Dye-Ligand Column Chromatography: Albumin Adsorption from Aqueous Media and Human Plasma with Dye-Affinity Microbeads

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ABSTRACT: Cibacron Blue F3GA was covalently coupled with poly(ethylene glycol-dimethacrylate-2-hydroxyethylmethacrylate) [poly(EGDMA-HEMA)] microbeads via the nucleophilic substitution reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA molecules under alkaline conditions. The affinity sorbent carrying 16.5 μmol Cibacron Blue F3GA/g polymer was then used for bovine serum albumin (BSA) adsorption from aqueous protein solutions and from human plasma in a packed-bed column. The BSA adsorption capacity of the microbeads decreased with an increase in the recirculation rate. High adsorption rates were observed at the beginning, then equilibrium was gradually achieved in about 60 min. The BSA concentration in the mobile phase was also effective on adsorption. BSA adsorption was first increased with BSA concentration, then reached a plateau that was about 57.3 mg BSA/g. Higher BSA adsorption was observed at lower ionic strength. The maximum adsorption was observed at pH 5.0, which is the isoelectric pH of BSA. Higher human serum albumin adsorption was achieved from human plasma (109.6 mg HSA/g). High desorption ratios (over 94% of the adsorbed albumin) were achieved by using 1.0*M* NaSCN (pH 8.0) in 30 min. It was observed that albumin could be repeatedly adsorbed and desorbed without a significant loss in adsorption capacity. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 74: 2803–2810, 1999

Key words: albumin separation; packed-bed column; dye-affinity sorbent; Cibacron Blue F3GA; poly(ethylene glycol-dimethacrylate-2-hydroxyethylmethacrylate) microbeads

INTRODUCTION

Bioaffinity chromatography is a highly specific separation technique used for the isolation and purification of biomolecules.[1] However, some limitations such as bioligand instability and leakage, desorption conditions, and cost confine its application. As an alternative, dye-ligand chromatography has been utilized as a powerful technique for the separation of a wide range of proteins, which can overcome these limitations and also presents versatile and important advantages over bioaffinity.[2–4] Dyes have been shown to interact with a large number of proteins, in some cases with remarkable degrees of specificities.[5–7] They are easily immobilized on the carrier matrices and are much less expensive and more stable than those bioligands.[8–10]

Recently, we prepared bioaffinity sorbents in which polyhydroxyethylmethacrylate or poly(ethyl-

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ene glycol-dimethacrylate-2-hydroxyethylmethacrylate) [poly(EGDMA-HEMA)] microbeads were used as the support, and several bioligands (e.g., protein A, DNA, heparin, collagen) and dye ligands (e.g., Cibacron Blue F3GA, Congo Red, Alkali Blue 6B) were incorporated into these microbeads for removal and separation of several substances (e.g., proteins, pathogenic antibodies, cholesterol, bilirubin) from aqueous media including plasma in a batch experimental setup.[11–16] In this study we attemped to prepare Cibacron Blue F3GA attached poly(EGDMA-HEMA) microbeads as a specific sorbent for albumin separation in a packed-bed column. Adsorption of albumin from both aqueous media and human plasma are presented in this article.

EXPERIMENTAL

Cibacron Blue F3GA Coupled Polymeric Microbeads

Poly(EGDMA-HEMA) microbeads were used as the affinity carrier for bovine serum albumin (BSA) adsorption. The microbeads were produced by a modified suspension polymerization of the respective comonomers (EGDMA, Rohm, Germany; HEMA, Sigma, St. Louis, MO) in an aqueous media as described in our previous article.[17] Benzoyl peroxide (BPO) and polyvinyl alcohol (PVA) (M_n: 100,000, 98% hydrolyzed, Aldrich, Rockford, IL) were used as the initiator and the stabilizer, respectively. Toluene (Merck AG, Darmstadt, Germany) was utilized as the pore former and was used as received. The dispersion medium was distilled water. To produce polymeric microbeads of about 150–200 μm in diameter and with a narrow size distribution, the amounts of EGDMA, HEMA, toluene, water, BPO, and PVA were 8 mL, 4 mL, 12 mL, 50 mL, 0.06 g, and 0.2 g, respectively. Polymerizations were carried out at 600 rpm at 65°C for 4 h and then at 90°C for 2 h. After cooling, the polymeric microbeads were separated from the polymerization medium by filtration, and the residuals (e.g., unconverted monomer, toluene) were removed by washing.

Cibacron Blue F3GA was supplied by Sigma and was used as received. It was covalently coupled to the poly(EGDMA-HEMA) microbeads via a nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA molecules under alkaline conditions.[15–17] Briefly, 3 g of poly(EGDMA-HEMA) microbeads was magnetically stirred (at 400 rpm) in a sealed reactor with 100 mL of the Cibacron Blue F3GA aqueous solution containing 4.0 g NaOH. The dye-coupling reaction was carried out at a constant temperature of 80°C for 4 h. Under these conditions, a chemical reaction takes place between the group of the Cibacron Blue F3GA having chloride and the hydroxyl group of the HEMA (with the elimination of NaCl), resulting in the coupling of Cibacron Blue F3GA to the poly(EGDMA-HEMA) microbeads. The initial concentration of the Cibacron Blue F3GA in the medium was 3.0 mg/mL. After incubation the solution cooled to room temperature and then the Cibacron Blue F3GA coupled microbeads were filtered and washed with distilled water and methanol several times until all the physically attached Cibacron Blue F3GA molecules were removed.

The amount of Cibacron Blue F3GA attached on the microbeads was evaluated by using an elemental analysis instrument (Leco, CHNS-932) by considering the nitrogen and sulfur stoichiometry. Note that the Cibacron Blue F3GA attached poly(EGDMA-HEMA) microbeads containing 16.5 μmol Cibacron Blue F3GA/g, which was the maximum value that we reached, was used in this study.[15–17] The leakage of the Cibacron Blue F3GA from the microbeads was checked spectrophotometrically at 630 nm by following the Cibacron Blue F3GA release in the adsorption and/or desorption media used. The Cibacron Blue F3GA attached microbeads were stored at 4°C with 0.02% sodium azide to prevent microbial contamination.

Albumin Adsorption–Desorption Studies

BSA (lyophilized, fraction V, Sigma) was selected as a model protein. Adsorption studies were performed in a packed-bed column system in which an adsorption column (internal diameter, 0.9 cm; height, 10 cm) equipped with a water jacket for temperature control was used. The column was filled with the unmodified microbeads or Cibacron Blue F3GA attached poly(EGDMA-HEMA) microbeads containing 16.5 μmol Cibacron Blue F3GA/g. In a typical continuous column system, 50 mL of the BSA solution was recirculated for 2 h through the column containing microbeads. The reservoir containing the BSA solution was stirred continuously at 100 rpm. The pH of the adsorption medium was changed between 4.0 and 7.5 by using different buffer systems (0.1M CH_3COONa—CH_3COOH for pH 4.0–6.0, 0.1M K_2HPO_4—KH_2PO_4 for pH 7.0–7.5). BSA adsorp-

tion was studied at two different ionic strength, 0.01 and 0.1 (adjusted by using NaCl). The initial concentration of BSA in the aqueous phase was varied between 0.5 and 4.0 mg/mL. In order to observe the effect of flow rate on adsorption, the recirculation rate of the aqueous phase was changed between 0.5 and 2.5 mL/min. Adsorption experiments were carried out at a constant temperature of 25°C. The BSA adsorption capacity was determined spectrophotometrically at 280 nm by measuring the initial and final concentrations of BSA within the reservoir.

In the desorption experiments, 50 mL of the NaSCN solution (1.0*M,* pH 8.0) was recirculated for 30 min through the column containing BSA adsorbed microbeads. The flow rate of the desorption agent (i.e., NaSCN) was 0.5 mg/mL. The final BSA concentration within the reservoir was determined by spectrophotometry. The desorption ratio was calculated from the amount of BSA adsorbed on the microbeads and the amount of BSA desorbed.

In order to obtain the reusability of the Cibacron Blue F3GA attached poly(EGDMA-HEMA) microbeads, the BSA adsorption–desorption cycle was repeated 6 times using the same affinity column.

Human Serum Albumin (HSA) Adsorption from Human Plasma

HSA adsorption studies were carried out in the same packed-bed column system. The column was filled with the Cibacron Blue F3GA attached poly-(EGDMA-HEMA) microbeads containing 16.5 μmol dye/g. The blood samples were obtained from a healthy human. Blood samples were centrifuged at 500 × *g* for 30 min at room temperature to separate the plasma. The original plasma of the healthy donor contained 39.7 mg HSA/mL as determined by the brom cresol green dye method.[18] In a typical packed-bed continuous column system, 50 mL of the plasma freshly separated from the human blood was recirculated for 2 h through the column containing dye-attached polymeric microbeads. The amount of HSA adsorbed was obtained by measuring the decrease in the HSA concentration in the plasma by the same assay. In this group of experiments, the volumetric flow rate of the human plasma was changed between 0.5 and 2.5 mL/min. The temperature was kept constant at 25°C. The same desorption procedure was applied for desorption of adsorbed HSA (see previous section).

RESULTS AND DISCUSSION

In this study, we attempted to prepare a specific dye-affinity sorbent for albumin separation from aqueous protein solution and human plasma in a packed-bed column system. Poly(EGDMA-HEMA) microbeads were selected as the carrier matrix. The poly(EGDMA-HEMA) microbeads used in this study were 150–200 μm in size. Cibacron Blue F3GA was used as the affinity dye ligand for specific attachment of albumin molecules. Weber et al. reported that Cibacron Blue F3GA has no adverse effect on biochemical systems.[19] However, all commercial reactive dyes (including Cibacron Blue F3GA) contain various impurities that may affect their biochemical and related use.[20–22] Reactive dyes are purified by a number of chromatographic procedures such as thin layer chromatography, high performance liquid chromatography, and column chromatography on silica gel or Sephadex.[22] However, Weber suggested that purification of reactive dyes is necessary only in certain free dyes.[20] In cases where immobilized dyes are used, purification of the dye before immobilization is not likely to be necessary. Because, few of the contaminants will be immobilized on the support matrix, proper washing of the matrix should remove adsorbed impurities.[22] Cibacron Blue F3GA was covalently coupled with the dyed microbeads. Ether linkages were formed between the reactive triazine ring of the dye and the hydroxyl groups of the microbead. Elemental analysis of the plain and Cibacron Blue F3GA attached microbeads was performed, and the dye loading was found as 16.5 μmol/g from the nitrogen and sulfur stoichiometry. Cibacron Blue F3GA leakage from the dyed microspheres was also investigated. Dye leakage was not observed from any of the dye-attached microbeads, even in a long period of time (more than 6 months). The details of preparation and characterization of the plain and Cibacron Blue F3GA attached poly-(EGDMA-HEMA) microbeads are given elsewhere.[15–17]

Column Performance

Effects of Flow Rate on BSA Adsorption

In this group of experiments the volumetric recirculation rate of the protein solution was changed between 0.5 and 2.5 mL/min; other parameters were kept constant. The BSA adsorption capacity of the Cibacron Blue F3GA attached microbeads

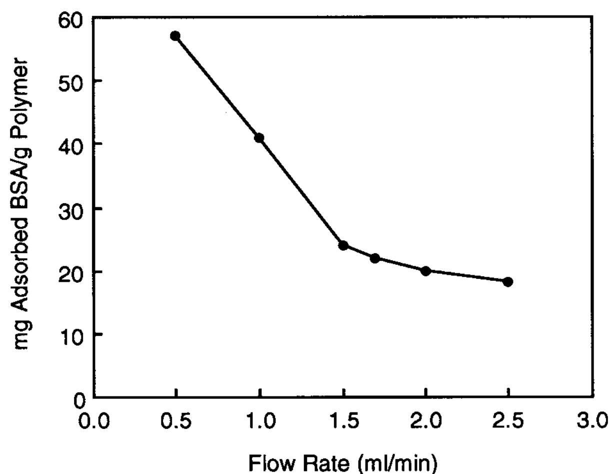

Figure 1 BSA adsorption at different flow rates: ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g polymer; BSA initial concentration, 4.0 mg/mL; pH 5.0; ionic strength, 0.01 (adjusted with NaCl); temperature, 25°C; and total volume of protein solution, 50 mL.

at different flow rates are given in Figure 1. As seen here, the adsorption capacity decreased significantly from 57.3 to 18.1 mg BSA/g polymer with the increase of the flow rate from 0.5 to 2.5 mL/min. This behavior may be explained as follows: the residence time in the column decreases with increasing flow rate, which does not give enough time for the BSA molecules to interact with the Cibacron Blue F3GA molecules on the microbeads. In addition, the increase in the flow rate may cause channeling in the sorbent bed, which results in a decrease in the effective use of the sorbent microbeads in the column. Therefore, low adsorption capacities are observed at high flow rates. Note that at the flow rates lower than 0.5 mL/min, we faced some technical problems in our chromatographic column system, such as fluctuation of the flow rate, difficulties in sampling, and so forth; therefore, we carried out all other adsorption tests at a flow rate of 0.5 mL/min.

Adsorption Rate

Figure 2 shows the adsorption rate curves obtained at two different ionic strengths (0.01 and 0.1). High adsorption rates were observed at the beginning of the adsorption, and then saturation values were then gradually achieved in about 120 min. This may be because of the decrease in the BSA concentration in the reservoir with time due to adsorption. As expected, when the BSA concentration in the mobile phase (i.e., the aqueous phase) decreases (which also corresponds to an increase in the stationary phase, i.e., the sorbent microbeads) the driving force (i.e., the concentration difference between the two respective phases) decreases, which in turn results in a drop in the adsorption rate.

The adsorption rate and capacity of BSA were increased by decreasing the ionic strength. This behavior may be explained by the formation of more compact structures of the BSA molecules at high ionic strengths because of conformational changes.[23] In this compact form, the binding sites (the proper sites for interaction with the dye-ligand molecules) on the BSA molecules may also turn toward the inside of the molecules, which may further reduce adsorption. In addition, it is also worth noting that more ions (such as Na^+ and Ca^{++}) may be attached to BSA molecules at high ionic strength.[24] This causes further stabilization of the BSA molecules (means higher solubility), which may lead to lower BSA adsorption on the Cibacron Blue F3GA attached microbeads.

BSA Adsorption Capacity

Effect of BSA Initial Concentration

Figure 3 shows the nonspecific and specific adsorption of BSA onto the plain and Cibacron Blue F3GA attached poly(EGDMA-HEMA) microbeads, respectively. Note that one of the main requirements in bioaffinity chromatography is

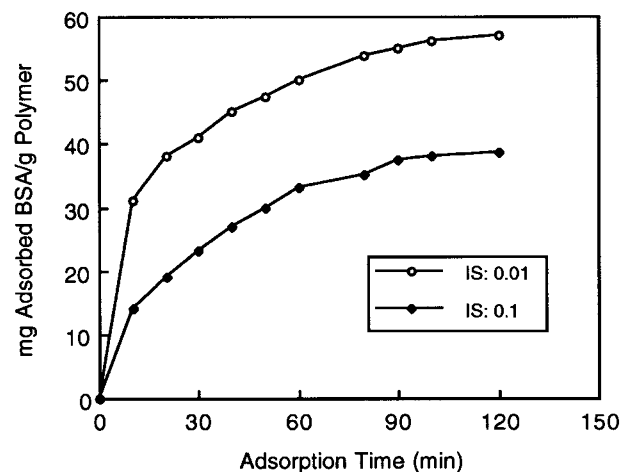

Figure 2 BSA adsorption rates: flow rate, 0.5 mL/min; BSA initial concentration, 4.0 mg/mL; pH 5.0; temperature, 25°C; and total volume of protein solution, 50 mL.

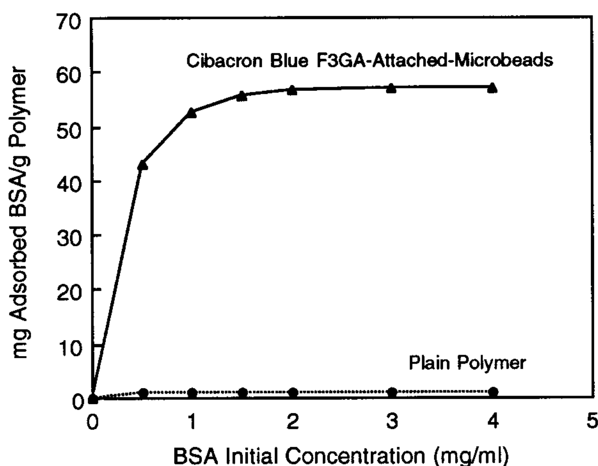

Figure 3 Effect of BSA initial concentration on BSA adsorption: ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g polymer; flow rate, 0.5 mL/min; pH 5.0; ionic strength, 0.01 (adjusted with NaCl); temperature, 25°C; and total volume of protein solution, 50 mL.

the specificity of the sorbent. The nonspecific interaction between the carrier matrix [poly-(EGDMA-HEMA) microbeads] and the molecules to be adsorbed (BSA) should be minimum for high specificity. As presented in this figure, with increasing BSA concentration in solution, the BSA adsorbed amount per gram by the Cibacron Blue F3GA attached microbeads increased. It became constant when the protein concentration was greater than 1.5 mg/mL. A negligible amount of BSA (1.1 mg/g) was adsorbed nonspecifically on the plain poly(EGDMA-HEMA) microbeads, while Cibacron Blue F3GA attachment significantly increased the BSA adsorption capacity of the microbeads (up to 57.3 mg BSA/g). This is evidence that this increase in the BSA adsorption capacity is due to specific interactions (both electrostatic and hydrophobic) between the Cibacron Blue F3GA and the BSA molecules.

In order to observe the dye attachment conditions on BSA adsorption, poly(EGDMA-HEMA) microbeads exposed to dye attachment conditions without Cibacron Blue F3GA were also used for BSA adsorption. The BSA adsorption observed was the same amount (1.1 mg BSA/g polymer) as that detected for plain microbeads. It can be said that the poly(EGDMA-HEMA) microbeads are impervious to the harsh alkaline conditions used for dye attachment. No carboxylic moieties are likely to be produced by hydrolysis of the ester linkage of HEMA molecules in the polymer chain, and hence they do not contribute to BSA adsorption. Therefore, it can be concluded that BSA molecules were adsorbed on the microbeads via attached Cibacron Blue F3GA groups.

Note that different sorbents with different adsorption capacities were reported in the literature for albumin adsorption. Nigel et al. used Sepharose CL-6B-200 as the carrier matrix and incorporated several dyes as specific ligands.[8] They reported BSA adsorption capacities of around 1–3 mg BSA/g moist gel. Denizli et al. obtained 35 mg BSA/g adsorption capacity by using Cibacron Blue F3GA attached PVA particles.[25] Nash and Chase modified poly(styrene-divinyl benzene) microspheres using PVA and Procion Blue MX-R, Procion Red HE-3B, Procion Yellow HE-3G, and Cibacron Blue F3GA dye ligands.[26] They presented adsorption capacities of 11.7–27 mg HSA/g. Horstmann et al. presented adsorption capacities of 5.4–11.2 mg BSA/g with the Cibacron Blue F3GA attached Sepharose CL-6B.[27] Boyer and Hsu used Sepharose beads carrying different amounts of Cibacron Blue F3GA (2–25 μmol/mL) and reported adsorption values up to 55.9 mg BSA/g polymer.[28] McCreath et al. showed 9.7 mg HSA/mL equilibrium adsorption capacity with the PVA coated particulate perfluoropolymer containing anion exchange and cation exchange groups.[29] Zeng and Ruckenstein reported 10.2 mg HSA/g adsorption capacity with Cibacron Blue F3GA attached polyethersulfone supported chitosan sorbents.[30] Li and Spencer used Cibacron Blue F3GA attached poly(ethylene-imine) coated titania and achieved 4.4 mg HSA/g.[31]

Chase reached 14 mg BSA/g with Cibacron Blue F3GA attached Sepharose CL-6B.[32] Janzen et al. developed porous and nonporous tentacle-type poly(ethylene-imine) anion exchanger sorbents, and they reported 4–40 mg/g BSA adsorption capacity.[33] Tuncel et al. reported 60 mg BSA/g polymer with Cibacron Blue F3GA attached PVA-coated monosize polystyrene microspheres.[34] Muller–Shulte et al. used several carrier matrices made of different polymers and Cibacron Blue F3GA as the ligand.[35] Their albumin adsorption values were in the range of 0.19–0.81 mg HSA/mL sorbent. The adsorption capacities of commercially available crosslinked agarose/Cibacron Blue F3GA sorbents (Bio-Rad) were reported as about 11 mg albumin/mL sorbent.[36] A comparison of these results shows that Cibacron Blue F3GA attached poly(EGDMA-HEMA) microbeads exhibit high albumin adsorption capacity in the packed-bed column system.

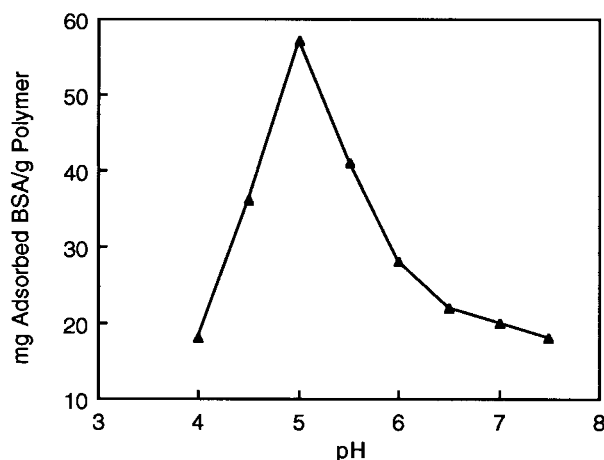

Figure 4 Effect of pH on BSA adsorption: ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g polymer; flow rate, 0.5 mL/min; BSA initial concentration, 4.0 mg/mL; ionic strength, 0.01 (adjusted with NaCl); temperature, 25°C; and total volume of protein solution, 50 mL.

As seen in Figure 3, BSA adsorption first increased significantly with the initial BSA concentration and then reached plateau values at around 1.5 mg/mL, at which we may assume that all the active binding sites available for BSA adsorption were occupied with BSA molecules. This is a typical Langmuir-type monolayer adsorption behavior, which can be described by the following equation:

$$q = q_m c/(K_d + c)$$

where q represents the amount of BSA adsorbed per unit mass of sorbent (mg/g), q_m is the maximum value of q (mg/g), c is the equilibrium concentration of BSA in the aqueous phase (mg/mL), and K_d is a constant. The values of K_d and q_m for our adsorption system (0.125 mg/mL and 60.14 mg/g, respectively) were found from the straight-line plot of c/q versus c by linear regression.

Effect of pH

Figure 4 shows the effects of pH. In all the cases investigated, the maximum adsorption of BSA was observed at pH 5.0. Significantly lower adsorption capacities were obtained in more acidic and more alkaline pH regions. It has been shown that proteins have no net electrical charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isolectric point.[37] The maximum adsorption was observed at pH 5.0, which is the isoelectric pH of BSA. These specific interactions may result from the ionization states of several groups on both the ligands (i.e., Cibacron Blue F3GA), the amino acid side chains in the BSA structure, and the conformational state of BSA molecules at this pH.

HSA Adsorption from Human Plasma

Table I shows the adsorption data for the HSA from human plasma obtained from a healthy donor. There was a very low nonspecific adsorption of HSA (2.2 mg/g) on the plain polymer, while much higher adsorption values (up to 109.6 mg/g) were obtained when the Cibacron Blue F3GA attached poly(EGDMA-HEMA) microbeads were used. Note that the adsorption of HSA onto the dye-attached polymeric microbeads was approximately 1.9-fold higher than those obtained in the studies in which aqueous solutions were used. This may be due to the conformational structure of HSA molecules within their native environment (i.e., human plasma), which is much more suitable for specific interaction with the Cibacron Blue F3GA molecules on the polymeric microbeads. The high HSA concentration (39.7 mg/mL) may also contribute to this high adsorption capacity. There is a competitive adsorption in human plasma. Other protein molecules may also be adsorbed and contribute to this high HSA adsorption capacity.

Desorption and Reuse

Desorption experiments were carried out in a packed-bed column system. Albumin adsorbed microbeads were placed in the column and

Table I HSA Adsorption from Plasma of Healthy Donor

HSA Concn (mg/mL)	Amount HSA Adsorbed[a] (mg/g)
2.5	9.9 ± 1.7
4.9	18.5 ± 3.1
9.9	34.2 ± 2.4
19.9	65.4 ± 2.2
39.7	109.6 ± 1.8

Ligand surface concentration, 16.5 μmol Cibacron Blue F3GA/g; flow rate, 0.5 mL/min; pH 5.0; temperature, 25°C; and total volume of protein solution, 50 mL.

[a] The average and the standard deviation of three parallel studies.

Table II Desorption of Albumin from Cibacron Blue F3GA Attached Microbeads

Medium	BSA Adsorbed (mg/g)	HSA Adsorbed (mg/g)	Desorption for BSA	Desorption for HSA
Protein solution	57.0 ± 0.5	—	94.9 ± 2.5	—
Human plasma	—	109.6 ± 6.8	—	95.6 ± 1.4

NaSCN solution (1.0*M,* pH 8.0) was recirculated, and the amount of albumin released in 30 min was determined. The desorption ratio for BSA was calculated by using the following expression:

$$\text{desorption ratio (\%)} = \frac{\text{amount of albumin desorbed}}{\text{amount of albumin adsorbed on microbeads}} \times 100$$

As shown in Table II, more than 94.9% of the adsorbed albumin could be easily desorbed from the Cibacron Blue F3GA attached microbeads. It is worth noting that the adsorbed BSA molecules easily desorbed. When the NaSCN was used, the electrostatic interactions between the positively charged groups of albumin and the negatively charged Cibacron Blue F3GA decreased. This forced the desorption of albumin. In addition, SCN^- ions easily bind albumin molecules. This also affects the conformational structure of albumin, and the hydrophobic interaction between the adsorbed albumin and Cibacron Blue F3GA could be decreased easily. From the desorption data given above, we concluded that NaSCN is a suitable desorption agent for the Cibacron Blue F3GA attached sorbents in all cases, and it allows repeated use of the affinity sorbents developed in this study.

In order to show the reusability of the dye-affinity column, the adsorption and desorption operation was repeated 6 times using the same column. The adsorption capacities did not change significantly during the repeated adsorption–desorption processes. Only a 5.3% decrease in albumin adsorption capacity was observed after 6 uses, which was an acceptable ranges in all cases.

REFERENCES

1. Turkova, J. Bioaffinity Chromatography; Elsevier: London, 1993.
2. Boyer, P. M.; Hsu, J. T. In Advances in Biochemical Engineering; Springer Verlag: Berlin, 1993; Vol. 49.
3. Lindner, N. M.; Jeffcoat, R.; Lowe, C. R. J Chromatog 1990, 508, 109.
4. Dean, P. D. G.; Watson, D. H. J Chromatog 1979, 165, 301.
5. McLoughlin, S. B.; Lowe, C. R. Prog Coloration 1988, 18, 16.
6. Travis, J.; Bowen, J. Biochem J 1976, 157, 301.
7. Clonis, Y. D.; Atkinson, T.; Burton, C. J.; Lowe, C. R., Eds. Reactive Dyes in Protein and Enzyme Technology; Stockton Press: New York, 1987.
8. Nigel, M.; Lindner, R.; Jeffcoat, R.; Lowe, C. R. J Chromatog 1989, 473, 227.
9. Müller–Schulte, D.; Manjini, S.; Vijayalakshmi, M. A. J Chromatog 1991, 539, 307.
10. Burton, S. J.; Stead, C. C.; Lowe, C. R. J Chromatog 1990, 508, 109.
11. Denizli, A.; Pişkin, E. J Chromatog B 1995, 666, 215.
12. Denizli, A.; Rad, A. Y.; Pişkin, E. J Chromatog B 1995, 668, 13.
13. Denizli, A.; Pişkin, E. J Chromatog B 1995, 670, 157.
14. Denizli, A.; Pişkin, E.; Dixit, V.; Arthur, M.; Gitnick, G. Int J Artif Organs 1995, 18, 105.
15. Denizli, A.; Kocakulak, M.; Pişkin, E. J Appl Polym Sci 1998, 68, 373.
16. Kocakulak, M.; Denizli, A.; Rad, A. Y.; Pişkin, E. J Chromatogr B 1997, 693, 271.
17. Denizli, A.; Kocakulak, M.; Pişkin, E. J Chromatogr B 1998, 707, 25.
18. Tietz, B. T. Textbook of Clinical Chemistry; W. B. Saunders: Philadelphia, 1986.
19. Weber, B.; Willeford, K.; Moe, J.; Piskiewicz, D. Biochem Biophys Res Commun 1979, 86, 252.
20. Stead, C. V. In Reactive Dyes in Protein and Enzyme Technology; Clonis, Y. D.; Atkinson, A.; Burton, C. J.; Lowe, C. R., Eds.; Macmillan: Basingstoke, 1987; p 13.
21. Boyer, P. M.; Hsu, J. T. Ad Biochem Eng 1993, 49, 1.
22. Lowe, C. R. In Topics in Enzyme and Fermentation Technology; Wiseman, A., Ed.; Ellis Horwood: Chichester, 1984; p 78.
23. Shirahama, H.; Suzawa, T. J Colloid Interface Sci 1985, 104, 416.

24. Shirahama, H.; Shikawa, T.; Suzawa, T. Colloid Polym Sci 1989, 267, 587.
25. Denizli, A.; Tuncel, A.; Kozluca, A.; Ecevit, K.; Pişkin, E. Separation Sci Technol 1997, 32, 1003.
26. Nash, D. C.; Chase, H. A. J Chromatog A 1997, 776, 55.
27. Horstmann, B. J.; Kenney, C. N.; Chase, H. A. J Chromatogr A 1986, 361, 179.
28. Boyer, P. M.; Hsu, J. T. Chem Eng J 1992, 47, 241.
29. McCreath, G. E.; Owen, R. O.; Nash, D. C.; Chase, H. A. J Chromatogr A 1986, 773, 73.
30. Zeng, X.; Ruckenstein, E. J Membr Sci 1996, 117, 271.
31. Li, Y.; Spencer, H. G. In Polymers of Biological and Biomedical Significance; Shalaby, W., Ed.; American Chemical Society: Washington, D.C., 1994; pp 297–305.
32. Chase, H. A. J Chromatog 1984, 297, 179.
33. Janzen, R.; Unger, K. K.; Muller, W.; Hearn, M. T. W. J Chromatog 1990, 522, 77.
34. Tuncel, A.; Denizli, A.; Purvis, D.; Lowe, C. R.; Pişkin, E. J Chromatog 1993, 634, 161.
35. Muller–Schulte, D.; Manjini, S.; Vijayalakshmi, M. A. J Chromatog 1991, 539, 307.
36. Bio-Rad Life Science Research Product Catalog; Bio-Rad: CA, 1995.
37. Denizli, A.; Pişkin, E. Turk J Chem 1994, 18, 188.