Biotechnol. Bioprocess Eng. **2001**, 6: 402-409

Methacrylamidohistidine in Affinity Ligands for Immobilized Metal-ion Affinity Chromatography of Human Serum Albumin

Mehmet Odaba, Bora Garipcan, Semir Dede, and Adil Denizli*

Department of Chemistry, Biochemistry Division, Hacettepe University, Ankara, Turkey

Abstract Different bioligands carrying synthetic adsorbents have been reported in the literature for protein separation. We have developed a novel and new approach to obtain high protein adsorption capacity utilizing 2-methacrylamidohistidine (MAH) as a bioligand. MAH was synthesized by reacting methacrylochloride and histidine. Spherical beads with an average size of 150-200 μm were obtained by the radical suspension polymerization of MAH and 2-hydroxyethylmethacrylate (HEMA) conducted in an aqueous dispersion medium. p(HEMA-co-MAH) beads had a specific surface area of 17.6 m^2/g. Synthesized MAH monomer was characterized by NMR. p(HEMA-co-MAH) beads were characterized by swelling test, FTIR and elemental analysis. Then, Cu(II) ions were incorporated onto the beads and Cu(II) loading was found to be 0.96 mmol/g. These affinity 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(HEMA-co-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 plasma (94.6 mg HSA/g). Adsorption 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 0.1 M Tris/HCl buffer containing 0.5 M NaSCN. High desorption ratios (up to 98% of the adsorbed HSA) were observed. It was possible to reuse Cu(II) chelated-p(HEMA-co-MAH) beads without significant decreases in the adsorption capacities.

Keywords: histidine, p(HEMA-co-MAH), human serum albumin, metal chelates, affinity beads

INTRODUCTION

Human serum consists of hundred of proteins and other biological substances of varying molecular shape, size, mass, charge, hydrophobicity, isoelectric point and function. These proteins are commercially and therapeutically important components of human serum. Human serum albumin (HSA), for example, 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, streoids, 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 pre cipitation of proteins using ethanol with varying pH, ionic stength 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].

Pseudo-specific ligands can be used to purify a wide range of biomolecules, thus offering more flexibility as compared with biospecific ligands [6]. Especially, aminoacid molecules may hold certain advantages as ligands for industrial bioaffinity separations since they are not likely to cause an immune response in case of leakage into the product [7]. These ligands are also much more stable than protein ligands because they don't require a specific tertiary structure for maintaining biological activity [8,9]. The only drawback of aminoacid ligands appears to be their moderate selectivity for target protein. This problem will be overcome by introducing a new selectively interacting materials on the basis of their affinities for chelated metal ions. Such separations are generally based on the selective interaction between proteins containing one or preferably several adjacent histidine residues and chelated metal ions [10]. The number of histidine residues in the protein is of primary importance in the overall affinity with che-

* **Corresponding author**
Tel: +90-312-2992162 Fax: +90-312-2992162
e-mail: denizli@hacettepe.edu.tr

lated metal ions. In addition, factors such as the accessibility, micro-environment of the binding residue (*i.e.*, histidine, cysteine, aspartic acid, glutamic acid and tyrosine), co-operation between neighboring amino acid side chains and local conformations play important roles in biomolecule adsorption. Aromatic amino acids, free carboxylic groups and the amino-terminus of the peptides also have some contributions [11]. The low cost of metal ions and the ease of regeneration of the polymer-based adsorbents are the attractive features of metal affinity separation.

This work studies the performance of a new pseudo-specific and/or metal-complexing ligand MAH containing affinity adsorbents for HSA purification from human serum. The purification of HSA is generally required for the treatment of hypoproteinemia. p(HEMA-co-MAH) beads were prepared by radical suspension polymerization of HEMA and MAH monomers in the presence of an initiator (benzoyl peroxide, BPO). We present the adsorption-desorption properties of HSA onto plain and Cu(II)-chelated p(HEMA-co-MAH) beads from aqueous solutions containing different amounts of HSA and at different pHs, and also from human serum.

MATERIALS AND METHODS

Materials

Histidine and methacrylochloride were supplied by Sigma (St Louis, USA) and used as received. The monomers, 2-hydroxyethylmethacrylate (HEMA) and ethyleneglycoldimethacrylate (EGDMA) were obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4°C until use. Benzoyl peroxide (BPO) was obtained from Fluka (Switzerland). Poly-(vinylalcohol) (PVAL; MW: 100,000, 98% hydrolyzed) was supplied from Aldrich Chem. Co. (USA). Human serum albumin (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 from Merck (Darmstadt, Germany). Coomassie Blue for the Bradford Protein assay was from BioRad (Richmond, CA, USA). All water used in the experiments was purified using a Barnstead (Dubuque, IA, USA) 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.

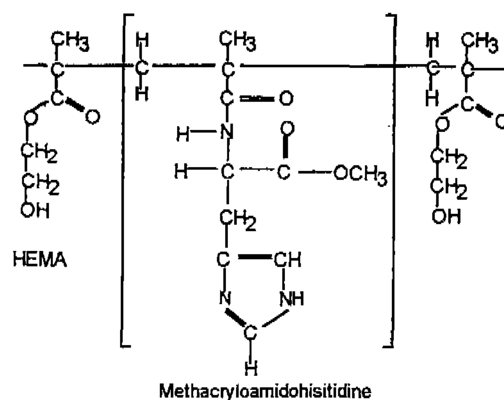
Synthesis of 2-Methacrylamidohistidine Monomer

For the synthesis of 2-methacrylamidohistidine (MAH) monomer, the following experimental procedure was applied: 5.0 g of histidine and 0.2 g of $NaNO_2$ were dissolved in 30 mL of K_2CO_3 aqueous solution (5%, v/v). This solution was cooled down to 0°C. Four mL of methacrylochloride was poured slowly into this solution under 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 ethylacetate. Aqueous phase was evaporated in a rotary evaporator. The residue (*i.e.*, MAH) was crystallized in ethanol and ethylacetate.

Preparation of p(HEMA-co-MAH) Beads

The comonomers, 2-hydroxyethylmethacrylate (HEMA) and MAH were copolymerized in suspension medium by using benzoyl peroxide (BPO) and poly(vinylalcohol) as the initiator and the stabilizer, respectively. Toluene and EGDMA were included in the polymerization recipe as the diluent (as a pore former) and crosslinker, respectively. Table 1 gives the polymerization recipe and experimental conditions to obtain copolymer beads with a swelling ratio of 65% and in the size range of 150-200 μm (in swollen form). At the end of the polymerization reaction, soluble components were removed from the polymer by repeated decantation with water and ethyl-alcohol. Polymer formula that was produced with HEMA and MAH monomers is as follows:

HEMA 2-methacrylamidohistidine



Cu(II) Loading

Loading of Cu(II) ions was carried out in a batch system. One hundred mL of aqueous 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 (NaOH) and nitric acid (HNO_3). The pH was maintained in a range of 0.1 units until equilibrium was attained. Investigations were made for pH value at 4.1. The concentration of the Cu(II) ions in the aqueous phase after the desired treatment periods was measured by using a graphite furnace atomic absorption spectrophotometer (AAS 5EA, Carl Zeiss Technology, Zeiss Analytical Systems, Germany). The wavelength used for copper for maximum absorbance was 324.8 nm. The instrument response was periodically

Table 1. Polymerization conditions for preparation of the p(HEMA-co-MAH) beads with a swelling ratio of 65% and in the size range of 150-200 μm

Aqueous Dispersion Phase	Organic Phase
50 mL of deionized water	1.0 g of MAH
0.2 g of PVAL	4.0 mL of HEMA
Polymerization Conditions	8.0 mL of EGDMA
Reactor volume: 100 mL	12 mL of toluene
Stirring Rate: 600 rpm	0.1 g of BPO

Temperature and Time: first at 65°C for 4 h, and then at 90°C for 2 h

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 in order to determine the margin of error. The amount of adsorption per unit mass of the beads was evaluated by using the following expression:

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

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

Cu(II) leakage from the Cu(II)-chelated p(HEMA-co-MAH) 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 of 8.0. The bead suspensions were stirred 24 h at room temperature. Cu(II) ion concentration was then determined in the supernatants using an atomic absorption spectrophotometer.

Characterization of p(HEMA-co-MAH) Beads

Surface Area Measurements

The specific surface area of the p(HEMA-co-MAH) beads was determined in BET apparatus. The average size and size distribution of the p(HEMA-co-MAH) beads were determined by screen analysis performed by using Tyler Standard Sieves.

Swelling Test

Water uptake ratio of the p(HEMA-co-MAH) bead was determined in distilled water. The experiment was conducted as follows: initially 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 ± 0.5°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(HEMA-co-MAH) beads was carried out at the TUBITAK Technical Services Laboratory (Ankara, Turkey), revealing that the incorporated MAPA density was as follow: 1.6 mmol/g.

FTIR Studies

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

NMR Studies

The proton NMR spectrum of MAH monomer was taken in $CDCl_3$ on a JEOL GX-400 300 MHz instrument. The residual non-deuterated 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). Human serum albumin concentration was varied between 0.5-6.0 mg/mL. In a typical adsorption experiment, HSA was dissolved in 10 mL of buffer solution, and 100 mg of 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(HEMA-co-MAH) beads was determined by measuring the initial and final concentration of HSA within the adsorption medium using Coomassie Brilliant Blue as described by Bradford [12].

HSA Adsorption from Human Serum

Human serum albumin adsorption from human plasma with p(HEMA-co-MAH) and Cu(II)-chelated p(HEMA-co-MAH) beads was studied batch wise. 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 bromocresol green (BCG) dye method at 628 nm [13]. Ten mL of the freshly separated human plasma was incubated with 100 mg of beads pre-equilibrated 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 polymer beads was determined by measuring the initial and final con-

centration of HSA in serum. Phosphate buffered saline (PBS, pH 7.4; NaCl, 0.9%) was used for dilution of human serum.

In order 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 plasma containing albumin (43.2 mg/mL), fibrinogen (2.1 mg/mL) and γ-globulin (14.6 mg/mL) at room temperature for 2 h. Total protein concentration was measured by using the total protein reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, UK) at 540 nm which based on Biuret reaction [13]. Chronometric determination of fibrinogen according to the Clauss method on plasma was performed by using Fibrinogene-Kit (bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France) [14]. Human serum albumin concentration was determined by using Ciba Corning Albumin Reagent (Ciba Corning Diagnostics Ltd., Halstead, Essex, UK) which based on bromocresol green (BCG) dye method [14]. 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 by using Coomassie Brilliant Blue as described by Bradford [12].

RESULTS AND DISCUSSION

Properties of Polymer Beads

The radical suspension polymerization procedure provided crosslinked p(HEMA-co-MAH) beads in the spherical form in the size range of 150-200 μm (Fig. 1). Specific surface area of the p(HEMA-co-MAH) beads was found to be 17.6 m^2/g. The p(HEMA-co-MAH) beads are crosslinked hydrophilic matrices. The equilibrium swelling ratio (the ratio of the masses of the beads before and after swelling) of the beads used in this study, which were prepared with the recipe given in Table 1, is 65% (w/w). Compared with pHEMA (55%) [15], the water uptake ratio of the p(HEMA-co-MAH) beads increases (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 more water molecules into polymer matrices. Second, reacting MAH with HEMA could effectively decrease the molecular weight of the resulting and reduce the crystallinity of the structure. Therefore, the water molecules penetrate into the polymer chains more easily, resulting in an improvement of polymer water uptake in aqueous solutions. However, it should be noted that these beads are quite rigid, and strong enough due to highly cross-linked structure therefore suitable for packed-bed column applications.

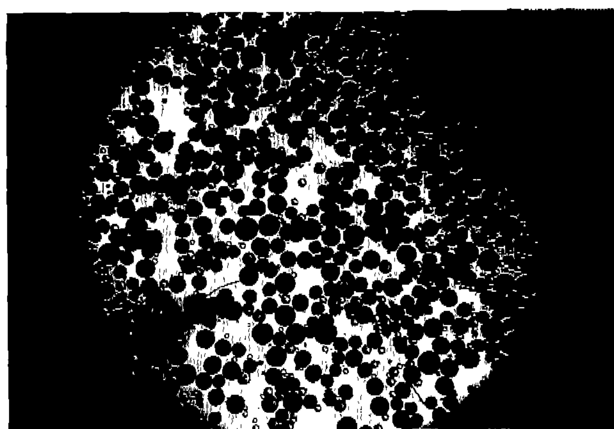


Fig. 1. Representative SEM micrograph of p(HEMA-co-MAH) beads.

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

To evaluate the degree of MAH incorporation, elemental analysis of the synthesized p(HEMA-co-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 MAH structure. ^{1}H-NMR spectrum is shown to indicate the characteristic peaks from the groups in MAH monomer. These characteristic peaks are as follows: ^{1}H-NMR ($CDCl_3$): d 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 (d; 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

Fig. 2 shows the effects of pH on HSA adsorption which is very significant. The amount of adsorbed HSA increased with increasing pH. This is due to the fact that histidine, cysteine and tryptophan residues in the

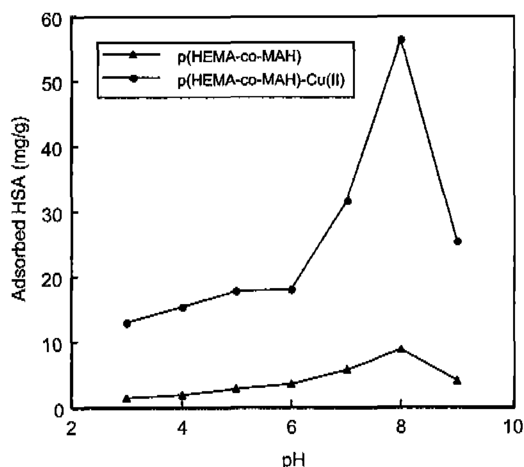


Fig. 2. Effect of pH on the HSA adsorption on p(HEMA-co-MAH) beads: MAH incorporation, 1.6 mmol/g; Cu(II) loading, 0.96 mmol/g; HSA Initial concentration, 5 mg/mL; T, 4°C. Each data is average of five parallel studies.

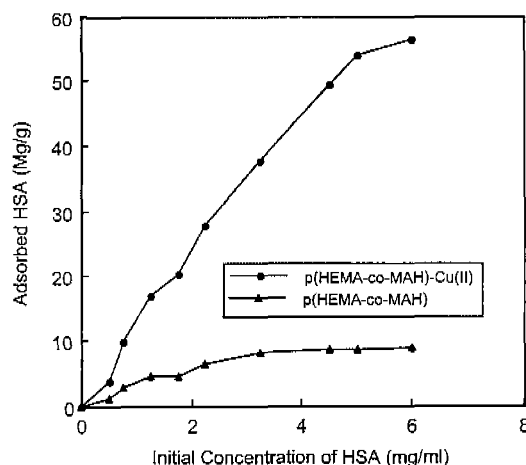


Fig. 3. Effect of the initial concentration of HSA on adsorption: MAH incorporation, 1.6 mmol/g; Cu(II) loading, 0.96 mmol/g; pH, 8.0; and T, 4°C. Each data is average of five parallel studies.

protein structure, which play an important role as a ligand, can coordinate to the Cu(II) ion at higher pH. As pH of the solution protein solution decreases, the degree of protonation of these amino acid residues increases and their coordination ability decrases. 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 behaviour was reported in literature for HSA adsorption with a cellulose affinity membrane carrying iminodiaceteic acid/Cu(II) [16]. On the other hand HSA adsorption on p(HEMA-co-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(HEMA-co-MAH) beads was found to be 8.8 mg/g.

Effect of HSA Initial Concentration

Fig. 3 shows the effects of initial concentration of HSA on the amount of HSA adsorbed. As seen in this figure, with increasing HSA concentration in solution, the adsorbed amount of HSA per unit mass of polymer beads until about 5.0 mg/mL, then approaches saturation. Small amounts of HSA adsorbed on the p(HEMA-co-MAH) beads (8.8 mg/g). Cu(II) incorporation significantly increased the HSA adsorption capacity of the polymer beads (up to 56.3 mg HSA/g). It is clear that this increase in adsorption capacity is due to specific interaction between immobilized Cu(II) ions and imidazole nitrogen donor atoms in the HSA molecules which promote the adsorption of protein.

Table 2. HSA adsorption from the plasma of a healthy donor: MAH Incorporation, 1.6 mmol/g; Cu(II) loading, 0.96 mmol/g; T, 4°C

HSA concentration (mg/mL)	Amount of HSA adsorbed* (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

* Each data is average of five parallel studies.

HSA Adsorption from Human Plasma

The adsorption of HSA from human plasma was performed in a batchwise. Table 2 shows the adsorption for human serum obtained from a healthy donor. There was a very low adsorption of HSA (12.4 mg/g) on the p(HEMA-co-MAH) beads, while much higher adsorption values (94.6 mg/g) were obtained when the Cu(II) chelated p(HEMA-co-MAH) beads were used. It is worth to note that adsorption of HSA onto the Cu(II) chelated p(HEMA-co-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 HSA molecule within their native environment (*i.e.* human plasma) much more suitable for specific interaction with the Cu(II) chelated p(HEMA-co-MAH) beads. The high HSA concentration (43.2 mg/mL) may also con tribute to this high adsorption due to the high driving force between the aqueous (*i.e.*, human plasma) and solid phases (*i.e.*, polymer beads).

Competitive protein adsorption was also carried out

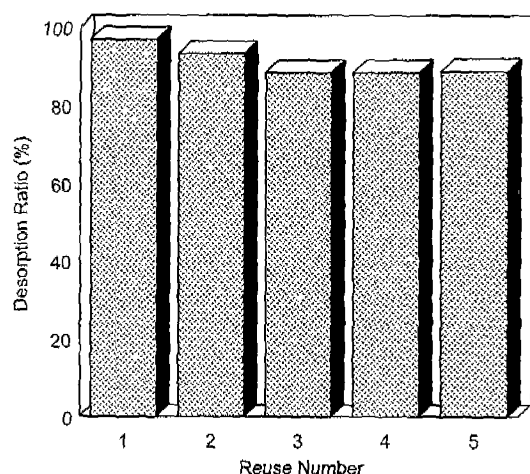


Fig. 4. Repeated use of p(HEMA-*co*-MAH)-Cu(II) beads: MAH incorporation, 1.6 mmol/g; Cu(II) loading, 0.96 mmol/g; pH, 8.0; and *T*, 4°C. Each data is average of five parallel studies.

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

Comparison with Other Adsorbents

Different pseudospecific and metal-chelate affinity adsorbents have been used for protein separation from various sources including human serum. Horstman *et al.* used dye-incorporated Sepharose CL-6B and they reported bovine serum albumin (BSA) adsorption capacities around 5.4-12 mg per gram moist gel [17]. Denizli *et al.* and his coworkers used different dye and metal-chelate polymeric adsorbents including monosize poly(methylmethacrylate-co-hydroxyethylmethacrylate), poly(vinyl alcohol) and poly(hydroxyethylmethacrylate) beads and they obtained 35-178 mg per gram polymer for HSA [18-22]. Nash and Chase used poly(vinyl alcohol) modified poly(styrene-diviniyl benzene) microbeads carring different dye ligands [23]. 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 [24]. Zeng and Ruckenstein reported 10.2 mg HSA/g adsorption capacity with Cibacron Blue F3GA-attached-polyethersulfone supported chitosan sorbents [25]. Li and Spencer used Cibacron Blue F3GA-attached polyethylene imine coated titania and achieved 4.4 mg HSA/g [26]. Chase reached 14 mg BSA/g with Cibacron Blue F3GA-attached Sepharose CL-6B [27]. Tuncel *et al.* reported 60 mg BSA/g adsorption capacity with Cibacron Blue F3GA-attached poly(vinyl-alcohol)-coated monosize polystyrene microbeads [28,29]. Muller-Shulte *et al.* used several polymeric carriers made of different polymers, and Cibacron Blue F3GA as the dye-ligand [30]. 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 [31]. Adsorption capacities of commercially available cross-linked agarose/Cibacron Blue F3GA sorbents (Bio-rad, California, USA) were reported as about 11 mg albumin per mL sorbent [32]. Comparison of these results shows that Cu(II)-chelated p(HEMA-co-MAH) beads exhibit higher human serum albumin adsorption capacities.

Desorption Studies

Desorption of HSA from the p(HEMA-co-MAH) beads was also carried out in batch system. The desorptions of HSA are expressed in % of totally adsorbed fibronectin. Up to 96.7% of the adsorbed HSA was desorbed by using 0.1 M Tris/HCl buffer containing 0.5 M NaSCN as elution agent. The addition of elution agent changed the charge of the peptide side groups due to their isoelectric points, resulting in the detachment of the HSA molecules from Cu(II) ions. Note that there was no Cu(II) release in this case which shows that Cu(II) ions are chelated strongly to MAH containing beads. With the desorption data given above we concluded that Tris/HCl buffer is a suitable desorption agent, and allows repeated use of the affinity beads used in this study. In order to show the reusability of the p(HEMA-co-MAH) beads, the adsorption-desorption cycle was repeated five times using the same affinity beads. There was no remarkable reduce in the adsorption capacity of the beads (Fig. 4). The HSA adsorption capacity decreased only 11.2% after five cycle. By taking into account the different experimental parameters studied above, it should be possible to scale up the process of HSA separation by bioaffinity chromatography on Cu(II)-chelated p(HEMA-co-MAH) beads.

CONCLUSION

Immobilized metal chelate affinity separation introduces a new approach 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 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 immobilized metal ions. In addition, factors such as the accessibility, micro-

environment 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 polymer based adsorbents are the attractive features of metal affinity separation. This study documents the use of PHEMA beads containing 2-methacryloamidohistidine in metal chelate affinity chromatography. A novel metal-chelating comonomer, 2-methacrylamidohistidine (MAH) was synthesized and then copolymerized with HEMA to have a loading up to 1.6 mmol MAH/g, which resulted a HSA adsorption of 8.8 mg/g from aqueous solutions. Cu(II) chelation significanlty increased HSA adsorption (56.3 mg/g). A remarkable increase in the HSA adsorption capacities were achieved from human plasma (up to 94.6 mg/g). Succesful desorption ratios (up to 98% of the adsorbed HIgG) 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 adsorbents without remarkable reduce in the adsorption capacities.

REFERENCES

[1] Putnam, F. W. (1975) *The Plasma Proteins-Structure, Function and Genetic Control*, Vol. 1. Academic Press, New York, USA.

[2] Norbert, W. (1976) *Fundamental of Clinical Chemistry*. W. B. Saunders, London, UK.

[3] He, X. M. and D. C. Carter (1992) Atomic structure and chemistry of human serum albumin. *Nature* 358: 209-315.

[4] Travis, J., J. Bowen, D. Tewksbury, D. Johnson, and R. Pannel (1976) Isolation of albumin whole human plasma and fractionation of albumin depleted plasma. *J. Biochem.* 157: 301-306.

[5] Zachariou, M. and M. T. W. Hearn (2000) Adsorption and selectivity characteristics of several human serum proteins with immobilised hard Lewis metal-ion chelate adsorbents. *J. Chromatogr.* A890: 95-116.

[6] Clonis, Y. D., N. E. Labrou, Y. Ph. Kotsira, C. Mazitsos, S. Melissis, and G. Gogolas (2000) Biomimetic dyes as affinity chromatography tools in enzyme purification. *J. Chromatogr. A* 891: 33-44.

[7] Bueno, S. M. A., C. Legallais, K. Haupt, and M. A. Vijayalakshmi (1996) Experimental kinetic aspects of hollow fiber membrane based pseudobioaffinity filtration: process for IgG separation from human plasma. *J. Membr. Sci.* 117: 45-56.

[8] El-Kak, A., S. Manjini, and M. A. Vijayalakshmi (1992) Interaction of immunoglobulin G with immobilized histidine: mechanistic and kinetic aspects. *J. Chromatogr.* A604: 29-37.

[9] Huang, P. Y. and R. G. Carbonell (1999) Affinity chromatographic screening of soluble combinatorial peptide libraries. *Biotechnol. Bioeng.* 63: 633-641.

[10] Porath, J., J. Carlsson, I. Olisson, and G. Belfrage (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 259: 598-599.

[11] Harding, M. M. (1999) The geometry of metal-ligand interactions relevant to proteins. *Acta Crys.* D55: 1432-1443.

[12] Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-251.

[13] Tietz, N. W. (1986) *Textbook of Clinical Chemistry*, WB Saunders Comp. Philadelphia, USA.

[14] Clauss, A. (1957) *Acta Haemat.* 17: 237-242.

[15] Denizli, A. (1999) Heparin immobilized poly(2-hydroxyethylmethacrylate) based microspheres. *J. Appl. Polym. Sci.* 74: 655-662.

[16] Kubota, N., Y. Nakagawa, and Y. Eguchi (1996) Recovery of serum proteins using cellulosic affinity membrane modified by immobilization of Cu(II) ion. *J. Appl. Polym. Sci.* 62: 1153-1160.

[17] Horstmann, B. J., C. N. Kenney, and H. A. Chase (1986) Adsorption properties on sepharose affinity adsorbents of varying particle size. *J. Chromtaogr.* 361: 179-190.

[18] Denizli, A. G. Köktürk, H. Yavuz, and E. Pikin (1999) Dye ligand column chromatography: albumin adsorption from aqueous media and human plasma with poly-(EGDMA-HEMA) microbeads. *J. Appl. Polym. Sci.* 74: 2803-2810.

[19] Denizli, A., A. Tuncel, A. Kozluca, K. Ecevit, and E. Pikin (1997) Cibacron Blue F3GA attached poly(vinylalcohol) particles for specific albumin adsorption. *Sep. Sci. Technol.* 32: 1003-1015.

[20] Denizli, A., F. Denizli, and E. Pikin (1999) Diamine-Plasma treated and Cu(II)-incorporated poly(2-hydroxyethylmethacrylate) microbeads for albumin adsorption. *J. Biomater. Sci. Polym. Ed.* 10: 305-318.

[21] Arıca, M. Y., H. N. Testereci, and A. Denizli (1998) Dye-ligand and metal chelate poly(2-hydroxyethyl-methacrylate) membranes for affinity separation of proteins. *J. Chromatogr.* A799: 83-91.

[22] Kassab, A., H. Yavuz, M. Odaba, and A. Denizli (2000) Human serum albumin chromatography by cibacron blue F3GA-derived microporous polyamide hollow fibre affinity membranes. *J. Chromatogr.* B746: 123-132.

[23] Nash, D. C. and H. A. Chase (1997) Modification of polystyrenic matrices for the purification of proteins II: effect of the degree of glutaraldehyde-poly(vinylalcohol) crosslinking on various dye ligand chromatography systems. *J. Chromatogr. A* 77: 55-63.

[24] Boyer, P. M. and J. T. Hsu (1992) Effects of ligand concentration on protein adsorption in dye ligand adsorbents. *Chem. Eng. J.* 47: 241-251.

[25] Zeng, X. and E. Ruckenstein (1996) Supported chitosan-dye affinity membranes and their protein adsorption, *J. Membr. Sci.* 117: 271-278.

[26] Y. Li and H. G. Spencer (1994) Dye-grafted poly(ethylene imine)-coated, formed in place class affinity membranes for selective separation of proteins. pp. 297-305, In: W. Shalaby (ed.). *Polymers of Biological and Biomedical Significance.* ACS, Washington, DC, USA.

[27] Chase, H. A. (1984) Prediction of the performance of

preparative affinity chromatography. *J. Chromatogr.* 297: 179-202.

[28] Tuncel, A., A. Denizli, D. Purvis, C. R. Lowe, and E. Pikin (1993) Cibacron Blue-F3GA attached monosize polyvinylalcohol-coated polystyrene microspheres for specific albumin adsorption. *J. Chromatogr.* 634: 161-168.

[29] Tuncel, A., A. Denizli, M. Abdelaziz, H. Ayhan, and E. Pikin (1992) Albumin adsorption onto large size monodisperse polystyrene latices having functional groups on their surfaces. *Clin. Mat.* 11: 39-144.

[30] Muller-Schulte, D., S. Manjini, and M. A. Vijayalakshmi (1991) Comparative affinity chromatographic studies using novel grafted polyamide and poly(vinyl alcohol) media. *J. Chromatogr.* 539: 307-314.

[31] McCreath, G. E., H. A. Chase, D. R. Purvis, and C. R. Lowe (1993) Novel affinity separations based on a pefluorocarbon emulsions. *J. Chromatogr.* 629: 201-213.

[32] Bio-Rad (1995) Life Science Research Product Catalog.

[Received July 30, 2001; accepted November 27, 2001]