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Application of Supermacroporous Monolithic Hydrophobic Cryogel in Capturing of Albumin

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Abstract Supermacroporous poly{2-hydroxyethyl methacrylate-*co*-[*N*,*N*-bis(2,6-diisopropylphenyl)-perylene-3,4,9,10-tetracarboxylic diimide]} [poly(HEMA-*co*-DIPPER)] monolithic cryogel column was prepared by radical cryocopolymerization of HEMA with DIPPER as functional comonomer and *N*,*N*′-methylene-bisacrylamide (MBAAm) as crosslinker directly in a plastic syringe for adsorption of albumin. The monolithic cryogel contained a continuous polymeric matrix having interconnected pores of 10–50 μm size. Poly(HEMA-*co*-DIPPER) cryogel was characterized by swelling studies, FTIR, scanning electron microscopy, and elemental analysis. The equilibrium swelling degree of the poly (HEMA-*co*-DIPPER) cryogel was 14.7 g H_2O/g dry cryogel. Poly(HEMA-*co*-DIPPER) cryogel was used in the adsorption/desorption of albumin from aqueous solutions. The nonspecific adsorption of albumin onto plain poly(HEMA) cryogel was very low (3.36 g/g polymer). The maximum amount of albumin adsorption from aqueous solution in acetate buffer was 40.9 mg/g polymer at pH 5.0. It was observed that albumin could be repeatedly adsorbed and desorbed with the poly(HEMA-*co*-DIPPER) cryogel without significant loss of adsorption capacity.

Keywords Cryogel · Affinity separation · Albumin · HEMA

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Introduction

The development of new techniques and methods for the separation and purification of proteins has been essential for many of the recent advancements in biotechnology and biomedicine [1]. The purification of a protein is a prerequisite for its structural and functional studies or its potential applications. A wide variety of purification techniques are available today. However, different types of chromatography have become dominant due to their high resolving power [2].

Macroporous polymeric materials have attracted great interest in biomedical, biotechnological, and pharmaceutical sciences [3–6]. In particular, porous hydrogels are prepared using several techniques, such as freeze-drying [7], porogenation [8], microemulsion formation [9], gas blowing technique [10], and phase separation [11,12]. Porous hydrogels adsorb and retain large amounts of water [13]. Macroporous hydrogels, called cryogels, are gel matrices that have interconnected macropores that are formed in moderately frozen solutions of monomeric and polymeric precursors [14]. The gel formation occurs in liquid microphase and the crystals of frozen solvents perform as porogen. After melting the ice crystals, a system of large interconnected pores with pore size up to 100 mm is formed [15]. Depending on the gel precursors and chemical reaction used, the micro- and macroporous structure of cryogels can vary to a large extent [16].

While conventional chromatography techniques, which uses gel beads column chromatography have certain limitations such as a high-pressure drop, low flow rates, and, hence, low productivities and difficulties in efficient scale up, cryogels provides a potential solution due to their low pressure drop and lack of diffusion resistances due to the macropores compared to traditional column chromatography [17].

Hydrophobic interaction chromatography (HIC) is one of the widely used protein purification techniques [18]. HIC is primarily based on the interaction between hydrophobic residues on the surface of the protein with the hydrophobic surface of a chromatographic resin [19]. During the last years, HIC has been used by many researchers and today is powerful bioseparation technique in laboratory scale. With development of new and various separation medium, HIC found to many applications in the purification of biomolecules, such as serum proteins, nuclear proteins, hormones, recombinant proteins, and enzymes [20].

Albumin is the most abundant protein present in blood plasma. Albumin has many physiological functions which contribute significantly to colloid osmotic blood pressure and aid in the transport, distribution, and metabolism of many endogeneous and exogeneous substance including bile acids, bilirubin, long-chain fatty acids, amino acids, steroids, metal ions such as copper, zinc, calcium, and magnesium and numerous pharmaceuticals. Albumin commonly used for therapeutic purposes such as shock, burns, hypoalbuminemia or hypoproteinemia, surgery, trauma, cardiopulmonary bypass, the acute respiratory distress syndrome, hemodialysis, acute nephrosis, hyperbilirubinemia, acute liver failure, ascites, and sequestration of protein-rich fluids in acute peritonitis, pancreatitis, mediastinitis, and extensive cellulites [21].

Albumin is at present commonly isolated from human plasma by Cohn's blood fractionation procedure [22]. Albumin is currently used in greater volume than any other biopharmaceutical solution that is available, and worldwide manufacturing is of the order of hundreds of tonnes annually. Cohn's method concerns precipitation of proteins using ethanol with varying pH, ionic strength, and temperature. But this technique, which is the oldest method of industrial fractionation of blood proteins, is not highly specific and can

give partially denaturated proteins [21]. Cryogels are very good alternative adsorbents for protein purification with many advantages such as large pores, short diffusion path, low-pressure drop, and very short residence time for both adsorption and elution. Cryogels are also cheap materials, and they can be used as disposable avoiding cross-contamination between batches [23]. And also, large pores of cryogel allow passing through of blood component without any occlusion. Thus, it is not necessary to separate the blood cells and other blood components.

In this study, supermacroporous poly(2-hydroxyethyl methacrylate-*co*-(*N*,*N*-bis(2,6-diisopropylphenyl)-perylene-3,4,9,10-tetracarboxylic diimide)) poly(HEMA-*co*-*N*,*N*-bis (2,6-diisopropylphenyl)-perylene-3,4,9,10-tetracarboxylic Diimide (DIPPER)) monolithic hydrophobic cryogel column was prepared by radical cryocopolymerization of HEMA with DIPPER as functional comonomer and MBAAm as crosslinker directly in a plastic syringe for adsorption of albumin. Poly(HEMA-*co*-DIPPER) cryogel was characterized by using FTIR, elemental analysis, scanning electron microscopy (SEM), and swelling test. Albumin adsorption on the poly(HEMA-*co*-DIPPER) cryogel from aqueous solutions containing different amounts of albumin, at different pHs and ionic strengths was performed. Desorption and reusability of poly(HEMA-*co*-DIPPER) cryogel were also investigated.

Experimental

Materials

Albumin, hydroxyethyl methacrylate, MBAAm, *N*,*N*,*N*′,*N*′-tetramethylene diamine (TEMED), and ammonium persulfate (APS) were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade and used as purchased without further purification.

Synthesis of DIPPER

N,*N*-bis-(2,6-diisopropylphenyl)-perylene-3,4,9,10-tetracarboxylic diimide was synthesized according to the literature procedure [24–26]. A mixture of 3.14 mL (15 mmol) of 2,6-diisopropylaniline and 0.33 g (1.5 mmol) of $Zn(CH_3COO)_2.2H_2O$ was added into a solution of 0.59 g (1.5 mmol) of perylene-3,4,9,10-tetracarboxylic dianhydride in 20 mL of quinoline. The mixture was stirred at 210 °C for 14 h under a nitrogen atmosphere. After cooling to room temperature, the mixture was added into 400 mL of methanol/20% hydrochloric acid (*v*/*v*=2:1) under stirring. The solid precipitate was filtered off and stirred in 100 mL of cold Na_2CO_3 solution (10%) for 2 h. Then, the precipitate was filtered again and washed with water until the filtrate was colorless. The solid was dried under vacuum at 90 °C for 16 h and purified by silica gel column chromatography with *n*-hexane/ethyl acetate (70:30) as eluent. Yield: 88%, mp>300 °C. FT-IR (KBr, cm^{-1}): 2,963, 2,873, 1,706, and 1,667 (imide group), 1,597, 1,404, 1,345, 1,255, 1,200, 959, 833, 813, 746 cm^{-1}. ^1H NMR ($CDCl_3$, δ 7.26 ppm): δ=8.78 and 8.73 (8 H, dd, perylene H); 7.50 (2 H, t); 7.36 (4 H, d); 2.75 (4 H, h); 1.17 (24 H, d) ppm. ^1HNMR spectrum was measured on a 400 MHz Bruker spectrometer. The FT-IR spectrum was determined on a Perkin–Elmer model Spectrum BX spectrophotometer by dispersing samples in KBr disks.

Preparation of Poly(HEMA-*co*-DIPPER) Cryogel

Production of poly(HEMA-*co*-DIPPER) monolithic cryogel was prepared as follows: 1.3 mL of HEMA and 20 mg of DIPPER monomers dissolved in 5.0 mL of deionized water. 0.283 mg of MBAAm dissolved in 10 mL of deionized water and mixed with first solution. The cryogel was produced by free radical polymerization initiated by TEMED (25 μL) and APS (20 mg). APS was added to this solution and cooled in an ice bath for 3 min. Then, TEMED was added, and the reaction mixture was stirred for 1 min. The reaction mixture was poured immediately into plastic syringe (total volume 5 mL, internal diameter 0.8 cm) with closed outlet at the bottom. The polymerization solution in the syringe was frozen at −12 °C for 24 h and then thawed at room temperature. In order to remove unreacted monomers and initiator, the cryogel was washed with 200 mL of water and stored in buffer containing 0.02% sodium azide at 4 °C. In order to investigate the nonspecific albumin adsorption onto poly(HEMA) cryogel (without DIPPER), the plain poly(HEMA) cryogel was prepared in the absence of DIPPER monomer using the same polymerization procedure which was given above.

Characterization of Cryogel

Swelling degree (S) of poly(HEMA-*co*-DIPPER) cryogel was determined in distilled water. Briefly; dry cryogel was carefully weighed (±0.0001 g) before being placed in a 50 mL of vial containing distilled water. The vial was put into an isothermal water bath with a fixed temperature (25±0.5 °C) for 2 h. The cryogel was taken out from water, wiped using a filter paper, and weighed. The swelling degree was calculated as:

$$S = \left(\frac{m_w - m_d}{m_d} \right)$$

where m_w and m_d are weights of wet and dry cryogels, respectively.

The surface morphology of the cryogel was examined using SEM. The crogel initially dried at 40 °C for 4 days. Dried cryogel mounted on a SEM sample mount and coated under vacuum with a thin layer of gold and examined by SEM (Phillips XL-30 S FEG, The Netherlands).

FTIR spectrum of poly(HEMA-*co*-DIPPER) cryogel was obtained by using a FTIR spectrophotometer (Varian FTS 7000, USA). The dry cryogel (about 0.1 g) was mixed with KBr (0.1 g) and pressed into a pellet form, and FTIR spectrum was then recorded.

To evaluate the degree of DIPPER incorporation to the poly(HEMA-*co*-DIPPER) structure, cryogel was subjected to elemental analysis by using a Leco Elemental Analyzer (Model CHNS-932, USA).

Adsorption of Albumin from Aqueous Solutions

Albumin adsorption studies were carried out in a column system. Poly(HEMA-*co*-DIPPER) cryogel was washed with 25 mL of distilled water and then equilibrated with 20 mM acetate buffer (pH 5.0) solution. Then, 10 mL of albumin solution was pumped through the cryogel column for 2 h by a peristaltic pump in a continuous system. The albumin adsorption capacity of poly(HEMA-*co*-DIPPER) cryogel was followed by monitoring the decrease in UV absorbance at 280 nm. Effects of albumin concentration, flow rate, medium pH, and ionic strength on the albumin adsorption capacity were also investigated. In order

to investigate the effect of albumin concentration on adsorption, the concentration was chanced in the range of 0.1–2.0 mg/mL. Chromatographic flow rate of albumin solution was changed between 0.36 and 2.14 cm/min. To determine effects of pH and ionic strength on the adsorption capacity, pH was changed between 3.0 and 8.0; ionic strength was varied in the range of 0.01–1.0 mM.

Desorption and Repeated Use

Adsorbed albumin was desorbed using 0.1 M ethylene glycol solution. Twenty milliliters of desorption agent was pumped through the cryogel column at a flow rate of 0.5 mL/min for 2 h. The final albumin concentration in the desorption medium was determined spectrophotometrically at 280 nm. After desorption, poly(HEMA-*co*-DIPPER) cryogel was cleaned with 50 mM NaOH and then re-equilibrated with 20 mM acetate buffer (pH 5.0) solution. The desorption was calculated from the amount of albumin adsorbed on poly (HEMA-*co*-DIPPER) cryogel and the final albumin concentration in the desorption medium. In order to investigate the repeated usage of poly(HEMA-*co*-DIPPER) cryogel, adsorption–desorption cycle of albumin repeated for five times. After desorption step, the cryogel was washed with 50 mM NaOH solution for regenerating and sterilize the poly (HEMA-*co*-DIPPER) cryogel.

All measurements were repeated three times, and the average values were shown.

Results and Discussion

Characterization of Cryogel

Poly(HEMA-*co*-DIPPER) cryogels were prepared as an affinity adsorbents for albumin adsorption. This supermacroporous cryogel was produced by polymerization in the frozen state of monomers, HEMA, and DIPPER with MBAAm as a cross-linker in the presence of APS/TEMED as an initiator/activator pair. The chemical structure of poly(HEMA-DIPPER) cryogel was shown in Fig. 1.

The polymerization proceeds in the frozen state, and after defrosting, cryogels had a macroporous structure with interconnected pores. The SEM micrographs of the internal structure of poly(HEMA-*co*-DIPPER) cryogel was shown in Fig. 2. Poly(HEMA-*co*-DIPPER) cryogel produced in such a way have porous and thin polymer walls, large continuous interconnected pores (10–50 μm in diameter) that provide channels for the

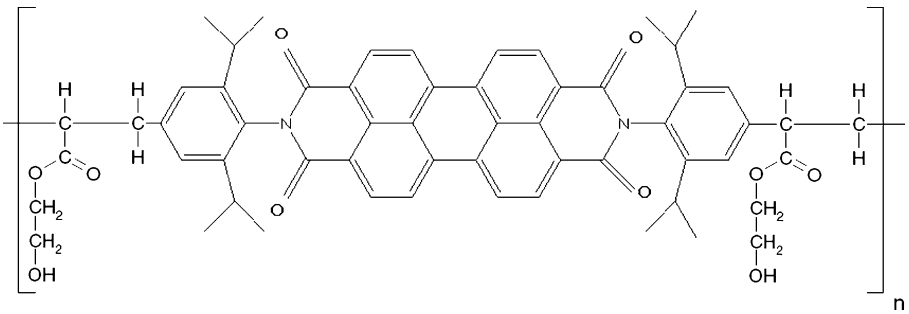

Fig. 1 Chemical structure of poly(HEMA-DIPPER) cryogel

mobile phase to flow through. Pore size of the cryogel matrix is much larger than that of albumin molecules, allowing them to pass easily. The general shape of albumin can be viewed as tree tennis balls in a can or cylinder. The Stokes radius is 3.9 nm [21].

As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible. The equilibrium swelling degree of the poly(HEMA-*co*-DIPPER) cryogel was 14.7 g H_2O/g dry cryogel. Poly(HEMA-*co*-DIPPER) cryogel is opaque, sponge-like, and elastic. This cryogel can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogel was submerged in water, it soaked in water and within 1 to 2 h restored its original size and shape. The FTIR spectrum of the poly(HEMA) and poly(HEMA-*co*-DIPPER) cryogel had the characteristic stretching vibration bands of hydrogen-bonded alcohol, O–H, around 3,400 cm^{-1} and carbonyl around 1,720 cm^{-1}, and poly(HEMA-*co*-DIPPER) cryogel had extra absorption bands of amide I and II, around 1,650 and 1,550 cm^{-1}, respectively (Fig. 3). Concentration of the incorporated DIPPER was found to be 36.3 μmol/g cryogel using nitrogen stoichiometry.

Adsorption of Albumin from Aqueous Solutions

The effect of pH on the adsorption of albumin onto poly(HEMA-*co*-DIPPER) cryogel was investigated in the range of pH 3–8 using 0.2 M acetate (for pH 3–5), and phosphate (for pH 6–8) buffers. Figure 4 shows the effect of pH on the adsorption of albumin. The maximum albumin adsorption was observed at pH 5.0, which is the isoelectric point of albumin. Albumin adsorption capacity decreased sharply with pH values above and below pH 5.0. This decrease may be attributing to electrostatic repulsion of charged groups of protein and crygel matrix. At the isoelectric point, net charge of proteins is zero; therefore, the maximum protein adsorption is usually observed at this pH values. In addition, these interactions between the cryogel matrix and protein molecules may result both from the ionization states of several groups on the both ligands (DIPPER) and amino acid side chains of albumin and from the conformational state of protein molecules at these pH values.

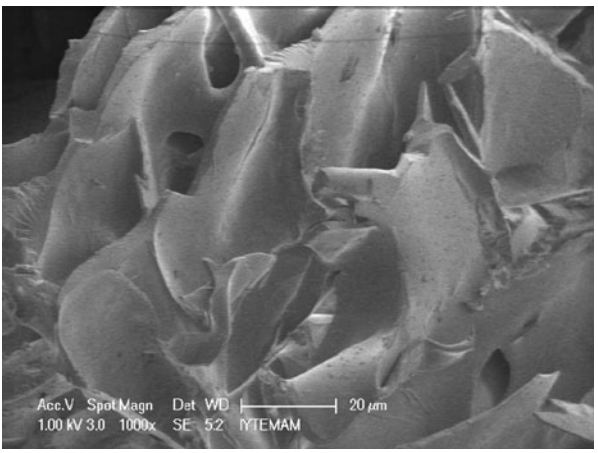

Fig. 2 Scanning electron micrographs of the supermacroporous poly(HEMA-*co*-DIPPER) cryogel

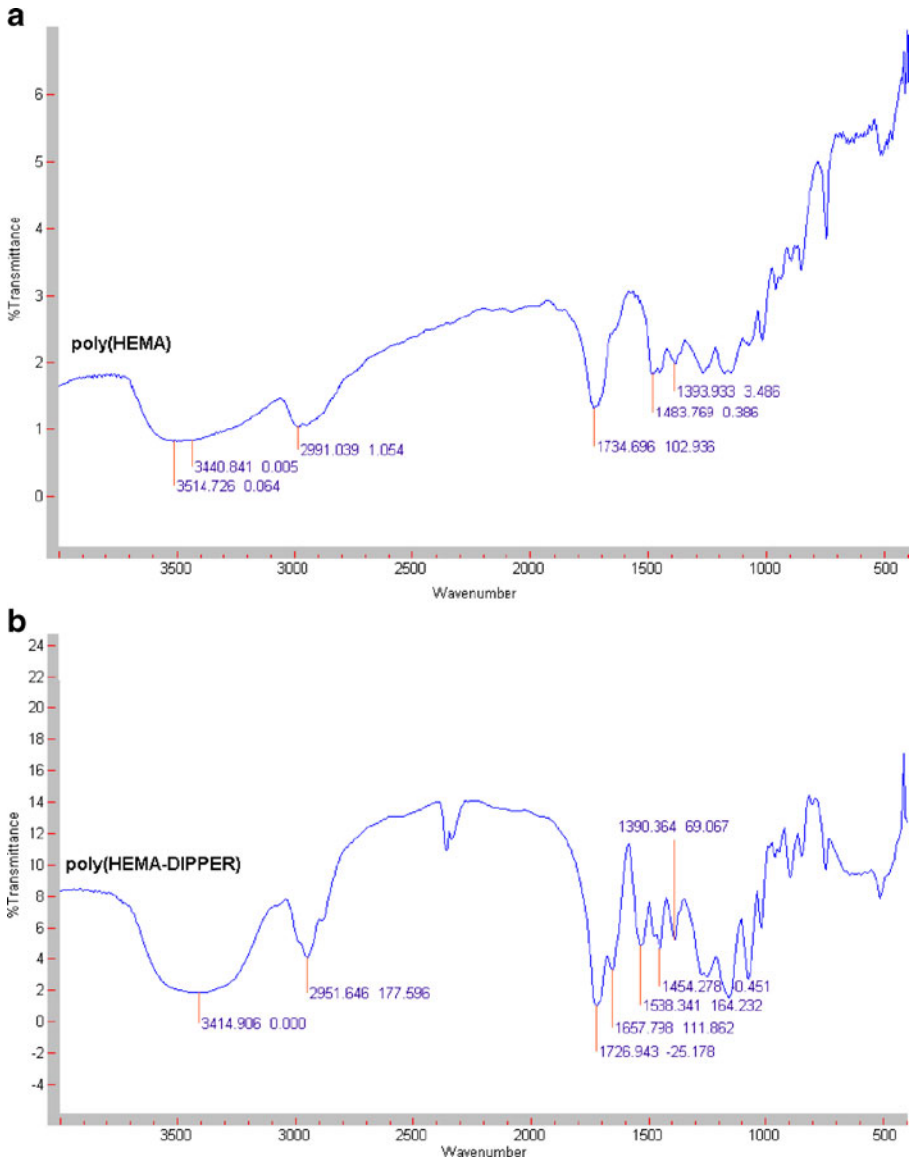

Fig. 3 FTIR spectrum of **a** poly(HEMA) and **b** poly(HEMA-*co*-DIPPER) cryogels

Figure 5 shows the effect of equilibrium concentration of albumin on the albumin adsorption capacity onto poly(HEMA-*co*-DIPPER) cryogel and plain poly(HEMA) cryogel. The adsorption values increased with increasing albumin concentration and a saturation value was achieved at 1.0 mg/mL of albumin concentration, which represents saturation of the active adsorption sites on the poly(HEMA-*co*-DIPPER) cryogel. Maximum adsorption capacity was found to be 40.9 mg/g polymer. The nonspecific interaction between the poly (HEMA-*co*-DIPPER) cryogel and albumin molecules should be minimum to consider the

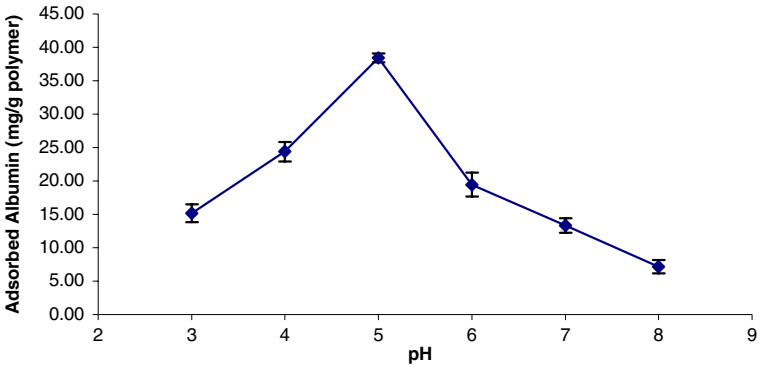

Fig. 4 Effect of pH on albumin adsorption. Albumin concentration 1.0 mg/mL; flow rate 0.36 cm/min; temperature 25 °C; loading time 2 h

interaction as specific. As seen in Fig. 5, nonspecific albumin adsorption onto plain poly (HEMA) cryogel was negligible (3.36 mg/g polymer).

Different support systems have been used for adsorption or purification of albumin from different sources. A comparison of the adsorption capacity of these support systems was summarized in Table 1. As seen in Table 1, the cryogelic affinity support described was quite comparable with the conventional and commercially available carriers.

One of the important phsycochemical aspects for evaluating of the adsorption process as a unit operation is the equilibria of adsorption. Modeling of the equilibrium data has been done using the Langmuir and Freundlich isotherms [27]. The Langmuir and Freundlich isotherms are represented as follows Eqs. 1 and 2, respectively.

$$1/q_e = (1/g_{max}) + [1/(g_{max}b)](1/C_e) \tag{1}$$

$$\ln q_e = 1/n(\ln C_e) + \ln K_F \tag{2}$$

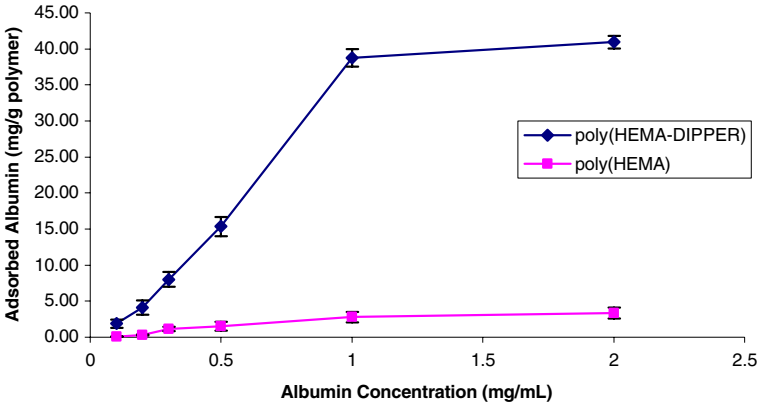

Fig. 5 Effect of albumin concentration on adsorption capacity. Flow rate 0.36 cm/min; pH 5.0 acetate buffer; temperature 25 °C, loading time 2 h

Table 1 Comparison of adsorption capacities of different adsorbents.

Adsorbent type	Ligand	Capacity	Ref.
Poly(HEMA-EGDMA)	Dyes	60.1 mg/g	[30]
m-p(EGDMA-*co*-MAH)	Aminoacid	19.2 mg/g	[31]
Chitosan	Cibacron Blue F3GA	95.2 mg/g	[32]
Perfluorocarbon	C.I. Reactive Blue 4	1.81 mg/mL	[33]
Poly(styrene-divinyl benzene)	Poly(vinyl alcohol)	11.7–27 mg/g	[34]

where b is the Langmuir isotherm constant, K_F is the Freundlich constant, and n is the Freundlich exponent; $1/n$ is a measure of the surface heterogeneity ranging between 0 and 1, becoming more heterogeneous as its value gets closer to zero. The ratio q_e gives the theoretical monolayer saturation capacity of polymer.

Figure 6 shows the graphs of Langmuir and Freundlich adsorption isotherms. Comparison of all theoretical approaches used in this study shows that Freundlich equation fits the experimental data best, and these results suggest that Freundlich adsorption model is applicable to this system.

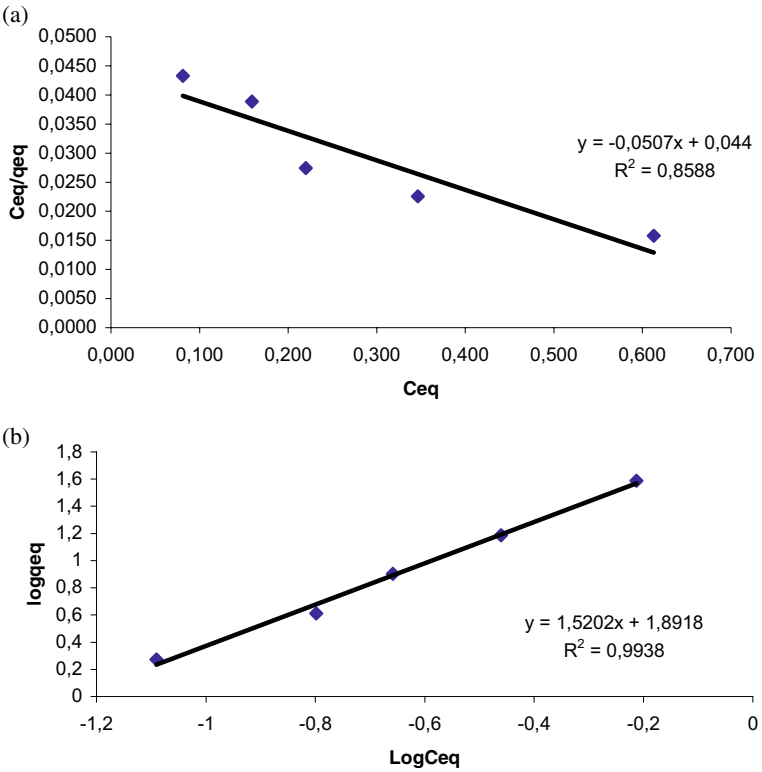

Fig. 6 Langmuir (**a**) and Fruendlich (**b**) adsorption isotherms

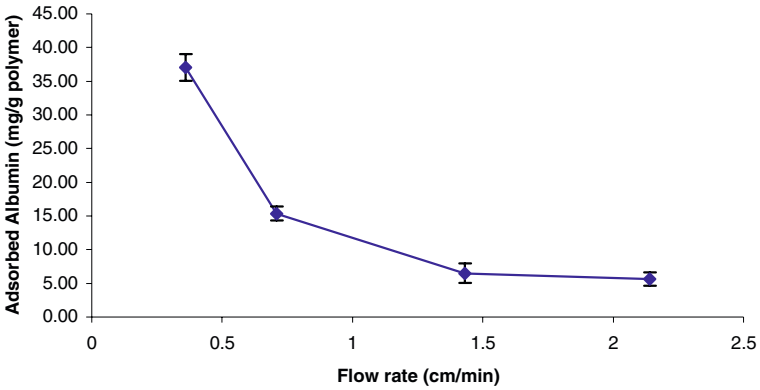

Fig. 7 Effect of flow rate on albumin adsorption. Albumin concentration 1.0 mg/mL; pH 5.0 acetate buffer; temperature 25 °C, loading time 2 h

The adsorption capacities at different flow rates are given in Fig. 7. The adsorption capacity decreased significantly from 40.9 to 5.6 mg/g polymer with increase of the flow rate from 0.36 to 2.14 cm/min. At high flow rates, contact time between albumin and poly (HEMA-*co*-DIPPER) cryogel is reduced, while the flow rate is decreased, contact time in the column becomes longer. These results agree with those referred to the literature [28]. At low flow rate, albumin has more time to diffuse to the pore walls of cryogel and to bind to the ligand; hence, a better adsorption capacity is obtained.

The effect of ionic strength on albumin adsorption was presented in Fig. 8. As shown in Fig. 8, albumin adsorption capacity increased with increasing ionic strength. The adsorption amount of albumin increased by about 44% as the ionic strength (NaCl concentration) changes from 0.01 to 1.0 M. In hydrophobic interaction chromatography, protein adsorption increases with increasing ionic strength. Because of the highly hydrophobic DIPPER monomers incorporated in the cryogel structure, it may be suggested that interaction between albumin and cryogel matrix should be hydrophobic.

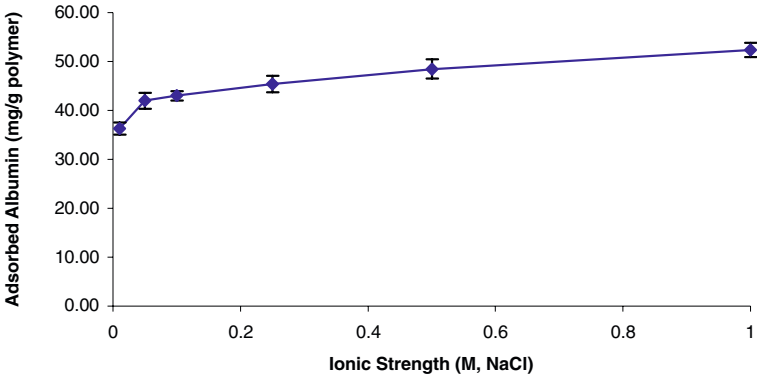

Fig. 8 Effect of the ionic strength on albumin adsorption. Albumin concentration 1.0 mg/mL; flow rate 0.36 cm/min; pH 5.0 acetate buffer; temperature 25 °C, loading time 2 h

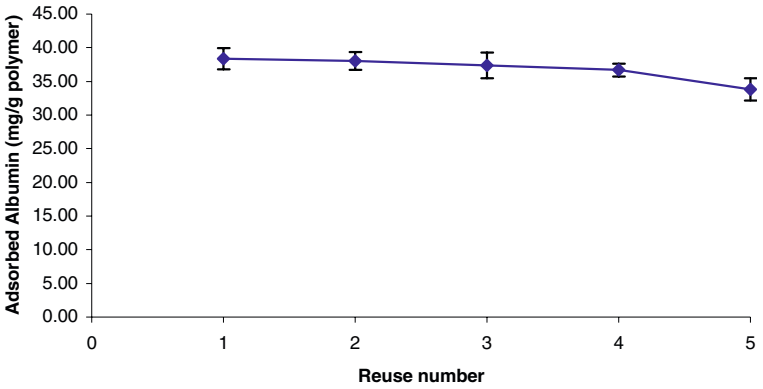

Fig. 9 Repeated use of poly(HEMA-*co*-DIPPER) cryogel. Albumin concentration 1.0 mg/mL; flow rate 0.36 cm/min; pH 5.0 acetate buffer; temperature 25 °C, loading time 2 h

Desorption and Repeated Use

Desorption of albumin from the poly(HEMA-*co*-DIPPER) cryogel was performed in a continuous system using 0.1 M ethylene glycol solution. Desorption of albumin is expressed in percentage of totally adsorbed albumin. Because of economic restraints, there is a growing interest in the preparation and use of effective low cost and reusable adsorbents [29] up to 92% of the adsorbed albumin desorbed by ethylene glycol. In order to show the repeated use of the poly(HEMA-*co*-DIPPER) cryogel, the adsorption–desorption cycle was repeated five times using the same column. At the end of the five adsorption–desorption cycles, albumin adsorption capacity decreased to only about 12% using 0.1 M ethylene glycol (Fig. 9).

Conclusion

Albumin has many physiological functions and commonly used for therapeutic purposes. Also, albumin is the most abundant protein present in blood plasma. Therefore, separation and adsorption of albumin have attracted great interest. A new monolithic cryogel was prepared by free radical polymerization of HEMA and DIPPER monomers. Experimental results indicate that this novel cryogel adsorbent can effectively adsorb albumin from aqueous solution, and its good adsorption/desorption behaviors promises for its practical applications. The adsorption of albumin is highly depends on pH, chromatographic flow rate, and ionic strength. The maximum albumin adsorption was observed at pH 5.0 while above, and below pH 5.0, the albumin adsorption capacity decreased. When the flow rate increased, the albumin adsorption capacity on poly(HEMA-*co*-DIPPER) cryogel decreased. The albumin adsorption capacity increased by increasing ionic strength. The albumin adsorption capacity decreased only 12% after five adsorption–desorption cycles. Compared with the poly(HEMA) cryogel, the adsorption capacity of poly(HEMA-*co*-DIPPER) cryogel is greatly increased. It can be concluded that DIPPER monomer plays key role for the adsorption of albumin on cryogel.

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