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Synthesis of penetrable poly(methacrylic acid-*co*-ethylene glycol dimethacrylate) microsphere and its HPLC application in protein separation

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ABSTRACT

In the present study, the narrow-dispersed penetrable poly(methacrylic acid-*co*-ethylene glycol dimethacrylate) (poly(MAA-*co*-EDMA)) microspheres were successfully synthesized based on the sacrificial support method. The poly(MAA-*co*-EDMA) microspheres mirrored the porous structure of the sacrificial support, i.e. penetrable silica, characteristic of copious mesopores and throughpores. In addition, they possessed large surface area, adjustable hydrophobicity and the cation-exchange ability. Owing to their multi functionalities, they were applied as chromatographic stationary phase to separate proteins in different separation modes, including reversed phase, hydrophobic interaction and weak cation exchange. Moreover, thanks to their throughpores, fast separation at low column backpressure could be achieved in these three modes. Both protein recovery and column stability were satisfactory. The penetrable poly(MAA-*co*-EDMA) microspheres were potential stationary phase matrix for fast protein separation.

1. Introduction

Diverse chromatographic methods have been adopted in purifying and isolating proteins, including reversed-phase chromatography (RPC) [1], ion-exchange chromatography (IEC) [2], affinity chromatography [3], size-exclusion chromatography (SEC) [4], hydrophobic interaction chromatography (HIC) [5] and hydrophilic interaction chromatography (HILIC) [6]. Among these, RPC, HIC and IEC were the three most common chromatographic modes for protein separation. IEC and HIC could maintain the structure and bioactivity of proteins under non-denaturing conditions, while RPC was more suitable for analytical purpose with relatively higher column efficiency. In particular, the mixed-mode chromatography (MMC) demonstrated increasing applications for protein separation [7], due to its enhanced selectivity and separation capacity compared to the traditional single-mode chromatography. Bai's group contributed a lot to combine HIC and IEC for protein separations [8–10].

No matter what kind of chromatographic modes is used, the stationary phases play an important role in protein separation. According to the matrices, the stationary phases can be classified into two categories, i.e. silicas and polymers. Silica-based matrices have ideal mechanical strength, controllable morphology and porous properties with desirable functionality. However, they are subject to insufficient hydrolytic stability of the Si-O-C linkage, especially under moderately acidic or slightly alkaline conditions; nonspecific adsorption is too strong to separate macromolecules, particularly proteins. In contrast, polymer-based packings could be "biocompatible" to prevent the irreversible adsorption of biomolecules, in addition to their appreciated wide pH applicability [11]. However, compared to the silica-based packings, polymer-based ones provided lower column efficiency, and had more difficulties in the control of the morphological and porous properties during preparation.

Perfusion chromatography [12,13] is well known for its suitability to separate macromolecules. It is mainly based on penetrable polymer matrix, which simultaneously possesses the macropores and mesopores. This combination of porosities guarantees fast mass transfer in macromolecule separation. However, the availability of stationary phases for perfusion chromatography is quite limited, which seriously hampers the development of this separation technology.

In the present study, to enrich the stationary phase for protein separation, penetrable poly(MAA-*co*-EDMA) microspheres were prepared. The penetrable poly(MAA-*co*-EDMA) was assumed to have many merits. First of all, it was biocompatible [14,15] and could be an ideal matrix for biomolecule separation. In addition, poly(MAA-*co*-EDMA) has a hydrophobic skeleton with plenty of carboxylic acid groups, which allows the material to be potentially applied in different chromatographic modes, i.e. RPC, HIC and IEC. This property was particularly appreciated, as the separation modes could be easily switched by only adjusting the mobile phase. Moreover, because of the penetrable macropores like the perfusion chromatography, the poly(MAA-*co*-EDMA)

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microspheres would offer fast mass transfer and low backpressure, turning the fast separation into a reality.

To better control the morphology and porosity, sacrificial support method was adopted to prepare poly(MAA-*co*-EDMA). Penetrable silica particles, which contain mesopores, penetrable macropores and interlaced skeletons [16–18], were used as the sacrificial support. The pre-polymerization mixture filled the pores of the silica particles, followed by polymerization and removing the silica support, leaving porosity-imprinted polymeric microspheres which were the "negative image" of the silica. In this way, the polymer maintained the same penetrable macropores and interlacing skeletons as the silica support. The as-prepared polymer was applied as the high performance liquid chromatographic (HPLC) stationary phase to separate proteins in different separation modes.

2. Experimental

2.1. Chemicals and reagents

The penetrable silica (ca. 5 μm diameter, 2 mL g^{-1} pore volume) was prepared as reported previously [16]. All protein standards including ribonuclease A (from bovine pancreas, RNase A), cytochrome C (from bovine heart), insulin (from bovine pancreas), transferrin, bovine serum albumin (BSA), myoglobin and α-chymotrypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catalase (from bovine liver, 2000 ~ 5000 U mg^{-1}) was purchased from Jiang Lai Chemicals Co., Ltd (Shanghai, China). Six polycyclic aromatic hydrocarbon (PAH) standards, i.e. naphthalene, diphenyl, phenanthrene, chrysene, pyrene and benzo(g,*h*,i)perylene, were purchased from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). Sodium chloride (NaCl), ammonium sulfate ($(NH_4)_2SO_4$), sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}2H_2O$), disodium phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), sodium hydroxide (NaOH), isopropanol, hydrochloric acid (HCl), trifluoroacetic acid (TFA), toluene, dodecanol, methacrylic acid (MAA), 2,2-azobis(isobutyronitrile) (AIBN) and ethylene glycol dimethacrylate (EDMA) were provided by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was produced by Milli-Q® reagent-grade water system (Millipore, MA, USA). Phosphate buffer saline (PBS) was prepared with $NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}12H_2O$ as specified concentration and pH.

2.2. Preparation of penetrable poly(MAA-co-EDMA) microspheres

Penetrable poly(MAA-*co*-EDMA) microspheres were synthesized by using MAA, EDMA and AIBN as the functional monomer, crosslinker and initiator, respectively. The schematic procedure is shown in Fig. 1.

Preparation of pre-polymerization mixture. AIBN (2% wt of total pre-polymerization mixture), MAA and EDMA in different ratios as listed in Table 1, were dissolved in porogen (toluene and dodecanol), and purged with N_2 for 10 min after sonication.

Preparation of the silica-polymer complex. In order to obtain discrete silica-polymer complex and to prevent sticky particle agglomerates, pre-polymerization mixtures were added to the penetrable silica in the ratio of 1.9 mL to 2 g; the volume of the former was slightly lower than the pore volume of the silica (2 mL g^{-1}). Mixed by vigorous shaking, the mixture was then gently stirred with a spatula until the free-flowing particles were obtained, reflecting that all the pre-polymerization mixture penetrated the silica pores. Finally, the mixture was sonicated and purged with N_2 to remove air bubbles, which was then sealed to polymerize at 65 °C for 24 h.

Sacrifice of the penetrable silica support. After polymerization, the resulting silica-polymer complex was immersed in the mixture of acetone and 2 M NaOH (10:90, v:v) at 65 °C overnight to dissolve the silica. The solid was then washed with abundant water and methanol before being dried in a vacuum oven at 120 °C overnight. Thermal gravimetric analysis (TGA) was carried out to confirm the complete removal of silica support, as listed in Table 1. Thus-obtained material was poly(MAA-*co*-EDMA), and ready for the following characterization and application.

2.3. Characterization of poly(MAA-co-EDMA)

The prepared poly(MAA-*co*-EDMA) was characterized by the following methods. The morphology was observed by scanning electron microscopy (SEM, ZEISS EVO18, Germany). The micro-/meso-pore properties of poly(MAA-*co*-EDMA) were characterized by a TriStar II surface area analyzer (Micromeritics, USA). The specific surface area value was calculated according to the Brunauer-Emmett-Teller (BET) method at P/P_0 between 0.05 and 0.2. The meso/micro-pore parameter was evaluated from the desorption branch of isotherm based on Barrett-Joyner-Halenda model. Mercury porosimeter was performed with Autopore IV 9500 instrument (Micromeritics, USA). TGA was performed on a thermal analyzer (TG 209 F1 Libra, Netzsch, Germany) at a heating rate of 10 °C min^{-1}. The contact angle measurement was conducted on Drop Shape Analysis System DSA 100 (Kruss, Germany).

The content of carboxyl acid groups and p*K*a of the prepared materials were determined by titration. Poly(MAA-*co*-EDMA) micropheres were suspended in 100 mL 0.1 M NaOH for 12 h, for the complete reaction of the carboxyl acid groups in the polymers with NaOH. Then back-titration was carried out to neutralize the residual NaOH with standardized 0.1 M HCl aqueous solution. Once every 1 mL HCl was dropped in, the corresponding conductivities or pH values of the solution were recorded by a conductivity meter (DDS-307 Lei Ci, Shanghai, China) or Mettler Toledo Delta 320 pH meter (Shanghai, China), respectively. Conductometric and acid-base titration curves were then plotted to obtain carboxyl acid groups' content and p*K*a of the polymers.

2.4. Column packing and HPLC analysis

The Poly A, B and C, as listed in Table 1, were separately homogenized with isopropanol and packed into the stainless steel columns (50 mm × 4.6 mm i.d., IDEX Health & Science LLC, MA, USA) with methanol at 40 MPa, resulting in the corresponding Column A, B and C, respectively.

HPLC analysis was performed on a Dionex Ultimate 3000 chromatographic system (California, USA) which consisted of a degasser, two

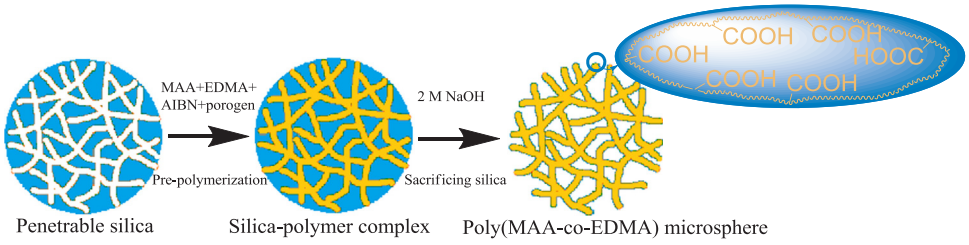

Fig. 1. The schematic procedure of penetrable poly(MAA-*co*-EDMA) microspheres.