Preparation of a Novel Metal-Chelate Affinity Beads for Albumin Isolation from Human Plasma

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ABSTRACT: In this study, we developed a novel approach to obtain a high protein-adsorption capacity utilizing 2-methacryloylamidohistidine (MAH) as a biollgand. MAH was synthesized by reacting methacryloyl chloride and histidine. Spherical beads, with an average size of 150–200 μm, were obtained by the radical suspension polymerization of MAH, ethyleneglycol dimethacrylate (EGDMA), and 2-hydroxyethyl methacrylate (HEMA) conducted in an aqueous dispersion medium. p(EGDMA–HEMA–MAH) beads had a specific surface area of 17.6 m^2/g. The synthesized MAH monomer was characterized by NMR. p(EGDMA–HEMA–MAH) beads were characterized by a swelling test, FTIR, and elemental analysis. Then, Cu(II) ions were incorporated into the beads and Cu(II) loading was found to be 0.96 mmol/g. These beads, with a swelling ratio of 65% and containing 1.6 mmol MAH/g, were used in the adsorption/desorption of human serum albumin (HSA) from both aqueous solutions and human serum. The adsorption of HSA onto p(EGDMA–HEMA–MAH) was low (8.8 mg/g). Cu(II) chelation onto the beads significantly increased the HSA adsorption (56.3 mg/g). The maximum HSA adsorption was observed at pH 8.0 Higher HSA adsorption was observed from human serum (94.6 mg HSA/g). Adsorptions of other serum proteins were obtained as 3.7 mg/g for fibrinogen and 8.5 mg/g for γ-globulin. The total protein adsorption was determined as 107.1 mg/g. Desorption of HSA was obtained using a 0.1*M* Tris/HCI buffer containing 0.5*M* NaSCN. High desorption ratios (to 98% of the adsorbed HSA) were observed. It was possible to reuse Cu(II)-chelated p(EGDMA–HEMA–MAH) beads without significant decreases in the adsorption capacities. J Appl Polym Sci 90: 2840–2847, 2003

INTRODUCTION

Serum proteins are commercially and therapeutically important components. Human serum albumin (HSA), for example, has represented approximately 50% of all sales of therapeutic plasma protein products.[1] HSA is the most abundant protein in serum. It has many important physiological functions which contribute significantly to colloid osmotic blood pressure and aid in the transport, distribution, and metabolism of many endogeneous and exogeneous substances including bile acids, bilirubin, fatty acids, amino acids, metal ions, and numerous pharmaceuticals.[2,3] Research on protein purification has attracted considerable attention for its great potential in blood protein manufacture. HSA is, at present, commonly isolated from human serum by Cohn's classical blood fractionation procedure.[4] Cohn's method concerns precipitation of proteins using ethanol with varying pH, ionic strength, and temperature. Although the Cohn procedure for the fractionation of albumin and other serum proteins is still widely used by industry, with the advent of improved methods of protein purification, there has been a continuous search during the past decades for more efficient protocols, particularly those employing novel chromatographic techniques.[5]

Immobilized metal chelate affinity chromatography has advanced and become a powerful technique for the isolation, separation, purification, and recovery of a wide range of peptides, proteins, nucleic acids, hormones, and enzymes.[6–10] It is based on the selective interactions between proteins containing one or preferably several adjacent histidine residues and metal-chelated ions. The number of histidine residues on the protein surface is of primary importance in the overall affinity with chelated metal ions. In addition, factors such as the accessibility, micro-environment of the binding residue (i.e., histidine, cysteine, aspartic acid, glutamic acid, and tryrosine), cooperation between neighboring amino acid side chains, and local conformations play important roles in biomolecule adsorption. Aromatic amino acid side chains, free carboxylic groups, and the amino-terminal groups of peptides also have some contributions.[11] Ligand stability, high protein loading, mild elution conditions, simple regeneration, and low cost are the attractive features of metal affinity separation.

This article shows the performance of a novel metal-complexing ligand meth-acryloylamidohistidine (MAH) containing affinity sorbents for HSA purifica-

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tion from human serum. The purification of HSA is generally required for the treatment of hypoproteinemia. p(EGDMA–HEMA–MAH)′ beads were prepared by suspension polymerization of ethyleneglycol dimethacrylate (EGDMA), 2-hydroxyethyl methacrylate (HEMA), and MAH monomers. Poly (HEMA) was selected as the basic support material, which is one of the most widely used hydrophilic polymers in biomedical applications, by considering possible applications in direct contact with plasma,[12–15] in which blood compatibility is one of the main concerns.[16,17] We present the adsorption–desorption properties of HSA onto plain and Cu(II)-chelated p(EGDMA–HEMA–MAH) beads from aqueous solutions, containing different amounts of HSA and at different pH, and also from human serum.

EXPERIMENTAL

Chemicals

L-Histidine methylester and methacryloyl chloride were supplied by Sigma (St Louis, MO) and used as received. HEMA and EGDMA were obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of a hydroquinone inhibitor and stored at 4°C until use. Benzoyl peroxide (BPO) was obtained from Fluka. Poly(vinyl alcohol) (PVAL; *Mw*, 100,000; 98% hydrolyzed) was supplied by Aldrich Chemical Co. (Milwaukee, WI). HSA (98% pure by gel electrophoresis, fatty acid free, 67 kDa) was purchased from Aldrich (Munich, Germany). Acetic acid and miscellaneous chemicals were of analytical reagent grade and supplied by Merck (Darmstadt, Germany). Coomassie Blue for the Bradford Protein assay was from BioRad (Richmond, CA). All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion-exchange packed-bed system. The resulting purified water (deionized water) has a specific conductivity of 18 μs.

Synthesis of MAH monomer

The following experimental procedure was applied for the synthesis of MAH: 5.0 g of L-histidine methylester and 0.2 g of $NaNO_2$ were dissolved in 30 mL of a K_2CO_3 aqueous solution (5%, v/v). This solution was cooled to 0°C. Methacryloyl chloride, 4.0 mL, was poured slowly into this solution under a nitrogen atmosphere and then this solution was stirred magnetically at room temperature for 2 h. At the end of the chemical reaction period, the pH of this solution was adjusted to 7.0 and then was extracted with ethyl acetate. The aqueous phase was evaporated in a rotary evaporator. The solid residue (i.e., MAH) was crystallized in ethanol and ethyl acetate.

Preparation of p(EGDMA–HEMA–MAH) beads

p(EGDMA–HEMA–MAH) beads were prepared by a suspension polymerization method. A typical procedure may be summarized as follows: The stabilizer, PVAL, was dissolved in 50 mL deionized water for the preparation of the continuous phase. The disperse phase was prepared by mixing EGDMA (8.0 mL), HEMA (4.0 mL), MAH (750 mg), and toluene (12.0 mL) in a test tube. The initiator, BPO (100 mg), was dissolved in this homogeneous solution. The disperse phase was added to the continuous medium in a glass-sealed polymerization reactor (100 mL) placed in a water bath equipped with a temperature-control system. The polymerization reactor was heated to 65°C within about 30 min by stirring the polymerization medium at 600 rpm. The polymerization was conducted at 65°C for 4 h and at 90°C for 1 h. After completion of polymerization, the reactor content was cooled to room temperature. A washing procedure was applied after polymerization to remove the diluent and any possible unreacted monomers from the beads. The polymer beads were filtered and resuspended in ethyl alcohol. The bead suspension was stirred for about 1 h at room temperature and the beads were separated by filtration. The beads were washed twice with ethyl alcohol and then four times with deionized water using the same procedure. When not in use, the beads were kept under refrigeration in a 0.02% sodium azide solution to prevent microbial contamination.

Cu(II) loading

Loading of Cu(II) ions was carried out in a batch system. An aqueous, 100 mL, solution containing Cu(II) ions (30 mg/L) was treated with the polymer beads. The polymer beads (1.0 g) were stirred with a copper nitrate salt solution at room temperature for 2 h. The suspensions were brought to the desired pH by adding sodium hydroxide and nitric acid. The pH was maintained in a range of 0.1 units until equilibrium was attained. Investigations were made with a pH value at 4.1. The concentration of the Cu(II) ions in the aqueous phase, after the desired treatment periods, was measured using a graphite furnace atomic absorption spectro-photometer (AAS 5EA, Carl Zeiss Technology, Zeiss Analytical Systems, Germany). At the end of the predetermined equilibrium period (i.e., 2 h), the beads were separated from the solution by centrifugation and washed several times with an ethanol–water mixture. The wavelength used for copper for maximum absorbance was 324.8 nm. The instru-

ment response was periodically checked with known Cu(II) solution standards. The loading experiments were performed in replicates of three and the samples were analyzed in replicates of three as well. For each set of data present, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples to determine the margin of error. The amount of adsorption per unit mass of the beads was evaluated using the following expression:

$$q = [(C_0 - C)V]/m \quad (1)$$

Here, q is the amount of Cu(II) ions adsorbed onto the unit mass of the beads (mmol/g); C_0 and C are the concentrations of the Cu(II) ions in the initial solution and in the aqueous phase after treatment for a certain period of time, respectively (mol/L); V, the volume of the aqueous phase (mL); and m, the mass of the beads used (g).

Cu(II) leakage from the beads was investigated with media containing NaCl at different ionic strengths (0.01 and 0.1) and pH (3.0–9.0) and also in a medium containing 0.5*M* NaSCN, at pH 8.0. The bead suspensions were stirred 24 h at room temperature. The Cu(II) ion concentration was then determined for the supernatants using an atomic absorption spectrophotometer.

Characterization of p(EGDMA–HEMA–MAH) beads

Surface-area measurements

The specific surface area of the p(EGDMA–HEMA–MAH) beads was determined in a BET apparatus. The average size and size distribution of the p(EGDMA–HEMA–MAH) beads were determined by screen analysis performed using Tyler standard sieves.

Surface morphology

The surface morphology of the polymeric beads was examined using scanning electron microscopy (SEM). The samples were initially dried in air at 25°C for 7 days before being analyzed. A fragment of the dried bead was mounted on an SEM sample mount and was sputter-coated for 2 min. The sample was then mounted in a scanning electron microscope (Raster Electronen Microscopy, Leitz-AMR-1000, Germany). The surface of the sample was then scanned at the desired magnification to study the morphology of the beads.

Swelling test

The swelling ratio of the p(EGDMA–HEMA–MAH) beads was determined in distilled water. The experiment was conducted as follows: Initially, the dry bead samples were carefully weighed before being placed in a 50-mL vial containing distilled water. The vial was put into an isothermal water bath with a fixed temperature (25°C) for 2 h. The bead sample was taken out from the water, wiped using a filter paper, and weighed. The weight ratio of dry and wet samples was recorded.

Elemental analysis

Elemental nitrogen analysis of the p(EGDMA–HEMA–MAH) beads was carried out at the TUBITAK Technical Services Laboratory (Ankara, Turkey), revealing that the incorporated MAH density was as follows: 1.6 mmol/g.

FTIR studies

FTIR spectra of the p(EGDMA–HEMA–MAH) beads was obtained using an FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry beads (about 0.1 g) were thoroughly mixed with KBr (0.1 g, IR grade, Merck) and pressed into pellet form. The FTIR spectrum was then recorded.

NMR studies

The proton NMR spectrum of the MAH monomer was taken in $CDCl_3$ on a JEOL GX-400 300-MHz instrument. The residual nondeuterated solvent ($CHCl_3$) served as an internal reference. Chemical shifts are reported in ppm (δ) downfield relative to $CHCl_3$.

HSA adsorption from aqueous solutions

The HSA adsorption experiments were carried out batchwise in the media at different pH values. The pH of the adsorption medium was varied between 3.0 and 9.0 using different buffer systems (0.1*M* CH_3COONa—CH_3COOH for pH 3.0–5.0, 0.1*M* K_2HPO_4—KH_2PO_4, for pH 6.0–7.0, and 0.1*M* $NaHCO_3$—H_2CO_3 for pH 8.0 and 9.0). The HSA concentration was varied between 0.5 and 6.0 mg/mL. In a typical adsorption experiment, HSA was dissolved in 10 mL of the buffer solution, and 100 mg of the polymer beads was added. Then, the adsorption experiments were conducted for 2 h (equilibrium time) at 4°C at a stirring rate of 100 rpm. At the end of this equilibrium period, HSA adsorption by the p(EGDMA–HEMA–MAH) beads was determined by measuring the initial and final concentrations of HSA within the adsorption medium using Coomassie Brilliant Blue as described by Bradford.[18]

HSA adsorption from human serum

HSA from human serum with p(EGDMA–HEMA–MAH) and Cu(II)-chelated p(EGDMA–HEMA–MAH) beads was studied batchwise. The blood was obtained from a healthy human donor. Blood samples were centrifuged at 500 g for 3 min at room temperature to separate the serum. The original serum of the donor contained 41.0 mg HSA/mL as determined by the bromocresol green (BCG) dye method at 628 nm.[19] The freshly separated human serum, 10 mL, was incubated with 100 mg of beads preequilibrated with phosphate buffer (pH 7.4) for 2 h. These experiments were conducted at 4°C and a stirring rate of 100 rpm. The amount of HSA adsorbed by the polymer beads was determined by measuring the initial and final concentration of HSA in the serum. Phosphate-buffered saline (PBS; pH, 7.4; NaCl, 0.9%) was used for dilution of the human serum.

To show dye specificity, competitive serum protein adsorption (i.e., albumin, fibrinogen, and immunoglobulin-G) was also studied. The polymer beads were incubated with a human serum-containing albumin (43.2 mg/mL), fibrinogen (2.1 mg/mL), and γ-globulin (14.6 mg/mL) at room temperature for 2 h. The total protein concentration was measured using the total protein reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, UK; Catalog Ref. No: 712076) at 540 nm, which is based on the Biuret reaction.[19] Chronometric determination of fibrinogen according to the Clauss method on serum was performed using a Fibrinogene-Kit (Ref No: 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France).[20] The HSA concentration was determined using a Ciba Corning albumin reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, UK; Catalog Ref. No: 229241), which is based on a BCG dye method.[20] The γ-globulin concentration was determined from the difference.

Desorption studies

The desorption of HSA was carried out using 0.05 Tris/HCl buffer containing 0.5*M* NaSCN at room temperature. The HSA-adsorbed polymer beads were placed in the desorption medium and stirred for 1 h, at 25°C, at a stirring rate of 100 rpm. The final HSA concentration within the desorption medium was determined using Coomassie Brilliant Blue as described by Bradford. The desorption ratio was calculated from the amount of HSA adsorbed on the beads and the final HSA concentration in the desorption medium. To test the reusability of the beads, adsorption–desorption was repeated five times using the same polymeric sorbent. After each HSA adsorption–desorption experiment, the Cu(II) ions was stripped with 25 m*M* EDTA at pH 4.9, and the Cu(II) adsorption procedure was applied again. For sterilization, the beads were washed with 50 a *M* NaOH solution after the desorption.

To examine the effects of the adsorption conditions on possible albumin denaturation, fluorescence spectra of the native albumin, heat-denaturated albumin, and desorbed albumin were obtained. A native albumin aqueous solution (1 mg/mL; pH, 7.4; ionic strength, 0.1) was incubated at 70°C for 90 min for heat denaturation. Fluorimetric measurements were taken with a Schimadzu RF-5301 spectro-fluorometer using 1-cm^2 quartz cells. Monochromatic readings were taken from a digital display with a 0.25-s time constant and a 2-nm band width on the excitation side and 2 nm on the emission side. Initial calibration was carried out with a standard solution of albumin in PBS with 280-nm fluorescence excitation and 342-nm emission wavelengths.

RESULTS AND DISCUSSION

Properties of polymer beads

The specific surface area of the p(EGDMA–HEMA–MAH) beads was found to be 17.6 m^2/g. The p(EGDMA–HEMA–MAH) beads are crosslinked hydrophilic matrices. The equilibrium swelling ratio (the ratio of the weights of the beads before and after swelling) of the beads used in this study was 65% (w/w). Compared with p(EGDMA–HEMA) (55%),[21] the swelling ratio of the p(EGDMA–HEMA–MAH) beads increase (65%). Several possible structural factors may contribute to this experimental result. First, incorporating MAH actually introduces more hydrophilic functional groups into the polymer chain, which can interact with more water molecules in the polymer matrices. Second, reacting MAH with EGDMA and HEMA could effectively decrease the molecular weight of the resulting polymer. Therefore, the water penetrates into the polymer chains more easily, resulting in an improvement of polymer swelling in aqueous solutions. However, it should be noted that these beads are rigid and strong enough, due to their crosslinked structure, to be suitable for column applications.

The surface morphology and internal structure of the p(EGDMA–HEMA–MAH) beads are exemplified by the electron micrographs in Figure 1. As clearly seen here, the beads have a spherical form and rough surface due to the pores which formed during the polymerization procedure. The photograph in Figure 1(B) was taken with broken beads to observe the internal part of the polymeric structure. The presence of pores within the bead interior is clearly seen in this photograph. It can be concluded that the p(EGDMA–HEMA–MAH) beads have a porous interior surrounded by a reasonably rough surface, in the dry

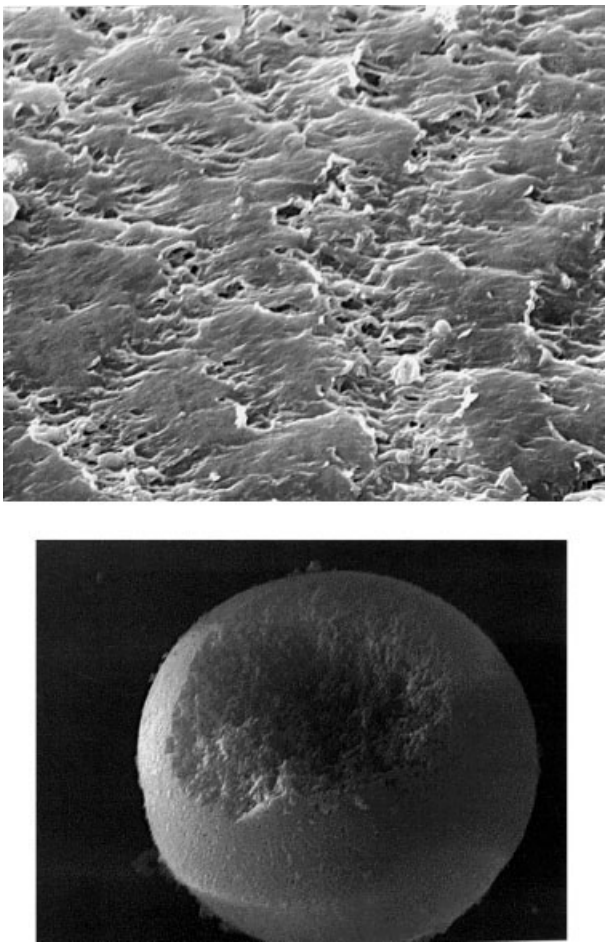

Figure 1 SEM micrographs of p(EGDMA–HEMA–MAH) beads: (A) surface; (B) internal structure.

state. The roughness of the surface should be considered as a factor providing an increase in the surface area. In addition, these pores reduce diffusional resistance and facilitate mass transfer because of their high internal surface area. This also provides higher metal chelation and albumin adsorption capacity.

MAH was synthesized as the metal-complexing ligand and/or comonomer. In the first step, MAH was synthesized from histidine and methacryloyl chloride. Then, MAH was incorporated into the bulk structure of the p(EGDMA–HEMA) beads. FTIR spectra of both MAH and p(EGDMA–HEMA–MAH) have the characteristic stretching vibration band of hydrogen-bonded alcohol, O—H, around 3480 cm^{-1}. The FTIR spectra of p(EGDMA–HEMA–MAH) have characteristic amide I and amide II absorption bands at 1645 and 1516 cm^{-1}, respectively. On the other hand, the hydrogen-bonded alcohol O—H stretching band intensity of plain pHEMA is higher than that of the p(EGDMA–HEMA–MAH) beads due to the incorporation of the MAH comonomer in the polymer structure.

To evaluate the degree of MAH incorporation into the polymer structure, elemental analysis of the synthesized p(EGDMA–HEMA–MAH) was performed. The incorporation of the MAH was found to be 1.6 mmol MAH/g from the nitrogen stoichiometry.

^{1}H-NMR was used to determine the synthesis of the MAH structure. The ^{1}H-NMR spectrum is shown to indicate the characteristic peaks from the groups in the MAH monomer. These characteristic peaks are as follows:

^{1}H-NMR ($CDCl_3$): δ 2.84 (t; 3H, J = 7.08 Hz, CH_3), 3.07–3.21 (m; 2H, CH_2), 4.82–4.87 (m; 1H, methin), 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl); 6.26 (δ; 1H, J = 7.4 Hz, NH), 7.06–7.22 (m; 5H, aromatic), 10.09 (s; 1H, OH).

HSA adsorption from aqueous solutions

Effects of pH

Figure 2 shows the effects of pH on HSA adsorption, which is very significant. The amount of adsorbed HSA increased with an increasing pH. This is due to that histidine, cysteine, and tryptophan residues in the protein structure, which play an important role as a ligand, can coordinate to the Cu(II) ion at higher pH. As the pH of the solution protein solution decreases, the degree of protonation of these amino acid residues increases and their coordination ability decreases. The maximum HSA binding capacity of affinity beads was found to lie at 56.3 mg HSA/g, which was observed at pH 8.0 HSA is negatively charged at pH 8.0 (isoelectric point of HSA: 4.9). But it is interesting to note that the amount of HSA adsorbed onto MAH-containing beads shows a maximum at pH 8.0, with a very significant decrease at lower and higher pH values. The same behavior was reported in the literature for HSA

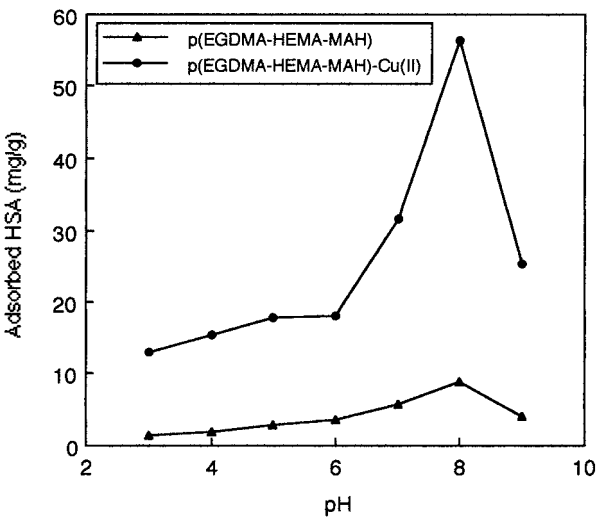

Figure 2 Effect of pH on the HSA adsorption on p(EGDMA–HEMA–MAH) beads: MAH incorporation: 1.6 mmol/g; Cu(II) loading: 0.96 mmol/g; HSA concentration: 5 mg/mL; T: 4°C. Each value is the average of five parallel studies.

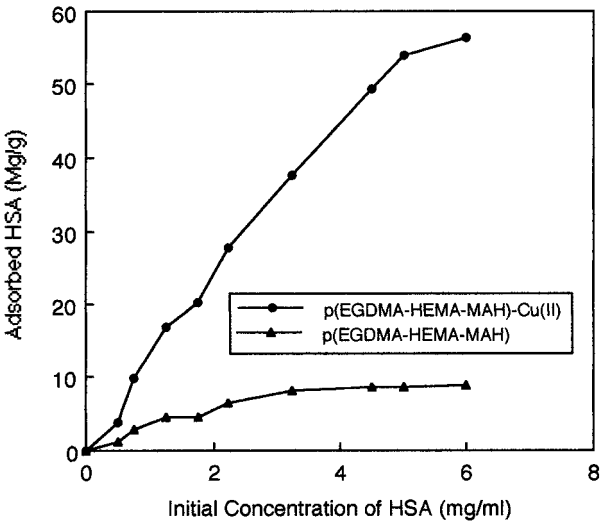

Figure 3 Effect of the concentration of HSA on adsorption: MAH Incorporation: 1.6 mmol/g; Cu(II) loading: 0.96 mmol/g; pH: 8.0; T: 4°C. Each value is the average of five parallel studies.

adsorption with a cellulose affinity membrane carrying iminodiaceteic acid/Cu(II).[22] On the other hand, HSA adsorption on p(EGDMA–HEMA–MAH) beads is independent of pH and it is observed almost at the same level at all the pH values studied. The adsorbed amount of HSA on p(EGDMA–HEMA–MAH) beads was found to be 8.8 mg/g.

Effect of HSA initial concentration

Figure 3 shows the effects of the initial concentration of HSA on the amount of HSA adsorbed. As seen in this figure, with increasing HSA concentration in the solution, the adsorbed amount of HSA per unit mass of polymer beads increases until about 5.0 mg/mL then approaches saturation. Small amounts of HSA were adsorbed on the p(EGDMA–HEMA–MAH) beads (8.8 mg/g). Cu(II) incorporation significantly increased the HSA adsorption capacity of the beads (to 56.3 mg HSA/g). It is clear that this increase in adsorption capacity is due to specific interactions between complexed Cu(II) ions and HSA molecules. Notably, the affinity between HSA molecules (especially the Imidazole group of the histidine residue, the thiol group of cysteine, and the Indoyl group of tryptophan) and attached Cu(II) Ions is primarily due to specific or electron donor–acceptor interactions. These specific interactions may result from the deprotonation of amino acid side chains (e.g., histidine and especially primary amines such as lysine) of HSA with immobilized Cu(II) Ions.

HSA adsorption from human serum

The adsorption of HSA from human serum was performed in a batchwise manner. Table I shows the adsorption for human serum obtained from a healthy donor. There was a low adsorption of HSA (12.4 mg/g) on the p(EGDMA–HEMA–MAH) beads, while much higher adsorption values (94.6 mg/g) were obtained when the Cu(II)-chelated p(EGDMA–HEMA–MAH) beads were used. It is worth noting that adsorption of HSA onto the Cu(II)-chelated p(EGDMA–HEMA–MAH) beads was approximately 1.7-fold higher than those obtained in the studies in which aqueous solutions were used. This may be explained as follows: The conformational structure of the HSA molecules within their native environment (i.e., human serum) is much more suitable for specific interaction with the Cu(II)-chelated p(EGDMA–HEMA–MAH) beads. The high HSA concentration (43.2 mg/mL) may also contribute to this high adsorption due to the high driving force between the aqueous (i.e., human serum) and solid phases (i.e., polymer beads).

Competitive protein adsorption was also carried out and interesting results were obtained in these studies. The adsorption capacities achieved were 94.6 mg/g for albumin, 3.7 mg/g for fibrinogen, and 8.5 mg/g for γ-globulin. The total protein adsorption was determined as 107.1 mg/g. It is worth noting that the adsorption of other blood proteins (i.e., fibrinogen and γ-globulin) on the p(EGDMA–HEMA–MAH) beads is negligible. It should be noted that HSA is the most abundant protein in serum. It generally makes up more than half of the total plasma proteins. It may be concluded that this low adsorption of fibrinogen and γ-globulin is due to the high concentration of HSA.

Comparison with other sorbents

Different metal-chelate affinity sorbents have been used for protein separation from various sources including human serum. Horstmann et al. used dye-attached Sepharose CL-6B and they reported bovine serum albumin (BSA) adsorption capacities around 5.4–12 mg per gram moist gel.[23] Denizli et al. used dye and metal-chelate sorbents including monosize poly(methyl methacrylate–hydroxyethyl methacrylate),

TABLE I
HSA Adsorption from the Serum of a Healthy Donor

HSA concentration (mg/mL)	Amount of HSA adsorbed[a] (mg/g)
1.4	11.3 ± 1.5
2.7	20.5 ± 1.9
5.4	33.5 ± 2.4
10.8	54.2 ± 2.2
21.6	78.6 ± 1.8
32.4	90.5 ± 2.1
43.2	94.6 ± 2.3

MAH Incorporation: 1.6 mmol/g; Cu(II) loading: 0.96 mmol/g; *T*: 4°C.

[a] Each value is the average of five parallel studies.

PVAL, and poly(hydroxyethyl methacrylate) beads and they obtained 35–178 mg per gram polymer for HSA.[24–28] Nash and Chase used PVAL-modified poly(styrene–divinylbenzene) beads carrying different dye ligands.[29] They presented adsorption capacities of 11.7–27 mg HSA/g. 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 per gram polymer.[30] Zeng and Ruckenstein reported a 10.2 mg HSA/g adsorption capacity with Cibacron Blue F3GA-attached polyethersulfone-supported chitosan sorbents.[31] Li and Spencer used Cibacron Blue F3GA-attached polyethyleneimine-coated titania and achieved 4.4 mg HSA/g.[32] Chase reached 14 mg BSA/g with Cibacron Blue F3GA-attached Sepharose CL-6B.[33] Tuncel et al. reported 60 mg BSA/g adsorption capacity with Cibacron Blue F3GA-attached PVAL-coated monosize polystyrene beads.[34] Muller-Schulte et al. used several polymeric carriers made of different polymers and Cibacron Blue F3GA as the dye-ligand.[35] Their albumin adsorption values were in the range of 0.19–0.81 mg HSA per mL sorbent. McCreath et al. developed liquid pefluorocarbon chromatographic supports carrying C.I. Reactive Blue 4 and the maximum capacity of the flocculated emulsion for HSA was found to be 1.81 mg/mL.[36] Uzun and Denizli modified microporous polyamide hollow fibers by acid hydrolysis to determine the reactive groups and subsequent binding of Cibacron Blue F3GA. Then, they loaded different metal ions (i.e., Cu^{2+}, Ni^{2+}, Co^{2+}). The maximum adsorption capacity was in the range of 195–289 mg/g.[37] These results shows that Cu(II)-chelated poly(EGDMA–HEMA–MAH) beads exhibit comparable HSA adsorption capacities.

Desorption studies

Desorption of HSA from the p(EGDMA–HEMA–MAH) beads was also carried out in a batch system. The desorptions of HSA are expressed in percent of totally adsorbed HSA. Up to 96.7% of the adsorbed HSA was desorbed by using 0.1*M* Tris/HCI buffer containing 0.5*M* NaSCN as an elution agent. The addition of an elution agent changed the charge of the peptide side groups due to their isoelectric points, resulting in the detachment of the HSA molecules from the Cu(II) ions. Note that there was no Cu(II) release in this case which shows that Cu(II) ions were chelated strongly to MAH-containing beads. With the desorption data given above, we conclude that Tris/HCI buffer is a suitable desorption agent and allows repeated use of the affinity beads used in this study.

To show the reusability of the p(EGDMA–HEMA–MAH) beads, the adsorption–desorption cycle was repeated five times using the same affinity beads. There was no significant reduction in the adsorption capacity of the beads (Fig. 4) The HSA adsorption capacity decreased only 11.2% after five cycles. It should be noted that after each HSA adsorption–desorption experiment the Cu(II) ions were stripped with 25 m*M* EDTA at pH 4.9, and the Cu(II) adsorption procedure was applied again. By taking into account the different experimental parameters studied above, it should be possible to scale up the process of HSA separation by affinity chromatography on Cu(II)-chelated p(EGDMA–HEMA–MAH) beads.

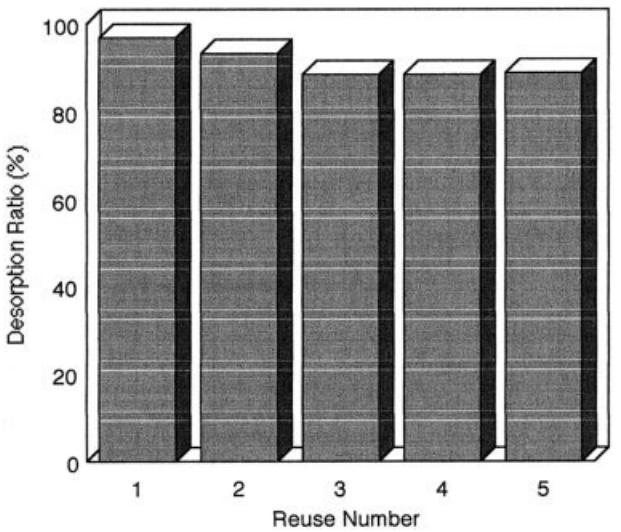

Figure 4 Repeated use of p(EGDMA–HEMA–MAH)–Cu(II) beads: MAH Incorporation: 1.6 mmol/g; Cu(II) loading: 0.96 mmol/g; pH: 8.0; T: 4°C. Each value is the average of five parallel studies.

To examine the effects of adsorption conditions on possible albumin denaturation, fluorescence spectrophotometry was employed. The fluorescence spectra of albumin samples obtained from the desorption step were recorded. The fluorescence spectra of native and heat-denaturated albumin (HSA reference materials) were also taken. The results showed a clear difference between the fluorescence spectra of native albumin and heat-denaturated albumin. An appreciable shift was seen in the maximum wavelength of denaturated albumin compared to the native one. On the other hand, fluorescence spectra of the samples withdrawn from the desorption step were very close to those of native albumin and no significant shift of maximum wavelength was detected in the spectra of these samples relative to that of native albumin. It may be concluded that metal chelate affinity chromatography with p(EGDMA–HEMA–MAH) beads can be applied for albumin separation without causing any denaturation.

CONCLUSIONS

Immobilized metal chelate affinity separation introduces a new approach for selectively interacting ma-

terials on the basis of their affinities for chelated transition metal ions. The separation is based on differential binding abilities of the biomolecules, to interact with chelated metal ions to a solid carrier. The number of histidine residues in the protein is of primary importance in the overall affinity for chelated metal ions. In addition, factors such as the accessibility, microenvironment of the binding residue (i.e., histidine, cysteine, and tryptophan), co-operation between neighboring amino acid side chains, and local conformations play important roles in bimolecule adsorption. Aromatic amino acids and the amino terminus of the peptides also have some contributions. The low cost of metals and the ease of regeneration of the sorbents are attractive features of metal affinity separation. This study documented the use of polymer beads containing MAH in metal chelate affinity chromatography. A novel metal-chelating ligand and/or comonomer, MAH, was synthesized and then polymerized with EGDMA and HEMA to have a loading up to 1.6 mmol MAH/g, which resulted in an HSA adsorption of 8.8 mg/g from aqueous solutions. Cu(II) chelation significantly increased HSA adsorption (56.3 mg/g). Remarkable increases in the HSA adsorption capacities were achieved from human serum (to 94.6 mg/g). High desorption ratios (to 98% of the adsorbed HlgG) were achieved by using 0.1*M* Tris/HCl buffer containing 0.5*M* NaSCN. It was possible to reuse these Cu(II)-chelated affinity sorbents without significantly reducing the adsorption capacities.

References

1. Putnam, F. W. The Plasma Proteins—Structure, Function and Genetic Control; Academic: New York, 1975; Vol. 1.
2. Norbert, W. Fundamentals of Clinical Chemistry; W. B. Saunders: London, UK, 1976.
3. He, X. M.; Carter, D. C. Nature 1992, 358, 209.
4. Travis, J.; Bowen, J.; Tewksbury, D.; Johnson, D.; Pannel A. J. Biochem 1976, 157, 301.
5. Zachariou, M.; Hearn, M. T. W. J Chromatogr A 2000, 890, 95.
6. Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. Nature 1975, 258, 598.
7. Şenel, S.; Kassab, A.; Arica, M. Y.; Say, R.; Denizli, A. Coll Surf B 2002, 24, 265.
8. Garipcan, B.; Bereli, N.; Patir, S.; Arica, Y.; Denizli, A. Macromol Biosci 2001, 1, 332.
9. Chaga, G. S. J Biochem Biophys Methods 2001, 49, 313.
10. Gaberc-Porekar, V.; Menart, V. J Biochem Biophys Methods 2001, 49, 335.
11. Harding, M. M. Acta Crystallogr D 1999, 855, 1432.
12. Odabasi, M.; Denizli, A. J Chromatogr B 2001, 760, 137.
13. Tighe, B. J. In Hydrogels in Medicine and Pharmacy; Peppas, N. A., Ed.; CRC: Boca Raton, FL, 1987; Vol. 2, p. 53.
14. Yavuz, H.; Arica, Y.; Denizli, A. J Appl Polym Sci 2001, 82, 186.
15. Kayirhan-Denizli, F.; Arica, Y.; Denizli, A. J Biomater Sci Polym Ed 2001, 12, 479.
16. Denizli, A. J Chromatogr B 2002, 772, 357.
17. Kayirhan, N.; Denizli, A.; Hasirci, N. J Appl Polym Sci 2001, 81, 1322.
18. Bradford, M. M. Anal Biochem 1976, 72, 248.
19. Tietz, N. W. Textbook of Clinical Chemistry; WB Saunders: Philadelphia, 1986.
20. Clauss, A. Acta Haemat 1957, 17, 237.
21. Denizli, A. J Appl Polym Sci 1999, 74, 655.
22. Kubota, N.; Nakagawa, Y.; Eguchi, Y. J Appl Polym Sci 1996, 62, 1153.
23. Horstmann, B.; J. Kenney, C. N.; Chase, H. A. J Chromtaogr 1986, 361, 179.
24. Denizli, A.; Köktürk, G.; Yavuz, H.; Pişkin, E. J Appl Polym Sci 1999, 74, 2803.
25. Denizli, A.; Tuncel, A.; Kozluca, A.; Ecevit, K.; Pişkin, E. Sep Sci Technol 1997, 32, 1003.
26. Denizli, A.; Denizli, F.; Pişkin, E. J Biomater Sci Polym Ed 1999, 10, 305.
27. Arica, M. Y.; Testereci, H. N.; Denizli, A. J Chromatogr A 1998, 799, 83.
28. Kassab, A.; Yavuz, H.; Odabaşi, M.; Denizli, A. J Chromatogr B 2000, 746, 123.
29. Nash, D. C.; Chase, H. A. J Chromatogr A 1997, 776, 55.
30. Boyer, P. M.; Hsu, J. T. Chem Eng J 1992, 47, 241.
31. Zeng, X.; Ruckenstein, E. J Membr Sci 1996, 117, 271.
32. Li, Y.; Spencer, H. G. In Polymers of Biological and Biomedical Significance: Shalaby, W., Ed., American Chemical Society: Washington, DC, 1994, pp 297–305.
33. Chase, H. A. J Chromatogr 1984, 297, 179.
34. Tuncel, A.; Denizli, A.; Purvis, D.; Lowe, C. R.; Pişkin, E. J Chromatogr 1993, 634, 161.
35. Muller-Schulte, D.; Manjini, S.; Vijayalakshmi, M. A. J Chromatogr 1991, 539, 307.
36. McCreath, G. E.; Chase, H. A.; Purvis, D. R.; Lowe, C. R. J Chromatogr 1993, 629, 201.
37. Uzun, L.; Denizli, A. J Appl Polym Sci 2002, 86, 3346.