Congo Red- and Zn(II)-Derivatized Monosize Poly(MMA-HEMA) Microspheres as Specific Sorbent in Metal Chelate Affinity of Albumin

ADİL DENİZLİ,[1,†] GÜLAY KÖKTÜRK,[1] BEKİR SALİH,[2] AHMET KOZLUCA,[1] and ERHAN PİŞKİN[1,*]

[1]Chemical Engineering Department and Bioengineering Division and [2]Department of Chemistry, Hacettepe University, Ankara, Turkey

SYNOPSIS

Monosize poly(methylmethacrylate-hydroxyethylmethacrylate) [poly(MMA-HEMA)] microspheres (4 μm in diameter) were produced by dispersion copolymerization of MMA and HEMA in an ethanol-water medium. Congo Red was attached to the poly(MMA-HEMA) microspheres, covalently. These Congo Red–derivatized microspheres were characterized by optical microscopy, Fourier transform infrared spectroscopy, and elemental analysis. Then, Zn(II) ions were incorporated by chelating with the immobilized Congo Red molecules. Different amounts of Zn(II) ions [1.2–17.6 mg of Zn(II)/g of polymer] were conjugated on the microspheres by changing the initial concentration of Zn(II) ions and pH. Bovine serum albumin (BSA) adsorption on these microspheres from aqueous solutions containing different amounts of BSA at different pH and ionic strengths was investigated in batch reactors. The nonspecific BSA adsorption on the plain poly(MMA-HEMA) microspheres was very low (0.7 mg of BSA/g of polymer). Congo Red derivatization significantly increased the BSA adsorption (up to 35.8 mg of BSA/g of polymer). A further increase in the adsorption capacity (up to 61.0 mg of BSA/g of polymer) was observed when Zn(II) ions were incorporated. More than 90% of the adsorbed BSA was desorbed in 1 h in the desorption medium containing 1.0*M* NaSCN at pH 8.0. © 1997 John Wiley & Sons, Inc.

INTRODUCTION

Metal chelate affinity chromatography offers a new possibility for selectively extracting proteins on the basis of their affinities for chelated metal ions. The separation is based on the differential binding abilities of the proteins (e.g., enzymes) to interact with chelated metal attached to a solid carrier. Metal chelate affinity chromatography of proteins, with metal chelate linked to Sepharose, was first described by Porath et al.[1] Those authors reported a model system using Zn(II) and Cu(II) columns in tandem for the fractionation of human serum 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.[2,3] Metal chelate affinity chromatography has been used to provide immunologically and physicochemically pure α_2-HS glycoprotein from plasma.[4,5] Plasminogen activators from both normal tissue (human uterus) and human melanoma cells have been isolated by metal chelate affinity chromatography,[6] as have ovalbumin,[7] nucleoside diphosphatase,[8] human lactoferrin,[9] lectin,[10] interferon,[11] carboxypeptidase B,[12] collagenase,[13] and human fibrinogen.[14]

As in metal chelate affinity, carrier matrices are usually made of polymer microspheres. They have attracted the most attention because they may be easily produced in a wide variety of compositions and modified into specific sorbents, by the introduction of a variety of ligands. In the conventional applications, nonporous or porous polymer microspheres with average diameters of usually more than

* To whom correspondence should be addressed.
† Present address: Hacettepe University, Department of Chemistry, Biochemistry Division.

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Table I Polymerization Recipe and Conditions for the Production of Poly(MMA-HEMA)

Ingredients	
MMA	4.0 mL
HEMA	1.0 mL
AIBN	0.06 g
PVP	4.0 g
Ethanol	50.0 mL
Water	50.0 mL
Conditions	
Temperature and time	65°C for 24 h
Stirring rate	400 rpm

100 μm are used. These sorbents are usually used in fixed-bed columns (rarely in fluidized-bed columns). When the nonporous microspheres are used, only the outer surface of the microspheres is available for the incorporation of the ligands, which will give the specificity of these sorbents. Low surface area means low adsorption capacity. Note that the surface area of these large-size sorbent particles reported in the literature is an approximate value because these microspheres always have a size distribution, narrow or wide, depending on the production process. The size distribution may even change from one batch to another in the same production process. This is of course an important disadvantage because we cannot define the adsorption capacities on the basis of unit surface area, which means that a comparison of the adsorption capacities of these type sorbents would be rather relative and poor.

In order to increase the active surface area, porosity may be created within the microspheres. Commercially available polymer-based porous sorbents exhibit surface areas 200–500 m^2 or even larger per unit mass of the sorbent. However, these type of sorbents also have important disadvantages. First, the adsorption rates are much slower, mainly because of the pore diffusion resistance. In addition, the high active surface area of these sorbents is mainly due to the fine pores in the matrix, which are not available for large solute molecules. In other words, large molecules cannot penetrate within these pores and therefore cannot use the active surface area available, which means low adsorption capacities for large molecules.

In this study, we attempted to prepare specific sorbents carrying Congo Red and Zn(II) ions (in chelate form) for metal chelate affinity separation of proteins. Here, we present the preparation and characterization of these specific sorbents and discuss the results of albumin adsorption/desorption studies.

EXPERIMENTAL METHODS

Poly(MMA-HEMA) Microspheres

Monosize poly(methylmethacrylate-hydroxyethylmethacrylate) (MMA-HEMA) microspheres were produced by dispersion polymerization of MMA and HEMA in an ethanol-water medium.[15] The monomer, i.e., MMA (Rohm and Haas, Darmstadt, Germany), was treated with aqueous NaOH to remove the inhibitor and stored in a refrigerator until use. The comonomer, HEMA, supplied from Sigma (St. Louis, MO), was purified by passing through active alumina. Azobisisobutironitrile (AIBN) and polyvinylpyrrolidone (PVP) (molecular weight 30,000; Aldrich Chem. Co., Rockford, IL) were used as the initiator and the stabilizer, respectively. Ethanol (Merck, Germany) was selected as the diluent and was used without further purification. The dispersion medium was distilled water. The dispersion polymerization recipe and conditions to obtain monosize microspheres with a diameter of 4 μm are given in Table I. Figure 1 shows a representative optical picture of the monosize (relative standard deviation < 1%) poly(MMA-HEMA) microspheres with a diameter of 4 μm, which were obtained at the polymerization conditions given in Table I.

Congo Red Incorporation

Congo Red (300 mg; BDH, Poole, United Kingdom) was dissolved in 10 ml of water. This dye solution was added to the aqueous medium prepared by dispersing 3.0 g of poly(MMA-HEMA) in 90 ml of distilled water; 4.0 g of NaOH was then added. The medium was heated to 80°C in a sealed reactor and kept at this temperature for 4 h at a stirring rate of 400 rpm. The microspheres were filtered and washed

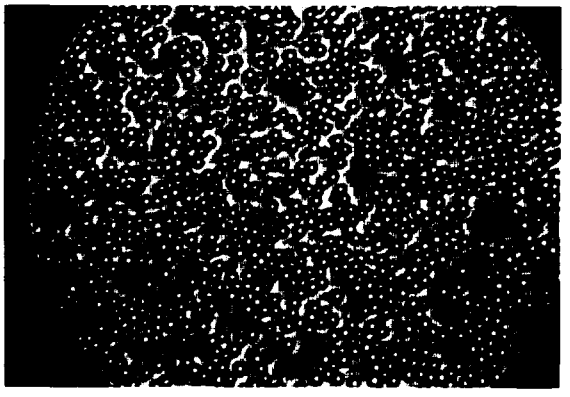

Figure 1 A representative optical micrograph of poly(MMA-HEMA) microspheres.

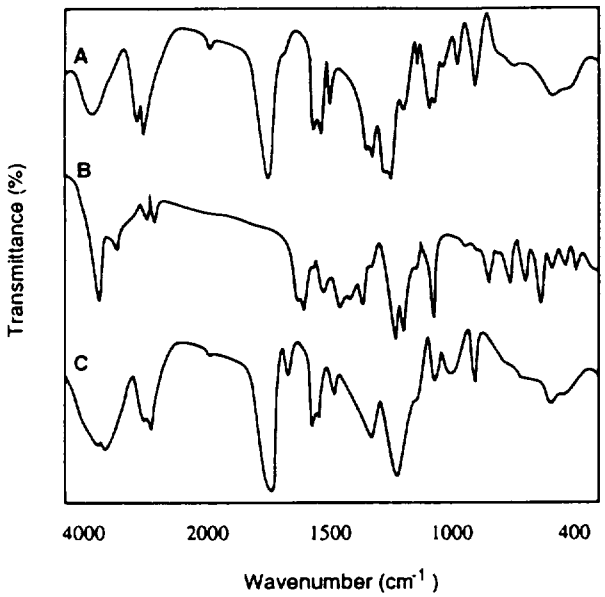

Figure 2 FT-IR spectra of: (A) poly(MMA-HEMA), (B) Congo Red, and (C) Congo Red–derivatized poly-(MMA-HEMA) microspheres.

with distilled water and methanol several times until all of the unbound dye was removed.

Dye leakage from the Congo Red–derivatized poly(MMA-HEMA) microspheres was investigated within the media containing NaCl at two different ionic strengths (i.e., 0.01 and 0.1) and at the selected pH in the range of 4.0–8.0. Note that these media were the same as those used in the protein adsorption experiments given below. Dye leakage was also determined in the medium at pH 8.0 and containing 1.0*M* NaSCN, which was the medium used in protein desorption experiments. The medium containing the Congo Red–derivatized microspheres was stirred for 24 h at room temperature, and then, polymeric microspheres were separated from the medium by filtration. The dye concentrations were measured in the liquid phase with a spectrophotometer at 497 nm.[16]

The size and size distribution of poly(MMA-HEMA) microspheres were measured with an optical microscope (Nikon, Alphapot YS, Chiyoda-Ku, Tokyo, Japan). The poly(MMA-HEMA) microspheres were filtered and dried in a vacuum oven, and then optical micrographs were taken.

The presence of Congo Red on the surface of the poly(MMA-HEMA) microspheres was confirmed by with a Fourier transform infrared (FTIR) spectrophotometer (Shimadzu, FTIR 8000 Series; Chiyoda-Ku, Tokyo, Japan). For FTIR studies, microspheres were washed several times with distilled water and dried in a vacuum oven. The microspheres (0.1 g) and dry KBr (0.1 g; IR Grade, Merck, Darmstadt, Germany) were througly mixed, the mixture was pressed to form a tablet, and the spectrum was recorded. In order to determine the amount of immobilized Congo Red on the poly(MMA-HEMA) microspheres, elemental analysis was performed with an elemental analysis instrument (CHNS-932, Leco).

Incorporation of Zn(II) Ions

Chelates of Congo Red microspheres with Zn(II) ions were prepared as follows: 100 mg of Congo Red–derivatized microspheres was mixed with aqueous solutions containing 2–350 ppm Zn(II) ion, at a constant pH of 6.8 (adjusted with universal buffer solution), which was the optimal pH for Zn(II) chelate formation at room temperature. $Zn(NO_3)_2$ 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, Victoria, Australia).

Zn(II) leakage from the Congo Red/Zn(II) microspheres was investigated in the media containing NaCl at two different ionic strengths (i.e., 0.01 and 0.1), at a pH value in the range of 4.0–8.0, and also in a medium containing 0.5*M* NaSCN, at a pH of 8.0. The microsphere suspensions were stirred for 24 h at room temperature. Zn(II) ion concentration was then determined in the supernatants with an atomic absorption spectrophotometer.

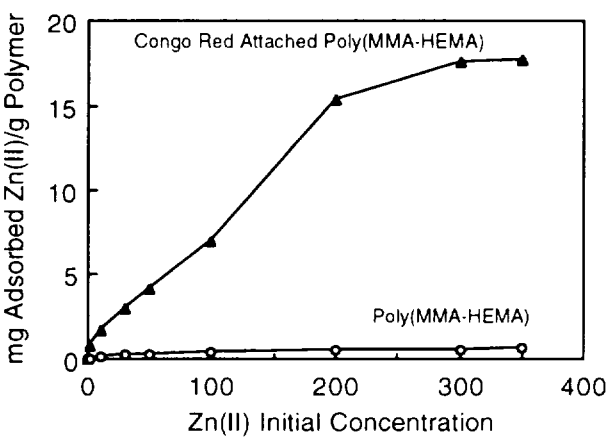

Figure 3 Incorporation of Zn(II) onto plain and Congo Red–derivatized poly(MMA-HEMA) microspheres as a function of Zn(II) ion concentration. Medium pH, 6.8.

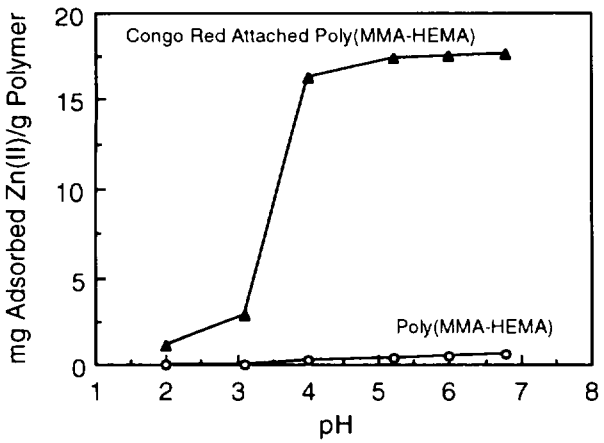

Figure 4 Incorporation of Zn(II) onto plain and Congo Red–derivatized poly(MMA-HEMA) microspheres as a function of medium pH. Zn(II) ion concentration, 300 ppm.

Albumin Adsorption and Desorption Studies

Bovine serum albumin (BSA, lyophilized, Fraction V; Sigma) was selected as a model protein. BSA adsorption on the plain, the Congo Red–derivatized, and the Congo Red/Zn(II)–derivatized poly(MMA-HEMA) microspheres was studied batchwise in the media containing different amounts of BSA with different ionic strengths at different pH. The pH of the adsorption medium was changed between 4.0 and 8.0 by the use of 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). Adsorption experiments were repeated at two different ionic strengths (0.01 and 0.1, adjusted by using NaCl). The initial BSA concentration was varied between 0.5 and 5.0 mg/mL. In a typical adsorption experiment, BSA was dissolved in 25 mL of buffer solution containing NaCl and 100 mg of microspheres was added. The adsorption experiments were conducted for 2 h at 25°C at a stirring rate of 100 rpm. At the end of the predetermined equilibrium period (i.e., 1 h), the microspheres were separated from the solution by centrifugation. The albumin adsorption capacity was determined by measuring the initial and final concentrations of BSA within the adsorption medium spectrophotometrically at 280 nm.[17]

The BSA desorption experiments were performed in a buffer solution containing 1.0*M* NaSCN at pH 8.0. The BSA-adsorbed microspheres 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 with a spectrophotometer. In the case of Zn(II)-carrying sorbents, the desorption of Zn(II) ions was also measured in the desorption media with an atomic absorption spectrophotometer. The desorption ratio was calculated from the amount of BSA adsorbed on the microspheres and the amount of BSA desorbed.

RESULTS AND DISCUSSION

Modified Poly(MMA-HEMA) Microspheres

FTIR spectroscopy was used to show the incorporation of Congo Red within poly(MMA-HEMA) microspheres. Figure 2 showed FTIR spectra of the plain and derivatized microspheres, with the dye itself for comparison. The bands observed at 1,630, 1,280, and 1,155 cm^{-1} indicated aromatic C=C vi-

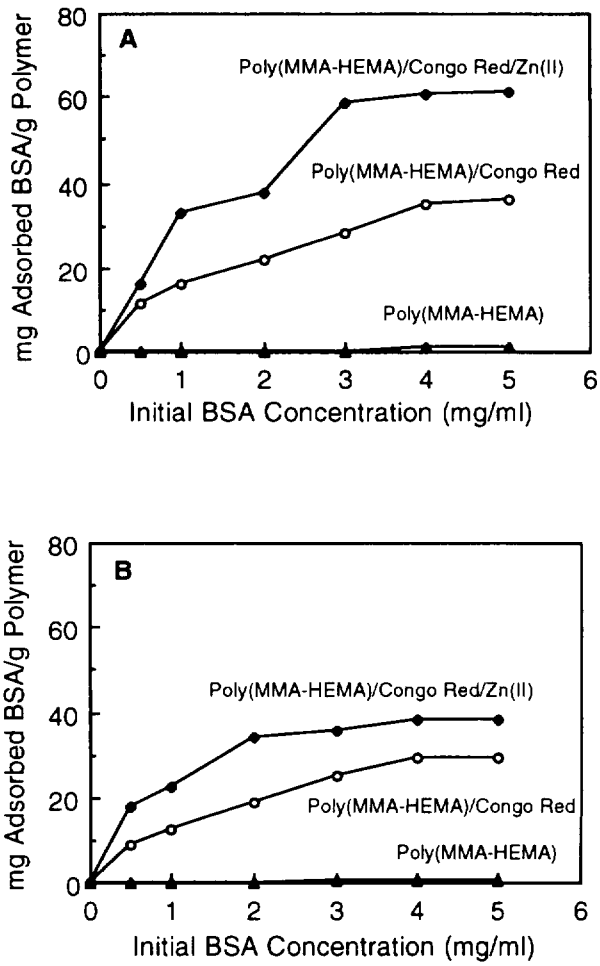

Figure 5 BSA adsorption on poly(MMA-HEMA) microspheres at two ionic strengths: (A) 0.01, (B) 0.1. pH, 5.0; salt used, NaCl; Congo Red loading, 4.2 μmol/g of polymer; Zn(II) loading, 17.6 mg/g of polymer.

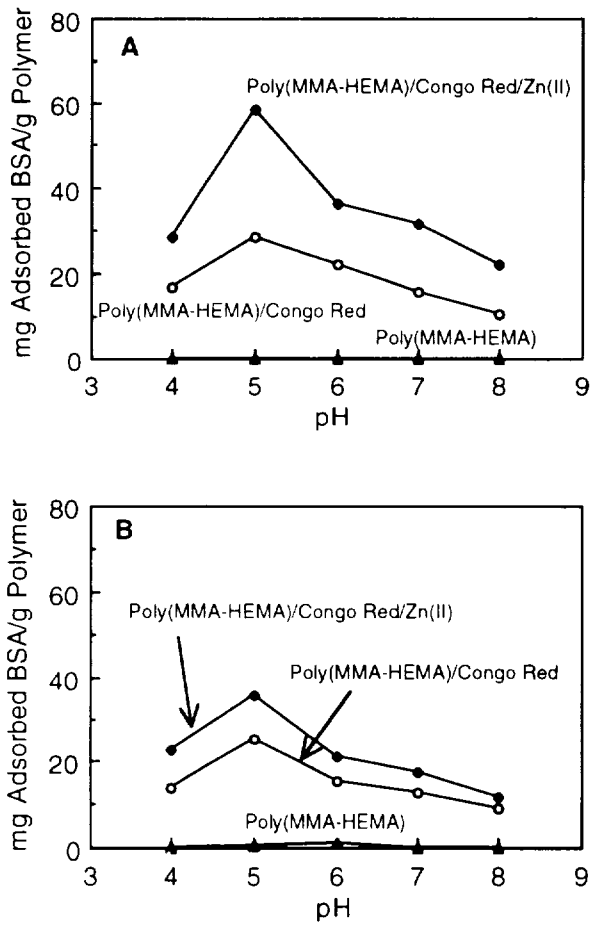

Figure 6 The variation of adsorption capacities of the poly(MMA-HEMA) microspheres with medium pH at two ionic strengths: (A) 0.01, (B) 0.1. BSA initial concentration, 3.0 mg/mL; salt used, NaCl. Congo Red loading, 4.2 μmol/g of polymer; Zn(II) loading, 17.6 mg/g of polymer.

bration, symmetric stretching of S=O, and asymmetric stretching of S=O [Fig. 2(C)]. In Figure 2(A), FTIR spectra of plain poly(MMA-HEMA) showed split bands at the same wave numbers, 1,280 and 1,155 cm^{-1}, which were observed on dye-derivatized polymer. However, FTIR spectra of dye-derivatized polymer indicated single bands at the same wave numbers because of the strong IR absorption of S=O groups on the structure of the dye. The band observed at 3,570 cm^{-1} indicated N—H and SO_3H groups, as was also pointed out on the chemical structure of Congo Red, given elsewhere.[18]

Plain and Congo Red microspheres were subjected to elemental analysis. The amount of Congo Red derivatized to the microspheres was evaluated from these data, by considering the stoichiometry, which was found as 4.2 μmol of dye/g of polymer.

The studies of Congo Red leakage from the derivatized microspheres showed that there was no leakage in any of the media described in the Experimental section, which assured that the washing procedure was quite satisfactory for the removal of uncovalently bound Congo Red molecules from the polymeric matrix.

Figure 3 shows the effects of Zn(II) ions concentration on the amount of Zn(II) ions adsorbed (chelated) on both the plain and the Congo Red–derivatized microspheres. There was very low Zn(II) adsorption [0.61 mg of Zn(II)/g of polymer] onto the plain poly(MMA-HEMA) microspheres, although the amount of ions adsorbed to the Congo Red microspheres increased with the Zn(II) ion concentration in solution. It reached a plateau value of 17.6 mg of Zn(II)/g of polymer at an aqueous concentration of 300 ppm, which represents saturation of the active points [which are available for Zn(II) ions] on the microspheres.

As seen in Figure 4, the adsorption of Zn(II) ions increased with increasing pH. The optimal pH value was 6.8. The nonspecific adsorption of Zn(II) ions was low, about 0.6 mg of Zn(II)/g of polymer, while the specific adsorption of Zn(II) ions, which was pH dependent, was much higher (17.6 mg of Zn(II)/g of polymer) than nonspecific adsorption. High adsorption at basic pH values implies that Zn(II) ions interact with Congo Red molecules not only through the nitrogen atoms by chelating, but also through $-SO_3H$ groups by cation exchange, which are inprotonated at high pH.

BSA Adsorption/Desorption

Adsorption

Figure 5 shows the effects of initial BSA concentration on adsorption, obtained at two different ionic strengths of 0.01 and 0.1, respectively, adjusted with NaCl. The pH of the adsorption medium was 5.0 (i.e., the isoelectric point of BSA), adjusted with the $CH_3COONa-CH_3COOH$ buffer system. Note that the amount of Zn(II) ions loaded on the Congo Red–derivatized poly(MMA-HEMA) microspheres was 17.6 mg of Zn(II)/g of polymer. As expected, BSA adsorption first increased with increasing initial concentration of BSA in the incubation medium and then reached a saturation value at an initial BSA concentration of 3.0 mg/mL, possibly as a result of the steric hindrance effect. The nonspecific BSA adsorption was very low (0.7 mg of BSA/g of polymer). Congo Red derivatization significantly increased the BSA adsorption capacity of the micro-

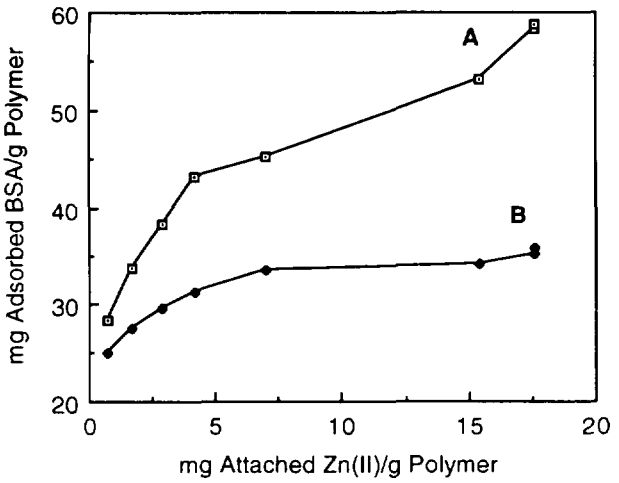

Figure 7 Effects of amount of Zn(II) conjugated onto microspheres on BSA adsorption at two different ionic strengths: (A) 0.01, (B) 0.1. BSA initial concentration, 3.0 mg/mL; salt used, NaCl; Congo Red loading, 4.2 μmol/g of polymer.

spheres (up to 35.8 mg of BSA/g of polymer), possibly because of the specific interactions between albumin and Congo Red molecules. A further significant increase (up to 61.0 mg of BSA/g of polymer) was noted when the Congo Red/Zn(II)–derivatized poly(MMA-HEMA) microspheres were used, in keeping with the objective of this study.

In order to show the effects of pH on BSA adsorption, adsorption experiments were repeated at different pH values between 4.0 and 8.0. Figure 6 shows the effects of pH obtained at two different ionic strengths, 0.01 and 0.1 (adjusted with NaCl). The maximum adsorption of BSA was observed at around its isoelectric point of pH 5.0. This may be because of preferential interaction between BSA molecules and Congo Red and Zn(II) ions at this pH. Significantly lower adsorption capacities were obtained with all microspheres 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.[19] These specific interactions may result both from the ionization states of several groups on both of the ligands [i.e., Congo Red and its chelator with Zn(II) ions] and albumin and from the conformational state of albumin at this pH, as discussed in the related literature.[20,21]

The BSA adsorption capacities of the Congo Red/Zn(II)-derivatized poly(MMA-HEMA) microspheres containing different amounts of Zn(II) [between 1.2 and 17.6 mg of Zn(II)/g of polymer] were investigated at pH 5.0. The initial concentration of BSA in the incubation solution was 3.0 mg/mL. Figure 7 shows the effects of Zn(II) conjugation onto poly(MMA-HEMA) microspheres on BSA adsorption. The amount of Zn(II) on the poly(MMA-HEMA) microspheres increased the amount of BSA adsorbed, which then reached an almost constant value. This may be the maximum amount of BSA that can be packed on the surface, owing to steric constraints.

The amount of BSA adsorbed on the microspheres was decreased by increasing the ionic strength in all cases discussed above. A similar tendency was also observed in our previous studies and by others.[22,23] This may be explained by the formation of more compact structures of the BSA molecules at high ionic strengths. More ions may also be attached to BSA molecules at high ionic strengths. This causes further stabilization of the protein molecules (higher solubility), which may lead to lower adsorption of albumin on the microspheres.

Desorption

The desorption of the adsorbed BSA from the Congo Red–derivatized poly(MMA-HEMA) and Congo Red/Zn(II)–derivatized poly(MMA-HEMA) microspheres was studied in a batch experimental set-up. The microspheres carrying different amounts of BSA were placed within the desorption medium containing 1.0*M* NaSCN at pH 8.0, and the amount of BSA and Zn(II) released in 1 h was determined. The desorption ratios for both BSA and Zn(II) were calculated by use of the following expression:

$$\text{desorption ratio (\%)} = \frac{\text{amount of BSA [or Zn(II)] released}}{\text{amount of BSA [or Zn(II)] adsorbed on the microspheres}} \times 100 \quad (1)$$

Table II gives the desorption data. More than 85% (up to 95%) of the adsorbed BSA was removed in all cases when NaSCN was used for desorption. Note that there was only negligable Zn(II) release in this case which shows that Zn(II) ions are attached to Congo Red molecules on the microsphere surface by strong chelate formation.

CONCLUSION

It can be concluded that the Congo Red— and Zn(II)-derivatized monosize poly(MMA-HEMA)

Table II Desorption of BSA and Zn(II) Ions

Microspheres	BSA Loaded (mg/g of polymer)	Zn(II) Loaded (mg/g of polymer)	Desorption Ratio for BSA (%)	Desorption Ratio for Zn(II) Ions (%)
Microsphere I[a]	35.8 ± 1.5	—	88 ± 1.5	—
Microsphere II[b]	61.0 ± 2.2	17.6 ± 0.1	91 ± 1.1	0

[a] Congo Red–carrying poly(MMA-HEMA) microspheres.
[b] Congo Red– and Zn(II)–carrying Poly(MMA-HEMA) microspheres.

microspheres may effectively [means with high adsorption capacities for both Zn(II) ions and BSA molecules] be used for metal chelate affinity separation of BSA. Adsorbed BSA can be desorbed with 1.0*M* NaSCN at pH 8.0.

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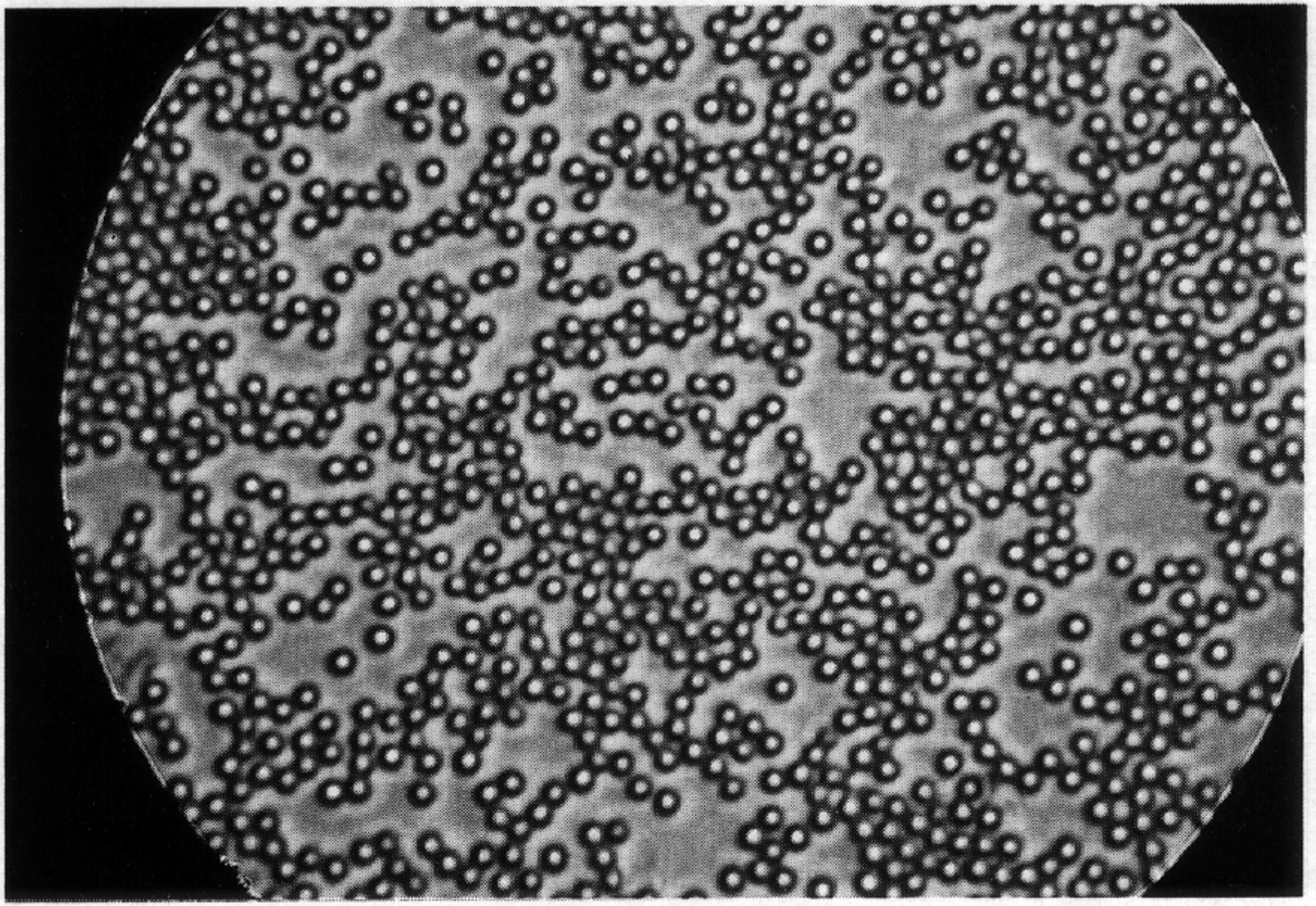

Figure 1 A representative optical micrograph of poly(MMA-HEMA) microspheres.