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Short communication

Polyethylene glycol diacrylate-based supermacroporous monolithic cryogel as high-performance liquid chromatography stationary phase for protein and polymeric nanoparticle separation

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Abstract

A supermacroporous monolithic cryogel was directly prepared by in situ cryo-copolymerization in a stainless steel cartridge (70 mm × 5.0 mm I.D.) using methacrylic acid (MAA) as functional monomer and polyethylene glycol diacrylate (PEGDA) as crosslinker. The highly crosslinked (90%, molar ratio) poly(MAA–PEGDA) cryogel had more uniform supermacropores with a mean diameter of 25 μm compared to the poly(acrylamide)-based cryogels. The viability of poly(MAA–PEGDA) cryogel as a medium was demonstrated for separations of lysozyme from chicken egg white (CEW) and water-soluble poly(*N*-isopropylacrylamide-co-3-(dimethylamino) propyl methacrylamide) (NIPAM-DMAPMA) nanoparticles from its crude reaction solution. The dynamic binding capacities of lysozyme and the polymeric nanoparticles were 4.51×10^{-3} μmol/ml and 33.4 μg/ml, respectively. The lysozyme recovered from the above separations had a purity of more than 85%, and retained 90% of its enzymatic activity.

Keywords: HPLC; Monolith; Cryogel; Nanoparticle; Protein

1. Introduction

Hydrophilic monolithic cryogels have recently been introduced as a promising matrix for separations of biological nano and microparticles such as plasmid DNA, recombinant proteins, and cells [1–6]. These supermacroporous cryogels are typically prepared by cryo-radical polymerization of acrylamide (AAm), along with other low molecular weight co-monomer, and *N*,*N*′-methylene bis-(acrylamide) (MBAAm) as a crosslinking reagent. Since MBAAm has a low solubility in water, most cryogels have low-crosslinking degrees (lower than 20%). Poly(AAm-MBAAm)-based cryogels are thus flexible but mechanically soft, and cannot be used as stationary phases for high-performance liquid chromatography (HPLC) separations.

In this preliminary study, highly crosslinked supermacroporous cryogels were prepared by in situ cryo-radical copolymerization in a stainless steel chromatographic cartridge, using polyethylene glycol diacrylate (PEGDA) as crosslinker and methacrylic acid (MAA) as functional monomer. Separations of protein mixture were tested using this novel cryogel column. Since water-soluble poly(*N*-isopropylacrylamide) (NIPAM)-based nanoparticles have received increasing attention in many interesting applications including controlled drug delivery, enzyme and cell immobilization and immunoassay [7], and the commonly used purification methods such as dialysis and precipitation are time-consuming, the separations of water-soluble poly(NIPAM-co-3-(dimethylamino) propyl methacrylamide) (NIPAM-DMAPMA) nanoparticles on this cryogel column were also investigated in this work.

2. Experimental

2.1. Materials

PEGDA (of number average molecular weight 575, liquid) and DMAPMA were purchased from Sigma-Aldrich

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(Milwaukee, WI, USA) and used as received. NIPAM (Sigma-Aldrich) was recrystallized in benzene/*n*-hexane. MAA (Lancaster, Morecambe, UK) was distilled under reduced pressure. MBAAm, ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), trypsin inhibitor (Try, isoelectric point (p*I*) 4.2), ovalbumin (OVA, p*I* 4.5), hemoglobin (Hb, p*I* 6.8–7.0), myoglobin (Myo, p*I* 7.3), ribonuclease A (RNase-A, p*I* 8.9), cytochrome *c* (Cyc, p*I* 10.2) and lysozyme (LZM, chicken egg white, CEW, p*I* 10.8) were all obtained from Sigma-Aldrich. Deionized water was produced by a Millipore water system composed of Milli-RO 60 and Milli-Q SP.

2.2. Preparation of monolithic cryogel

The supermacroporous monolithic cryogel was prepared by an in-situ cryo-polymerization technique in a stainless steel chromatographic cartridge (70 mm × 5.0 mm I.D.). The mixture of monomers solution (MAA/PEGDA = 1/9, molar ratio) and deionized water (2.5 ml, monomers/water = 2/8, v/v), in which APS (5 mg) and TEMED (5 μl) were dissolved, was purged with nitrogen for 15 min in an ice bath and injected into the stainless steel cartridge. The column was sealed and frozen under −80 °C for 1 h and −20 °C for 24 h. Then, the column was thawed by gradually increasing the temperature to room temperature. The column was intensively washed with 800 ml of water at a flow rate of 1.0 ml/min.

2.3. Porosity and morphology of monolithic cryogel

The cryogel morphology was observed by environmental scanning electron microscopy (ESEM) (FEI Quanta 200FEG). The water content and the bound water content of cryogel were estimated according to reference [5].

2.4. Protein separation by cryogel monolith

All chromatographic experiments were performed on an HPLC (Agilent 1100, Palo Alto, CA, USA) with the above poly(MAA–PEGDA) cryogel monolithic column at room temperature. Protein solutions (1.0 μg/μl) were prepared with a loading solution (10 mM sodium phosphate buffer, pH 7.0). A CEW solution diluted with sodium phosphate buffer (10 mM, pH 7.0) was obtained as described in the literature [6]. The chromatographic profiles were monitored at 220 nm. Prior to injection, the samples were filtered through a membrane (0.22 μm). After a protein solution (20 μl) was injected, the separations were carried out typically by passing the loading solution through the column for 6 min, and then the elution solution (10 mM sodium phosphate buffer containing 1 M NaCl, pH 7.0). Triplicate runs were performed to examine the reproducibility. The purity and enzyme activity of lysozyme were determined following the procedures as described in reference [6].

2.5. Polymeric nanoparticle separation by cryogel monolith

Water-soluble poly(NIPAM-DMAPMA) nanoparticles were prepared by precipitation polymerization of 200 mg of NIPAM (1.77 mmol), 8 μl of DMAPMA (0.04 mmol), 4 mg of MBAAm (0.03 mmol) and 10 mg of APS in 20 ml of deionized water at 70 °C for 48 h as described in reference [8]. After cooling, 5 ml of the crude reaction solution was collected and analyzed by HPLC (Agilent 1100) with the poly(MAA–PEGDA) cryogel column. The remaining reaction solution was thoroughly dialyzed (molecular weight cut off 3500; Visking membrane, Carl Roth, Karlsruhe, Germany) for 4 days against distilled water. Finally, the particles were freeze-dried in vacuum at −50 °C in an ALPHA1-4 freezer (Leybold, Cologne, Germany) for at least 48 h. The morphology of the resultant nanoparticles was observed by ESEM. The hydrodynamic nanoparticle sizes were measured by the dynamic light scattering (DLS) method using a Brookhaven ZetaPlus (Holtsville, NY, USA).

3. Results and discussion

3.1. Preparation and characterization of poly(MAA–PEGDA) cryogel

Since PEGDA has good water solubility, it was chosen as a crosslinker for the preparation of the highly crosslinked cryogel. MAA is usually used as an acidic functional monomer, thus the resultant cryogel column can be used as a cation exchange media. Another modification in the preparation protocol was freezing the monomer solution at −80 °C for 1 h and then at −20 °C until the completion of the polymerization, instead of freezing at a constant temperature (mostly at −12 °C) for preparation of polyAAm-based cryogels. This modification was expected to increase the rate of ice crystal formation. As shown in Fig. 1, a uniform tubular macropore structure with a mean pore size of about 25 μm and pore distribution of 5–100 μm was clearly observed. This mean pore size was slightly lower than those of polyAAm-based cryogels, but larger than other classical polymeric organic monoliths [9–11].

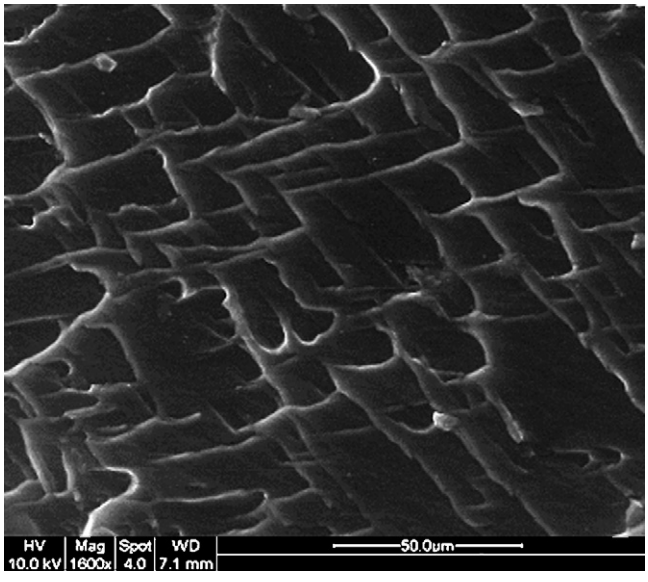

Fig. 1. ESEM image of poly(MAA–PEGDA) monolithic cryogel.

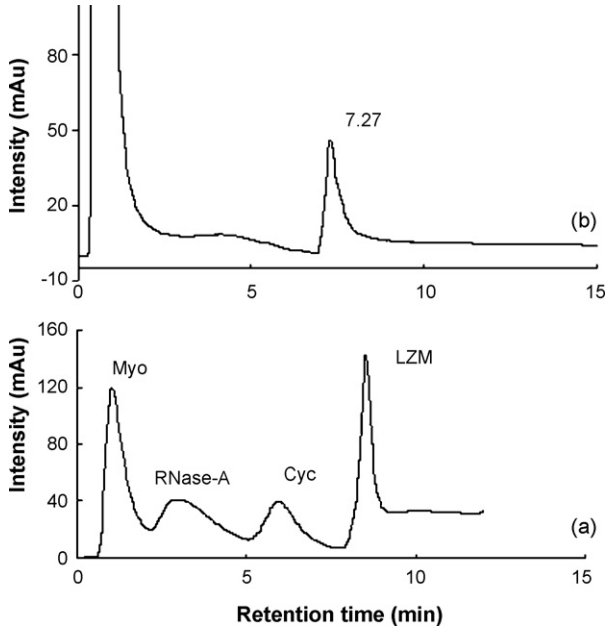

Fig. 2. Chromatograms of protein mixtures on the poly(MAA–PEGDA) monolithic cryogel. UV detector: 220 nm. Mobile phase A: loading buffer (sodium phosphate buffer, 10 mM, pH 7.0); B: elution buffer (sodium phosphate buffer containing 1 M NaCl, 10 mM, pH 7.0). (a) A mixture of Myo, RNase-A, Cyc and LZM. Salt gradient: 0–3 min, from 100% A to 95% A + 5% B; 3–6 min, 90% A + 10% B; 6 min, 100% B. Flow rate: 0.5 ml/min. Backpressure: 0.5 MPa. Total protein injection: 20 μg. (b) A solution containing 4.0 mg/ml of chicken egg white. Injection volume: 20 μl. Salt gradient: 0–6 min, 100% A; 6 min, 100% B. Flow rate: 1.0 ml/min. Backpressure: 0.9 MPa.

Another important feature of the chromatographic material is the pore volume. The dry polymer constituted only 18.2% (w/w) of the total weight of completely swollen cryogel, and the total water content was 81.8%, in which the bound water content was 5.8%. This indicated that the main part of the cryogel volume was composed of the interconnected supermacropores. These large pores provided most of the fluid flowing channels within the cryogel, which endowed the cryogel-packed columns with interesting chromatographic properties such as low backpressure and mass transfer by convection rather than by diffusion [10]. The cryogel allowed water to pass through at a flow rate of up to 3.0 ml/min with a backpressure of only 2.9 MPa, though it could not sustain a flow rate higher than 4.0 ml/min or a backpressure higher than 4.0 MPa. These pressure drops are lower than those of classical polymeric organic monoliths (2–10 MPa) [9–11], but higher than those of other reported polyAAm-based cryogels (0.001–0.02 MPa) at equivalent flow rates [5].

3.2. Protein separation

To confirm the ion exchange mechanism of the poly(MAA–PEGDA) cryogel used as media for separation of proteins, Try (p*I* 4.2), OVA (p*I* 4.5), Hb (p*I* 7.0), Myo (p*I* 7.3), RNase (p*I* 8.9), Cyc (p*I* 10.2) and LZM (p*I* 10.8) were chosen as the model proteins. All the proteins with p*I*s lower than 7.0 could hardly be retained on the cryogel column. The eluting order of the four basic proteins tested was in good accordance with the order of their p*I* values as shown in Fig. 2a. These results are consistent with those observed on other cation ion exchange columns [10,12]. The resultant cryogel showed modest peak efficiency compared with beaded exchange columns with carboxyl groups [12], which is partly due to its supermacroporous structure resulting in a lower surface area. However, since LZM is the only basic protein in the highly abundant proteins of CEW [6], LZM can be effectively separated from other CEW proteins using the resultant cryogel (Fig. 2b). The recovered LZM had a purity of more than 85% and retained 90% of its enzymatic activity. The dynamic protein capacity of LZM was 4.51×10^{-3} μmol/ml column volume according to the dynamic measurement methods [12]. The chromatograms of the seven tested standard proteins were shown in Figure S1 in the supplementary material.

3.3. Nanoparticle separation

Water-soluble poly(NIPAM-DMAPMA) nanoparticles were obtained by precipitation polymerization. The resultant nanoparticles showed a size of 231 ± 19 nm in diameter (mean ± SD, $n = 3$) and a narrow size distribution (polydispersity < 0.1) by DLS method, which were consistent with ESEM image (Fig. 3A). Fig. 3B shows the separations of cation water-soluble poly(NIPAM-DMAPMA) nanoparticles on the poly(MAA–PEGDA) cryogel column. As can be seen, cation nanoparticles purified by a dialysis process had similar chromatographic performance to lysozyme on the resultant cryogel since they could be eluted with similar retention time under salt gradient elution. NIPAM as the major monomer was hardly retained on the resultant cryogel, mainly because NIPAM was not protonated under a neutral pH condition. Interestingly, DMAPMA as a basic monomer was hardly retained on the cryogel either. This result may be attributed to the difference

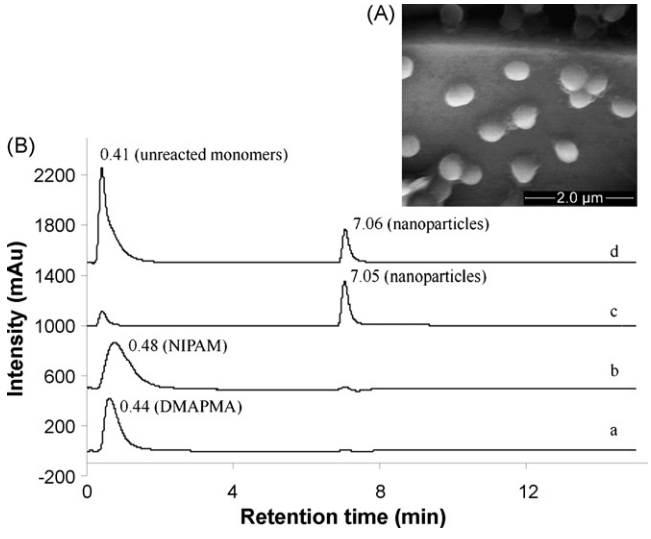

Fig. 3. Polymeric nanoparticles separation. (A) ESEM image of the purified poly(NIPAM-DMAPMA) nanoparticles. (B) Chromatograms of water-soluble poly(NIPAM-DMAPMA) nanoparticles and the corresponding monomers on the poly(MAA–PEGDA) monolithic cryogel. Chromatographic conditions were the same as in Fig. 2b. (a) DMAPMA, 47 ng; (b) NIPAM, 500 ng; (c) purified poly(NIPAM-DMAPMA) nanoparticles by dialysis, 2.0 μg; (d) unpurified preparation solution of poly(NIPAM-DMAPMA) nanoparticles diluted by 10-fold-volume of deionized water, 20 μl.

in the binding strength between solutes and the cryogel. A single-site ionic bond between DMAPMA and COO^- of the poly(MAA–PEGDA) column could break in a loading solution with an ionic strength of 10 mM phosphate, whereas a multiple-sites ionic bond between the nanoparticles and the cryogel would break only when the ionic strength of the solution is significantly increased. Therefore, cation nanoparticles can be retarded and separated from the corresponding monomers on the cryogel monolith. The dynamic binding capacity of the resultant nanoparticles was 33.4 μg/ml column volume. Compared with the commonly used dialysis and precipitation methods for purification of nanoparticles, the cryogel column offers a rapid and convenient surrogate. More recently, size-exclusion chromatography (SEC) has been applied to characterize the size distribution of polymeric nanogels [13]. Due to their relatively lower exclusion limits (100 nm), commercial SEC columns are not suitable for separation of particles with diameters of several-hundred nanometers. Monoliths based on glycidyl methacrylate–ethylene dimethacrylate were also investigated for the separation of viruses from suspensions [14] or the adsorption behavior of protein-modified latex particles on the support [15]. With the supermacroporous structure, the presented monolithic cryogel provided a promising alternative to the existing media for purification of nanoparticles from crude product solutions. This is particularly useful in dealing with nanoparticles in the size range from several nanometers to hundreds of nanometers.

4. Conclusion

In this study we demonstrated the potential of a highly crosslinked poly(MAA–PEGDA) supermacroporous cryogel as a medium for separations of proteins and nanoparticles with diameters ranging from several nanometers to hundreds of nanometers. Since the procedure of making the PEGDA-based supermacroporous cryogel is technically simple, the continuous cryogels are very promising alternatives to existing media for nano and microparticles purification from crude particulate materials.

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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.2007.12.084.

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