-30-30D configuration, and, 57% higher for the 30-30-50 kDa configuration for mAb2, compared to the 30-30-30D membrane configuration.

While discussing any pressure driven filtration operation like ultrafiltration, it is common to report the hydraulics as a function of protein concentration using the differential pressure between the feed and retentate (ΔP) and the feed flow-rate to get the differential pressure. While the ultrafiltration behavior of a given module and/or configuration can be described completely using these two metrics, it is operationally important to understand the absolute values of the retentate pressure or the feed pressure along with the ΔP. The absolute magnitude of the retentate pressure for the highest protein concentration for both mAb1 and mA2 is provided in Table 4. From this information, the feed pressure can also be calculated.

Discussion

This work examined several important aspects of the ultrafiltration behavior of partially retained solutes and completely retained proteins using single pass TFF. The dataset provided in this work is the first experimental data reported for the ultrafiltration behavior of a partially retained solute using SPTFF, and the first for dataset for a completely retained protein (mAb) using more open membranes and hybrid membrane configurations. As reported in the above sections "Sieving behavior of partially retained lysozyme

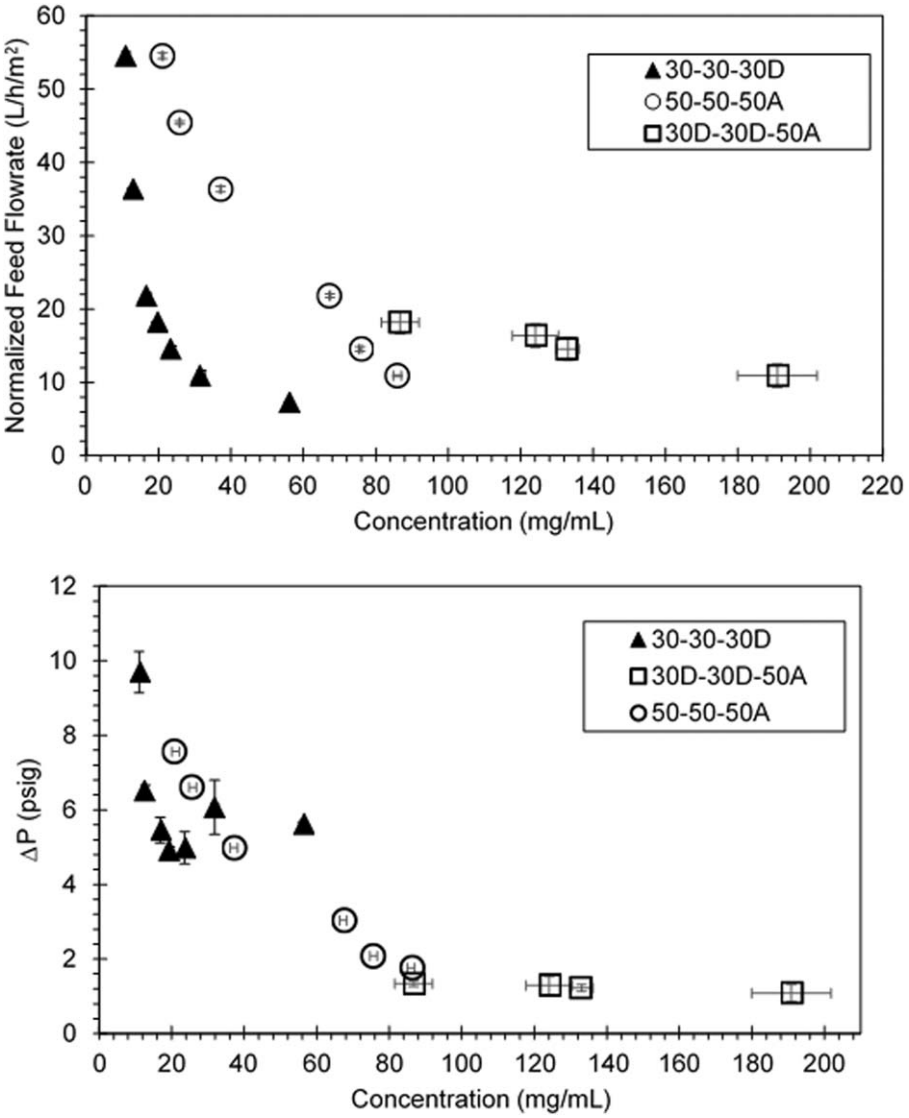

Figure 5. (A) Normalized feed flow rate vs. final retentate concentration using different SPTFF arrangements for mAb2. The feed concentration was 5 ± 1 g/L. (B) Differential pressure vs. final retentate concentration for different SPTFF arrangements for mAb2.

using conventional TFF" and "Sieving behavior of lysozyme in single-pass TFF," the sieving behavior is complicated compared to conventional TFF. This behavior will translate to other more complex systems where SPTFF is being explored—like isolation of monoclonal antibodies in the permeate during primary clarification[20] and for purification of other modalities where high sieving into the permeate is desired. While the operation itself is simpler compared to TFF, the sieving behavior is more complicated. In case of completely retained proteins, SPTFF is already in place in industry to provide inline concentration of in-process pools as reported in literature.[9,21] One of the major limitations with SPTFF is the need to explore complicated staging arrangements or expand the membrane as higher concentrations are targeted, because the normalized feed flow rates are significantly lower (<10 L/h/m^2). This work explored the use of a hybrid staging arrangement that used retentive membranes of two different molecular weight-cutoffs to achieve the target concentrations at higher flow-rates than currently obtained using SPTFF.

Comparison of sieving coefficients of lysozyme using TFF and SPTFF

The trend in the sieving coefficients of lysozyme as a function of flux using the TFF mode at a normalized feed flow-rate of 100 L/h/m^2 was not surprising, but the sieving behavior of lysozyme as a function of molecular weight cut-off was. The flow-rate of 100 L/h/m^2 was chosen because TFF is typically operated at a normalized feed-flow rate of 200–800 L/h/m^2, and 100 L/h/m^2 was a normalized-feed-flow rate that represented polarization conditions more typical of SPTFF. The TFF data in Figure 2 indicated that the 10 kDa CRC and the 50 kDa PES membranes were very similar in sieving behavior toward lysozyme, while the 30 kDa CRC membrane had the highest sieving coefficients. This agreed with the finding by Bakshayeshi[22] that the rating of membranes using dextran retention tests is not a standardized practice yet, and different vendors rate their membranes differently. Nevertheless, the 10 kDa CRC, 30 kDa CRC, and 50 kDa PES membranes were all manufactured by MilliporeSigma, and the molecular weight cut-off rating based on

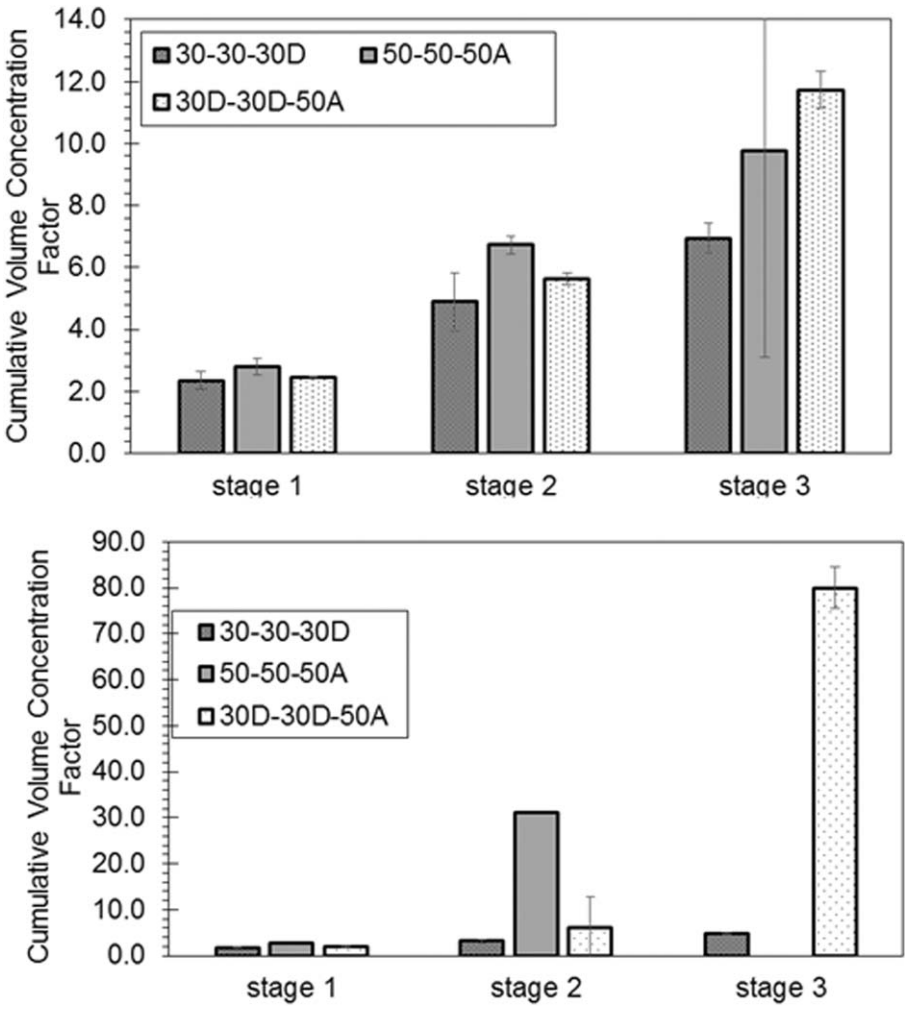

Figure 6. Cumulative volume concentration factor at each stage for (A) mAb1 and (B) mAb 2 using the 30 kDa, 50 kDa, and the 30-30-50 kDa hybrid configurations. The 50 kDa membrane for mAb1 was not used beyond stage 2 for the 50-50-50A configuration. Normalized feed flow rates were: (A) mAb1: 3 g/L feed solution 7.8 L/h/m^2 for the 30 kDa, 11.7 L/h/m^2 for the 50 kDa, and 12.1 L/h/m^2 for the 30-30-50 kDa hybrid, (B) 16 g/L feed solution 7.5 L/h/m^2 for the 30 kDa, 6.6 L/h/m^2 for the 50 kDa, and 21.1 L/h/m^2 for the 30-30-50 kDa hybrid.

Table 4. Retentate Pressures for mAb1 and mAb2 at the Highest Protein Concentrations Achieved Using Different Combinations, Averaged Over all the Buffer Compositions Used in This Work

	30-30-30D (3 × 30 kDa with D screen)			50-50-50A (3 × 50 kDa with A screen)			30D-30D-50A (Hybrid configuration)		
Monoclonal antibody	Maximum concentration (g/L)	ΔP (psig)	Retentate pressure (psig)	Maximum concentration (g/L)	ΔP (psig)	Retentate pressure (psig)	Maximum concentration (g/L)	ΔP (psig)	Retentate pressure (psig)
mAb1	172 ± 16	4.1 ± 0.6	17 ± 1	243 ± 40	4.8 ± 0.1	15.1 ± 2.5	223 ± 10	1.9 ± 0.1	10.1 ± 0.3
mAb2	56 ± 1	5.6 ± 0.1	38 ± 3	86 ± 0	1.8 ± 0.1	11.7 ± 0.1	191 ± 11	1.1 ± 0.2	11.5 ± 0.3

Data is presented as average ± SD.

dextran sieving is expected to hold true for proteins as well, regardless of the membrane material. The choice of the material (CRC vs. PES) is based on its compatibility with the solution to be ultrafiltered. A 50 kDa CRC membrane would have had the higher permeability compared to a 10 kDa or a 30 kDa CRC membrane, but a 50 kDa CRC membrane was not commercially available by any vendor that makes ultrafiltration membranes.

The trend for SPTFF somewhat qualitatively agrees with the classical stagnant film model: decreasing the feed flow rate increases the residence time of the protein in the retentate channel and hence the concentration at every section of the SPTFF module. This increases the accumulation of lysozyme at the membrane wall, C_W and results in a higher sieving coefficient (Eq. (1)). This is very apparent for the 30 kDa CRC membrane where the sieving coefficient increased at stage 2 compared to stage 1, but not so for the 10 kDa CRC and the 50 kDa PES membranes (Figure 3). In fact, the sieving coefficients of the 50 kDa PES membrane decreased as a function of the stage, as the protein concentration increased through the module while that of the 10 kDa CRC membrane did not change as a function of the stage. The fact that decreasing normalized feed-flow rate increases the residence time and hence the wall concentration is reflected

in the trend of stage-wise sieving coefficients being higher at 18 L/h/m^2 compared to 55 L/h/m^2 for both the 30 and 50 kDa membranes. The trend of decreasing sieving coefficients using the 50 kDa membrane as a function of the stage at a given feed flow rate was attributed to membrane fouling. The 50 kDa PES membranes had to be cleaned thoroughly using 400 ppm of bleach in 0.1 M NaOH to restore the permeability.

The classical stagnant film model cannot sufficiently explain the differences in the sieving coefficients for SPTFF and the TFF mode. According to the classical stagnant film model, the sieving coefficient observed during TFF (Figure 2) should be the lowest, since the feed flow-rate is the highest (100 L/h/m^2 compared to 55 and 18 L/h/m^2 for SPTFF) and the concentrations are lower and more uniform (10 mg/mL for TFF vs. an increasing concentration in SPTFF). This means the wall concentration should have been lower at every flux tested for the TFF, and hence the sieving coefficients also lower. The reverse is observed: The sieving coefficient is highest using the TFF mode for all the membranes. The differences in the sieving coefficients could be attributed to differences in the mechanism of concentration polarization using SPTFF vs. conventional TFF. Moreover, a more complex dependence on protein concentration is indicated looking at the data in Figure 3 for the 30 kDa CRC membrane, where the sieving coefficient decreases with increasing concentration in stage 3, regardless of the normalized feed flow rate. This behavior indicates that the ultrafiltration behavior of partially retained solutes may be complicated using SPTFF compared to TFF, and any separation process that intends to separate macromolecular solutes using SPTFF will have to be studied very carefully as the sieving behavior expected from TFF or stirred cells will most likely not hold for SPTFF. Examples of such separations include microfiltration of clarified harvest,[20] separation of individual proteins from bioprocess streams,[13,15] separation of PEGylated proteins from PEG and non-PEGylated proteins,[14,18] ultrafiltration of other therapeutic modalities like viral vectors and plasmid DNA, and fractionation of dairy protein fractions in the food industry.[15,17] These separation processes to obtain proteins in the permeate rely on concentration polarization to boost the separation.

The data also indicates that a modified concentration polarization model would be required to be developed for SPTFF, and recirculation-TFF or stirred cell behavior cannot be conveniently leveraged to be used in SPTFF. This is a topic of future investigation by the authors.

The sieving behavior of lysozyme using TFF and SPTFF was an unexpected finding: it was expected that the 10 kDa CRC membrane would be much tighter toward lysozyme and the 50 kDa PES membrane would be most open to lysozyme, with the 30 kDa CRC membrane lying in between the two. In fact, the hydraulic permeability of the 50 kDa PES membrane was the highest ($L_P = 425 \pm 20$ LMH/bar), compared to the 30 kDa CRC ($L_P = 142 \pm 18$ LMH/bar) and 10 kDa CRC membranes ($L_P = 98 \pm 4$ LMH/bar). The permeability and the rating as "50 kDa" alone indicated that the 50 kDa membrane would be completely permeable to lysozyme. This could be attributed to the differences in structure between the Biomax® and Ultracel® membranes, and also in the method of rating these membranes.[22] Recent work by Manzano[23] demonstrate similar differences in results for RNA transmission through 100 kDa CRC and PES membranes. This is also an important observation and warrants future investigation from the context of SPTFF, because the molecular weight cut-off as "50 kDa" is misleading, when it compared more closely with the 10 kDa with regard to sieving behavior but had a high permeability, indicative of a more open membrane. This observation also calls the methodology to rate ultrafiltration into question: current methods for rating membranes are agnostic to the membrane surface chemistry. With the industry expanding the use of ultrafiltration and membrane technology, more rigorous characterization techniques will be required to accurately rate ultrafiltration membranes.

Behavior of completely retained proteins (monoclonal antibodies) using SPTFF

The data presented in Figures 4 and 5 are significant in context to work published by several groups on highly concentrated protein solutions[5,6,24] where the module screen type and the buffer composition significantly affected the ability to reach high concentrations for BSA, mAbs, and Fc-Fusion proteins. The general conclusion from all these studies was that the axial pressure drop in TFF cassettes caused reverse filtration at high protein concentrations because of loss of retentate pressure at the module exit. Furthermore, the intermolecular interactions between highly concentrated mAbs was a strong function of the buffer: The viscosity and osmotic pressure effects were significantly different and higher in the histidine matrix compared to the phosphate matrix.[6] The data in Figure 4 for mAb1 using SPTFF indicates that the volume concentration factor was not affected by the buffer matrix, the membrane screen type or the molecular weight cut-off between 10 and 30 kDa CRC membranes. Higher flow-rates were possible using a 50 kDa membrane and the 30-30-50 kDa configuration, which was likely related to the higher permeability of the 50 kDa PES membrane and the non-uniform pressure drop from the 30-30-50 kDa configuration. The wall concentration will increase throughout the module, with the intermolecular interactions arising from buffer and protein interactions becoming significant only at the final sections where flow-rates are already low and the retentate pressure is still finite (non-zero), giving a positive TMP, in contrast to conventional TFF, where the osmotic pressure contributions result in reverse flow at the module exit.

Taken together, this indicated that a 10 and 30 kDa membrane did not matter in single pass concentration for retentive mAbs. The screen type and buffer composition did not affect the maximum concentration achieved. However, there was a significant difference between the 30 and 50 kDa membranes, with the 50 kDa or the 30-30-50 hybrid configurations providing higher normalized feed flow-rates to achieve the same target concentrations. A complete 1 h concentration performed using all these membranes also indicated that the 30-30-50 kDa was the most stable in terms of the consistency. The 50 kDa system alone required lesser area (0.22 m^2 compared to 0.33 m^2) than the 30 kDa system to achieve a given target concentration. However, the 50 kDa system (50-50-50A) by itself also exhibited inconsistent performance and membrane fouling became an issue during concentration and the process had to be interrupted when concentrated beyond 1 h. The inconsistency for the 50 kDa membrane is reflected in Figure 6 in the high standard deviation for the volume concentration factor. The high standard

deviation for the 50 kDa membrane for protein concentration reflects the change in protein concentration during the 30 min measurement time period, and also a variability that resulted from the fouling of the more open membrane. The 30 kDa system and the 30-30-50 kDa systems were tested to operate up to 4 h without any change in hydraulics or conversions (protein concentrations) for both mAb1 and mAb2. Material limitations prevented operating for longer periods.

The 30-30-50 kDa system did not foul in between runs and was able to operate at a higher flow-rate compared to the 30 kDa membrane system or the 50 kDa membrane system alone. The data in Figure 6 illustrates what happens to the cumulative volume concentration factor in different stages of the SPTFF. The first two stages perform majority of the conversion. As the concentrated protein enters the third stage, it is highly concentrated and approaches the wall concentration, C_W in the last stage for the 10 and 30 kDa membrane, limiting the maximum achievable concentration realistically. When the third stage was replaced with a 50 kDa membrane, the first two stages performed the majority of the conversion, but the 50 kDa membrane in the third stage was more permeable, had a higher wall concentration, and pushed the concentration more than what the 10 kDa or the 30 kDa membrane could. Moreover, the higher permeability of the 50 kDa membrane allowed operation at 2–4× higher normalized feed-flow rates than the 30 kDa membrane alone, reducing the processing time significantly. The ability of the hybrid system to achieve very high volume concentration factors (approximately 80× for mAb2 at a 57% higher normalized feed flow-rate compared to the 30 kDa membrane) and operate at significantly higher feed flow-rates is a very important outcome of this study.

Connecting the pieces together

This paper reported the single-pass ultrafiltration behavior of partially retained proteins like lysozyme and completely retained proteins like monoclonal antibodies using 10 kDa CRC, 30 kDa CRC, and 50 kDa PES membranes. While these types of proteins were found to behave differently in terms of sieving, the data from both these types of proteins can be combined to make several conclusions. Firstly, the sieving behavior of lysozyme using 10 kDa CRC and 50 kDa PES membranes was similar in the total recycle TFF mode. The 30 kDa CRC membrane gave an approximately 2.8× higher sieving coefficient ($S_o = 0.70$ at $J_V = 30$ LMH) compared to the 10 kDa CRC membrane ($S_o = 0.25$ at $J_V = 26$ LMH) or the 50 kDa PES membrane ($S_o = 0.28$ at $J_V = 28$ LMH). If a separation were to be performed to separate a solute like lysozyme from a larger protein, the data using total recycle experiments would suggest using the 30 kDa CRC membrane to be optimal. However, data obtained from SPTFF experiments suggest that the sieving coefficients can be about 1.6–2.5× lower depending on the feed flow rate ($\langle S_O \rangle = 0.55 \pm 0.04$ at a feed flow-rate of 18 L/h/m^2 and $\langle S_o \rangle = 0.37 \pm 0.03$ at a feed-flow rate of 55 L/h/m^2 for the 30 kDa membrane) (Table 3), indicating that the process to perform the separation would require either more open membranes or an optimization study to find the ideal feed flow rate and the ideal configuration. The sieving coefficients measured and calculated using SPTFF were also more sensitive compared to that measured using TFF. The 10 kDa CRC membranes and the 50 kDa PES membranes did not differ significantly regardless of the mode of operation (TFF vs. SPTFF). Thus, the 50 kDa PES membrane would be a better option than the 10 kDa CRC membrane considering the flux increase that the 50 kDa PES membrane offered at the same or better retention.

With the industry moving toward continuous bioprocessing, and with other emerging therapeutic modalities that necessarily require a single pump pass through microfiltration and ultrafiltration membranes for fractionation, this is a significant finding that will impact such separations. An example of such a separation process valid in the context of this work would be the use of single pass TFF to separate PEGylated (or conjugated) proteins from unreacted PEG using ultrafiltration. The viscosity of the PEGylated proteins could be high, limiting the use of TFF mode, but allowing the use of an SPTFF configuration. With the implementation of a continuous diafiltration strategy[10] and using the correct membrane configuration, this separation may be performed using SPTFF. However, the choice of the membrane and expected sieving behavior from TFF experiments will have to be reevaluated in terms of the data presented in this work.

For completely retained solutes, the conversions were much more predictable. As expected, the 50 kDa PES membrane was more permeable and allowed higher conversions. The 50 kDa system alone led to overconcentration in the first and second stages, leading the third stage to be redundant. However, a tight control on the feed flow-rate was difficult to obtain, and the 50 kDa membranes by themselves fouled severely. This is reflected in the larger error bars for protein concentration and conversion (Figure 6). The 30-30-50 kDa system allowed operation between 2–4× higher feed flow-rates and higher conversions. In addition to using the hybrid approach for protein concentration alone, such an approach may be effective for selective fractionation of proteins by tailoring the sieving coefficients to the desired value.

Conclusions

This is the first reported work that studied the behavior of a partially retained solute like lysozyme and a completely retained solute like monoclonal antibodies using commercially available equal area staged SPTFF modules. The sieving coefficients measured using TFF and SPTFF indicated a more complicated concentration polarization behavior for SPTFF compared to TFF. The retention of lysozyme was higher using SPTFF compared to TFF for all the types of membranes. This observation was counter-intuitive in the context of the classical stagnant film model. In case of retained solutes like monoclonal antibodies, the 10 and 30 kDa membranes gave the same conversions at the same feed flow-rates and the screen type did not matter. Moreover, the effects of the buffer matrix were not significant for the SPTFF mode. The low flow rates required to achieve high conversions were improved by hybridizing the module with a 50 kDa membrane as the third stage after two 30 kDa membranes. The overall results of this study indicate that SPTFF is an attractive process for concentration of proteins in a single pump pass. However, the sieving behavior of partially retained solutes is complicated compared to TFF, and will require further understanding. This is a topic of future exploration by the authors. The molecular weight cut-off of the membrane did not correlate with the sieving characteristics for partially retained protein but it did so for the completely retained protein.

Conflicts of Interest

The authors declare no conflicts of interest.

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Manuscript received Jan. 30, 2018, and revision received May 31, 2018.