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Macroporous poly(glycidyl methacrylate–triallyl isocyanurate–divinylbenzene) matrix as an anion-exchange resin for protein adsorption

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Abstract

A novel macroporous poly(glycidyl methacrylate–triallyl isocyanurate–divinylbenzene) matrix was prepared by a radical suspension copolymerization. The matrix contained epoxy groups, so diethylaminohydroxypropyl groups were coupled to the matrix, leading to an anion-exchange resin. We studied the components, surface and pore structures of the anion-exchange resin by Fourier transform infared spectroscopy and scanning electron microscopy (SEM). SEM observations showed that the resin abounded in macropores as large as 3 to 8 μm both in the surface and the interior. The back-pressure of the column packed with the resin was modest even at a high flow-rate (60.2 cm/min). Then, bovine serum albumin (BSA) was used as a model protein to examine the adsorption properties of the anion-exchange resin. The results showed that under optimum conditions the resin had a capacity as high as 22.8 mg BSA/g wet resin, or 68.7 mg/g dry resin. The adsorbed protein could be desorbed by increasing the liquid phase ionic strength. Most importantly, the matrix had little nonspecific adsorption for BSA before introducing the ion-exchange groups.

Keywords: Poly(glycidyl methacrylate–triallyl isocyanurate–divinylbenzene) resins; Stationary phases, LC; Proteins; Albumin

1. Introduction

Ion-exchange chromatography (IEC) has been widely used as an integral part of separation and purification method of proteins for many years since Sober and Peterson synthesized ion-exchanging cellulose [1]. From then on, soft gels based on agarose and dextran have been developed and extensively used as supporting materials for protein purification by affinity [2], ion-exchange [3], and hydrophobic interaction [4] chromatographies. The gel structure of the conventional soft beads enables proteins to diffuse into the gel interior via a concentration gradient until protein binding becomes saturated. If an acceptable resolution is to be achieved, operations should be carried out at very low flow-rate to meet the diffusion of proteins and increase the dynamic binding capacity of the column. Furthermore, the packing volume of gel-based matrices may also be affected by changes in flow-rate, and the mobile phase properties. To overcome the drawbacks of the soft gel-based column chromatography, the development of packing materials that are mechanically stable, and resistant to matrix deformation under

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various mobile phase conditions or usable at high flow-rates have attracted increasing attentions [5]. The packing materials are almost porous beads with various functional groups [6,7]. The uses of mechanically stable beads with pore diameters greater than 200 Å have led to high-performance ion-exchange chromatography (HPIEC) of biopolymers [8]. For example, to receive the same or a superior resolution, separation of proteins can be achieved 10~100-times faster than the conventional gel columns [9]. These dramatic achievements have made HPIEC a powerful fractionation technique for proteins.

In the present work, we synthesized a cross-linked copolymer of glycidyl methacrylate, triallyl isocyanurate and divinylbenzene. To our knowledge, this kind of matrix has not been reported in the literature. Anion-exchange groups were introduced by epoxy opening reaction with diethylamine. The morphological structure and the polymer composition were analyzed by scanning electron microscopy (SEM) and Fourier transform infared spectroscopy (FT-IR), respectively. In addition, the mechanical property of the resin was investigated by examining the pressure drop of a column packed with the resin on a high-performance liquid chromatography (HPLC) system. Finally, to evaluate the matrix as an adsorbent for proteins, factors presumably affecting the adsorption capacity of the resin such as cross-linking degree, amount of porogenic solvent, particle size and salt concentration were studied using bovine serum albumin (BSA) as a model protein.

2. Materials and methods

2.1. Materials

Glycidyl methacrylate (GMA) (99%) was purchased from Luoyang Chengguang (Henan, China) and used without further purification. BSA was purchased from Sigma (St. Louis, MO, USA), and was dissolved in 0.01 *M* Tris–HCl buffer (pH 7.6). 2,2′-Azobis-(isobutyronitrile) (AIBN) was obtained from the Chemical Factory of Tianjin University (Tianjin, China) and recrystallized in ethanol before use. Triallyl isocyanurate (TAIC) was kindly donated by Professor X.Q. Guo, Nankai University (Tianjin, China). Divinylbenzene (DVB) (56% divinyl monomer) obtained from the chemical plant of Nankai University was extracted with 10% aqueous sodium hydroxide and distilled water, dried over anhydrous magnesium sulfate, and distilled under vacuum. Other reagents were all of analytical grade.

2.2. Preparation of macroporous matrix

The basic copolymer (GMA–TAIC–DVB) was prepared by a radical suspension copolymerization in the presence of an inert phase (porogenic solvents) consisting of toluene and *n*-heptane. Fig. 1 describes the copolymerization. Typically, a mixture of GMA (20 ml), TAIC (5.56 g), DVB (5.88 ml) and the porogenic solvents (toluene: 7.7 ml; *n*-heptane: 10.6 ml), in which AIBN (0.3 g) was dissolved, was suspended in 150 ml 1% poly(vinyl alcohol) (PVA)

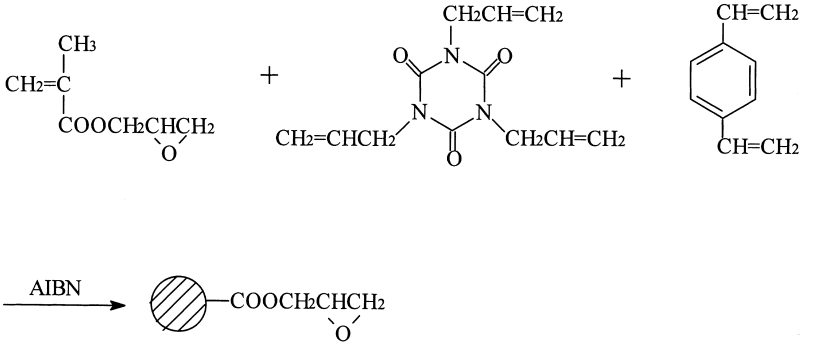

Fig. 1. The preparation route of macroporous poly(GMA–TAIC–DVB) matrix.

solution by agitation. The size of the beads was controlled by adjusting the stirring speed. When the desired droplet size was obtained, the polymerization was allowed to proceed under nitrogen atmosphere at 65°C for 4 h, 75°C for 4 h and finally at 85°C for 4 h, at which time the beads solidified. The programme of temperature changes was chosen to ensure that polymerization did not occur too rapidly and to avoid formation of bubbles in the beads. This reaction led to the formation of white microbeads. The solidified beads were recovered by filtration on a No. 4 (5~12 μm) sintered glass funnel and thoroughly washed with 1000 ml distilled water. Then, the beads were placed in a Soxhlet extraction apparatus, and the porogenic solvents were extracted out of the beads with acetone under reflux for at least 24 h. The porous beads were dried under vacuum (<1 Torr; 1 Torr=133.322 Pa) at room temperature. The dried resins were fractionated by sieving with standard test sieves.

2.3. Preparation of anion exchanger

The modification of microbeads for the preparation of an anion exchanger is based on the reaction of the epoxide groups of the copolymer with diethylamine as shown in Fig. 2. Typically, 5.0 g resin was mixed with 50 ml dioxane and 10 ml diethylamine. The mixture was stirred and heated at 80°C for 7 h. The reaction product was washed thoroughly with 1000 ml distilled water and 250 ml ethanol and then dried at room temperature in vacuum (<1 Torr). Direct modification of macroporous resins with aqueous diethylamine solutions appears to be a poor reaction. This is because that aqueous solution is a bad solvent for the polymer matrix, and it might suppress the reaction of the active groups inside the matrix. If the reaction is performed in dioxane, a good solvent for polymer and able to swell the cross-linked polymer [10], a reasonable amount of functional groups can be bound. In this case, not only the pore surface but also the interior of the matrix may be modified.

2.4. FT-IR and SEM

FT-IR was performed on a Nicolet 5DX FT-IR instrument (USA) at room temperature. A KBr disc was prepared with powders ground with an agate mortar, and dried under vacuum (<1 Torr) at room temperature. SEM observations were performed using a microscope (Leitz-AMR-1000, Germany) after coating the beads with gold–palladium alloy. For the inner structure of the resin, the beads were cut into pieces for inspection.

2.5. Flow characteristics

The flow resistance of the column (150 mm×4.6 mm I.D.) packed with the resin (10~30 μm) was determined by measuring the back-pressure as a function of linear flow velocity. Buffer (0.01 *M* Tris–HCl, pH 7.6) and tetrahydrofuran (THF) were used as mobile phases.

2.6. Determination of adsorption isotherms

Adsorption experiments were performed by adding the wet resin (0.2 g) to 10 ml previously prepared BSA solutions (concentrations were from 0.2 mg/ml to 2.0 mg/ml). The suspension systems were incubated on a shaking incubator (25°C, 120 rpm) for 24 h. The decrease in the optical density at 280 nm of the supernatant solutions was recorded, and the equilibrium concentration and the amount of protein adsorbed to the resin was calculated according to a BSA standard curve. Desorption experiments were performed by adding NaCl to a final NaCl concentration of 1.0 *M*. The result showed that about 80% of the adsorbed proteins could be desorbed.

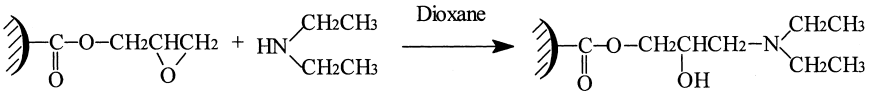

Fig. 2. The preparation of anion exchanger by modifying the matrix with diethylamine.

3. Results and discussion

3.1. Characterization of resins

The anion exchangers were extensively characterized to obtain ion-exchange capacity, water content, SEM and FT-IR spectrum. The ion-exchange capacity obtained by acid–base titration according to the method described previously [11] was 1.24 mmol/g dry resin. The water content was determined according to Wu and Brown [12]. The equilibrium water content was 66.8%, indicating the resin presents hydrophilic skeleton. The SEM photographs of the beads are shown in Fig. 3. The shape and surface structure of the bead can be clearly seen (Fig. 3a). At a larger magnification, the highly reticular, three-

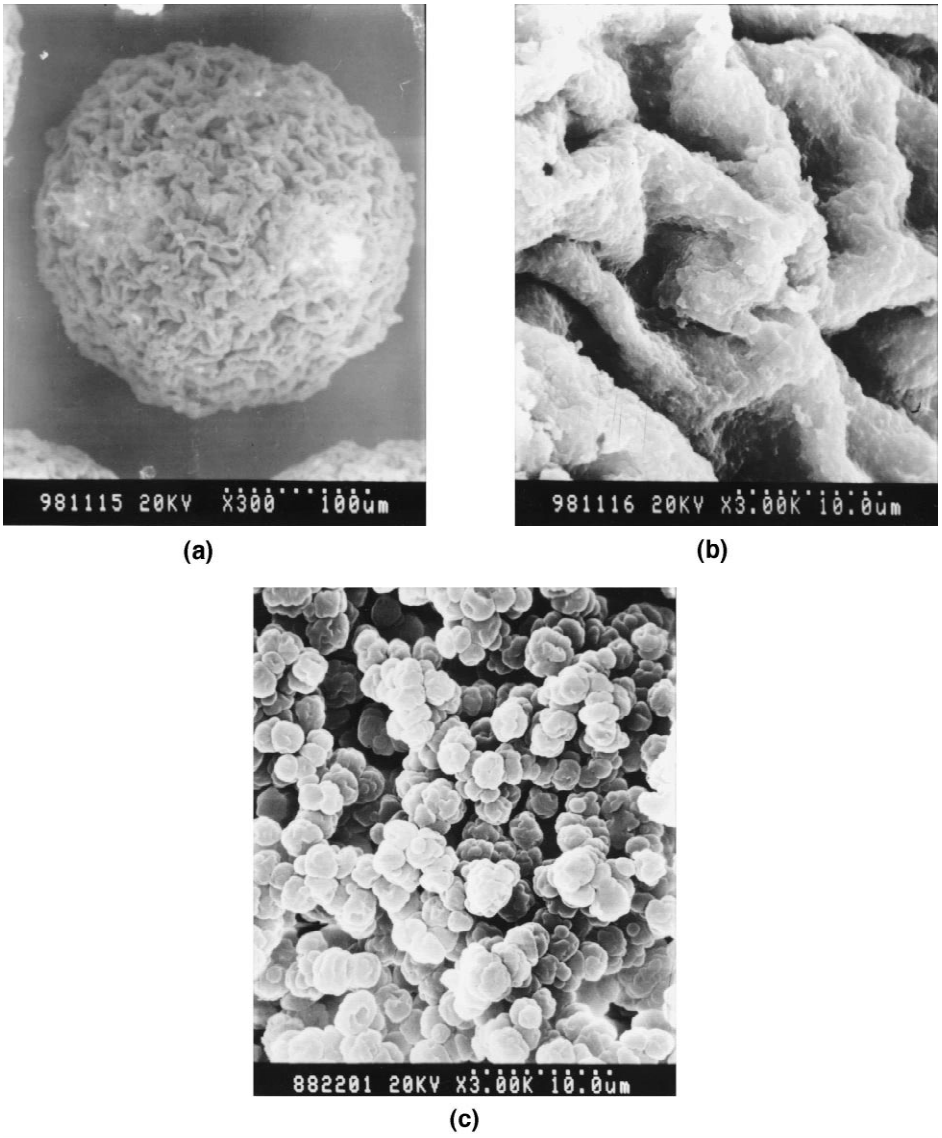

Fig. 3. SEM photomicrographs of: (a) macroporous bead surface (300×); (b) bead surface (3000×); (c) inner part of the resin.

dimensional porous matrix of the bead is particularly evident (Fig. 3b). The pores show a rather large size distribution and appear to be interconnected by numerous channels in the pore walls, and pore diameter reaches between 3 to 8 μm. A scanning electron micrograph of a cross section of the bead reveals clusters of globules isolated by large pores (Fig. 3c).

We analyzed the constitution of the copolymer matrix by FT-IR. The spectrum of the resin sample is shown in Fig. 4. The peaks at 1728.5 cm^{-1} and 1697.5 cm^{-1} are the characteristics of –C=O stretching vibration come from GMA and TAIC, respectively, which indicated that both GMA and TAIC took part in the polymerization. The peak at 760 cm^{-1} is attributed to isotriazine ring of TAIC, while a characteristic absorption band at 711.8 cm^{-1} is due to the benzene ring, which also indicated that DVB participated in the polymerization.

3.2. Effect of pH and NaCl concentration on bed volume

In order to investigate the effect of ionic strength and pH conditions on the bed volume of the resins, the resins were subjected to solutions of different sodium chloride concentrations (from 0 to 1.0 *M*) or different pH values (4.0~12.0). The experimental results showed that the bed volume of the resin kept nearly constant in all the fluids tested, indicating little effect of the ionic strength and pH of the medium on the bed volume. This means that this kind of anion exchanger has a good chemical stability.

3.3. Flow behavior of a packed column

For a resin to be used in a HPLC, it should be rigid and stable when its packed column is operated at a high flow-rate. This can be investigated by examining the dependence of column pressure drop on the flow-rate of mobile phases. Fig. 5 shows the effect of the mobile phase flow-rate on back-pressure or pressure drop in a column (150 mm×4.6 mm I.D.) packed with 10~30 μm beads prepared in this work. A linear dependency of the back-pressure on the mobile phase flow-rate was observed for the two kinds of mobile phases used, up to a superficial velocity of 60.2 cm/min. The results indicate that little compression or damage of the particles occurred under the operation conditions. The differ-

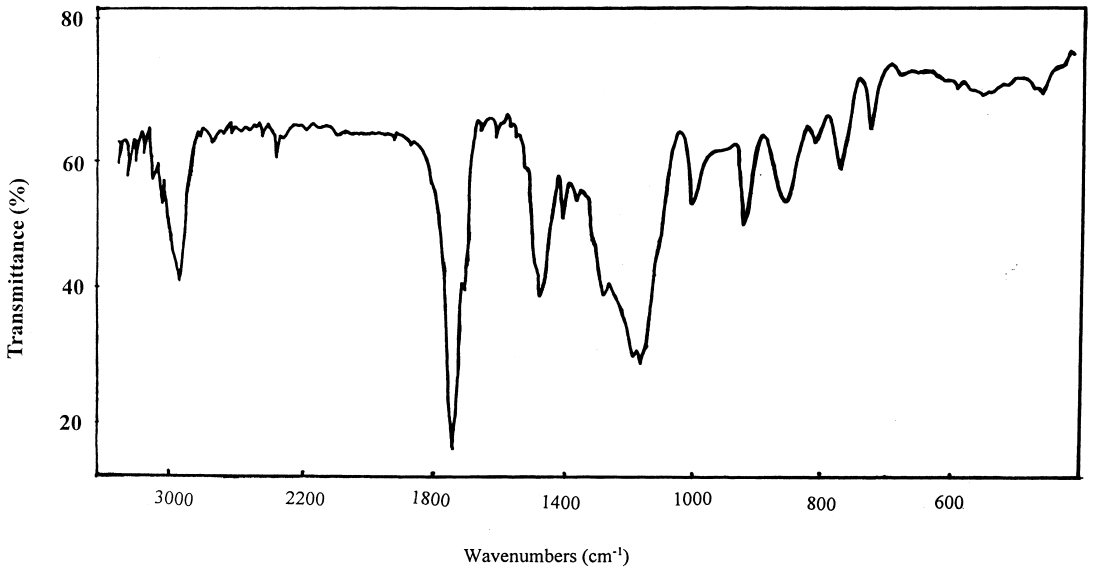

Fig. 4. A typical FT-IR spectrum of the anion exchanger.

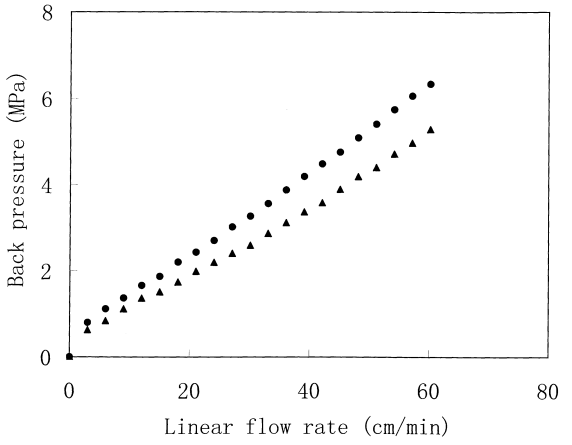

Fig. 5. Effect of liquid flow-rate on back-pressure in a column (150×4.6 mm I.D.) packed with 10~30 μm beads. The mobile phases were THF (▲) and 0.01 *M* Tris–HCl buffer (pH 7.6) (●), respectively.

ence in the slops of the plots was due to the difference in the mobile phase viscosities.

3.4. Non-specific adsorption of the resin before modification

It is of importance for a good matrix to have little non-specific adsorption for proteins. In order to investigate the extent of the non-specific adsorption, two individual experiments were performed using the resins before ionization with diethylamine. The results are shown in Fig. 6. Before the ring opening reaction, the copolymer resin with epoxide groups had higher adsorption capacity for BSA, that is, 7.8 mg/g wet resin. The adsorption may be contributed to either non-specific adsorption of the resin matrix or the reaction of BSA with the epoxide groups. By hydrolyzing the epoxide groups with 0.5 *M* aqueous sulfuric acid, we obtained a diol groups substituted resin [13]. Because the highly hydrophilic property of the resin surface, its adsorption capacity for BSA was reduced to 1.4 mg/g wet resin (Fig. 6).

3.5. Adsorption behavior of the anion resins

The adsorption of BSA can be described by the Langmuir equation:

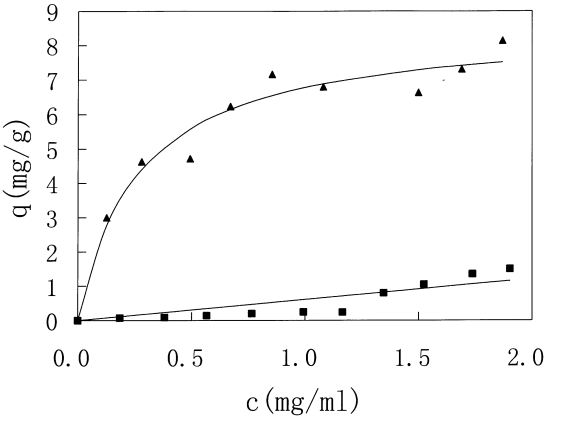

Fig. 6. Non-specific adsorption for BSA of anion exchanger; (▲) resins with epoxide groups; (■) resins with diol groups.

$$q = \frac{q_m c}{K_d + c}$$

where c (mg/ml) is the equilibrium concentration of BSA in bulk solution, q (mg/g wet resin) is the adsorbed density of protein to the adsorbent, q_m is the adsorption capacity, and K_d is the dissociation constant. Parameters in the Langmuir equation were estimated by fitting the equation to the experimental results by the Simplex method. We studied the effect of cross-linking degree, the amount of porogenic solvents, particle size and salt concentration on the adsorption capacity of the resin.

Cross-linking degree could affect the swelling ratio and the porous structure of a copolymerized resin. Keeping the amount of pore-forming agents (porogenic solvents) constant, resins with various cross-linking degrees were manufactured by changing the amount of cross-linking agents (TAIC and DVB). Fig. 7 shows the adsorption of the resins with different cross-linking degrees for BSA. The results indicate that the adsorption capacity increased as the cross-linking degree decreased. This may be due to the decrease of the micropores accessible for BSA inside the matrix when the cross-linking degree was decreased.

Fig. 8 shows the influence of the amount of porogenic solvents on BSA adsorption to the resins. For the three resins tested, the resin capacity for BSA had its maximum at 120% (molar ratio to monomers) of the porogenic solvents. This indicates

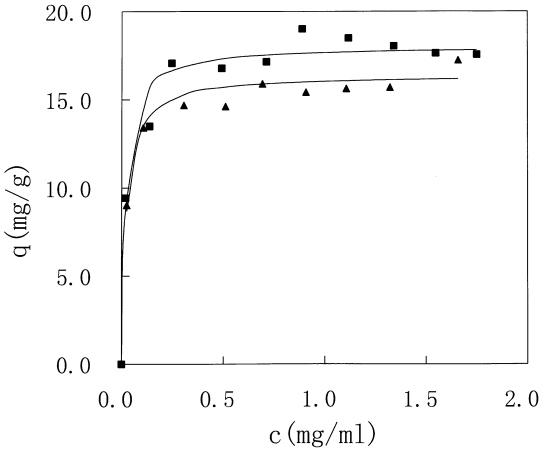

Fig. 7. Effect of cross-linking degree on BSA adsorption to the anion-exchange resin. Porogenic agent: 80% (molar ratio to monomers). Cross-linking degrees were (■) 15% (molar ratio to monomers), $q_m = 18.0$, $K_d = 0.021$; (▲) 20%, $q_m = 16.3$, $K_d = 0.022$.

that the pore diameters and specific surface area of the resin were related to the amount of porogenic solvents. Suitable amount of porogenic solvents is beneficial in forming balanced pore size and large specific surface area for effective protein adsorption.

The isotherms of BSA adsorption to resins with two different particle sizes were shown in Fig. 9. The binding capacity hardly depended on particle size.

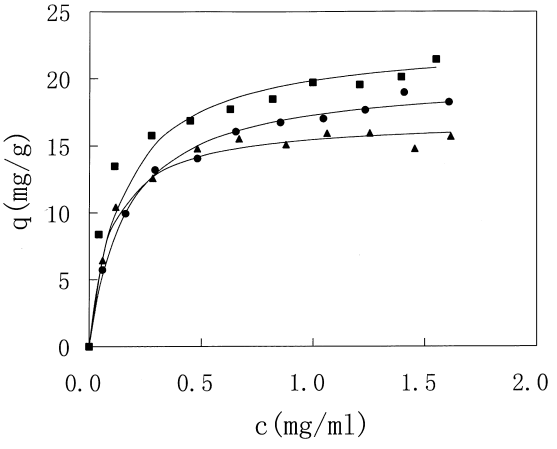

Fig. 8. Effect of the amount of porogenic solvents on BSA adsorption to the resin. Cross-linking degree was 30%; porogenic solvents were (■) 120%, $q_m = 22.8$, $K_d = 0.15$; (●) 140%, $q_m = 20.1$, $K_d = 0.16$; (▲) 100%, $q_m = 16.9$, $K_d = 0.094$.

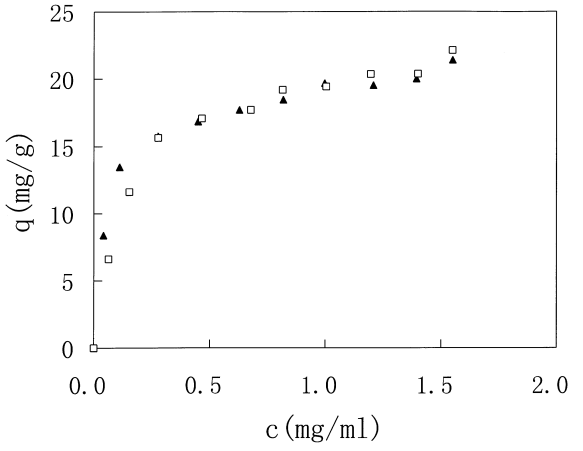

Fig. 9. Adsorption isotherms of BSA to resins of different sizes, (□) $d = 227$ μm, $q_m = 20.6$, $K_d = 0.067$, (▲) $d = 114$ μm, $q_m = 22.8$, $K_d = 0.15$.

This demonstrated that the resin structure was independent of the particle size.

The effect of salt concentration on BSA adsorption is shown in Fig. 10. With an increase of the aqueous phase concentration of sodium chloride, the adsorption capacity decreased drastically. This is a feature of ion-exchange resins, that is, the ionic interactions decrease with increasing ionic strength due to the Debye screening effect. Therefore, proteins adsorbed to the resin can be easily desorbed or eluted by increasing the bulk phase ionic strength.

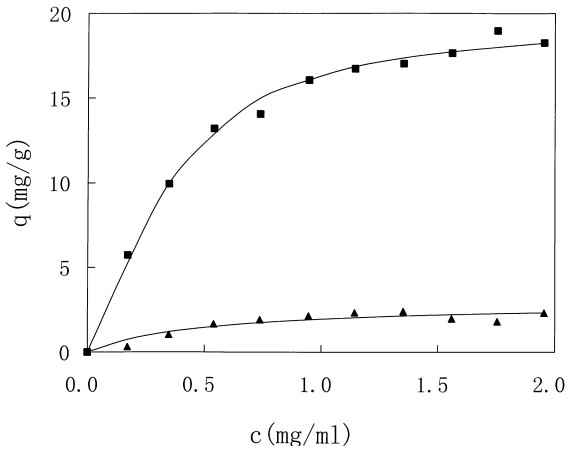

Fig. 10. Effect of salt concentration on adsorption capacity of the resin. NaCl concentrations are (■) 0, $q_m = 19.5$, $K_d = 0.25$, (▲) 1.0, $q_m = 2.97$, $K_d = 0.5$.

4. Conclusions

In this paper, a novel anion exchanger with diethlylanime hydroxypropyl groups was prepared by the reaction of macroporous poly(GMA–TAIC–DVB) resin with diethylamine. FT-IR spectroscopy demonstrated that the matrix was a copolymer of GMA, TAIC and DVB. The macroporous structure of the resin matrix was directly observed by SEM. The SEM photographs showed an evenly distributed network of macropores with diameters of 3 to 8 μm both in the surface and the interior. Column flow experiments indicated that a column packed with the anion-exchange resin of 10~30 μm could be operated at a mobile phase flow-rate as high as 60.2 cm/min. Under optimum conditions the adsorption capacity of the resin for BSA was 22.8 mg/g wet resin. Moreover, the non-specific adsorption was minimized to 1.4 mg BSA/g wet resin. No mechanical deterioration or deformation was observed during modification and recycled uses of the resin. These characteristics make this matrix useful in the chromatographic purification of proteins.

At the present stage however, the capacity of the resin is not as high as conventional agarose beads such as Sepharose [14]. Therefore, further work should direct towards increasing the capacity of the resin. Because the macropores of the resin are extremely large, reducing the diameter of the macropores, leading to the increase of specific area for protein adsorption, is considered to be an effective approach. To this end, the conditions for synthesizing the copolymer matrix should be further optimized.

Acknowledgements

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