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Congo Red and Cu(II) carrying poly(ethylene glycol dimethacrylate–hydroxyethyl methacrylate) microbeads as specific sorbents Albumin adsorption/desorption

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Abstract

Poly(ethylene glycol dimethacrylate–hydroxyethyl methacrylate) [poly(EGDMA–HEMA)] microbeads, in the size range 150–200 μm, were produced by a modified suspension copolymerization of EGDMA and HEMA. Congo Red was attached covalently to the poly(EGDMA–HEMA) microbeads, then Cu(II) ions were incorporated within the microbeads by chelating with the immobilized dye molecules. Different amounts of Cu(II) ions [0.5–2.9 mg Cu(II)/g polymer] were conjugated on the microbeads by changing the initial concentration of Cu(II) ions, pH and ionic strength. Bovine serum albumin (BSA) adsorption on these microbeads from aqueous solutions containing different amounts of BSA at different pH and ionic strengths was investigated in batch reactors. The non-specific BSA adsorption on the poly(EGDMA–HEMA) microbeads was almost zero. Congo Red derivatization significantly increased the BSA adsorption (up to 90 mg BSA/g polymer). A further increase in the adsorption capacity (up to 136 mg BSA/g polymer) was observed when Cu(II) ions were incorporated. More than 90% of the adsorbed BSA was desorbed in 1 h in a desorption medium containing 0.5 *M* NaSCN at pH 8.0.

Keywords: Sorbents; Stationary phases, LC; Metal chelate affinity chromatography; Congo Red; Poly(ethylene glycol dimethacrylate–hydroxyethyl methacrylate); Copper; Albumin; Proteins

1. Introduction

Purification of proteins requires several steps involving methods which select on the basis of size, charge, hydrophobicity or specific affinity. The overall procedure will be efficient if the techniques separate according to those properties which best discriminate between the material of interest and the impurities.

Metal chelate affinity chromatography offers a new possibility for selectively extracting materials on the basis of their affinities for chelated metal ions. The separation is based on differential binding abilities of the proteins or enzymes to interact with chelated metal attached to a solid carrier.

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Metal chelate affinity chromatography of proteins, with the metal chelate linked to Sepharose, was first described by Porath et al. [1]. They 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 physico-chemically 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 [6,7], as have nucleoside diphosphatase [8], human lactoferrin [9], lectin [10], interferon [11] and carboxypeptidase B [12].

Recently, we prepared swellable poly(ethylene glycol dimethacrylate–hydroxyethyl methacrylate) [poly(EGDMA–HEMA)] microbeads for diverse applications [13,14]. We immobilized dye molecules (i.e., Congo Red and Alkaline Blue 6B) as ligands on these microbeads for the specific removal of heavy metal ions by chelation from aqueous solutions [15–18]. In this study, we attempted to prepare specific sorbents carrying Congo Red and Cu(II) ions (in chelate form) for dye affinity and metal chelate affinity separation of proteins. Bovine serum albumin (BSA) was selected as a model protein. Here we report the preparation and characterization of these specific sorbents, and discuss their BSA adsorption/desorption properties.

2. Experimental

2.1. Production of poly(EGDMA–HEMA) microbeads

Poly(EGDMA–HEMA) microbeads were selected as the base material for the synthesis of chromatographic microbeads, and produced by a modified suspension polymerization of the respective monomers, i.e., ethylene glycol dimethacrylate (EGDMA; Rohm) and hydroxyethyl methacrylate (HEMA; Sigma) in an aqueous medium. Benzoyl peroxide (BPO) and poly(vinyl alcohol) (PVAL) (M_r 100 000, 98% hydrolysed; Aldrich) were used as the initiator and the stabilizer, respectively. Toluene (Merck) was selected as the diluent and used without further purification. The dispersion medium was distilled water. The modified suspension polymerization recipe and conditions are given in Table 1. Other details relating to polymerization system and procedure were given elsewhere [13,14].

2.2. Congo Red derivatization of poly(EGDMA–HEMA) microbeads

Congo Red (BDH) (300 mg) was dissolved in 10 ml of water. This dye solution was added to the aqueous medium prepared by dispersing 3.0 g of poly(EGDMA–HEMA) in 90 ml of distilled water, then 4.0 g of NaOH were 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 microbeads were filtered and washed with distilled water and methanol several times until all the unbound dye was removed.

The leakage of the dye from the Congo Red-derivatized poly(EGDMA–HEMA) microbeads was investigated in media containing NaCl at two different ionic strengths (0.01 and 0.1) and at a selected pH in the range 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 a medium at pH 8.0

Table 1
Polymerization recipe and conditions for the production of poly(EGDMA–HEMA)

Parameter	Value
EGDMA	8.0 ml
HEMA	4.0 ml
BPO	0.06 g
PVAL	0.2 g
Toluene	12.0 ml
Temperature and time	65°C for 4 h and 90°C for 2 h
Stirring rate	600 rpm

and containing 0.5 *M* NaSCN, which was the medium used in protein desorption experiments. The medium containing the dye-derivatized microbeads was stirred for 24 h at room temperature, then polymeric microbeads were separated from the medium. The dye concentrations were measured in the liquid phase spectrophotometrically at 497 nm [15–18].

2.3. Characterization of microbeads

In order to obtain the size and size distribution of the poly(EGDMA–HEMA) microbeads, optical photographs were taken with an optical microscope (Nikon; Alphapot YS) equipped with a dark-field phase-contrast attachment.

The presence of Congo Red on the surface of the poly(EGDMA–HEMA) microbeads was confirmed using a Fourier transform (FT) IR spectrophotometer (Shimadzu; FTIR 8000 Series). For FTIR studies, the microbeads were washed several times with distilled water and dried in a vacuum oven. The microbeads (0.1 g) and dry KBr (IR Grade; Merck) (0.1 g) were thoroughly mixed, the mixture was pressed to form a tablet and the spectrum was recorded.

In order to determine the amount of immobilized dye on the poly(EGDMA–HEMA) microbeads, elemental analysis was performed by using an elemental analysis instrument (Leco; CHNS-932).

2.4. Chelation of Congo Red and Cu(II) ions

Chelates of Congo Red microbeads with Cu(II) ions were prepared as follows: 100 mg of Congo Red microbeads were mixed with aqueous solutions containing 1–30 ppm of Cu(II) ion at a constant pH of 4.1 (adjusted with universal buffer solution), which was the optimum pH for Cu(II) chelate formation [15,16,18] and at room temperature. $Cu(NO_3)_2$ was used as the source of Cu(II) ions. The flasks were agitated magnetically at 600 rpm for 1 h (sufficient to attain equilibrium). The concentration of the Cu(II) ions in the resulting solutions was determined with an atomic absorption spectrometer (GBC 932 AA) [15,18].

Cu(II) leakage from the Congo Red–Cu(II) microbeads was investigated in media containing NaCl at two different ionic strengths (0.01 and 0.1) and pH in the range 4.0–8.0, and also in a medium containing 0.5 *M* NaSCN at pH 8.0. The microbead suspensions were stirred for 24 h at room temperature. The Cu(II) ion concentration was then determined in the supernatants using the atomic absorption spectrometer.

2.5. Albumin adsorption and desorption studies

BSA (lyophilized, fraction V; Sigma) was selected as a model protein. BSA adsorption on the plain and the Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA) microbeads was studied batchwise in the media at different pH. The pH of the adsorption medium was varied between 4.0 and 8.0 by using different buffer systems (0.1 *M* CH_3COONa–CH_3COOH for pH 4.0–6.0, 0.1 *M* K_2HPO_4–KH_2PO_4 for pH 7.0 and 0.1 *M* aq. NH_3–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 7.0 mg/ml. In a typical adsorption experiment, BSA was dissolved in 25 ml of buffer solution containing NaCl and 0.2 g of microbeads were 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 microbeads 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 [19,20].

The BSA desorption experiments were performed in a buffer solution containing 0.5 *M* NaSCN at pH 8.0 or 25 m*M* EDTA at pH 4.9. The BSA-adsorbed microbeads were placed in the desorption medium and stirred for 1 h at 25°C at a stirring rate of 100 rpm. The final BSA concentration within the desorption medium was determined by spectrophotometry. In the case of Cu(II)-carrying sorbents, desorption of Cu(II) ions was also measured in the desorption media

by means of the atomic absorption spectrometer. The desorption ratio was calculated from the amount of BSA adsorbed on the microbeads and the amount of BSA desorbed.

3. Results and discussion

3.1. Poly(EGDMA–HEMA) microbeads

A representative optical micrograph of poly(EGDMA–HEMA) microbeads is given in Fig. 1A. Over 90% of these microbeads, which were produced by following the recipe given in Table 1, were in the size range 150–200 μm (i.e., swollen size). The other 10% containing smaller or larger microbeads was removed by sieving.

The swelling ratio, which is defined as

$$\text{swelling ratio (\%)} = \frac{\text{volume in swollen form} - \text{volume in dry form}}{\text{volume in dry form}} \cdot 100$$

obtained with this recipe was 55% [15,16]. Note that the microbeads are rigid and very strong because of the highly cross-linked matrix (due to EGDMA), and therefore they are suitable for column applications in chromatographic separations.

3.2. Congo Red derivatization of poly(EGDMA–HEMA) microbeads

A representative optical micrograph of Congo Red-derivatized poly(EGDMA–HEMA) microbeads is given in Fig. 1B. The light red colour of the microbeads clearly indicated the presence of dye on/in the microbeads.

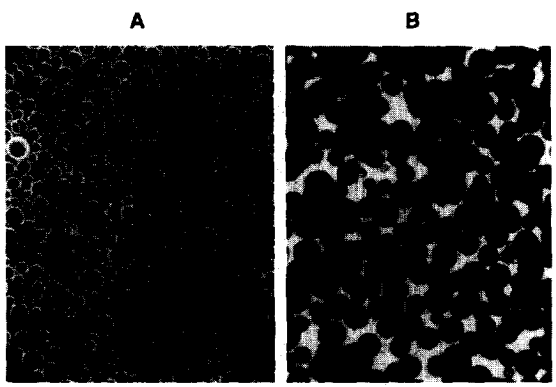

Fig. 1. Optical micrograph of poly(EGDMA–HEMA) microbeads: (A) plain; (B) Congo Red derivatized.

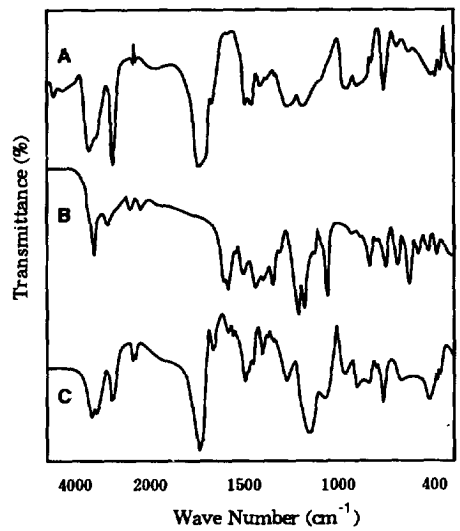

Fig. 2. FTIR spectra of (A) poly(EGDMA–HEMA); (B) Congo Red; (C) Congo Red-derivatized poly(EGDMA–HEMA) microbeads.

FTIR spectrometry was used to show the incorporation of Congo Red within the poly(EGDMA–HEMA) microbeads. Fig. 2 shows the FTIR spectra of the plain and derivatized microbeads, with the dye itself for comparison. The bands observed at 1075, 1155, 1280 and 3570 cm^{-1} indicated symmetric stretching of S=O, asymmetric stretching of S=O, aromatic C–N vibration and N–H and SO_3H groups, respectively, as also shown on the structure of Congo Red given in Fig. 3.

Plain and Congo Red-derivatized microbeads were subjected to elemental analysis. The amount of Congo Red derivatized on the microbeads was evaluated from these data, by considering the stoichiometry, which was found to be 14.5 μmol dye/g polymer.

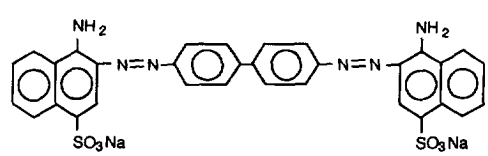

Fig. 3. Structure of Congo Red.

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

3.3. Congo Red/Cu(II)-derivatized poly(EGDMA–HEMA) microbeads

Fig. 4 shows the effects of Cu(II) ion concentration on the amount of Cu(II) ions adsorbed (chelated) on both the plain and Congo Red-derivatized microbeads. The amount of ions adsorbed on the latter increased with increasing concentration in solution. It reached a plateau value of 2.9 mg Cu(II)/g polymer at an aqueous concentration of 30 ppm. This is much greater than the adsorption capacity of the plain microbeads for Cu(II).

3.4. Albumin adsorption/desorption

3.4.1. Adsorption

Fig. 5 shows the effects of initial BSA concentration on adsorption, obtained at two different ionic strengths (0.01 and 0.1), adjusted with NaCl. The pH of the adsorption medium was 6.0, adjusted with the CH_3COONa–CH_3COOH buffer system. Note that the amount of Cu(II) ions loaded on the Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA) microbeads was 2.9 mg Cu(II)/g polymer. As expected, adsorption first increased with increasing initial concentration of BSA and then reached a saturation value at an initial BSA concentration of 3.0 mg/ml. The non-specific BSA adsorption was almost zero. Congo Red derivatization significantly increased the BSA adsorption capacity of the microbeads (up to 90 mg BSA/g polymer), possibly because of the specific interactions between albumin and Congo Red molecules. A further significant increase (up to 136 mg BSA/g polymer) was noted when the Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA) microbeads were used, in keeping with the objective of this study.

In order to establish the effects of pH on BSA adsorption, adsorption experiments were repeated at different pH values between 4.0 and 8.0. In these studies the initial concentration of BSA was 3 mg BSA/ml, and the Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA) microbeads containing 2.9 mg Cu (II)/g polymer were used. Fig. 6 shows the effects of pH obtained at two different ionic strengths, 0.01 and

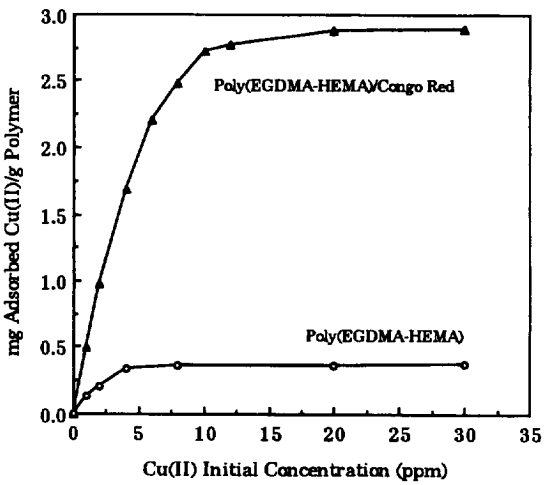

Fig. 4. Cu(II) adsorption capacities of plain and Congo Red-derivatized poly(EGDMA–HEMA) microbeads.

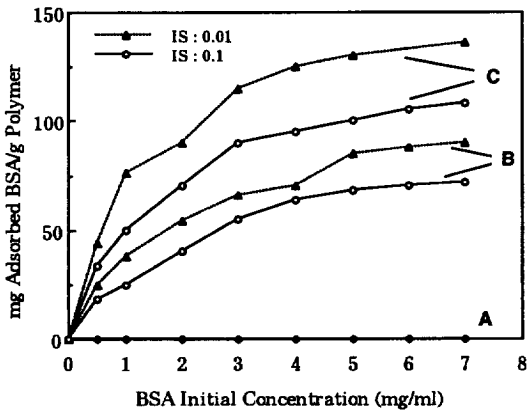

Fig. 5. BSA adsorption on microbeads at two different ionic strengths: (A) plain poly(EGDMA–HEMA); (B) Congo Red-derivatized poly(EGDMA–HEMA); (C) Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA).

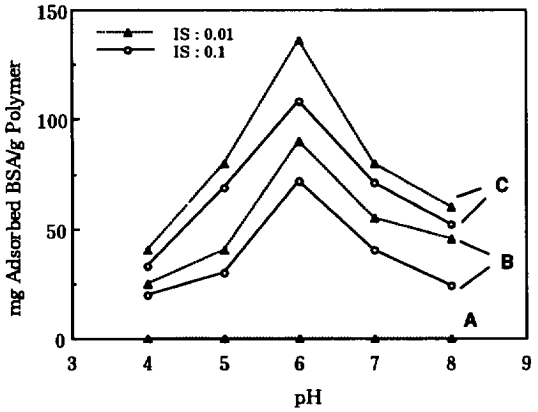

Fig. 6. Effects of pH on BSA adsorption at two different ionic strengths: (A) plain poly(EGDMA–HEMA); (B) Congo Red-derivatized poly(EGDMA–HEMA); (C) Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA).

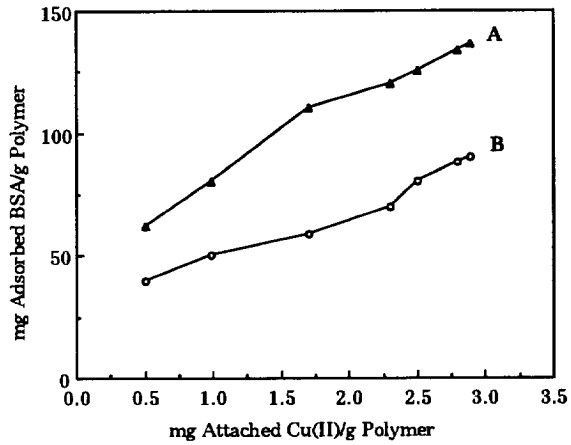

Fig. 7. Effects of amount of Cu(II) conjugated on microbeads on BSA adsorption at two different ionic strengths: (A) 0.01; (B) 0.1.

0.1 (adjusted with NaCl). In all the cases investigated, the maximum adsorption of BSA was observed at pH 6.0. Significantly lower adsorption capacities were obtained with all microbeads at more acidic and more alkaline pH. 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 isoelectric point [19,20]. The isoelectric pH of BSA is 5.0. In our case, the maximum adsorption was not at this pH, but rather shifted to a more alkaline value. This may be because of preferential interaction between albumin molecules and Congo Red and Cu(II) ions at this pH. These specific interactions may result both from the ionization states of several groups on both the ligands [i.e., Congo Red and its chelator with Cu(II) ions] and albumin, and from the conformational state of albumin at this pH, as usually discussed in the relevant literature [21,22].

Albumin adsorption capacities of the Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA) microbeads containing different amounts of Cu(II) (between 0.5 and 2.9 mg Cu(II)/g polymer) were investigated at pH 6.0. The initial concentration of BSA in the incubation solution was 3.0 mg/ml. Fig. 7 shows the effects of Cu(II) conjugated on to the microbeads on BSA adsorption. When the amount Cu(II) on the microbeads increased, the amount of BSA adsorbed first increased and then reached an almost a constant value. This may be the maximum amount of BSA that can be packed on the surface, owing to steric constraints.

The amount of albumin adsorbed on the microbeads was decreased by increasing the ionic strength in all cases discussed above. A similar tendency was also observed in our previous studies and also by others [23–25]. This may be explained by the formation of more compact structures of the albumin molecules at high ionic strengths. More ions may also be attached to albumin 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 microbeads.

3.4.2. Desorption

The desorption of the adsorbed BSA from the Congo Red-derivatized poly(EGDMA–HEMA) and Congo Red–Cu(II)-derivatized poly(EGDMA–HEMA) microbeads was studied in a batch experimental set-up. The microbeads carrying different amounts of BSA were placed in a desorption medium containing 0.5 *M* NaSCN at pH 8.0 or 25 m*M* EDTA at pH 4.9, and the amounts of BSA and Cu(II) released in 1 h were determined. The desorption ratios for

Table 2
Desorption of BSA and Cu(II) ions

Microbeads	BSA loaded (mg/g polymer)	Cu(II) loaded (mg/g polymer)	Desorption ratio for BSA (%)		Desorption ratio for Cu(II) ions (%)	
			With NaSCN	With EDTA	With NaSCN	With EDTA
I[a]	90	–	90	35	–	–
II[b]	136	2.9	94	98	0	100

[a] Congo Red-carrying poly(EGDMA–HEMA) microbeads.
[b] Congo Red–Cu(II)-carrying poly(EGDMA–HEMA) microbeads.

both BSA and Cu(II) were calculated by using the following expression:

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

Table 2 gives the desorption data. More than 90% (up to 95%) of the adsorbed BSA was removed in all cases when NaSCN was used for desorption. Note that there was only negligible Cu(II) release in this case, which shows that Cu(II) ions are attached to Congo Red molecules on the surface of the microbeads by strong chelate formation. However, when EDTA was used for desorption, only 35% of BSA was removed from Congo Red microbeads, maybe because of the salting-out effect. Under the same desorption conditions, about 98% of the BSA was desorbed from the Congo Red–Cu(II)-carrying microbeads. Note that in the latter case almost all of the Cu(II) ions initially loaded came out (were released) from the microbeads. This means that EDTA breaks down the chelates between Cu(II) ions and Congo Red.

With the desorption data given above, we concluded that NaSCN is a suitable desorption agent especially for the Congo Red–Cu(II)-carrying sorbents, and allows repeated use of the affinity sorbents developed in this study.

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