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Poly(Styrene-Hydroxyethyl Methacrylate) Monodisperse Microspheres as Specific Sorbent in Dye Affinity Adsorption of Albumin

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ABSTRACT

In this study, human serum albumin (HSA) adsorption properties of reactive green HE-4BD-attached monodisperse poly(styrene-2-hydroxyethyl methacrylate) [poly(St-HEMA)] microspheres were investigated. Poly(St-HEMA) microspheres with an uniform size of 4.0 μm in diameter were produced by the dispersion copolymerization of St and HEMA in an ethanol–water medium. Reactive green HE-4BD was covalently attached onto the poly(St-HEMA) microspheres via a nucleophilic substitution reaction between the hydroxyl groups of HEMA and triazinyl chloro

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groups of dye with the equilibrium coupling capacity of 45.8 μmol dye (g polymer)$^{-1}$. The poly(St-HEMA) monodisperse microspheres were characterized by scanning electron microscopy. The effect of concentration of HSA, medium pH, and ionic strength on the adsorption efficiency of dye-attached microspheres were studied in a batch system. The nonspecific adsorption of HSA on the poly(St-HEMA) microspheres was 2.1 mg g^{-1}. Reactive green HE-4BD attachment significantly increased the HSA adsorption up to 221 mg g^{-1}. The langmuir adsorption model was found to be applicable in interpreting HSA adsorption by reactive green HE-4BD attached microspheres. Significant amount of the adsorbed HSA (up to 92%) was eluted in 1 hr in the elution medium containing 1.0-M NaCl. To determine the effects of adsorption conditions on possible conformational changes of HSA structure, fluorescence spectrophotometry was employed. We resulted that poly(St-HEMA) dye-affinity microspheres can be applied for HSA adsorption without causing any significant conformational changes. Repeated adsorption/elution processes showed that these dye-attached monodisperse microspheres are suitable for HSA adsorption.

Key Words: Microspheres; Reactive green HE-4BD; Human serum albumin; Dye-affinity adsorbents; Styrene; 2-Hydroxyethyl methacrylate.

INTRODUCTION

Bioaffinity chromatography is already a well-established method for identification, purification, and separation of biomolecules, and based on highly specific molecular recognition or biorecognition.[1,2] In this method, the molecule having specific recognition capability is immobilized on a suitable insoluble support, which is usually a polymeric material in bead or membrane form. A wide variety of biofunctional molecules, including enzymes, coenzymes, cofactors, antibodies, amino acids, oligopeptides, several other proteins, nucleic acids (DNA, RNA), and oligonucleotides may be used as ligands in the design novel biosorbents.[3–8] These ligands are extremely specific in most cases. However, they are expensive due to high cost of production and/or extensive purification steps. In the process of biospecific sorbent preparation, it is difficult to immobilize them onto the supporting matrix with retention of their original biological activity. High precautions are also needed in their use and storage. Dye-ligands have been considered as one of the important alternatives to the natural biological counterparts for bioaffinity chromatography to circumvent many of their drawbacks mentioned above.[9–11] Dye-ligands are able to bind most type of proteins, especially enzymes, in some cases in a remarkably specific manner.[12–14] They are

commercially available, inexpensive, and can easily be immobilized, especially matrices bearing hydroxyl groups. Although dyes are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many proteins and enzymes by mimicking the structure of the substrates, cofactors, or binding agents for those biomolecules.

Chromatographic techniques have a number of drawbacks, such as the compressibility of the column packaging materials (i.e., softgel and macrobeads) and the fouling.[15] To increase the protein loading capacity, the particle size has been reduced to 0.1–1.0 μm, but such carriers require high-pressure equipment. Due to these reasons, nonporous affinity sorbents of small-particle diameter (i.e., micron size) have been gaining more attention since the mid-1980s for the rapid high-performance liquid chromatography (HPLC) of biomolecules.[16] 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.[17] 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.[18]

In the present work, a dye-affinity monodisperse microspheres using poly(styrene-2-hydroxyethyl methacrylate) [poly(St-HEMA)] as the support matrices was prepared. A dye-ligand, Reactive green HE-4BD, was covalently attached to the monodisperse microspheres and the adsorption–elution behavior of human serum albumin (HSA) was investigated. System parameters, such as the adsorption conditions (i.e., concentration of HSA, medium pH, and ionic strength), were varied to evaluate their effects on the performances of dye-affinity microspheres.

EXPERIMENTAL

Materials

HSA (98% pure by gel electrophoresis, fatty acid free, 67 kDa) was purchased from Aldrich Chem. Co., (Milwaukee, WI) and used as received. Reactive green HE-4BD was obtained from Polyscience (Warrington) and used without further purification. Styrene (St, Yarpet AS, Turkey) was distilled under vacuum and stored in a refrigerator until use. 2-Hydroxyethyl methacrylate (HEMA) supplied from Sigma (St. Louis) was purified by passing through active alumina and stored in a refrigerator until use. Azobisisobutyronitrile (AIBN) and poly(vinyl pyrrolidone) (PVP) (MW: 30,000, BDH Chemicals Ltd., Poole, England) were selected as the initiator and the stabilizer, respectively. AIBN was recrystallized from methanol.

Ethanol (Merck, Germany) was used as the diluent and used without further purification. All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure organic/colloid removal and ion exchange packed bed system. The resulting purified water (deionized water) had a specific conductivity of 18 MΩ.

Preparation of Poly(St-HEMA) Microspheres

The dispersion polymerization was performed in a magnetic driven, sealed cylindrical reactor (volume: 500 mL) equipped with a temperature control system. A typical procedure applied for the dispersion copolymerization of styrene and 2-hydroxyethyl methacrylate is given later. The monomer phase was comprised of 40-mL styrene and 10-mL HEMA. AIBN, 250 mg was dissolved into this monomer mixture. The resulting medium was sonicated for about 5 min at 200 W within an ultrasonic water bath (Bransonic 2200, England) for the complete dissolution of AIBN in the polymerization medium. Four gram of PVP was dissolved in a homogeneous solution of ethanol (100 mL) and water (100 mL) placed in a polymerization reactor. The reactor content was stirred at 500 rpm during the monomer addition completed within about 5 min and the heating was started. Then the reactor was purged with bubbling nitrogen for about 5 min. Then, the sealed reactor was placed in a shaking water bath at room temperature. The initial polymerization time was defined when the reactor temperature was raised to 65°C. The dispersion copolymerization was performed for 4 hr at 500-rpm stirring rate and a temperature of 65 ± 0.1°C. After completion of the polymerization period, the reactor content was cooled down to room temperature and centrifuged at 5000 rpm for 10 min for the removal of dispersion medium. Poly(St-HEMA) microspheres were redispersed within 10 mL of ethanol and centrifuged again under similar conditions. The ethanol washing was repeated three times for complete removal of unconverted monomers and other components. Finally, poly(St-HEMA) microspheres were redispersed within 10 mL of water (0.10%, by weight) and stored at room temperature.

Dye Attachment to Poly(St-HEMA) Microspheres

Reactive green HE-4BD was covalently attached to the poly(St-HEMA) monodisperse microspheres via the nucleophilic substitution reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA comonomer, with the elimination of NaCl under alkaline conditions. First,

reactive green HE-4BD was dissolved in 10 mL of water (dye concentration: 0.5 mg mL^{-1}). This aqueous dye solution was transferred to poly(St-HEMA) microspheres (total mass: 10 g) in 90 mL of distilled water, and then 4.0 g of NaOH were added. The medium was heated at 80°C in a sealed reactor and was stirred magnetically for 4 hr. Under these experimental conditions, a chemical reaction took place between the chlorine-containing group of the reactive green HE-4BD and the hydroxyl groups of the HEMA comonomer, with the elimination of NaCl, resulting in covalent attachment of reactive green HE-4BD onto the poly(St-HEMA) microspheres. To remove the nonspecifically attached dye molecules, an extensive cleaning procedure was applied, which was as follows: the microspheres were first washed with deionized water. The microspheres were dispersed in methanol, and the dispersion was sonicated for 2 hr in an ultrasonic bath (200 W, Bransonic 200, USA). At the last stage, microspheres were washed again with deionized water. It must be pointed out that any remaining chlorine atoms in the reactive green HE-4BD (dichlorotriazinyl dye) structure, after attachment, converted to amino groups by treating them with 2-M NH_4Cl at pH 8.5 for 4 hr at room temperature. Reactive green HE-4BD-attached poly(St-HEMA) microspheres were stored at 4°C with 0.02% sodium azide to prohibit microbial contamination.

The release of the reactive green HE-4BD from the dye-attached microspheres was investigated at different pH values in the range of 4.0–8.0. It should be noted that these media were the same that were used in the HSA adsorption experiments given as follows. Reactive green HE-4BD release was also determined in the medium at 1.0-M NaCl, which was the medium used the HSA elution experiments. The medium with the reactive green HE-4BD-attached microspheres was incubated for 24 hr at room temperature. Then, microspheres were removed from the medium, and the reactive green HE-4BD concentration in the supernatant was measured by spectrophotometry at 630 nm.

Characterization of Monodisperse Microspheres

Elemental Analysis

The amounts of attached reactive green HE-4BD on the microspheres were obtained by using an elemental analysis instrument (Leco, CHNS-932, USA). The amount of reactive green HE-4BD attachment on the microspheres was calculated by considering the sulfur stoichiometry.

Microscopic Observations

Microscopic observations and photographs of the microspheres were performed by using a scanning electron microscope (JEOL, JEM 1200 EX,

Tokyo, Japan). Microspheres were dried at room temperature and coated with a thin layer of gold (about 100 Å) in vacuum and photographed in the electron microscope).

FTIR Studies

FTIR spectra of the poly(St-HEMA), reactive green HE 4BD, and reactive green HE-4BD-attached poly(St-HEMA) microspheres were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry microspheres (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany) and pressed into a pellet. The FTIR spectrum was then recorded.

HSA Adsorption from Aqueous Solutions

In these experiments, the effects of medium pH and ionic strength on the adsorption capacity of reactive green HE-4BD derived poly(St-HEMA) microspheres were studied. The adsorption experiments were carried out batchwise in the media at different pH values. The pH of the adsorption medium was varied between 4.0 and 8.0 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 Tris/HCl for pH 8.0). Ionic strength of the adsorption media was changed in the range of 0.01–0.2. HSA concentration was varied between 0.5–3.0 mg mL^{-1}. In a typical adsorption experiment, HSA was dissolved in 10 mL of buffer solution, and microspheres (total mass: 100 mg) were added. Then the adsorption experiments were conducted for 2 hr (equilibrium time) at 4°C at a stirring rate of 100 rpm. At the end of this equilibrium period, HSA adsorption by the reactive green HE-4BD derived microspheres was determined by measuring the initial and final concentration of HSA within the adsorption medium using Coomassie brilliant blue as described by Bradford.

Elution Studies

The HSA elution experiments were performed in 1.0-M NaCl solution. The HSA adsorbed microspheres were placed in the elution medium and magnetically stirred for 1 hr at 25°C at a stirring rate of 100 rpm. The final HSA concentration within the elution medium was determined by spectrophotometry. The elution ratio was calculated from the amount of HSA adsorbed on the microspheres and the amount of HSA desorbed. To evaluate the effects of adsorption conditions on HSA structure, fluorescence spectra of the native HSA, heat-denaturated HSA, and desorbed HSA were obtained.

Native HSA aqueous solution (1 mg mL^{-1}, pH: 7.0) was denaturated at 70°C for 4 hr. Measurements were taken with a Shimadzu spectrofluorometer using 1-cm^2 quartz cells. Monochromatic readings were taken from a digital display with a 0.2-sec time constant and a 2-nm bandwidth on the excitation side and 5 nm on the emission side. Initial calibration was carried out with standard solution of HSA in phosphate buffered saline (PBS) with 280-nm fluorescence excitation and 340-nm emission wavelengths.

RESULTS AND DISCUSSION

Poly(St-HEMA) Microspheres

Figure 1 shows a representative optical picture of the monodisperse (RSD $< 1\%$) poly(St-HEMA) microspheres with a diameter of 4 μm. As seen here, micropheres were obtained in the highly monodisperse form. The polydispersity index (PDI) value of poly(St-HEMA) microspheres was calculated to be around 1.001. FTIR spectroscopy was used to show incorporation of reactive green HE-4BD within poly(St-HEMA) microspheres (Fig. 2). The bands observed at 1620, 1275, and 1150 cm^{-1} indicated aromatic C═C vibration, symmetric stretching of S═O, and asymmetric stretching of S═O. FTIR spectra of plain poly(St-HEMA) showed split bands at the same wave numbers, 1275 and 1150 cm^{-1}, which were observed on dye-derivatized

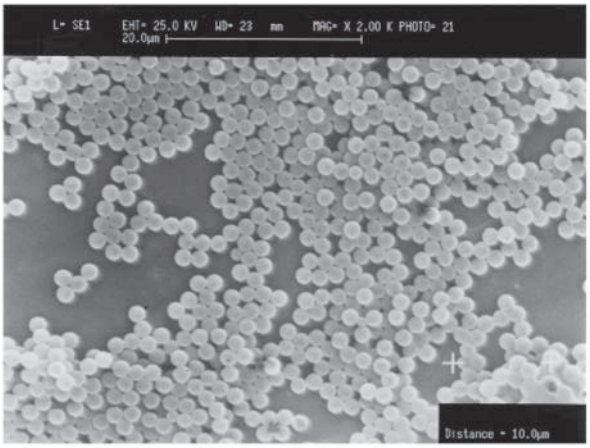

Figure 1. Scanning electron micrography of the monodispersed poly(St-HEMA) microspheres.

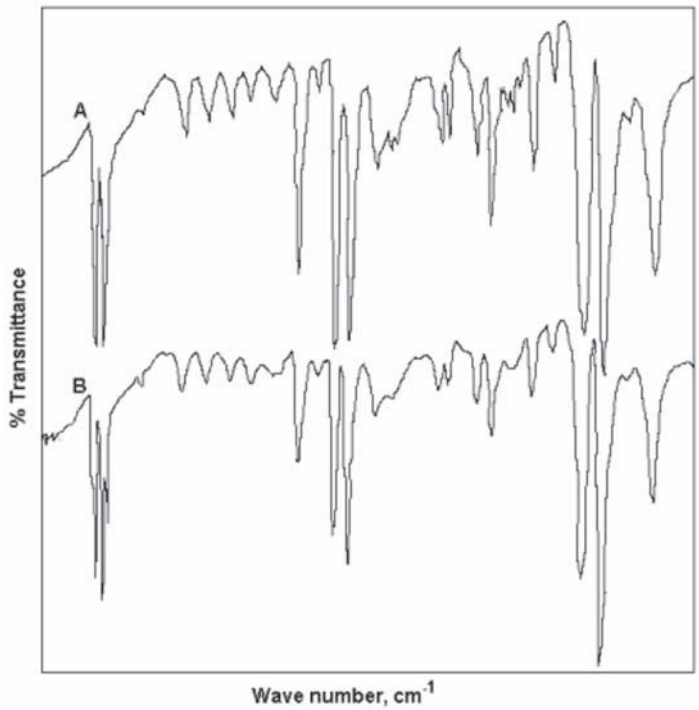

Figure 2. FTIR spectra: (A) poly(St-HEMA) microspheres; (B) reactive green HE-4BD attached poly(St-HEMA) microspheres.

polymer. But FTIR spectrum 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 dye. The band observed at 3570 cm^{-1} indicated N–H and SO_3H groups on the chemical structure of reactive green HE-4BD.

Reactive green HE-4BD is a dichlorotriazine dye (Fig. 3). It contains six sulfonic acid groups, and five basic primary and secondary amino groups. The

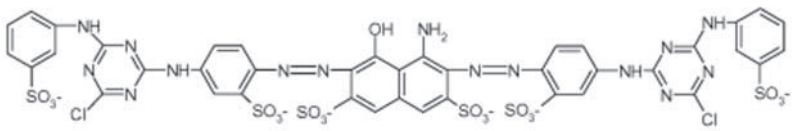

Figure 3. Chemical structure of reactive green HE-4BD.

strong binding of the dye-ligands to proteins may have resulted from cooperative effect of different mechanisms, such as hydrophobic and/or ion-exchange interactions 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. The dye-ligands are not very hydrophobic overall, but they do have planar aromatic surfaces that prefer to interact with hydrophobic residues in proteins. The reactive green HE-4BD molecules were covalently attached to the monodisperse poly(St-HEMA) microspheres. The visual observations (the color of the monodisperse microspheres) ensured attachment of dye molecules. Note that the reactive green HE-4BD-attached microspheres were extensively washed and there is no detectable dye-ligand leakage from any of the dye-attached microspheres and in any media used at adsorption and/or elution steps.

Normal and reactive green HE-4BD-attached microspheres were subjected for elemental analysis. The amount of reactive green HE-4BD attached to the microspheres was evaluated from these data, by considering the stoichiometry, which was found as 45.8 μmol dye (g polymer)$^{-1}$. The studies of reactive green HE-4BD leakage from the dye-attached microspheres showed that there was no leakage in any of the media described in Experimental section, which assured that the washing procedure was quite enough for removal of uncovalently bound reactive green HE-4BD molecules from the polymeric matrix.

HSA ADSORPTION AND ELUTION

Adsorption Isotherm

Figure 4 shows the adsorption isotherm of HSA. As presented in this figure, with increasing HSA equilibrium concentration in solution, the amount of HSA adsorbed by the monodisperse poly(St-HEMA) microspheres increases and approaches saturation. This adsorption isotherm represents a high affinity between HSA and reactive green HE-4BD. It becomes constant when the HSA concentration is greater than 2.5 mg mL^{-1}. A negligible amount of HSA adsorbed nonspecifically on the poly(St-HEMA) (2.1 mg g^{-1}). Reactive green HE-4BD attachment significantly increased HSA adsorption capacity of the microspheres (up to 221 mg g^{-1}). This result is due to the fact that a strong specific interaction between HSA and dye molecules.

Langmuir Adsorption Model

An adsorption isotherm is used to characterize the interaction of the each protein with the adsorbents. This provides a relationship between the

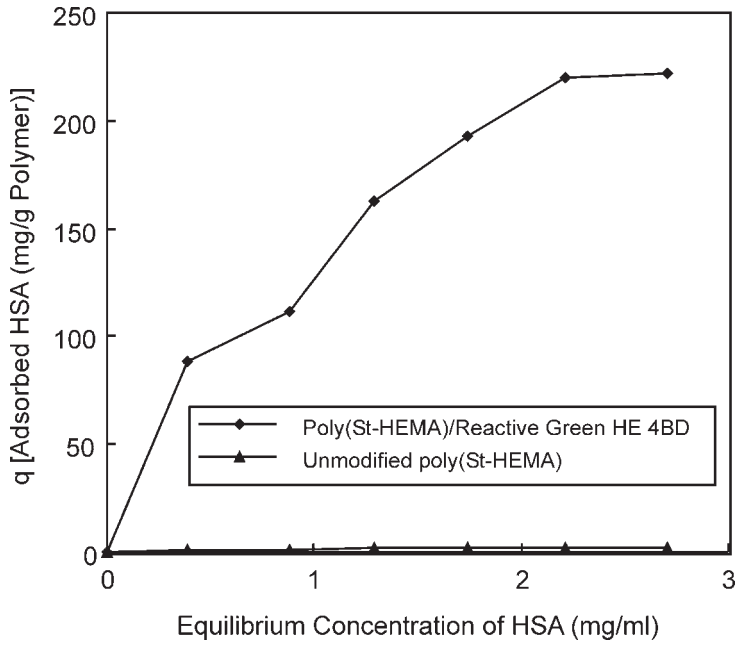

Figure 4. HSA adsorption on reactive green HE-4BD microspheres as a function of HSA equilibrium concentration; reactive green HE-4BD loading: 45.8 μmol g^{-1}; pH: 5.0; *T*: 25°C.

concentration of protein in the solution and the amount of protein adsorbed onto solid phase when the two phases are at equilibrium. The Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which can only hold one molecule. These sites are also assumed to be energetically equivalent, and distant to each other so that there is no interactions between molecules adsorbed to adjacent sites.

During the batch experiments, adsorption isotherms were used to evaluate adsorption properties. For the systems considered, the Langmuir model was found to be applicable in interpreting HSA adsorption by reactive green HE-4BD-attached microspheres. The Langmuir adsorption isotherm is expressed by Eq. (1). The corresponding transformations of the equilibrium data for HSA gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

$$Q = \frac{Q_{\max} b C_{\text{eq}}}{1 + b C_{\text{eq}}} \tag{1}$$

where Q is the concentration of bound HSA in the adsorbent (mg g^{-1}), C_{eq} is the equilibrium HSA concentration in solution (mg L^{-1}), b is the Langmuir constant (L mg^{-1}), and Q_{max} is the maximum adsorption capacity (mg g^{-1}). This equation can be linearized so that

$$\frac{1}{Q} = \left[\frac{1}{(Q_{max}b)}\right]\left[\frac{1}{C_{eq}}\right] + \left[\frac{1}{Q_{max}}\right] \tag{2}$$

From the plot of $1/C_{eq}$ vs. $1/Q$ was employed to generate the intercept of $1/Q_{max}$ and the slope of $1/Q_{max}b$ (Fig. 5). The maximum capacity (Q_{max}) data for the adsorption of HSA was obtained from experimental data. The correlation coefficient (R^2) was 0.91 for reactive green HE-4BD attached microspheres, indicating that the Langmuir adsorption model can be applied in this affinity adsorbent system. It should be also noted that the maximum adsorption capacity (Q_{max}) and the Langmuir constant were found to be 271 mg g^{-1} and 1.2 L mg^{-1} from the Langmuir model.

Effect of pH

Figure 6 reveals the effect of pH on the adsorption of HSA onto reactive green HE-4BD derived poly(St-HEMA) microspheres. In all the investigated

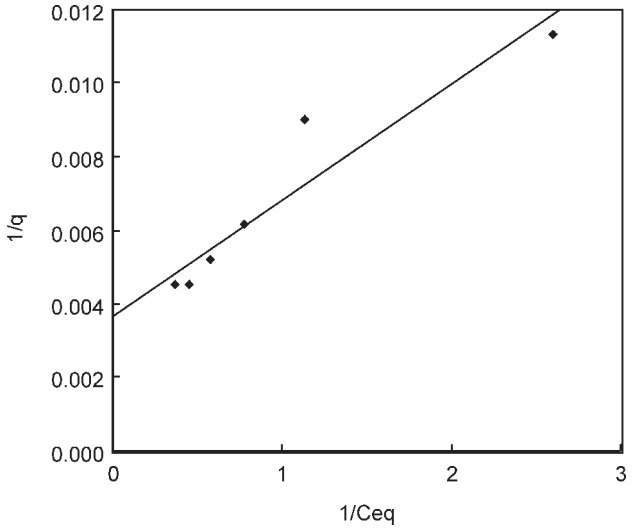

Figure 5. Linear representation of Langmuir equation of HSA with reactive green HE-4BD microspheres: reactive green HE-4BD loading: 45.8 μmol g^{-1}; pH: 5.0; T: 25°C.

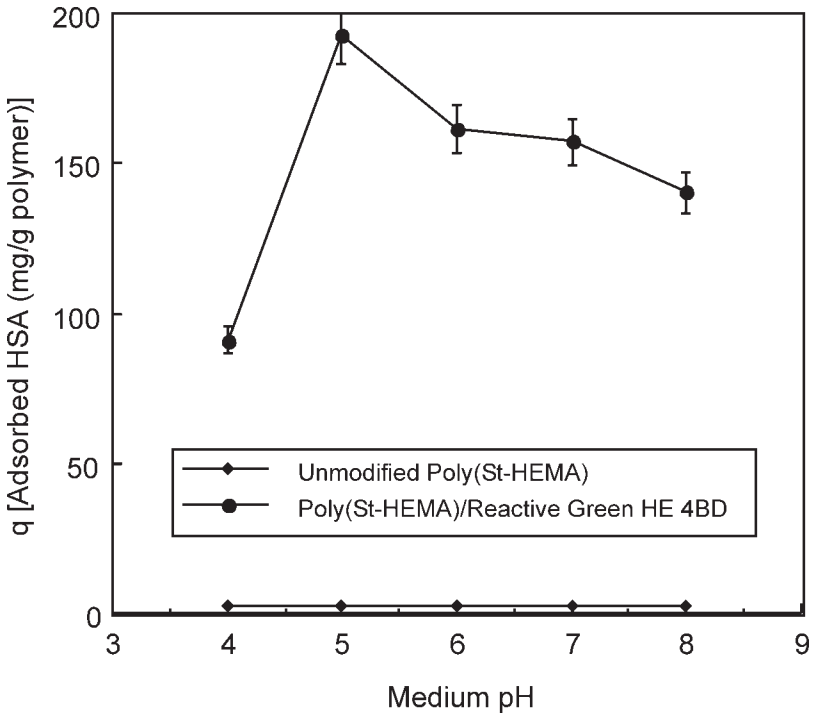

Figure 6. The variation of HSA adsorption capacities of the monodisperse microspheres as a function of pH; reactive green HE-4BD loading: 45.8 μmol g^{-1}; HSA initial concentration: 1.0 mg mL^{-1}; *T*: 25°C.

cases, the maximum adsorption of HSA was observed at pH 5.0, which is the isoelectric point of HSA. Significantly lower adsorption capacities were obtained with all microspheres in acidic and in alkaline pH regions. This is also expected because, as it is known, below or above IEP values, proteins are charged positively or negatively, respectively. They are more hydrated, which increases their stability and solubility in aqueous phase (i.e., means lower adsorption). The decrease in the HSA adsorption capacity can be also attributed to electrostatic repulsion forces between the identically charged groups. In addition, these interactions between the dye and protein molecules may result both from the ionization states of several groups on both the ligands (i.e., reactive green HE-4BD) and amino acid side chains in HSA structure, and from the conformational state of protein molecules at this pH. It should be also noted that nonspecific adsorption is independent of pH and it is observed at the same at all the pH values studied.

Effect of Ionic Strength

The effect of ionic strength (adjusted by adding NaCl) on HSA adsorption is presented in Fig. 7, which shows that the adsorption capacity decreases with increasing ionic strength of the binding buffer (acetate buffer, pH = 5.0). The adsorption of HSA decreases by about 72% as the NaCl concentration changes from 0.01 to 0.2 M. The decrease in the adsorption capacity as the ionic strength increases can be attributed to the repulsive electrostatic interactions between the dye-attached monodisperse microspheres and protein (i.e., HSA) molecules. When the salt concentration increases in the adsorption medium, this can lead to coordination of the deprotonated sulfonic acid groups of the dye with sodium ions of the salt (NaCl), which leads to low protein adsorption. The distortion of existing salt bridges in the presence of salt also contributed to this low protein adsorption at high ionic strength.

Comparison with Related Literature

Different dye-affinity sorbents with different adsorption capacities were reported in literature for albumin adsorption. Nigel et al. used dye-incorporated

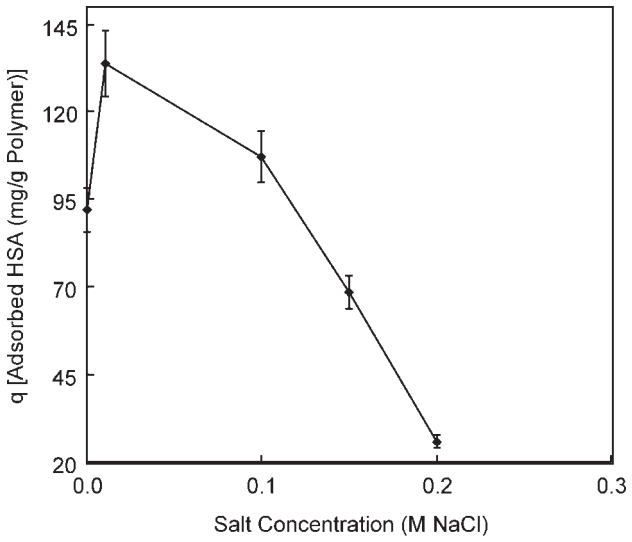

Figure 7. Effect of the ionic strength on HSA adsorption on the cibacron blue F3GA attached poly(St-HEMA) microspheres; reactive green HE-4BD loading: 45.8 μmol g^{-1}; HSA concentration: 0.5 mg mL^{-1}; pH: 5.0; *T*: 25°C.

sepharose CL-6B-200. They reported bovine serum albumin adsorption capacities around 1–3 mg (g moist gel)$^{-1}$.[19] Denizli et al. used different dye-affinity polymeric sorbents including monodisperse poly(methyl-methacrylate-hydroxyethyl methacrylate), poly(vinyl alcohol) and poly (hydroxyethyl methacrylate) microspheres. They obtained 35–178 mg (g polymer)$^{-1}$ for bovine and HSA.[20–22] Nash and Chase used poly(vinyl alcohol) modified poly(styrene-divinyl benzene) beads caring different dye-ligands.[23] They presented adsorption capacities of 11.7–27 mg HSA g^{-1}. Horstman et al. used cibacron blue F3GA-incorporated sepharose CL-6B with different narrow–range mean particle diameters. They reported bovine serum albumin (BSA) adsorption capacities around 5.4–12 mg (g moist gel)$^{-1}$.[24] Zeng and Ruckenstein reported 10.2 mg HSA g^{-1} adsorption capacity with cibacron blue F3GA attached poly(ether sulfone) supported chitosan.[25] Li and Spencer used cibacron blue F3GA attached poly(ethylene imine) coated titania and achieved 4.4 mg HSA g^{-1}.[26] Chase reached 14 mg BSA g^{-1} with cibacron blue F3GA attached sepharose CL-6B.[27] Tuncel et al. reported 60 mg BSA g^{-1} polymer with cibacron blue F3GA attached poly(vinyl alcohol)-coated monodisperse polystyrene beads.[28] Muller-Schulte et al. used several carriers made of different polymers, and cibacron blue F3GA as the dye-ligand.[29] The albumin adsorption values were in the range of 0.19–0.81 mg HSA (mL sorbent)$^{-1}$. Adsorption capacities of commercially available agarose-cibacron blue F3GA sorbents (BioRad, California) were reported as about 11 mg albumin (mL sorbent)$^{-1}$.[30] Comparison of these results show that reactive green HE-4BD attached monodisperse poly(St-HEMA) microspheres exhibit much higher HSA adsorption capacities.

Elution and Stability of Reactive Green HE-4BD Microspheres

The elution of HSA from reactive green HE-4BD microspheres was studied in a batch system. Reactive green HE-4BD microspheres carrying HSA were placed in an elution medium containing 1.0-M NaCl and the amount of HSA released in 60 min was determined. The elution ratios for HSA were calculated by using the amount of HSA released and amount of HSA adsorbed. More than 90% of the adsorbed HSA was desorbed in all cases when NaCl was used for elution. Note that there was no reactive green HE-4BD release in this case, which shows that dye molecules are attached to poly(St-HEMA) microspheres by covalent bonding. With the elution data just given, we concluded that NaCl is a suitable elution agent and allows repeated use of the affinity adsorbents prepared in this study.

For practical applications, the stability of the prepared dye-affinity microspheres is very important. The results of repeated use of the reactive green HE-4BD microspheres are shown in Fig. 8. After the first run, a decrease of about 5% in HSA adsorption capacity was observed. This value remained stable in subsequent runs. This result is probably due to noncovalently bound dye molecules being washed out after first run rather than dye-ligand leakage. This is an important feature, indicating the possibility of recycling monodisperse microspheres for reuse in the process.

To evaluate the effects of adsorption conditions on HSA structure, fluorescence spectrophotometry was employed. The fluorescence spectra of HSA samples obtained from the elution step were recorded (Fig. 9). The fluorescence spectra of native and heat-denaturated HSA were also taken. A clear difference was observed between the fluorescence spectra of native HSA and heat-denaturated HSA. An appreciable shift was seen in the maximum wavelength of denaturated HSA according to the native one. On the other hand, the fluorescence spectra of the samples withdrawn from the elution step were very close to those of native HSA and no

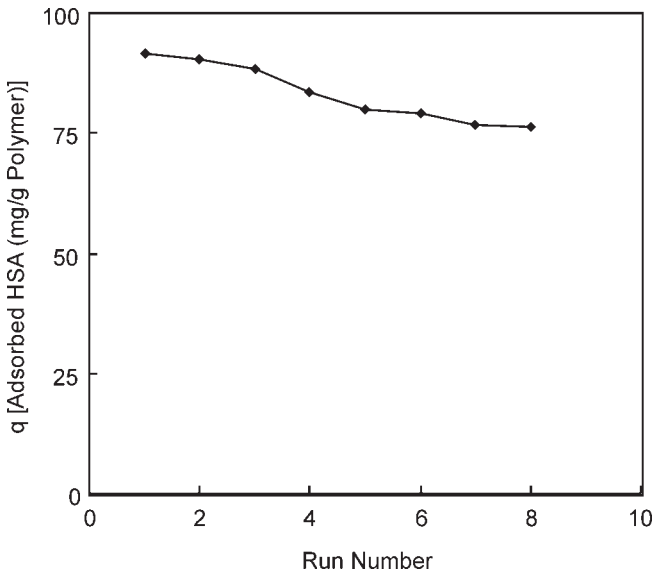

Figure 8. Repeated use of reactive green HE-4BD microspheres; reactive green HE-4BD loading: 45.8 μmol g^{-1}; HSA concentration: 0.5 mg mL^{-1}; pH: 5.0; *T*: 25°C. After washing with deionized water and elution with 1.0-M NaCl, the microspheres were re-equilibrated and used again.

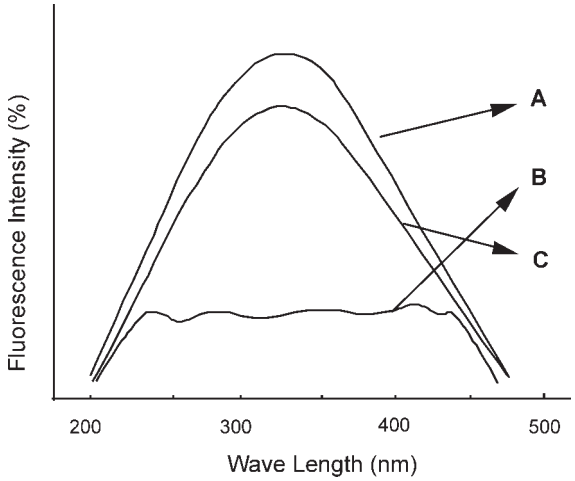

Figure 9. Fluorescence spectra of HSA; (A) native HSA; (B) heat-denaturated HSA; and (C) eluted HSA.

significant shift of maximum wavelength was detected in the spectra of these samples relative to that of native HSA. It may be concluded that dye-affinity chromatography with monodisperse poly(St-HEMA) can be applied for HSA separation without causing any conformational changes and denaturation.

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