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Separating Proteins with Activated Carbon

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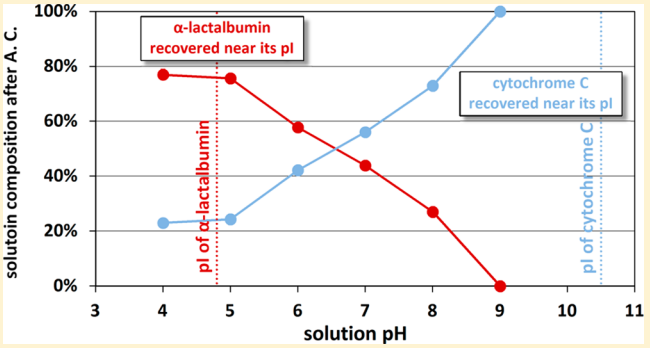
Supporting Information

ABSTRACT: Activated carbon is applied to separate proteins based on differences in their size and effective charge. Three guidelines are suggested for the efficient separation of proteins with activated carbon. (1) Activated carbon can be used to efficiently remove smaller proteinaceous impurities from larger proteins. (2) Smaller proteinaceous impurities are most efficiently removed at a solution pH close to the impurity's isoelectric point, where they have a minimal effective charge. (3) The most efficient recovery of a small protein from activated carbon occurs at a solution pH further away from the protein's isoelectric point, where it is strongly charged. Studies measuring the binding capacities of individual polymers and proteins were used to develop these three guidelines, and they were then applied to the separation of several different protein mixtures. The ability of activated carbon to separate proteins was demonstrated to be broadly applicable with three different types of activated carbon by both static treatment and by flowing through a packed column of activated carbon.



1. INTRODUCTION

Activated carbon is a porous material with a high surface area that physically adsorbs molecules through noncovalent interactions.[1] It is used in water purification and in the food and beverage industry for the nonselective removal of lower concentrations of proteins. It is also commonly used in applications such as oral poisoning treatment[2] and the process of hemoperfusion.[3] Additionally, activated carbon is used for the removal of small molecules from protein solutions,[4−6] and a dextran coating is often employed to prevent contact between the proteins and the activated carbon's surface.[7,8] However, despite the numerous applications of activated carbon that involve proteins, there is a limited understanding of the factors that influence these interactions.[9−14]

We recently found that certain grades of activated carbon could be used to selectively remove host cell protein (HCP) impurities from monoclonal antibody containing feed streams.[15] We were intrigued that this relatively inexpensive adsorbent material was useful for the separation of proteins and pursued an in-depth study to understand why activated carbon was selectively adsorbing the HCP instead of the monoclonal antibody. Thus, we examined the adsorption of several model polymers and proteins with activated carbon under different conditions to identify the key factors that govern their interactions. On the basis of our adsorption studies with activated carbon, we suggest three guidelines for the efficient separation of proteins and were able to demonstrate their utility in the separation of several different protein mixtures.

Protein size was the first factor identified as influencing the separation of proteins with activated carbon. Early research into activated carbon established that its binding strength for small molecules under aqueous conditions increases as the number of units in a homologous series is increased.[16] This relationship follows Traube's surface tension rule, and it works well to explain the increasing strength of adsorption for a series of small molecules, such as formic acid, acetic acid, propionic acid, butyric acid, etc.[17] However, despite the strong binding strength of large molecules, such as polymers and proteins, their capacity is limited by their inability to access the significant portions of the activated carbon's surface area that is contained within mesopores (2−50 nm) and micropores (< 2 nm).[18] The exclusion of large molecules from portions of the activated carbon's internal surfaces should allow it to selectively remove smaller proteins from larger proteins if the pores are of a sufficient size. This conclusion is consistent with an investigation by Kopper et al. into the purification of peanut proteins, which found that when activated carbon was saturated with a large protein, it still retained its binding capacity for a smaller protein.[12] However, it is difficult to make definitive conclusions on how a protein's size influences its adsorption on activated carbon by examining proteins of varying size, since they will also differ in their charge, shape, and hydrophobicity. Previous studies of activated carbon with polymers are instructive since polymers do not differ in their chemical characteristics as a function of size.[19,20] These studies established that activated carbon's capacity is significantly reduced as the molecular weight of the polymer is increased above a certain critical limit. To investigate if the size selectivity

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was important to the separation of proteins, we examined the binding of different lengths of polymers under aqueous conditions for a specific type of activated carbon that was found to be effective for the selective removal of HCP from monoclonal antibodies.[15]

The influence of a protein's effective charge on its interaction with activated carbon will also be influenced by the functional groups on its surface. The types of activated carbons investigated in this study were derived from wood that was chemically activated with phosphoric acid. It has been previously reported that these types of activated carbons have a surface primarily composed of aromatic groups and oxygen-containing functional groups that vary in concentration and type depending on the activation process.[21,22] Under aqueous conditions proteins should be able to bind to the aromatic structures on the activated carbon surface through hydrophobic interactions, but this interaction is also influenced by any charged groups on the surface. One method to examine the interaction between activated carbon and proteins is to measure capacity as a function of solution pH. For instance, if the solution pH is below the isoelectric point of the protein, then it will have an overall positive charge and should therefore be more readily adsorbed by negatively charged surfaces. There are reports in the literature that indicate that the charge on the protein does influence the activated carbon's protein binding capacity. Wu reported that activated carbon's binding capacity for bovine serum albumin (BSA) and lysozyme increased as the solution pH approached the isoelectric point of these proteins, although the study was conducted over a relatively limited pH range (pH 1.7−7.3).[9] In contrast, a study by Sekaran et al.[11] reported that a rice bran-based activated carbon had a maximum binding capacity for BSA at pH 7.0, which is much further away from BSA's reported isoelectric point of 4.9.[23] In that case, the ionic strength of the solution was 0.6 M, and it is unclear how the concentration of salt influenced the protein's interaction with activated carbon. Studies of molecular sieves found that they have a maximum adsorption for lysozyme[24,25] and cytochrome *c*[26] at a solution pH where the proteins have a minimum overall charge, suggesting that electrostatic repulsion strongly influences the binding of proteins with mesoporous materials. In the current investigation, we sought more conclusive evidence to establish the relationship between the protein's effective charge and activated carbon's protein binding capacity. Thus, activated carbon's protein binding capacity for five different proteins was measured over a range of different pHs. In addition, the influence of sodium chloride concentration was examined for both highly charged proteins and minimally charged proteins.

The separation of various proteins was performed to determine if an understanding of a protein's size and effective charge could be practically applied to the separation of two different proteins. First, the removal of a smaller protein from a larger monoclonal antibody with activated carbon was examined as a function of solution pH. Second, the separation of two smaller proteins with activated carbon was investigated as a function of solution pH to see if differences in the two proteins' effective charges would allow the selective removal of one of the proteins. Third, we examined three different types of activated carbon to establish that the separation of proteins with activated carbon was not limited to a single type. Finally, two proteins were separated by flowing a solution through a packed column of activated carbon to determine if the separation could also be efficiently accomplished under dynamic conditions.

2. EXPERIMENTAL SECTION

2.1. Materials. Dextran polymer standards were purchased from Pharmacosmos A/S (Roervangsvej 30, DK-4300, Holbaek, Denmark). The molecular weights of the dextrans used were 1000 Da (Dextran T1, 1 kg, 5510 0001 4000), 3500 Da (Dextran T3.5, 500 g, 5510 0003 4007), 10 000 Da (Dextran T10, 500 g, 5510 0010 4007), 40 000 Da (Dextran T40, 500 g, 5510 0040 4007), 70 000 Da (Dextran T70, 500 g, 5510 0070 4007), 500 000 Da (Dextran T10, 100 g, 5510 0500 4006), and 2 000 000 Da (Dextran T200, 500 g, 5510 2000 4007).

The sulfonated polystyrene standards (poly(styrenesulfonate) Na Salt Kit, Mp 891-1 020 000 (Da), 10 × 0.50 g, code: pss-pskit) were purchased from Polymer Standards Service-USA, Inc. (Warwick, RI). The standards used had weight-average molecular weights of 1100, 3610, 6520, 14 900, 32 900, 63 900, 148 000, 282 000, 666 000, and 976 000 Da.

Cytochrome *c* from equine heart (≥95% by SDS-PAGE, product number: C2506, lot number: 84H7135), α-lactalbumin from bovine milk (≥85% by PAGE, product number: L5385, lot number: 110M7003V), lysozyme from chicken egg white (≥98% SDS-PAGE, product number: L4919, lot number: 088 K13582), and albumin from bovine serum (≥98% by agarose gel electrophoresis, product number: A7906, batch number: 038 K0668) were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

MAb I was obtained as a solution from EMD Serono Biodevelopment. It was dialyzed into water to remove buffer salts with dialysis tubing (Standard RC Dialysis Trial Kits, Spectra/Por 1-3, 3.5K MWCO, 54 mm flat width, serial number: 132725, Spectrum Laboratories, Inc., Rancho Dominguez, CA). The dialysis tubing containing 0.5 L of the MAb I solution was submerged in 40 L of water for 24 h. The dialysis tubing was then moved into a new container containing 40 L of fresh water where it remained submerged for an additional 24 h. A portion of the dialyzed MAb I solution was then concentrated using Amicon Ultra-15 centrifugal filter units (3 kDa, catalogue number: UFC900324, EMD Millipore Corporation, Billerica, MA). The concentrated portion of the solution was recombined with the rest of the dialyzed MAb I solution. The concentration of monoclonal antibody was determined by measuring its absorbance at 280 nm. The combined solution was then diluted with water to give a stock solution with a concentration of 10.0 mg/mL. The MAb I stock solution was then filtered through a 0.22 μm membrane (0.22 μm Millipore Express PLUS membrane, 1.0 L, catalogue number: SCGPU11RE, EMD Millipore Corporation, Billerica, MA).

Nuchar HD activated carbon (lot number: 1339-R-09) was obtained from MeadWestVaco Corporation (Richmond, VA). Darco KB-G activated carbon (lot number: 0545.0) and Norit CGP Super activated carbon (lot number: 0592.0) were obtained from Norit Americas Inc. (Marshall, TX).

2.2. General Static Binding Procedure. A stock solution of polymer(s) or protein(s) at twice the desired concentration in water was prepared and then filtered through a 0.22 μm membrane (Stericup-GP filter with 0.22 μm Millipore Express PLUS membrane, 250 mL, catalogue number: SCGPU02RE, EMD Millipore Corporation, Billerica, MA). Centrifuge tubes (15 mL) were loaded with dry activated carbon or left empty to act as a control. Then 2.5 mL of buffer at the appropriate pH (50 mM acetate for pH 4.0, 5.0, 6.0, or 50 mM Tris for pH 7.0, 8.0, and 9.0) was added to each tube, and the tubes were subjected to vortexing. Next 2.5 mL of the polymer or protein stock solution was added to each tube. The tubes were allowed to rotate for 20 h at room temperature. The tubes were subsequently subjected to centrifugation, and the supernatant solutions were filtered through a 0.22 μm membrane (Millex syringe filter units, Millex-GV, 0.22 μm, PVDF, 33 mm, gamma sterilized, catalogue number: SLGV033RB, EMD Millipore Corporation, Billerica, MA) in order to remove any activated carbon particles that might remain suspended in solution. The amount of polymer(s) or protein(s) in the resulting

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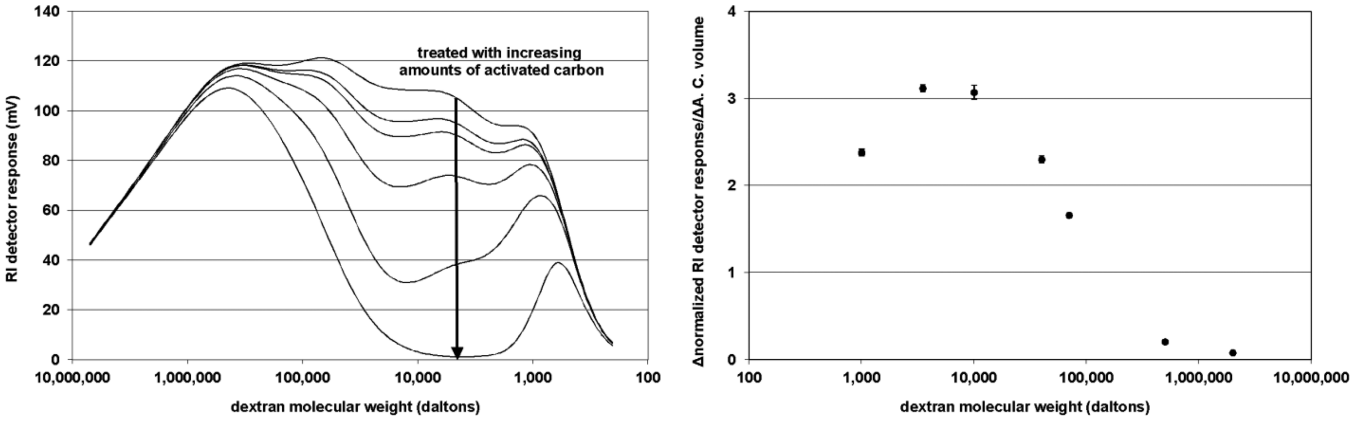

Figure 1. SEC chromatograms of aqueous solutions containing varying molecular weights of dextrans that were treated with increasing amounts of Nuchar HD activated carbon (left). The Δnormalized RI detector response/Δactivated carbon volume represents the ability of Nuchar HD activated carbon to adsorb a specific molecular weight of dextran and was plotted as a function of molecular weight (right).

solution was then determined by measuring the UV absorbance or by subjecting the solution to analytical chromatography.

2.3. General Flow-Through Binding Procedure. A stock solution of two proteins at twice the desired concentration in water was prepared and then filtered through a 0.22 μm membrane (Stericup-GP filter with 0.22 μm Millipore Express PLUS membrane, 250 mL, catalogue number: SCGPU02RE, EMD Millipore Corporation, Billerica, MA). The solution was then diluted in half with a buffer of the desired pH (50 mM acetate for pH 4.0 or 50 mM Tris for pH 9.0) and then filtered through a 0.22 μm membrane (Stericup-GP filter with 0.22 μm Millipore Express PLUS membrane, 250 mL, catalogue number: SCGPU02RE, EMD Millipore Corporation, Billerica, MA). A glass chromatography column (Omnifit benchmark column 10 mm/100 mm, 10 mm diameter, 100 mm length, SKU: 006BCC-10-10-AF, Diba Industries, Danbury, CT) was loaded with 200 mg of Nuchar HD activated carbon (lot number: 1339-R-09, MeadWestVaco Corporation, Richmond, VA) slurried in water. The column was packed by flowing water through it, which resulted in packed column volume of 0.8 mL. The column was equilibrated with a buffer corresponding to the desired pH (25 mM acetate for pH 4.0 or 25 mM Tris for pH 9.0). Then 100 mL of the buffered protein solution was passed through the activated carbon column at a flow rate of 0.4 mL/min, resulting in a residence time of 2.0 min. Eight 12.5 mL fractions were collected. Then an additional 12.5 mL of the equilibration buffer was flowed through the column while a ninth 12.5 mL fraction was collected. The concentrations of the two proteins in the individual fractions and a pooled sample of all nine fractions were subjected to analytical reverse-phase HPLC.

2.4. Analytical Size-Exclusion Chromatography. Analytical size-exclusion chromatography of dextrans was performed with a Waters 2695 separation module and a Waters 2414 refractive index detector using a Phenomenex Shodex OH pak 13 μm SB-806 M HQ gel filtration column (part number: SB-806MHQ, column size: 300 × 8 mm, Phenomenex Inc., Torrance, CA). The isocratic mobile phase was composed of 50 mM potassium phosphate at pH 7.0 with 10 mg/L of sodium azide. The column was run at a flow rate of 1.0 mL/min for 20 min at a temperature of 35 °C.

Analytical size-exclusion chromatography of proteins was performed with an Agilent 1260 HPLC using a Tosoh Biosciences TSK-Gel Super SW3000 column (part number: 18675, column size: 300 × 4.6 mm, Tosoh Bioscience LLC, King of Prussia, PA). The isocratic mobile phase was composed of 0.2 M sodium phosphate at pH 7.0. The column was run at a flow rate of 0.35 mL/min for 15 min at a temperature of 25 °C. The UV detector was set to a wavelength of 230 nm with 550 nm used as a reference. The recovery of the proteins was calculated based on the areas measured in the HPLC peaks.

2.5. Analytical Reverse-Phase HPLC. Reverse-phase HPLC analysis was performed with an Agilent 1290 UPLC using a Higgins Analytical Targa C18 5 μm column (part number: TS-2546-C185, column size: 250 × 4.6 mm, Higgins Analytical, Inc., Mountain View, CA). The gradient mobile phase was a combination of solution A which was composed of 0.1% trifluoroacetic acid in water and solution B which was composed of 0.1% trifluoroacetic acid in acetonitrile. The composition of the mobile phase was increased from 5% to 95% of solution B in solution A over a period of 15 min at a flow rate of 1.0 mL/min and a temperature of 25 °C. The UV detector was set to a wavelength of 230 nm with 550 nm used as a reference. The recovery of the proteins was calculated based on the areas measured in the HPLC peaks.

3. RESULTS

3.1. Probing the Size-Selective Range of Activated Carbon. Our investigation of the size-selective range of activated carbon employed Nuchar HD activated carbon, which was previously observed to efficiently remove HCP from monoclonal antibodies.[15] Prior studies with neutral polymers, such as polyethylene glycol and dextrans, reported that certain activated carbons had their highest binding capacity at intermediate molecular weights.[19,20] Below this molecular weight range, the activated carbon's binding strength for the polymers decreases as the molecular weight is decreased in agreement with Traube's rule. However, above a certain molecular weight the polymer is too large to access surface areas contained within smaller pores. In this study, the ability of Nuchar HD activated carbon to adsorb different molecular weights of both neutral dextrans as well as hydrophobic and negatively charged sulfonated polystyrenes was measured under aqueous conditions.

An aqueous solution composed of several different dextrans ranging in molecular weight from 1000 to 2 000 000 Da was treated with varying amounts of Nuchar HD activated carbon under static binding conditions. The composition of the dextran solution after treatment with activated carbon was then analyzed with size exclusion chromatography (SEC) using a refractive index detector (Figure 1). The ability of activated carbon to adsorb a particular molecular weight of dextran from the mixture was represented by the change in the normalized refractive index response divided by the change in the activated carbon volume.

The ability of Nuchar HD activated carbon to adsorb different molecular weights of dextran was consistent with previously reported studies that investigated other types of activated carbon.[19,20] The ability of Nuchar HD activated carbon to adsorb dextran increased from 1000 to 3500 Da,

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which is predicted by Traube's rule that indicates the binding strength should increase with the molecular weight. However, a slight decrease was observed at 10 000 Da, and then a steep decrease occurred as the molecular weight was increased further. The decrease is consistent with the exclusion of dextrans from the surface area contained within activated carbon's smaller pores.

The size range of molecules excluded by Nuchar HD activated carbon was also examined by measuring the activated carbon's static binding capacity for sulfonated poly(styrene) standards under aqueous conditions as a function of their weight-average molecular weight. The aromatic rings on the sulfonated poly(styrene)s are nonpolar and are expected to strongly bind with the activated carbon's surface through hydrophobic interactions. Nuchar HD activated carbon's static binding capacity for a specific molecular weight of sulfonated polystyrene was measured under static binding conditions and plotted as a function of molecular weight (Figure 2).

A clear trend was observed that as the molecular weight of the sulfonated polystyrene was increased the activated carbon's binding capacity was reduced. This trend is in contrast to the polar dextrans that followed Traube's rule at lower molecular weights. The sulfonated polystyrene's nonpolar aromatic rings appear to strongly bind with the activated carbon's surface through hydrophobic interactions even at 1100 Da or approximately five repeat units. The size-based exclusion of sulfonated polystyrene occurred at lower molecular weights compared to the dextrans, which might be attributed to differences in the shape of the two polymers. However, it might also indicate that electrostatic repulsion between the negatively charged sulfonate groups is an important influence.

3.2. Influence of Solution pH on Activated Carbon's Binding Capacity for Proteins. Nuchar HD activated carbon's binding capacity for several different proteins was measured as the solution pH was varied to determine the influence of the protein's effective charge. The net effective charge on a protein is reduced as the solution pH is adjusted closer to the protein's isoelectric point. Cytochrome *c*, *α*-lactalbumin, lysozyme, BSA, and a monoclonal antibody were chosen as model proteins after they were confirmed to be soluble in an aqueous buffer at 1.0 mg/mL from pH 4.0 to pH 9.0. Nuchar HD activated carbon's static binding capacities for these proteins was determined and plotted as a function of solution pH (Figure 3).

The activated carbon was found to have its highest binding capacity for the lower molecular weight proteins including cytochrome *c* (13 400 Da),[31] *α*-lactalbumin (14 175 Da),[32] lysozyme (14 307 Da),[33] and BSA (66 430 Da)[34] when the solution pH was closest to their isoelectric point. At this pH the proteins have a weak overall charge, which suggests that activated carbon's binding capacity for proteins is reduced by electrostatic repulsion. The activated carbon's binding capacity for the lower molecular weight proteins was significantly reduced as the solution pH was adjusted further away from the protein's isoelectric point. This was observed both when the proteins were positively charged, as was the case for cytochrome *c* and lysozyme, or when they were negatively charged as was the case for *α*-lactalbumin and BSA.

The influence of the solution pH on the activated carbon's binding capacity for BSA was particularly strong. Its binding capacity for BSA ranged from 77.8 mg/mL at pH 5.0 down to only 1.5 mg/mL at pH 9.0. The activated carbon's binding capacity for BSA at higher pHs might also be influenced by the formation of more well-ordered aggregates that would greatly increase its effective hydrodynamic volume in solution.[35]

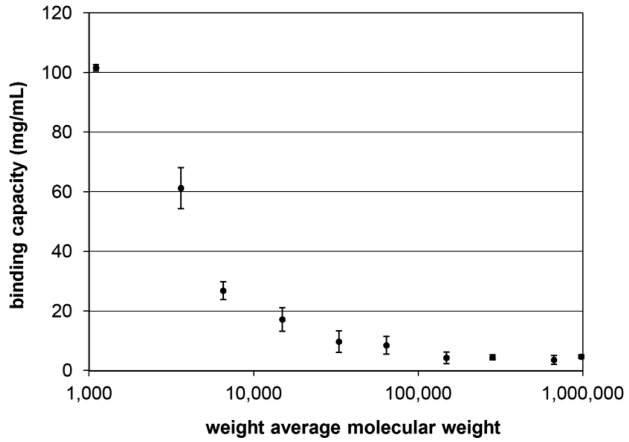

Figure 2. Nuchar HD activated carbon's static binding capacity (mg/mL) for an individual sulfonated polystyrene standard under aqueous solution conditions plotted as a function of weight-average molecular weight.

The activated carbon's maximum binding capacity for the higher molecular weight MAb I (145 000 Da) was significantly lower than the lower molecular weight proteins. It also differed from the lower molecular weight proteins since activated carbon's binding capacity for MAb I did not vary greatly with solution pH. The results are consistent with previous polymer experiments described above that indicated higher molecular weight compounds are too large to access a significant percentage of the activated carbon's internal surface area.

3.3. Influence of Intermolecular Electrostatic Repulsion on Activated Carbon's Capacity for Proteins. If electrostatic repulsions reduce activated carbon's capacity at a solution pH where the proteins are highly charged, then it would be predicted that increasing the concentration of sodium chloride should increase its binding capacity by weakening these interactions. In contrast, increasing the salt concentration should not greatly influence the activated carbon's binding capacity at a solution pH where the protein is weakly charged. In this experiment the influence of salt concentration on Nuchar HD activated carbon's static binding capacity was examined for a strongly negatively charged *α*-lactalbumin at pH 9.0 and a strongly positively charged lysozyme at pH 4.0. These results were contrasted with *α*-lactalbumin at pH 5.0 and lysozyme at pH 9.0 where the proteins are weakly charged. Nuchar HD activated carbon's static binding capacities for the strongly and weakly charged *α*-lactalbumin or lysozyme were plotted as a function of sodium chloride concentration (Figure 4).

It was found that increasing the sodium chloride concentration at a solution pH where the proteins were strongly charged increased the activated carbon's binding capacity for both the negatively charged *α*-lactalbumin at pH 9.0 and the positively charged lysozyme at pH 4.0. In contrast, increasing the sodium chloride concentration at a solution pH where the proteins were weakly charged had little influence on the activated carbon's binding capacity for that protein. It is unlikely that the increase in binding capacity resulted from an increase in the hydrophobic driving force since no increase was observed for the proteins at a solution pH where they were more weakly charged. The results suggest that activated

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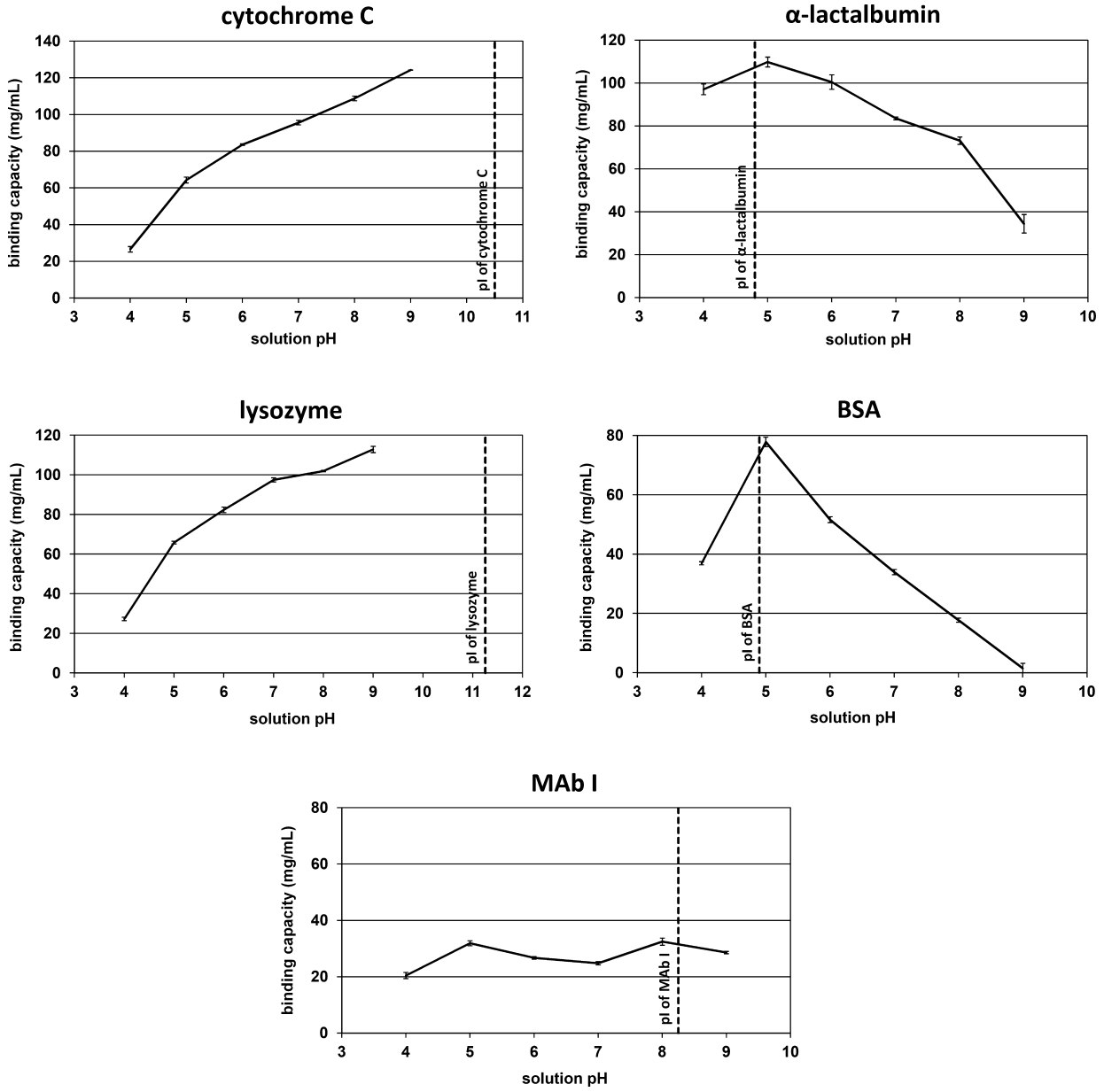

Figure 3. Nuchar HD activated carbon's static binding capacity for cytochrome *c*, *α*-lactalbumin, lysozyme, BSA, and a monoclonal antibody plotted as a function of solution pH. The reported isoelectric point is 10.5 for cytochrome *c*,[27] 4.8 for *α*-lactalbumin,[28] 11.2−11.3 for lysozyme,[29,30] and 4.9 for BSA.[23] An isoelectric point of 8.3 was calculated for MAb I based on its amino acid composition.

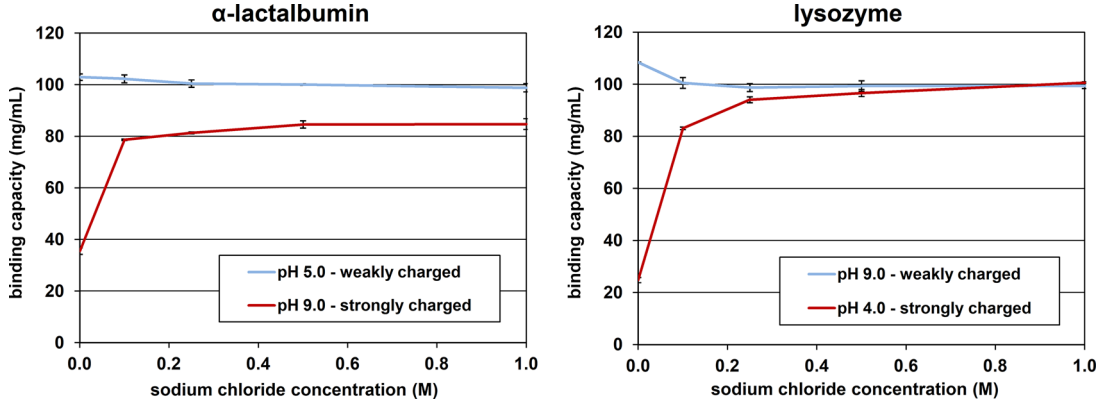

Figure 4. Nuchar HD activated carbon's static binding capacity for *α*-lactalbumin (left) and lysozyme (right) at solution pHs where the proteins are weakly or strongly charged plotted as a function of sodium chloride concentration.

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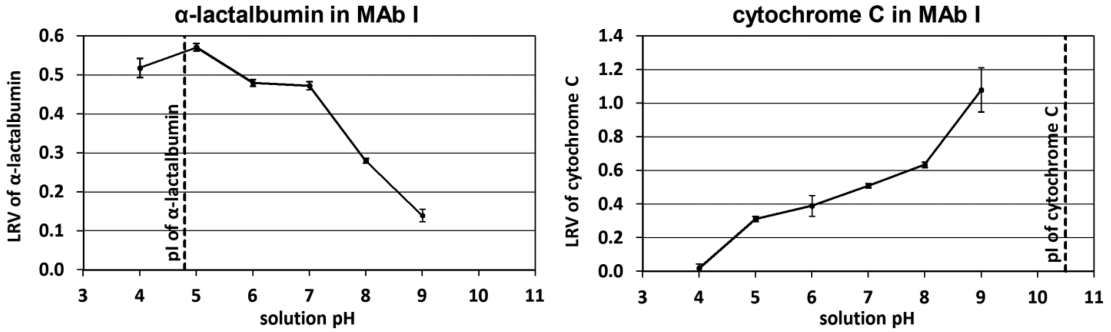

Figure 5. LRV of α-lactalbumin (left) or cytochrome c (right) removed from solutions of MAb I with Nuchar HD activated carbon plotted as a function of solution pH.

carbon's binding capacity for a highly charged protein is significantly reduced by electrostatic repulsion.

3.4. Separation of a Higher Molecular Weight Monoclonal Antibody from a Lower Molecular Weight Proteinaceous Impurity with Activated Carbon. Previous experiments demonstrated that activated carbon has a low binding capacity for higher molecular weight polymers, suggesting that it should provide excellent recoveries when employed for the purification of higher molecular weight proteins. Nuchar HD activated carbon was also shown to have a maximum binding capacity for lower molecular weight proteins when the solution pH is closest to that protein's isoelectric point. These two findings indicate that a lower molecular weight proteinaceous impurity should be most efficiently removed from a higher molecular protein at a solution pH close to the isoelectric point of the impurity. To test this hypothesis, we examined the removal of cytochrome c (13 400 Da)[31] or α-lactalbumin (14 175 Da)[32] from a higher molecular weight monoclonal antibody (145 000 Da) as the solution pH was varied. Solutions containing 5.0 mg/mL of MAb I and either 1.0 mg/mL of cytochrome c or α-lactalbumin were treated with Nuchar HD activated carbon under static binding conditions. The compositions of the activated carbon treated solutions were then determined by analytical size-exclusion chromatography. The log reduction values (LRVs) of cytochrome c or α-lactalbumin from MAb I are plotted as a function of solution pH (Figure 5).

The results of this experiment demonstrate that activated carbon most efficiently purifies a higher molecular weight protein when the solution pH is closest to the isoelectric point of the lower molecular weight proteinaceous impurity. Activated carbon removed significantly greater amounts of α-lactalbumin at pH 5.0 (0.52 LRV) and cytochrome c at pH 9.0 (1.08 LRV) where these two proteins are weakly charged. In contrast, very little of the proteinaceous impurity was removed where α-lactalbumin had a strong negative charge at pH 9.0 (0.12 LRV) or where cytochrome c had a strong positive charge at pH 4.0 (0.02 LRV). The results are instructional for the application of activated carbon to the downstream purification of monoclonal antibodies, where it could be employed to selectively remove lower molecular weight HCP.[36]

3.5. Separation of Lower Molecular Weight Proteins with Activated Carbon. While the size-selective nature of activated carbon makes it ideal for the purification of higher molecular weight proteins, we were curious if it could also be employed to purify lower molecular weight proteins. This might be possible if the lower molecular weight protein product and the proteinaceous impurity have significantly different isoelectric points. A selective purification might then be accomplished at a solution pH that is near the isoelectric point of the proteinaceous impurity and further away from the isoelectric point of the protein product. At this pH, electrostatic repulsion will lower the activated carbon's binding capacity for the more strongly charged protein product and also maximize its binding capacity for the weakly charged proteinaceous impurity. The charge-selective nature of activated carbon was investigated by treating solutions composed of 1.0 mg/mL of cytochrome c and 1.0 mg/mL of α-lactalbumin at pHs ranging from 4.0 to 9.0 with Nuchar HD activated carbon under static binding conditions. The percentages of cytochrome c and α-lactalbumin remaining in solution after treatment with activated carbon were determined by analytical reverse-phase HPLC and plotted as a function of solution pH (Figure 6).

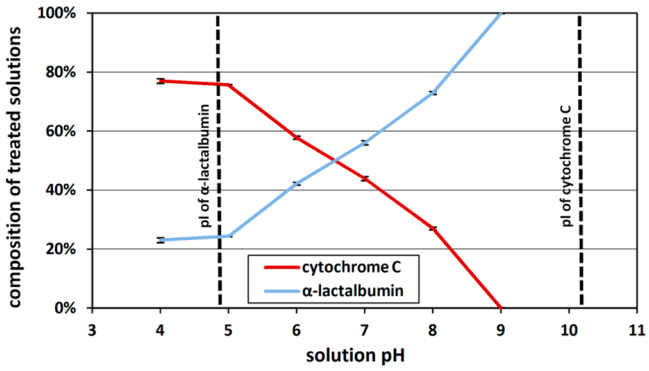

Figure 6. Percentage of cytochrome c and α-lactalbumin remaining after treatment of a 1:1 solution with Nuchar HD activated carbon under static binding conditions plotted as a function of solution pH.

The percentages of cytochrome c and α-lactalbumin remaining after treatment of the 1-to-1 solution with Nuchar HD activated carbon were found to vary greatly with solution pH. The maximum amount of α-lactalbumin was removed at pH 4.0 and 5.0, near its isoelectric point of 4.8.[28] In contrast, the maximum amount of cytochrome c was removed at pH 9.0, nearest that protein's isoelectric point of 10.5.[27] At an intermediate pH of 6.0 or 7.0 the activated carbon removed similar amounts of both proteins.

Next, the ratios of the two proteins were altered to resemble a typical purification application where the proteinaceous impurity is a small percentage of the mixture to evaluate if activated carbon would provide reasonable recoveries of a lower molecular weight protein product. Two solutions were prepared with a 10-to-1 ratio of cytochrome c (5.0 mg/mL)

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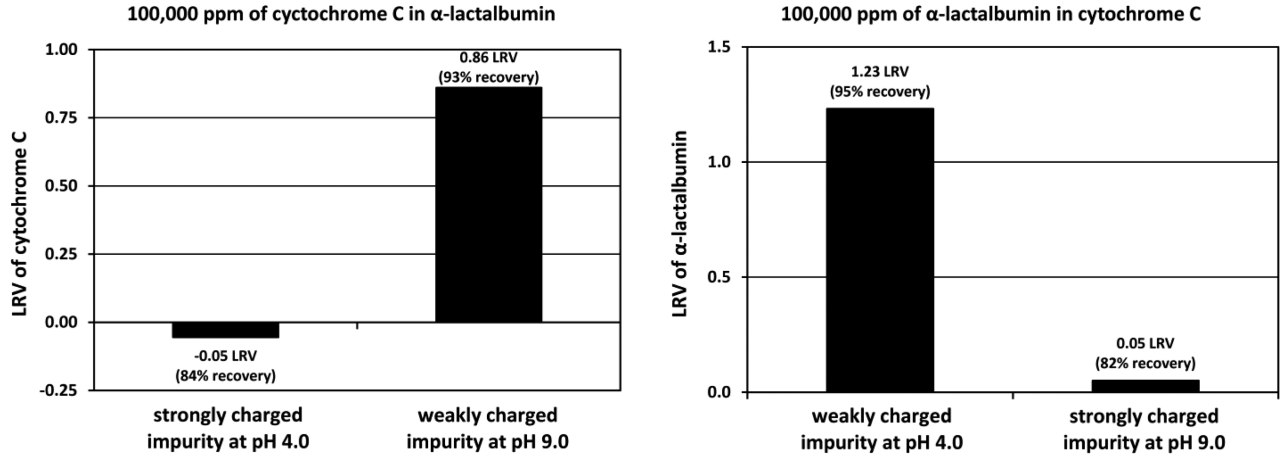

Figure 7. LRV of the proteinaceous impurity removed by the static treatment of a solution composed of 100 000 ppm of cytochrome *c* in *α*-lactalbumin (left) or 100 000 ppm *α*-lactalbumin in cytochrome *c* with Nuchar HD activated carbon.

to *α*-lactalbumin (0.5 mg/mL) or *α*-lactalbumin (5.0 mg/mL) to cytochrome *c* (0.5 mg/mL). The protein at the higher concentration represented the product and the protein at the lower concentration represented the proteinaceous impurity. These were treated with Nuchar HD activated carbon under static binding conditions. The compositions of the activated carbon-treated solutions were determined by analytical reverse-phase HPLC, and the LRV of cytochrome *c* and *α*-lactalbumin are shown in Figure 7.

The proteinaceous impurity was efficiently removed, and good product recoveries were obtained at a solution pH where the product was strongly charged and the proteinaceous impurity was weakly charged. In contrast, at a solution pH where the product was weakly charged and the impurity was strongly charged, the proteinaceous impurity was not efficiently removed and the recovery of the product was reduced. The results demonstrate that activated carbon can be employed to selectively remove a lower molecular weight proteinaceous impurity with a sufficiently different isoelectric point from a lower molecular weight product by selecting the appropriate solution pH.

3.6. Separation of Proteins with Different Types of Activated Carbon. Since the investigations of activated carbon described above were limited to Nuchar HD activated carbon from MeadWestvaco, we wanted to confirm that the separation of proteins with activated carbon was not limited to this particular type. Therefore, the ability of activated carbon to remove cytochrome *c* from *α*-lactalbumin was also evaluated using CGP Super and Darco KB-G activated carbons (both from Norit), which were confirmed to have reasonable capacities for proteins. Solutions having 1.0 mg/mL of cytochrome *c* representing the proteinaceous impurity and 5.0 mg/mL of *α*-lactalbumin representing the product were treated with equal masses of either Nuchar HD, CGP Super, or Darco KBG activated carbon under static binding conditions at pH 4.0 or pH 9.0. The concentrations of cytochrome *c* and *α*-lactalbumin remaining in solution after treatment with activated carbon were determined by analytical reverse-phase HPLC, and the LRV of cytochrome *c* was calculated (Figure 8).

The results of the experiment demonstrate that the cytochrome *c* impurity is efficiently removed from the *α*-lactalbumin at pH 9.0 closest to cytochrome *c*'s isoelectric point (10.5)[27] for all three types of activated carbon examined. In contrast, the cytochrome *c* impurity is not efficiently removed

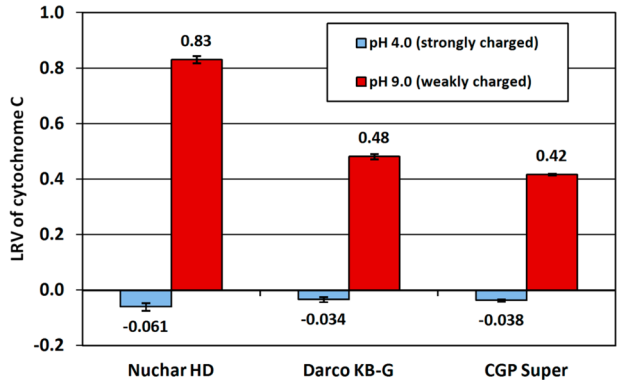

Figure 8. LRV of cytochrome *c* removed by Nuchar HD, Darco KB-G, or CGP Super activated carbon from a solution of 1.0 mg/mL of cytochrome *c* and 5.0 mg/mL of *α*-lactalbumin at pH 4.0 or pH 9.0.

with all three types of activated carbon further away from cytochrome *c*'s isoelectric point at pH 4.0. Differences between the LRVs of the three types of the activated carbon at a particular pH might be attributed to differences in their pore size or surface area. However, this example does demonstrate that the ability of activated carbon to separate proteins is not limited to a single type, but appears to be a property of several different commercially available activated carbons.

3.7. Flow-Through Separation of Proteins with Activated Carbon. In previous experiments it was demonstrated that activated carbon could be used to separate proteins under static binding conditions; however, it would be more convenient to treat a protein solution by flowing through a packed column of activated carbon. Flowing through an adsorbent media is advantageous since it reduces processing times and eliminates the solid/liquid separation step needed to remove the media after static treatment. In this experiment, a solution composed of 0.5 mg/mL of cytochrome *c* representing the proteinaceous impurity and 5.0 mg/mL of *α*-lactalbumin representing the product at pH 4.0 or 9.0 was flowed through a chromatography column packed with Nuchar HD activated carbon. The concentrations of cytochrome *c* and *α*-lactalbumin in the column fractions were determined by analytical reverse-phase HPLC and plotted as a function of the mass loading of *α*-lactalbumin divided by the volume of activated carbon (Figure 9).

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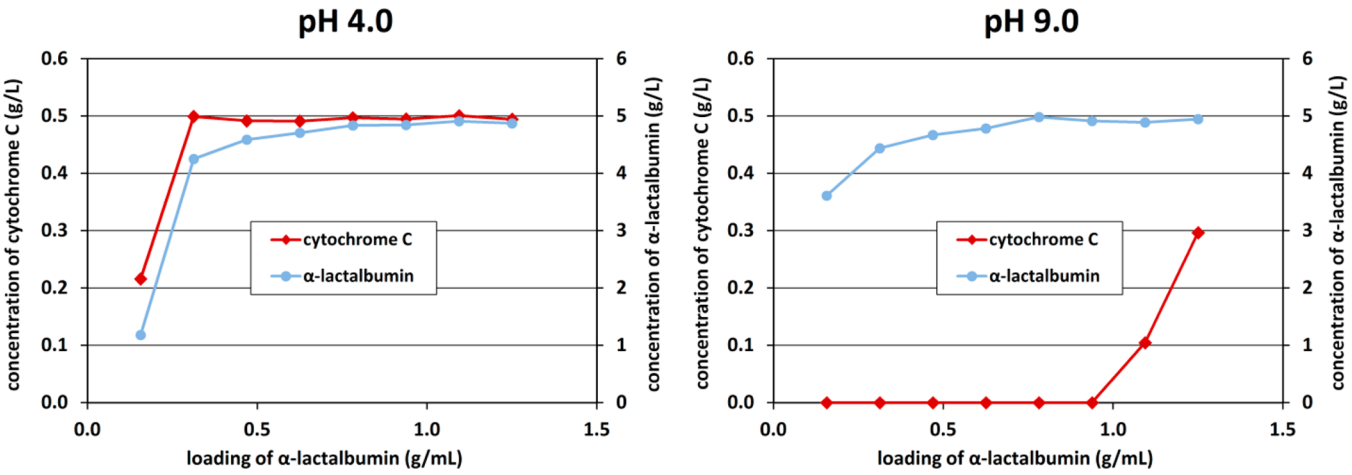

Figure 9. Concentration of cytochrome *c* and *α*-lactalbumin in 12.5 mL fractions that were collected after a solution of 0.5 mg/mL of cytochrome *c* and 5.0 mg/mL of *α*-lactalbumin at pH 4.0 or pH 9.0 was passed through a column of Nuchar HD activated carbon.

The results of the flow-through experiment with a packed column of activated carbon are in good agreement with the static binding studies that showed a strong dependence on solution pH. At pH 4.0 the cytochrome *c* impurity broke through in the first fraction. Conversely, at pH 9.0, the breakthrough of the cytochrome *c* impurity was not observed until the seventh fraction, where the column had been loaded with 1.09 g of *α*-lactalbumin per mL of activated carbon. The overall recovery of *α*-lactalbumin was measured to be only 88% at pH 4.0 while the recovery was 94% at pH 9.0. After the solution was passed through the activated carbon column at pH 4.0, the concentration of the cytochrome *c* impurity was increased from 100 000 to 106 125 ppm, resulting in only a −0.03 LRV. In contrast, at pH 9.0 the concentration of the cytochrome *c* impurity was significantly decreased from 100 000 to 10 584 ppm, resulting in a 0.98 LRV.

4. DISCUSSION

Our investigation has shown that activated carbon's protein binding capacity is significantly influenced by both the protein's size and effective charge. The first experiments with dextrans and sulfonated polystyrenes demonstrated that Nuchar HD activated carbon had a greatly reduced ability to bind polymers above a critical molecular weight (section 3.1). The size preference was also observed in the relative binding capacities of five different proteins. The activated carbon had the highest capacities for the lowest molecular weight proteins (cytochrome *c*, *α*-lactalbumin, lysozyme) and much lower capacities for the higher molecular weight proteins, including BSA and a monoclonal antibody (section 3.2). These results can be intuitively understood since larger molecules will be unable to access a significant portion of an activated carbon's total surface area that is contained within the smaller mesopores and micropores.

Activated carbon was found to have its highest binding capacity for a protein when the solution pH was closest to its isoelectric point, where the protein has a minimum overall charge (section 3.2). The results indicate that electrostatic repulsion significantly reduces activated carbon's binding capacity for proteins. Further evidence to support this conclusion was obtained by examining the activated carbon's protein binding capacity as a function of salt concentration (section 3.3). Increasing the salt concentration was found to significantly increase the activated carbon's protein binding capacity at a solution pH where the protein was highly charged. In contrast, increasing the salt concentration at a solution pH where the proteins were weakly charged had little influence on activated carbon's protein binding capacity. The significant difference in activated carbon's protein binding capacity as a function of protein charge is likely due to some combination of intramolecular repulsion that will increase the diameter and/or change the shape of the protein and intermolecular repulsion between proteins that will reduce their packing density on the surface of the activated carbon. The effect of intermolecular electrostatic repulsion might be amplified by restricting passage of the proteins through the pores as they become covered by charged proteins. Similarly charged proteins would be electrostatically repelled from accessing surface areas deeper within the pores. This electrostatic gating effect has been well documented for charged ultrafiltration membranes that selectively exclude the passage of similarly charged proteins.[37−41]

The influence of a protein's size and effective charge on activated carbon's protein binding capacity led us to suggest three guidelines for the efficient separation of proteins using activated carbon. (1) Activated carbon can be used to efficiently remove smaller proteinaceous impurities from larger proteins. (2) Smaller proteinaceous impurities are most efficiently removed at a solution pH close to the impurity's isoelectric point, where they have a minimal effective charge. (3) The most efficient recovery of a small protein from activated carbon occurs at a solution pH further away from the protein's isoelectric point, where it has a strong effective charge.

The first and second guidelines were demonstrated to be useful when applied in combination for the removal of two different lower molecular weight proteinaceous impurities from a higher molecular weight monoclonal antibody (section 3.4). This separation takes advantage of the size differences between the two proteins according to the first guideline. It was found that the most efficient removal of the impurity was observed at the solution pH closest to the proteinaceous impurity's isoelectric point. In fact, very little separation was observed at a solution pH further away from the impurity's isoelectric point, highlighting the importance of the second guideline.

The third guideline allows activated carbon to be employed for the purification of lower molecular weight proteins, if the

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proteinaceous impurity has a sufficiently different isoelectric point. In this case, the proteins are separated not by a difference in size, but by a difference in the amount of charge. This separation can be accomplished by selecting a pH close to the isoelectric point of the proteinaceous impurity in order to maximize activated carbon's binding capacity by minimizing the protein's charge. The selected pH will then also be far away from the product's isoelectric point, thus maximizing the recovery of the highly charged protein to be purified. This concept was applied to the removal of cytochrome *c* from α-lactalbumin by selecting pH 9.0 and the removal of α-lactalbumin from cytochrome *c* by selecting pH 4.0 (section 3.5). The purification was not dependent on the polarity of the charge, but instead the relative amount of the charge on the two proteins.

The application of activated carbon to the separation of proteins using these three guidelines appears to be rather general. Although not every grade of activated carbon has a pore size distribution that is sufficiently large to bind proteins, three types of commercially available activated carbon from two different manufacturers have been shown to be effective for the separation of two proteins (section 3.6). The separation of proteins was also demonstrated by flowing a protein mixture through a packed column of activated carbon (section 3.7). Both of these results suggest that activated carbon has the potential to be used as a single-use media for the separation of proteins at a manufacturing scale. Further investigation of activated carbon for the separation of greater than two proteins would be of great value for optimizing the purification of more complex protein mixtures. It is our hope that the results of our investigation will stimulate an interest in the application of this relatively inexpensive media for the chromatographic purification of proteins.

5. CONCLUSIONS

In summary, we have demonstrated that both the size and the effective charge of a protein strongly influence its adsorption on activated carbon. Binding studies with various molecular weights of dextrans and sulfonated polystyrenes confirmed that activated carbon has a lower binding capacity for larger molecules. This suggests that larger proteins are not able to access a significant percentage of the activated carbon's internal surface area that is contained within smaller pores. Activated carbon was found to have a maximum binding capacity when the solution pH was near the protein's isoelectric point, where there is a minimum effective charge on the protein. This understanding was applied to the purification of a higher molecular weight monoclonal antibody where it was found that lower molecular weight proteinaceous impurities are most efficiently removed at a solution pH closest to the impurity's isoelectric point. It was also possible to purify lower molecular weight proteins with activated carbon at a solution pH where the product is strongly charged and the proteinaceous impurity is weakly charged. The separation of proteins was demonstrated with three different types of activated carbon. Protein separation was also accomplished by flowing a protein mixture through a packed column of activated carbon.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and data for all binding capacities measurement and protein separations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

HCP, host cell protein; MAb, monoclonal antibody; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LRV, log reduction value.

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