Protein Separations by Ultrafiltration: Exploiting Small Charged Ligands

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Introduction

Affinity ultrafiltration can provide high resolution separations by exploiting the selectivity of biomolecular binding interactions while maintaining the high throughput characteristic of conventional ultrafiltration processes. Mattiasson and Ramstrop (1984) demonstrated the feasibility of using affinity ultrafiltration for the purification of Concanavalin A using heat killed Saccharomyces cerevisae as the affinity ligand (Mattiasson and Ramstrop, 1984). The separation was performed using membranes with a molecular weight of 1,000 Kd which were fully retentive to the very large binding complex formed between Concanavalin A and the S. cerevisiae while allowing unbound species to pass relatively freely through the membrane. Subsequent studies have examined the isolation of urokinase using N-acryloyl-m-aminobenzamide copolymerized with acrylamide as an affinity macroligand (Male et al., 1990) and the purification of avidin using biotinylated liposomes (Powers et al., 1990) among others. Affinity ultrafiltration has also been used to separate chiral molecules using stereoselective macroligands. For example, BSA has been used for the optical resolution of racemic mixtures of tryptophan (Poncet al., 1997) and HSA for ibuprofen (Itoh et al., 1997).

These applications of affinity ultrafiltration all used very large macroligands, relative to the size of the desired product, to obtain the desired retention of the ligand-product complex using conventional ultrafiltration membranes. However, these macroligands tend to be very expensive, which has severely limited the viability of affinity ultrafiltration for commercially relevant separation processes. In addition, the use of small ligands bound to large carriers, e.g., Cibacron Blue bound to agarose (Herrak and Merrill, 1989), can result in a loss of ligand accessibility, mass transfer limitations, and/or problems with leaching of the ligand from the large carrier over time. The net result is that there are currently no large-scale commercial applications of affinity ultrafiltration for the purification of high value products.

Affinity ultrafiltration would be much more attractive commercially if one could employ a relatively small inexpensive affinity ligand to effect the separation. Recent work by Rao and Zydney (2005) has demonstrated that small charged dyes can dramatically alter the rate of protein transport through electrically charged ultrafiltration membranes due to the strong electrostatic interactions between the dye-protein complex and the charged membrane. For example, the addition of 1 g/L of Cibacron Blue to an 8 g/L solution of bovine serum albumin reduced the BSA sieving coefficient by more than two orders of magnitude. The objective of this study was to demonstrate the feasibility of using affinity ultrafiltration with the small charged ligand Cibacron Blue for separation of the model system of BSA and ovalbumin (OVA). Ovalbumin has a similar size and surface charge as BSA, but previous data suggest that it has limited binding interactions with Cibacron Blue (Lascu et. al., 1984). The results clearly demonstrate that high resolution protein separations can be achieved using a small charged affinity ligand with high binding specificity in combination with an electrically-charged ultrafiltration membrane to retain the like-charged affinity complex.

Material and Methods

Protein Solutions

Experiments were performed using bovine serum albumin (BSA) that is essentially fatty acid free (catalogue # A-6003 from Sigma Chemical, St. Louis, MO). BSA has an isoelectric point (pI) of approximately 4.9 and a molecular weight of 67 kD. Ovalbumin also known as hen egg albumin (catalogue # A-5503, Grade V, minimum 98%, agarose gel electrophoresis, from Sigma Chemical, St. Louis, MO) has an isoelectric point (pI) of approximately pH 4.5.

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 sulfonic acid groups attached to an aromatic ring structure. Previous work by Rao and Zydney (2005) indicates that as many as 10 molecules of Cibacron blue can bind to a single molecule of BSA, probably at both the fatty acid and anion binding sites

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 resistivety 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 solution pH was measured using a 420APlus pH meter (Thermo Orion, Beverly, MA). The ionic strength was adjusted by adding NaCl (Mallinckrodt Baker, Inc., Phillipsburg, NJ), with the solution conductivity measured using a 105A plus conductivity meter (Thermo Orion, Beverly, MA). All buffer solutions were prefiltered through 0.2 μm pore size Super-200 membranes (Pall Corp., Ann Arbor, MI) to remove particulates and undissolved salts.

Protein solutions were prepared by 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.

Assays

Concentrations of the individual proteins in the absence of the dye were determined spectrophotometrically with a UV-VIS spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan) using the natural absorbance at 280 nm.

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 (13 μm particle size, 10^5 MW exclusion limit, obtained from GE Healthcare, Piscataway, NJ). Column calibration was done using binary protein solutions of known concentration. The column was first equilibrated with fresh buffer at a flow rate of approximately 0.3 mL/min for 180 minutes. This also served to flush both the sample and the reference cells in the refractive index detector (Agilent 1100 series). Column equilibration was confirmed by tracking the base line 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 (100 μl) were

injected by an autosampler, with the data analyzed using Agilent ChemStation software on a Dell Celeron Computer.

Membrane Preparation

All filtration experiments were performed using Composite Regenerated Cellulose (CRC) membranes obtained from Millipore Corp. (Bedford, MA) with nominal molecular weight cut offs of 30 kD or 100 kD. These cellulosic membranes are nearly uncharged and have very low protein adsorption due to their high degree of hydrophilicity. Individual membrane discs (25 mm diameter) were cut from large flat sheets using a specially designed cutting device.

A negatively-charged version of the 100 kD membrane was made in our laboratory by the covalent attachment of negatively charged sulphonic acid groups to the surface of the membrane using the base-activated chemistry developed by van Reis (2001). Membranes were first flushed with deionized distilled water to remove any residual storage agents. The membranes were then equilibrated with 0.1 N NaOH and immersed in a 2 M solution of 3-Bromopropanesulfonic acid sodium salt (Catalogue #B2912, Sigma Chemical) in 0.1 N NaOH for fixed periods of time ranging from 2 to 24 hours to vary the extent of surface modification. The membranes were then flushed with approximately 100 L/m^2 of distilled water followed by storage in 0.1 N NaOH.

Equilibrium Binding Experiments

Binding interactions were evaluated using a stirred ultrafiltration cell which allowed protein-free samples to be collected through the UF membrane. A protein – dye mixture was added to a 25 mm diameter stirred UF cell (Model 8010, Amicon Corp., Beverly, MA) which contained either a 30 kD nominal molecular weight cut-off unmodified CRC membrane (for experiments with BSA) or a 10 kD membrane (for ovalbumin). The stirrer was set at 600 rpm using a Strobotac Type 1531-AB strobe light (General Radio Co., Concord, MA). The device was then air pressurized to ΔP = 21 kPa (3 psig), and small samples were withdrawn through the membrane for off-line analysis of the free Cibacron Blue concentration. Data were also obtained with pure Cibacron Blue (in the absence of protein) to evaluate the sieving coefficient of free Cibacron Blue through the 30 kD membrane.

Protein Filtration

Protein filtration experiments were also conducted in the 25 mm diameter stirred ultrafiltration cell. The device was air pressurized to the desired value and the membrane hydraulic permeability (L_p) was evaluated from the slope of data for the filtration velocity (J_v) as a function of the transmembrane pressure drop (ΔP):

$$L_p = \frac{J_v \mu}{\Delta P} \qquad (1)$$

where μ is the solution viscosity. The stirred cell was then emptied and refilled with a protein - Cibacron Blue solution at the desired ionic strength and pH. The cell was air-pressurized, and the filtrate flux was determined by timed collection using a digital balance (Model AG104, Mettler Toledo, Columbus, OH) with an accuracy of 0.1 mg. A 200 μL filtrate sample was then collected for subsequent analysis of the protein and Cibacron Blue concentrations. The filtrate

port was immediately clamped and a small (approximately 200 μL) sample of the bulk solution was obtained directly from the stirred cell. The stirred cell was then emptied, rinsed, and refilled with buffer, with the hydraulic permeability of the used membrane evaluated to provide a measure of the extent of fouling. All experiments were performed at room temperature (22 ± 3°C) using the same lot of proteins.

Diafiltration

The actual separation of ovalbumin and BSA was performed using the Amicon Stirred cell. The stirred cell was filled with 10 ml of the protein mixture. A separate reservoir was filled with a protein-free buffer solution (containing no dye) and attached to the stirred cell with silicone tubing. The filtrate velocity was maintained at nearly a constant value over the course of the diafiltration by a peristaltic pump connected to the filtrate line. The filtrate flux created a vacuum in the stirred cell causing fresh buffer from the reservoir to be drawn into the cell from the solution reservoir at a rate equal to the volumetric filtration rate. Filtrate solution was collected continuously with periodic samples taken from the stirred cell to evaluate the protein concentrations in the retentate.

Results

Binding Isotherms

Before examining the effect of Cibacron Blue on the separation of a mixture of proteins, we first evaluated the extent of Cibacron Blue binding to BSA and ovalbumin over a range of conditions using the approach described by Rao and Zydney (2005). The amount of bound Cibacron Blue was determined from a simple mass balance:

$$C_{bound} = C_{total} - C_{free} \qquad (2)$$

where the total concentration of Cibacron Blue in the solution was evaluated directly from the mass of dye added to a known volume of buffer. The concentration of free Cibacron Blue was evaluated directly from the measured filtrate concentrations as:

$$C_{free} = \frac{C_{filtrate}}{S} \qquad (3)$$

where the observed sieving coefficient (S), for free Cibacron Blue, defined as the ratio of concentration of the solute in the filtrate to the bulk solution, was evaluated immediately prior to performing the binding experiment using the same membrane and the same concentration of pure Cibacron Blue that was added to the protein solution.

Typical equilibrium binding parameters for BSA and ovalbumin at pH 5 and 10 mM ionic strength are shown in Table 1. These data were obtained from two separate sets of experiments, one performed by varying the Cibacron Blue concentration in a solution containing 5 g/L BSA and one performed with a solution containing 15 g/L ovalbumin. The BSA data are consistent with previous studies which have clearly demonstrated that Cibacron Blue

can bind to multiple fatty acid binding sites on the surface of BSA (Leatherbarrow and Dean, 1980; Antoni et al., 1980)

Table 1: Equilibrium binding parameters

Protein	Conditions	n	$K_{eq} \times 10^3$ (M^{-1})
BSA	pH 5.0, 10 mM	11	110
OVA	pH 5.0, 10 mM	3	1.7

Experiments performed over a range of pH showed a sharp reduction in the amount of Cibacron Blue binding per mole of BSA at higher pH, which is probably due to the increased electrostatic repulsion between the more negatively-charged protein and the negatively charged dye. The maximum degree of binding selectivity was thus obtained around pH 5 and at relatively low Cibacron Blue concentrations (with most of the binding sites on BSA being saturated when Cibacron Blue was added in a molar ratio of approximately 12 moles of Cibacron Blue or greater per mole of BSA), conditions which gave binding levels to BSA that were approximately 3.6 times those to ovalbumin.

Protein Sieving

Figure 1 shows experimental data for the sieving coefficients of BSA and ovalbumin as a function of the Cibacron Blue concentration using a negatively charged 100 kD CRC membrane. All experiments were performed with binary solutions of the two proteins with 10 g/L BSA and 6 g/L ovalbumin in a pH 5.0, 10 mM ionic strength buffer. The data show a clear decrease in BSA transmission upon the addition of Cibacron Blue, with a distinct minimum in protein transmission (S=0.0064) at a Cibacron Blue concentration of 1.4 g/L, which corresponds to approximately 12 moles of dye per mole of BSA. Further addition of the ligand causes an increase in BSA transmission due to the increase in the ionic strength of the solution, resulting in a shielding of the electrostatic interactions between the negatively-charged protein and the negatively-charged membrane. This effect has been discussed previously by Rao and Zydney (2005). The observed sieving coefficient of ovalbumin increases with the addition of Cibacron Blue, reflecting the absence of any significant change in the net electrical charge of ovalbumin due to the minimal binding of the dye in the presence of BSA.

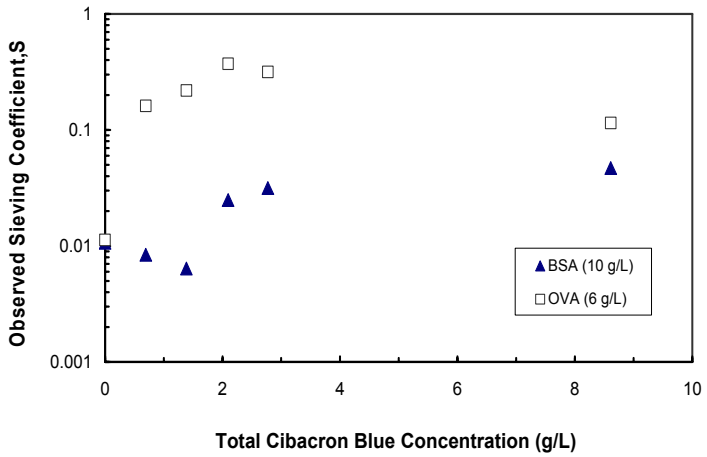

Figure 1: Observed sieving coefficients for BSA and ovalbumin in a binary mixture through the negatively charged 100 kD CRC membrane as a function of total Cibacron Blue concentration in 10 mM acetate buffer at pH 5.0.

The data in Figure 1 have been replotted in Figure 2 in terms of the selectivity, which is defined as the ratio of the transmission of ovalbumin to BSA (S_{OVA}/S_{BSA}). In the absence of Cibacron Blue there is essentially no selectivity for the protein separation. The selectivity increases dramatically upon addition of Cibacron Blue to a maximum value of 34 at a Cibacron Blue concentration of 1.4 g/L, corresponding to a molar concentration of 12 moles of Cibacron Blue per mole BSA. The selectivity decreases on further addition of ligand due to the decrease in the transmission of ovalbumin and the large increase in the transmission of BSA at high Cibacron Blue concentrations. This latter effect is primarily due to the increase in solution ionic strength, and the corresponding reduction in electrostatic repulsion, under conditions where there is a significant amount of free Cibacron Blue in the solution.

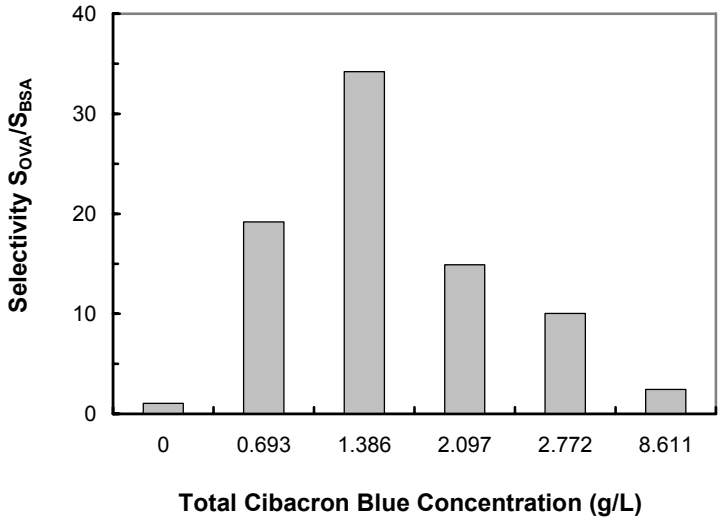

Figure 2: Selectivity between ovalbumin and BSA as a function of the Cibacron Blue concentration

Based on the results Figures 1 and 2, a diafiltration process was designed for the actual separation of BSA and ovalbumin. The diafiltration was performed at pH 5.0 and 10 mM ionic strength using a 100 kD CRC membrane, with 12 moles of Cibacron Blue added per mole of BSA, conditions which gave the maximum selectivity for this system. Parallel experiments were performed under the same conditions but in the absence of Cibacron Blue as a control. The feed solution contained 2 g/L BSA and 1g/L ovalbumin, with 0.28 g/L of Cibacron Blue added directly to the protein solution for one experiment; no Cibacron Blue was used in the diafiltration buffer in either case. A small amount of NaCl was added to the feed solution for the experiment performed in the absence of Cibacron Blue so that the solution ionic strength was approximately the same for both runs. Experimental data for the membrane hydraulic permeability evaluated after the diafiltrations were within 15% of the initial value, demonstrating fouling was minimal under the conditions of the experiment.

Figure 3 shows the normalized protein concentrations in the stirred cell as a function of the number of diavolumes (N), which is equal to the total collected filtrate volume divided by the constant volume in the stirred cell. The left panel shows results in the absence of Cibacron Blue while the right panel shows data in the presence of the dye. In both cases, the solid lines represent the calculated values of the protein concentration evaluated by integration of the differential mass balance assuming a constant observed sieving coefficient (van Reis and Saksena, 1997):

$$C/C_o = \exp(-NS_o) \qquad (4)$$

where the observed sieving coefficients were evaluated from data in Figure 1.

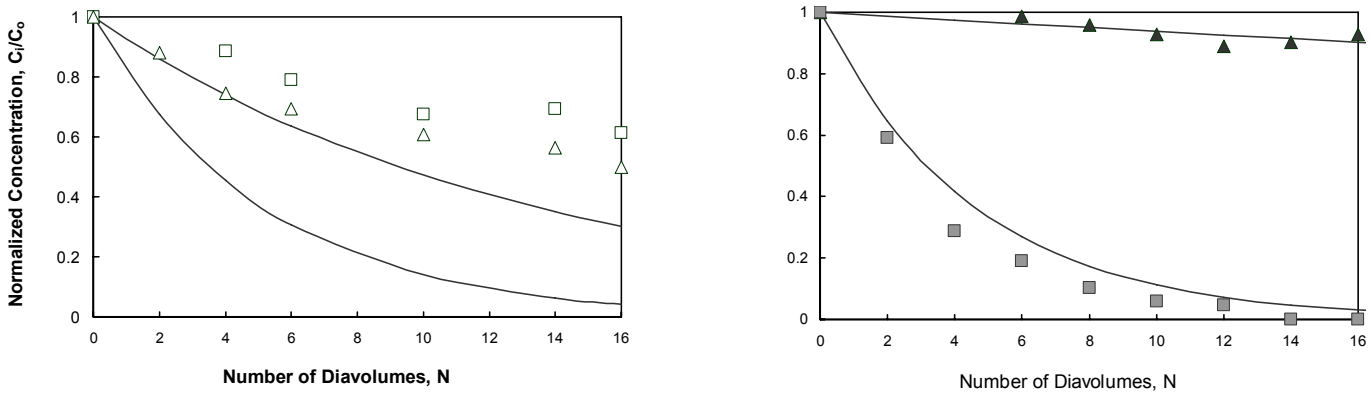

Figure 3: Normalized concentrations of ovalbumin (Δ) and BSA ( ) as a function of the number of diavolumes both in the absence, open symbols (left panel) and in the presence of Cibacron Blue, filled symbols (right panel).All experiments were performed using a negatively charged 100 kD membrane and a buffer solution of pH 5.0 and 10 mM. Solid curves are model calculations as described in the text.

There is minimal separation between BSA and ovalbumin in the absence of Cibacron Blue, with the concentration of BSA decreasing from 2 g/L to 1.26 g/L while that for ovalbumin decreases from 1g/L to 0.5 g/L. In contrast, the ovalbumin concentration for the experiment performed with 0.28 g/L Cibacron Blue decreased by over two orders of magnitude while the BSA was very highly retained, with the BSA concentration decreasing only to 1.75 g/L after14 diavolumes . The net result was a final BSA product having a purification factor of over 900 and a yield of over 90%. Ovalbumin was also produced in the purified filtrate stream, with a purification factor of over 10-fold and a yield of close to 100%.

Discussion

Although a number of previous studies have demonstrated the feasibility of affinity ultrafiltration for protein purification, all those studies have been performed using a large expensive macroligand to retain the protein of interest. The data obtained in this study provide the first demonstration that it is possible to use a small charged ligand such as the dye Cibacron Blue to enable protein separations, with the dye selectively binding to the protein product allowing the resulting complex to be retained by an electrically-charged ultrafiltration membrane.

In contrast to results obtained with macroligands, there exists an optimal concentration of the charged dye for maximizing the selectivity of the separation. In this case, there was essentially no separation between BSA and ovalbumin at low dye concentrations since the two proteins are of similar size and electrical charge. The separation was also lost at high concentrations of Cibacron Blue since the presence of a high concentration of free dye causes a significant increase in the solution ionic strength thereby shielding the electrostatic interactions between the membrane and the protein-dye complex. A 30-fold increase in selectivity was observed upon addition of approximately 12 moles of Cibacron Blue per mole of BSA due to the strong electrostatic repulsion of the highly-charged BSA-Cibacron Blue complex under these conditions.

A diafiltration process was used to obtain both BSA and ovalbumin as highly purified products in the retentate and the filtrate streams, respectively. The purification factor for BSA after a 14 diavolume process was greater than 900-fold with a recovery of more than 90 % of the protein. The Cibacron Blue could then be separated from the BSA by shifting the solution conditions to reverse the protein-dye binding; previous studies suggest the use of high pH and the addition of NaSCN leads to the nearly complete inhibition of Cibacron Blue binding to BSA. The small dye could easily be recovered by a classical diafiltration under these conditions, with the very large protein simply retained by steric interactions using a small molecular weight cut-off membrane. This could potentially provide a highly selective but low cost affinity ultrafiltration process exploiting the biospecific binding of a small charged affinity ligand to achieve high resolution separations.

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