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IgG-BSA separation and purification by internally staged ultrafiltration

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ABSTRACT

Purification of IgG from residual host cell proteins (HCPs) in post-Protein A chromatography is important since some HCPs bind with Protein A and elute with the monoclonal antibody (mAb); removal of HCPs from CHO cell lines is essential. To that end, an advanced separation and purification technique in biopharmaceutical manufacturing, namely, internally staged ultrafiltration (ISUF), is investigated here. Choosing BSA as a model for HCPs in post-protein A eluate, separation of a binary mixture of IgG and BSA containing 1.0 mg/ml IgG and 0.1 mg/ml BSA is successfully demonstrated here using a modified ISUF technique: two Omega 100 kDa membranes on top followed by one Omega 70 kDa membrane at the bottom. This modified configuration demonstrated exceptional performance with almost *complete* rejection, 99 % purity, and 99.5 % retention of IgG, along with 96.5 % recovery of BSA over 10 diavolumes. This modified membrane stacking resulted from strategic considerations of membrane stacking and careful selection of molecular weight cutoffs and materials, and performance analysis of different membranes and stacking configurations using rejection behaviors, purity levels, and recovery rates under varying diavolume and pressure differential. The approach adopted here enhances flexibility in membrane choices in ISUF and provides valuable insights for optimizing membrane-based biopharmaceutical separation techniques.

1. Introduction

In biopharmaceutical manufacturing, separation and purification of proteins or monoclonal antibodies (mAbs) from their binary/multi-component mixtures is carried out by ion exchange chromatography, affinity chromatography, and size exclusion chromatography. Membrane filtration is used for viral filtration and then ultrafiltration (UF)/diafiltration (DF) [1]. UF-based protein mixture separation is undertaken if the molecular weight ratio (MWR) of two proteins is at least ~7–10 [2]. To separate protein mixtures having MWR<7, novel cascade configurations employing separate UF devices each having individual pumps were studied [3]. High-performance tangential flow filtration (HPTFF) technique is employed now to achieve higher purification separation of protein mixtures having lower MWRs. In HPTFF, (1) pH used coincides with the pI of the smaller species preferentially passing through; (2) membrane charge repulses the larger protein with a similar charge at the pH used; (3) buffer ionic strength is low to avoid shielding species charges; (4) optimal operating flux is in the linear region [4–7]. HPTFF achieves high selectivity [5]; but almost pure protein is not obtained in the permeate; internally-staged ultrafiltration (ISUF) [8,9] yields almost pure protein.

In the current ISUF version (Fig. 1a, Fig. 1b.), three identical UF membranes are stacked with no gaskets or spacers in-between. Membrane 1 permeate (composition C_{p1}) with the selective membrane skin facing the feed is fed to the selective skin side of membrane 2 yielding C_{p2} in the permeate and then yielding C_{p3} in the permeate similarly from membrane 3. Each additional membrane amplifies the protein rejection by membrane 1 resulting in almost a completely rejected species after membrane 3 [8,9]. This is as if we were achieving progressively an ideal isoporous membrane [10] having a step function pore size distribution (Fig. 1c) using commercially available UF membranes. The pH, ionic strength, membrane charge and the operating pressure should be such that membrane 1 yields a selectivity of ~15–20 between the two proteins so that membrane 2 faces minimum concentration polarization. Such a system run for over 10–15 hr followed by membrane cleaning in situ reproduced results before and after on-line cleaning [9]. Various UF-based bioseparations [11–15] were studied using this technique and variations thereof involving different membrane combinations instead of the same membrane.

To separate hemoglobin (Hb; MW 64.7 kDa; pI, 6.8) from bovine serum albumin (BSA; MW 66.4 kDa; pI 4.7) (MWR, 1.03), ISUF was studied recently using 3 Ultracel 100 kDa membranes in an Amicon®

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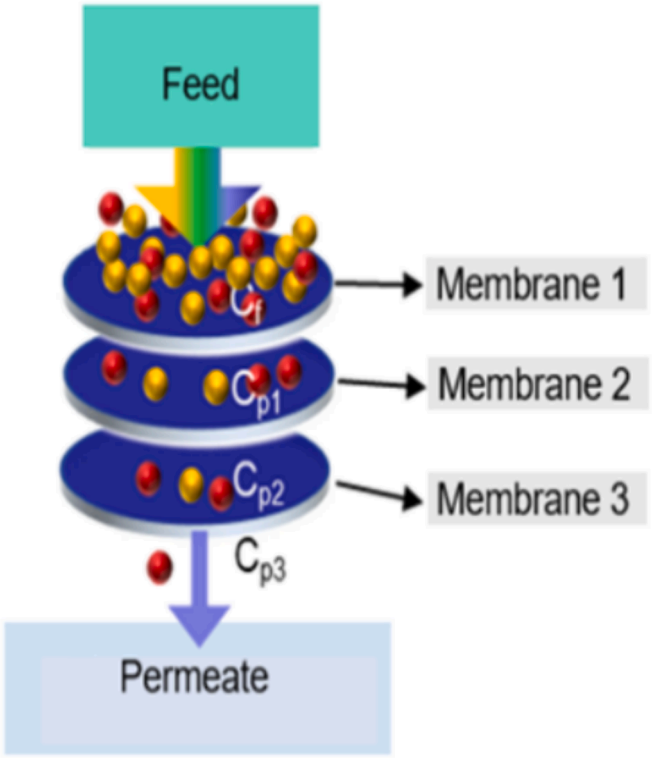

Fig. 1a. Internally staged UF (ISUF) concept.

$$R_1 = \left(1 - \frac{C_{p_1}}{C_f}\right)$$

$$C_{p_1} = 0.3C_f$$

$$C_{p_2} = 0.3C_{p_1}$$

$$= 0.3 \times 0.3C_f = 0.09C_f$$

$$C_{p_3} = 0.3C_{p_2} = 0.027C_f$$

$$\frac{C_{p_3}}{C_f} = 0.027 \Rightarrow R_3 = 1 - 0.027 = 0.973$$

Fig. 1b. Idealized rejection achieved in ISUF if a single membrane has a rejection of 0.7.

stirred cell as a function of diavolume [16]. The results show that BSA was almost completely rejected; 99.5 %+ BSA was retained over a diavolume up to 10+; hemoglobin purity in the permeate was 97 %+over 10+ diavolumes only because BSA concentration in feed was 5–20 times higher; Hb selectivity over BSA in the permeate varied in the range of 6000–1000 up to a diavolume of 6 and came down to 100 up to a diavolume of 10 [16]. This is to be contrasted with a recent study [17] of Hb-HSA (human serum albumin) separation employing four hollow fiber UF modules and individual complexation of Hb with haptoglobin (Hp) and HSA with Immunoglobulin G (IgG) followed by dissociation of each complex; recovery of HSA-IgG was ~50 % and recovery of Hb-Hp was ~10–15 %. Since the ISUF concept is being scaled up, it is useful to

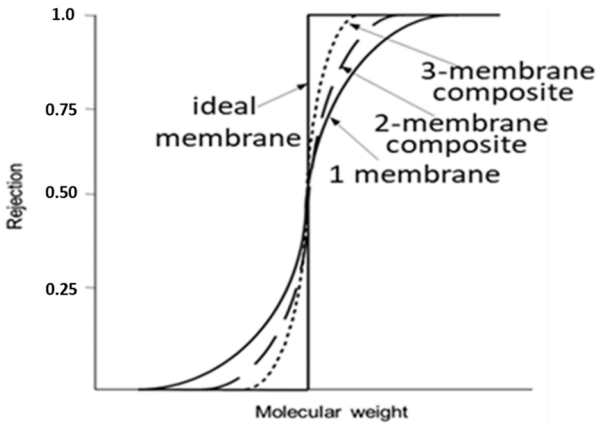

Fig. 1c. Molecular weight cut off profile of multi-membrane composites.

investigate other important biopharmaceutical separation problems in downstream processing: IgG purification from residual host cell proteins (HCPs) in the purification of mAbs post-Protein A chromatography [18,19] since some HCPs bind with Protein A and elute with the mAb; removal of HCPs from CHO cell lines is essential in mAb bioprocessing [20].

For clearance of process-related impurities (e.g., HCPs, residual DNA, virus) in mAb purification, various technologies have been explored. For mAbs, bovine serum albumin (BSA) was used as a model protein in HCP binding studies in adsorptive depth filters and adsorptive hybrid filters [21]. In anion exchange membrane chromatography for effective removal of viruses needed in mAb purification, BSA was also selected as a model protein mimicking acidic HCPs competitive for virus binding [22]. For a particular cell line producing a mAb, there may be more than 1000 HCPs in the harvested cell culture fluid (HCCF) [23,24]. In spite of 2–3 additional chromatographic purification steps beyond Protein A chromatography, some HCPs persist in the product, generate immunogenic reaction, show catalytic activity for product fragmentation and cause product aggregation [25].

Particular HCPs are somewhat more abundant in post-Protein A eluate. Table 6 in [25] identified 18 types of proteins having 8 types of functions; a few are mentioned here with their molecular weight in parenthesis: peroxiredoxin-1(22–27 kDa); cathepsins (25–36 kDa); clusterin (34–39 kDa); alpha-enolase (42 kDa); actin (42 kDa); vimentin (42 kDa); lipoprotein lipase (56 kDa); pyruvate kinase (228 kDa); nidogen-2 (200 kDa). During processing of three different mAbs, five HCPs were detected in the pools of the polishing chromatography steps with considerable abundance (Table 2 in [23]); four of them or types were identified above– clusterin, vimentin, peroxiredoxin-1, peroxiredoxin-2. A large majority of such proteins have molecular weight less than that of BSA which was chosen here as a model for HCPs. Therefore, their membrane clearances are likely to be higher except when their aggregates are present.

An important aspect of ISUF is: volume flux is not reduced 3 times by 3 membranes rather it is reduced by ~2–2.5 times [9,16]. However, since membranes in ISUF have much larger pores than that needed for high rejection, volume fluxes can be higher. Generally, for IgG separation and concentration, 30 kDa membranes are used [26,27] to eliminate virtually any loss of IgG. We studied here using much larger molecular weight cutoff (MWCO) membranes (e.g., 100 kDa); that will also allow much higher transmission of HCPs represented here by BSA as a model HCP. This may lead to some leakage of IgG to prevent which the third 100 kDa membrane (membrane 3, in Fig. 1a) was replaced with a 70 kDa membrane. Our modified ISUF enhances membrane stacking flexibility.

An important point needs to be made here. The separation problem in the IgG-BSA system from post-Protein A eluate is unlike other binary protein mixture separation problems. Most protein mixture studies are

satisfied once 95–96 % separation, recovery and purity are achieved. Our earlier protein mixture separation applications using ISUF as well as those of others [4,28–30] fall in this category. Here we have to retain virtually all of IgG because of its high value. Further, its purity has to be extraordinarily high because of possible effects outlined earlier [25]. We report here performances of a modified ISUF membrane stack and conventional ISUF stacks for separation and purification of a mixture of IgG-BSA. An important issue valid for all configurations is: what should be the solution pH for the known pIs of IgG and BSA for the negatively charged commercial membranes. Note: the MWR for separation of BSA from IgG is ~2.26.

2. Experimental

2.1. Materials and reagents

Bovine immunoglobulin (IgG, MW 150,000; pI, 5.5–8.3 [31,32,33]) and bovine serum albumin (BSA, MW 66,430; pI, 4.7 [34]) were obtained from Sigma (St. Louis, MO). Acetic acid (Sigma Aldrich) and sodium hydroxide (Sigma Aldrich) were used to prepare 2.3 mM sodium acetate buffer at pH 4.8. Citric acid and sodium citrate (Sigma Aldrich) were used to prepare 2.3 mM sodium citrate buffer at pH 4.8. Then the buffer pH was adjusted also to 4.2, 4.5, 4.8, 5.1, and 5.4 by 0.1 M HCl or 0.1 M NaOH. Buffer solution and protein solutions were prefiltered through a 0.45 μm polyethersulfone (PES) membrane (VWR International, Radnor, PA; now Avantor). Most experiments used a feed of 1.0 mg/ml IgG and 0.1 mg/ml BSA;a few used 0.2 mg/mL IgG, 0.2 mg/mL BSA. A N_2 gas cylinder was provided by Airgas (Piscataway, NJ).

Polyethersulfone (PES) flat membrane (Omega®, MWCO 100,000 (100 kDa) and 70,000 (70 kDa)) by Pall Corp. (East Hills, NY) and Ultracel® (PLHK062) 100 kDa membranes of regenerated cellulose by MilliporeSigma (Bedford, MA) were used. The UF membranes of diameter 63.5 mm were put into 2.3 mM sodium acetate buffer solution in a petri dish for 24 h to ensure that the membranes were thoroughly wetted and equilibrated with the ion concentration; a similar step was taken with the citrate buffer. The membrane filtration area was 28.4 cm^2.

2.2. Experimental setup and procedure

Ultrafiltration experiments were conducted with a 200 mL Amicon® stirred cell (UFSC20001, MilliporeSigma, Bedford, MA). The experiments were conducted at room temperature which was around 25 °C. The UF membranes were placed in the cell and sealed by the O-ring. A mixed proteins solution of 200 mL volume was added into the cell at the beginning. The buffer solution, cleaning solution, and deionized (DI) water were stored in separate reservoirs. Amicon® stirred cell selector valve (Cat. #: 6003, MilliporeSigma, Bedford, MA) was installed between nitrogen gas inlet, the reservoir, and the stirred cell. Buffer solution flow into the cell was driven by N_2 gas.

The pressure in the system was adjusted to 10.34 kPag for 1 piece (pc) of membrane placed in the cell, and 31.02 kPag for 3 pieces (pcs) of membranes unless otherwise mentioned. Correspondingly, the applied pressure difference (ΔP) values were 10.34 kPa for 1 pc of membrane placed in the cell, and 31.02 kPa for 3 pcs of membranes in the cell unless otherwise mentioned. The stirring speed was set at 500 rpm. The skin side of each membrane was up facing the feed solution.

Continuous recording of the permeate solution volume coming out from the cell was done; the permeate was pipetted into a clean cuvette. The protein concentrations in feed and permeate solutions were measured by the dual-wavelength method using a Varian Cary® 50 UV–vis spectrophotometer (Agilent, Santa Clara, CA) at 280 nm and 628 nm. See Figures S1 and S2 for the calibration curves. For testing a protein mixture at 628 nm, the protein sample was added to 3 mL of 0.1 g/L bromocresol green (BCG) solution and 1 mL of 0.5 g/L ethylenediaminetetraacetate acid (EDTA) solution for 1 h. The feed concentrations of proteins were measured before and at the end the experiment. For the first 30 min, permeate concentrations of proteins were measured every 10 min; then concentration recording time was changed to every 30 min, 1 h and 2 h etc. Nitrogen gas cylinder valve was closed when diavolume reached ~6 unless mentioned otherwise. The number of diavolumes was different in individual separation experiments. The concentration of the remaining protein solution was recorded in the UV–vis spectrophotometer. Protein rejection at any time is reported with respect to the bulk feed protein concentration in the cell at that time calculated by taking into account the total amount of protein that has permeated out by that time.

The definitions of various quantities reported and their calculation methods are reported below.

The rejection, R_i, of any protein species i is calculated by

$$R_i = 1 - \frac{C_{P_i}}{C_{f_i}} \tag{1}$$

where C_{P_i} is the concentration of protein i in the permeate solution at time t, and C_{f_i} is its concentration in the retentate at that time. An experiment was started at $t = 0$ with C_{f_0} as the protein concentration in the cell; C_{f_i} varies with filtrate volume, V, collected over time t:

$$C_{f_i} = C_{f_0} - \frac{1}{V_0}\int_0^t C_{P_i}(t)dV(t) \tag{2}$$

In Eq. (2) V_0 is the volume of feed solution in the stirred cell, (200 mL); V(t) is the total volume of the filtrate up to time t; $C_{P_i}(t)$ is the protein concentration in the permeate solution at time t and is the same as C_{P_i}.

From the volumetric filtration rate Q, through a membrane of area A_m, the volume flux, J_v, is calculated as:

$$Jv = Q/A_m \tag{3}$$

The Diavolume (N) at any time t is obtained from the ratio of total permeate volume at any time t, ($V_{totalp}(t)$), and the volume of feed solution in stirred cell (V_{cell}):

$$N = \frac{V_{totalp}(t)}{V_{cell}} \tag{4}$$

The % purity of IgG in the cell (retentate) and the % recovery of BSA in the permeate are calculated by the equations given below:

$$\%\ Purity\ of\ IgG\ inretentate = \frac{C_{f-IgG}}{C_{f-total}} = \frac{C_{f-IgG}}{C_{f-IgG} + C_{f-BSA}} \times 100\% \tag{5}$$

$$\%\ Recovery\ of\ BSA = \frac{C_{f-0} - C_{f-BSA}}{C_{f-0}} \times 100\% \tag{6}$$

The % purity of BSA in the permeate and the % retention of IgG are calculated as follows:

$$\%\ Purity\ of\ BSA\ inpermeate = \frac{C_{p-BSA}}{C_{p-BSA} + C_{p-IgG}} \times 100\% \tag{7}$$

$$\%\ Retention\ of\ IgG = \frac{C_{f-IgG}}{C_{f-0}} \times 100\% \tag{8}$$

The selectivity, Ψ, of BSA over IgG is calculated using the following equation:

$$\Psi = \left(\frac{C_{P_{BSA}}}{C_{P_{IgG}}}\right)/\left(\frac{C_{f_{BSA}}}{C_{f_{IgG}}}\right) \tag{9}$$

To check reproducibility, experiments were repeated; a few times some experiments were done three times. The following procedure was adopted after each experiment. The protein solution was disposed of first. Then the cell was cleaned by a 0.5 % Tergazyme® cleaning solution introduced from a separate reservoir holding 700 mL cleaning solution introduced earlier. Employing the N_2 gas pressure from the cylinder, the

cell was run with the cleaning solution through the membranes in situ for 4 h. Then the cleaning solution in the reservoir was replaced by 1L DI water and the cell was run for another 4 h before conducting an experiment. This in-situ cleaning allowed the same membrane stack to be repeatedly used; usually the membranes in a stack were replaced by new membranes after 6–7 uses even though the distilled water flux did not change much after cleaning from the flux level after previous cleaning.

Stirred UF cells are designed for single membranes. However, we have been able to use such a cell for a stack of three membranes. One has to ensure that the O-ring based seal is working well.

3. Results and discussion

Fig. 2A has two figures in it. The bottom one provides all data. The top figure focuses only on the very top section of the bottom figure and illustrates the data there in a much-expanded vertical scale. The bottom figure illustrates the performance of a modified ISUF configuration where two Omega 100 kDa membranes were at the top followed by an Omega 70 kDa membrane at the bottom at a pH of 4.8. Except during an initial diavolume of up to ~1, the rejection of IgG was maintained throughout at around 0.999 up to ~10 diavolume when the experiments were stopped.

Correspondingly, the purity of IgG achieved increased with diavolume from around 90 % to a very high value at ~10 diavolume. These performance levels are important since mAb loss during downstream processing is avoided almost at all cost; further, the highest mAb purity is desirable.

The rejection of BSA after an initial increase to 0.8 up to 1 diavolume decreased steadily to 0.6. The % BSA recovery in the permeate increased steadily and went up to 90 %.

Fig. 2B shows the values of the percentage retention of IgG in retentate and the percentage purity of BSA in permeate for this experimental system in the batch cell whose other results are described in Fig. 2A. It is clear that there is excellent retention of IgG in the retentate reflecting the almost complete rejection of IgG by the 3-membrane composite. Further the purity of BSA in the permeate continues to rise to a value of 96.5 % at 10 diavolume.

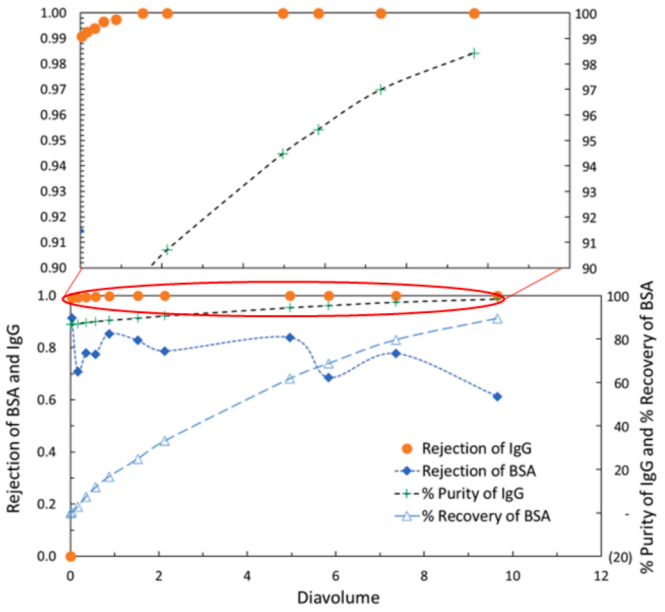

Fig. 2A. *Bottom figure:* Rejection of IgG and BSA, % purity of IgG in cell and *% recovery of BSA* as a function of diavolume in 3-membrane configuration at 31.02 kPag. *Top figure: Expanded scale of bottom figure for rejection of IgG and % purity of IgG as a function of diavolume up to 10 diavolume.* Batch *UF* feed: 1.0 mg/mL IgG and 0.1 mg/mL BSA, 2.3 mM acetate buffer: pH 4.8; *2 Omega®100 kDa membranes on top and 1 Omega®70 kDa membrane at bottom.*

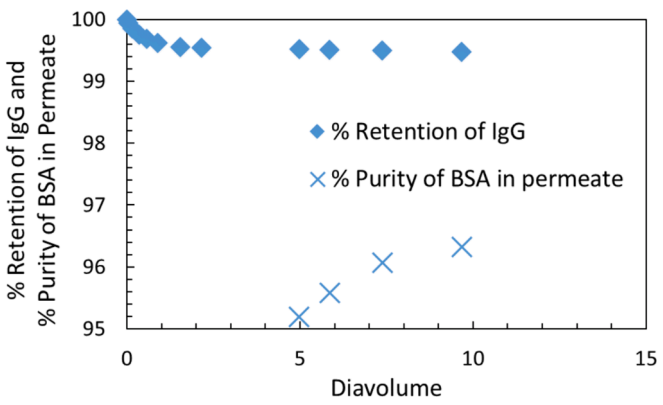

Fig. 2B. Effect of diavolume on % retention of IgG and % purity of BSA in permeate in 3- membrane configuration at 31.02 kPag for a feed of 1.0 mg/mL IgG and 0.1 mg/mL BSA, 2.3 mM acetate buffer at pH 4.8; *2 Omega 100 kDa membranes (top), 1 Omega 70 kDa membrane (bottom).*

Fig. 2C illustrates as a function of diavolume the selectivity achieved between BSA and IgG from the data shown in Fig. 2A. Except for somewhat low values of around 40–60 over the first diavolume, the selectivities achieved were in general above 1500 and going up to 4000. Earlier literature results indicate a BSA-IgG selectivity of around 50 through a single 100 kDa PES membrane at a pH of 4.8 and low ionic strength [4]; for HSA and HIgG it was around 260 at a low ionic strength [28]. Employment of the ISUF configuration at the same pH and a low ionic strength has drastically enhanced the selectivity to the level of 1500–4000. Whereas similar selectivities were achieved earlier between hemoglobin in the permeate and BSA in the retentate in the ISUF configuration [16], the retention of BSA was continuously decreasing with diavolume [16]. Here we achieve a constant 99.5 % retention of IgG with continuously increasing diavolume using a modified ISUF configuration.

A comparison of these performances with those reported in the literature on a variety of chromatographic methods is useful. Employing mixed-mode chromatography for separation of IgG from BSA using four different ligands, Wang et al. [31] obtained the following best performance level: IgG purity level of 92.3 % and IgG recovery of 95.6 %. Using hydrophobic charge-induction chromatography (HCIC) with 4-

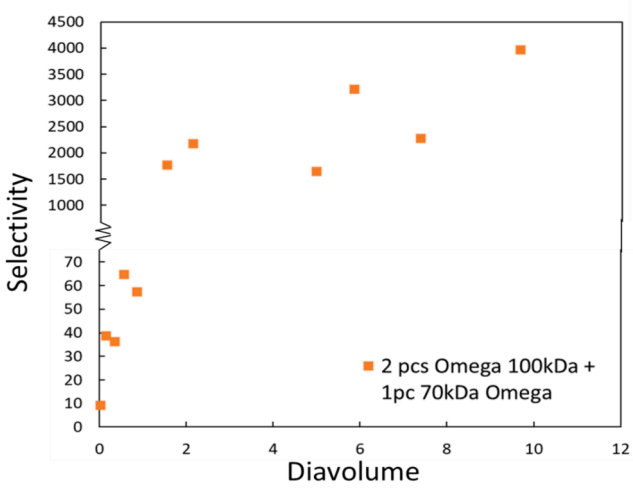

Fig. 2C. The BSA-IgG selectivity as a function of diavolume in *3-membrane configuration* at 31.02 kPag. Batch ultrafiltration feed: 1.0 mg/mL IgG and 0.1 mg/mL BSA, 2.3 mM acetate buffer: pH 4.8; *2 Omega® 100 kDa membranes at the top and 1 Omega® 70 kDa membrane at the bottom.*

mercaptoethyl-pyridine (MEP) as the ligand, Tong et al. [32] determined that the purity of IgG could be improved to about 95 % by controlling the loading pH or the addition of NaCl in the buffer. It is clear from the results shown here that ISUF performances are quite high; further, the purity level achieved depends on the diavolume used and the initial mixture composition. As the diavolume increases, the purity level achieved here keeps increasing linearly with the diavolume.

Fig. 3A illustrates the corresponding performances when a single 70 kDa Omega membrane is used. The rejection of IgG is around 99 % and rises quickly to virtually complete rejection while the rejection of BSA increases from 0.91 to 0.945 over 2.5 diavolume. This is a high value. The % purity of IgG increases from 92.5 % to 94.5 % over the same diavolume range. Clearly, the separation and purification performances of the 3-membrane composite of two 100 kDa and one 70 kDa membrane are doing much better (Fig. 2A): although IgG rejection is almost similar, purity of IgG achieved is significantly higher since BSA rejection is much lower. The BSA rejection in Fig. 2A starts ~0.8 but drops to 0.6 and lower as diavolume increases. Thus, diavolumes needed to achieve a certain level of IgG purification is considerably higher with a single 70 kDa membrane.

In the ISUF studies of reference [16], the pH employed was 7 to 7.4+ for two reasons: (1) BSA would be strongly negatively charged and the 100 kDa membranes employed namely, Ultracel (primarily) and Omega, were also negatively charged resulting in strong repulsion of BSA and its high rejection. On the other hand, hemoglobin with its pI around 6.8 was only mildly negatively charged and could easily go through the membrane in [16]. (2) Further its binding/association with the negatively charged BSA was strongly reduced by its slight negative charge [16]. Here on the other hand, we want BSA to go through the membrane and so we use a pH of 4.8 very close to its pI of 4.7; it has close to a net zero charge and should therefore have a significant permeability through 100 kDa membranes used earlier in ISUF [16]. Further its agglomeration is also low.

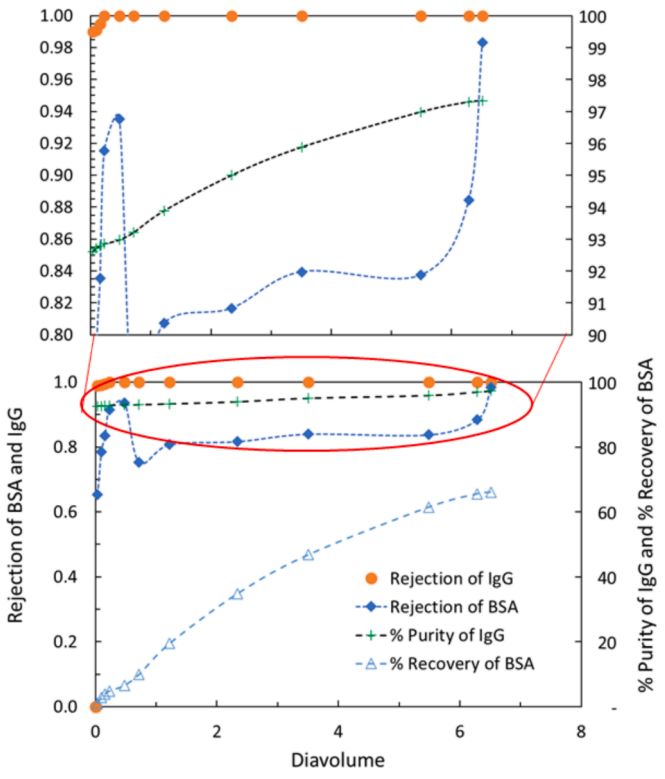

Fig. 3A. Rejection of IgG and BSA, % purity of IgG in cell and % recovery of BSA as a function of diavolume in *one Omega® 70 kDa membrane* configuration at 20.68 kPag. Batch ultrafiltration feed: 1.0 mg/mL IgG and 0.1 mg/mL BSA, 2.3 mM acetate buffer: pH 4.8.

Using a pH of 4.8 very close to the pI 4.7 of BSA becomes even more important with a 70 kDa Omega membrane (used at the bottom of the membrane stack in the present study) through which BSA permeability will be significantly reduced compared to that through a 100 kDa membrane. We illustrate the effect of pH at pH values of 4.2, 4.5, 4.8, 5.1, 5.4 for such a 70 kDa membrane in Fig. 3B. Except at a pH of 4.8, the rejection of BSA is always above 0.9 and going up to complete rejection for various pH values. At a pH of 4.8, BSA rejections are however going down to around 0.6–0.7. In this experiment, we avoided other complications by not using any other protein to avoid any other interactions.

The membrane stack configuration in Fig. 2A differs from the original stack concept of three identical UF membranes in the stack. It is therefore necessary to explore what happens when three membranes are identical in the membrane stack. Fig. 4 illustrates the results of IgG retention and BSA purity in the cell having a stack of three Omega 100 kDa membranes and compares the results with those for a single Omega 100 kDa membrane.

Fig. 4 results show that although % IgG retention with a 3-membrane stack is high, the expanded scale figure at the top shows that, it falls short and there is significant and continuing leakage of IgG. Further, the rejection of IgG with 3 Omega 100 kDa membranes is high *~0.98–0.99* but not high enough for this separation. Whereas Fig. 2B shows a flattening out of IgG retention with diavolume at 99.5 %, Fig. 4 shows that it keeps on decreasing. That is the beneficial effect of a 70 kDa membrane with a very high intrinsic rejection of IgG. The rejection requirements vary depending on the protein; whereas the performance shown may be acceptable for another protein, for mAbs, almost complete/total rejection is required. During concentration processes for mAbs [26], the feed concentration becomes much higher and total rejection is absolutely needed.

The original idea behind ISUF was that one could achieve a very high rejection by using three membranes stacked one over the other in one device even though one membrane has a limited rejection for a protein. This configuration allows the possibility of utilizing existing membranes which have inadequate protein rejection and creates a membrane stack that achieves very high rejections for particular proteins in one device. To achieve such a performance, the ISUF technique rides on the shoulders of the conditions employed in HPTFF. In the present experiments, the pH employed was 4.8 where BSA (pI, 4.7 [33]) is only slightly negatively charged and therefore passes through without much hindrance through the larger pores of a membrane having a negative charge [16]. On the other hand, under the optimized conditions of HPTFF, one would employ a pH such that the preferentially rejected protein (here, IgG) has considerable negative charge and encounters repulsion by the negatively charged membrane used here. To achieve such a condition for IgG (pI, 5.5–8.3 [31,32,33]) over a usable pH range, we need a positively charged UF membrane [35]. That would however create a problem for BSA facilitating its binding with the membrane. Hence, we

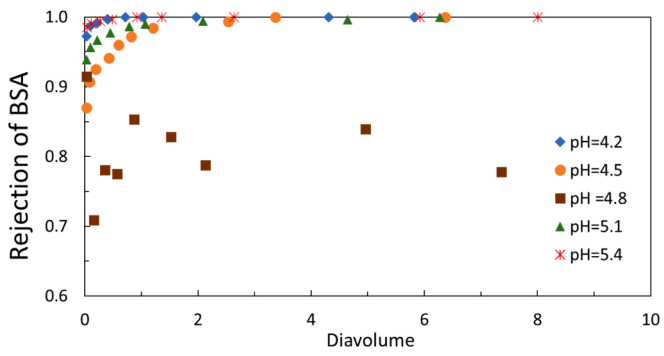

Fig. 3B. Rejection behavior of BSA at different pHs as a function of diavolume in *one Omega® 70 kDa membrane* configuration at 20.68 kPag. Batch ultrafiltration feed: 0.1 mg/mL BSA, 2.3 mM acetate buffer: pH 4.2, 4.5, 4.8, 5.1, 5.4.

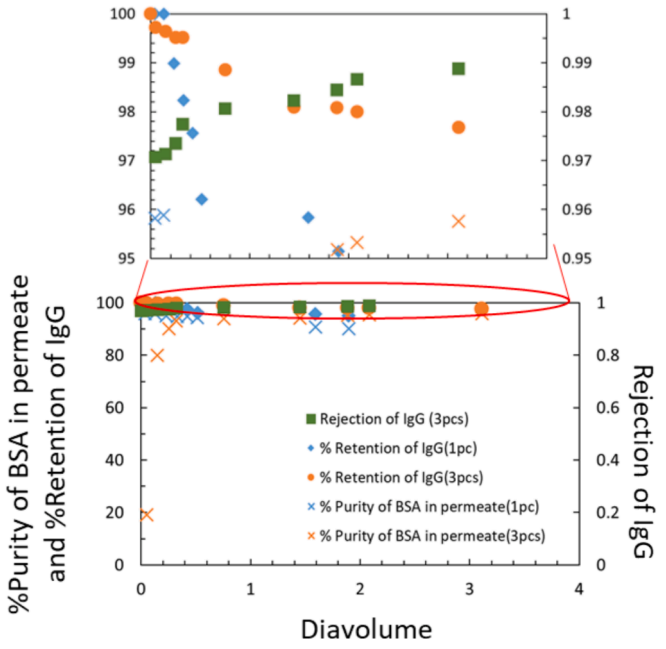

Fig. 4. Diavolume dependence of % IgG retention and % BSA purity in permeate for 1- and 3- membrane systems using *one and three Omega 100 kDa membranes*. Batch UF feed: 0.2 mg/mL IgG, 0.2 mg/mL BSA, pH 4.8, 2.3 mM citrate buffer; applied ΔP is 10.34 kPa for 1 membrane and 31.02 kPa for 3 membranes.

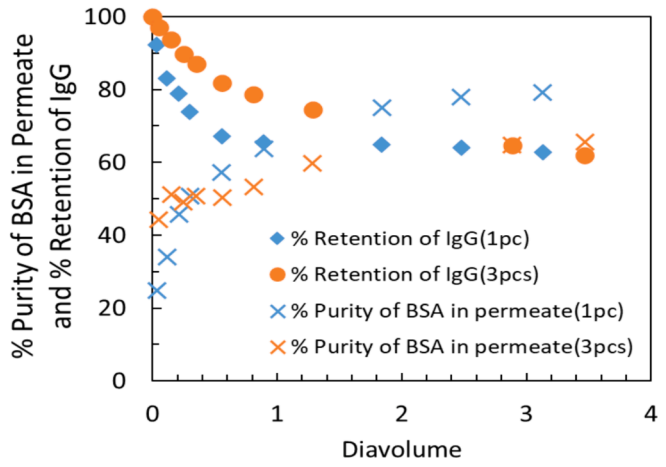

Fig. 5A. % Purity of BSA in permeate and % retention of IgG as a function of diavolume in *1- and 3- membrane configurations* at 10.34 kPag for *Ultracel 100 kDa membrane*. Batch UF feed: 0.2 mg/mL IgG, 0.2 mg/mL BSA, pH 4.8, 2.3 mM citrate buffer.

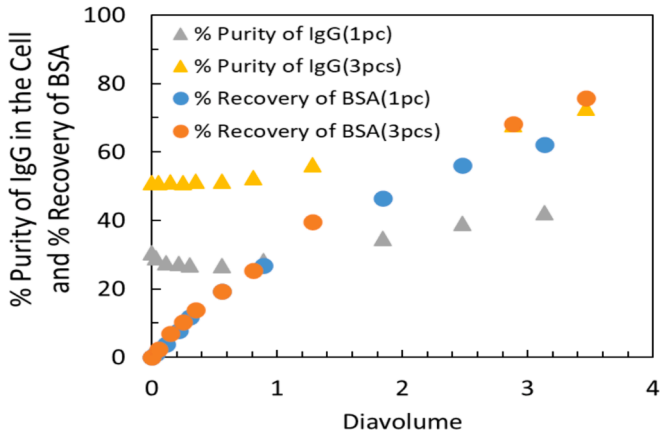

Fig. 5B. % IgG Purity and % BSA recovery as a function of diavolume in *1- and 3- membrane configurations* at 10.34 kPag for *Ultracel 100 kDa membrane*. Batch UF feed: 0.2 mg/mL IgG, 0.2 mg/mL BSA, pH 4.8, 2.3 mM citrate buffer.

have opted for focusing on BSA removal since IgG purification is the goal without its loss through the membrane. That is being achieved here at a pH~4.8.

How does a pH of 4.8 compare with that of the eluate from Protein A chromatography? The pH of this eluate is known to have low values varying over a range of 2.5 to 4[36]. Here are some examples: IgG_4 was eluted with 17 column volume of elution buffer (10 mM Na_2HPO_4 PBS at pH 3) [19]; mAb1 elution was performed using 0.1 M sodium acetate buffer, pH 3.5, using 10–15 column volumes [23] (also [31,32]). If another buffer at a different pH other than the eluate pH is needed to take care of the dominant HCPs, buffer exchange needs to be used.

We have seen earlier [16] that the ISUF configuration with three stacked membranes of 100 kDa Ultracel membranes was successful in achieving high purification and high recovery of each of hemoglobin and BSA from a solution in a system having a MWR of 1.03. Both 100 kDa membranes, Ultracel and Omega, have negative surface charges [16]. We explored therefore 100 kDa Ultracel membrane based conventional ISUF configuration of three stacked membranes here also for IgG-BSA separation using experimental conditions shown in Fig. 4. Fig. 5A illustrates the performance which shows that the % retention of IgG by this 3-membrane stack is considerably lower than those shown in Fig. 4 for any given diavolume. Correspondingly, the % purity of BSA is significantly lower than that in Fig. 4. Fig. 5B shows the corresponding results for % purity of IgG and the % recovery of BSA.

Regarding buffers, we started our investigation using both acetate and citrate buffers. Fig. 6 illustrates the results from our earlier studies; the results for both buffers are somewhat close to each other. However, for diavolumes greater than 6, the citrate buffer showed a certain amount of cloudiness. Hence for high diavolume runs, acetate buffer which has been used also by others [23,31,32] was adopted. Earlier studies in literature used also equal concentrations of BSA and IgG [4,28] as in Fig. 4 and Fig. 5. On the other hand, with eluates from post-protein A chromatography, the HCP concentrations are much reduced [19]; hence in Fig. 2 and Fig. 3, HCP concentrations are 1/10th of the IgG concentration.

It is useful to have an idea of the solvent fluxes obtained for these two membrane configurations, a three-membrane stack of two 100 kDa membranes supported by a 70 kDa membrane and just a single 70 kDa membrane. Fig. 7A illustrates the solvent volume flux achieved during the separation studies whose results are illustrated in Fig. 2A for the stack of two Omega 100 kDa membranes followed by a 70 kDa Omega membrane. Fig. 7B provides the corresponding data for a single 70 kDa Omega membrane. It appears that even after making allowances for 31.02 kPag pressure used in the 3- membrane composite, the flux for the single 70 kDa Omega membrane is lower reflecting the effect of higher concentration polarization on the 70 kDa Omega membrane being the only membrane. This needs a bit of deliberation.

In the ISUF method, two 100 kDa membranes on top of a 70 kDa membrane was subjected to 31.05 kPag; for the configuration of a single 70 kDa membrane, 20.68 kPag was applied. Since permeability of 100 kDa membranes is not that much higher than that of a 70 kDa membrane, the pressure drop for each membrane in ISUF is low around 10.35 kPag. Therefore, the flux in ISUF should be lower. Instead, it is higher.

The observed results are due to concentration polarization effects. A single 70 kDa membrane rejects both IgG and BSA effectively causing high concentration polarization which reduces the flux considerably. The top 100 kDa membrane in ISUF does not cause that much polarization since it passes BSA quite a bit and does not reject IgG that

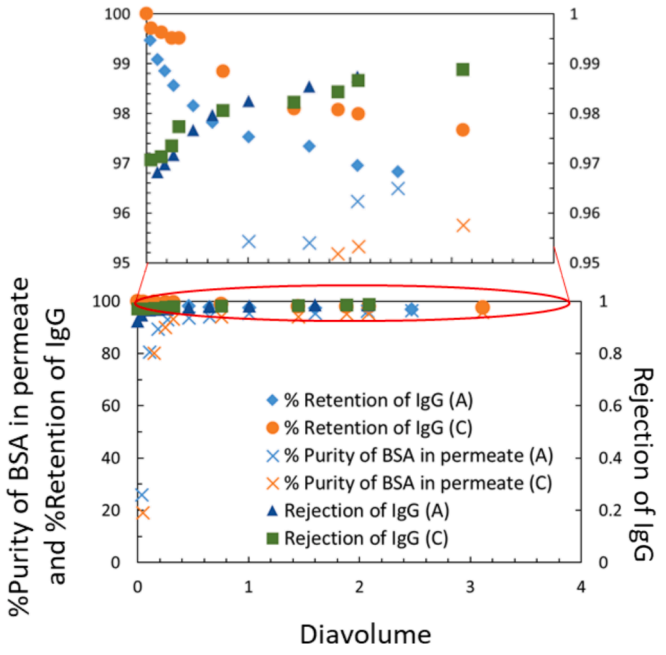

Fig. 6. Diavolume dependence of %IgG retention, IgG rejection and %BSA purity in permeate for 3- membrane systems using *three Omega 100 kDa membranes*. Batch UF feed: 0.2 mg/ml IgG, 0.2 mg/ml BSA, pH 4.8, 2.3 mM acetate buffer (A) and citrate buffer (C); applied ΔP is 31.02 kPa for 3 membranes.

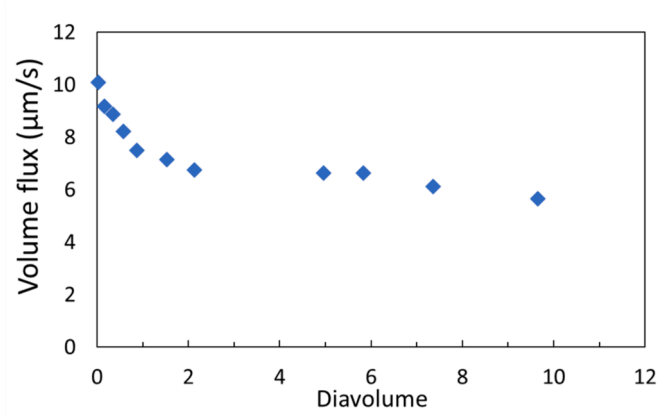

Fig. 7A. *Solvent volume* flux of *2pc Omega 100 kDa (top) and 1pc Omega 70 kDa (bottom)* at 31.02 kPag for a feed of 1.0 mg/mL IgG and 0.1 mg/mL BSA, 2.3 mM acetate buffer at a pH of 4.8.

strongly. Polarization levels in the second and the third membrane are much lower due to much lower concentrations in solutions on top of the second and the third membrane. As a result, volume fluxes are higher under ISUF.

Another comparison of flux is also useful namely, one 100 kDa Omega membrane and a stack of three 100 kDa Omega membranes. Fig. 7C illustrates the performance. The difference is limited.

Protein adsorption on the membranes during separation runs is also an item of interest. We therefore conducted additional experiments to investigate protein adsorption during membrane separation, specifically focusing on different membrane configurations for separating IgG and BSA. This was prompted by our finding that the rejection of IgG by one Omega 70 kDa was very high, and the flux was low. To dig deeper into this phenomenon, the weights of the membranes both before and after regular separation experiments were determined. After an experiment was over, the membrane(s) was(were) washed just a bit with the buffer

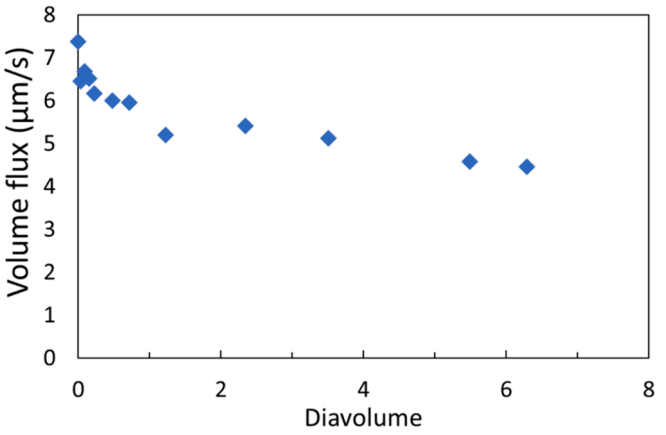

Fig. 7B. *Solvent volume* flux of *one Omega 70 kDa membrane* at 20.68 kPag for a feed of 1.0 mg/mL IgG and 0.1 mg/mL BSA, 2.3 mM acetate buffer at a pH of 4.8.

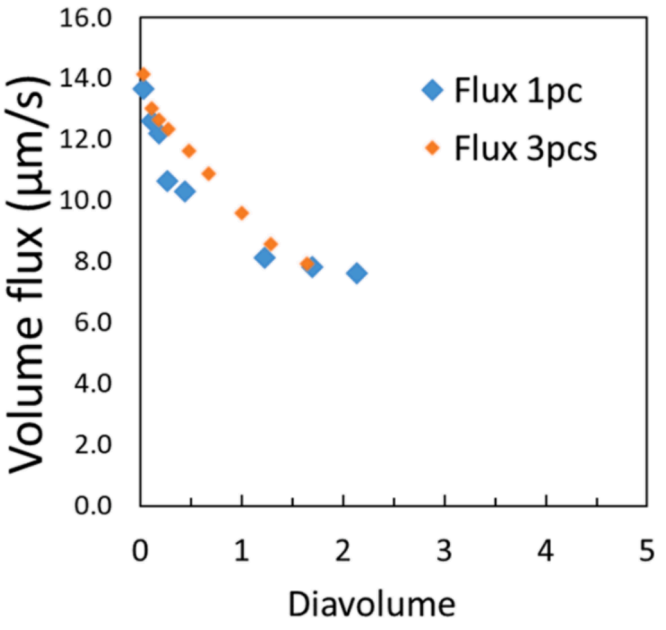

Fig. 7C. *Solvent volume* flux for *a single 100 kD Omega membrane* and *a 3-membrane composite* at 10.34 and 31.02 kPag UF feed respectively: 0.2 mg/mL IgG, 0.2 mg/mL BSA, pH 4.8, 2.3 mM citrate buffer.

and then allowed to dry for a day. Surprisingly, no significant change in the membrane weight was observed for pre- and post- experiments, as indicated in Table 1. This suggests that relatively loose adsorption of proteins might only transpire during the separation process rather than strong adsorption and membrane fouling.

We have focused here on removing BSA as a model for HCPs. Simultaneously, other proteins among HCPs need to be removed to achieve high purification of the IgG. A 70 kDa Omega membrane allows considerable permeation of BSA representing a model HCP. Membranes with a lower MWCO such as 50 kDa will not allow higher permeation of larger HCPs. In one example of residual HCPs in post-protein A chromatography eluate from Chinese hamster ovary-based cell culture [19], the molecular weights of most HCPs were around and less than that of BSA; however, their pIs varied over quite a range. Therefore, considerable purification is possible via ISUF. However, there will be HCP impurities with molecular weights higher than that of BSA. Final polishing will then require a polishing adsorption/chromatographic step.

Table 1
Weights of Omega 70 kDa and 100 kDa membranes, and feed concentrations of IgG and BSA before and after separation processing with 2.3 mM acetate buffer at pH 4.8

Membrane	Weight of membranes (g)		Difference	Feed concentration of IgG (mg/mL)		Difference	Feed concentration of BSA (mg/mL)		Difference
	Before	After		Before	After		Before	After	
One Omega 70 kDa	0.3269	0.3268	−0.0001	1.025	1.021	−0.004	0.122	0.041	−0.081
Two Omega 100 kDa & One Omega 70 kDa	0.9846	0.9842	−0.0004	1.028	1.023	−0.005	0.156	0.015	−0.141

4. Additional considerations

Successful removal of chemical foulants of protein A chromatographic columns generated from nonspecific chemical interactions of chromatin of host cells have substantially improved the performance of protein A columns [37]. It has also been suggested that coordinated ultrafiltration-adsorption processing of protein A eluate can lead to extraordinary purification of an mAb [38]. For those larger HCPs remaining in the concentrate from the ISUF process for a protein A eluate feed hypothetically considered here because their MWs are significantly larger than that of BSA, such a process will be useful to explore.

5. Concluding remarks

High performance of a modified ISUF technique has been demonstrated for purifying IgG from its mixture with BSA representing a model HCP in post-Protein A chromatography eluate. The modified ISUF configuration, utilizing larger MWCO membranes with a stack of two Omega 100 kDa membranes on an Omega 70 kDa membrane, achieved exceptional IgG rejection while allowing higher transmission of HCPs represented by BSA as a model HCP. There was almost 100 % rejection, 99 % purity, and 99.5 % retention of IgG, and 96.5 % BSA recovery over 10 diavolumes for separation of a binary mixture of IgG and BSA containing 1.0 mg/ml IgG and 0.1 mg/ml BSA. Factors such as pH, ionic strength, and operating pressure played crucial roles in optimizing the separation. The pH of 4.8 for example ensured that BSA had a small negative charge and could pass through a negatively charged membrane without much hindrance; optimized conditions for IgG in HPTFF would have however required a positively charged UF membrane, leading to a challenge on BSA removal for successful IgG purification. Of the various membrane configurations explored, the superior performance of a three-membrane stacked composite with two Omega 100 kDa membranes on top followed by one Omega 70 kDa membrane was demonstrated. The performances of alternative configurations, including a single Omega 70 kDa membrane and a stack of three Omega 100 kDa membranes, were poorer in terms of IgG purity, BSA recovery and higher flux in the separation process. This study recognizes the need for additional steps, like polishing via adsorption or chromatography, to address residual HCPs having molecular weights higher than that of BSA.

Declaration of competing interest

The authors 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.

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Appendix A. Supplementary material

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