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Suma Rao[a]; Kimberly Ager[a]; Andrew L. Zydney[a]
[a] Department of Chemical Engineering, The Pennsylvania State University, University Park, PA

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High Performance Tangential Flow Filtration Using Charged Affinity Ligands

Suma Rao, Kimberly Ager, and Andrew L. Zydney
Department of Chemical Engineering, The Pennsylvania State University, University Park, PA

Abstract: High performance tangential flow filtration is an emerging technology for protein separations. The objective of this work was to examine the use of small charged affinity ligands to control the protein charge and thus extend the potential applicability of this separation technique. Experiments were performed using binary mixtures of bovine serum albumin and ovalbumin, with Cibacron Blue added as an affinity ligand. The addition of Cibacron Blue caused a large increase in the selectivity due to the increase in BSA retention associated with the strong electrostatic repulsion between the negatively-charged protein-ligand complex and the negatively-charged cellulosic membrane. Appropriate conditions for the separation were identified using total recycle experiments in a small-scale tangential flow filtration device. A diafiltration process was designed to separate BSA from ovalbumin, with the Cibacron Blue recovered in a second stage operated with a buffer containing NaSCN to displace the dye from the protein. The results demonstrate the ability to use small charged affinity ligands with bio-specific binding characteristics to enhance separations by high performance tangential flow filtration.

Keywords: Ultrafiltration, affinity separations, bioprocessing, protein purification

INTRODUCTION

Microfiltration and ultrafiltration have been traditionally limited to separating solutes that differ by at least an order of magnitude in size. High Performance Tangential Flow Filtration (HPTFF) is an emerging technology that is able to

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Address correspondence to Andrew L. Zydney, Department of Chemical Engineering, The Pennsylvania State University, University Park, PA 16802. Tel.: 814-863-7113; Fax: 814-865-7846; E-mail: zydney@engr.psu.edu

separate solutes irrespective of the relative size of the product and impurity (1). HPTFF obtains high selectivities for protein separations by:

1. operation in the pressure-dependent regime to minimize fouling and exploit concentration polarization effects,
2. proper selection of pH and ionic strength to maximize differences in effective volume of the product and impurity,
3. using an electrically charged membrane to enhance repulsion of like-charged species, and
4. using a diafiltration mode to obtain high purification factors and yields (1).

Several recent studies have demonstrated the potential of HPTFF for protein purification. For example, van Reis et al. (2) were able to achieve 900-fold purification at more than 90% yield for the separation of an antigen-binding fragment (Fab) of a monoclonal antibody from bovine serum albumin (BSA) using high performance tangential flow filtration. High selectivities were obtained by operating close to the isoelectric point of the Fab (pH 8.4) and using low salt concentrations and a negatively charged membrane, conditions that gave high retention of the negatively charged BSA. It was also possible to purify the Fab in the retentate by operating near the isoelectric point of BSA using a positively-charged membrane. van Einhoven et al. (3) reported purification factors of close to 100 and yields of nearly 70% for the separation of BSA and hemoglobin, with the separation driven by the large difference in isoelectric point for the hemoglobin (pI = 7.0) and BSA (pI = 4.8). Lu et al. were able to separate lysozyme from ovalbumin using high performance ultrafiltration at pH 10–11, again exploiting the large difference in surface charge for the two proteins (4). Kanani et al. (5) showed that the selectivity for separating lysozyme and myoglobin could be significantly enhanced by the addition of BSA as a "dual-facilitating agent." The increase in selectivity was due to electrostatic effects. Lysozyme retention increased due to the electrostatic attraction between the positively-charged lysozyme and the negatively-charged BSA, while the myoglobin transmission increased due to the Donnan effect.

Applications of HPTFF for the separation of proteins with similar electrical charge are much more limited. Ebersold and Zydney (6) obtained moderate purification of protein variants differing at only a single amino acid residue, in this case exploiting the difference in the protein charge arising from the substitution of a negatively-charged carboxylic acid moiety for the positively-charged amine group on the amino acid lysine. A two-stage system was required to obtain reasonable yield and purification factor due to the relatively low selectivity between the product and variant associated with the small charge difference in this system. Wan et al. (7) used ultrafiltration to remove dimers and higher order oligomers from a monoclonal antibody product based primarily on the difference in physical size, although the purification factor for the single stage system was less than four.

Recent studies by Rao and Zydney (8, 9) have demonstrated that the effective protein charge can be "controlled" through the use of small charged ligands that selectively bind to the protein of interest. Experimental studies performed with BSA and the dye Cibacron Blue showed that each molecule of BSA could bind up to 11 molecules of the dye, causing the net protein charge at pH 5 to shift from approximately +1 to −12 (8). This shift in charge caused a dramatic change in the rate of protein transport. For example, the addition of 1.1 g/L of Cibacron Blue to an 8 g/L BSA solution caused the transmission of BSA to drop by more than a factor of 100 due to the strong electrostatic repulsion between the negatively-charged complex of BSA and Cibacron Blue and the negatively-charged membrane. Rao and Zydney (9) demonstrated that the bio-specific binding characteristics of Cibacron Blue could be used to dramatically increase the selectivity between BSA and ovalbumin during ultrafiltration.

Although the studies by Rao and Zydney (8, 9) clearly demonstrate that small charged affinity ligands can be used to alter the rate of protein transport, all of the experimental data were obtained in a small stirred ultrafiltration cell which is not representative of the tangential flow filtration (TFF) systems used for large-scale protein purification (2). In addition, these studies never examined the recovery of the dye or the preparation of the dye-free protein product. The objective of this work was to extend these previous studies to TFF, providing an appropriate framework for the analysis of HPTFF systems using small charged affinity ligands to enhance the selectivity. Experiments were performed using the model system of ovalbumin and BSA with Cibacron Blue used as the affinity ligand. Data were obtained in a Pellicon XL tangential flow filtration module, which is linearly scalable to commercial bioprocesses (10). The results clearly demonstrate that high resolution protein separations can be achieved using HPTFF by exploiting a small charged affinity ligand to control the net protein charge.

MATERIAL AND METHODS

Protein Solutions

Experiments were performed using bovine serum albumin (>96% pure, essentially fatty acid free, catalogue # A-6003 from Sigma Chemical, St. Louis, MO) and ovalbumin (>98% pure, A-5503, Sigma Chemical). BSA has an isoelectric point (pI) of approximately 4.9 and a molecular weight of 67 kD, while ovalbumin has an isoelectric point (pI) of approximately 4.5 and a molecular weight of 44 kD.

Cibacron Blue 3GA (Catalogue # C-9534, Sigma Chemical) was used as the small affinity ligand. Cibacron Blue has a molecular weight of 0.774 kD and contains three negatively-charged sulfonic acid groups attached to an aromatic ring structure (11). Previous work by Rao and Zydney (8, 9)

indicated that as many as 11 molecules of Cibacron Blue can bind to a single molecule of BSA, probably through the fatty acid and anion binding sites on the protein. Ovalbumin displays minimal binding of Cibacron Blue up to fairly high ligand concentrations (12).

Buffer solutions were prepared by dissolving pre-weighed amounts of the appropriate salts in deionized water obtained from a NANOpure Diamond water purification system (Barnstead Thermolyne Corporation, Dubuque, IA) with a resistivity greater than 18 MΩ–cm. An acetate buffer composed of CH_3COONa and CH_3COOH (EM Science, Gibbstown, NJ) was used for experiments at pH 5.0. The dye was separated from the BSA by ultrafiltration at pH 8.0 using Tris–HCl buffer with sodium thiocyanate (Sigma Chemical, St Louis, MO) at a total ionic strength of 1 M. The solution pH was measured using a 420APlus pH meter (Thermo Orion, Beverly, MA), and the solution conductivity was measured using a 105A plus conductivity meter (Thermo Orion, Beverly, MA). All buffer solutions were prefiltered through 0.2 μm pore size Supor-200 membranes (Pall Corp., Ann Arbor, MI) to remove particulates and undissolved salts.

Protein solutions were prepared by slowly dissolving the protein powder in the desired buffer, with the resulting solution filtered through a 0.22 μm syringe filter (Costar Corp., Cambridge, MA) to remove any protein aggregates immediately prior to use. Protein solutions were used within 24 hours of preparation to minimize the likelihood of protein aggregation or denaturation during storage.

Assays

Protein concentrations in the binary mixture were analyzed by size exclusion chromatography (Agilent 1100 series quaternary HPLC system). Assays were performed using a Superdex 75 column with 13 μm particle size and 10^5 MW exclusion limit (GE Healthcare, Piscataway, NJ). Column calibration was done using binary protein solutions of known concentrations. The column was first equilibrated with fresh buffer at a flow rate of approximately 0.3 mL/min for 180 min. This also served to flush both the sample and reference cells in the refractive index detector (Agilent 1100 series). Column equilibration was confirmed by tracking the baseline refractive index (RI). The mobile phase was a 50 mM phosphate buffer with 0.15 M NaCl at a flow rate of 0.2 mL/min. Protein samples (50 μl) were injected by an autosampler with the data analyzed using Agilent ChemStation software on a Dell Celeron Computer.

Cibacron Blue concentrations were determined spectrophotometrically using the natural absorbance at 616 nm. The presence of protein had no effect on the absorbance of the dye at 616 nm over the range of concentrations examined in this study.

Membrane Preparation

All filtration experiments were performed using Ultracel composite regenerated cellulose membranes in Pellicon XL tangential flow filtration modules provided by Millipore Corp. (Bedford, MA). These cellulosic membranes are nearly uncharged and have very low protein binding due to their high degree of hydrophilicity. Protein separations were performed using a charge-modified version of the 100 kD nominal molecular weight cut-off membranes with the Cibacron Blue recovered using a conventional 10 kD membrane. All membranes/modules were thoroughly flushed with deionized distilled water prior to use to remove any residual storage agents.

The negatively-charged version of the 100 kD membrane was made in our laboratory by the covalent attachment of negatively charged sulfonic acid groups to the surface of the membrane using the base-activated chemistry developed by van Reis (13). Membranes were first flushed with deionized distilled water followed by 0.1 N NaOH. The charging solution, a 2 M solution of 3-Bromopropanesulfonic acid sodium salt (Catalogue # B2912, Sigma Chemical) in 0.1 N NaOH, was pumped through the module at a feed flow rate of 20 mL/min using a Rabbit-Plus peristaltic pump (Rainin Instrument Co., Emeryville, CA). Charging was done for 8 hours with the charging solution flushed through the system in a total recycle mode. The membranes were flushed with approximately 100 L/m^2 of distilled water to quench the reaction and were then stored in 0.05 N NaOH until use.

Protein Filtration

Initial protein filtration experiments were conducted using the Pellicon XL module with the charge-modified 100 kD Ultracel membrane in total recirculation mode to determine the appropriate conditions for the protein separation. The feed tank was filled with the binary protein mixture containing a specified concentration of Cibacron Blue. The feed was pumped through the module using a Rabbit-Plus peristaltic pump (Rainin Instrument Co., Emeryville, CA) at flow rates from 18 ml/min to 25 ml/min. The transmembrane pressure was monitored using pressure gauges (Millipore Corp, Bedford, MA) in both the feed and filtrate lines. The filtrate and retentate solutions were recycled back to the feed tank to maintain a constant concentration of the proteins and affinity ligand. The filtration velocity was measured by timed collection, with periodic samples taken from both the filtrate and bulk samples. Data were obtained over a range of filtrate flux, set by varying the transmembrane pressure drop. All experiments were performed at room temperature ($22 \pm 3^{\circ}C$) using the same lots of protein.

Diafiltration

A two stage separation process was performed using a diafiltration mode as shown schematically in Fig. 1. Stage 1 employed a Pellicon XL module with a negatively charged version of the 100 kD Ultracel membrane. A binary mixture of BSA and ovalbumin, with an appropriate concentration of Cibacron Blue, was added to the feed tank. The inlet flow was pumped through the module using a peristaltic pump at a flow rate of 300 L m^{-2} hr^{-1} (volumetric flow rate normalized by the membrane area). Initially, the filtrate and retentate lines were both returned to the feed tank, providing total recycle until the system had stabilized (typically 10 min). At this point, the diafiltration was begun, with the filtrate directed to a second collecting vessel. The total fluid volume in the feed tank was maintained at a constant level by continuous addition of fresh diafiltration buffer (10 mM acetate, pH 5.0) at a rate equal to the ultrafiltration rate. Samples were taken from the feed and filtrate reservoir at appropriate time points throughout the diafiltration for subsequent analysis of the protein concentrations.

The final retentate from Stage 1 was further purified in Stage 2 to recover the Cibacron Blue and yield a dye-free BSA product. Experiments were performed using a Pellicon XL module with an unmodified (essentially neutral) 10 kD Ultracel membrane. The diafiltration buffer was 1 M NaSCN

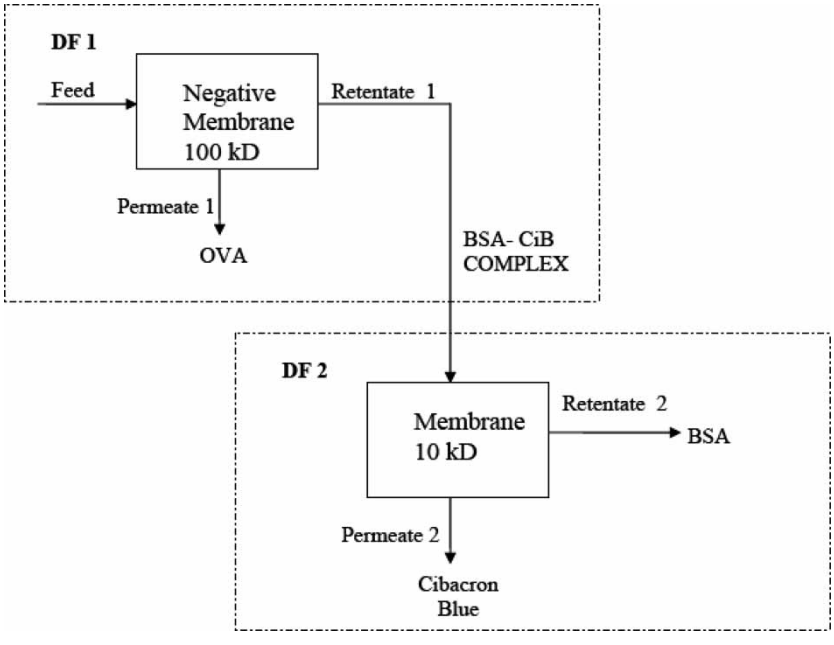

Figure 1. Schematic of the 2-stage separation process.

at pH 8, conditions which are known to cause the dissociation of Cibacron Blue from BSA (14). The filtrate flux in the second stage was maintained at approximately 50 L m^{-2} h^{-1} throughout the diafiltration process.

RESULTS AND DISCUSSION

In order to understand the basis for the enhanced separation in the presence of Cibacron Blue, model calculations were performed to evaluate the effective net charge of the protein-dye complex as a function of the Cibacron Blue concentration for both BSA and ovalbumin. The number of molecules of Cibacron Blue bound to each protein was evaluated using a multisite Langmuir-type binding isotherm (8):

$$C_{bound} = \frac{nK_{eq}C_{protein}C_{free}}{1 + K_{eq}C_{free}} \tag{1}$$

where n is the maximum number of molecules of Cibacron Blue bound per protein molecule, K_{eq} is the equilibrium binding constant, and $C_{protein}$ is the total concentration of protein in the solution. Experimental binding data obtained by Rao and Zydney (9) for the individual proteins gave $n = 11$ and $K_{eq} = 110 \times 10^3$ M^{-1} for BSA and $n = 2.8$ and $K_{eq} = 1.7 \times 10^3$ M^{-1} for ovalbumin, both at pH 5.0 in a 10 mM acetate buffer. The total concentration of bound Cibacron Blue in the binary protein mixture was evaluated by accounting for the binding to both BSA and ovalbumin:

$$C_{bound} = \frac{n_{BSA}K_{BSA}C_{BSA}C_{free}}{1 + K_{BSA}C_{free}} + \frac{n_{OVA}K_{OVA}C_{OVA}C_{free}}{1 + K_{OVA}C_{free}} \tag{2}$$

The net protein charge was calculated using a charge regulation model that accounts for the binding of the negatively-charged Cibacron Blue and the change in the local pH at the surface of the protein associated with electrostatic interactions between the charged protein and the H^+ ions (see Appendix 1). Figure 2 shows the model calculations for a solution containing 10 g/L BSA solution and 6 g/L ovalbumin at pH 5.0 in a 10 mM acetate buffer. These calculations are approximate since they neglect the detailed geometry and charge distribution over the surface of the protein, as well as the affects of protein hydration. However, the results should provide an accurate semi-quantitative description of the affect of the charged dye on the net protein charge.

The addition of Cibacron Blue causes the predicted charge of BSA to decrease from about +1.1 in the absence of Cibacron Blue to a value of −12 at a Cibacron Blue concentration of 1.4 g/L, corresponding to approximately 12 molecules of Cibacron Blue per molecule of BSA. Although BSA is able to bind 11 molecules of Cibacron Blue under these conditions, with each molecule of the dye having a net negative charge of −3, the net charge of the

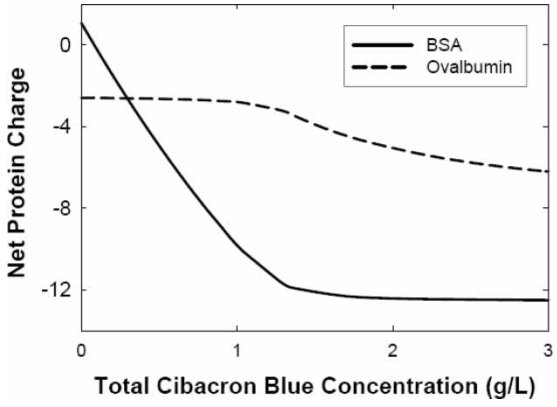

Figure 2. Predicted values of the effective charge for BSA (solid curve) and ovalbumin (dashed curve) in a pH 5.0, 10 mM acetate buffer in the presence of Cibacron Blue accounting for competitive ligand binding and charge regulation.

BSA-Cibacron Blue complex decreases by only about 13 electronic charge units due to the effects of charge regulation. The increase in the negative charge of the protein causes an increase in the local concentration of the positively-charged H^+ ions which in turn leads to the protonation of approximately 15 carboxylic acid groups each with a pK_a of 4.02 as well as a small change in the protonation of several other amino acid residues (Table A1). In contrast to the results with BSA, Cibacron Blue has a relatively small effect on the predicted charge of ovalbumin due to the much smaller value of K_{eq} (and n) for ovalbumin relative to that for BSA. Thus, a Cibacron Blue concentration of 1.4 g/L causes the effective charge on ovalbumin to decrease by less than 1 electronic charge since almost all of the dye is bound by BSA under these conditions. Even the addition of 2.9 g/L of Cibacron Blue causes the effective charge of ovalbumin to decrease by only 3.5 charge units. These calculations clearly indicate that the addition of Cibacron Blue can selectively shift the net protein charge, in this case providing conditions in which there is a very large difference in the effective charge on ovalbumin and BSA.

Initial protein sieving experiments were performed in a total recycle mode using the Pellicon XL module with a negatively charged version of the 100 kD Ultracel membrane. The negative membrane was produced by recirculating a 2 M solution of 3-Bromopropanesulfonic acid at pH 11 for 8 hrs. Previous data obtained with small membrane discs charged in a stirred ultrafiltration cell under these conditions yielded a membrane with a zeta potential of −12.4 mV. The sieving data were obtained with a solution containing a binary mixture of 10 g/L BSA and 6 g/L of ovalbumin, both in the absence of any ligand and with a Cibacron Blue concentration of 1.4 g/L (corresponding to a molar ratio of 12 molecules of dye per molecule of BSA). The solution pH was adjusted to 5.0 using a 10 mM acetate buffer based on data obtained

previously in a stirred ultrafiltration cell (8, 9). Results are shown in Table 1 at a feed flow rate of 25 mL/min, equal to an area normalized feed flow rate of 300 L m^{-2} h^{-1}, and a filtrate flow rate of 35 L m^{-2} h^{-1}. The observed sieving coefficients for BSA and ovalbumin, defined as the ratio of the protein concentration in the filtrate solution to that in the bulk (feed) solution, differ by less than a factor of five in the absence of Cibacron Blue with values of $S_o = 0.020$ for BSA and $S_o = 0.098$ for ovalbumin. The greater sieving coefficient for ovalbumin under these conditions is likely due to the smaller size (MW of 44 kD versus 67 kD for BSA) and possibly the different surface charge. The addition of Cibacron Blue caused a 10-fold reduction in the observed sieving coefficient for BSA, with the sieving coefficient for ovalbumin decreasing by less than 35%. The net result is that the addition of Cibacron Blue causes the selectivity for the BSA-ovalbumin system:

$$\psi = \frac{S_{o,OVA}}{S_{o,BSA}} \tag{3}$$

to increase from less than 5 to more than 30. This large increase in selectivity is a direct result of the strong electrostatic exclusion of the negatively-charged complex formed between BSA and Cibacron Blue from the negatively-charged Ultracel membrane. Data obtained by Rao and Zydney (9) with a neutral membrane in a stirred cell ultrafiltration cell showed only a small increase in selectivity upon the addition of Cibacron Blue due to the much weaker electrostatic repulsion.

The effects of the feed and filtrate flow rates on the separation characteristics are examined in Fig. 3. Data were again obtained using a protein mixture containing BSA at a concentration of 10 g/L, ovalbumin at a concentration of 6 g/L, and Cibacron Blue at a concentration of 1.4 g/L. The solution pH was adjusted to pH 5.0 using a 10 mM acetate buffer. Experiments were performed with the negatively charged 100 kD Ultracel membrane at feed flow rates of 25 and 18 mL/min, corresponding to area normalized feed flow rates of 300 and 216 L m^{-2} h^{-1}. Results for the selectivity (ψ) are shown in the top panel of Fig. 3. The selectivity initially increases with increasing filtrate flux, attaining its largest value of $\psi = 36$ at a filtrate flux of 50 L m^{-2} h^{-1} and a feed flow rate of 300 L m^{-2} h^{-1}. The selectivity decreases at very high filtrate flux, which is likely due to the greater increase in the observed

Table 1. Observed sieving coefficients at a normalized feed flux of 300 L m^{-2} h^{-1} and a filtrate flux of 35 L m^{-2} h^{-1}

Cibacron Blue Concentration (g/L)	$S_{o,BSA}$	$S_{o,OVA}$
0	0.020	0.098
1.4	0.002	0.064

sieving coefficient of BSA associated with the greater degree of concentration polarization for the more highly retained species. The selectivity at a feed flow of $216\ L\ m^{-2}\ h^{-1}$ was significantly smaller than that obtained at $300\ L\ m^{-2}\ h^{-1}$ at intermediate values of the filtrate flux, but the reverse behavior was seen at the lowest flux ($20\ L\ m^{-2}\ h^{-1}$) where the selectivity at $216\ L\ m^{-2}\ h^{-1}$ was more than twice that at a feed flux of $300\ L\ m^{-2}\ h^{-1}$. These differences are likely due to the different degrees of concentration polarization arising from the flow rate dependence of the mass transfer coefficient along with the different variations in the protein concentration with axial position associated with the different conversions (ratio of filtrate to feed flow rate) at the two feed flow rates.

The bottom panel of Fig. 3 shows the calculated values of the mass throughput parameter, $J\Delta S$, which determines the economics and practicality of the separation (15). High values of $J\Delta S$ allow effective separations to be achieved using less membrane area, shorter diafiltration times, and/or with less diafiltration buffer. In contrast to the results for the selectivity, the $J\Delta S$ values are uniformly higher at the lower feed flux due to the higher values

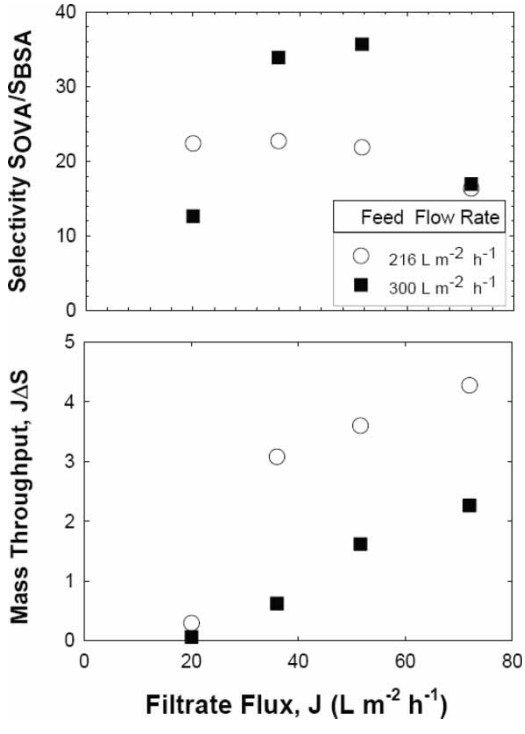

Figure 3. Selectivity (top panel) and mass throughput (bottom panel) for separation of a binary mixture of 10 g/L BSA and 6 g/L ovalbumin with 1.4 g/L Cibacron Blue for a negatively charged 100 kD Ultracel membrane.

of the ovalbumin sieving coefficient obtained under these conditions. This increase in S_o arises from the greater degree of concentration polarization at the lower feed flow rate due to the lower value of the mass transfer coefficient. $J\Delta S$ increases monotonically with increasing filtrate flux, although the rate of increase becomes much less pronounced at high values of the filtrate flux due to the reduction in ΔS at high flux, particularly at the lower feed flow rate.

The most appropriate conditions for separating ovalbumin and BSA using the affinity HPTFF process were identified using process optimization diagrams following the approach developed by van Reis and Saksena (15). The process optimization involves a trade-off between the purification factor, yield, membrane area, process time, and buffer requirements, all of which are functions of the selectivity and the mass throughput parameters. The product yield is defined as the percent recovery of the desired product in either the final retentate or filtrate stream, while the purification factor is equal to the yield of the desired product divided by the yield of the impurity. In this case, ovalbumin is the impurity for the BSA product collected in the retentate while BSA is the impurity for the ovalbumin product collected in the filtrate solution. Theoretical calculations were performed by fixing the BSA yield in the retentate at a value of 95%, with the purification factor for BSA and the required number of diavolumes (N) for the separation evaluated as (15):

$$P_{BSA} = Y_{BSA}^{1-\psi} \tag{4}$$

$$N = \frac{\ln(P_{BSA})}{\Delta S} \tag{5}$$

where N is the ratio of the cumulative filtrate volume to the constant volume of the feed reservoir (tank) during a constant volume diafiltration. The corresponding values for the yield and purification factor for ovalbumin collected in the filtrate solution were calculated as:

$$P_{OVA} = \frac{Y_{OVA}}{1 - (1 - Y_{OVA})^{1/\psi}} \tag{6}$$

$$N = \frac{\ln[(Y_{OVA}/P_{OVA} - 1)/(Y_{OVA} - 1)]}{\Delta S} \tag{7}$$

The results are summarized in Table 2 for calculations performed at the feed flow rate of 300 L m^{-2} h^{-1}over the full range of filtrate flux. Similar trends were seen using other values for the BSA yield. The purification factor for BSA is fairly small at low values of the filtrate flux due to the small selectivity under these conditions. In addition, the required number of diavolumes is very large (N = 49 at a feed flow rate of 300 L m^{-2} h^{-1}), indicating that this separation would be very difficult to effect in a large scale commercial process. The use of very large filtrate flux significantly reduces the required number of diavolumes, but the purification factor for BSA

Table 2. Calculated values of the yield, purification factor, and number of diavolumes for a diafiltration process providing 95% yield of BSA

Filtrate flux ($L\ m^{-2}\ h^{-1}$)	Selectivity (Ψ)	Mass throughput ($L\ m^{-2}\ h^{-1}$)	N	P_{BSA}	Y_{OVA}	P_{OVA}
20	13	0.24	49	2	47	10
36	34	2.26	27	5	82	16
50	36	5.83	15	6	83	17
72	17	8.15	7	2	56	12

becomes very small and the yield and purification factor for ovalbumin are also reduced. The optimal conditions for the separation would depend on the detailed economics and separation constraints for the commercial process, including the value for the two protein products, the required degree of purification, and the overall process design. However, the use of a feed flow rate of $300\ L\ m^{-2}\ h^{-1}$ and a filtrate flow rate of $50\ L\ m^{-2}\ h^{-1}$ should provide a reasonable combination of purification factor and yield for both BSA and ovalbumin with moderate requirements for the number of diavolumes. These conditions were thus used in all subsequent experiments.

The separation of BSA and ovalbumin was performed using a constant volume diafiltration to effectively wash the more permeable ovalbumin through the membrane and into the filtrate while the highly charged BSA – Cibacron Blue complex was retained in the feed reservoir. Figure 4 shows experimental data obtained during a diafiltration experiment using the negatively-charged version of the 100 kD Ultracel membrane in the Pellicon XL tangential flow filtration module at a feed flow rate of $300\ L\ m^{-2}\ h^{-1}$ and a filtrate flow rate of $50\ L\ m^{-2}\ h^{-1}$. The initial (feed) was 2 g/L BSA and 1 g/L ovalbumin, with 0.28 g/L of Cibacron Blue added as the affinity ligand. Experiments were performed using 10 mM acetate at pH 5 as the diafiltration buffer; the retention of Cibacron Blue (bound to BSA) was sufficiently high that the Cibacron Blue concentration in the feed reservoir remained nearly constant throughout the diafiltration even without the presence of additional Cibacron Blue in the diafiltration buffer.

The data in Fig. 4 have been plotted as the normalized protein concentration in the feed reservoir (with C normalized by the initial feed concentration) as a function of the total number of diavolumes. The normalized protein concentration decreases with increasing number of diavolumes, with the rate of decrease being much more dramatic for ovalbumin due to the much greater degree of ovalbumin transmission through the negatively-charged membrane. The ovalbumin concentration at the end of the 20-diavolume process was less than 0.05 g/L, corresponding to removal of more than 95% of the ovalbumin present in the original feed solution. In

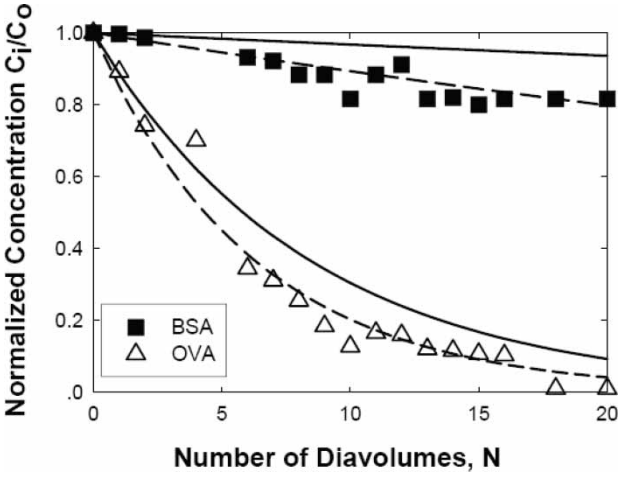

Figure 4. Normalized protein concentrations during diafiltration of a feed containing 2 g/L BSA and 1 g/L ovalbumin in the presence of 0.28 g/L Cibacron Blue using a negatively charged 100 kD Ultracel membrane. Solid and dashed curves are model calculations as described in the text.

contrast, the BSA concentration at the end of the diafiltration was greater than 1.6 g/L providing a BSA yield of more than 80% in the retentate solution. Greater values of the BSA yield could be obtained by stopping the diafiltration after a smaller number of diavolumes, with the yield of BSA being greater that 90% for $N < 7$.

The solid and dashed curves in Figure 4 represent the calculated values of the normalized protein concentration evaluated by integration of a differential mass balance assuming a constant observed sieving coefficient (15)

$$C/C_o = \exp(-NS_o) \tag{8}$$

The solid curves were generated using the protein sieving coefficients evaluated from data obtained in the total recycle experiments, with the model over-predicting the protein concentrations throughout the diafiltration. The dashed curves in Fig. 4 represent the model calculations using the best fit values of the sieving coefficients determined by fitting the experimental data in Fig. 4 to Eq. (8) yielding $S_{o,BSA} = 0.01$ and $S_{o,OVA} = 0.16$. These values for the sieving coefficients were both somewhat larger than those determined from the total recycle experiments causing the model to give a more rapid reduction in the concentrations of both BSA and ovalbumin during the diafiltration. This discrepancy is likely due to the inherent variability between membranes/modules used in these experiments

The behavior of the BSA – ovalbumin separation was examined in more detail by replotting the data from Fig. 4 as the protein yield as a function of the purification factor. Results for BSA (in the retentate

solution) are shown in the top panel of Fig. 5 while those for ovalbumin (in the filtrate solution) are shown in the bottom panel. The dashed curves are model calculations given by Eqs. (4) and (6) with the selectivity calculated from the best fit values of the observed sieving coefficients as determined from the data in Fig. 4. The diafiltration process for BSA starts in the upper left corner with a yield of 100% and a purification factor of 1 because both proteins are fully contained in feed reservoir at the start of the process. The purification factor for BSA increases throughout the diafiltration due to the rapid removal of ovalbumin, with the BSA yield decreasing slightly due to the slow leakage of BSA into the filtrate. The final purification factor for BSA is nearly 15-fold, with the BSA yield remaining greater than 80% throughout the diafiltration due to the strong retention of the charged complex formed between BSA and Cibacron Blue. Note that a separate diafiltration experiment performed in the absence of Cibacron Blue gave a maximum purification factor for BSA of less than 1.7

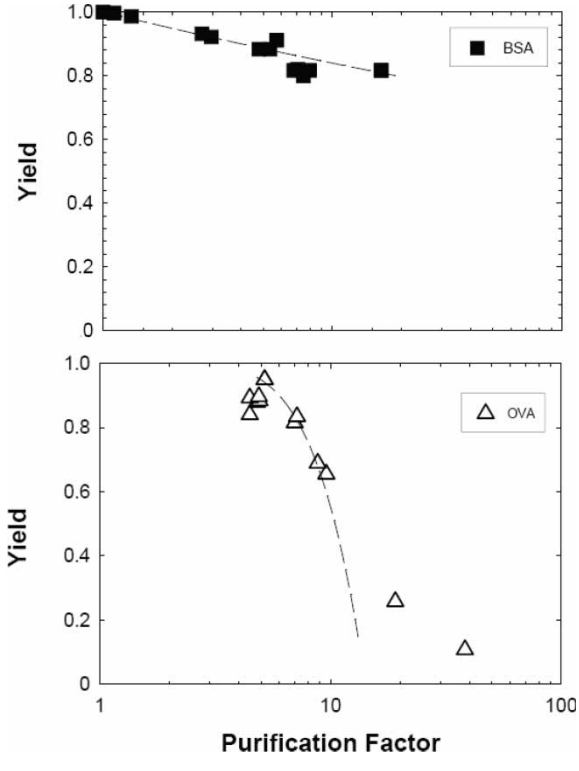

Figure 5. Yield as a function of purification factor for BSA collected in the retentate (top panel) and ovalbumin collected in the filtrate (bottom panel). Dashed curves are model calculations as described in the text.

with a final yield less than 30% due to the significant reduction in BSA retention in the absence of Cibacron Blue.

The diafiltration process for ovalbumin begins in the lower right corner with a yield of zero and a maximum purification factor of about 35 for the data and closer to 16 for the model. The origin of this discrepancy is unclear, although the data suggest that there may have been a rapid reduction in the selectivity during the initial phase of the diafiltration. The ovalbumin yield increases throughout the diafiltration process as the ovalbumin passes into the filtrate solution, but there is a corresponding reduction in the purification factor due to the continual leakage of BSA through the membrane. This effect is relatively small near the start of the diafiltration, but it becomes very pronounced as the ovalbumin yield approaches 100%. The final ovalbumin product had a yield of more than 95% although the purification factor for this process was less than 5.

Figure 6 shows size exclusion chromatograms for the feed (top panel) and final retentate (bottom panel) for the separation process presented in Fig. 5. The small peak seen in the feed solution at a retention time of 62.5 min is due to the presence of a small amount of BSA dimer in the feed, corresponding to approximately 13% of the main BSA peak (which appears at a retention time of 71 min). The final retentate product has a BSA purity of greater than 96%, with the ovalbumin peak at 77 min just barely visible in the chromatogram.

In order to develop an effective commercial process for protein purification using HPTFF employing an affinity ligand, it would be necessary to recover the ligand while at the same time generating a ligand-free protein product. Experiments were thus performed to examine the separation of BSA and Cibacron Blue using a second diafiltration employing a 10 kD unmodified (neutral) Ultracel membrane in a Pellicon XL module. It was impractical to perform this diafiltration at pH 5 because of the strong binding between BSA and Cibacron Blue; model calculations indicated that more than 1000 diavolumes would be needed to recover 99% of the Cibacron Blue under these conditions. Previous studies of Cibacron Blue binding have shown that the binding strength is significantly reduced at higher pH due to repulsive electrostatic interactions between the negatively-charged dye and the negatively-charged protein (9). In addition, NaSCN is known to displace Cibacron Blue from BSA (14). Diafiltration experiments were thus performed at pH 8.0 using a 10 mM Tris buffer with 1 M NaSCN. The feed solution was the final retentate obtained from the first stage protein separation without any pre-conditioning.

Figure 7 shows results for the normalized concentration of Cibacron Blue as a function of the number of diavolumes for this second diafiltration. The rate of Cibacron Blue removal increased during the initial stages of the diafiltration due to the increase in solution pH and NaSCN concentration associated with the displacement of the original acetate buffer from the feed reservoir. The Cibacron Blue concentration then decreases

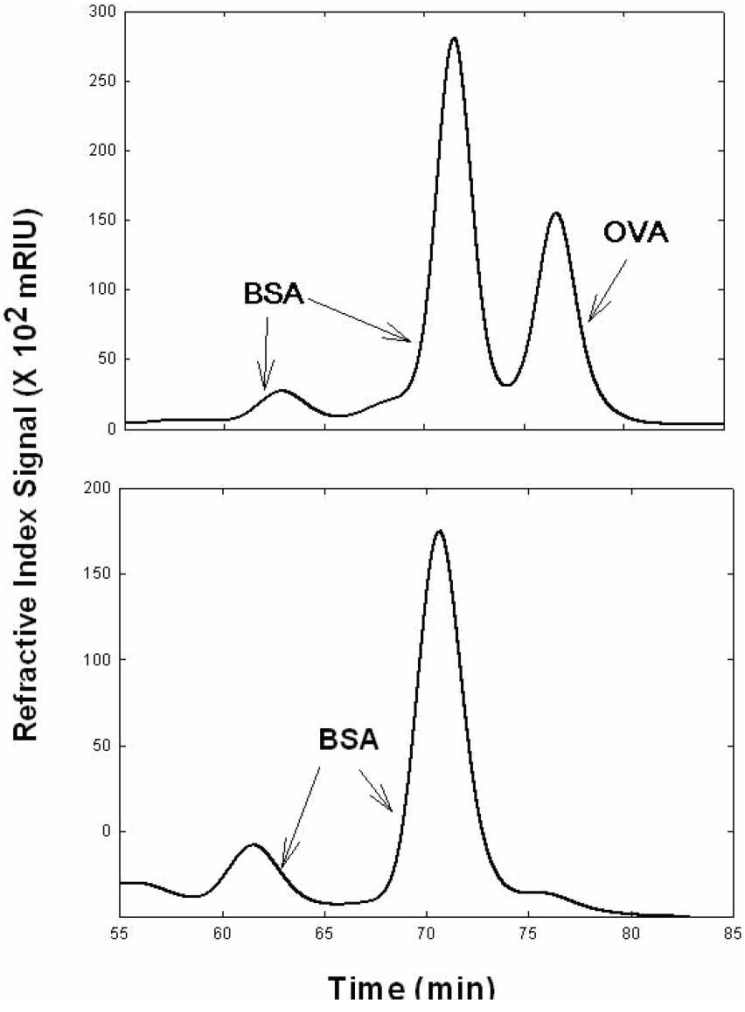

Figure 6. Size exclusion chromatograms for the initial feed (top panel) and final retentate (bottom panel) for the diafiltration process examined in Figure 5.

exponentially with increasing number of diavolumes, consistent with the expected washout of the unbound dye from the protein solution. The final concentration of Cibacron Blue after 10 diavolumes was only 0.23 mg/L, corresponding to 99.9% removal of the Cibacron Blue. The BSA yield for this process was greater than 98%; there was no detectable BSA (detection limit of approximately 0.05 g/L) in the filtrate solution collected through the 10 kD membrane.

The solid curve in Fig. 7 is the model calculation using a Cibacron Blue sieving coefficient through the 10 kD membrane of 0.59. The model is in good

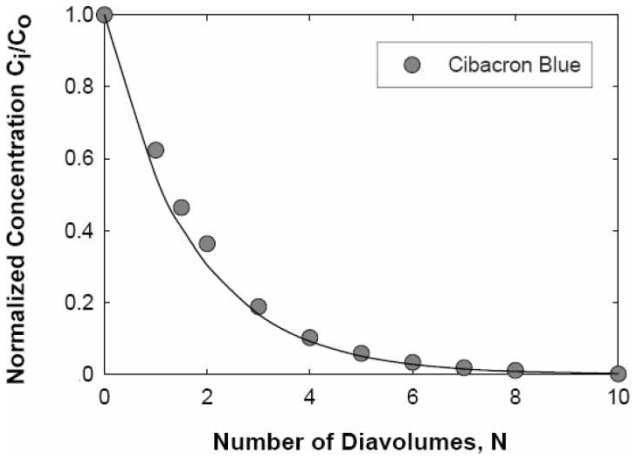

Figure 7. Normalized ligand concentration in the second stage diafiltration using a 10 kD unmodified Ultracel membrane with a pH 8.0, 10 mM tris, 1 M NaSCN diafiltration buffer. Solid curves are model calculations as described in the text.

agreement with the experimental data; there is a small discrepancy at large numbers of diavolumes which is likely due to the inherent difficulties in accurately measuring the very small concentrations of the dye under these conditions. Model calculations indicate that it should be possible to reduce the concentration of Cibacron Blue in the product solution to less than 1 ppm (based on BSA) using a 20-diavolume process.

CONCLUSIONS

Although a number of studies have demonstrated the potential of high performance tangential flow filtration for protein separations, most of the results have been obtained with proteins having significantly different electrical charge allowing high selectivities to be generated by exploiting differences in electrostatic interactions. The data presented in this manuscript clearly demonstrate that it is possible to use small charged affinity ligands to selectively shift the charge of the product (or impurity), greatly enhancing the performance of the HPTFF process. Experiments performed with a binary mixture of BSA and ovalbumin, using Cibacron Blue as the affinity ligand, gave a retentate product with greater than 80% yield and 15-fold purification factor of BSA. The ovalbumin was collected in the filtrate with 95% yield and several-fold purification. The performance characteristics could be easily adjusted by altering the number of diavolumes to achieve the desired yield and purification factor.

A second stage diafiltration process was used to produce a dye-free protein while at the same time recovering the affinity ligand, which could potentially be recycled for use in subsequent protein purification. The small affinity ligand was easily separated from the BSA using a conventional ultrafiltration process operated with a diafiltration buffer in which the dye-protein binding was minimal. In this case, we used a high pH to enhance the repulsive interactions between the negatively-charged protein and dye with NaSCN added to facilitate the displacement of the dye from the protein surface. 99.9% recovery of the Cibacron Blue was obtained after only 10 diavolumes, with even greater removal possible by simply increasing the number of diavolumes.

The affinity HPTFF process was performed using Pellicon XL tangential flow filtration modules that have been specifically designed to be linearly scalable to commercial processes with more than 80 m^2 of membrane area (10). The protein separation was accomplished at a flux of 50 L m^{-2} h^{-1}, which is similar to the flux currently used in large-scale ultrafiltration systems. These results indicate that there should be no significant technical barriers to the implementation of this type of affinity HPTFF system in commercial protein purification. However, future studies will be needed to demonstrate the actual practicality and overall economics of these systems.

APPENDIX

The effective charge on BSA and ovalbumin was evaluated using the charge regulation model originally developed by Tanford (16) and Scatchard (17) but modified to account for the binding of Cibacron Blue. The net charge was evaluated from the difference in the number of protonated amino acids, the number of bound acetate anions, and the number of bound molecules of Cibacron Blue:

$$Z = \upsilon_{H^+} - \upsilon_{acetate} - 3\upsilon_{CiB} \tag{A1}$$

where each bound Cibacron Blue (CiB) has a charge of −3 due to the three sulphonic acid groups.

The number of positively charged amino acid residues was calculated by summing over the different types of ionizable residues

$$\upsilon_{H^+} = \upsilon_{\max} - \sum_i \frac{n_i K_i^{\text{int}}}{K_i^{\text{int}} + [H_b^+]\exp(-e\psi_s^*/kT)} \tag{A2}$$

where $[H_b^+]$ is the bulk hydrogen ion concentration. The number (n_i) and intrinsic equilibrium constants (K_i^{int}) for each binding site are given in Table A1 for BSA and Table A2 for ovalbumin. The total number of positively charged amino acid residues at very low pH, i.e. where all the available sites are protonated, is $\upsilon_{\max} = 96$ for BSA and $\upsilon_{\max} = 42$ for ovalbumin. The exponential term in Eq. (A2) is the Boltzmann factor and

Table A1. Type and number of titratable groups on BSA (16)

Type (i)	Number (n_i)	pK^i_{int}
α-Carboxyl	1	3.75
β,γ-Carboxyl	99	4.02
Imidazole	16	6.9
α-Amino	1	7.75
ε-Amino	57	9.8
Phenolic	19	10.35
Guanidine	22	12

accounts for the partitioning of the hydrogen ions into the region immediately adjacent to the protein surface due to electrostatic interactions. Thus, the H^+ concentration near a negatively-charged protein will be greater than the bulk value, with the reverse being true for a positively-charged protein. $\psi_s{}^*$ is the electrostatic potential at the protein surface which can be related to the net protein charge as (18):

$$\sigma_s^* = \frac{eZ_{Protein}}{4\pi r_s^2} = \frac{\varepsilon_0 \varepsilon \psi_s^* (1 + \kappa r_s)}{r_s} \tag{A3}$$

where e is the electron charge, r_s is the radius of the (spherical) BSA or ovalbumin, ε_0 is the permittivity of free space, ε is the dielectric constant of the medium, and κ is the inverse of the Debye length:

$$\kappa^{-1} = \left(\frac{\varepsilon_0 \varepsilon RT}{F^2 \sum z_i^2 c_i} \right)^{1/2} \tag{A4}$$

The number of bound acetate ions (CH_3COO^-) was evaluated similarly as:

$$\upsilon_{actetate^-} = \sum_j \frac{m_j K_j \gamma [CH_3COO^-] \exp(e\psi_s^*/kT}{1 + K_j \gamma [CH_3COO^-] \exp(e\psi_s^*/kT} \tag{A5}$$

The parameters m_j and K_j for the three distinct acetate binding sites are given in Table A3 (19). These parameters account for the binding of acetate ions to

Table A2. Ionizable residues and pK_a values for ovalbumin (20)

Residue	Number (n_i)	pK^i_{int}
Aspartic acid	16	3.9
Glutamic acid	33	4.2
Histidine	7	6.0
Lysine	20	10.5
Arginine	15	12.5

Table A3. Values of parameters m_j and K_j in the acetate binding model (19)

Binding site j	M_j	K_j (L/mol)
1	1	530
2	6	50
3	14	2

BSA; the binding of other ions was neglected. Acetate binding to ovalbumin was also described using Eq. (A5) given the high degree of homology between the proteins and the lack of any available data. Differences in the extent of acetate binding would simply shift the net charge values but would not alter any conclusions about the affects of Cibacron Blue on the ovalbumin charge. γ is the activity coefficient of CH_3COO^- and was evaluated as (18):

$$-\log \gamma = \frac{0.5\sqrt{[CH_3COO^-]/2}}{(1+2\sqrt{[CH_3COO^-]/2})} \tag{A6}$$

The net protein charge in a given solution was evaluated by simultaneously solving Eqs. (A1) to (A6) with the number of bound molecules of Cibacron Blue given by Eq. (1) for the individual proteins. The protein radii were estimated as $r_s = 3.48$ nm for BSA and $r_s = 3.0$ nm for ovalbumin, both independent of the number of molecules of bound Cibacron Blue.

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