New Metal Chelate Sorbent for Albumin Adsorption: Cibacron Blue F3GA-Zn(II) Attached Microporous Poly(HEMA) Membranes

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ABSTRACT: Poly(2-hydroxyethyl methacrylate) [poly(HEMA)] membranes were prepared by UV-initiated photopolymerization of HEMA in the presence of an initiator (α-α'-azobis-isobutyronitrile, AIBN). The triazine dye Cibacron Blue F3GA was attached as an affinity ligand to poly(HEMA) membranes, covalently. These affinity membranes with a swelling ratio of 58% and containing 10.7 mmol Cibacron Blue F3GA/m^2 were used in the albumin adsorption studies. After dye-attachment, Zn(II) ions were chelated within the membranes via attached-dye molecules. Different amounts of Zn(II) ions [650–1440 mg Zn(II)/m^2] were loaded on the membranes by changing the initial concentration of Zn(II) ions and pH. Bovine serum albumin (BSA) adsorption on these membranes from aqueous solutions containing different amounts of BSA at different pH was investigated in batch reactors. The nonspecific adsorption of BSA on the poly(HEMA) membranes was negligible. Cibacron Blue F3GA attachment significantly increased the BSA adsorption up to 92.1 mg BSA/m^2. Adsorption capacity was further increased when Zn(II) ions were attached (up to 144.8 mg BSA m^2). More than 90% of the adsorbed BSA was desorbed in 1 h in the desorption medium containing 0.5*M* NaSCN at pH 8.0 and 0.025*M* EDTA at pH 4.9. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 68: 657–664, 1998

Key words: albumin adsorption; affinity membranes; poly(HEMA); metal chelate adsorption; Cibacron Blue F3GA

INTRODUCTION

Chromatographic separation and purification of biomolecules such as proteins and enzymes needs several steps involving methods which select on the basis of molecular size (gel permeation chromatography), electrical charge (ion-exchange chromatography), hydrophobicity (hydrophobic interaction chromatography), or biological recognition (bioaffinity chromatography). The overall procedure will be efficient if the techniques separate according to those properties which best discriminate between the material of interest and the impurities. Metal chelate affinity chromatography introduces a new possibility for selectively interacting materials on the basis of their affinities for chelated transition-metal ions. The separation is based on differential binding abilities of the proteins or enzymes to interact with chelated metal ions to a solid carrier.[1–4]

Metal chelate affinity chromatography of proteins, with metal chelate linked to Sepharose, was first introduced by Porath et al.[5] They reported a model system using Zn(II) and Cu(II) columns in tandem for the fractionation of human serum

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proteins. Subsequent studies have shown the wide applicability of the technique and consistency of the methodology. The plasma proteins α_2-macroglobulin and α_1-proteinase inhibitor, for example, have been purified to homogeneity on zinc chelate columns.[6,7] Metal chelate affinity chromatography has been used to provide immunologically and physicochemically pure α_2-HS glycoprotein from plasma.[8,9] Plasminogen activators from both normal tissue (human uterus) and human melanoma cells have been isolated by metal chelate affinity chromatography[10,11] as have nucleoside diphosphatase,[12] human lactoferrin,[13] lectin,[14] interferon,[15] and carboxypeptidase B.[16]

Poly(hydroxyethyl methacrylate) [poly(HEMA)] is among the major synthetic polymers approved by federal agencies like the Food and Drug Administration in the United States for biomedical, pharmaceutical, and industrial applications. It is a nontoxic, hydrophilic, and biocompatible material which is often employed as a support for diverse biomedical applications.[17–19] In addition, it has been used in previous enzyme and protein immobilization studies either by entrapment into or by covalent binding onto its membrane and microspheres.[20,21]

The purpose of the present study was to prepare an affinity membrane containing Cibacron Blue F3GA and Zn(II) ions (in chelate form) for dye affinity and metal chelate affinity separation of proteins. Bovine serum albumin (BSA) was selected as a model protein. In this article, we present BSA adsorption/desorption properties of these Cibacron Blue F3GA/Zn(II) attached microporous poly(HEMA) membranes.

EXPERIMENTAL

Materials

Bovine serum albumin (BSA, lyophilized, Fraction V) was purchased from the Sigma Chemical Co. (St Louis, MO) and used as received. 2-Hydroxyethyl methacrylate (HEMA) was obtained from the Sigma Chemical Co. and distilled under reduced pressure in the presence of hydroquinone and stored at 4°C until use. Ethyleneglycoldimethacrylate (EGDMA) was purchased from the Sigma Chemical Co. and the inhibitor in EGDMA was removed by alkaline salt extraction (20% NaCl and 5% NaOH were used as the alkaline salt). α-α'-Azobisisobutyronitrile (AIBN) was purchased from Fluka AG (Buchs, Switzerland) and used as received. Cibacron Blue F3GA (CB) was obtained from Polyscience (Warrington, USA). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany).

Methods

Preparation of Poly(HEMA) Membrane

The membrane preparation mixture (5 mL) contained 2 mL (HEMA), 0.01 mL EGDMA as a crosslinker, 5 mg AIBN as a polymerization initiator, and 3 mL 0.1M $SnCl_4$ as a pore former. The mixture was then poured into a round glass mold ($\phi = 4.5$ cm) and exposed to ultraviolet radiation (12 W lamp, P.W. Allen and Co.) for 10 min, while a nitrogen atmosphere was maintained in the mold. The membrane was washed several times with distilled water and cut into circular pieces ($\phi = 0.5$ cm) with a perforator. The other details related to the polymerization system and procedure were given elsewhere.[22,23]

Dye Attachment to Poly(HEMA) Membrane

Cibacron Blue F3GA was covalently attached to the poly(HEMA) membrane via the nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the poly(HEMA), under alkaline conditions. The attachment procedure previously used was employed.[24,25] First, 300 mg of Cibacron Blue F3GA was dissolved in 10 mL of water. This dye solution was transferred to poly(HEMA) membrane pieces ($\phi = 0.5$ cm, thickness ca. 0.06 cm) in 90 mL distilled water, and then 4 g of NaOH were added. The medium was heated at 80°C in a sealed reactor and was stirred magnetically for 4 h. The membrane pieces were washed several times with distilled water and methanol until all the unattached dye was removed. They were then stored at 4°C until use.

Incorporation of Zn(II) Ions to Dye-Attached Poly(HEMA) Membranes

Chelates of Cibacron Blue F3GA membranes with Zn(II) ions were prepared as follows: Cibacron Blue F3GA membranes were mixed with aqueous solutions containing 10–350 ppm Zn(II) ion, at a constant pH of 6.8 (adjusted with a universal buffer solution), which was the optimum pH for Zn(II) chelate formation[25] and at room temperature. A 1000-ppm atomic absorption standard solution (containing 10% concentrated HNO_3) was

used as the source of Zn(II) ions. The flasks were agitated magnetically at 600 rpm for 1 h (sufficient to attain equilibrium). The concentration of the Zn(II) ions in the resulting solutions was determined with an atomic absorption spectrophotometer (GBC 932 AA, Australia).

Zn(II) leakage from the Cibacron Blue F3GA-Zn(II) membranes was investigated with media containing NaCl at 0.01 ionic strength and pH in the range 4.0–8.0, and also in a medium containing 0.5*M* NaSCN, at pH 8.0. The membrane suspensions were stirred 24 h at room temperature. After this period, the leached Zn(II) was determined in the supernatants using an atomic absorption spectrophotometer.

Adsorption Studies with BSA

BSA was selected as a model protein. BSA adsorption of the plain and the dye-attached [poly-(HEMA)–Cibacron Blue F3GA] and Zn(II) chelated–[poly(HEMA)–Cibacron Blue F3GA–Zn(II)] membrane disks were studied at various pH. The pH of the adsorption medium was changed between 3.0 and 8.0 using different buffer systems (0.1*M* CH_3COONa—CH_3COOH for pH 4.0–6.0, 0.1*M* K_2HPO_4—KH_2PO_4 for pH 7.0, and 0.1*M* NH_4OH—NH_4Cl for pH 8.0). The initial BSA concentration was changed between 0.5 and 7.0 mg/mL. In a typical adsorption experiment, BSA was dissolved in 25 mL of the buffer solution containing NaCl and membranes were added. The adsorption experiments were carried out for 2 h at 25°C at a stirring rate of 100 rpm. At the end of the equilibrium period (i.e., 1 h), the membranes were separated from the solution. The BSA-adsorption capacity was determined by measuring the initial and final concentrations of BSA within the adsorption medium spectrophotometrically at 280 nm.[25]

Desorption of BSA from Derivatized Poly(HEMA) Membrane

The BSA-desorption experiments were performed in a buffer solution containing 0.5*M* NaSCN at pH 8.0 or 0.025*M* EDTA at pH 4.9. The BSA-loaded membranes were placed in the desorption medium and stirred for 1 h at 25°C, at a stirring rate of 100 rpm. The final BSA concentration within the desorption medium was determined by spectrophotometry. In the case of Zn(II)-carrying sorbents, the desorption of Zn(II) ions was also measured in the desorption media using atomic absorption. The desorption ratio was calculated from the amount of BSA adsorbed on the membranes and the amount of BSA desorbed.

Characterization of Poly(HEMA) Membranes

Water Content of Poly(HEMA) Membranes. The swelling behavior of poly(HEMA) membranes was determined in distilled water. Dry membrane pieces were placed into distilled water and kept at a constant temperature of 25 ± 0.5°C. Swollen membranes were periodically removed and weighed by an electronic balance (Shimadzu, Japan, EB.280 ± 1.10^{-3} g). The water content of the swollen membranes was calculated using the following expression:

$$\text{Swelling ratio } \% = [(W_s - W_0)/W_0] \times 100 \quad (1)$$

where W_0 and W_s are the weights of membrane before and after swelling, respectively.

FTIR Spectra. FTIR spectra of the Cibacron Blue F3GA plain and Cibacron Blue F3GA attached poly(HEMA) membranes were obtained by using a FTIR spectrophotometer (Shimadzu, FTIR 8000 Series, Japan). The poly(HEMA) membrane (0.1 g) and KBr (0.1 g) were thoroughly mixed and this mixture was pressed to form a tablet, and the spectrum was recorded.

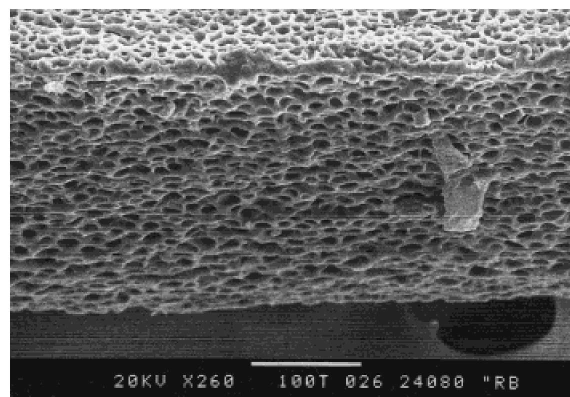
Scanning Electron Microscopy. Scanning electron micrographs of the poly(HEMA) membrane were obtained using a Leitz AMR-1000 (Germany) after coating with gold under a vacuum.

Elemental Analysis. The amount of Cibacron Blue F3GA attached covalently to the poly-(HEMA) membrane was obtained from the elemental analysis device (Leco, CHNS-932, USA).

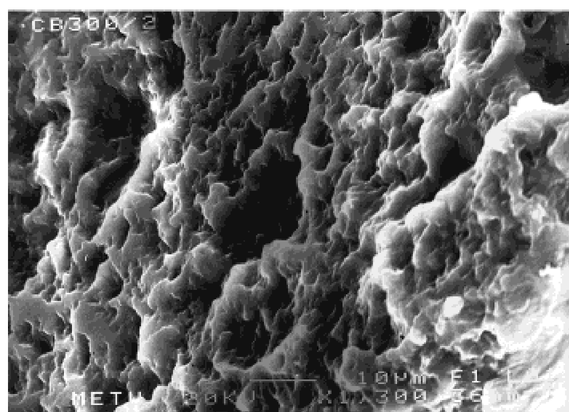
RESULTS AND DISCUSSION

Cibacron Blue-Derivatized Poly(HEMA) Membrane

Preparation and characterization details of the poly(HEMA) membrane were given in our previous articles.[22,23] The membranes prepared in this study are highly swellable (the swelling ratio 58%). The membrane shows a homogeneous and highly open pore structure, which may lead to a high internal surface area (means high adsorp-



(A)



(B)

Figure 1 SEM photographs of poly(HEMA) membrane: (A) cross section; (B) surface.

tion capacity) with low diffusional resistance in the matrix (Fig. 1).

To examine the nature of the interaction between the dye (Cibacron Blue F3GA) and the poly(HEMA) membrane, FTIR spectra of plain Cibacron Blue F3GA, poly(HEMA), and poly(HEMA)–Cibacron Blue F3GA were obtained. As shown in Figure 2, the FTIR spectra of both poly(HEMA) and poly(HEMA)–Cibacron Blue F3GA have the characteristic stretching vibration band of hydrogen-bonded alcohol, O—H, around 3500 cm^{-1}. The FTIR spectra of the dye-attached poly(HEMA) has some absorption bands different from those of poly(HEMA). These are at 3375, 1520, and 650 cm^{-1} and characteristic N—H stretching, N—H bending (scissoring), and S—O stretching, respectively, were observed also in Cibacron Blue F3GA (Fig. 3). The dye-attached poly(HEMA) spectrum has a sharp shoulder absorption band at about 3380 cm^{-1} and is interpreted as the N—H absorption. The bands at 1075, 1155, and 1280 cm^{-1} representing the symmetric stretching of S=O, asymmetric stretching of S=O, and aromatic C—N vibration, respectively, are due to Cibacron Blue F3GA bonded to poly(HEMA). These bands, however, do not appear, because plain poly(HEMA) also has some absorption bands in the same region. Thus, the absorption bands of plain poly(HEMA) overlap with those of the Cibacron Blue F3GA at around these wavenumbers. For dye-attached poly(HEMA), the adsorption band intensities in this region are higher than those of poly(HEMA), but the intensity increase is quite small because of the low concentration of Cibacron Blue F3GA on the polymeric surface. On the other hand, the hydrogen-bonded alcohol O—H stretching band intensity of plain poly(HEMA) is higher than that of the poly(HEMA)–Cibacron Blue F3GA membrane. The reason for the loss of the —OH groups is as a result of the condensation reaction between —OH groups of poly(HEMA) and $—NH_2$ groups of Cibacron Blue F3GA.

Plain and Cibacron Blue F3GA-attached poly(HEMA) membranes were subjected to elemental analysis. The amount of Cibacron Blue F3GA attached on the membrane was calculated from this data (by considering the stoichiometry) to be 10.7 mmol Cibacron Blue F3GA/m^2. Studies aimed at detecting leakage of Cibacron Blue F3GA and Zn(II) from the dye-attached and Zn(II)-derivatized poly(HEMA) membrane revealed no leakage in any of the adsorption and desorption media and implied that the washing procedure was satisfactory for the removal of the physically adsorbed Cibacron Blue F3GA molecules and Zn(II) ions from poly(HEMA) membrane.

Cibacron Blue F3GA/Zn(II)–Attached Poly(HEMA) Membranes

Figure 4 shows the effects of the Zn(II) ion concentration on the amount of Zn(II) ions adsorbed (chelated) on both the plain and Cibacron Blue F3GA-attached poly(HEMA) membranes. The amount of ions adsorbed to the Cibacron Blue F3GA-attached membranes increased with the concentration in the solution. It reached a plateau value of 1.44 g Zn(II)/m^2 membrane at an aqueous concentration of 150 ppm. This is much greater than the adsorption capacity of the plain poly(HEMA) membranes for Zn(II) [0.125 g Zn(II)/m^2].

As seen in Figure 5, the adsorption of Zn(II)

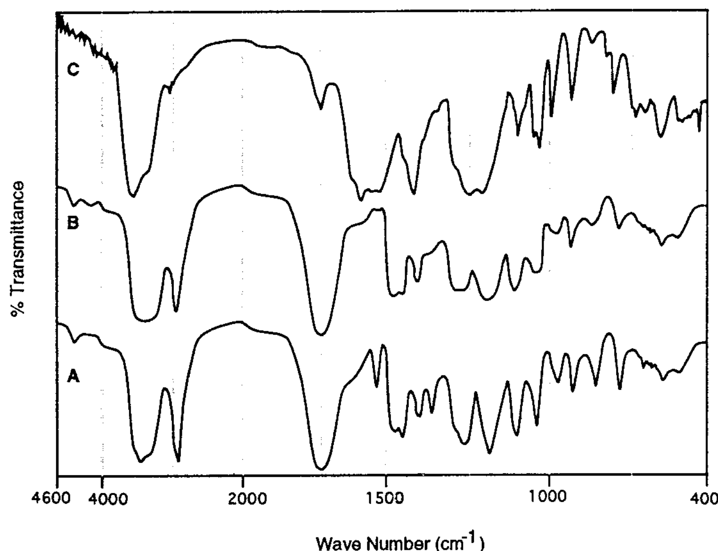


Figure 2 FTIR spectra: (A) plain poly(HEMA); (B) Cibacron Blue F3GA; (C) poly-(HEMA)–Cibacron Blue F3GA.

ions increased with increasing pH. The optimal pH value was 6.8. The nonspecific adsorption of Zn(II) ions was low, about 0.125 g/m^2 membrane, while the specific adsorption of Zn(II) ions, which was pH-dependent, was much higher (1.44 g/m^2 membrane) than was nonspecific adsorption. High adsorption capacity at basic pH values implies that the Zn(II) ions interact with the Cibacron Blue F3GA molecules not only through the nitrogen atoms by chelating, but also through —SO_3H groups by cation exchange, which are inprotonated at high pH.

Albumin Adsorption/Desorption

Figure 6 shows the effects of the initial BSA concentration on adsorption. As presented in this figure, with increasing BSA concentration in the solution, the amount per unit area of albumin adsorbed by the membrane increases almost linearly at low concentrations, below about 2.0 mg/mL, then increases less rapidly and approaches saturation. It becomes constant when the protein concentration is greater than 3.0 mg/mL. A negligible amount of BSA adsorbed nonspecifically on the plain poly(HEMA) membrane is 1.2 mg/m^2. Cibacron Blue F3GA attachment significantly increased the BSA-adsorption capacity of the membranes (up to 92.1 mg BSA/m^2), possibly because of the specific interactions between albumin and Cibacron Blue F3GA molecules. A further significant increase (up to 144.8 $mg/BSA/m^2$ membrane) was noted when the Cibacron Blue F3GA–Zn(II)-attached poly(HEMA) membranes were used. It is clear that this increase is due to chelate

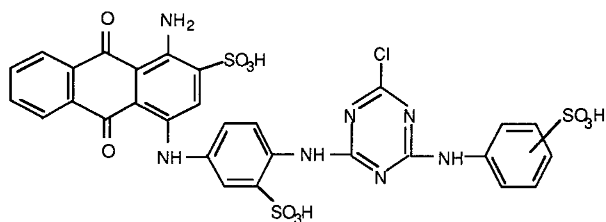


Figure 3 Chemical Structure of Cibacron Blue F3GA.

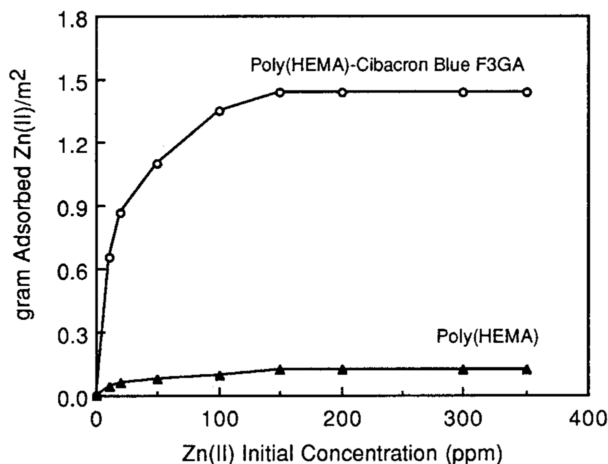


Figure 4 Incorporation of Zn(II) onto plain and Cibacron Blue F3GA-attached poly(HEMA) membranes as a function of Zn(II) ion concentration; pH 6.8.

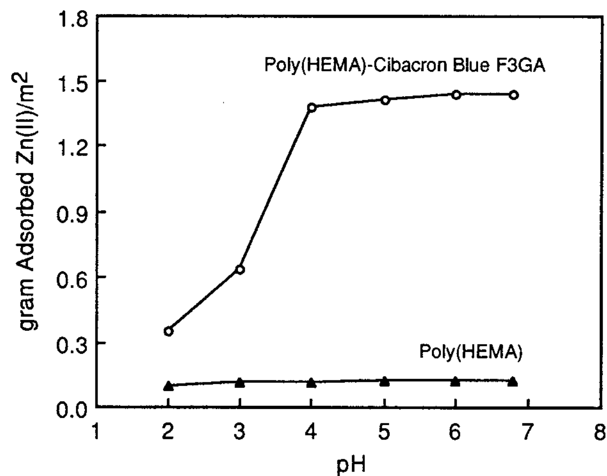


Figure 5 Incorporation of Zn(II) onto plain and Cibacron Blue F3GA-attached poly(HEMA) membranes as a function of pH; Initial Zn(II) concentration: 150 ppm.

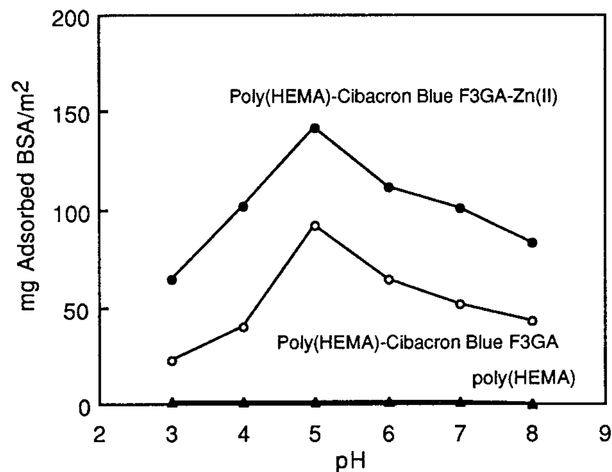


Figure 7 Effects of pH on BSA adsorption; ionic strength: 0.01 (adjusted with NaCl); BSA initial concentration: 3.0 mg/mL; Cibacron Blue F3GA loading: 10.7 mmol/m^2; Zn(II) loading: 1.44 g/m^2 membrane.

formation between the Zn(II) ions and BSA molecules [i.e., Zn(II) ions promote the adsorption of albumin to attached Cibacron Blue F3GA].

Figure 7 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 with all membranes in more acidic and in more alkaline pH regions. It has been shown that proteins have no net charge at their isoelectric points, and, therefore, the maximum adsorption from aqueous solutions is usually observed at their isolectric point.[26,27] The maximum adsorption was observed at pH 5.0, which is the isoelectric pH of BSA. These specific interactions may result both from the ionization states of several groups on both the ligands [i.e., Cibacron Blue F3GA and its chelator with Zn(II) ions] and amino acid side chains in the albumin structure and from the conformational state of albumin molecules at this pH, as usually discussed in the related literature.[28,29]

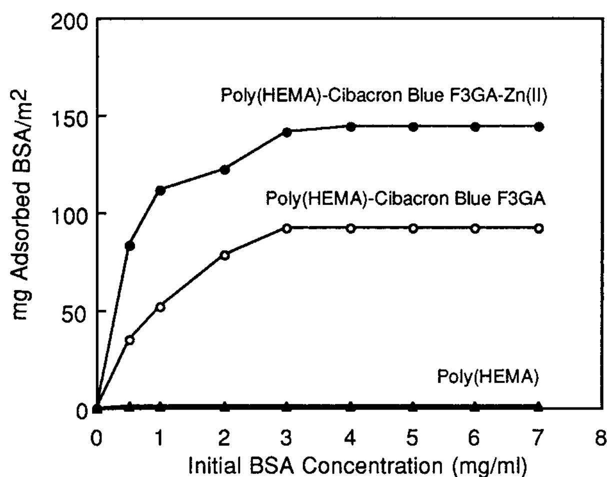


Figure 6 Effects of BSA initial concentration on BSA adsorption; ionic strength: 0.01 (adjusted with NaCl); pH 5.0; Cibacron Blue F3GA loading: 10.7 mmol/m^2; Zn(II) loading: 1.44 g/m^2 membrane.

The albumin-adsorption capacities of the Cibacron Blue F3GA–Zn(II)-attached poly(HEMA) membranes containing different amounts of Zn(II) [between 0.65 and 1.44 g Zn(II)/m^2] were investigated at pH 5.0. The initial concentration of BSA in the incubation solution was 3.0 mg/mL. Table I shows the effects of Zn(II) attached onto membranes on BSA adsorption. When the amount Zn(II) on the membranes increased, the amount of BSA adsorbed first increased and then reached an almost constant value. This may be the maximum amount of BSA that can be packed on the surface, owing to steric hindrance.

Table I Effects of Amount of Zn(II) Attached onto Membranes on BSA-adsorption Capacity

g Attached Zn(II)/m^2	mg Adsorbed BSA/m^2
0.65	64.2 ± 3.5
0.86	91.6 ± 3.7
1.10	110.5 ± 2.6
1.35	129.2 ± 1.5
1.44	144.8 ± 2.1

Ionic strength: 0.01 (adjusted with NaCl).

Table II Desorption of BSA and Zn(II) Ions

Membranes	BSA-loaded (mg/m^2)	Zn(II)-loaded (g/m^2)	Desorption Ratio for BSA (%)		Desorption Ratio for Zn(II) Ions (%)	
			With NaSCN	With EDTA	With NaSCN	With EDTA
Membrane I[a]	92.1 ± 2.5	—	91	25	—	—
Membrane II[b]	144.8 ± 2.7	1.44 ± 0.015	94	100	0	100

[a] Poly(HEMA)–Cibacron Blue F3GA membranes.
[b] Poly(HEMA)–Cibacron Blue F3GA–Zn(II) membranes.

Note that a wide variety of sorbents with a wide range of adsorption capacities were reported in the literature for albumin adsorption. Denizli et al. found a 41 mg/m^2 adsorption capacity with Congo Red-attached monosize poly(MMA–HEMA) microspheres.[27] Tuncel et al. reached the adsorption capacity of 40 mg BSA/m^2 with poly(vinyl alcohol)-coated-Cibacron Blue F3GA-immobilized monosize polystyrene microspheres.[30] Zeng and Ruckenstein reported 139 mg HSA/m^2 with the Cibacron Blue F3GA-attached microporous chitosan membranes.[31] Denizli et al. showed 264 mg BSA/m^2 with the poly(vinyl alcohol) particles containing Cibacron Blue F3GA.[32] Li and Spencer presented an adsorption capacity of 400 mg HSA/m^2 with Cibacron Blue F3GA-attached poly(ethylene imine)-coated titania.[33] Ratnayake and Regnier reported very low protein adsorption capacities between 1.22–2.16 mg/m^2 with carboxylic acid-attached polyacrylate cation-exhange sorbents.[34] Kang et al. immobilized 62.5 mg albumin/m^2 on the poly(methyl methacrylate) surface.[35] The maximum BSA adsorption that we achieved with the sorbent system developed in this study was 92.1–144.8 mg/m^2 membrane, which was quite comparable with the related literature.

Desorption

The desorption of the adsorbed BSA from the Cibacron Blue F3GA-attached poly(HEMA) and Cibacron Blue F3GA/Zn(II)-attached poly(HEMA) membranes was studied in a batch experimental setup. The membranes loaded with different amounts of BSA were placed within the desorption medium containing 0.5M NaSCN at pH 8.0 or 0.025M EDTA at pH 4.9, and the amount of BSA and Zn(II) released in 1 h was determined. The desorption ratios for both BSA and Z(II) were calculated by using the following expression:

$$\text{Desorption ratio (\%)} = \frac{\text{amount of BSA \{or Zn(II)\} released}}{\text{amount of BSA \{or Zn(II)\} adsorbed on the membrane}} \times 100$$

Table II gives the desorption data. More than 90% of the adsorbed BSA was removed when NaSCN was used for desorption. Note that there was no Zn(II) released in this case which shows that the Zn(II) ions are attached to the Cibacron Blue F3GA molecules on the membrane surface by strong chelate formation. However, when EDTA was used for desorption, only 25% of BSA was removed from the Cibacron Blue F3GA-attached membranes, maybe because of a salting-out effect. While under the same desorption conditions, about 94% of the BSA was desorbed from the Cibacron Blue F3GA/Zn(II)-carrying membranes. Note that in this later case almost all of the Zn(II) ions initially loaded came out (released) from the membranes. This means that EDTA breaks down the chelates between Zn(II) ions and Cibacron Blue F3GA. With the desorption data given above, we concluded that NaSCN and EDTA are suitable desorption agents especially for the Cibacron Blue F3GA–Zn(II) carrying sorbents and allows repeated use of the affinity sorbents developed in this study.

CONCLUSION

Microporous poly(HEMA) membranes were prepared by UV-initiated photopolymerization of the the hydroxyethyl methacrylate monomer. An affinity dye-ligand, that is, Cibacron Blue F3GA, was covalently immobilized into these membranes with a surface concentration of 10.7 mmol m^2 membrane. Then, Zn(II) ions were incorporated with Cibacron Blue F3GA molecules. Che-

lated amounts of Zn(II) ions were obtained between 0.65 and 1.44 g Zn(II)/m^2 membrane. The results presented in this communication showed that up to 144.8 mg albumin per unit area of the membrane can be adsorbed. As previously mentioned, the poly(HEMA) membrane modified by attachment of Cibacron Blue F3GA and Zn(II) ions revealed good properties as an affinity membrane.

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