A novel stationary phase derivatized from hydrophilic gigaporous polystyrene-based microspheres for high-speed protein chromatography

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Using agarose coated gigaporous polystyrene microspheres as a base support, a novel anion exchanger (DEAE-AP) has been developed after functionalization with diethylaminoethyl chloride. The gigaporous structure, static adsorption behavior, and chromatographic properties of DEAE-AP medium were characterized and compared with those of commercially available resin DEAE Sepharose Fast Flow (DEAE-FF). The results implied that there existed some through pores in DEAE-AP microspheres, which effectively reduced resistance to stagnant mobile phase mass transfer by inducing convective flow of mobile phase in the gigapores of medium. As a consequence, the column packed with DEAE-AP exhibited low column backpressure, high column efficiency, high dynamic binding capacity and high protein resolution at high flow velocity up to 2600 cm/h. In conclusion, all the results suggested that the gigaporous absorbent is promising for high-speed protein chromatography.

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1. Introduction

With the quick development of biotechnology, more and more bioproducts with large molecule size such as protein and plasmid DNA need to be separated. Up to present, liquid chromatography (LC) is an important tool and a necessary step for the separation and purification of biological macromolecules owing to its high resolution and mild separation conditions. It is well known that long time exposure in artificial environment often leads to the loss of protein activity and fragmentation of the target, so the short chromatographic time is preferred [1].

The properties of stationary phase are crucial to the performance of LC, and a key aspect of LC is choice of a suitable stationary packing. Several packing materials (e.g., polysaccharides, inorganic compound and polymers) have been used to improve the separation of proteins during the past decades [2]. Among them poly(styrene-divinylbenzene) (PS) microspheres are of increasing interest to chromatographers over silica and conventional separation media (e.g., dextran and agarose) as chromatographic packing materials for proteins and antibodies owing to their excellent mechanical properties and a good chemical stability over a wide pH range [2–4]. However, for conventional porous microspheres, slow mass transfer rate is the limiting factor that restricts their application in biomacromolecules separation [2]. In 1990s, a novel PS-based medium, referred as POROS perfusion absorbent, was introduced to mend the stagnant mass transfer resistance [5]. POROS microspheres were prepared by nanomicrospheres' agglomeration method, which have two sets of pores: through pores (600–800 nm) and diffusive pores (80–150 nm). The interconnected through pores bring intraparticle convective transport into the bead and diffusive pores provide a substantial surface area. So, this kind of stationary phase can be operated at high speed while maintaining high column efficiency and dynamic binding capacity [2,5–8]. Even though, the preparation method of POROS is complicated and the diameter of the two sets of pores is difficult to control, there are few reports about application of POROS in protein separation in recent years. Inspired by high internal phase emulsion (HIEP) method [9,10], our research group has developed a novel surfactant reverse micelles swelling method to prepare gigaporous PS microspheres with pore size of ca. 300–500 nm [11], which overcomes the preparation problem of POROS medium described above and can be used as perfusion chromatographic supports. Unfortunately, the native PS beads restrict their application in the chromatography for proteins, except in the reversed-phase mode, because of their high hydrophobic properties, which will lead to non-specific adsorption and denaturation of proteins. In previous study, we have successfully modified the gigaporous PS microspheres with agarose and poly(vinyl alcohol), respectively [12,13], and the coatings of gigaporous PS microspheres are hydroxyl-rich and biocompatible. The hydrophilic gigaporous PS microspheres are good chromatographic
“base supports” for different types of chromatography such as ion-exchange or affinity chromatography since the coatings may be easily derivatized by classical methods.

Since ion-exchange chromatography (IEC) medium was first used in protein chromatography [14], it has been widely used in protein separation, and nearly 75% protein purification includes the step of IEC [15]. In this paper, taking agarose coated gigaporous PS (Agap-co-PS) microspheres [13] as a base support, we prepared diethylaminoethyl (DEAE) anion exchanger (DEAE-AP) by Williamson reaction. The chromatographic properties, such as flow hydrodynamics, static and dynamic adsorption behavior, and column efficiency, were investigated in detail. Also, protein separation was performed to further testify its feasibility in high-speed protein chromatography.

2. Experimental

2.1. Materials

The Agap-co-PS microspheres used in this study were prepared in our previous study [9]. The coating amount is 81.97 mg/g dry microspheres, the specific surface area was 24.09 m²/g, the average diameter was 55 μm (30–85 μm range), and the average pore size was 300 nm (100–500 nm range). The Agap-co-PS microspheres were stored in 20% ethanol–water solution at room temperature for further process.

DEAE Sepharose Fast Flow (DEAE-FF) was purchased from Amersham Biosciences (ionic capacity 0.16 mmol/ml gel, Sweden); bovine serum albumin (BSA, MW 67,000) and myoglobin (MYO, MW 17,800) were obtained from Sigma (St. Louis, MO, USA); transferrin (TRA, MW 80,000) were ordered from Merck (USA); dextran standards (T-10, T-40, T-70, T110, T200, T500 and T2000) were purchased from Pharmacia Biotech (USA); diethylaminoethyl chloride (DEAE·HCl, CR) was from Shanghai Nanxiang Reagent Co., Ltd. (China); Tris(hydroxymethyl) aminomethane (Tris base, AR) was obtained from Shanghai BOAO Biotechnology Co., Ltd. (China). Other reagents were all of analytical grade from local sources.

In all the experiments, commercially available medium DEAE-FF was used as a control and the average value of at least triplicate samples was presented for all data.

2.2. Preparation of anion exchanger (DEAE-AP)

DEAE groups were introduced onto Agap-co-PS microspheres according to the method reported by Wang et al. [16]. Typically, the gigaporous Agap-co-PS microspheres were soaked and swollen in deionized water for 24 h aforesaid, then filtered through sintered glass funnel to remove the external water. Taking 2 g of drained microspheres suspended in 10 ml of DEAE·HCl (3.5 mol/l) in an incubator at 70 °C and shaking at 130 rpm for 10 min, 10 ml of pre-heated NaOH solution (5 mol/l) at 70 °C was added. After reaction for 1 h, the microspheres were rinsed with deionized water thoroughly to remove the residual DEAE·HCl on the microspheres, then collected with a sintered glass funnel and stored in 20% ethanol–water solution.

2.3. Characterization of ion-exchange media

The pore structures of dehydrated DEAE-AP and DEAE-FF microspheres were observed by scanning electron microscopy (SEM, JEM-6700F, JEOL, Japan). Solvent-replacement method could maintain the porous structure of soft gel as much as possible [17]. The microspheres were dehydrated by stepwise transfer into aqueous solutions of ethanol (10% increment, from 10% to 90% ethanol), anhydrous ethanol and diethyl ether. Mercury porosimetry measurements (MPM) were conducted by an AutoPore IV 9500 mercury porosimetry (Micromeritics, USA) to study the difference between DEAE-AP and Agap-co-PS microspheres. Hydrated density of wet microspheres was measured with a 25 ml pycnometer. The effective porosities ($\varepsilon_p$) of the DEAE-AP and DEAE-FF medium for BSA were measured by the method reported elsewhere [18].

The total ionic capacity of the anion exchanger was detected by the following method. The microspheres were packed into a glass column (20 cm × 1 cm I.D.) and transformed with 10 column volumes (CVs) of 0.5 mol/l NaOH at a flow rate of 1 ml/min, then the column was washed with deionized water until the pH value of eluate decreased to neutralization. Afterwards, 10 CVs of 0.05 mol/l standard HCl solution was used to elute the column and the eluate was collected. Finally, hydrogen ions in the eluate were titrated with 0.1 mol/l standard NaOH solution and the total ionic capacity was calculated by mass balance. The same volume of fresh HCl solution was used as control.

2.4. Static adsorption isotherms

The batch adsorption experiment was utilized to determine the static adsorption isotherms of DEAE-AP and DEAE-FF microspheres with BSA as model protein. After equilibration of the microspheres with 20 mmol/l Tris–HCl buffer (pH 8.0), they were drained by a sintered glass funnel. Then, 0.1 g of the drained ion exchanger was weighed into a 25 ml Erlenmeyer flask containing 10 ml BSA solution with a protein concentration up to 2 mg/ml. Adsorption was conducted at 25 °C in an incubator, stirring for 24 h at 120 rpm. At the end of adsorption equilibrium, the microspheres were separated from the solution by centrifugation. The residual protein concentrations (equilibrium concentration) were determined at 280 nm by UV spectrophotometer (Ultrospec2100 pro, Amersham Biosciences, USA). The bound amount of BSA per milliliter of wet microspheres ($q$) was calculated by the mass balance of proteins.

$$ q = \frac{(c_0 - c)V\rho_w}{W} $$

(1)

where $c_0$ and $c$ are initial and equilibrated concentration of BSA, respectively (mg/ml), $V$ the solution volume (ml), $W$ the mass of wet microspheres (g), and $\rho_w$ the density of wet microspheres (g/ml).

2.5. Chromatography

The Agap-co-PS, DEAE-AP and DEAE-FF media were respectively packed into stainless column (250 mm × 4.6 mm I.D. and 100 mm × 4.6 mm I.D.) by slurry packing method at Shimadzu LC-8A preparative liquid chromatography system (Shimadzu Corporation). The chromatography of dextran standards was performed on an Agilent Technologies 1200 Series System equipped with Alltech 3300 evaporative light scattering detector (ELSD) to study the porous character of Agap-co-PS microspheres before and after functionalization under inverse size-exclusion chromatography (ISEC) mode. All the other chromatography experiments were conducted on the LC-8A system. The mechanical stability and permeability of medium was evaluated through the effect of flow rate on the backpressure of column, and the mobile phase was high-purity water. Furthermore, the bed permeability ($K$) can be evaluated by the Darcy’s law in a laminar flow region [19].

$$ K = \frac{\mu L}{\Delta \Pi} $$

(2)

where $\mu$ is the viscosity of the mobile phase (Pa s), $u$ the superficial velocity (cm/s), $L$ the length of column (cm), and $\Delta \Pi$ the column pressure-drop (Pa).

The column efficiency was evaluated in terms of the height equivalent to a theoretical plate (HETP). The experiments were con-
ducted at flow velocities ranging from 180 to 2528 cm/h under a non-retained condition. The mobile phase was 1.0 mol/l NaCl in 20 mmol/l Tris–HCl buffer (pH 8.0) and BSA was used as a probe protein. After equilibrating the column with 10 CVs of mobile phase, 200 μl of protein sample was injected and the chromatogram was recorded at the column exit by UV detector at 280 nm. The dead volume of the system was measured by injecting 200 μl of 20% acetone solution via the injection loop.

Frontal analysis was conducted in 20 mmol/l Tris–HCl buffer (pH 8.0) to determine the dynamic binding capacity of the two media. After equilibrating the column with 10 CVs of the mobile phase, 2 mg/ml BSA in 20 mmol/l Tris–HCl buffer (pH 8.0) was loaded and protein concentration at the outlet was measured with a UV monitor. The breakthrough profile was recorded until the absorbance of the outlet stream reached that of the inlet stream. Finally, the column was eluted with 5 CVs of 1.0 mol/l NaCl in the Tris–HCl buffer. The dynamic binding capacity at 10% breakthrough was calculated from the following equation:

\[ q_{10} = \frac{c_{f}(t_{10} - t_{0})}{V_B} \]

where \( q_{10} \) is the dynamic binding capacity at 10% breakthrough (mg/ml wet resin), \( c_{f} \) the feed concentration of BSA (mg/ml), \( F \) the volumetric flow rate (ml/min), \( V_B \) the bed volume (ml), \( t_{10} \) the time at 10% breakthrough (min), and \( t_{0} \) the retention time under non-retained condition (min).

### 2.6. Protein separation

To compare the high-speed chromatographic separation performance of DEAE-AP and DEAE-FF columns on proteins, a protein mixture comprising MYO, TRA and BSA was used under identical chromatographic conditions. After equilibrating the column with 10 CVs of 20 mmol/l Tris–HCl buffer, pH 8.0 (buffer A), 100 μl of sample solution containing 5 mg/ml MYO, 5 mg/ml TRA and 10 mg/ml BSA was injected via the injection loop. Then, linear gradient elution was carried out by a change from 100% buffer A to 50% buffer B (1.0 mol/l NaCl in buffer A) in 30 ml eluate. Protein concentration was detected at 280 nm. After each run the column was eluted with 10 CVs of buffer B. The column was then equilibrated with buffer A until a constant UV baseline was obtained.

### 3. Results and discussion

#### 3.1. Physical properties of the resins

Fig. 1 shows the SEM images of DEAE-AP and DEAE-FF microspheres at different magnifications. Compared to the smooth surface of DEAE-FF microspheres (Fig. 1c), the rough surface of DEAE-AP microspheres (Fig. 1a) implies that there is significant difference on pore structure between the two types of microspheres. At larger magnification, some highly reticular gigapores larger than 500 nm could be observed on DEAE-AP microspheres (Fig. 1b) as compared to the small pores on DEAE-FF micro-

![SEM images of DEAE-AP](a, 1900×; b, 10,000×) and DEAE-FF (c, 1900×; d, 10,000×) microspheres.
Fig. 2. Pore size distribution curves of gigaporous Agap-co-PS and DEAE microspheres. These gigapores in DEAE-AP microspheres are expected to provide an interconnected path for convective flow in chromatography.

Fig. 2 shows the pore size distributions of gigaporous microspheres before and after functionalization. The pores with a diameter between 100 and 600 nm occupied most of the pore volume in the medium and the greatest incremental pore volume occurred at a pore diameter of around 300 nm. From 100 to 600 nm the incremental pore volumes are almost equal in both medium types indicated that the gigaporous structure of Agap-co-PS microspheres was well maintained after functionalization. The calibration curves for a series of dextran standards on Agap-co-PS microspheres and on DEAE-AP medium are shown in Fig. 3. In SEC, the plot of elution volume versus molecular size can be related to the pore size distribution of the media. Compared with Agap-co-PS column, the retention volume of the dextran on DEAE-AP column only slightly decreased, which also indicated that the gigaporous structure of Agap-co-PS microspheres was well maintained after grafting DEAE groups.

The physical properties of DEAE-AP and DEAE-FF media are listed in Table 1. Because the backbone of DEAE-AP microspheres is hydrophobic polystyrene material, it is evident that the water content of DEAE-AP microspheres was far lower than that of DEAE-FF microspheres. Owing to the gigaporous structure of DEAE-AP microspheres, it could provide more pore spaces to allow the entrance of BSA. As a result, the effective porosity of DEAE-AP microspheres for BSA was about 22% higher than that of DEAE-FF microspheres.

3.2. Flow hydrodynamics

Fig. 4 shows the effect of flow velocity on the backpressures of DEAE-FF and DEAE-AP columns. As shown in Fig. 4, the linear relationship was obtained on DEAE-AP column for flow velocity up to 3612 cm/h. However, for DEAE-FF column, the linear relationship was only obtained for flow velocity up to 1668 cm/h owing to DEAE-FF resins' low mechanical stability. Moreover, it can be seen that the backpressure of DEAE-AP column was much lower than that of DEAE-FF column under the same flow velocity. The lower backpressure of DEAE-AP column is an evidence for the presence of flow-through pores which reduced the flow resistance. In previous study [13], we have confirmed that mobile phase in the column flowed not only through the spaces between matrix but also through the gigapores of particles, i.e., the existence of through pores in gigaporous microspheres. For DEAE-AP column, we calculated 63.38% of flow was through the inter-spaces between the particles, and about 36.62% of the flow was possibly through the intra-pores of the particles (see Supplementary Content), which could not only induce convective flow within particles but also greatly shorten the diffusive distance from outer particle to inner particle.

According to experimental data, the bed permeability ($K$) can be calculated from Eq. (2), which further confirmed this consideration. As listed in Table 1, the value of $K$ for DEAE-AP column was 3.34 times higher than that for DEAE-FF column.

3.3. Column efficiency

HETP is a typical criterion to describe the overall column efficiency. In this study, BSA was used as a probe solute and the HETP was measured under the non-retained condition. Fig. 5 shows the relationship between HETP and flow velocity for both DEAE-FF and DEAE-AP columns. At the lowest velocity tested (180 cm/h), the HETP of DEAE-FF column (2.95 mm) was 1.24 times larger than that of DEAE-AP column.
of DEAE-AP column (2.38 mm). With increasing the flow velocity, the difference of HETP values between the two columns was further augmented. For DEAE-FF columns, HETP rapidly increased with the increase of flow velocity, which is in accordance with the Van Deemter equation [20]. It indicated that the mass transfer resistance in the DEAE-FF resins was dominated by the intraparticle term. However, the HETP of DEAE-AP column only increased slightly with flow velocity up to 2528 cm/h, that is, HETP was nearly independent of flow velocity in a wide range of flow rate. We speculated that the presence of flow-through pores in DEAE-AP microsphere could induce intraparticle convective mass transfer for BSA and significantly lessened the stagnant mobile phase mass transfer of particle by reducing the diffusive distance [2].

3.4. Static and dynamic protein adsorption

The static BSA adsorption isotherms of DEAE-FF and DEAE-AP microspheres are presented in Fig. 6. We fitted the adsorption values with Langmuir model by using non-linear regression method and found the simulated curve could fit experimental data well.

\[
q = \frac{q_m C}{K_d + C}
\]

where \(C\) is the equilibrium concentration of BSA in bulk solution (mg/ml), \(q\) the adsorbed density of protein (mg/ml wet resin), \(q_m\) the adsorbed capacity in equilibrium (mg/ml wet resin), and \(K_d\) the dissociation constant (mg/ml). The static BSA adsorption capacity of DEAE-FF and DEAE-AP microspheres were simulated from Eq. (4) to be 83.33 and 52.08 mg/ml wet resin, respectively. Though the ion-exchange capacity of DEAE-AP microspheres was similar to that of DEAE-FF microspheres, the static adsorption capacity of the former was smaller than the latter. This indicated that the effective adsorption sites (i.e., the specific surface areas) of DEAE-AP microspheres were lower than that of DEAE-FF microspheres due to the presence of gigapores in DEAE-AP microspheres, which would reduce the specific surface areas of microspheres more or less.

It is more significant for a medium to present rather high dynamic binding capacity than high static adsorption capacity in practical application. Frontal analysis by breakthrough experiments can provide information about the dynamic binding capacity (DBC) of a medium. The DBC values of DEAE-AP and DEAE-FF columns were calculated from Eq. (3). The effects of flow velocity on the DBC values of the two columns are shown in Fig. 7. In contrast to static adsorption capacity, the DBC of DEAE-AP column was 1.8–2.1 times higher than that of DEAE-FF column at all flow velocities tested. With increasing the mobile phase velocity, the DBC of DEAE-FF column decreased drastically from 33.97 mg BSA/ml bed (253 cm/h) to 15.6 mg BSA/ml bed (1445 cm/h). However, the DBC of DEAE-AP column decreased slowly from 44.95 mg BSA/ml bed (361 cm/h) and kept a value of 27.49 mg BSA/ml bed up to a flow velocity of 2000 cm/h. In addition, the DBC of DEAE-AP column was 36.8 mg BSA/ml bed at a flow velocity of 722 cm/h, which is similar to that of commercially available bioporous polymer-based anion-exchange resin POROS Q/M (38 mg BSA/ml bed) [21]. The results indicated that the presence of flow-through pores in DEAE-AP microspheres could induce convective flow within particles and thus the rate of mass transfer was accelerated [22], leading to the high column efficiency (Fig. 5) and DBC at elevated flow velocity.

3.5. Protein separation

In order to further verify the advantages of DEAE-AP column in high-speed protein chromatography, a mixture of model proteins, MYO, TRA and BSA, was separated at different flow velocities. The chromatograms obtained are shown in Fig. 8. DEAE-AP column enabled complete separation of the three components of the mixture at the mobile phase velocity up to 2600 cm/h within 3 min, and the separation chromatograms and resolution were barely reduced. For DEAE-FF column, however, the resolution decreased significantly with increasing the flow velocity. At the flow velocity of 722 cm/h, the separation chromatogram changed markedly, which
indicated that the column probably slightly collapsed and formed flow channeling owing to the poor mechanical strength of agarose beads. Combined with the data of column efficiency and DBC of DEAE-AP column, all the results demonstrated that DEAE-AP column is promising for high-speed protein chromatography.

4. Conclusions

Using hydrophilic gigaporous Agap-co-PS microspheres we prepared in previous work as a base support, a novel gigaporous anion-exchange medium has been developed after functionalization with diethylaminoethyl chloride. The gigaporous structure of DEAE-AP microspheres was identified by effective porosity for protein measurement, pore size analysis and SEM images. Accelerated mass transfer induced by convective flow of mobile phase through the gigapores in the medium has also been demonstrated by its low column backpressure, high column efficiency, high dynamic binding capacity and high protein resolution at high flow velocity. In conclusion, all the results indicated that the gigaporous absorbent is promising for high-speed protein chromatography. Especially, the novel stationary phase could potentially play important roles in large scale protein quick separation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.07.059.

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