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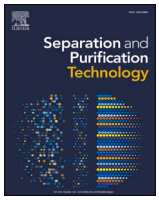

Hemoglobin-BSA separation and purification by internally staged ultrafiltration

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A B S T R A C T

Separation and purification of a binary protein mixture having close molecular weights was studied using ultrafiltration (UF) membranes. The binary system of hemoglobin (Hb; MW 64.7 kDa; isoelectric point (pI) 6.8) and bovine serum albumin (BSA; MW 66.4 kDa; pI 4.7) having a molecular weight ratio (MWR) of only 1.03 was studied. It has been demonstrated that the internally staged ultrafiltration (ISUF) technique, which used a stack of three UF membranes without any gaskets/spacers in-between, can yield nearly pure Hb in the permeate from its mixture with BSA in an Amicon® stirred cell. Further, the highest purity of BSA, 99 %, was achieved at a pH 7.8 at 10.66 diavolume. This study achieved these results by avoiding membrane fouling by adjusting the pH of the buffer/solution close to 7.8 and operating at a low applied pressure difference of 10.34 kPa. When pH is at around 7.8, BSA is much more negatively charged than Hb; so BSA was much more strongly repelled by the negatively charged UF membranes. A slight net negative charge on the Hb will affect the permeability of Hb to a small extent through the negatively charged UF membrane. However, this can considerably reduce the attraction between BSA and Hb, avoid higher protein agglomeration and alleviate membrane fouling. Low membrane fouling improved UF membrane performance; the recovery of Hb, retention of BSA, and purification of Hb and BSA show good results. Besides, the reusability of UF membranes by in situ cleaning continued to show good performance even after undergoing regeneration 7 times. The results suggest ISUF technique may be used in practical systems for separating and purifying protein mixtures.

1. Introduction

Separation and purification of individual proteins or monoclonal antibodies (mAbs) is a significant activity in the manufacturing processes in the biopharmaceutical industry. Conventionally, the key step utilized to separate and purify targeted proteins/mAbs from other constituents involves chromatography e.g., affinity chromatography, and size exclusion chromatography. For example, currently, mAbs are purified by sequential processes of Protein A-based affinity chromatography, viral inactivation, polishing chromatography, and a viral filtration step followed by ultrafiltration (UF)/diafiltration (DF) [1]. Ultrafiltration has been generally employed for size-based separation of protein mixtures where the molecular weight ratio (MWR) of two proteins is at least ~ 7–10 [2]; the MWR for separation of host cell proteins (HCPs) (represented by say, bovine serum albumin (BSA)) from immunoglobulin G (IgG) is ~ 2.16. Therefore, novel cascade configurations in separate devices with individual pumps were investigated to achieve higher protein purification where the MWR is < 7 [3]; these authors numerically illustrated a 3-stage process for protein fractionation using two proteins with apparent sieving coefficients (S_i) of 0.5 (preferentially transmitted) and 0.01 (preferentially retained).

To achieve better UF-based purification of similarly sized biomolecules, considerable past research focused on “fine-tuning” operating and physicochemical conditions to attain higher selectivity [4–6]. These researchers exploited the size difference between two proteins via increased or decreased hydrodynamic radius resulting from changes in buffer conditions (i.e., ionic strength and pH). Repulsion by membrane charge for similarly charged species was also exploited to enhance selectivity between the permeating species and the rejected species. Van Reis et al. [7] utilized these concepts along with an optimal operating flux or transmembrane pressure drop (ΔP) to illustrate the high-performance tangential flow filtration (HPTFF) technique. The HPTFF technique achieves high selectivity but cannot yield almost pure protein in the permeate. A new concept termed internally-staged ultrafiltration (ISUF) was introduced [8,9] which could result in almost pure protein in the permeate.

In the ISUF technique, a multi-membrane stack of three identical UF membranes is employed without any gaskets or spacers in-between the membranes. Permeate from membrane 1 with the selective skin facing the feed solution is fed to the selective skin side of membrane 2 and so on

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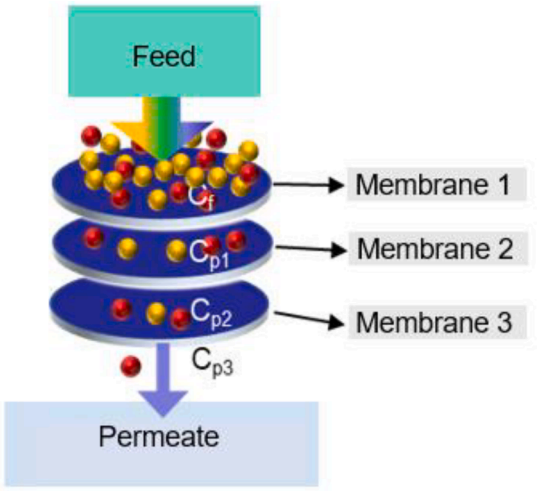

Fig. 1. The basic configuration of Internally Staged Ultrafiltration (ISUF).

(Fig. 1). Protein rejection by the first membrane is amplified with each additional membrane, ultimately resulting in almost a wholly rejected species [8,9]. It is however necessary to choose pH, ionic strength, the membrane charge and the operating pressure such that the first membrane stage has a selectivity of ~ 15–20 between the two proteins to minimize concentration polarization over membrane 2. The system was run for over 10–15 hr. Membrane cleaning in situ was achieved with reproducible experimental results before and after on-line cleaning [9]. This technique and variations thereof involving different membrane combinations instead of the same membrane have been investigated for various UF-based bioseparations [10–14].

The suggested relatively high selectivity of the first membrane in ISUF reduces the permeate concentration of the rejected species substantially and this permeate solution is fed to the second membrane. Further since we are dealing with molecules that are close to each other in their molecular weights, the concentration of the preferentially passing protein in the permeate from the first membrane is also reduced. In effect, the solution strength in the feed to the second membrane is drastically reduced which compensates considerably for the lack of stirring on top of it. This technique yielded nearly pure hemoglobin (Hb) (MW, 64.7 kDa) in the permeate from its mixture with BSA (MW, 66.4 kDa) in one stirred UF cell [9]; the MWR of the two proteins is 1.03.

However, there is no information on the purity of BSA, the retained protein, and the recovery of the more permeable protein (Hb) in the permeate and the corresponding dependencies on the diavolume (DV) among others. These aspects are important if the ISUF technique is to be applied to the removal of residual host cell proteins (HCPs) in the purification of mAbs post-Protein A chromatography [15,16] since some of the HCPs bind with Protein A and elute with the mAb; elimination of problematic HCPs in CHO cell lines used for mAb bioprocessing is essential [17]. It is however likely that a chromatographic polishing step may be needed to remove one/two HCPs after such an ISUF treatment.

Here we focus on BSA-Hb separation [5,9] in the ISUF configuration covering the separation performance vis-a-vis a number of important variables. The membranes studied have a molecular weight cutoff (MWCO) of 100 kDa. There are no other separation studies for such a system. Vardanega et al. [18] explored the effect of a magnetic field on BSA ultrafiltration under various pH conditions; a higher operating pH over the IEP of BSA decreased membrane fouling.

According to the principles of HPTFF, the pH of operation should be around the IEP of the protein to be preferentially permeated so that its transmission is unaffected by membrane charge. Therefore, our pH is around 6.8 (IEP of Hb) where BSA is negatively charged and is repelled by the negatively charged membrane. Further the ionic strength is very low to prevent shielding of the charges on BSA molecules. Finally, the applied pressure difference is low so that we operate in the linear region and prevent membrane fouling as best as we can. Earlier studies [4–7] and that by Burns and Zydney [19] provide guidance and insights into various effects especially charge effects. This reference provides the zeta potential of Omega 100 membranes as a function of solution pH.

2. Experimental

2.1. Materials and reagents

Hemoglobin (Hb, MW 64.7 kDa) and bovine serum albumin (BSA, MW 66.4 kDa) were obtained from Sigma (St. Louis, MO). The isoelectric point (pI) for Hb is 6.8 [20] and the pI value for BSA is 4.7 [21]. Sodium dihydrogen phosphate (Alfa Aesar) and disodium hydrogen phosphate (Sigma Aldrich) were used to prepare a 2.3 mM sodium phosphate buffer at pH from 6.8 to 7.4. Tris hydrochloride (Tris-HCl) and the Tris base were used to prepare 2.3 mM Tris buffer at pH 7.2 to 8.2. Then buffer pH was adjusted by 0.1 M HCl or 0.1 M NaOH. For Hb-BSA separation, reference [5] used a buffer using KH_2PO_4 and Na_2HPO_4; two ionic strengths were used: 0.0023 M and 0.10 M; the pH was varied between 6.8 and 7.1. In earlier ISUF of Hb-BSA [9],20 mM sodium phosphate buffer at pH 6.8 and 2.3 mM sodium phosphate buffer at pH 6.8 were used. The buffer and protein solutions were prefiltered through a 0.45 μm polyethersulfone (PES) membrane (VWR International, Radnor, PA; now Avantor).

Two ultrafiltration membranes were used in this study as shown in Table 1. The UF membranes were put in 2.3 mM sodium phosphate or Tris buffer solution in a petri dish for 24 hr to ensure that the membranes were thoroughly wetted and equilibrated. The membrane filtration area was 28.4 cm^2. The primary focus was on the Ultracel® membrane.

2.2. Experimental setup and procedure

Ultrafiltration experiments were conducted in a 200 ml Amicon® stirred cell (UFSC20001, MilliporeSigma, Bedford, MA). The UF membranes were placed in the cell and sealed by an O-ring. A mixed protein solution of 200 ml was added into the cell at the beginning. The buffer, 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.

The pressure in the system was adjusted to 10.34 kPag for 1 membrane disk placed in the cell, and 31.02 kPag for 3 membrane disks unless otherwise mentioned. Correspondingly, the applied pressure difference (ΔP) values are 10.34 kPa for 1 membrane disk placed in the cell, and 31.02 kPa for 3 membrane disks unless otherwise mentioned. For Omega membranes, an appropriately sized membrane was cut out of a much larger membrane sample sheet. The stirring speed was set at 500 rpm. The skin side of each membrane was up facing the feed solution.

The volume of the permeate solution from the cell was recorded continuously; the permeated solution was pipetted into a clean cuvette. Then protein concentrations in the feed and permeate solutions were measured by the dual-wavelength method using a Varian Cary® 50 UV–vis spectrophotometer (Agilent, Santa Clara, CA) at 407 nm and 280 nm [8]. The protein concentrations in the feed solution were measured before the experiment and those in the retentate at the end of the experiment. The permeate concentrations of proteins were measured every 5 min for 30 min, then recording time was changed to every 10 min for 30 min, 30 min for 2 hr, 1 hr for 3 hr and 2 hr for 6 hr. When the

Table 1
Ultrafiltration membranes.

Membranes	Material	MWCO	Company
Ultracel® (PLHK062)	Regenerated cellulose	100 kDa	MilliporeSigma
Omega (OT100SD)	Modified polyethersulfone	100 kDa	Pall

diavolume reached about 12, the nitrogen gas cylinder valve was closed. The concentration of the remaining protein solution was recorded via the UV–vis spectrophotometer. Protein rejection is reported as a function of time with respect to the bulk protein concentration in the cell at that time by taking into account the total amount of protein that has permeated.

Rejection, R_i, and the sieving coefficient S_i of the solutes (proteins: Hb and BSA) for any membrane were calculated respectively by Equations (1a) and (1b):

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

$$S_i = \frac{C_{P_i}}{C_{fi}} \tag{1b}$$

Here C_{P_i} is the concentration of Hb/BSA in the permeate solution at time t, and C_{fi} is the concentration of Hb/BSA in the retentate solution. C_{fi} is a function of the filtrate volume, V, collected over time t:

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

Here C_{f_0} is the concentration of Hb/BSA in the cell at the beginning of the experiment when the concentration of Hb is 0.2 mg/ml, and that of BSA is 1.0 mg/ml (unless otherwise mentioned); V_0 is the initial volume of feed solution in the stirred cell, (200 ml); V(t) is the total volume of the solution filtered by the membrane up to time t; $C_{P_i}(t)$ is the concentration of Hb/BSA in the permeate solution at any time t, which is the same as C_{P_i}.

Volume flux, J_v, is defined as the volumetric filtration rate, Q, divided by the membrane area, A_m:

$$J_v = \frac{Q}{A_m} \tag{3}$$

Diavolume (N) at any time t is calculated by the equation given below; it is the ratio of total permeate volume at any time t, ($V_{totalp}(t)$), and the initial volume of feed solution in stirred cell (V_{cell}):

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

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

$$Purity\ of\ BSA\ in\ retentate = \frac{C_{f-BSA}}{C_{f-total}} = \frac{C_{f-BSA}}{C_{f-BSA} + C_{f-Hb}} \times 100\% \tag{5}$$

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

The purity of Hb in permeate and the retention of BSA are calculated by the equations given below:

$$Purity\ of\ Hb\ in\ permeate = \frac{C_{p-Hb}}{C_{p-BSA} + C_{p-Hb}} \times 100\% \tag{7}$$

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

Selectivity, Ψ, of Hb and BSA is calculated by Equation (9):

$$\Psi = \left(\frac{C_{p_{Hb}}}{C_{p_{BSA}}}\right) \Big/ \left(\frac{C_{f_{Hb}}}{C_{f_{BSA}}}\right) = (S_{Hb}/S_{BSA}) \tag{9}$$

To clean the cell after disposing of the remaining protein solution, a 0.5 % Tergazyme® cleaning solution was introduced from 700 ml cleaning solution introduced earlier in a separate reservoir. Then using the nitrogen gas cylinder, the cell was run with the cleaning solution through the membranes for 4 hr. Then the cleaning solution in the reservoir was replaced by 1L DI water and the cell was run for another 4 hr.

The surface zeta potentials of virgin Omega and Ultracel membranes were tested at different pH by a Zetasizer Nano ZS using a surface zeta potential cell kit in the Malvern Instrument (Malvern, United Kingdom). A rectangular membrane sample 3.8 mm × 4.8 mm was placed on the holder, and the holder was mounted on the surface zeta potential cell kit; 10 μL of zeta potential transfer standard (latex colloids) of − 42 ± 4.2 mV from Malvern Instruments was added into the buffer for measurement. The skin side of the UF membrane faced outward and was submerged in the buffer at different pH. The membrane surface zeta potential was obtained from the measurement of five sequence displacements.

2.3. Experiments with regenerated membranes

After cleaning, the regenerated membranes were flushed with the 2.3 mM sodium phosphate buffer at the specified pH in the reservoir for 1 h. The buffer was placed in the feed reservoir and the filtration rate of the buffer solution was recorded during this run. The regenerated membrane-based experiment was started when the filtration flow rate reached 90 % of that achieved with the previous virgin membrane-based experiment. Then protein solution filtration experiments were conducted as described earlier using the regenerated membrane. After recording the results with the rest of the protein solution, three Ultracel® UF membranes were cleaned with 0.5 % Tergazyme® solution and then uninstalled. Finally, the membranes were stored in 10 % ethanol solution in a petri dish in the refrigerator at 3 °C.

2.4. Experiments on agglomeration of proteins

Solutions of BSA at 1.0 mg/ml level and Hb at 0.2 mg/ml were prepared in sodium phosphate buffer solution at pH 6.7, 7.0, 7.4 and in Tris buffer at pH 7.3, 7.6, 7.8, 8.2. The size distribution of the agglomerates in the protein mixtures at different pH and at different times was obtained by a Zetasizer Nano ZS (Malvern Panalytical ltd., UK) employing dynamic light scattering technology. The measurements were done at 0.5, 6.5, 18 and 50 hr.

3. Results and discussion

3.1. Zeta potential of membranes

The performance of the separation and purification of proteins from the mixture is affected by the membrane charge which depends on the membrane material and pH of the buffer. The Ultracel and Omega UF membranes used in this study are negatively charged naturally in a neutral environment. The negative charge becomes stronger when the pH increases slightly due to the adsorption of anions to the membrane [19]. Surface zeta potential data (Table 2) indicate that both membranes had ~ -20 mV negative charge at around pH 7.0. At this pH, BSA has a strong negative charge since its pI is 4.7 and is therefore strongly rejected by the negatively charged UF membranes. However, Hb has no net charge at a pH equal to its pI 6.8; at a pH of 7.0, it has a very low level of net negative charge and easily passes through the negatively charged UF membrane possibly yielding almost pure Hb in permeate.

Table 2
Surface zeta potential of Omega and Ultracel membranes.

pH	Omega 100 kDa membrane Surface Zeta Potential (mV)	Ultracel 100 kDa membrane Surface Zeta Potential (mV)
5.0	−10.4 ± 3.61	–
7.0	−18.1 ± 3.16	−19.9 ± 1.91
10.0	−21.5 ± 2.27	–

3.2. Flux of Ultracel and Omega membranes

The fluxes of Ultracel and Omega membrane in 1- and 3- membrane configurations at different pressures with a protein mixture containing 1.0 mg/ml BSA and 0.2 mg/ml hemoglobin are shown in Fig. 2. Due to the differences in materials and the porous structure between Ultracel and Omega membranes, Ultracel membranes showed a higher flux than Omega membranes at a ΔP of 10.34 kPa for 1 membrane and 31.02 kPa for 3 membranes. Interestingly, Ultracel flux at ΔP = 10.34 kPa for 3 membranes was stable around 12 μm/s which was considerably less than the value at ΔP = 31.02 kPa. In addition, the flux values of Omega membranes for 1 membrane at ΔP = 10.34 kPa and 3 membranes at 31.02 kPa were similar around 6 μm/s.

As an experiment commences, a protein solution of reduced concentration begins to pass through the membrane pores. Membrane fouling increases with increasing protein adsorption on the membrane and the flux decreases as shown. At the very beginning, for 3 disks of Ultracel® membranes, the flux under 31.02 kPa pressure differential is higher than the flux under 10.34 kPa pressure differential. Under constant stirring speed, higher pressure operation having a higher flux leads to a higher amount of rejected proteins and their increased deposition on the membrane surface. The membrane fouling rate is slightly slower under the lower pressure difference; correspondingly the flux decline is relatively lower at 10.34 kPa pressure differential. When a pseudo-steady state is reached, the degree of membrane fouling and the concentration polarization in the low differential pressure experiment is less. So, the plateau flux level at 10.34 kPa for 3 disks of Ultracel® membrane reaches 60 % of the flux at 31.02 kPa (the pressure difference 10.34 kPa is 33 % of 31.02 kPa).

Another item needs to be kept in mind. The solutions flowing through the second and subsequently the third membrane have much less protein and are less viscous. Further, there is a lower incidence of concentration polarization and protein adsorption on these membranes in addition to the reduced viscosity and therefore less flow resistance.

3.3. Agglomeration of Hb and BSA

Fig. 3 illustrates the results obtained on the evolution of protein agglomerates and their size distribution as a function of pH and time in two different buffers. It is clear that at pH 6.8 corresponding to the pI of Hb, the level of agglomeration is high. It is well known that proteins aggregate significantly at their isoelectric point without any net charge. the effect of electrostatic repulsions is reduced allowing hydrophobic interactions to facilitate agglomeration. It is also clear that as time

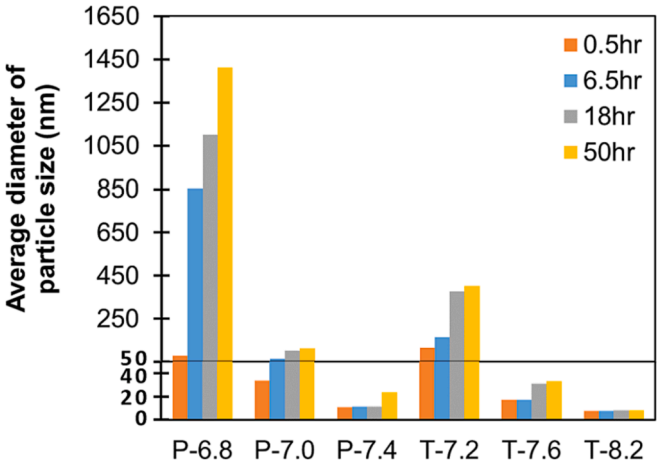

Fig. 3. Average diameter of agglomerate particle size in Hb-BSA solution at pH from 6.8 to 7.4 with sodium phosphate buffer (indicated by P- 6.8, P- 7.0, P-7.4 corresponding to 3 pHs, 6.8, 7, and 7.4) and at pH from 7.2 to 8.2 with Tris buffer (indicated by T- 7.2, T-7.6, T-8.2 corresponding to 3 pHs, 7.2, 7.6, and 8.2) in the time range from 0.5 hr to 50 hr. Concentration of protein:1.0 mg/ml BSA and 0.2 mg/ml hemoglobin.

increases, the agglomeration level increases. Fig. 3 further shows that as pH goes beyond 6.8, the agglomeration decreases drastically. Part of the reason is that Hb molecules develop a net negative charge and therefore encounter repulsion from BSA molecules having a net negative charge.

The agglomeration level depends also on the buffer type. Increasing pH reduces agglomeration in both buffers. The Tris buffer is basic and has a pK_a of 8.08; a significant fraction will be positively charged for the pH range used. Such positively charged species will associate with the net negatively charged BSA and lead to a higher agglomeration level compared to that with the phosphate buffer which will have primarily negatively charged dihydrogen phosphate ion and monohydrogen phosphate ion.

The development of Fig. 3 relies on the actual protein aggregate particle size distribution measurements developed in the Zetasizer instrument. Fig. 4 illustrates the type of size distribution data obtained for say, 0.5 hr contact time. Such data were generated for various times for the Hb-BSA solution.

We had also made UV–vis based measurements of the standard deviations in the measured concentrations of Hb and BSA at different pH levels with sodium phosphate buffer and Tris buffer for different time

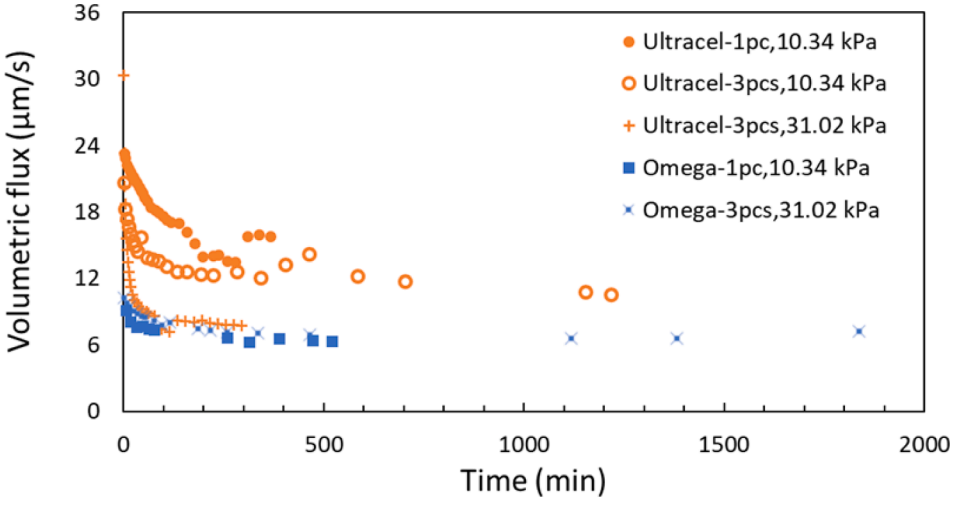

Fig. 2. Volumetric fluxes of Ultracel and Omega 100 kDa membranes in 1-and 3-membrane configurations at different applied pressure differences. Batch ultrafiltration:1.0 mg/ml BSA and 0.2 mg/ml hemoglobin; pH 7.4, 2.3 mM sodium phosphate buffer.

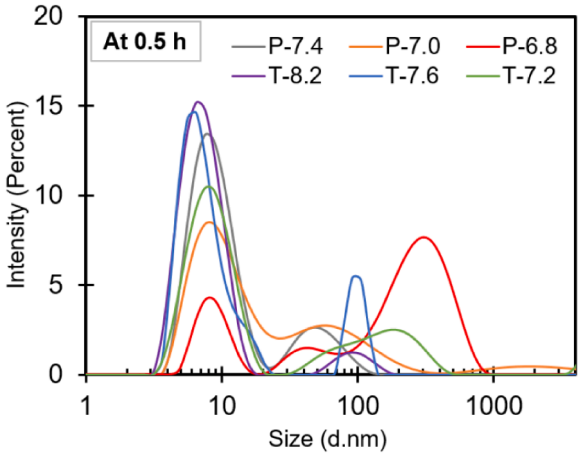

Fig. 4. Actual data of the size distribution of protein agglomerates obtained from Hb and BSA solution at various pHs in two buffer solutions identified in Fig. 3 for 0.5 hr.

periods. Those results are provided in Figures A.1 and A.2 in the Appendix; the related method is also described there.

3.4. A stack of three UF membranes vs A single UF membrane with Ultracel®

As mentioned earlier, the ISUF configuration of a stack of 3 UF membranes has higher rejection than the 1-membrane configuration because of the amplification of rejection with additional membranes. Fig. 5A, 5B and 5C show the feed and permeate concentration profiles of BSA and HB, rejection behaviors of Hb and BSA and fluxes in 1- and 3-membrane configurations as a function of the diavolume. As more layers of membranes were used, permeate concentrations of BSA (Fig. 5A) and Hb (Fig. 5B) were reduced, and their rejections (Fig. 5C) were improved. Fig. 5A and 5C show that BSA is virtually completely rejected with 3 disks of membranes. Conventionally, one would use a 30 kDa membrane to reject BSA completely; however, the flux would be much lower. Here the flux will be much higher for the same rejection: the fluxes of 3 disks of membranes are almost two times smaller than those with one membrane (Fig. 5C) at different diavolumes. Fig. 5D shows that the selectivity for Hb over BSA in the 3-membrane configuration is at least an order of magnitude higher than those with the 1-membrane configuration; here, the feed concentration changes with time.

The data from Eijndhoven et al. [5] for 1-membrane system are similar to our 1-membrane system; the maximum selectivity they obtained was around 70. With 3 membranes in ISUF, we are achieving a selectivity that varies between 6000 and 1000 + depending on the bulk

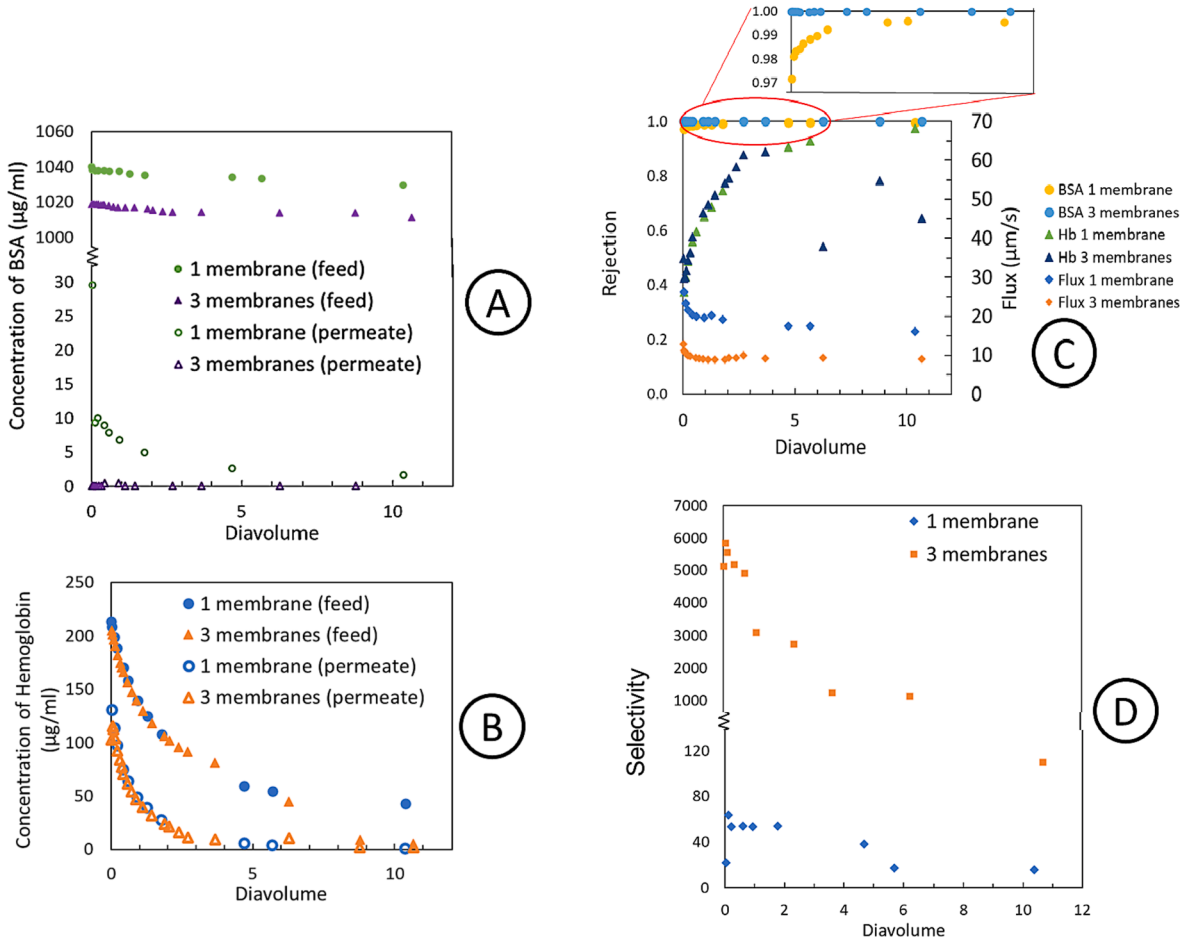

Fig. 5. Feed and permeate concentrations of (A) BSA, (B) hemoglobin, (C) their rejection and flux behaviors, and (D) their selectivity in 1- and 3- membrane configurations as a function of diavolume. Batch ultrafiltration:1.0 mg/ml BSA and 0.2 mg/ml hemoglobin; pH 7.8, 2.3 mM Tris buffer, Ultracel® 100 kDa membranes, 10.34 kPag.

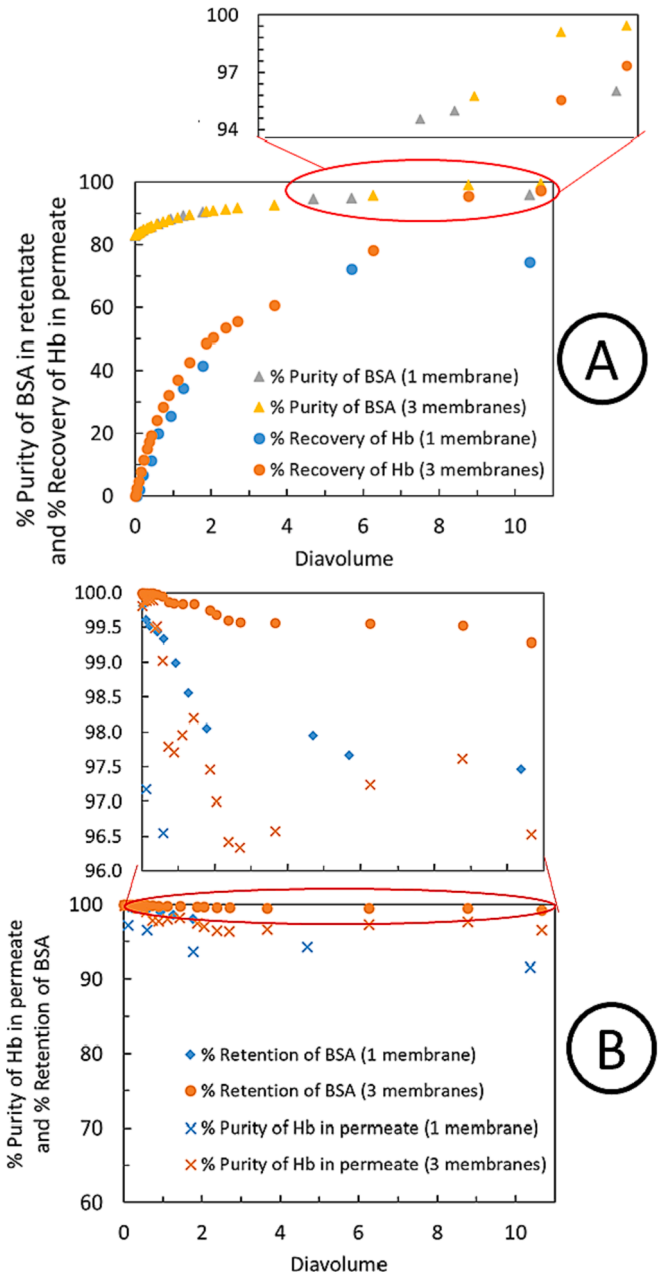

Fig. 6. (A) Purity of BSA in retentate and recovery of Hb in permeate and (B) purity of Hb in permeate and retention of BSA as a function of diavolume in 1-membrane and 3-membrane configurations. Batch ultrafiltration:1.0 mg/ml BSA and 0.2 mg/ml hemoglobin; pH 7.8, 2.3 mM Tris buffer, Ultracel® 100 kDa membranes, 10.34 kPag.

concentration up to a diavolume of 6.

Fig. 6A illustrates the purity of BSA in retentate and the recovery of Hb in permeate as a function of diavolume. In contrast, Fig. 6B shows the purity of Hb in permeate and the retention of BSA in 1- and 3- membrane configurations. The purity of Hb in permeate in 3-membrane configuration is maintained above 95 %, while the corresponding value in 1-membrane configuration varies from 80 % to 95 %. With increasing diavolume, these figures show that a much greater amount of BSA can be retained/blocked, and a greater amount of Hb can pass though the 3-membrane configuration than those in the 1-membrane configuration. The results indicate that we can achieve BSA purity in 1-and 3-membrane configurations to reach 95 % at ~ 5 diavolume; further, as the diavolume increases, the purity of BSA is increased vis-à-vis the 1-membrane system. The retention of BSA in 3-membrane configuration is also higher, and the recovery of Hb in 3-membrane system is higher. These results also show that Hb recovery with 3 Ultracel membranes approach 95 %+ at 10 diavolume+; the earlier study using Omega 100 kDa membrane [9] went up to 60 % at 4.45 diavolume. The earlier study [9] did not generate any data on BSA retention and BSA recovery.

To further study Hb-BSA separation and obtain a higher purity of proteins, we set the initial purity of BSA to be 95 % for a batch ultrafiltration feed containing 1.0 mg/ml BSA and 0.05 mg/ml Hb, since we achieved a 95 % purity level of BSA previously after 8 diavolume. Fig. 7A illustrates the purity of BSA in the retentate and the recovery of Hb in permeate during further processing. Fig. 7B shows the purity of Hb correspondingly in the permeate and retention of BSA in 1- and 3-membrane configurations. The results indicate that we can achieve the purity of BSA in 1-and 3-membrane configurations to reach 99 % at around 5 diavolume. Still the retention of BSA in 3-membrane is higher, while the recovery of Hb in 1-membrane is slightly higher. As the feed concentration of Hb was extremely low at 0.05 mg/ml, the purity of Hb in the permeate in 3-membrane configuration only reached 30–40 % after 2 diavolume and maintained a higher level than the one with 1-membrane configuration. All in all, the ISUF technique in Hb-BSA separation performed distinctly better than that in the 1-membrane configuration. Fig. 7C shows that the selectivity in the 3-membrane configuration is significantly higher than that in the 1-membrane configuration.

3.5. *Virgin vs Regenerated membranes and pressure effect in Ultracel® membrane*

The results in Fig. 8 show that the virgin and regenerated membranes have a similar performance; however, the regenerated membrane performance is somewhat better. At lower pressure drops, the purity of Hb in the permeate reached higher than 90 %; the recovery of Hb also can reach a high value of 60 % at 4 diavolume. In addition, the retention of BSA was maintained at a high level above 98 %. In Table 3, the purity of BSA, the recovery of Hb, purity of Hb in permeate, the retention of BSA and UF flux are shown for different pressures in 3-membrane configuration at around 1.1 diavolume. With decreasing pressure, BSA retention and Hb purity in permeate were also increased; operation at lower pressure leads to higher purity of retained BSA, higher recovery and purity of Hb in permeate and higher retention of BSA. This supports a basic aspect of HPTFF operation in terms of operating at lower pressures preferably in the linear region.

3.6. *Effects of pH and diavolume for Ultracel® membrane*

Figs. 9 and 10 illustrate the purity of BSA, the recovery of Hb, the purity of Hb in permeate and retention of BSA in a 3-membrane configuration at the following pH values: 6.8, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2. The recovery of Hb and purity of BSA and Hb were improved as pH was increased from 6.8 to 8.0. At a pH of 6.8, the net charge of Hb is zero; at the macromolecular level, the total charge contribution by positively charged amino groups is the same as those of the negatively charged carboxyl groups. The positively charged amino groups attract the negatively charged BSA to form an agglomerate, which can lead to membrane fouling. A gel layer may form if membrane fouling becomes severe. Under this condition, the UF membrane cannot continue to achieve effectively the type of high-quality separation being achieved here. To avoid fouling, Hb was allowed to develop a small amount of net negative charge. However, when pH is above 8.0, the recovery of Hb is apparently reduced due to the excess negative charge of Hb. Even though the rejection of BSA is close to 1, the separation performance of BSA and Hb was impaired when pH

was over 8.0 due to the reduced permeability of Hb. According to these results with UF membranes in Hb-BSA separation, a pH range of 7.4 – 7.8 provides the best separation performance. One can collect the

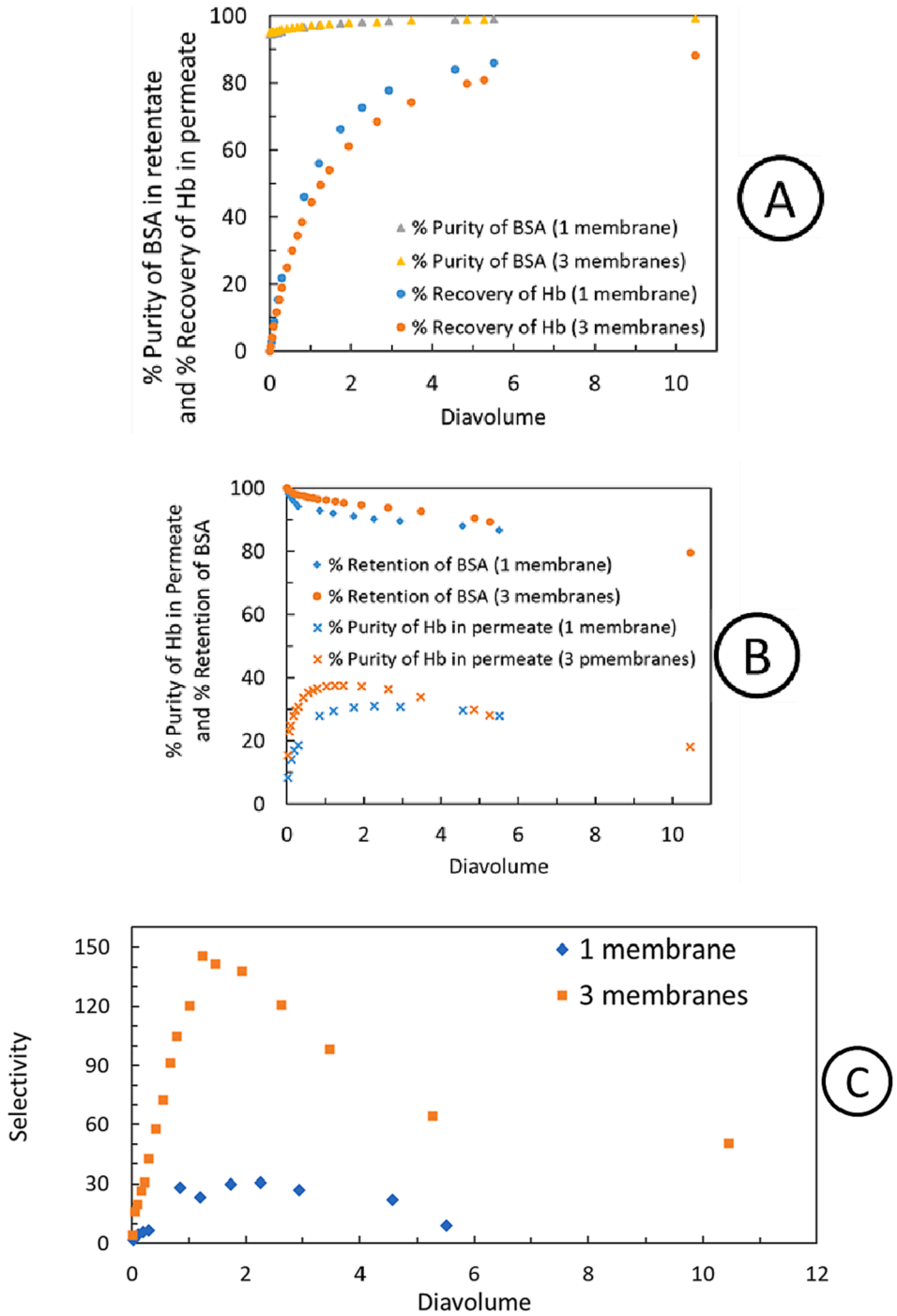

Fig. 7. Effect of diavolume for 1-membrane and 3-membrane configurations: (A) Purity of BSA in retentate and recovery of Hb in permeate; (B) purity of Hb in permeate and retention of BSA; (C) the Hb-BSA selectivity in permeates. Batch ultrafiltration:1.0 mg/ml BSA and 0.05 mg/ml hemoglobin; pH 7.4, 2.3 mM sodium phosphate buffer, Ultracel® 100 kDa membranes, 10.34 kPag.

first 2–4 diavolume of permeate to obtain high purity of Hb. One also can use high diavolume to achieve high purity of BSA. For example, the highest purity of BSA was achieved at pH 7.8: 99 % at 10.6 diavolume.

3.7. Performance of Omega membrane

Fig. 11 shows similar results in the 3-membrane configuration for Omega® 100 kDa membrane at pH 7.4, 2.3 mM sodium phosphate buffer and 31.02 kPag. The purity of BSA can reach around 92 % and the recovery of Hb can reach around 60 % at around 8 diavolume. Due to its different properties compared to the Ultracel® 100 kDa membrane, the pressure used was 31.02 kPag to maintain the flux at least around 6 μm/s. The above results from the Omega 100 kDa membrane show that the ISUF technique works. Although we have done extensive measurements using the Omega membrane, only the best separation performance is provided here for the sake of brevity. Using the principles described here, one needs to consider the intrinsic properties of other UF membranes and then achieve optimized separation performance for the Hb-BSA system.

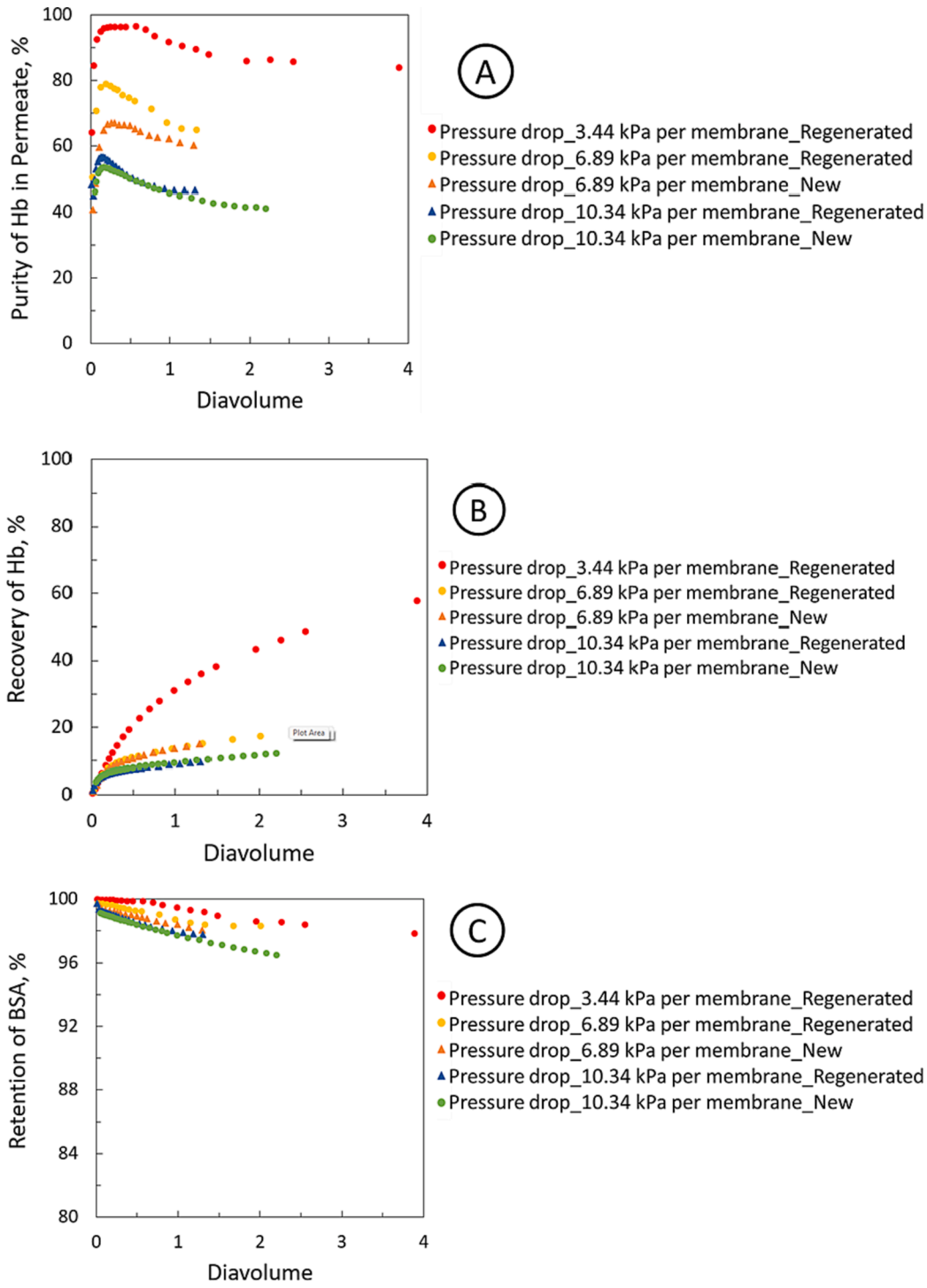

Fig. 8. (A) Purity of Hb in permeate, (B), recovery of Hb, and (C) retention of BSA as a function of diavolume in 3-membrane configuration at different pressures with virgin and regenerated membranes. Batch ultrafiltration:1.0 mg/ml BSA and 0.20 mg/ml hemoglobin; pH 7.2, 2.3 mM Sodium phosphate buffer, Ultracel® 100 kDa membranes.

Table 3
Summary of BSA-Hb separation in 3-membrane configurations (regenerated membranes) at different applied pressures.

Applied pressure difference	Number of membranes	Pressure drop per membrane	Diavolume	Purity of Hb in permeate	Purity of BSA	Recovery of Hb	Flux (μm/s)	Retention of BSA
10.34 kPa	3	3.44 kPa	1.16	90.35%	88.43 %	33.62 %	6.5	99.30 %
20.68 kPa	3	6.89 kPa	1.15	65.19%	85.27 %	14.13 %	6.7	98.48 %
31.02 kPa	3	10.34 kPa	1.18	46.48%	84.37 %	9.40 %	7.3	97.83 %

Batch ultrafiltration:1.0 mg/ml BSA and 0.20 mg/ml hemoglobin; pH 7.2, 2.3 mM sodium phosphate buffer, Ultracel® 100 kDa membranes.

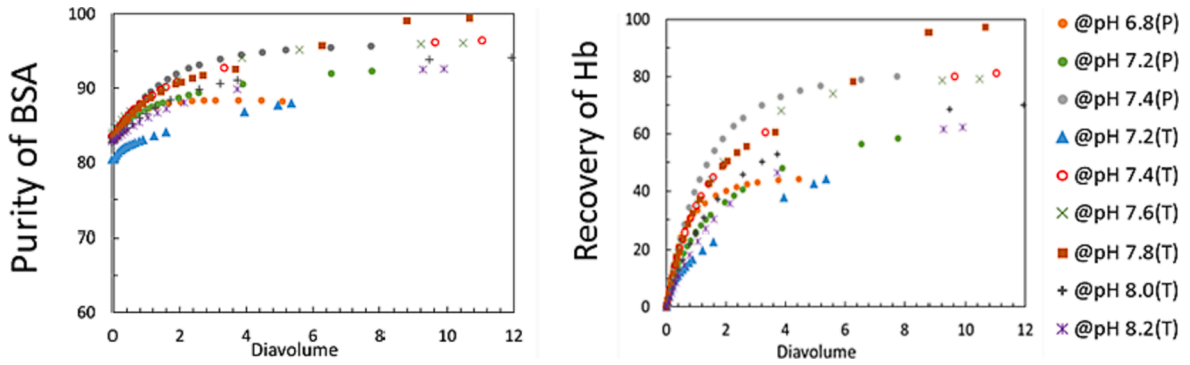

Fig. 9. Effect of pH: Purity of BSA in cell and recovery of Hb in permeate as a function of diavolume in 3-membrane configuration at 10.34 kPag. Batch ultrafiltration feed:1.0 mg/ml BSA and 0.2 mg/ml Hb, 2.3 mM sodium phosphate buffer pH 6.8, 7.2 and 7.4; 2.3 mM Tris buffer: pH 7.2, 7.4, 7.6, 7.8, 8.0 and 8.2, Ultracel® 100 kDa membranes.

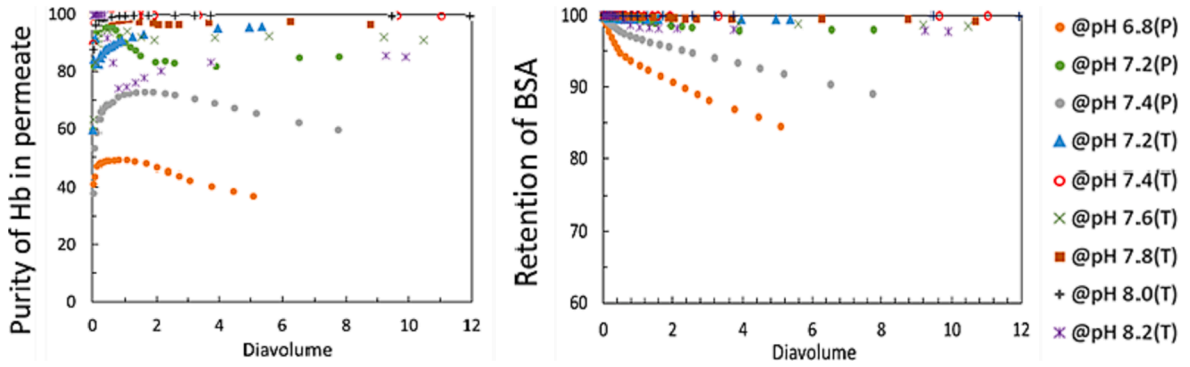

Fig. 10. Effect of pH: Purity of Hb in permeate and retention of BSA as a function of diavolume in 3-membrane configuration at 10.34 kPag. Batch ultrafiltration feed:1.0 mg/ml BSA and 0.2 mg/ml Hb, 2.3 mM sodium phosphate buffer pH 6.8, 7.2 and 7.4; 2.3 mM Tris buffer: pH 7.2, 7.4, 7.6, 7.8, 8.0 and 8.2, Ultracel® 100 kDa membranes.

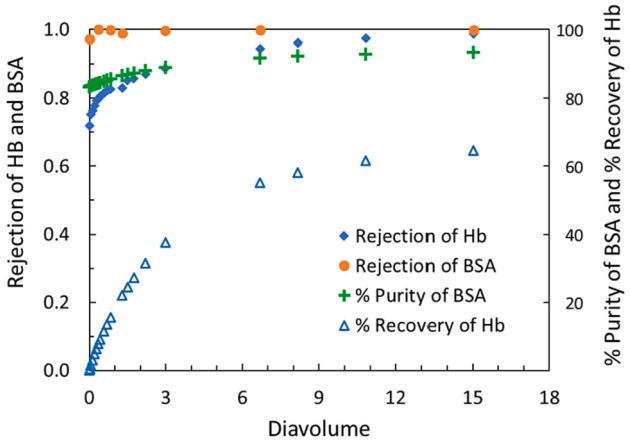

Fig. 11. Rejection behaviors of BSA and Hb, purity of BSA, and recovery of Hb as a function of diavolume in 3-membrane configurations. Batch ultrafiltration feed:1.0 mg/ml BSA and 0.2 mg/ml Hb, pH 7.4, 2.3 mM sodium phosphate buffer; Omega® 100 kDa membranes, 31.02 kPag.

4. Concluding remarks

High performance of the ISUF technique in Hb-BSA separation was validated for Ultracel and Omega 100 kDa membranes. Even though the molecular weight ratio of these two proteins is around 1.03, almost pure Hb is obtained in the permeate from Hb-BSA separation by ISUF and considerable purity and retention of BSA are also achieved. The best separation performance by ISUF was obtained over a pH range of 7.4 – 7.8, at an applied pressure difference of 10.34 kPa with Ultracel 100 kDa membrane and 31.02 kPa with Omega 100 kDa membrane. High purity of Hb is achieved during the first 2–4 diavolume of the permeate being collected due to lower Hb concentration in the starting feed solution. We can also use high diavolumes to achieve high purity of BSA. For example, the highest purity of BSA was achieved at pH 7.8: 99 % at 10.66 diavolume. Despite seven times in situ membrane regeneration, Ultracel® membrane performances were reproducible. In future research studies, the potential of improving the separation performance in a continuous flow system by ISUF in one UF device such as a cassette will be explored using various protein mixtures with molecular weight ratios in the range of 1 to 3.5. A key issue among others is the diavolume level needed for the desired level of purification and recovery of the two proteins. That information will be important for cost estimation.

There are additional potential applications of such a technique worthy of exploration. How useful is the ISUF technique for virus removal applications where a stack of two membranes is usually studied in a different configuration with the membrane skin facing downstream [22] as if those were depth filters? There are patents [23,24] with a concept very similar to that explored in earlier ISUF studies [8–9], where a two-layer membrane device is claimed to have improved membrane performance in terms of support defect or membrane to membrane variability vis-à-vis virus filtration.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2023.123363.

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