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Cooperation of solid granule and solvent as porogenic agents Novel porogenic mode of biporous media for protein chromatography

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Abstract

A novel porogenic mode, cooperation of solid granule and solvent, has been introduced to prepare a biporous medium for protein chromatography. The matrix, a ternary copolymer of glycidyl methacrylate, triallylisocyanurate and divinylbenzene, was produced by a simple in situ polymerization with granules of sodium sulfate and cyclohexanol and dodecanol as porogenic agents. Functionalized with diethylamine, the resin (denoted as Resin C) was used as an anion exchanger. The pore structure, specific surface area and chromatographic properties of Resin C were determined and compared with those of the resin with only the solvents as porogen (Resin A) and the resin with only the salt granules as porogen (Resin B). The results indicated that Resin C contained regions of micropores with a maximum at approximately 55 nm and regions of macropores with a distinct maximum near 340 nm, which swelled to about 1 μm in aqueous solution. Compared with Resins A and B, the biporous medium Resin C simultaneously possessed a high specific surface area of 37.2 m^2/g and a low back-pressure at mobile phase flow velocity up to 720 cm/h. The result of dynamic porosity showed that mobile phase was able to convectively flow through the macropores in Resin C. The dynamic adsorption capacity of Resin C for bovine serum albumin was as high as 57.0 mg/ml column volume (95.0 mg/g wet resin), basically identical to its static capacity, while that of Resin A was only 1.95 mg/ml column volume (3.12 mg/g wet resin), about 3% that of its static capacity. In addition, the column efficiency of Resin C was comparable to that of Resin B, but much higher than that of Resin A, indicating that the mass transfer behavior of proteins in the column was greatly improved by convective flow through the macropores.

Keywords: Stationary phases, LC; Anion exchangers; Proteins

1. Introduction

Macroporous polymers have been broadly applied as liquid chromatographic media to separate biological products since diethylaminoethyl derivatized cellulose was introduced into the separation of proteins by Sober and Peterson in 1954 [1]. Generally, the polymers are prepared by suspension polymerization in a water-organic two-phase reaction system, and the reaction mixture consists of monomers, crosslinking monomer and polymerization initiator, as well as suitable porogenic agents, whose type and amount affect greatly the pore size of the polymers [2]. Organic solvents are the most common

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porogen, and linear polymers can also be used [3–6]. Different applications of the macroporous polymers require different pore size distributions. Successful use of superporous agarose and perfusion chromatography in protein separation proved that an ideal medium for protein separation is a biporous material with high specific surface area and large pores required for high capacity, low flow resistance and rapid solute transport [7–17]. However, it is difficult to prepare the biporous matrix with traditional porogen. The superporous agarose gel was prepared by a double emulsification procedure [7], and the currently preferred method for fabrication of perfusion chromatographic matrices involves preparing small particles using suspension, emulsion, or hybrid polymerization techniques and building up the particles into matrices of 5–10 μm [11]. Moreover, the hydrophobic character of the perfusion chromatographic skeleton polymer, poly(styrene–divinylbenzene) matrix is a serious limitation to its suitability for macromolecule separations other than a reversed-phase mode since the hydrophobic interaction between polymers and proteins can be so strong that the proteins may be denatured. Therefore, the matrix needs to be masked using coating technology before applying in the chromatographic separation of biological macromolecules in all retention modes except reversed-phase mode [16,18]. Obviously, the production of the biporous chromatographic media is time-consuming and costly.

Is it possible to produce bidisperse porous media with one simple preparation step? The novel porogenic way, the cooperation of solid granule and solvent to be described here will prove that the answer is 'yes'. The authors' laboratory has prepared novel macroporous poly(glycidyl methacrylate–divinylbenzene–triallyl isocyanurate) anion exchangers for protein adsorption [19,20]. In the present work, we introduce the skeleton polymer to prepare biporous separation media by in situ polymerization with granules of sodium sulfate and cyclohexanol and dedocanol as porogenic agents. After functionalized with diethlamine, the matrix (denoted as Resin C) was used as anion exchanger. The pore structure, pore size distribution and specific surface area of Resin C were characterized and compared with those of Resin A (with only solvents as porogen) and Resin B (with only salt granules as porogen). The chromatographic properties, such as dynamic porosity, static and dynamic adsorption behavior, and column efficiency, were determined, and the differences in the properties between these three resins were discussed.

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 Chemical (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. Cyclohexanol and dodecanol were products of Tianjin Chemical (Tianjin, China). Sodium sulfate was obtained from Tianjin Yifa Chemical Factory (Tianjin, China). Granular sodium sulfate with a diameter of approximately 1 μm was produced by recrystallizing in ethanol. 2,2′-Azobis(isobutyronitrile) (AIBN) was purchased from Tianjin Dagu Chemical Factory (Tianjin, China) and recrystallized in ethanol before use. Other reagents were all of analytical grade.

2.2. Preparation of solid media

The free radical initiator AIBN (2 mol% with respect to monomers) was dissolved in a mixture of monomers (GMA, DVB and TAIC, 1:0.2:0.1 mol/mol/mol), and then porogenic agents–solvents (cyclohexanol–dodecanol, 6.5:1, v/v) and/or granules of sodium sulfate were added. Compositions of the polymerization mixtures for preparing Resins A, B and C were 30/70/0, 70/0/30 and 30/40/30 (monomers/solvent/solid granule, %, v/

v/v), respectively. After degassed and blended in an ultrasonicator for 30 min, the mixture was poured into a glass tube (100×10-mm I.D.) sealed at one end. Then, the other end of the tube was sealed and the copolymerization described by Yu and Sun [19] was allowed to proceed at 70°C for 12 h. This procedure led to the formation of a white solid rod within the tube. After the reaction, the seals at the two ends were removed, and the polymer rod was pushed out of the tube and ground into small particles. The solvent porogenic agents, cyclohexanol and dodecanol, were removed by extracting the particles with ethanol under reflux for at least 24 h in a Soxhlet extraction apparatus, while the solid porogenic agent, sodium sulfate, by washing the particles with 30% ethanol solution until no precipitation in the eluent could be detected by the addition of $BaCl_2$. The porous particles were dried under vacuum (<1 Torr; 1 Torr=133.322 Pa) at room temperature, and fractionated by sieving with standard test sieves.

2.3. Preparation of anion exchangers

The modification of the particles for the preparation of anion exchangers is based on the reaction of the epoxide groups of the copolymers with diethylamine as described elsewhere [19]. Typically, 5.0 g resin was mixed with 25 ml dioxane and 25 ml diethylamine, and the mixture was stirred and heated at 60°C for 6.5 h. Thereafter, 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).

2.4. Characterization of the ground particles

The pore structures of the adsorbents in dry state were characterized using a JEM-100 CXII scanning electron microscopy (SEM) instrument. All samples were sputter-coated with gold before the SEM examination. The pore size distributions were determined by mercury porosimetry using a Micromeritics Autopore II 9220 mercury porosimeter. Meanwhile, the pore structures of the adsorbents in 0.01 mol/l Tris–HCl buffer, pH 7.6, after swelling in ethanol were observed with a charged couple detecting device (CCD). The procedure was performed as follows: the images of the particles obtained by an optical microscope with a magnification of 640 were collected using a CCD camera (Minton MS-368P), and then input into a computer and transferred to pictures by a acquisition card (CA-CPE-2000, 786×576×24 bits). The specific surface areas of the particles were calculated from the BET isotherm of nitrogen, measured with a BET ST-03 instrument.

2.5. Static adsorption isotherms

The standard batch adsorption system was utilized to determine the static adsorption isotherms of the anion exchangers for bovine serum albumin (BSA) [21]. In general, 0.2 g wet resin was contained in 10 ml BSA solution. Adsorption was performed at 25°C in a shaker at 160 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 anion exchangers were calculated according to a BSA calibration curve and by mass balance.

2.6. Chromatography

All chromatography experiments were performed as described earlier [20]. The particles of Resins A, B and C with diameters ranging from 37 to 74 μm were, respectively, packed into Column A, B and C (30×4.6-mm I.D.). A Waters HPLC system 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 experiments. The data were acquired and processed with the PC 800 software (Waters, USA).

The flow behaviors of the columns were determined by measuring the back-pressure as a function of liquid-phase flow velocity with 0.01 mol/l Tris–HCl buffer, pH 7.6, as the mobile phase.

The lysozyme solution in 0.01 mol/l Tris–HCl buffer, pH 7.6, was used as a probe to evaluate the column properties of the packing columns, including

dynamic porosity and column efficiency. The dynamic porosity of the columns (ε) is defined as:

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

where V_R is the retention volume of the protein, and V_B is the bed volume. The column efficiency was measured by elution chromatography. It was expressed as the height equivalent to a theoretical plate (HETP) and calculated from the following equations:

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

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, and $W_{1/2}$ is the peak width at half peak-height.

Frontal analysis was carried out to determine the dynamic adsorption behavior of the anion exchangers for BSA. BSA solution 0.01 mol/l Tris–HCl buffer, pH 7.6 (1.0 mg/ml), was used for the frontal analysis and the dynamic adsorption density to the packed bed (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 5% that in the feed stream, and V_0 is the dead volume out of the HPLC system.

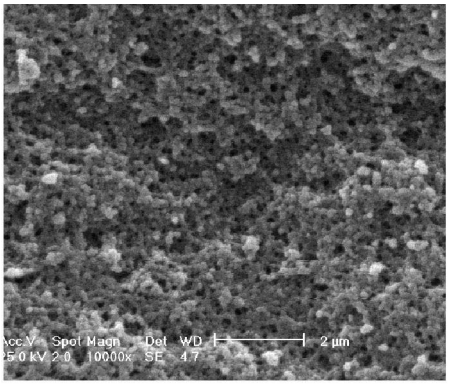
3. Results and discussion

3.1. Characterization of the matrix

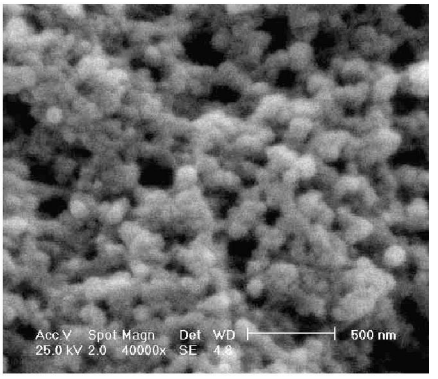
Fig. 1 shows the scanning electron micrographs of the particles. It can be seen that Resin A, with solvent as the porogenic agent, consists of clusters of microspheres fused into a continuous three-dimensional structure and irregular micropores between the clusters. Resin B, with solid granules as the porogen, is composed of a continuous three-dimensional polymer phase and irregular macropores. Compared with Resins A and B, Resin C with both the solvents and the solid granules as the porogen contains regions of micropores similar to Resin A and regions of macropores similar to Resin B. Based on the electron micrographs, it can be concluded that the material with bidisperse porous structure can be prepared by the cooperation of the solvents and the solid granules as the porogenic agents.

Fig. 2 shows the pore size distribution curves obtained from mercury intrusion porosimetry measurements. Obviously, Resins A and B contain one family of pores with size ranging from about 8 to 238 and 70 to 700 nm with a distinct maximum near 60 and 190 nm, respectively. In contrast, Resin C consists of two families of pores: the large channels with size ranging from about 120 to 1630 nm with a distinct maximum near 340 nm, and the relatively small pores below 120 nm in size with a maximum at approximately 55 nm. The results also confirm that Resin C possesses a bidisperse pore size distribution, which corresponds well to those observed by the SEM. Meanwhile, it can be found that the solvent porogenic agents are the main contributors to the regions of the micropores while the solid granules to the regions of the macropores. Thus, similar to the traditional media with solvents as porogen, the size of micropores in Resin C can be adjusted by varying the composition of the polymer mixture, such as the percentage of cross-linking monomer, the type and amount of porogen, and the concentration of free-radical initiator [22]. The distinction between Resins B and C in the size of macropores suggests that the volume shrinkage of the particles that occurs during drying is not identical for the two resins. It may be due to the difference of the skeleton structures of the two solid media, which can be clearly seen from the SEM micrographs.

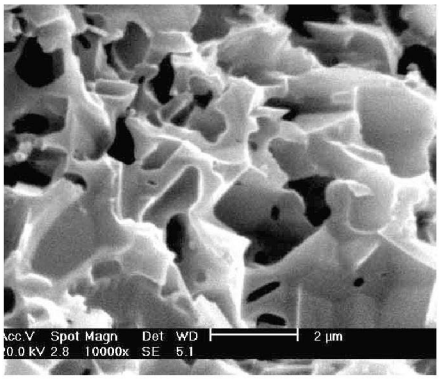
It is also worth while checking the porous structures of the media in aqueous solution. A detecting technique, CCD detecting, is utilized to observe the configuration of the particles in 0.01 mol/l Tris–HCl buffer, pH 7.6, after swelling in ethanol and the



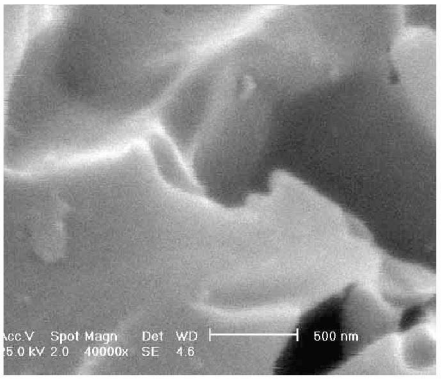
Resin A (10,000×)



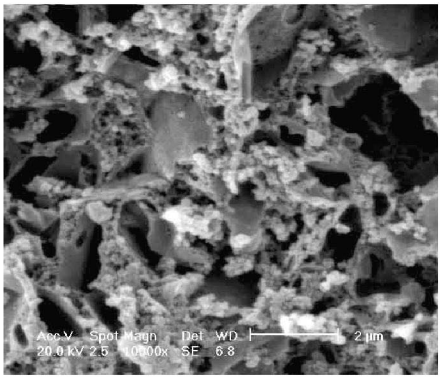
Resin A (40,000×)



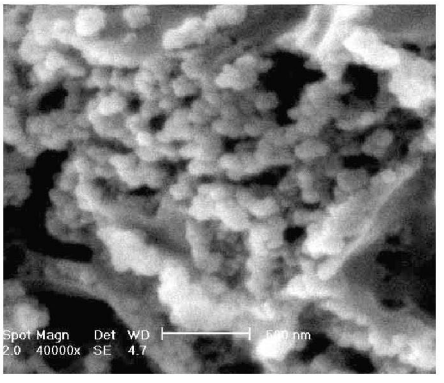
Resin B (10,000×)



Resin B (40,000×)



Resin C (10,000×)



Resin C (40,000×)

Fig. 1. Scanning electron micrographs of the matrices at two magnifications.

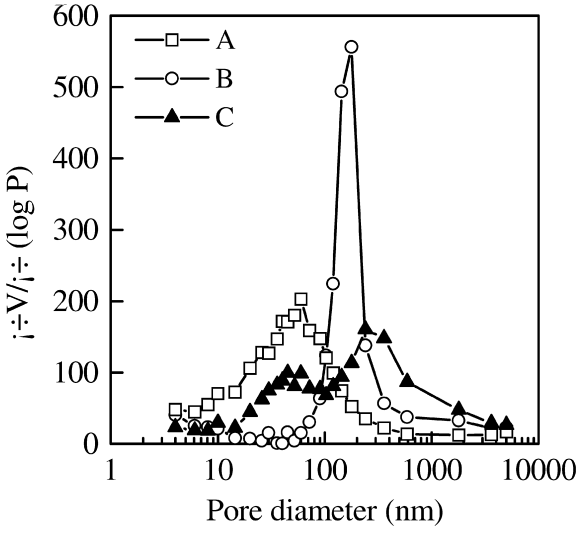
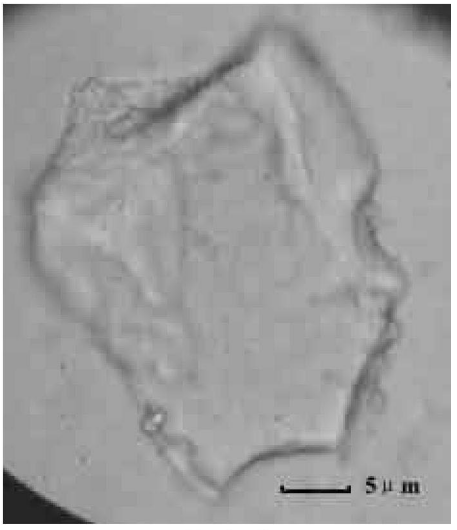


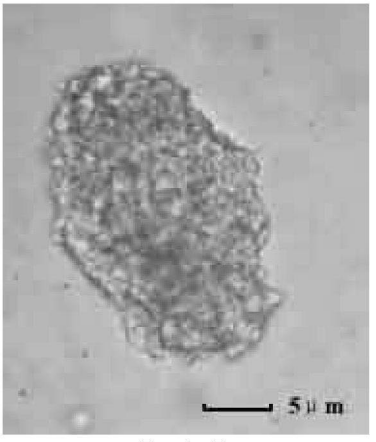
Fig. 2. Pore size distribution curves of the matrices obtained from mercury intrusion porosimetry measurements.

results are shown in Fig. 3. This technique has also been employed for the characterization of superporous agarose matrix [10]. It can be seen that in Resins B and C there are interconnected channels that transect the particles formed by macropores with size around 1 μm. The results indicate that the granular sodium sulfate can be used as porogenic agent to produce macropores with size corresponding to its diameter, i.e., the size of the macropores in the biporous medium can be changed by altering the diameter of the solid porogenic agent.

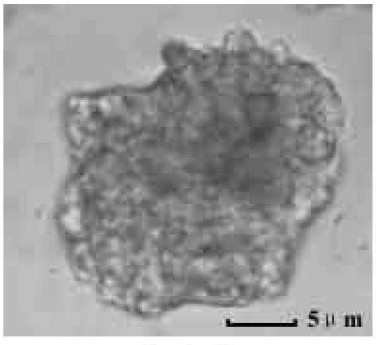
When the separation media is used to a large-scale chromatography, a high protein binding capacity is desired. The specific surface area of the matrix is one of the main factors influencing the protein binding capacity. Determined by the BET method, the specific surface areas of Resins A, B and C are 39.9, 5.0 and 37.2 m^2/g, respectively. The results reveal that the solvent porogenic agent is the main contributor to the high specific surface area of Resin C. Combined with the results of pore structures, it can be concluded that the cooperation of solid granules and solvents as porogenic agents can produce the separation media with high specific surface area and large pores required for both high capacity and convective flow through the pores in chromatography.



Resin A



Resin B



Resin C

Fig. 3. Images of the resins in aqueous solution recorded by CCD detection.

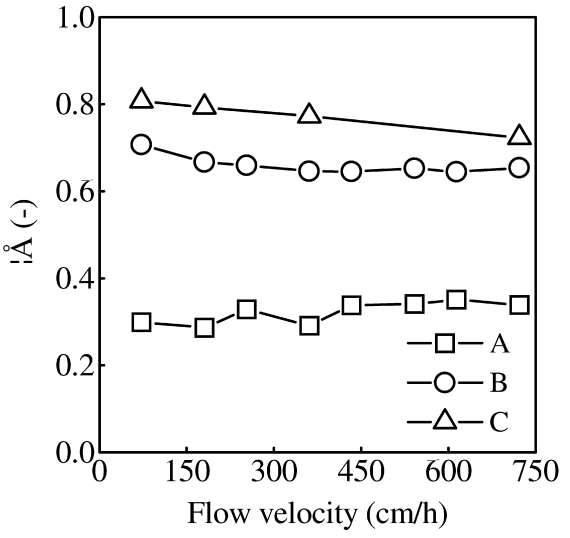


Fig. 4. Effect of flow-rate on the dynamic porosity of the resins for lysozyme with 0.01 mol/l Tris–HCl buffer, pH 7.6, as mobile phase.

3.2. Dynamic porosity and back-pressure

Fig. 4 shows the dynamic porosity of the resins for lysozyme as a function of liquid flow-rate. Lysozyme, with an isoelectric point (p*I*) of 11.0 [23], positively charged in the buffer, was not adsorbed to the anion exchange resin [20]. The porosities of Resins B and C are much higher than that of Resin A at the flow velocity range tested. The result reveals that the protein is able to convectively flow through the macropores in Resins B and C. Therefore, it can be expected that due to the presence of the flow-through pores, the resistance to mass transport of macromolecules in Resins B and C is substantially smaller than that in Resin A. This will be discussed later.

The effect of flow-rate on back-pressure shows that at 720 cm/h, the back-pressures of Columns A, B and C are 7.9, 3.5 and 2.8 MPa, respectively. It can been seen that the flow resistance of Columns B and C is much lower than that of Column A, which is due to the presence of macropores around 1 μm [24]. However, at flow-rates higher than 1000 cm/h, the back pressure increases drastically. It is considered due to the breakage of the irregularly shaped resin, which can result in the blockage of the column. This is a drawback of the current resin, so further effort should be made to enhance its mechanical strength.

3.3. Adsorption properties

Fig. 5 shows the static BSA adsorption isotherms of the media. The adsorption of BSA to the resins can be described by the Langmuir equation:

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

where q (mg/g wet resin) is the adsorbed BSA density to the adsorbent, q_m is the adsorption capacity, c (mg/ml) is the equilibrium concentration of BSA in bulk solution, and K_d is the dissociation constant. By fitting the equilibrium data to the Langmuir equation with the nonlinear Simplex method, q_m and K_d are determined. The static BSA capacities of Resins A, B and C are estimated to be 107.7, 9.5 and 95.5 mg/g wet resin, respectively. The results agree well with the data of the specific surface area described above.

Frontal analysis, providing the breakthrough

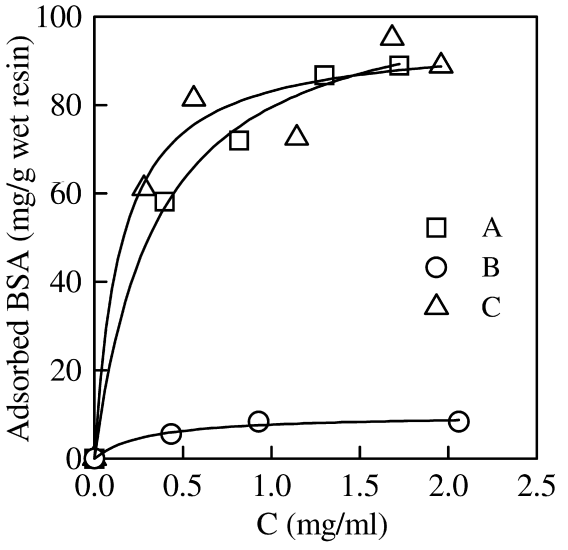


Fig. 5. Static adsorption isotherms of BSA to the anion exchange resins.

curves of a protein, can be used to evaluate the dynamic capacity of a chromatographic medium for a protein. Differences in the static and dynamic binding capacity of an adsorbent are a sensitive measure of mass transfer limitations [25,26]. Fig. 6 shows BSA breakthrough curves at 72 cm/h for Columns A, B and C. The 5% breakthrough adsorption capacity of Columns A and B are 1.95 and 8.34 mg BSA/ml column (or 3.12 and 10.3 mg/g wet resin), respectively, while that of Column C is as high as 57.0 mg BSA/ml column (or 95.0 mg/g wet resin). The adsorption capacity of Column A (diffusion-limited case) descends considerably compared with the static capacity determined by batch experiments that allow long adsorption time. In contrast, the dynamic capacities of Resins B and C are basically identical to the respective static capacities. The results prove that in Resin C with diameter ranging from 37 to 74 μm, the flowthrough pores considerably reduce mass transport resistance and the diffusion equilibration for BSA is able to be achieved within 2.5 min (the mobile phase retention time). The result is in good agreement with those reported with very high porosity throughpore materials in perfusion chromatography [12].

It has been reported that the commercially available biporous adsorbent, Poros Q, has a dynamic capacity of 25 mg BSA/ml column, lower than those of Mono Q, Resource Q and Q HyperD (i.e., 80, 70 and 200 mg BSA/ml column, respectively), at 80 cm/h (determined at 50% breakthrough) [27]. It can be found that Resin C possesses a capacity higher than the commercial biporous adsorbent, comparable with those of Mono Q and Resource Q. In contrast, the dynamic capacities of Resins A and B are much lower than those of the high-performance ion exchangers, including Resin C.

3.4. Column efficiency (HETP)

The chromatographic efficiency of the resins is determined using lysozyme in 0.01 mol/l Tris–HCl buffer, pH 7.6 (Fig. 7). Several important features of these plate height curves should be noted. First, Columns B and C are of much greater efficiency at high mobile phase velocity than Resin A. Second, the HETP values of Columns B and C are only weakly related to mobile phase velocity, while that of Column A exhibits an obvious rise when the flow-rate increases. Third, the HETP values of Columns B and C are basically equivalent, even with increasing flow-rate. The results manifest that the presence of flow-through pores in Resin C substan-

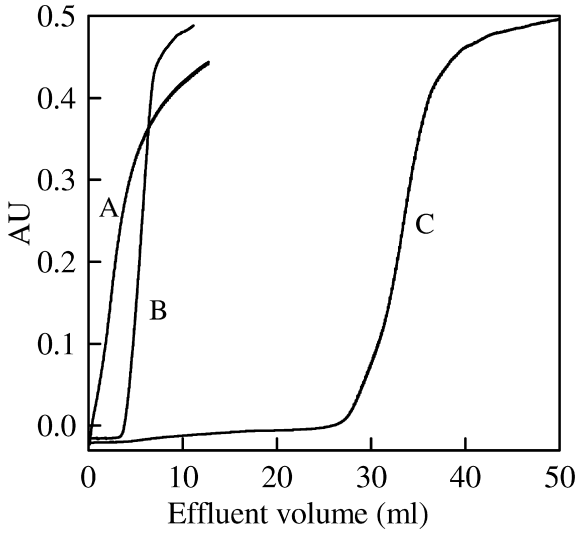


Fig. 6. Breakthrough curves for BSA at 72 cm/h. Conditions: column 30×4.6-mm I.D.; mobile phase 0.01 mol/l Tris–HCl, buffer pH 7.6; feed BSA concentration, 2 mg/ml; UV detection at 280 nm.

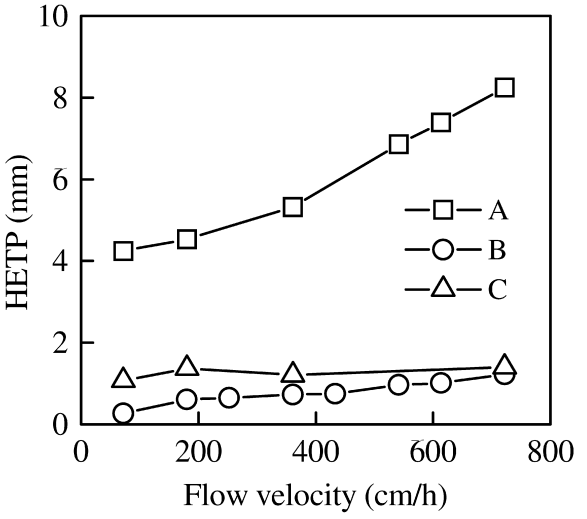


Fig. 7. Dependence of the column efficiency on mobile phase flow velocity. The injection size was 20 μl of 5 mg/ml lysozyme in 0.01 mol/l Tris–HCl buffer, pH 7.6.

tially increases the mass transfer rate of the large molecules and minimizes the negative effects of diffusion [13,14].

HETP for macromolecules of the columns packed with 20 μm Poros Q, a strong anion exchange perfusion chromatographic material, was about 0.4 mm [13], while that of the columns packed with 140 μm superporous agarose was approximate 2 mm [7]. The HETP of the present resin (Resin C) is between those values.

4. Conclusions

An ideal media for protein separation is biporous materials with high specific surface area and large pores required for high capacity and low flow resistance. However, the preparation of biporous resins with conventional solvent porogenic agent is costly and time-consuming. We have developed a novel porogenic mode, i.e., cooperation of solid granules and solvents, which simplifies the preparation of biporous media into a simple in situ polymerization. A biporous copolymer of GMA, DVB and TAIC is produced using granules of sodium sulfate and cyclohexanol and dedocanol as porogenic agents, and employed as anion exchanger after functionalized with diethlamine. Data from SEM, mercury intrusion porosimetry measurements and CCD detection reveal that the matrix contains regions of micropores below 120 nm in size with a maximum at approximately 55 nm and regions of macropores with size ranging from about 120–1630 nm, with a distinct maximum near 340 nm, which can swell to above 1 μm in aqueous solution. The result obtained from dynamic porosity indicates that proteins can convectively flow through the macropores in the biporous medium. Compared with the resins using only solvents or solid granules as porogenic agent, the biporous medium simultaneously possesses a high specific surface area of 37.2 m^2/g and a low back-pressure at mobile-phase flow velocity up to 720 cm/h. The dynamic adsorption capacity is as high as 57.0 mg/ml, basically identical to the static capacity. In addition, the effect of flow velocity on HETP of the resins manifests that the flow-through pores in the biporous resin substantially increase the mass transfer rate of the large molecules and minimize the negative effects of diffusion. All the results confirm that the cooperation of solid granules and solvents provides a new porogenic mode for the preparation of biporous separation media with high specific surface area and large pores, leading to a chromatographic medium with high capacity, low flow resistance and high column efficiency.

Acknowledgements

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