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Preparation and evaluation of poly(polyethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate) monolith for protein analysis

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Abstract

A poly(polyethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate) monolith was prepared by UV-initiated polymerization. Methanol and ethyl ether were selected as porogens from a variety of organic solvents to achieve the desirable characteristics of the monolith. The preparation of the monolith could be achieved within 10 min. The monolith was macroscopically homogeneous, had low flow resistance, and did not swell or shrink significantly in tetrahydrofuran. Inverse size-exclusion data indicate that the monolith had a total porosity of 75.4% and an internal porosity of 9.1%. The monolith could be used for size-exclusion separation of peptides, although it could not separate proteins with molecular masses between 10 and 100 K due to its unique pore size distribution. It was found to resist adsorption of proteins in capillary liquid chromatography when using 100 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl. Complete recovery of both acidic and basic proteins was achieved. The monolith can be used for applications in which inert materials are required for protein analysis.

Keywords: Monolith; Proteins; Peptides; Biocompatible; Liquid chromatography; Polyethylene glycol acrylate; Capillary columns

1. Introduction

Minimal interaction of support matrix and analytes is often desirable for separations such as gel electrophoresis and size-exclusion chromatography of proteins. Proteins are well known to exhibit hydrophobic and/or ionic interactions with a variety of surfaces. Therefore, an inert material, which can significantly reduce or eliminate adsorption of proteins, would be very useful.

Known materials that resist protein adsorption include polysaccharide and polyacrylamide polymers; these enjoy wide application in gel electrophoresis and size-exclusion separation of proteins [1]. An efficient method to address adsorption problems in capillary electrophoresis is to coat the capillary surface with such polymers [2,3]. In addition to polysaccharide and polyacrylamide, other neutral hydrophilic polymers have been investigated and found useful in capillary electrophoresis, such as polyvinyl alcohol [4], polyethylene oxide [5,6], polyvinylpyrrolidinone [7] and a copolymer of polyethylene glycol and polypropylene glycol [8]. All of these polymers are neutral and hydrophilic. A systematic study of protein adsorption with a variety of surface structures resulted in the conclusion that materials are protein compatible if they are neutral, hydrophilic, proton acceptors and not proton donors [9–11].

Other materials used in gel electrophoresis reported in 1992 by Zewert and Harrington are polyhydroxy methacrylate, polyhydroxy acrylate, polyethylene glycol methacrylate and polyethylene glycol acrylate [12,13]. To avoid the toxicities of acrylamide and bisacrylamide, and the difficulties associated with polyacrylamide gel electrophoresis of very hydrophobic proteins, such as bovine serum albumin or zein, polyethylene glycol methacrylate 200 in hydroorganic solvents was evaluated. Although there was no direct evidence to show the inertness of this material, successful electrophoresis of proteins demonstrated the protein compatibility of such polymers.

The inert polymers mentioned above are polymer gels that are soft in nature. These polymers can only be used in

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their swollen states because such polymers lose their permeabilities upon drying. Attempts have been made to prepare rigid beads with permanent porous structures from such polymers. Among these hydrophilic polymers, polyacrylamide is the only one that can form rigid beads by inverse suspension techniques using a high content of bisacrylamide as a crosslinker [14]. The use of a higher level of crosslinker accounts for the formation of rigid beads instead of soft particles.

Monolithic materials offer an alternative to columns packed with small particles or beads. A monolith (originally called a continuous bed or continuous polymer bed [15]) is a continuous rod with canal-like large through-pores and nanometer-sized pores in the skeletal structure. Preparation of a monolith is typically performed in a mold, such as in a tube or capillary where only one phase of the monomer mixture is used. Two types of monolithic materials have been developed to date. The first type is based on a silica backbone [16,17] in which a continuous sol–gel network can be created by the gelation of a sol solution within a mold. Silica monoliths are mainly used for the separation of small molecules because of their hydrophobic characteristics after derivatization.

The second category includes polymer monoliths [15,18] normally prepared by in situ polymerization of monomer solutions, which are composed of a monomer, crosslinker, porogen and initiator. They can be initiated either by a redox system, e.g., TEMED and APS, or by a free radical initiator. For free radical initiation, both thermally and, more importantly, UV-initiated polymerization can be used. By the use of UV-initiated polymerization, a spatially defined monolith in a capillary or microchip can be prepared using a suitable mask. Furthermore, UV-initiated polymerization is typically much faster than thermally-initiated polymerization.

The first demonstration of a polyacrylamide monolith was performed in 1989 by Hjertén's group [15]. Acrylic acid and *N*,*N*′-methylenebisacrylamide were used as monomer and crosslinker, respectively, to prepare a macroporous gel plug for cation-exchange chromatography of proteins. Favorable chromatographic behavior (i.e., high efficiency at high mobile phase flow rate) was observed although the polymer monolith was compressible.

The preparation of a rigid polyacrylamide-co-bisacrylamide monolith was performed in 1997 by Svec's group [19]. Several variables were studied to prepare a flow-through monolith with a mean pore diameter of ∼1 μm. The porogens used for preparing the acrylamide-co-bisacrylamide monolith were dimethyl sulfoxide and a long-chain alcohol, such as heptanol or dodecanol. The concentration of initiator was also investigated to adjust the medium pore diameter of the monolith; a lower concentration of initiator increased the permeability of the resulting monolith as expected. Unfortunately, thermally-initiated polymerization was used to prepare the monolith. As a result, 24 h was required to complete the polymerization at 1% initiator concentration.

In this work, a protein compatible poly(polyethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate) monolith (PEGMEA/PEGDA) was prepared by photoinitiated polymerization. Physical properties, such as pressure drop and swelling or shrinking in organic solvents, were characterized first, and then inertness in LC was evaluated by using a series of both acidic and basic model proteins under a variety of buffer conditions.

2. Experimental

2.1. Chemicals

Anhydrous methanol, anhydrous ethyl ether and ACS reagent hexanes were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA), Fisher Scientific (Fair Lawn, NJ, USA) and EMD Chemicals (Gibbstown, NJ, USA), respectively. HPLC-grade toluene and THF were from Mallinckrodt Chemicals and Curtin Matheson Scientific (Houston, TX, USA), respectively. All other solvents (cyclohexanol, dodecanol and dimethyl sulfoxide) were of analytical grade or better. Phosphate buffer solutions were prepared with deionized water from a Millipore water purifier (Molsheim, France) and filtered through a 0.22 μm filter. Thiourea (99.9%), 2,2-dimethoxy-2-phenylacetophenone (99%), 3-(trimethoxysilyl)-propyl methacrylate (98%), ethylene dimethacrylate (98%), poly(ethylene glycol) methyl ether acrylate (PEGMEA, average molecular weight, Mn ∼454), and poly(ethylene glycol) diacrylate (PEGDA, Mn ∼575 and ∼258) were supplied by Sigma–Aldrich (Milwaukee, WI, USA) and used without further purification. Proteins [pepsin from porcine stomach mucosa, bovine serum albumin (>99%), myoglobin from horse skeleton muscle, α-chymotrypsinogen A from bovine pancreas, lysozyme from turkey egg white, and bovine serum albumin fluorescein isothiocyanate conjugate (FITC–BSA)] and peptides (neurotensin, angiotensin II fragment 3–8 and leucine enkephalin) were also obtained from Sigma–Aldrich.

2.2. Capillary liquid chromatography

UV transparent fused silica capillary tubing with 75 μm I.D. and 365 μm O.D. was supplied by Polymicro Technologies (Phoenix, AZ, USA). Capillary LC experiments were performed with an ISCO Model 100 DM syringe pump (Lincoln, NE, USA), 60 nl Valco internal sample loop (Houston, TX, USA), a Linear Scientific UVis 203 detector (Reno, NV, USA) and a Thermo Separations PC 1000 V3.0 software work station (Fremont, CA, USA) for data collection and treatment. The PC 1000 provided retention times, peak heights, peak areas, asymmetry factors and column plate counts. On-column UV detection was performed at 214 nm. Chromatograms were transferred to an ASCII file and redrawn using Microsoft Excel (Redmond, WA, USA).

2.3. Preparation of polymer monoliths

Before filling the UV transparent capillary with monomer mixture, the capillary inner surface was treated with 3-(trimethoxysilyl)-propyl methacrylate (commercial identification number Z-6030) to ensure covalent bonding of the monolith to the capillary wall [3,20]. Briefly, the capillary was rinsed sequentially with acetone, water, 0.2 M NaOH, water, 0.2 M HCl, water and acetone using a syringe pump for 30 min each at a flow rate of 5 μl/min. The washed capillary was then dried in an oven at 120 °C for 1 h, filled with a 30% Z-6030 acetone solution, sealed with a rubber septum and placed in the dark for 24 h. The vinylized capillary was then washed with acetone at a flow rate of 5 μl/min for 10 min, dried using a stream of nitrogen for 3 h, and sealed with a rubber septum until used.

Four monolith recipes as indicated in Table 1 were prepared to test protein compatibility. The monomer mixture was prepared in a 1 dram (4 ml) glass vial by admixing in sequence the initiator, monomer, crosslinker and porogens, and ultrasonicating for 5 min before use. Because of the low viscosity of the monomer solution, the introduction of monomer solution into the UV transparent capillary was facilitated by capillary surface tension. The capillary was then placed under a Dymax 5000AS UV curing lamp (Torrington, CT, USA) for 10 min. For measurement of polymerization conversion (see below), a series of irradiation times was used. The UV curing lamp can produce an irradiation intensity of 200 mW/cm^2 in the wavelength range of 320–390 nm.

2.4. Laser-induced fluorescence imaging of FITC–BSA

Laser-induced fluorescence (LIF) imaging of FITC–BSA in a series of capillary columns was performed in a device described elsewhere [21]. Briefly, a 488 nm line from an Ar ion laser was used to excite the sample, and the fluorescence was imaged using a Nikon Coolpix 995 digital camera (Tokyo, Japan).

2.5. Pressure drop measurements

Pressure drop measurements were performed using a Fisons Phoenix 20 CU HPLC pump (Milan, Italy) in the constant flow mode. Methanol and tetrahydrofuran (THF) were pumped through the monolithic column at flow rates of 4, 6, 8 and 10 μl/min, respectively, and the pressure drop for water was measured at 4 μl/min. After stabilizing, the pump pressure was recorded.

2.6. Polymerization conversion evaluation and scanning electron microscopy (SEM)

A bulk solution of 10 g optimized monomer mixture (monolith 4, Table 1) was prepared based on the procedure outlined in Section 2.3. An aliquot of 0.3 g of the monomer mixture was dispensed into a series of 1 dram (4 ml) glass vials and irradiated under the UV lamp for 10 s, 20 s, 30 s, 1 min, 2 min, 5 min, 10 min and 30 min, respectively. The bulk monolith was carefully removed by breaking the glass vial, and it was sliced into sections, Soxhlet extracted with methanol overnight and placed in a vacuum oven at 60 °C overnight. The dried monolith material was weighed and compared with the combined weight of the monomer and crosslinker to obtain the conversion of monomer to polymer.

One of the dry monoliths (i.e., with 10 min irradiation time) was also used to obtain the SEM images. The monolith was sputtered with ∼20 nm gold, and SEM images were taken using an FEI Philips XL30 ESEM FEG (Hillsboro, OR, USA).

2.7. Inverse size-exclusion chromatography (ISEC)

The same liquid chromatographic system as described in Section 2.2 was used for ISEC. The mobile phase was THF and detection was made at 254 nm. Polystyrene standards with narrow molecular weight distributions and average molecular masses of 201, 2460, 6400, 13 200, 19 300, 44 100, 75 700, 151 500, 223 200, 560 900, 1 045 000, 1 571 000 and 1 877 000 were purchased from Scientific Polymer Products (Ontario, NY, USA). Solutions of 1 mg/ml polystyrene and toluene each in THF were prepared.

2.8. Protein recovery determination

A monolithic column with a total length of 80 cm and effective length of 60 cm was prepared with one detection window at 19 cm and the other at 60 cm from the column inlet. The detection window at 19 cm was created by carefully introducing an air bubble during introduction of the monomer solution. A mixture of protein and thiourea (an internal standard to calibrate any detection window re-

Table 1
Composition of reagent solution for various monoliths used in this study[a,b]

No.	DMPA	PEGMEA	EDMA	PEGDA	Ethyl ether	Other
1	0.008	0.32	0.48	–	–	0.38 Cyclohexanol + 0.58 dodecanol + 0.24 hexanes
2	0.008	–	0.8	–	1.20	–
3	0.006	–	–	0.6	1.40	–
4	0.006	0.15	–	0.45	1.10	0.30 Methanol

[a] Units are in g.
[b] Recipes for monoliths 1 and 4 were optimized.

sponse variation due to different background absorbances of the two detection windows) was injected into the monolithic column. Protein recovery was calculated by comparison of the calibrated protein peak area from the second detection window with that from the first one. The calibrated peak area of a protein was obtained by dividing the protein peak area by that of thiourea from the same detection window.

3. Results and discussion

3.1. Crosslinker influence on inertness of the monolith

Initially, ethylene dimethacrylate (EDMA) was chosen as a crosslinker to prepare the PEGMEA monolith because EDMA has been widely used in the preparation of rigid porous polymer monoliths, such as butyl methacrylate, glycidyl methacrylate and hydroxylethyl methacrylate [22]. However, the resultant monolith (monolith 1, Table 1) exhibited strong adsorption of FITC–BSA as shown in the LIF images (see Fig. 1A). To investigate the cause of adsorption of BSA in the poly(PEGMEA-co-EDMA) monolith, monolith 2 composed of pure EDMA was prepared with ethyl ether as porogen. Not surprisingly, the EDMA monolith had a strong fluorescence residue after introducing FITC–BSA and flushing with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl buffer (Fig. 1B). Because polyethylene glycol is known not to adsorb proteins, PEGDA was chosen as a crosslinker for the preparation of the PEGMEA monolith. Results of the use of PEGDA with Mn ∼575 as crosslinker showed that the PEGMEA/PEGDA monolith did resist the adsorption of proteins (data not shown). Unfortunately, the resultant monolith was compressible upon application of >1000 psi buffer even though 75% crosslinker was used in the monomer recipe. This indicates that the PEGMEA monolith with long-chain PEGDA crosslinker yielded a soft monolith. However, replacement of PEGDA Mn ∼575 with PEGDA Mn ∼258 dramatically improved the rigidity of the monolith. From the fluorescence images (Fig. 1C) of this new polymer monolith 3, no obvious adsorption of FITC–BSA was observed. Therefore, PEGDA Mn ∼258 was finally selected as the crosslinker to prepare the PEGMEA/PEGDA monolith (monolith 4, Table 1). A fluorescence test of the optimized PEGMEA/PEGDA monolith also showed no adsorption of FITC–BSA (see Fig. 1D).

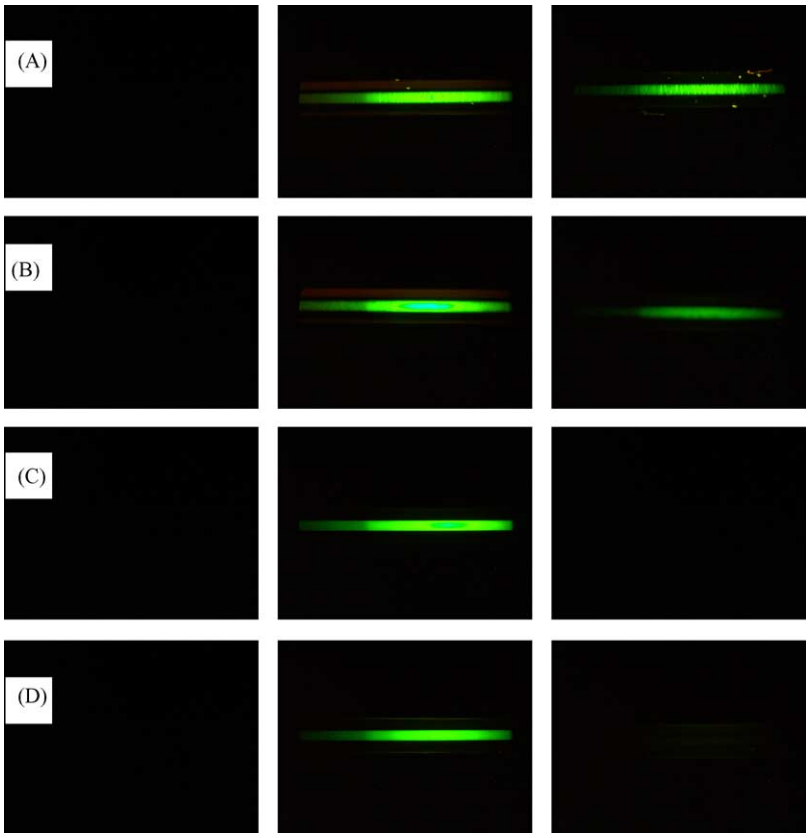

Fig. 1. LIF images of the monolith before, during and after loading of FITC–BSA. The LIF image was first recorded before loading of FITC–BSA for which a dark background was obtained for all monoliths. The monolithic column was loaded with 0.01 mg/ml FITC–BSA and the fluorescence image was taken. The monolithic column was then flushed with 100 mM (pH 7.0) phosphate buffer containing 0.5 M NaCl for 5 min under a linear flow velocity of ∼4 mm/s, and the LIF image was obtained again. (A) PEGMEA/EDMA monolith; (B) EDMA monolith; (C) PEGDA Mn ∼258 monolith; (D) PEGMEA/PEGDA monolith. The monomer recipes for all of the monoliths are listed in Table 1.

3.2. Optimization of porogen composition

To be useful in flow-through applications, the monolith must have low flow resistance. Furthermore, for chromatographic use, a homogeneous monolith is critical for achieving high efficiency. Here, homogeneity refers to the uniformity of the monolithic bed along both radial and axial directions. Because polymer monoliths are made of tiny globules which are connected together to form a continuous rod, they are microscopically heterogeneous. Thus, homogeneity in this study refers to the uniformity of the monolithic bed macroscopically. If the monolith was free of voids or cracks and its color was uniform upon examination under a microscope, the monolith was considered to be homogeneous. Therefore, optimization involved preparing a homogeneous monolith with as low flow resistance as possible.

Five factors can be adjusted to change the pressure drop of the polymer monolith: initiator concentration, total monomer to total porogen ratio, monomer to crosslinker ratio, porogen types and ratio between porogens. Although a decrease in initiator can decrease the pressure drop of the monolith, a longer time is required to complete the polymerization. A decrease in total monomer to total porogen ratio is a straightforward method to decrease the pressure drop of the monolith, however, it decreases the homogeneity and rigidity of the monolith as well. A change in monomer to crosslinker ratio can have an effect on the pressure drop of the resulting monolith, although it also changes the rigidity and homogeneity of the monolith. The most powerful factors to engineer the pressure drop of the monolith are the selection of porogen types and the ratio between porogens, because they do not affect the rigidity of the monolith.

For the preparation of the PEGMEA/PEGDA monolith, when ethyl ether was used as porogen, the crosslinker had to be greater than 70% to make a rigid monolith. As a result, 75% PEGDA (crosslinker) and 25% PEGMEA (monomer) were used throughout the optimization of the monolith. The total monomer to porogen ratio was kept constant at 3:7 and the initiator concentration was 1% of the monomers. A variety of solvents were evaluated to prepare the PEGMEA/PEGDA monolith. First, 30% PEGMEA or PEGDA solutions (containing 1% photo-initiator, 2,2-dimethoxy-2-phenylacetophenone (DMPA)) in ethyl ether, hexanes, cyclohexanol, dodecanol, dimethyl sulfoxide, methanol, toluene or THF were prepared and placed under the UV lamp to find the potential porogens for the PEGMEA/PEGDA monolith. PEGMEA and PEGDA both dissolved well in all solvents except hexanes. For PEGMEA, dodecanol formed a white solid material, and dimethyl sulfoxide resulted in a transparent soft gel. All other solvents formed a dense liquid after 10 min UV irradiation. For PEGDA, dimethyl sulfoxide and THF resulted in transparent solid materials, which indicate the formation of an extremely small pore structure. All other solvents yielded a white solid, except toluene which formed a yellow rigid solid.

A 2 cm long monolith prepared in a UV transparent capillary was used to test the pressure drop of the monolith composed of only PEGDA. Ethyl ether and methanol porogens yielded a porous monolith, whereas all others would not allow flow at 4500 psi methanol. This is also in contrast to other reported monoliths for which a long-chain alcohol, such as cyclohexanol or dodecanol, was used to prepare a porous monolith [18,19,23]. Therefore, methanol and ethyl ether were selected as porogens to optimize the preparation of the PEGMEA/PEGDA monolith. Since both PEGMEA and PEGDA do not dissolve in hexanes, and both dissolve in mixtures of hexanes and methanol or ethyl ether, hexanes was selected as a macroporogen for the monolith. Thus, the final porogens selected were methanol, ethyl ether and hexanes.

Three porogen mixtures, i.e., methanol/hexanes, ethyl ether/hexanes and methanol/ethyl ether, were optimized for the desired homogeneity and flow resistance of the monolith. The pressure drop of the monolith was found to be insensitive to the ratio of methanol and hexanes or ethyl ether and hexanes. Fortunately, the flow resistance of the monolith was found to be strongly dependent on the ratio of methanol and ethyl ether (see Fig. 2A). For the optimized recipe (monolith 4), i.e., 7.5% PEGMEA, 22.5% PEGDA, 15% methanol and 55% ethyl ether, the pressure drop was 21 psi/(cm μl/min) when methanol was used as pumping liquid in a 75 μm I.D. monolithic capillary. For a 20 cm × 75 μm I.D. capil-

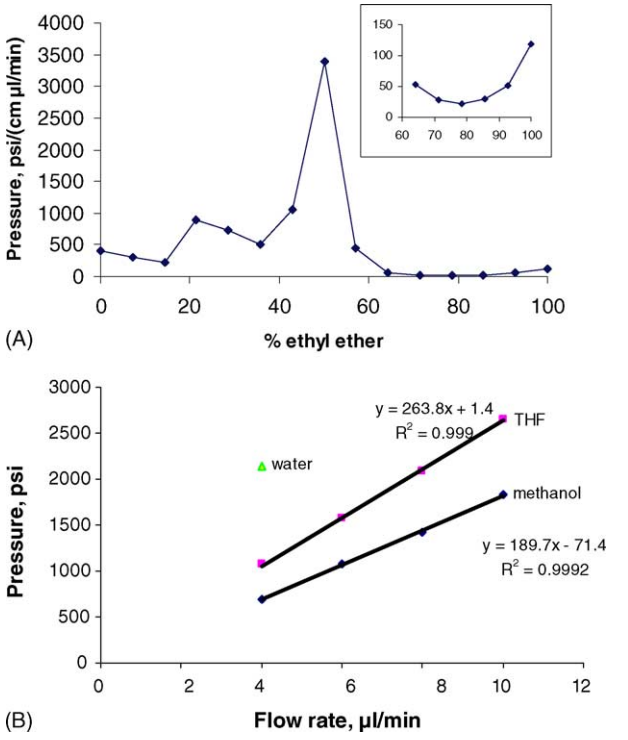

Fig. 2. Flow resistance of the PEGMEA/PEGDA monolith. (A) Pressure drop dependence of the monolith on the percent of ethyl ether. Inset is the magnification of the section for ethyl ether of 60–100%. (B) Linear pressure dependence of the optimized PEGMEA/PEGDA monolith on the flow rates of water, THF and methanol.

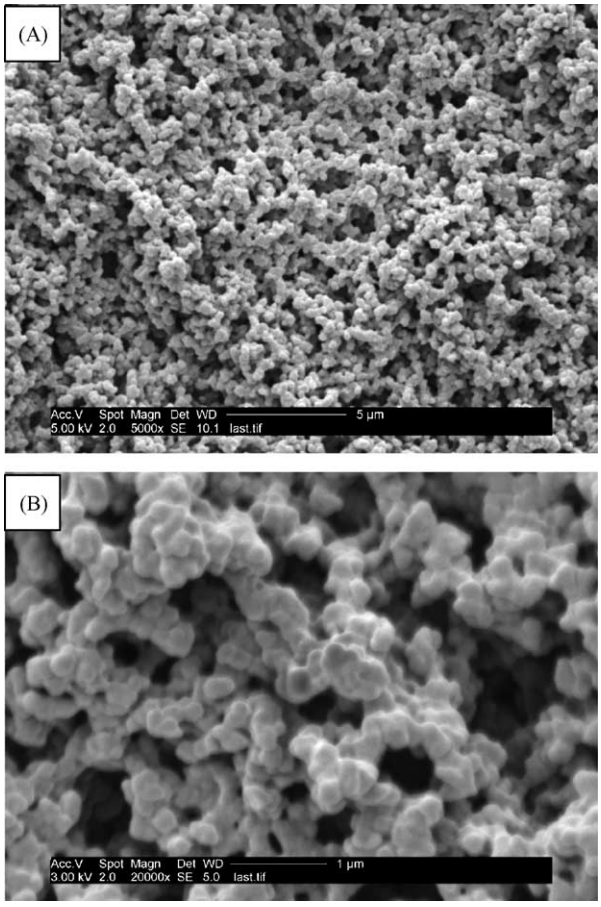

Fig. 3. SEM images of the optimized PEGMEA/PEGDA monolith: (A) 5000× magnification; (B) 20 000× magnification. From image (B), it is clearly seen that the polymer monolith is composed of microglobules interconnected to form clusters that form the skeleton of the monolith. Between clusters are through-pores, which determine the permeability of the monolith.

lary, this corresponds to a linear flow velocity of 3.78 mm/s of methanol at a pressure of 420 psi.

SEM images of the optimized PEGMEA/PEGDA monolith are shown in Fig. 3. From the images, a rough estimation of 0.2–0.3 μm diameter globule size could be made. If these globules were tightly packed as in a packed column, the pressure drop would be tremendously high. Therefore, the low flow resistance of 21 psi/(cm μl/min) was due to the large through-pores or high porosity of the monolith. It may also have been a result of a high degree of connectivity of the through-pores, which has been shown to be an important factor affecting the permeability of a monolith in theoretical studies [24,25]. The shrinking of the monolith in methanol (vide infra), could also lead to low flow resistance.

3.3. Kinetics of polymerization of PEGMEA/PEGDA

Both thermal and UV-initiated polymerization can be used to prepare polymer monoliths. Typically, thermally-initiated polymerization uses AIBN as initiator, and polymerization proceeds slowly, normally taking 24 h [18,19]. In contrast,

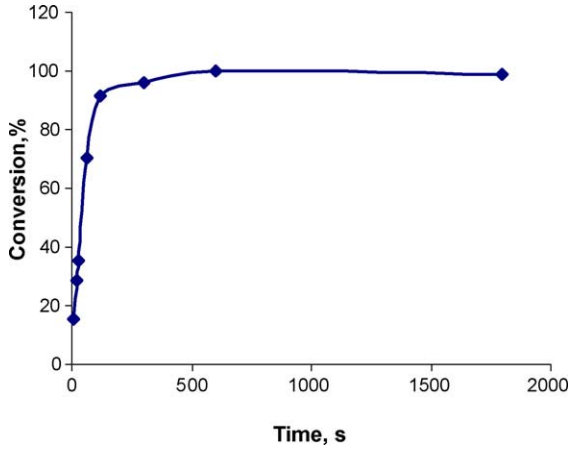

Fig. 4. Rate of conversion of monomers to polymer. For experimental conditions, see Section 2.6.

photo-initiated polymerization can be finished in minutes [23]. The kinetics of polymerization of PEGMEA/PEGDA is shown in Fig. 4. Over 90% of the monomer was converted into polymer in 2 min, and complete conversion of the monomer was finished in ∼10 min. The high irradiation intensity (200 mW/cm^2) used in our experiments, which is ∼10-fold greater than a previously reported UV curing system [23], contributed to the fast polymerization of the monomer solution.

3.4. Physical properties of the PEGMEA/PEGDA monolith

A quantitative index, the swelling propensity (SP), was defined by Nevejans and Verzele [26] to characterize the swelling and shrinking properties of a packed bed:

$$\mathrm{SP} = \frac{p(\mathrm{solvent}) - p(\mathrm{H_2O})}{p(\mathrm{H_2O})}$$

where p takes into account the viscosities of the solvent, and is defined as the ratio of pressure over solvent viscosity. By definition, SP = 0 if no swelling or shrinking occurs, SP > 0 if there is swelling, and SP < 0 if the packed bed shrinks. From Fig. 2, the SP values for methanol and THF were calculated to be −0.44 and −0.08, respectively, assuming viscosities for water, methanol and THF of 1.025, 0.59 and 0.55 cP, respectively, at room temperature (data from the online CRC Handbook at 25 °C). This indicates that no significant shrinking or swelling of the PEGDA/PEGMEA monolith in THF was observed. Since THF can dissolve most hydrophobic polymers, the stability of the monolith in THF indicates that the monolith is relatively non-hydrophobic. However, shrinking of the monolith did occur in methanol, which unexpectedly had a positive effect because it improved the column permeability while maintaining a rigid structure. As shown in Fig. 2, when 2600 psi THF was applied to the monolithic column (4 cm × 75 μm I.D.), no change in pressure drop was observed. This indicates high stability of the monolith, which

is a result of the high concentration of crosslinker used in the monomer recipe.

3.5. Chromatographic evaluation of the monolith

Proteins were carefully selected to investigate the possibility of hydrophobic or ionic interaction with the monolithic material. Acidic (pepsin), basic (lysozyme) and hydrophobic (BSA) proteins were included. Several peptides with different molecular masses were also used to explore the elution mechanism of the monolithic column. Table 2 lists the molecular masses and p*I* values of the proteins and peptides used in this study.

Phosphate buffers (a) pH 7.0 with concentrations of 10, 20, 50, 100, 200 and 500 mM; (b) 10 mM concentration with pH values of 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0; and (c) 100 mM concentration (pH 7.0) with additives of 0.5 M Na_2SO_4, 0.5 M NaCl, 10% ethylene glycol or 10% acetonitrile were used to elute the proteins. Buffers (a) and (c) were used to explore the possible hydrophobic interaction of the proteins with the monolith, and buffer (b) was used to investigate the possibility of any ionic interactions. In all cases, the proteins eluted earlier than thiourea. This indicates a SEC elution mechanism.

When buffer (a) was used, splitting of all of the protein peaks was observed when the buffer concentration was increased to 500 mM. However, the elution time was kept nearly constant for the proteins investigated within experimental error (except for the 500 mM buffer, because two retention times were obtained due to splitting of the peaks). For buffer (c), 0.5 M Na_2SO_4 in 100 mM (pH 7.0) also caused splitting of the protein peak. This indicates possible hydrophobic interaction of the proteins with the monolith. However, 10% ethylene glycol or even 10% acetonitrile (α-chymotrypsinogen A formed a precipitate in the buffer with acetonitrile as an additive and, thus, could not be chromatographed) in buffer (c) provided elution of proteins in a similar way as 0.5 M NaCl additive. Not only were protein profiles similar to each other when buffer (c) was used, but the elution times were also close to each other. This strongly suggests that hydrophobic interaction, if any, would not be very significant.

The pH of buffer (b) was found to strongly affect the protein peak profiles. At pH 2.0, all proteins showed some degree of tailing, and α-chymotrypsinogen A and lysozyme exhibited peak splitting. Above pH 4.0, the symmetry of the protein peaks improved, except that lysozyme split into two peaks at all pH values. This indicates a possible ionic interaction between lysozyme and the monolith. However, as shown above, this weak ionic interaction disappeared when buffer (c) with 0.5 M NaCl additive (weak buffer ionic strength) was used.

In summary, good peak symmetries for all of the proteins were obtained with the use of buffer (c) with 0.5 M NaCl additive, i.e., 100 mM phosphate (pH 7.0) buffer containing 0.5 M NaCl, a condition often employed in high performance SEC of proteins. This indicates that the PEGMEA/PEGDA monolith had insignificant hydrophobic or ionic interactions with the proteins. It should be mentioned that all of the experiments described above employed high mobile phase flow rate (∼1.10 mm/s) so that proteins eluted within ∼3 min from a ∼20 cm monolithic column. Such a flow rate facilitates the screening of buffers at the expense of skewing protein peaks. If a lower flow rate was used, improvement in peak symmetry could be achieved.

Fig. 5A shows a chromatogram of a mixture of proteins and thiourea using low mobile phase flow rate. No separation between these proteins was observed. Injections of each protein under the same chromatographic conditions revealed that all five proteins with different molecular masses and p*I* values had almost the same elution time. In contrast, for the three peptides, a moderate separation was achieved, although they were not baseline resolved (see Fig. 5B). A mixture of α-chymotrypsinogen A, the three peptides and thiourea was also injected into the column, and the chromatogram is shown in Fig. 5C. Although the elution time for the protein was a little earlier than neurotensin (compare Fig. 5A and B), coelution of α-chymotrypsinogen A and neurotensin was observed. Since we aimed to develop an inert, homogeneous monolith with pressure drop as low as possible, no further optimization of pore size distribution was attempted for SEC of proteins.

It should be mentioned that the peak shown in Fig. 5A was a coelution profile of five proteins and thus, it was relatively broad. Chromatography of each of the five proteins revealed a column efficiency of 6000–8000 plates/m and an asymmetric factor of 1.3–1.5 for a single protein. For peptides and thiourea, elution of each of them separately resulted in column plate counts of 9000–20 000 plates/m and an asymmetric factor of <1.1. This roughly follows the trend of SEC, in which significantly lower plate counts for proteins than for small molecules have been observed due to the lower diffusion coefficients of the macromolecules. Typical plate counts in modern SEC (column dimensions of 250 mm × 4.6 mm I.D.) ranged from 8000 plates/m for proteins (i.e., amylase) to 34 000 plates/m for small molecules (i.e., glycyl tyrosine) [27]. For example, a plate count in SEC for α-chymotrypsinogen A was estimated to be ∼5600 plates/m

Table 2
Proteins and peptides used in this study

Analyte	Molecular mass	p*I*
Bovine serum albumin[a]	68000	4.7
Pepsin[a]	34000	<1
α-Chymotrypsinogen A[a]	24000	8.8
Myoglobin[a]	17500	7.1
Lysozyme[a]	14000	11.0
Neurotensin[b]	1672.9	9.5
Angiotensin II fragment 3–8[b]	774.9	7.8
Leucine enkephalin[b]	555.6	5.9

[a] The molecular masses and isoelectric point p*I* values of proteins were obtained from [32].

[b] The molecular masses of peptides were read from the labels of the chemicals provided by Sigma–Aldrich, and the p*I* values were obtained from the EMBL Heidelberg European Molecular Biology Laboratory Program http://www.embl-heidelberg.de/cgi/pi-wrapper.pl).

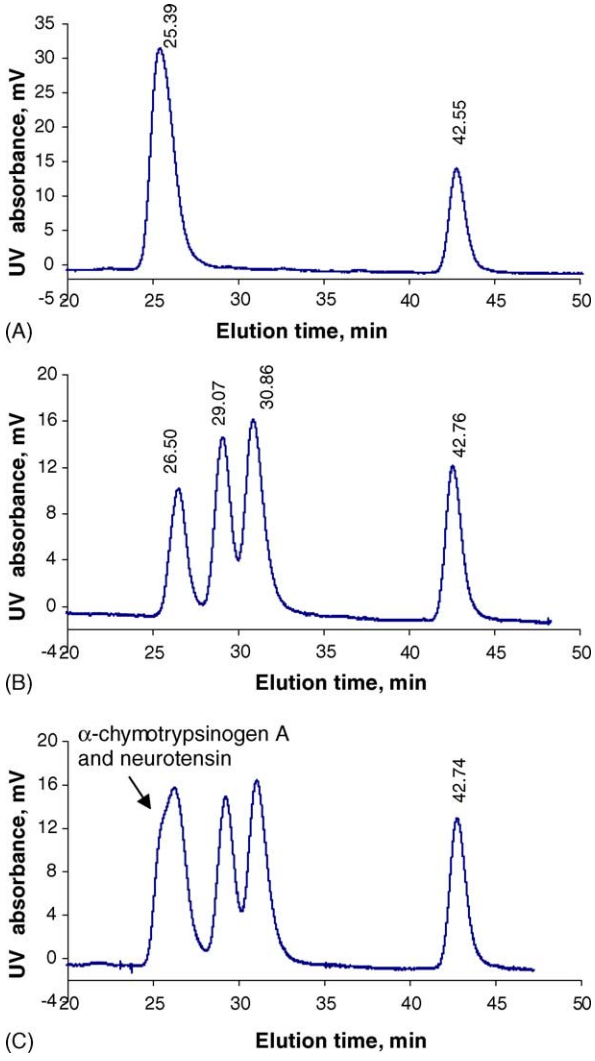

Fig. 5. Chromatograms of mixtures of several peptides, proteins and thiourea under isocratic elution conditions. The mobile phase was 100 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl, operated at a constant pressure of 600 psi (accurate flow rate was not measured). The stationary phase was 75 μm I.D., 60 cm effective length of PEGMEA/PEGDA monolith. Concentrations were thiourea, 0.15 mg/ml, proteins, 0.8 mg/ml each, and peptides, 0.5 mg/ml each. (A) Mixture of bovine serum albumin, pepsin, α-chymotrypsinogen A, myoglobin, lysozyme and thiourea; (B) mixture of neurotensin, angiotensin II fragment 3–8, leucine enkephalin and thiourea (in elution order); (C) mixture of α-chymotrypsinogen A, neurotensin, angiotensin II fragment 3–8, leucine enkephalin and thiourea. For physical properties of the proteins and peptides, see Table 2.

based on a previously published chromatogram [28]. Thus, the plate counts achieved for proteins in this study with the use of the polymer monolith is acceptable. Furthermore, plate counts of 2240–6400 plates/m were reported for monolithic SEC of polystyrenes in THF [29].

3.6. ISEC characterization of the PEGMEA/PEGDA monolith

To further understand the separations of proteins and peptides shown in Fig. 5, the porosity and pore size distribution of the PEGMEA/PEGDA monolith were investigated by ISEC. ISEC was originally used to characterize the structure of a packed bed with known probe compounds, e.g., polystyrene standards with narrow molecular mass distribution [30]. Guiochon and co-workers were among the first to use ISEC to characterize the porous structure of silica monoliths [31]. They defined several terms to describe the structure of a monolithic bed, such as total porosity (ε_t), external porosity (ε_e) and internal porosity (ε_i). Based on ISEC, a pore size distribution of a monolith could also be derived assuming a simple correlation of $Mw = 2.25(10d)^{1.7}$, where Mw is the molecular mass of the polystyrene standard and d is the diameter of the polystyrene standard in nm. Following the method of Guiochon and co-workers [31], we obtained an ISEC plot for the PEGMEA/PEGDA monolith, which is shown in Fig. 6A. The retention volumes, shown in Fig. 6 were the corrected retention volumes, taking into account the extracolumn volume of the chromatographic system, which

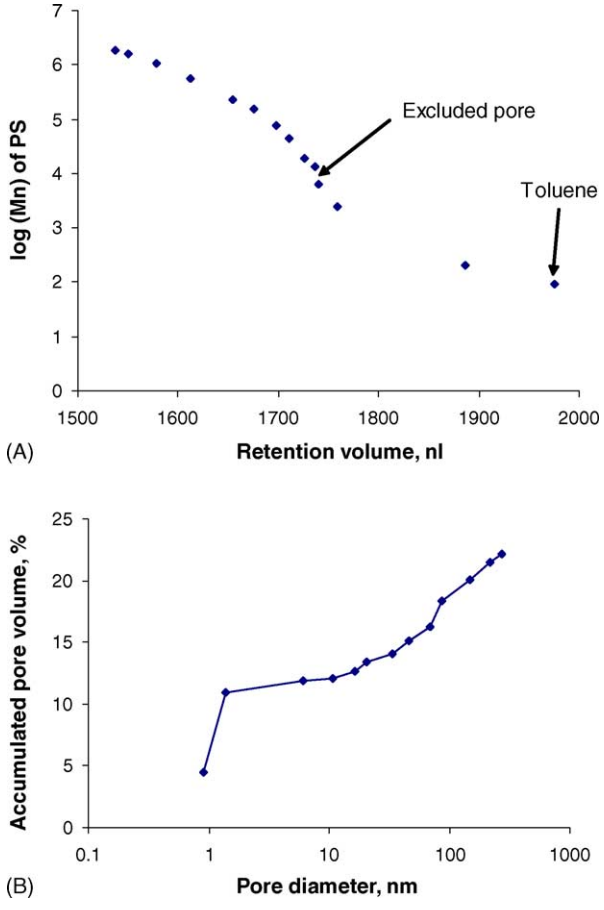

Fig. 6. ISEC plot (A) and accumulated pore size distribution (B) for the PEGMEA/PEGDA monolithic column. THF was used as mobile phase under a constant pressure of 1500 psi, and the mobile phase flow rate was measured to be 0.45 μl/min by monitoring the movement of liquid meniscus in the capillary. A 75 μm I.D., 59.3 cm long monolithic column with online detection at 254 nm was used. In panel (A), toluene (Mn 92) was used as a small molecule to determine the total porosity of the column. The exclusion pore volume was approximately the intersection point of the interpolated straight lines corresponding to the internal and external pore zones.

was measured to be 248 nl, including the 60 nl internal sample loop. From Fig. 6A, the total porosity was calculated to be 75.4%, which is in agreement with the percent of porogen content in the monomer recipe (monolith 4 in Table 1, 70% porogen used). The excluded molecular mass was estimated to be 10^4, which corresponds to 14 nm. The external porosity was thus calculated to be 66.3% and the internal porosity was 9.1%. The relatively large total porosity (75.4%) accounts for the low flow resistance of the monolithic column.

The accumulated pore size distribution curve was derived from the ISEC calibration curve, and is shown in Fig. 6B. The pore volume fraction corresponding to pores larger than 304 nm was 77.8% (not drawn in the figure), and 7.0% for pores between 50 and 304 nm. The pore volume fraction for micropores (<2 nm) was 10.9%, and only 4.2% for mesopores (2–50 nm). It can be seen that most of the pore volume fraction came from pores larger than 304 nm. The mesopore volume fraction was very small (4.2%), and the pore volume fraction in the range of 1.4–10.8 nm was only 1.1%. Since the Stokes' radii for proteins in the molecular mass range of 10–70 K are between 1.5 and 3.6 nm (data from http://itsa.ucsf.edu/~hdeacon/Stokesradius.html), the monolith would predict no separation of the proteins used in this study. This explains the coelution of the proteins shown in Fig. 5A. In contrast, the pore volume fraction of micropores was relatively large (10.9%), and the curve (Fig. 6B) in this pore size range was sharp. These two characteristics explain the separation of peptides (Fig. 5B). Although the molecular mass difference between proteins and peptides was large, the difference between the pore volumes which excluded proteins and peptides was small, as can be seen in Fig. 6B. This unique pore size distribution of the monolith explains why α-chymotrypsinogen A coeluted with neurotensin (Fig. 5C).

In summary, the PEGMEA/PEGDA column shows SEC elution of peptides and proteins. The larger the molecule, the earlier the elution. However, due to the small pore volume fraction in the mesopores range of the monolith, separation between proteins could not be achieved using such monolithic columns.

3.7. Protein recovery evaluation

To further evaluate the protein adsorption properties of the PEGMEA/PEGDA monolith, a protein recovery experiment was performed. In conventional HPLC, the peak areas of a compound eluted from a packed column and stainless steel tubing were compared [28,32]. Because a strong dependence of peak area on mobile phase flow rate was observed in our capillary liquid chromatographic experiments, a direct comparison of the protein peak areas from monolithic and open tubular fused silica capillaries would not provide reliable data for calculating protein recovery. In contrast, the two detector method [33] or modified two detection window method [34,35] in capillary electrophoresis would be applicable for measuring protein recovery in the capillary format because peak areas are measured in one run and variations in detector or detection window responses are taken into account.

In our work, the two detection window method was used to perform recovery experiments. Thiourea was used as an internal standard to calibrate the detection window response variation. The recoveries for pepsin, BSA, myoglobin, α-chymotrypsinogen A, and lysozyme were 98.0, 99.6, 103.5, 99.2 and 98.7%, respectively. This provides direct evidence that the PEGMEA/PEGDA monolith does not adsorb any significant amount of proteins under the conditions of 100 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl.

4. Conclusions

A non-adsorptive monolith for proteins, PEGMEA/PEGDA, was prepared using methanol and ethyl ether as porogens. Complete conversion of the monomer to the polymer monolith could be finished in 10 min. The polymer monolith had very low flow resistance, and was macroscopically homogeneous. Protein recovery approached 100% if 100 mM phosphate (pH 7.0) buffer containing 0.5 M NaCl was used as mobile phase. No significant ionic or hydrophobic interactions with proteins were found.

Another feature of this monolith is that it did not discriminate the elution of several proteins (molecular weight from 14 to 67 K) studied. Together with the homogeneity and low flow resistance characteristics, the monolith would be very useful in situations requiring an inert material for protein analysis, such as in flow counteracting capillary electrophoresis [36,37] or electric field gradient focusing [21], in which the required hydrodynamic flow produces band broadening. By incorporating an inert material in the separation channel, sharpening of the protein bands is expected while maintaining the original separation/focusing mechanism. Currently, the incorporation of such a monolith into the separation/focusing channels of electric field gradient focusing devices [21] is under investigation. For SEC of proteins using this monolith, a reduction in through-pore diameter and optimization of the pore volume in the mesopore range must be accomplished. Unfortunately, this would be accompanied by a concomitant increase in flow resistance of the monolith.

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