Nonporous Monosize Polymeric Sorbents: Dye and Metal Chelate Affinity Separation of Lysozyme

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ABSTRACT: Lysozyme adsorption onto dye-attached nonporous monosize poly(2-hydroxyethyl-methacrylate-methylmethacrylate) [poly(HEMA-MMA)] microspheres was investigated. Poly(HEMA-MMA) microspheres were prepared by dispersion polymerization. The monochloro-triazine dye, Cibacron Blue F3GA, was immobilized covalently as dye–ligand. These dye-affinity microspheres were used in the lysozyme adsorption–desorption studies. The effect of initial concentration of lysozyme and medium pH on the adsorption efficiency of dye-attached and metal-chelated microspheres were studied in a batch reactor. Effect of Cu(II) chelation on lysozyme adsorption was also studied. The nonspecific adsorption of lysozyme on the poly(HEMA-MMA) microspheres was 3.6 mg/g. Cibacron Blue F3GA attachment significantly increased the lysozyme adsorption up to 247.8 mg/g. Lysozyme adsorption capacity of the Cu(II) incorporated microspheres (318.9 mg/g) was greater than that of the Cibacron Blue F3GA-attached microspheres. Significant amount of the adsorbed lysozyme (up to 97%) was desorbed in 1 h in the desorption medium containing 1.0*M* NaSCN at pH 8.0 and 25 m*M* EDTA at pH 4.9. In order to examine the effects of separation conditions on possible conformational changes of lysozyme structure, fluorescence spectrophotometry was employed. We conclude that dye- and metal-chelate affinity chromatography with poly(HEMA-MMA) microspheres can be applied for lysozyme separation without causing any significant changes and denaturation. Repeated adsorption/desorption processes showed that these novel dye-attached monosize microspheres are suitable for lysozyme adsorption.
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Key words: nonporous sorbents; cibacron Blue F3GA; lysozyme; dye affinity microspheres; poly(HEMA-MMA), metal chelates

INTRODUCTION

The rapid development of biotechnology, biochemistry, pharmaceutical science, and medicine requires more reliable and efficient separation techniques for isolation and purification of biomolecules such as proteins, enzymes, peptides, nucleic acids, and hormones.[1] Dye–ligand chromatography is an effective and widely used method for separation of biomolecules, because inexpensive, stable, and group-specific dye ligands are available.[2] However, conventional chromatographic techniques have a number of drawbacks, such as the compressibility of the column packaging materials (i.e., softgel and macrobeads) and the fouling.[3] In order to overcome these problems and to increase the loading capacity, the particle size has been reduced to 1–10 μm, but such carriers require high-pressure equipments. Due to these reasons, nonporous affinity sorbents of small particle diameter (i.e., micron

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size) have been gaining more attention since the mid-1980s for the rapid high-performance liquid chromatography of biomolecules.[4] A major advantage of the nonporous sorbents is that significant intraparticle diffusion resistances are absent; this is particularly useful for the rapid analysis of proteins with high efficiency and resolution.[5] The rapid separation makes it very useful for quality control, on-line monitoring, and purity check of biomolecules such as peptide mapping of recombinant products.[6]

Different types of micron-size/nonporous affinity microsphere applications have been already published: *p*-aminophenyl-*b*-D-glucopyranosidase and *p*-amino-benzamidine modified monosize polystyrene microspheres were used for affinity chromatography of concanavalin A.[7] Dinitrophenylamino acids immobilized crosslinked polystyrene microspheres were used for the affinity chromatography of immunoglobulin E.[8] Cibacron Blue attached polyvinylalcohol coated monosize nonporous polystyrene dye-affinity sorbents were used for albumin adsorption.[9] Triazine dyes and monoclonal antibody mouse immunoglobulin-G (IgG) immobilized nonporous silica-based affinity matrices were utilized in packed columns for adsorption of several proteins.[10] Nonporous silica carrying Protein A was used for the high performance affinity chromatography of IgG.[11]

In the present article, a dye- and metal-chelate affinity microspheres using nonporous monosize poly(2-hydroxyethylmethacrylate-methylmethacrylate) [poly(HEMA-MMA)] microspheres as the support matrices has been prepared. A dye–ligand Cibacron Blue F3GA was covalently attached to the poly(HEMA-MMA) microspheres and the adsorption–desorption behavior of lysozyme was investigated. System parameters such as microsphere properties [i.e., dye loading, Cu(II) attachment] and the adsorption conditions (i.e., initial concentration of lysozyme and medium pH) were varied to evaluate their effects on the performances of dye-affinity microspheres.

EXPERIMENTAL

Production of Poly(HEMA-MMA) Microspheres

Monosize poly(HEMA-MMA) microspheres were produced by dispersion polymerization of HEMA and MMA in an ethanol–water medium.[12] The monomer, i.e., 2-hydroxyethylmethacrylate (HEMA) supplied from Sigma, USA, was purified by passing through active alumina. The comonomer methylmethacrylate (MMA) (Rohm and Haas, Darmstad, Germany) was treated with aqueous NaOH/NaCl to remove the inhibitor and stored at 4°C until use. Azobisisobutironitrile and polyvinylpyrrolidone (PVP) (molecular mass: 30.000, Aldrich Chem. Co., Rockford, IL, USA) were used as the initiator and the stabilizer, respectively. Ethanol (Merck, Darmstad, Germany) was selected as the diluent and used without further purification. The dispersion medium was comprised of ethanol, water, and PVP (50.0 mL/50.0 mL/4.0 g). The monomer phase contained MMA/HEMA mixture (2.0 mL/4.0 mL) and AIBN (80 mg). The dispersion polymerization was carried out in a glass reactor and charged with polymerization mixture. The resulting homogeneous solution was purged with bubbling nitrogen for 10 min at room temperature. The Pyrex glass cylinders having a total volume of 200 mL were used as the polymerization reactor. The sealed reactor was placed at the horizontal position within a shaking water bath equipped with a temperature control system. The polymerizations were performed at 65°C for 24 h with 400 cycles/min.

After completion of the polymerization reaction, the poly(HEMA-MMA) microspheres was cleaned by using the serum replacement and ion-exchange methods.[9] The microspheres taken from the polymerization reactor was centrifuged and the supernatant was removed. Distilled water (100 mL) was added onto the microspheres and was stirred for 24 h at room temperature to remove any unreacted ingredients on the surface of polymeric microspheres. This procedure was repeated three times. The poly(HEMA-MMA) microspheres dispersed in distilled water and was treated with a mixed bed of anion and cation-exchange resins (H^+ and OH^- type, Amberlite, BDH, England) at the last step.

Dye Attachment to Poly(HEMA-MMA) Microspheres

Cibacron Blue F3GA (Sigma, St Louis, MO, USA) was covalently attached to the poly(HEMA-MMA) microspheres via the nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the poly(HEMA-MMA), under alkaline conditions. The attachment procedure previously used was employed.[13,14] First, Cibacron Blue F3GA was dissolved in 10 mL of water. This aqueous dye solution was transferred to poly(HEMA-MMA) microspheres in 90 mL dis-

tilled water, and then 4 g of NaOH were added. The medium was heated at 80°C in a sealed reactor and was stirred magnetically for 4 h. In order to adjust attached amount of ligand, Cibacron Blue F3GA concentration was changed between 0.5 and 5.0 mg/mL. After dye attachment, the microspheres were washed several times with distilled water and methanol until all the physically adsorbed dye molecules was removed. Cibacron Blue F3GA attached poly(HEMA-MMA) microspheres were stored at 4°C with 0.02% sodium azide to prohibit microbial contamination.

The amounts of attached Cibacron Blue F3GA on the microspheres were obtained by using elemental analysis. The amount of Cibacron Blue F3GA attachment on the microspheres was calculated from these data, by considering the nitrogen and sulfur stoichiometries.

The release of the Cibacron Blue F3GA from the dye-attached microspheres was investigated at different pH values in the range of 4.0–8.1. It should be noted that these media were the same used in the lysozyme adsorption experiments given below. Cibacron Blue F3GA release was also determined in the medium at 1.0*M* NaSCN, which was the medium used the lysozyme desorption experiments. The medium with the Cibacron Blue F3GA attached microspheres was incubated for 24 h at room temperature. Then, microspheres were removed from the medium, and the Cibacron Blue F3GA concentration in the supernatant was measured by spectrophotometry at 630 nm.

Incorporation of Cu(II) Ions

Cibacron Blue F3GA carrying poly(HEMA-MMA) microspheres were transferred to aqueous solution containing 30 ppm Cu(II) ions, at constant pH of 4.1 (adjusted with HCl and NaOH) at room temperature and magnetically stirred for 1 h. $Cu(NO_3)_2$ was used as the source of Cu(II) ions. The concentration of the Cu(II) ions in the resulting solution was determined with a graphite furnace atomic absorption spectrophotometer, AAS (GBC 932 AA, Australia). All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. For each sample, the mean of 10 AAS measurements was recorded. Cu(II) leakage from the dye-attached and metal-chelated poly(HEMA-MMA) microspheres was also investigated in both lysozyme adsorption (pH range 4.0–8.1) and desorption media (1.0*M* NaSCN, pH 8.0). The solution containing the dye-attached and metal-chelated poly(HEMA-MMA) microspheres was stirred for 24 h at room temperature. After this period, the released Cu(II) was determined in these solutions by using AAS. After each lysozyme adsorption–desorption experiment, the Cu(II) ions were stripped with 25 m*M* EDTA at pH 4.9, and the Cu(II) adsorption procedure was applied again.

Lysozyme Adsorption–Desorption Studies

Lysozyme (chicken egg white, EC 3.2.1.7) was supplied from Sigma Chemical Co. (St Louis, MO, USA). Lysozyme adsorption on the plain poly(HEMA-MMA), the Cibacron Blue F3GA-attached poly(HEMA-MMA), and Cibacron Blue F3GA/Cu(II) derived poly(HEMA-MMA) microspheres were studied at various pH. The pH of the adsorption medium was changed between 4.0 and 8.1 by using different buffer systems (0.1*M* CH_3COONa-CH_3COOH for pH 4.0–6.0, 0.1*M* Na_2HPO_4-NaH_2PO_4 for pH 7.0–8.1). The initial concentration of lysozyme was changed between 0.05 and 1.0 mg/mL. In a typical adsorption experiment, lysozyme was dissolved in 25 mL of buffer solution and microspheres were then added (100 mg). The adsorption experiments were carried out for 2 h at 20°C at a magnetic stirring rate of 100 rpm. The time to reach equilibrium adsorption with continuous stirring was found to be 120 min and in the rest of the study this adsorption duration was therefore employed. At the end of the equilibrium time (i.e., 2 h), the microspheres were separated from the solution by centrifuging. The amount of lysozyme adsorbed at various time periods was obtained by measuring the difference between the initial and the residual lysozyme concentration in the solution spectrophotometrically at 280 nm. It also should be noted that all adsorption curves are averages of at least duplicated experiments.

Lysozyme desorption was performed in a buffer solution containing 1.0*M* NaSCN or 25 m*M* EDTA at pH 4.9. The lysozyme 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 lysozyme concentration within the desorption medium was determined by spectrophotometry. In the case of Cu(II)-chelated microspheres, desorption of Cu(II) ions was also measured in the desorption media by means of the atomic absorption spectrophotometer. The desorption ratio was calculated from the amount of lysozyme adsorbed on the microspheres and the amount of lysozyme desorbed.

To examine the effects of separation conditions on lysozyme denaturation, fluorescence spectra of the native lysozyme, heat-denaturated lysozyme, and desorbed lysozyme were obtained. Native lysozyme aqueous solution (1 mg/mL, pH: 7.0, ionic strength: 0.1) was incubated at 70°C for 4 h for heat denaturation. Fluorimetric measurements were taken with a Jasco FP-550 Spectrofluorometer using 1 cm^2 quartz cells. Monochromatic readings were taken from a digital display with a 0.25 s time constant and a 3 nm band width on the excitation side and 5 nm on the emission side. Initial calibration was carried out with standard solution of lysozyme in phosphate buffered saline with 280 nm fluorescence excitation and 340 nm emission wavelengths.

RESULTS AND DISCUSSION

Dye-Attached Poly(HEMA-MMA) Microspheres

Details of production and characterization of monosize and nonporous poly(HEMA-MMA) microspheres were given in our previous paper.[12] Figure 1 shows a representative optical picture of the monosize (Root Mean Square Deviation, RSD $< 1\%$) poly(HEMA-MMA) microspheres with a diameter of 4 μm which were obtained at the polymerization conditions given in Experimental section. It should be mentioned that no significant deformation of the poly(HEMA-MMA) microspheres was observed after dye-attachment.

Cibacron Blue F3GA is a monochlorotriazine dye (Fig. 2), and it contains three sulfonic acid groups, four basic primary groups, and secondary amino groups. Cibacron Blue F3GA is fully deprotonated in the pH range from 1 to 14, and hence carries three negatively charged SO_3 groups. The strong binding of the dye–ligands to proteins may have resulted from cooperative effect of different mechanisms such as hydrophobic interactions, electrostatic interactions, and hydrogen bonding caused by the aromatic structures and acidic groups on the dye–ligand and by groups on the side chains of amino acids on the protein molecules.[15] The dye–ligands are not very hydrophobic overall, but they do have planar aromatic surfaces that prefer to interact with hydrophobic residues in proteins. On the other hand, the binding of Cu(II) ions to the Cibacron Blue F3GA molecules occurs especially through oxygen and nitrogen atoms. The Cibacron Blue F3GA molecules were covalently attached to the poly(MMA-HEMA) microspheres. It is a widely used dye–ligand (even in the commercial sorbents). It is accepted that ether linkages are formed between the reactive triazine ring of the dye and the functional hydroxyl groups of the sorbent (such as OH groups of HEMA). Note that the Cibacron Blue F3GA attached microspheres were extensively washed as described in the experimental section to ensure that there was no dye leakage from any of the dye-attached microspheres and in any media used at adsorption and/or desorption stages.

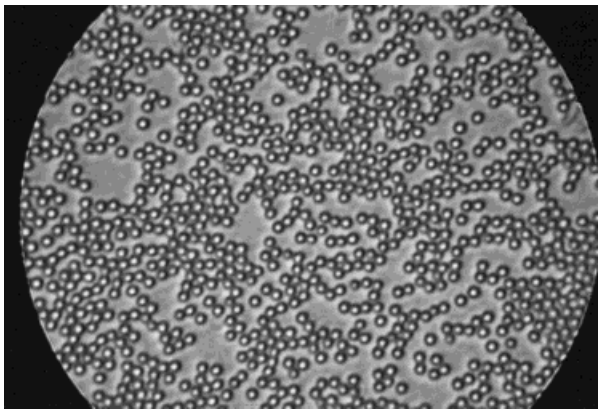

Figure 1 A representative optical micrograph of poly(HEMA-MMA) microspheres.

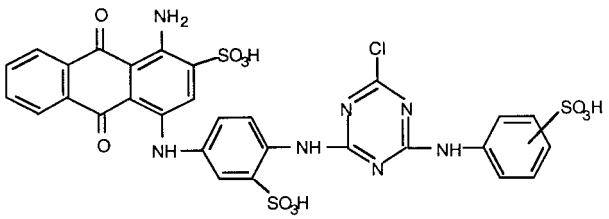

Figure 2 Chemical Structure of Cibacron Blue F3GA.

Fourier transform IR (FTIR) spectra of poly(HEMA-MMA), Cibacron Blue F3GA and poly(HEMA-MMA)–Cibacron Blue F3GA are given in Figure 3. FTIR spectra of both poly(HEMA-MMA) and poly(HEMA-MMA)-Cibacron Blue F3GA have the characteristic stretching vibration band of hydrogen-bonded alcohol, O—H, around 3500 cm^{-1}. The FTIR spectra of Cibacron Blue F3GA-incorporated poly(HEMA-MMA) has some absorption bands different than those of poly(HEMA-MMA). These are at 3515–3535, 1465, and 1575 cm^{-1} characteristic N—H stretching, conjugated C═N, and N═N vibration, respectively, also observed in Cibacron Blue F3GA (Fig. 2). Cibacron Blue F3GA incorporated poly(HEMA-MMA) has a sharp shoulder absorption

band at about 3500 cm^{-1} and interpreted as the N—H absorption. The bands at 1065 and 1135 cm^{-1} represent symmetric stretching of S═O and asymmetric stretching of S═O, respectively, which are due to Cibacron Blue F3GA bonded to poly(HEMA-MMA). In addition, for dye-attached poly(HEMA-MMA), absorption band intensities in this region are higher than those of plain poly-(HEMA-MMA), but the intensity increase is quite small because of the low concentration of Cibacron Blue F3GA on the polymer surface. On the other hand, hydrogen-bonded alcohol O—H stretching band intensity of plain poly(HEMA-MMA) is higher than that of poly(HEMA-MMA)–Cibacron Blue F3GA. The reason for the loss of the —OH groups is as a result of the nucleophilic substitution reaction between —OH groups of poly(HEMA-MMA) and Cl atoms of Cibacron Blue F3GA.

Lysozyme Adsorption/Desorption Studies

Adsorption Rate

Figure 4 gives the adsorption rate curves obtained by following the decrease of the concentration of lysozyme within the protein solution with time. As seen here, relatively faster adsorption rates were observed at the beginning of adsorption process, and then adsorption equilibrium was established in about 30 min. A point worth noting

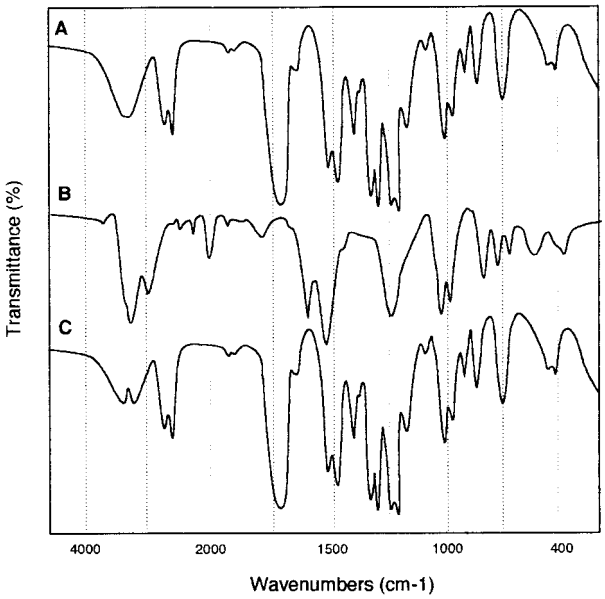

Figure 3 FTIR spectrums: (A) poly(HEMA-MMA); (B) Cibacron Blue F3GA; (C) Cibacron Blue F3GA-attached poly(HEMA-MMA).

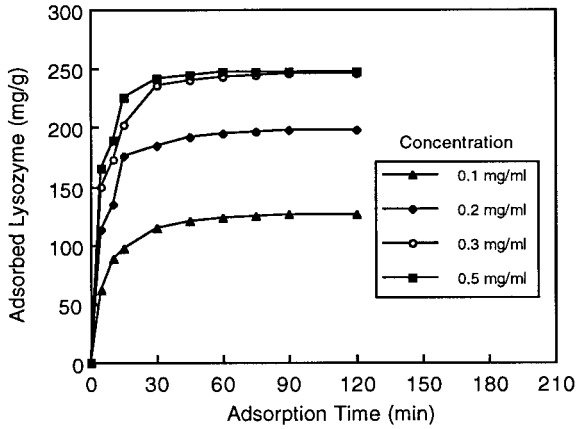

Figure 4 Adsorption rates of lysozyme: Cibacron Blue F3GA loading: 41.7 μmol/g; pH: 7.0; T: 20°C.

that, in Figure 4 of the kinetic curves, a similar tendency was observed for all the concentrations. Compared with 2–3 h for most polymeric sorbents, this fast adsorption could be an advantage for separation and may be attributed to the zero pore diffusion resistance of poly(HEMA-MMA) microspheres because of nonporous structure.[9–11] Nonspecific lysozyme adsorption [i.e., the adsorption onto the plain poly(HEMA-MMA) microspheres] was about 3.6 mg/g microsphere. Lysozyme has been reported to have a higher structural stability and hydrophobicity.[16] There are hydrophobic MMA groups on the polymer structure that interact with lysozyme molecules—hence, this adsorption could have resulted from relatively hydrophobic interactions between lysozyme and hydrophobic groups on the surface of poly(HEMA-MMA) microspheres. On the other hand, much higher adsorption rates were observed when the Cibacron Blue F3GA-attached poly(HEMA-MMA) microspheres were used. Notice that adsorption rates increased with increasing lysozyme concentration. This could have resulted from the relatively high driving force, which is the lysozyme concentration difference between the protein solution and the microsphere phases, in the case of high lysozyme concentration. A point worth noting that, in Figure 4 of the kinetic curves, a similar tendency was observed for all the concentrations.

Effect of Cibacron Blue F3GA Loading

Dye loading is an important parameter in the adsorption of proteins. Figure 5 provides the relationship between the lysozyme adsorption ca-

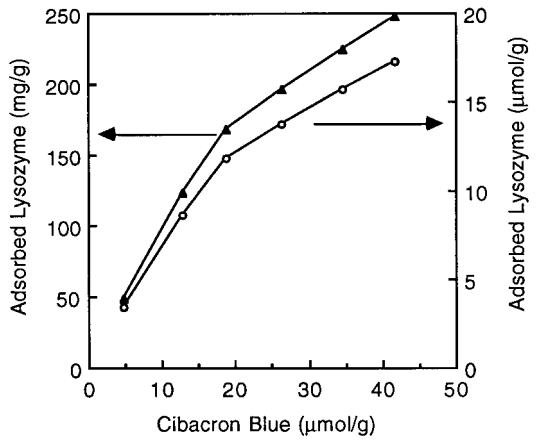

Figure 5 Effect of Cibacron Blue F3GA loading on lysozyme adsorption upon dye-attached poly(HEMA-MMA) microspheres; initial concentration of lysozyme: 0.4 mg/mL, pH: 7.0; *T*: 20°C.

pacity and the Cibacron Blue F3GA loading in poly(HEMA-MMA) microspheres. The lysozyme adsorption capacity increases rapidly at low dye loadings (<20 μmol/g) but much slower at higher ones. Note that the Cibacron Blue F3GA attached poly(HEMA-MMA) microspheres contained 41.7 μmol Cibacron Blue F3GA/g, which was the maximum value that we achieved. The maximum lysozyme adsorption that corresponding this dye derivatization was 247.8 mg/g (17.3 μmol/g). According to this result, each lysozyme molecule can interact with up to 3 Cibacron Blue F3GA molecules. This shows that steric hindrances between the Cibacron Blue F3GA and lysozyme molecules were not important and in this case all the Cibacron Blue F3GA molecules could be used for lysozyme attachment.

Effect of Initial Concentration of Lysozyme

Figure 6 shows the effect of initial lysozyme concentration on adsorption. As presented in this figure, with increasing lysozyme concentration in solution, the amount of lysozyme adsorbed by the microspheres increases almost linearly at low concentrations, below about 0.20 mg/mL, then increases less rapidly and approaches saturation. The steep slope of the initial part of the adsorption isotherm represents a high affinity between lysozyme and Cibacron Blue F3GA molecules. It becomes constant when the protein concentration is greater than 0.35 mg/mL. A negligible amount of lysozyme adsorbed nonspecifically on the nonderivatized poly(HEMA-MMA) microspheres (3.6 mg/g). Cibacron Blue F3GA attachment significantly increased lysozyme adsorption capacity of the microspheres (up to 247.8 mg/g). It is clear that this increase is due to specific interaction between Cibacron Blue F3GA and lysozyme molecules.

In order to determine the effect of protein size on the adsorption process with these dye-affinity microspheres, identical experiments were carried out using bovine serum albumin (BSA) and the corresponding adsorption isotherms obtained for BSA adsorption to Cibacron Blue F3GA attached poly(HEMA-MMA) microspheres. To compare the adsorption behaviors of these two proteins with the same dye loading, the adsorption curve for the binding of BSA to Cibacron Blue F3GA attached poly(HEMA-MMA) microspheres is shown in Figure 6 as a small graph. The molecular weight of lysozyme (molecular mass: 14.300, molecular diameter: 27.3 Å) is much smaller than that of BSA (molecular mass: 67.000, molecular diameter: 83.5 Å); the adsorption capacity for lysozyme (247.8 mg/g) is about 2.3-fold higher than that of BSA (108 mg/g) on a weight basis due to molecular size. In addition, Cibacron Blue F3GA attached poly(HEMA-MMA) shows much higher affinity for lysozyme than BSA.

Effect of pH

The amount of lysozyme adsorbed onto the Cibacron Blue F3GA-attached poly(HEMA-MMA) microspheres as a function of pH exhibits two adsorption domains, as shown in Figure 7. (1) Lysozyme is highly positively charged at pH 7.0

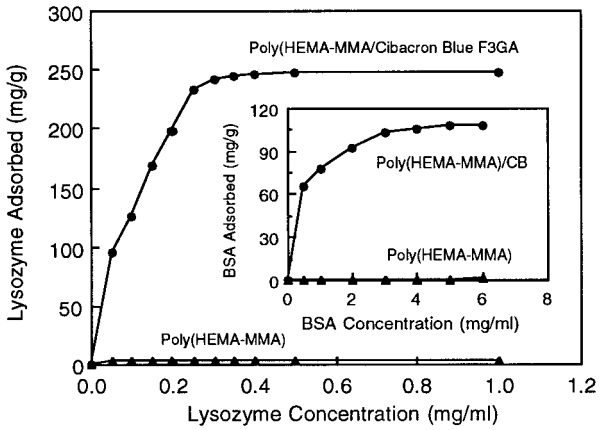

Figure 6 Effect of lysozyme initial concentration on lysozyme adsorption; Cibacron Blue F3GA loading: 41.7 μmol/g; pH: 7.0; *T*: 20°C.

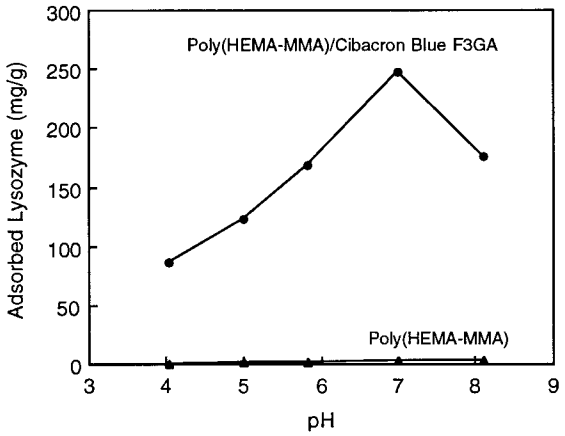

Figure 7 Effects of pH on lysozyme adsorption; initial concentration of lysozyme: 0.4 mg/mL; Cibacron Blue F3GA loading: 41.7 μmol/g; T: 20°C.

(isoelectric point of lysozyme: 11.2). But it is interesting to note that the amount of lysozyme adsorbed onto Cibacron Blue F3GA attached poly(HEMA-MMA) microspheres shows a maximum at pH 7.0, with a very significant decrease at lower and higher pH values. Specific interactions (hydrophobic, electrostatic, and hydrogen bonding) between lysozyme and dye–ligand molecules at pH 7.0 may result from both the ionization states of several groups on both the Cibacron Blue F3GA (i.e., sulfonic acid and amino) and amino acid side chains in lysozyme, and from the conformational state of lysozyme molecules (more folded structure) at this pH. (2) At pH values lower and higher than pH 7.0, the adsorbed amount of lysozyme drastically decreases. This could be created from the ionization state of lysozyme and could be caused by repulsive electrostatic forces between lysozyme and the dye-ligand molecules. In increase in conformational size and the lateral electrostatic repulsions between adjacent adsorbed lysozyme molecules may also cause a decrease in adsorption efficiency.

Effect of Cu(II)-Incorporation

Different interaction mechanisms of metal ions with proteins have been proposed,[17] but the molecular recognition of proteins with immobilized metal ions obviously remains still unclear. In one proposed mechanism, the formation of a coordination complex structure between protein and immobilized metal ion is considered to be the major binding mode. However, more than one type of interaction mechanism is operational.[17] In immobilized metal chelate affinity chromatography, the exposed electron-donating amino acid residues on the protein surface—such as the imidazole group of histidine, the indoyl group of tryptophan, and the thiol group of cysteine—contribute to the binding of proteins to immobilized metal ions. While maintaining a free cysteine residue in a natural protein is rare,[18] the exposed histidine residue is the dominant binding site in protein adsorption with an immobilized metal ion.[19] Chicken egg white lysozyme consists of 129 amino acids and contains four disulfide bridges. Factors influencing the interactions include the number of electron-donating groups on the protein surface, medium pH, concentration of protein, type of metal ions, ligand density, and type and size of chelating ligand.

Figure 8 shows the effects of Cu(II) incorporation on the lysozyme adsorption. Note that the amount of Cu(II) incorporated on the Cibacron Blue F3GA attached poly(HEMA-MMA) microspheres was 5.2 mg/g (81.9 μmol/g). Thus each dye molecule can interact with up to 2 copper ions. Theoretically one expects that one heterofunctional affinity chelating ligand Cibacron Blue F3GA coordinated more than 2 copper ions, giving different geometrical structures such as planar, tetrahedral, or octahedral arrangements around the metal ion (coordination number of copper ion is four). Other free coordination valances of the copper ions are occupied by water molecules.

As expected, adsorption increased with Cu(II) loading. The maximum adsorption capacity of Cibacron Blue F3GA attached poly(HEMA-MMA) microspheres was 247.3 mg/g. Cu(II) incorpora-

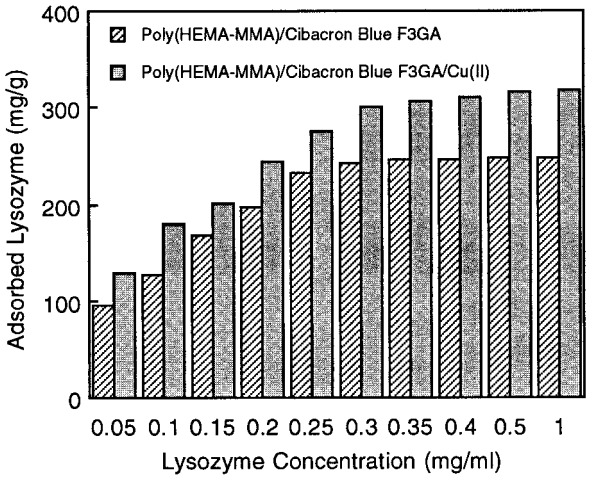

Figure 8 Effect of Cu(II) incorporation onto lysozyme adsorption; pH: 7.0; T: 20°C.

Table I Desorption of Lysozyme and Cu(II) Ions

Microspheres	Lysozyme Loaded (mg/g)	Cu(II) Loaded (mg/g)	Desorption Ratio for Lysozyme (%)		Desorption Ratio for Cu(II) Ions (%)	
			With NaSCN	With EDTA	With NaSCN	With EDTA
Microsphere I[a]	247.8	—	98	23	—	—
Microsphere II[b]	318.9	5.2	91	97	0	100

[a] Cibacron Blue F3GA carrying poly(HEMA-MMA) microspheres.
[b] Cibacron Blue F3GA and Cu(II) carrying poly(HEMA-MMA) microspheres.

tion significantly increased the lysozyme adsorption capacity of the nonporous microspheres (318.9 mg/g). This is due to the preferential interaction between lysozyme molecules (especially imidazole side chains of histidine residue in lysozyme structure) and chelated Cu(II) ions.

Only a few studies on the application of nonporous microspheres in affinity chromatography of proteins have been reported. Chen et al. investigated lysozyme adsorption capacity on the hydrophilic gel; the maximum lysozyme adsorption capacity of the adsorbents was 84 mg/g.[19] Horstman et al. reported 15.1–16.6 mg/g lysozyme adsorption on the Cibacron Blue F3GA–Sepharose CL-6B.[20] Nash and Chase modified the poly(styrene-divinylbenzene) chromatography matrices by the adsorption and crosslinking of poly(vinyl alcohol), and they attached Procion Yellow HE-3G dye–ligand for human serum albumin and lysozyme adsorption.[21] Their lysozyme adsorption efficiencies were in the range of 11.2–20 mg/mL. Champluvier and Kula used nylon-based microfiltration membranes containing various pseudo-affinity ligands (triazine dyes) for lysozyme adsorption, and they reported a 8.6 mg/mL adsorption capacity.[22] Nash et al. investigated lysozyme adsorption onto Procion MX-R attached poly(styrene-divinylbenzene) matrices, and they achieved 68 mg/g adsorption capacity.[23] The macroporous chitin membrane was used as an affinity membrane for lysozyme separation with high selectivity and high adsorption capacity at saturation (50 mg/mL membrane).[24] Chetty and Burns investigated Macrosorb.KAX.CB for protein separation, and they reported a 23 mg/mL lysozyme adsorption capacity.[25] It should be mentioned that the commercial dye-affinity resin Macrosorb.KAX.CB is a composite of silica and agarose onto which the ligand Cibacron Blue F3GA has been derivatized. The maximum lysozyme adsorption that we achieved with the sorbent system developed in this study was in the range of 247.8–318.9 mg/g, which was quite comparable with the related literature.

Desorption

The desorption of lysozyme from Cibacron Blue F3GA attached poly(HEMA-MMA) and Cibacron Blue F3GA/Cu(II) derived poly(HEMA-MMA) microspheres was studied in a batch system. Microspheres carrying different amounts of lysozyme were placed in a desorption medium containing 1.0*M* NaSCN or 25 m*M* EDTA at pH 4.9, and the amount of lysozyme and Cu(II) released in 60 min was determined. The desorption ratios for both lysozyme and Cu(II) were calculated by using the following expression:

$$\text{Desorption Ratio (\%)} = \frac{\text{Amount of lysozyme [or Cu(II)] released}}{\text{Amount of lysozyme [or Cu(II)] adsorbed}} \times 100$$

Table I gives the desorption data. From the lyothropic series, SCN^- is a chaotropic anion, which could enhance protein desorption.[26] More than 95% (up to 97%) of the adsorbed lysozyme was desorbed in all cases when NaSCN was used for desorption. Note that there was no Cu(II) release in this case, which shows that Cu(II) ions are attached to Cibacron Blue F3GA molecules on the microspheres surface by strong chelate formation. However, when EDTA was used alone for desorption, only 23% of lysozyme was removed from Cibacron Blue F3GA attached microspheres, possibly because of a salting-out effect. While under the same desorption conditions, about 97% of the lysozyme was desorbed from the Cibacron Blue F3GA/Cu(II) carrying microspheres. Note

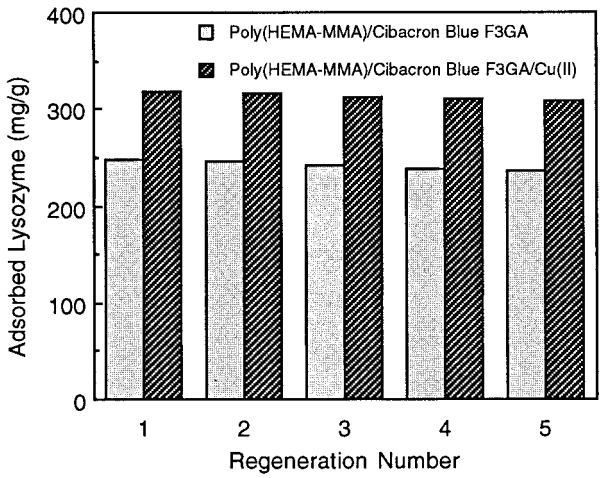

Figure 9 Repeated use of dye-derived and metal-chelated microspheres. Cibacron Blue F3GA loading: 41.7 μmol/g; initial concentration of lysozyme: 0.4 mg/mL; Cu(II) loading: 5.2 mg/g; pH: 7.0 and T: 20°C.

that in this later case almost all of the Cu(II) ions initially loaded were released from the microspheres. This means that EDTA breaks down the chelates between Cu(II) ions and Cibacron Blue F3GA molecules. With the desorption data given above, we concluded that NaSCN is a suitable desorption agent especially for the Cibacron Blue F3GA/Cu(II) carrying microspheres, and allows repeated use of the affinity sorbents developed in this study.

In order to show the reusability of the poly(HEMA-MMA)–Cibacron Blue F3GA and poly(HEMA-MMA)–Cibacron Blue F3GA-Cu(II) microspheres, adsorption–desorption cycles of lysozyme were repeated five times using the same microspheres. As seen from Figure 9, adsorption capacities for both polymeric microspheres did not change noticeable during the repeated adsorption–desorption operations.

In order to examine the effects of separation conditions on lysozyme denaturation, fluorescence spectrophotometry was employed. The fluorescence spectra of lysozyme samples obtained from the desorption step were recorded. The fluorescence spectra of native and heat-denaturated lysozyme were also taken. A clear difference was observed between the fluorescence spectra of native lysozyme and heat-denaturated lysozyme. An appreciable shift was seen in the maximum wavelength of denaturated lysozyme according to the native one. On the other hand, the fluorescence spectra of the samples withdrawn from the desorption step were very close to those of native lysozyme and no significant shift of maximum wavelength was detected in the spectra of these samples relative to that of native lysozyme. It may be concluded that dye- and metal-chelate affinity chromatography with poly(HEMA-MMA) microspheres can be applied for lysozyme separation without causing any denaturation.

CONCLUSION

Nonporous and monosize poly(HEMA-MMA) microspheres were prepared by dispersion polymerization of HEMA and MMA monomers. Cibacron Blue F3GA was then covalently attached to these microspheres with a solid phase concentration of 41.7 μmol/g as a dye–ligand. Adsorption/desorption studies of lysozyme on Cibacron Blue F3GA and Cu(II) derived poly(HEMA-MMA) microspheres led to the following conclusions: Lysozyme adsorption capacity of dye-attached microsphere was 247.8 mg/g. When the Cu(II) chelated with Cibacron Blue F3GA molecules, lysozyme adsorption capacity was increased up to 318.9 mg/g. Adsorbed lysozyme were desorbed up to 97% by using 1.0M. NaSCN as the desorption agent. In order to examine the effects of separation conditions on conformational changes of lysozyme molecules (i.e., denaturation), fluorescence spectrophotometry was employed. It appears that dye- and metal-chelate affinity chromatography with modified poly(HEMA-MMA) microspheres can be applied for separation of proteins without causing any denaturation. Repeated adsorption/desorption processes showed that these novel dye-attached and metal-chelated monosize microspheres are suitable for protein adsorption/desorption.

REFERENCES

1. Turkova, J. Bioaffinity Chromatography; Elsevier: New York, 1993.
2. Clonis, Y. D.; Atkison, T.; Bruton, C. J.; Lowe, C. R., Eds. Reactive Dyes in Protein and Enzyme Technology; Stockton Press: New York, 1987.
3. Langlotz, P.; Kroner, K. H. J Chromatogr A 1992, 591, 107.
4. Lee, W. C. J Chromatogr B 1997, 699, 29.
5. Maa, Y. F.; Horwath, Cs. J Chromatogr 1988, 445, 71.
6. Yamasaki, Y.; Kitamura, T.; Nakatani, S.; Kato, Y. J Chromatogr 1989, 481, 391.

7. Lee, W. C.; Lin, C. H.; Ruaan, R. C.; Hsu, K. Y. J Chromatogr A 1995, 704, 307.
8. Wongyai, S.; Varga, J. M.; Bonn, G. K. J Chromatogr 1991, 536, 155.
9. Tuncel, A.; Denizli, A.; Purvis, D.; Lowe, C. R.; Pişkin, E. J Chromatogr A 1993, 634, 161.
10. Mao, Q. M.; Johnston, A.; Prince, I. G.; Hearn, M. T. W. J Chromatogr 1991, 548, 147.
11. Lee, W. C.; Chuang, C. Y. J Chromatogr A 1996, 721, 31.
12. Denizli, A.; Köktürk, G.; Salih, B.; Kozluca, A.; Pişkin, E. J Appl Polym Sci 1997, 63, 27.
13. Arica, M. Y.; Testereci, H. N.; Denizli, A. J Chromatogr A 1998, 799, 83.
14. Denizli, A.; Şenel, S.; Arica, M. Y. Colloids and Surfaces B 1998, 11, 113.
15. Sulkowski, E. Trends Biotechnol 1985, 3, 1.
16. Arai, T.; Norde, W. Colloids and Surfaces 1990, 51, 1.
17. Hemdan, E. S.; Porath, J. J Chromatogr 1985, 323, 255.
18. Arnold, F. H. Bio/Technology 1991, 9, 151.
19. Chen, W. Y.; Wu, C. F.; Liu, C. C. J Colloid Interf Sci 1996, 180, 135.
20. Horstman, B. J.; Kenny, C. N. J Chromatogr A 1986, 361, 179.
21. Nash, D. C.; Chase, H. A. J Chromatogr A 1997, 776, 65.
22. Champluvier, B.; Kula, M. R. J Chromatogr 1991, 539, 315.
23. Nash, D. C.; McCreath, G. E.; Chase, H. A. J Chromatogr A 1997, 738, 53.
24. Ruckenstein, E.; Zeng, X. F. Biotechnol Bioeng 1997, 56, 610.
25. Chetty, A. E.; Burns, M. A. Biotechnol Bioeng 1991, 38, 963.
26. Porath, J. Biotechnol Prog 1987, 3, 14.