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CIBACRON BLUE F3GA AND Zn(II) CONTAINING POLY(ETHYLENE GLYCOL DIMETHACRYLATE-HYDROXYETHYL METHACRYLATE) MICROBEADS FOR ALBUMIN ADSORPTION

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ABSTRACT

Swellable poly (ethylene glycol dimethacrylate-hydroxyethyl methacryate) [poly(EGDMA-HEMA] microbeads, in the size range of 150-200 μm, were produced by a modified suspension copolymerization of EGDMA and HEMA. Cibacron Blue F3GA was attached, then Zn(II) ions were incorporated within the microbeads by chelating with the Cibacron Blue F3GA molecules. The maximum amount of Cibacron Blue F3GA attachment was 16.5 μmol/g polymer. Different amounts of Zn(II) ions (0.4-32.4 mg/g polymer were incorporated on the microbeads by changing the initial concentration of Zn(II) ions and medium pH. Bovine serum albumin (BSA) adsorption onto unmodified, Cibacron Blue F3GA-attached and Cibacron Blue F3GA-Zn(II)-derivatized microbeads was investigated. The non-specific adsorption of BSA was very low (0.051 mg/g polymer). Cibacron Blue F3GA attachment significantly increased the BSA adsorption (60.5 mg/g polymer). The maximum BSA adsorption (122 mg/g polymer) was observed

at pH 6.0 when Cibacron Blue F3GA-Zn(II)-derivatized microbeads were used. Desorption of BSA was achieved by using 25 mM EDTA (pH 4.9). High desorption ratios (up to 95% of the adsorbed BSA) were observed. It was possible to reuse Cibacron Blue F3GA-Zn(II)-derivatized microbeads without significant losses in the adsorption capacities.

INTRODUCTION

The chromatographic separation of proteins is important not only for analyzing proteins, but also for separating them in large scale industries, such as the food and drug industries. A number of chromatographic methods for separating proteins have been developed. Among these methods, metal chelate affinity technique has, in the past decade, found wide acceptance and application in the recovery of proteins due mainly to their high selectivity. Metal chelate affinity chromatography introduces an entirely new basis for separating proteins based on their affinity for transition metal ions. Many transition metal ions, i.e., zinc, copper, can coordinate to the amino acids such as histidine, cysteine and tryptophan via electron donor groups on the amino acid side chains [1-4].

The interaction of metal chelates with proteins containing surface exposed amino acids such as histidine, cysteine and tryptophan was first described by Porath et al [5]. Subsequent studies have shown the wide applicability of the technique and consistency of the methodology. The plasma proteins α_2-macroglobulin and α_1-proteinase inhibitor, for example, have been purified to homogeneity on zinc chelate columns [6,7]. Metal chelate affinity chromatography has been used to provide immunologically and physicochemically pure α_2-HS glycoprotein from plasma [8,9]. Plasminogen activators from both normal tissue (human uterus) and human melanoma cells have been isolated by metal chelate affinity chromatography [10,11] as have nucleoside diphosphatase [12],human lactoferrin [13], lectin [14], interferon [15], carboxypeptidase B [16].

In our recent studies, we have developed a series of swellable poly(EGDMA-HEMA) microbeads containing different chelating dyes (Alkali Blue 6B and Congo Red) and evaluated them as affinity sorbents for removal of heavy metal ions [Cd(II), Cu(II), Pb(II), and Zn(II)] (17-20). We also used Congo Red and Alkali Blue 6B attached poly(EGDMA-HEMA) microbeads for BSA adsorption [21, 26]. The purpose of this study was to further extend our earlier attempts to use the metal chelate affinity chromatography for adsorption of BSA.

We used Cibacron Blue F3GA and Zn(II) ions (in chelate form). Bovine Serum Albumin (BSA) was selected as a model protein. Here, we present BSA adsorption/desorption properties of these derivatized microbeads.

EXPERIMENTAL

Production of Cibacron Blue F3GA-Derivatized Microbeads

Poly(EGDMA-HEMA) microbeads were selected as the base material for the synthesis of chromatographic affinity matrix, and produced by a modified suspension polymerization of the respective monomers i.e., ethylene glycol dimethacrylate (EGDMA, Rohm, Germany) and 2-hydroxyethyl methacrylate (HEMA, Sigma, USA) in an aqueous media [17-19]. Benzoyl peroxide (BPO) and polyvinyl alcohol (PVAL) (M_n: 100.000, 98% hydrolyzed, Aldrich Chem. Co., USA) were used as the initiator and the stabilizer, respectively. Toluene (Merck, Germany) was selected as the diluent and used as received. Dispersion medium was distilled water. In order to produce polymeric microbeads of about 150-200 μm in diameter and with a narrow size distribution, the amounts of EGDMA, HEMA, toluene, water, BPO and PVAL were 8 ml, 4 ml, 12 ml, 50 ml, 0.06 g, 0.2 g, respectively. Polymerizations were carried out at an agitation rate of 600 rpm at 65°C for 4 hours and at 90°C for 2 hours. After cooling, the polymeric microbeads were separated from the polymerization medium by filtration, and the residuals (e.g. unconverted monomer, toluene) were removed by a cleaning procedure given in detail elsewhere [22].

Cibacron Blue F3GA was purchased from Sigma (USA). Three grams of poly(EGDMA-HEMA) microbeads were magnetically stirred (at 400 rpm) in a sealed reactor at a constant temperature of 80°C for 4 hours with 100 ml of the Cibacron Blue F3GA aqueous solution containing 4.0 g NaOH. In order to change the immobilized Cibacron Blue F3GA concentration on the microbeads, the initial concentration of the Cibacron Blue F3GA was varied between 0.1 and 4.0 mg/ml. After derivatization, the Cibacron Blue F3GA-derivatized microbeads were filtered, and washed with distilled water and methanol several times until all the physically attached Cibacron Blue F3GA molecules were removed. The dyed gels were stored at 4°C with 0.02% sodium azide to prohibit microbial contamination.

The amounts of immobilized Cibacron Blue F3GA on the microbeads were obtained by using an elemental analysis instrument (Leco, CHNS-932, USA). The amount of Cibacron Blue F3GA derivatization on the microbeads was evaluated from these data, by considering the nitrogen and sulfur stoichiometry.

The leakage of the Cibacron Blue F3GA from the Cibacron Blue F3GA-derivatized microbeads was investigated within the media containing NaCl at ionic strength 0.01, and at the selected pH in the range of 4.0-8.0. Note that these media were the same which were used in the BSA adsorption experiments given below. Cibacron Blue F3GA leakage was also determined in the medium at pH of 4.9 and containing 25 mM EDTA, which was the medium used in BSA desorption experiments. The medium with the Cibacron Blue F3GA-attached microbeads was stirred for 24 hours at room temperature. Then polymeric microbeads were separated from the medium, and the Cibacron Blue F3GA concentrations were measured in the liquid phase by spectrophotometry at 630 nm for Cibacron Blue F3GA.

Zn(II) Incorporation

Adsorption of Zn(II) ions onto Cibacron Blue F3GA-attached microbeads was carried out as follows: 100 mg of Cibacron Blue F3GA-attached microbeads were mixed with aqueous Zn(II) solutions. Initial concentration of Zn(II) ions and medium pH were changed between 1-400 ppm and 3-7.5 (adjusted with universal buffer solution), respectively. A 1000 ppm atomic absorption standard solution (containing 10% concentrated HNO_3) was used as the source of Zn(II) ions. The flasks were agitated magnetically at 600 rpm for 1 hour (sufficient to attain equilibrium). The concentration of the Zn(II) ions in the resulting solutions was determined with an atomic absorption spectrophotometer (GBC 932 AA, Australia) [17-20].

Zn(II) leakage from the Cibacron Blue F3GA-Zn(II)-derivatized microbeads was investigated with media containing NaCl (ionic strength 0.01), and pH in the range 4.0-8.0. The microbead suspensions were stirred 24 hours at room temperature. Zn(II) ion concentration was then determined in the supernatants using an atomic absorption spectrophotometer.

BSA Adsorption/Desorption

Bovine serum albumin (BSA, lyophilized, Fraction V, Sigma, USA) was selected as a model protein. BSA adsorption on the unmodified, Cibacron Blue F3GA-attached and Cibacron Blue F3GA/Zn(II)-derivatized poly(EGDMA-HEMA) microbeads was studied batch wise in the media at different pH. The pH of the adsorption medium was changed 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 NH_4OH-NH_4Cl for pH 8.0). Ionic strength of the

adsorption media was 0.01 (adjusted by using NaCl). The BSA initial concentration was changed between 0.5-7.0 mg/ml. In a typical adsorption experiment, BSA was dissolved in 25 ml of buffer solution containing NaCl and 200 mg of microbeads were added. The adsorption experiments were conducted for 2 hours at 25°C and at a stirring rate of 100 rpm. At the end of this equilibrium period, the microbeads were separated from the solution by centrifugation. The BSA adsorption capacity was determined by measuring the initial and final concentrations of BSA within the adsorption medium by spectrophotometry at 280 nm [23].

The BSA desorption experiments were performed in a buffer solution containing 25 mM EDTA at pH 4.9. The BSA adsorbed microbeads were placed in the desorption medium and stirred for 1 hour at 25°C, at a stirring rate of 100 rpm. The final BSA concentration within the desorption medium was determined by spectrophotometry. In the case of Zn(II) incorporated microbeads, desorption of Zn(II) ions were also measured in the desorption media by means of an atomic absorption spectrometry. The desorption ratio was calculated from the amount of BSA adsorbed on the microbeads and the amount of BSA desorbed.

In order to obtain the reuse of the Cibacron Blue F3GA-Zn(II) derivatized poly(EGDMA-HEMA) microbeads, BSA adsorption-desorption cycle was repeated three times by using the same polymeric microbeads. After each experiment, the Zn(II) was desorbed with 25 mM EDTA, pH 4.9 and the Zn(II) loading was repeated.

RESULTS AND DISCUSSION

Characteristics of Poly(EGDMA-HEMA) Microbeads

Details of production and characterization of poly(EGDMA-HEMA) microbeads were given in our previous papers [17-21]. The poly(EGDMA-HEMA) microbeads are highly swellable (due to HEMA) in aqueous media (the swelling ratio is 55%), and are in the size range 150-200 μm (i.e., swollen size). They are hard and very strong because of the highly cross-linked structure (due to EGDMA), and therefore they are suitable for column applications in chromatographic separations. A representative optical photograph of poly-(EGDMA-HEMA) microbeads is given in Figure 1.

In this study, we aimed to prepare a specific affinity sorbents containing Cibacron Blue F3GA and Zn(II) ions (in chelate form) for metal chelate affinity

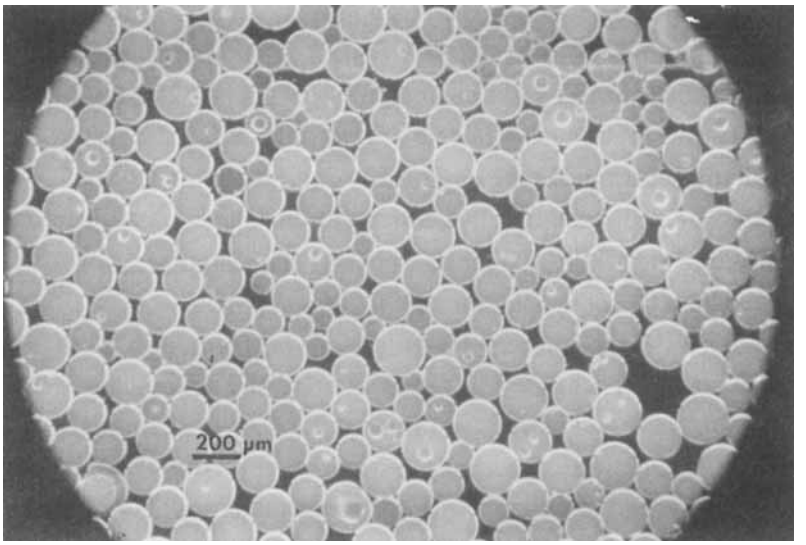

Figure 1. Optical photograph of poly(EGDMA-HEMA) microbeads.

adsorption of BSA. Cibacron Blue F3GA is a widely used dye-ligand which was the first reactive dyes found to be able to interact with proteins and metal ions [24]. Chemical reaction between Cibacron Blue F3GA and poly(EGDMA-HEMA) microbeads are shown in Figure 2. The covalent coupling of Cibacron Blue F3GA to the poly(EGDMA-HEMA) microbeads results from the formation of an ether linkage between the reactive triazine ring of the dye and the hydroxyl groups of the HEMA [25]. The blue color of the microbeads also clearly indicated the presence of Cibacron Blue F3GA molecules on/in the microbeads. FTIR spectra also ensured incorporation of Cibacron Blue F3GA molecules [26]. The studies of Cibacron Blue F3GA and Zn(II) leakage from the microbeads showed that there was no leakage from any of the Cibacron Blue F3GA and Zn(II) derivatized microbeads and in any media described in the previous section.

Zn(II) Incorporation

Influence of Dye Loading on Zn(II) Adsorption

We changed the initial concentration of Cibacron Blue F3GA in the dye-attachment medium in the range of 0.1-4.0 mg/ml, and obtained the extent of Cibacron Blue F3GA loading (i.e., µmol attached dye per gram of the microbeads) by elemental analysis. Table I shows the influence of Cibacron Blue F3GA

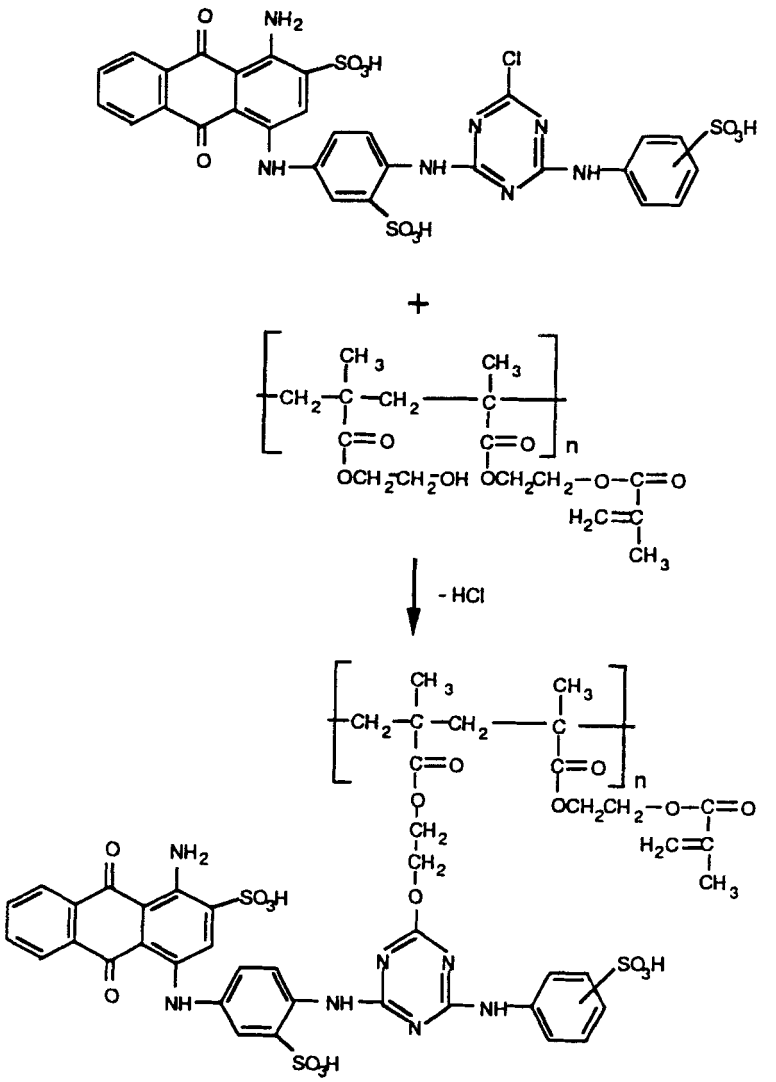

Figure 2. Chemical reaction between Cibacron Blue F3GA and poly(EGDMA-HEMA) microbeads.

concentration on Cibacron Blue F3GA loading and Zn(II) incorporation. Cibacron Blue F3GA loading increased with the concentration of the Cibacron Blue F3GA, then reached a plateau around 2 mg/ml Cibacron Blue F3GA concentration. Maximum loading of Cibacron Blue F3GA was 16.5 μmol dye/g polymer.

TABLE I. Influence of Cibacron Blue F3GA Concentration on Dye and Zn(II) Incorporation.

Cibacron Blue F3GA Concentration (mg/ml)	Cibacron Blue F3GA Attachment (μmol/g)	Zn(II) Adsorption (μmol/g)
0.1	5.0 ± 0.10	128
0.5	10.1 ± 0.21	227
1.0	14.5 ± 0.19	417
2.0	16.3 ± 0.25	484
3.0	16.5 ± 0.22	495
4.0	16.5 ± 0.24	495

When the number of Cibacron Blue F3GA molecules on the microbeads increased (means higher dye loadings) the amount of Zn(II) incorporation onto the Cibacron Blue F3GA-derivatized microbeads also increased. The maximum amount of Zn(II) incorporation on the Cibacron Blue F3GA-derivatized microbeads was 435 μmol Zn(II)/g polymer.

Influence of Zn(II) Initial Concentration on Zn(II) Incorporation

Figure 3 shows the influence of Zn(II) ions concentration on the amount of Zn(II) ions incorporation on the Cibacron Blue F3GA-derivatized microbeads. When the initial concentration of Zn(II) increased, the amount of Zn(II) chelated to the Cibacron Blue F3GA-derivatized microbeads also increased, in the studied range.

It should be noted that the non-specific adsorption of Zn(II) ions (adsorption on the unmodified poly(EGDMA-HEMA) microbeads) was very low (22.48 μmol Zn(II)/g polymer). In order to observe the influence of dye grafting conditions, Zn(II) adsorption onto the poly(EGDMA-HEMA) microbeads were repeated atCibacron Blue F3GA-grafting conditions without dye. The amount of Zn(II) adsorption was the same as unmodified polymeric microbeads. It is clear that the poly(EGDMA-HEMA) microbeads are completely stable to the harsh alkaline conditions used for Cibacron Blue F3GA grafting. Some carboxylic moieties are likely to be produced by hydrolysis of ester linkage of HEMA, but they don't contribute to Zn(II) adsorption. Hence, it can be said that all the Zn(II) ions were adsorbed via Cibacron Blue F3GA molecules.

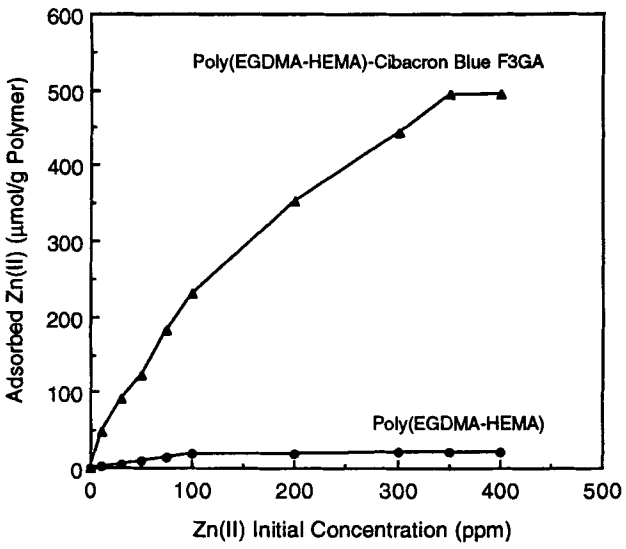

Figure 3. Influence of Zn(II) ions concentration on Zn(II) incorporation: Cibacron Blue F3GA loading: 16.5 μmol/g; Medium pH 6.5; temperature: 20°C.

Influence of Medium pH on Zn(II) Incorporation

Metal ions adsorption both on non-specific and specific sorbents is pH dependent. In the absence of complexing (i.e. chelating) agents, the hydrolysis and precipitation of the metal ions are affected by the concentration and form of soluble metal species. The solubility of metal ions is governed by hydroxide or carbonate concentration [27]. Hydrolysis of Zn(II) ions becomes significant at approximately pH 7.5. Therefore, in order to establish the influence of pH on the incorporation of Zn(II) ions onto the Cibacron Blue F3GA-derivatized microbeads, we repeated the batch equilibrium adsorption studies at different pH in the range of 3.0-7.5. Figure 4 shows the influence of pH on Zn(II) incorporation on the Cibacron Blue F3GA-derivatized microbeads.

There was almost no interaction between the immobilized Cibacron Blue F3GA molecules and Zn(II) ions at pH 3.0, while incorporations were observed at pH 4.0 and above. Most likely, the functional groups on the immobilized Cibacron Blue F3GA molecules ionize and interact with Zn(II) ions above pH 4.0.

BSA Adsorption

In the second part of the study, we investigated the influence of the amounts of incorporated Zn(II) ions, the BSA initial concentration and medium pH upon the

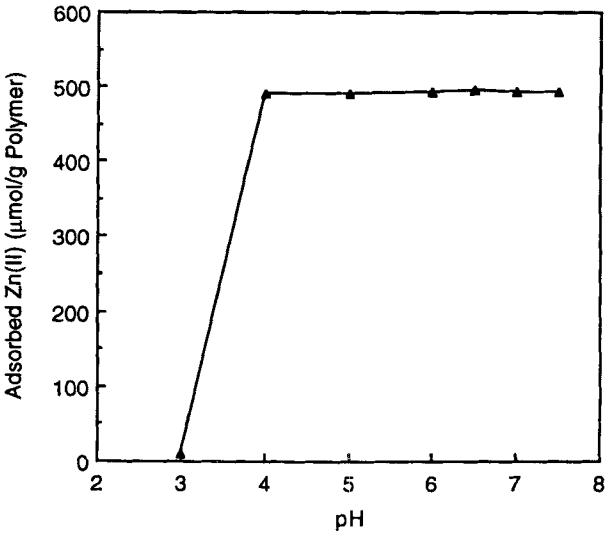

Figure 4. Influence of medium pH on Zn(II) adsorption: Cibacron Blue F3GA loading:16.5 μmol/g; Zn(II) initial concentration: 400 ppm; temperature: 20°C.

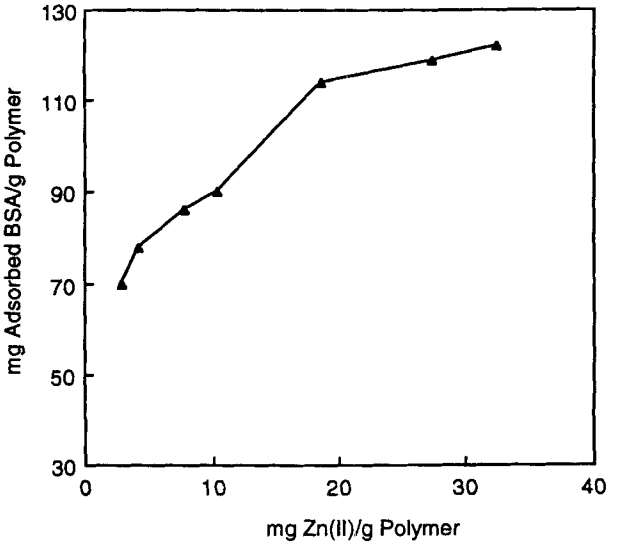

Figure 5. Influence of amount of Zn(II) loading on BSA adsorption: Cibacron Blue F3GA loading: 16.5 μmol/g, BSA initial concentration: 5.0 mg/ml, pH: 6.0, and temperature: 20°C.

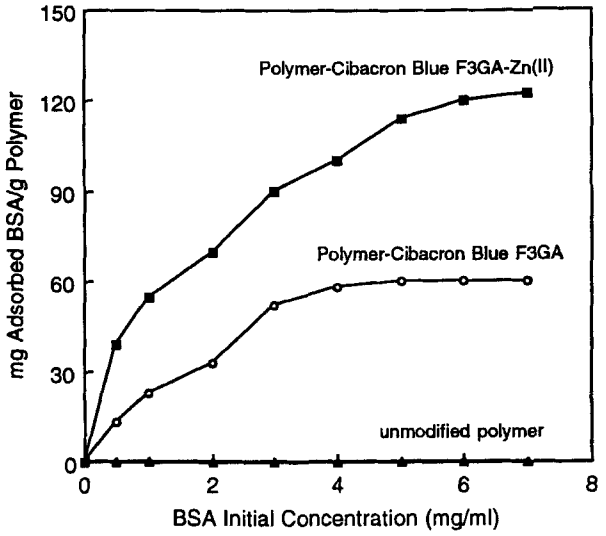

Figure 6. Influence of BSA initial concentration on BSA adsorption: Cibacron Blue F3GA loading: 16.5 μmol/g, Zn(II loading: 32.4 mg/g, pH: 6.0, and temperature: 20°C.

BSA adsorption on the Cibacron Blue F3GA-Zn(II)-derivatized microbeads. Desorption of BSA and reusability of microbeads were also studied.

Influence of Zn(II) Loading on BSA Adsorption

BSA adsorption capacities of the Cibacron Blue F3GA-Zn(II)-derivatized poly(EGDMA-HEMA) microbeads containing different amounts of Zn(II) (between 2.9 and 32.4 mg Zn(II)/g polymer) were investigated at pH 6.0. The initial concentration of BSA in the incubation solution was 5.0 mg/ml. Figure 5 shows the influence of Zn(II) incorporation on BSA adsorption. When the amount Zn(II) on the microbeads increased the amount of BSA adsorbed first increased and then reached an almost constant value. This may be the maximum amount of BSA that can be packed on the surface, due to the steric constraints.

Influence of BSA Initial Concentration on BSA Adsorption

Figure 6 shows the BSA-adsorption curves for the Cibacron Blue F3GA-Zn(II) derivatized microbeads. Similarly, in all cases, BSA adsorption first increases and reaches a plateau (the first plateau observed, around 2 mg/ml BSA initial concentration), may be considered as a typical example of the occupation of all of

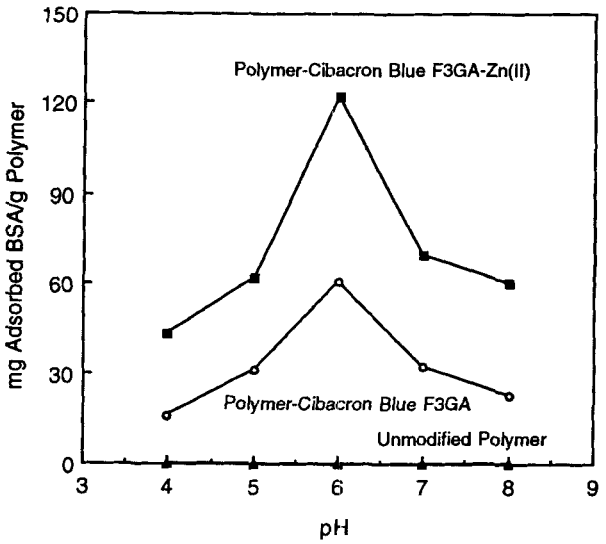

Figure 7. Influence of medium pH on BSA adsorption: Cibacron Blue F3GA loading: 16.5 μmol/g, Zn(II) loading: 32.4 mg/g, BSA initial concentration: 5.0 mg/ml, and temperature: 20°C.

the active surface groups on the sorbent surface, which are available for BSA adsorption. The number of these groups were increased by the incorporation of Zn(II) ions, therefore, the maximum adsorption capacity of the sorbent was increased as expected. The maximum BSA adsorption was 122 mg BSA/g polymer which was comparatively high in comparison with the related literature data [28-31].

Influence of pH on BSA Adsorption

Figure 7 shows the influence of medium pH on BSA adsorption onto the Cibacron Blue F3GA-Zn(II) derivatized microbeads. In all the cases investigated, the maximum adsorption of BSA was observed at pH 6.0. Significantly lower BSA adsorption capacities were obtained with all microbeads in lower and higher pH values. 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 [32]. The isoelectric pH of BSA is 5.0. In our case, the maximum adsorption pH was not 5.0, but rather shifted to higher pH values than isoelectric point of BSA. This may be due to preferential interaction between BSA and Cibacron Blue F3GA-Zn(II) at this pH. These specific interactions may be

TABLE II. BSA desorption from Cibacron Blue F3GA-Zn(II)-derivatized microbeads: Cibacron Blue F3GA loading: 16.5 μmol/g, Zn(II) loading: 32.4 mg/g, BSA initial concentration: 5.0 mg/ml, pH: 6.0, and temperature: 20°C.

BSA Initial Concentration (mg/ml)	Desorption Ratio of BSA (%)	Desorption Ratio of Zn(II) (%)
0.5	90.3 ± 2.7	99.4 ± 0.2
1.0	88.6 ± 1.7	99.6 ± 0.1
2.0	89.2 ± 2.0	99.7 ± 0.1
3.0	91.6 ± 1.4	99.1 ± 0.3
4.0	90.7 ± 2.2	99.3 ± 0.2
5.0	93.5 ± 3.2	99.5 ± 0.4
6.0	94.2 ± 1.5	99.6 ± 0.3
7.0	92.7 ± 3.3	99.7 ± 0.1

resulted both from the incorporated Zn(II) ions and BSA molecules, and from the conformational state of BSA at this pH, as discussed in the related literature [33,34].

Desorption and Repeated Use

The desorption of BSA from the Cibacron Blue F3GA-Zn(II)-derivatized microbeads was studied in a batch experimental setup.The microbeads were loaded with different amounts of BSA were placed within the desorption medium containing 25 mM EDTA at pH 4.9. Then, the amount of BSA and Zn(II) released in 1 hour was determined. The desorption ratios for both BSA and Zn(II) were calculated by using the following equation:

$$\text{Desorption Ratio (\%)} = \frac{\text{amount of BSA desorbed \{or Zn(II)\}}}{\text{amount of BSA adsorbed \{or Zn(II)\}}} \times 100$$

Table II shows the desorption ratios for BSA and Zn(II) ions. A significant amount of the adsorbed BSA (around 90%) was easily desorbed from the Cibacron Blue F3GA-Zn(II)-derivatized poly(EGDMA-HEMA) microbeads in all cases when EDTA was used for desorption. Note that almost all of the Zn(II) ions

TABLE III. Repeated use of Cibacron Blue F3GA-Zn(II)-derivatized microbeads: Cibacron Blue F3GA Loading: 16.5 μmol/g, Zn(II) Incorporation: 32.4 mg/g, BSA initial concentration: 5.0 mg/ml, pH: 6.0, and temperature: 20°C.

Cycle No	BSA Adsorption (mg/g)	Desorption (%)
1	114.0 ± 0.7	93.5 ± 3.2
2	106.6 ± 1.7	98.0 ± 1.4
3	104.5 ± 2.0	99.1 ± 1.1
4	102.4 ± 1.4	96.5 ± 0.9
5	98.8 ± 2.2	97.7 ± 1.5

initially loaded were desorbed from the microbeads. This means that EDTA breaks down the chelates between Zn(II) ions and Dyes.

In order to show the reuse of the Cibacron Blue F3GA-Zn(II)-derivatized poly(EGDMA-HEMA) microbeads, adsorption-desorption cycle was repeated five times by using the same affinity sorbent (Table III). BSA adsorption capacities of poly(EGDMA-HEMA) microbeads were decreased only 13.4% relative to the original (unused) adsorption capacity of the sorbents after 4 times reuse.

CONCLUSION

High BSA adsorption capacities were obtained with Cibacron Blue F3GA-attached poly(EGDMA-HEMA) microbeads. Incorporation of Zn(II) ions onto the Cibacron Blue F3GA-attached microbeads further increased BSA adsorption capacities up to 122 mg BSA/g polymer. It was possible to use of the Cibacron Blue F3GA-Zn(II) derivatized microbeads in BSA adsorption-desorption cycles.

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