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Poly(glycidyl methacrylate–divinylbenzene–triallylisocyanurate) continuous-bed protein chromatography

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Abstract

A novel continuous bed with high dynamic adsorption capacity for protein has been developed. It is a macroporous poly(glycidyl methacrylate–divinylbenzene–triallylisocyanurate) rod prepared by in situ copolymerization in a chromatographic tube. The bed matrix contained epoxy groups, so diethylaminohydroxypropyl groups were coupled to the matrix, leading to an anion-exchange continuous bed. The component, specific surface area, and the pore structure of the bed matrix were characterized by Fourier transform infrared spectroscopy, BET method and scanning and transmission electron microscopies, respectively. The flow properties, column efficiency and the dynamic adsorption behavior of the bed were studied. The results show that the continuous bed, a ternary copolymer of glycidyl methacrylate (GMA), divinylbenzene (DVB) and triallylisocyanurate (TAIC) with a specific surface area of 56.4 m^2/g, consisted of a three-dimensional structure made up of continuous clusters of microspheres (300 nm) and interconnected irregular pores. The rate of mass transfer is enhanced by the convection of the mobile phase through the pores. The dynamic adsorption isotherm of the anion-exchange column for bovine serum albumin was expressed by the Langmuir equation with a dynamic capacity as high as 76.0 mg/g. Moreover, the separation of proteins, i.e. lysozyme, hemoglobin and bovine serum albumin, is little affected by mobile-phase velocity up to 902.5 cm/h; it was completed within 5 min at 902.5 cm/h.

Keywords: Continuous bed; Stationary phase, LC; Poly(glycidyl methacrylate–divinylbenzene–triallylisocyanurate); Proteins

1. Introduction

Polymers have long been introduced as liquid chromatographic media to separate biological products [1–6]. Although polymers can be shaped into various forms, spherical beads have been the primary configuration of the polymeric media. However, the production of a bead-packing column is often time-consuming and costly because it is a multistep process, involving polymerization itself, removal of suspension media, sizing of the beads and packing of the column. Thus, the concept of continuous-bed chromatography was introduced by Hjerten et al. [7], which considerably simplified the procedure for producing a chromatographic column. The bed is a continuous rod synthesized by polymerizing water-soluble monomers (e.g. derivatives of acrylamide) directly in a chromatographic tube. Following compression, the bed is ready for use, and consists of hydrophilic and non-porous bundles and voids of

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3–4 μm between them [8]. The continuous beds have been successfully used to separate proteins and peptides by several chromatographic methods [9–13]. Also non-compressed continuous gel beds have been synthesized for capillary electrochromatography [14,15]. In addition, Svec and Frechet developed continuous rods by in situ polymerization of oil-soluble monomers such as methacrylate and styrene with organic solvents as porogenic agents within the confines of the chromatographic tube [16,17]. The rods are rigid, incompressible and highly porous monoliths fully filling the chromatographic tube. The polymeric rods are composed of irregular clusters of microglobules and pores between the clusters, including large flow-through pores and smaller diffusion pores [18]. The separation media have been used for the chromatographic separation of biological compounds by reversed-phase [17,19], affinity [20] and ion-exchange chromatography [21–23].

The authors have prepared a novel macroporous poly(glycidyl methacrylate–divinylbenzene–triallyl isocyanurate) bead as an anion-exchange resin for protein adsorption [24]. Crosslinking agents, a mixture of triallyl isocyanurate, with low radical polymerization reactivity, and divinylbenzene, an active monomer, were employed, which provided the polymer skeleton with high mechanical strength and hydrolytic stability. The matrix abounded in macropores as large as 3–8 μm both in the surface and in the interior. In the present work, we introduce the skeleton polymer to prepare an anion-exchange continuous bed within the confine of a 100×4.6-mm I.D. chromatographic stainless-steel tube. The composition and specific surface area of the bed were characterized. The dynamic porosity of the continuous bed was determined as a function of liquid-phase flow velocity. In addition, the dynamic adsorption behavior and the column efficiency were investigated, and the continuous bed was applied to the chromatographic separation of proteins.

2. Materials and methods

2.1. Materials

The proteins were purchased from Sigma (St. Louis, MO, USA). Glycidyl methacrylate (GMA) (99%) was purchased from Luoyang Chenguang (Henan, China) and used without further purification. 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. 2,2′-Azobis-(isobutyronitrile) (AIBN) was obtained from Tianjin Dagu (Tianjin, China) and recrystallized in ethanol before use. Other reagents were all of analytical grade.

2.2. Preparation of continuous bed

The mixture of monomers (GMA, DVB and TAIC, 1:0.2:0.1 mol/mol/mol) (1 ml), porogenic agents (toluene and heptane 1.8:1, w/w) (0.7 ml) and AIBN (2 mol% with respect to monomers) was poured into a stainless steel tube (100×4.6-mm I.D.) sealed at one end, and degassed in an ultrasonicator for 15 min. Then the other end was sealed and the polymerization was allowed to proceed at 65°C for 4 h, 75°C for 4 h and finally 85°C for 4 h. This procedure led to the formation of a white solid rod within the tube. The copolymerization has been described by Yu and Sun [24].

2.3. Preparation of anion-exchange column

After the copolymerization described above, the seals at the two end of the tube were removed. The column was provided with fittings and attached to an HPLC system (see below). The column was washed to remove the porogenic agents by pumping tetrahydrofuran (THF) (50 ml) at 0.5 ml/min. For derivation of diethylaminohydropropyl groups, a mixture of diethylamine and THF (1:1, v/v) was continuously pumped through the column heated to 60°C for 7 h at 0.2 ml/min. This reaction has been described earlier [24]. Thereafter, the column was washed routinely with THF, water and 0.01 mol/l Tris–HCl buffer, pH 7.6 (buffer A).

2.4. Characterization of the rod

The polymer rod was push out of the tube after the

chromatographic experiments described below had been completed. It was cut into small pieces and dried at 70°C for 24 h. The specific surface area of the rod was calculated from the BET isotherm of nitrogen, measured with a BET ST-03 instrument. The pore structure of the rod was characterized by electron microscopies. Scanning electron micrographs were obtained using a JEM-100 CXII scanning electron microscope (SEM). All samples were sputter-coated with gold before the SEM examination. Transmission electron microscopy (TEM) was performed on a XL30 ESEM transmission electron microscope. The pieces of the rod matrix were dispersed on a carbon-coated Formvar film copper grid for the TEM observation.

2.5. Chromatography

A Waters HPLC with a system controller, a 600E pump, a manual injection valve (Rheodyne 7725i) and a 2478 UV detector was used to carry out all the chromatography experiments. The data were acquired and processed with the PC 800 software (Waters, USA).

The flow behavior of the continuous bed was determined by measuring the back-pressure as a function of liquid-phase flow velocity. Buffer A mentioned above was used as the mobile phase.

The solutions of lysozyme in buffer A and bovine serum albumin (BSA) in buffer A plus 1.0 mol/l NaCl (buffer B) were used as probes to evaluate the column properties of the continuous-bed chromatography, including dynamic porosity and column efficiency. The dynamic porosity of the bed (ϵ) is defined as:

$$\epsilon = \frac{V_R}{V_B} \quad (1)$$

where V_R is the retention volume of the proteins under unretained conditions, and V_B is the bed volume. The column efficiency of the anion-exchange continuous bed was measured by elution chromatography. It was expressed as the height equivalent to a theoretical plate (*HETP*) and calculated from the following equations:

$$HETP = \frac{L}{N} \quad (2)$$

and

$$N = 5.54\left(\frac{V_R}{W_{1/2}}\right)^2 \quad (3)$$

where L is the column length, N is the theoretical plate number of the column, and $W_{1/2}$ is the peak width at the half peak-height.

Frontal analysis was carried out to determine the dynamic adsorption behavior of the anion-exchange continuous bed for BSA. The BSA solutions of different concentrations were used for the frontal analysis and the dynamic adsorption density to the continuous rod (q) was obtained from:

$$q = \frac{c(V - V_0)}{V_B} \quad (4)$$

where c is the feed BSA concentration, V is the volume of the protein solution pumped into the column when the concentration of protein in the column effluent reaches 50% of that in the feed stream, and V_0 is the dead volume out of the HPLC system.

3. Results and discussion

3.1. Characterization of the rod matrix

The mechanism of pore formation during a typical polymerization in the presence of porogenic agents (precipitators) has been analyzed in the literature [25,26]. The scanning electron micrograph of the rod (Fig. 1) reveals that the rod matrix consists of clusters of microspheres fused into a continuous three-dimensional structure and irregular pores between the clusters. The size of the microspheres is ~300 nm and there are macropores of ~1 μm in the rod. Fig. 2 shows a transmission electron micrograph of the rod. The sites of high exposure are the result of electron diffraction through large pores that transect the medium [27], which provides evidence for the presence of flow-through pores. Based on the electron micrographs, it can be concluded that the rod matrix was of three-dimensional structure made up of continuous clusters of microspheres and interconnected irregular pores. However, some caution is warranted in deducing the structure of the rod from

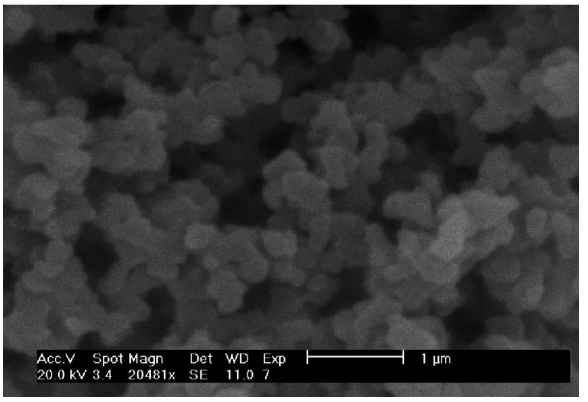

Fig. 1. A scanning electron micrograph of the continuous-bed matrix.

the photos, since the preparation of the rod for microscopy including drying and cutting may have changed its appearance [8].

When the continuous bed is used for large-scale chromatography, a high protein binding capacity is indispensable. The specific surface area of the bed matrix is one of the main factors influencing the protein binding capacity. It is determined by the BET method that the bed matrix possesses a specific surface area of 56.4 m^2/g.

3.2. Dynamic porosity and back-pressure

In addition to the porous properties of the polymer rod in the dry state described above, it is worth while checking the porous properties of the polymer medium immersed in a solution since its practical use involves liquid mobile phase. However, no literature data are available for the dynamic porosity of a continuous bed. Fig. 3 shows the dynamic porosity of the continuous rod as a function of liquid flow-rate. Lysozyme (p*I* 11.0) [28], positively charged in buffer A, was not adsorbed to the anion-exchange resin. With increasing flow velocity, the porosity of the rod for lysozyme, i.e. the retention volume of lysozyme, decreases until a flow velocity of 400 cm/h is reached. This is considered to be due to the presence of micropores in the rod, into which lysozyme can diffuse. The elution volume of the protein decreases with increasing flow velocity, since less time is available for the protein to achieve diffusion equilibrium as the flow velocity increases [29]. On the other hand, the ratio of the pores through which proteins can flow increases with increasing flow velocity/back-pressure. Hence, the dynamic porosity tends to remain unchanged at flow-rates higher than 450 cm/h. It was confirmed that BSA was unretained in the mobile phase containing

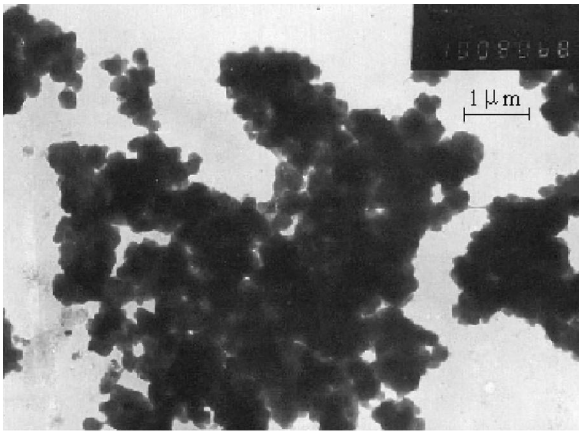

Fig. 2. A transmission electron micrograph of the continuous-bed matrix.

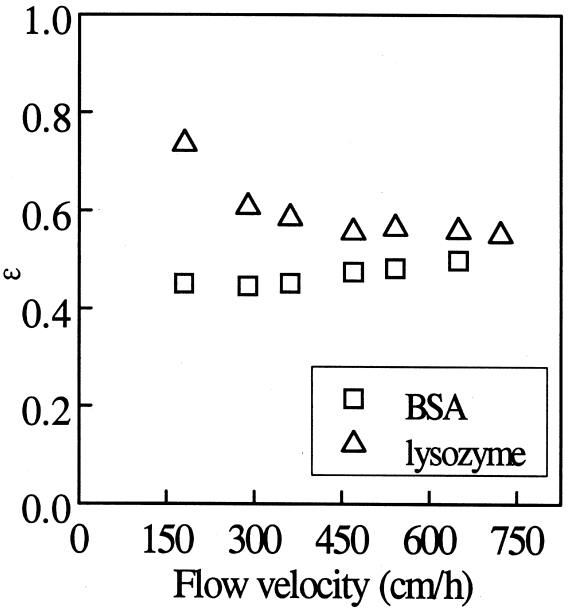

Fig. 3. Effect of flow-rate on the dynamic porosity of the continuous bed for lysozyme and BSA. The buffer for lysozyme was buffer A and for BSA was buffer B.

buffer B. Fig. 3 indicates that the dynamic porosity for BSA keeps nearly constant at 150–361 cm/h. Thereafter, it increases a little with increased flow velocity. The results indicate that lysozyme and BSA show different behaviors regarding dynamic porosity as a function of flow velocity. This is considered to be due to the difference in their molecular sizes. Lysozyme molecule is smaller than BSA, so lysozyme can diffuse into more micropores than BSA, and the effect of diffusive mass transfer for lysozyme is greater than for BSA. For BSA, however, the increase of the dynamic porosity with increasing flow-rate/back-pressure is dominant.

The effect of flow-rate on back-pressure is shown in Fig. 4. It can be seen that the mobile phase can flow through the continuous bed at a modest flow resistance. The linearity of the back-pressure versus flow velocity confirms that the bed is incompressible and can withstand a back-pressure higher than 9.0 MPa.

3.3. Dynamic adsorption properties

Frontal analysis, providing the breakthrough curves of a protein, can be used to evaluate the dynamic capacity of a chromatographic medium for the protein. Fig. 5 shows the results of the frontal analysis at different flow-rates. It can be found that the 5% breakthrough volume decreases a little, from 15.2 to 13.9 ml, as the flow velocity increases seven times, from 36 to 504 cm/h. This result is in direct contrast to those obtained using the traditional packed columns (diffusion-limited case), whose dynamic loading capacity decreases considerably with increasing mobile-phase velocity [30]. From the result, it can be concluded that the flow-through pores contributed greatly to protein adsorption, and thus the resistance to mass transport is substantially smaller in the continuous bed.

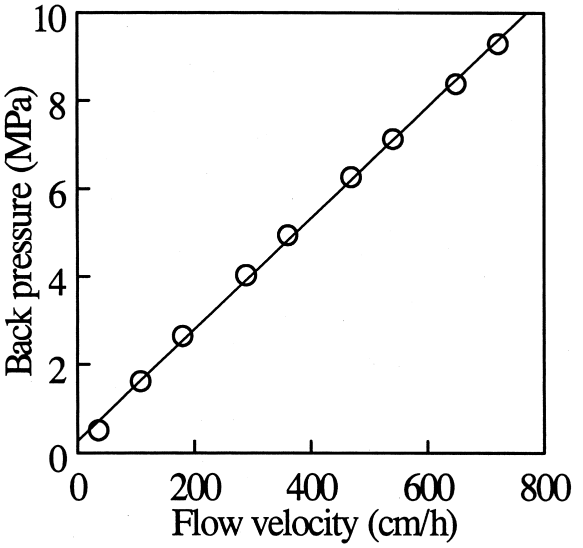

Fig. 4. Effect of liquid flow velocity on back-pressure in the continuous bed with buffer A as the mobile phase.

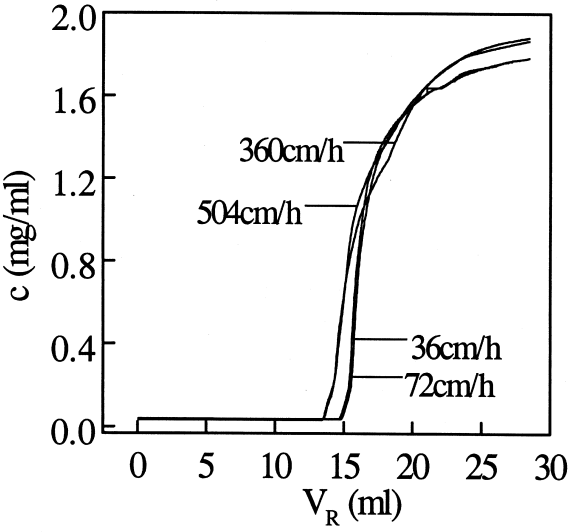

Fig. 5. BSA breakthrough curves at different flow velocities. Conditions: mobile phase buffer A; feed BSA concentration 2 mg/ml; UV detection at 280 nm.

The dynamic adsorption isotherm can be determined using the dynamic adsorption method with different BSA loading concentrations. The data detected at 72 cm/h are shown in Fig. 6. The dynamic adsorption isotherm can be described by the Langmuir equation:

$$q = \frac{q_m c}{K_d + c} \quad (5)$$

where q is the dynamic adsorbed BSA density, q_m is the dynamic capacity, c is the BSA concentration in the feed steam, and K_d is the dissociation constant. By fitting the equilibrium data to the Langmuir equation with the non-linear Simplex method, q_m and K_d were determined to be 23.9 mg/ml bed (or

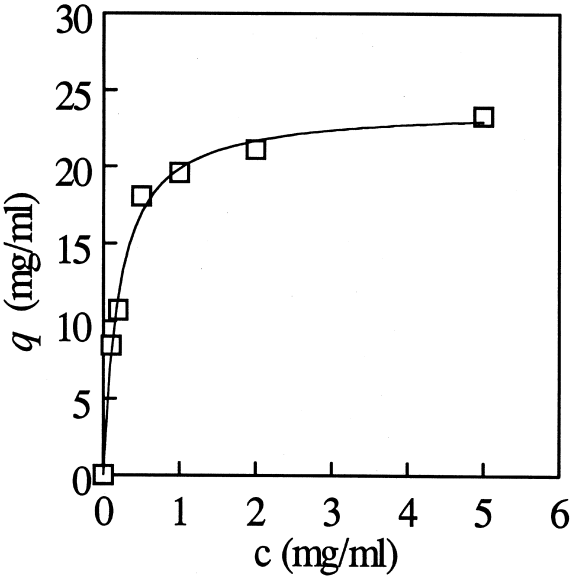

Fig. 6. Dynamic adsorption isotherm of the anion-exchange continuous bed for BSA. Conditions: mobile phase buffer A; flow velocity 72 cm/h; UV detection at 280 nm.

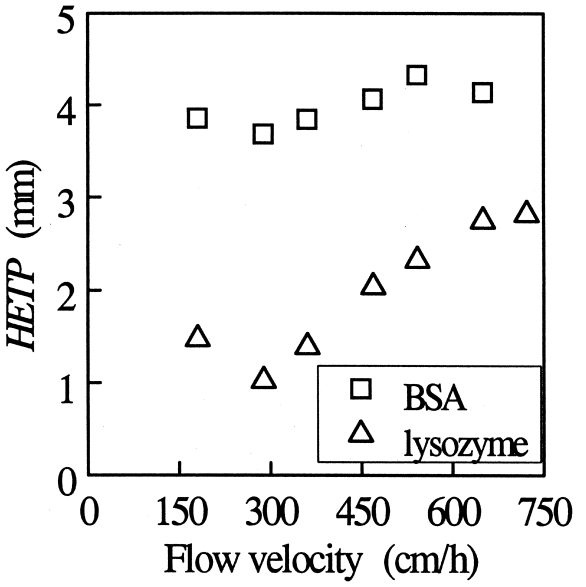

Fig. 7. Dependence of the column efficiency on mobile-phase flow velocity. The injection size was 20 μl of 5 mg/ml lysozyme or of 10 mg/ml BSA solution. Lysozyme was dissolved in buffer A, while BSA was dissolved in buffer B.

76.0 mg/g separation medium) and 0.205 mg/ml, respectively. This is a capacity value comparable to or higher than the highest adsorption capacities of rigid continuous beds for proteins reported so far in literature [21,22].

Svec and Frechet prepared a continuous rod of poly(glycidyl methacrylate-co-ethylene dimethacrylate) using dodecanol and cyclohexanol as porogenic agents [21]. The rod had a specific surface area as high as 295 m^2/g, much higher than in the present work, while the adsorption capacity for BSA was 66.4 mg/g separation medium, comparable to that in the present work. This suggests that micropores that cannot accommodate proteins contributed greatly to the specific surface area of their rod.

3.4. Column efficiency (HETP)

The chromatographic efficiency of the continuous bed was determined using lysozyme and BSA under the unretained conditions of the proteins. It can be seen from Fig. 7 that the highest efficiency is achieved at 300 cm/h and the relationship between the *HETP* and flow-rate follows the typical van Deemter profile in the range of lower flow-rate [31]. The *HETP* increases slowly with a tendency to reach a plateau at higher flow velocity. This is in contrast to the behavior of packed columns, which exhibit a linear increase in the *HETP* when the flow-rate increases beyond an optimum value [32]. At low flow-rates, protein molecules can sufficiently diffuse into the micropores of the rod, therefore, *HETP* controlled by the film mass transfer decreases with increasing flow-rate. However, at a moderate flow-rate, the intrapore diffusion becomes the key step of mass transfer, and *HETP* increases with increasing flow-rate. The phenomenon that *HETP* reaches a plateau at high flow-rate results from the fact that the ratio of the pores through which proteins can flow increases with increasing flow velocity/back-pressure. Therefore, the mass transfer rate of the large molecules substantially increases and the negative effects of diffusion are minimized [33,34]. The results also suggest that the resolution will not decrease greatly with the increase of flow-rate, and a rapid separation of proteins can be obtained by increasing mobile-phase flow-rate. Compared with the *HETP* value of a continuous bed, in which the bed matrix is non-porous and has an interstitial

distance of 3.0 μm [35], the *HETP* value of the present bed is higher. This is ascribed to the presence of micropores (diffusion pores) that contributed to the high adsorption capacity of the present bed. There are no *HETP* data available for rigid continuous beds with high protein adsorption capacities [21,22].

3.5. Separation of proteins

Separations of a model protein mixture (lysozyme, hemoglobin and BSA) in buffer A were performed by gradient elution chromatography with the anion-exchange continuous bed. The chromatograms at different flow velocities are shown in Fig. 8. The results indicate that the proteins are essentially baseline separated, and the shape of the chromatographs and the resolution of the proteins are little affected by mobile-phase velocity up to 902.5 cm/h. Namely, a good separation of proteins is obtained at high flow-rate without loss of resolution. The proteins are separated within 5 min at 902.5 cm/h.

The column was washed with 0.1 *M* NaOH at room temperature to clean the column after each use. During the 2 months of the experimental period, no change occurred in back-pressure or chromatograms, and the adsorption properties of the column did not change with the number of injections. The results show that the column is stable and can withstand long-term use for protein separations.

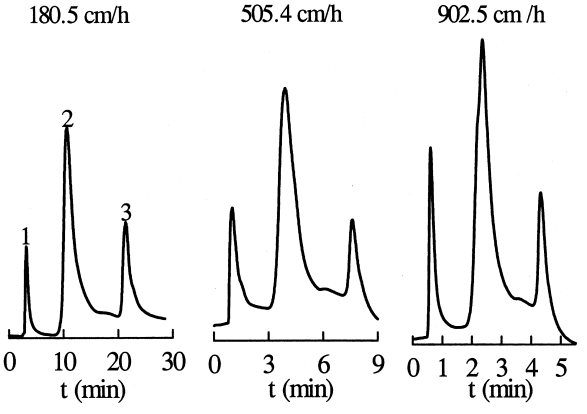

Fig. 8. Separation of lysozyme (1), hemoglobin (2) and BSA (3) on the anion-exchange column at different flow velocities. Conditions: gradient produced with buffers A and B, 0–20% buffer B in 0.5 ml, 20% buffer B in 5 ml, and 20–100% buffer B in 0.5 ml; protein injection size, 0.02 ml; protein concentrations: lysozyme 2 mg/ml, hemoglobin 10 mg/ml, BSA 10 mg/ml; UV detection at 280 nm.

4. Conclusions

For preparative separations, high throughput is one of the most important indexes when evaluating chromatography. It needs a rigid column with high adsorption capacity at high flow-rates. We have prepared such a continuous bed. The bed matrix, a ternary macroporous copolymer of GMA, DVB and TAIC, possessed a specific surface area of 56.4 m^2/g and exhibited a moderate back-pressure at mobile-phase flow velocity up to 720 cm/h. Functionalized with diethylamine, the anion-exchange continuous bed showed a dynamic adsorption capacity as high as 76.0 mg/g and the dynamic adsorption isotherm was expressed by the Langmuir equation. In addition, the dynamic porosity of the bed for lysozyme and BSA was first determined and discussed. The effect of flow velocity on *HETP* of the continuous bed, showing a tendency to approach a plateau at high flow velocity, was not as significant as that of conventional packed columns. This suggests that the continuous bed can be used at high flow-rate in protein separations. The separation of the synthesized protein mixture demonstrated this concept; it was completed within 5 min at 902.5 cm/h.

Acknowledgements

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