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Size-exclusion separation of proteins using a biocompatible polymeric monolithic capillary column with mesoporosity

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ABSTRACT

Biocompatible poly(ethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate) monoliths were prepared for size exclusion chromatography (SEC) of proteins in the capillary format using Brij 58P in a mixture of hexanes and dodecanol as porogens. The monolithic columns provided size separation of four proteins in 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl, and there was a linear relationship between the retention times and the logarithmic values of the molecular weights. Compared to SEC monoliths previously synthesized using a triblock copolymer of polyethylene oxide and polypropylene oxide, an increase in mesoporosity was confirmed by inverse size exclusion chromatography. As a result, improved protein separation in the high molecular weight range and reduced column back-pressure were observed.

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1. Introduction

Size exclusion chromatography (SEC) is an important technique for the separation and purification of proteins, for which it would be ideal if no interaction between the proteins and the stationary phase occurred [1,2]. Conventional SEC media are hydrophilic polyacrylamide, agarose, dextran or silica/polymer beads, and mobile phases are usually aqueous buffers with pH values close to physiological conditions. Thus, degradation of proteins and consequent loss of biological activity can be avoided. This characteristic of a chromatographic stationary phase has been referred to as biocompatibility in a recent review [3].

The monolithic column format is a good alternative to typical spherical particle packed columns for capillary LC with regard to ease of preparation and reduced flow resistance [4,5]. Polymer-based monoliths, in particular, have covered the most popular biomolecule separation modes, such as reversed-phase [6,7], ion-exchange [8,9], affinity [10,11], hydrophobic interaction [12,13], and hydrophilic interaction [14,15] as a result of the availability of a wide variety of functional monomers and biocompatible skeletal structures. However, polymer monoliths are rarely used for SEC because they typically have low percentages of mesopores in their pore size distributions [16–18]. SEC requires the monolith to have the right mesopore pore size distribution and large pore volume to achieve high separation selectivity and resolution.

Polyethylene glycol (PEG) is a mildly hydrophilic and biocompatible polymer. Copolymerization of the diacrylate monomer containing PEG units has resulted in porous monoliths that resist non-specific adsorption of proteins, and they have exhibited size exclusion properties of small peptides and proteins [19,20]. In contrast to conventional porogens, the nonionic surfactant, poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) created more mesopores for separating proteins by SEC [20]. The pore size could be tuned to some extent by varying the type and concentration of surfactant. Despite these promising results, our initial use of high MW porogenic solvents led to porous polymers with relatively limited resolution and high flow impedance.

Brij is another polyoxyethylene surfactant that has been used in developing polyacrylamide media with appropriate pore size dis-

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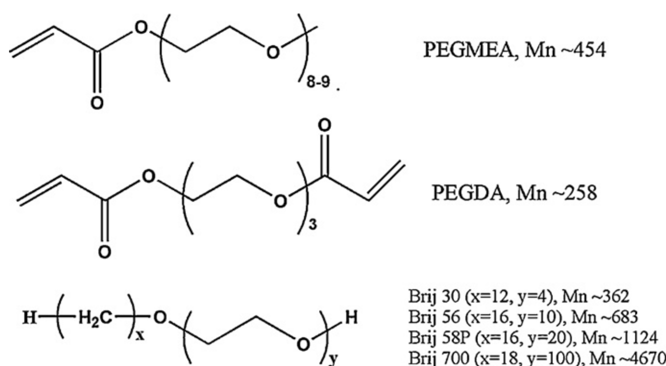

Fig. 1. Molecular structures of PEG monomers and Brij porogens used for synthesizing the monoliths.

tributions for globular protein separation [21]. In this study, a new poly(polyethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate) monolith that was more suitable for SEC of proteins was developed using Brij 58P as the mesoporogen. The new monolith demonstrated improved resolution of proteins and increased permeability compared to our previous work [20].

2. Experimental

2.1. Chemicals

2,2-Dimethoxy-2-phenylacetophenone (DMPA, 99%), polyethylene glycol methyl ether acrylate (PEGMEA, Mn ~454), polyethylene glycol diacrylate (PEGDA, Mn ~258), 3-(trimethoxysilyl)propyl methacrylate (TMSPMA, 98%), Brij 30 (Mn ~362), Brij 56 (Mn ~683), Brij 58P (Mn ~1124), Brij 700 (Mn ~4670), 1-dodecanol, hexanes, uracil (U) and HPLC water (CHROMASOLV®) were purchased from Sigma–Aldrich (Milwaukee, WI). Fig. 1 shows the molecular structures of the PEG monomers and Brij porogens. The reported Mns of these compounds are related to the lengths of the PEG chains. All of the monomers were used without further purification. Phosphate buffer solutions were prepared with HPLC water and filtered through a 0.22-μm membrane filter. All proteins and peptides were purchased from Sigma–Aldrich, including thyroglobulin (TG, MW = 670,000), catalase (CAT, MW = 250,000), bovine serum albumin (BSA, MW = 66,000), soybean trypsin inhibitor (STI, MW = 20,100), and leucine enkephalin (LE, MW = 555).

2.2. Preparation of polymer monoliths

Fused silica capillaries (150 μm i.d.) clad with UV-transparent fluorinated hydrocarbon polymer (Polymicro Technologies, Phoenix, AZ) were silanized using TMSPMA in order to anchor the polymer monolith to the capillary wall as described previously [8]. The polymer precursor was prepared by dissolving DMPA in a solution containing PEGMEA and PEGDA (1:3 weight ratio) and porogen, consisting of a ternary mixture of varying amounts of Brij, 1-dodecanol and hexanes. After sonication for ~10 min, the polymer precursor was heated in a GC oven for several min at 50 °C until a clear solution was observed. The mixture was not purged with an inert gas in our experiments because high MW Brij precipitates easily when purged with N_2, and UV-initiated polymerization is so efficient that the presence of trace oxygen does not interfere. Subsequently, the reaction mixture was quickly introduced into the silanized capillary by vacuum and irradiated for 6 min using a PRX 1000-20 Exposure Unit (TAMARACK Scientific, Corona, CA). During the irradiation, a heater was placed under the capillary and was set to maintain a constant temperature of ~50 °C around the capillary to ensure that the Brij 58P remained dissolved. After polymerizations were completed, the monolithic columns were connected to an LC pump and extensively rinsed with methanol to remove porogenic solvents and unreacted reagents. During rinsing, the monolithic columns were placed in a stirred water bath (~50 °C) in order to increase the solubility of Brij in the rinse solvent.

2.3. Analysis

An Eksigent Nano 2D LC system (Dublin, CA) with a K-2600 UV detector (Sonntek, Upper Saddle River, NJ) and a 3-nL detection cell from LC Packings/Dionex (Sunnyvale, CA) were used to conduct all chromatographic experiments. Standard proteins or peptides dissolved in 20 mM phosphate (pH 7.0) were used to evaluate the monolithic columns. Ultraviolet absorbance detection was carried out at 214 nm. ISEC and SEC calibration curves were determined for the monoliths using protein and peptide standards (i.e., TG, CAT, BSA, STI, LE) and uracil, with a mobile phase consisting of 20 mM phosphate buffer containing 0.15 M NaCl. To investigate the permeability and rigidity of these monolithic columns, pressure drop measurements were made at room temperature (~23 °C) using pure water as the permeating fluid at flow rates ranging from 50 to 300 nL/min.

For selected capillary columns, a section containing monolithic polymer was cut and placed on a sticky carbon foil, which was attached to a standard aluminum specimen stub for characterization by scanning electron microscopy (SEM, FEI Philips XL30 ESEM FEG, Hillsboro, OR). The surface areas of prepared bulk monoliths were measured using a Quantachrome NOVA 4000 analyzer (Boynton Beach, FL) based on nitrogen adsorption BET measurements.

3. Results and discussion

3.1. Preparation and characterization of the SEC monoliths

Our previous work demonstrated the generation of mesopores within poly(PEGMEA-co-PEGDA) monoliths for size separation of proteins using high MW copolymers as porogens. However, the size-exclusion selectivity for proteins was not high, and a relatively high proportion of mesopores resulted in decreased permeability of the monolithic bed. Therefore, we evaluated another option, the use of Brij surfactant as porogenic solvent. We found that Brij 58P with MW 1124 in a mixture with a long chain alcohol (such as 1-dodecanol) and hexanes as the porogen system resulted in a good monolith. The use of lower MW Brij (i.e., 362 and 683) usually resulted in transparent or translucent monolithic structures that did not allow flow-through, and Brij with higher MW of 4670 was difficult to dissolve in most organic solvents.

The flow resistance of monolithic columns is conveniently characterized by the column permeability, K, using Darcy's equation [22]. Polymer monoliths having a large proportion of small pores always exhibit excessively low permeabilities due to the typical monomodal pore size distribution. Hence, permeability data can provide useful information relating to pore structure. As shown in Table 1, the polymerization mixtures contained 0.006 g DMPA, 0.6 g monomers (PEGMEA and PEGDA, 0.15:0.45) and 1.4 g porogenic solvent mixture (Brij 58P, 1-dodecanol and hexanes). To investigate the influence of porogen composition on permeability, the weight fraction of one porogen was kept constant, while the weight fractions of the other two porogens were varied. The data for M1–M3 showed that an increase in ratio of hexanes to dodecanol (0.3:0.8, 0.6:0.5, and 0.7:0.4, w/w) while keeping the Brij concentration constant increased the permeability. However, an increase in ratio of Brij to hexanes (M3–M5; 0.3:0.7, 0.5:0.5, and 0.7:0.3, respec-

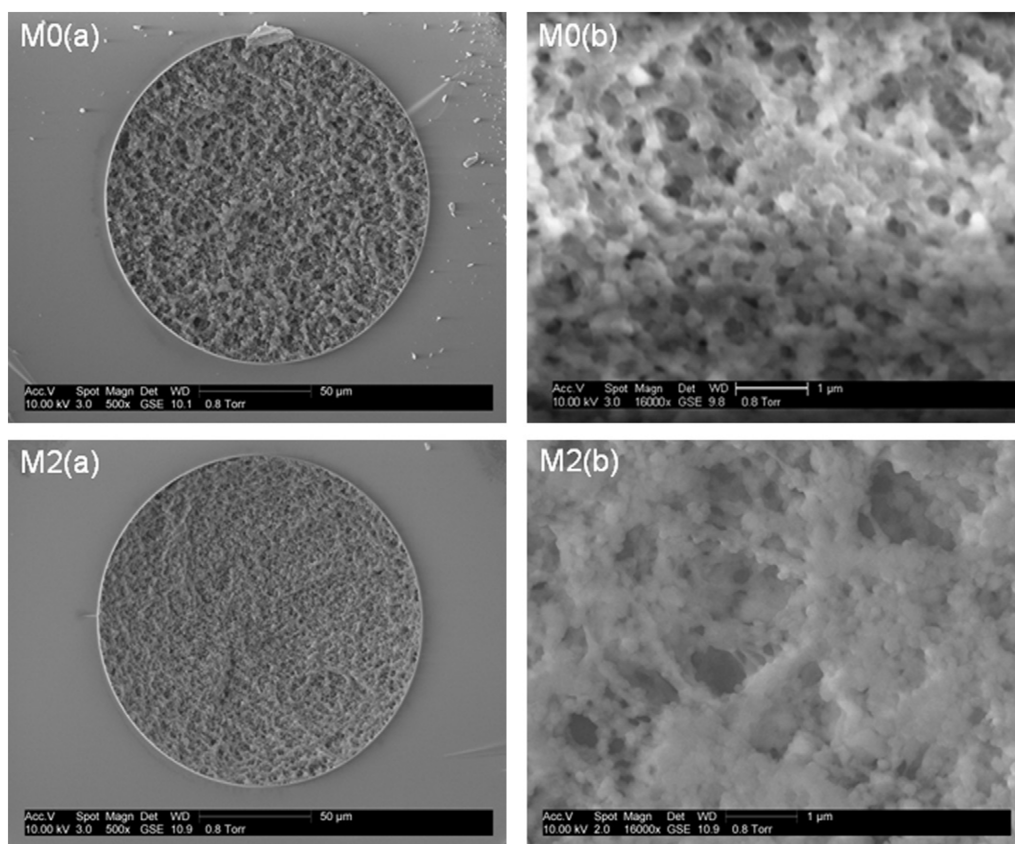

Fig. 2. SEM images of M0 and M2 monoliths with magnification of (a) 500, (b) 16,000.

tively) resulted in a decrease in permeability. Additionally, when the content of hexanes was kept constant, the permeabilities of the monoliths remained almost constant, even when changing the weight ratio of Brij 58P to dodecanol (M6, 0.5:0.2; M3, 0.3:0.4; and M7, 0.2:0.5). These data showed that both Brij 58P and dodecanol served as microporogens to produce small pores, while hexanes produced macropores for good bulk flow properties. To investigate the role of Brij in generating mesopores, another column, M0, was also prepared without the addition of Brij (dodecanol/hexanes, 0.5:0.6) for reference. Comparing M2 with M0, the column permeability decreased by adding Brij 58P to the porogenic mixture, which indicated that Brij may produce more mesopores. The surface area is also a good indication of the presence of mesopores as measured in the dry state. M0 without any Brij had a specific surface area of only 12 m^2/g; however, the addition of Brij yielded a monolith (M2) with a surface area of 187 m^2/g due to the creation of mesopores.

Since the monolithic materials in this study were used in combination with liquids in LC, determination of their pore properties in the wet state should be more valuable than properties measured in the dry state. ISEC was a convenient method to use, since it is based on liquid chromatography. Since the poly(PEGMEA-co-PEGDA) monoliths exhibit negligible interaction with proteins and peptides, real analytes (i.e., proteins and peptides) could be used as probes to estimate the pore size distributions. As shown in Table 1, the total porosity (ε_T) was determined according to the elution volume of uracil, and the porosity representing pores that permit passage of the largest protein (TG) through the monolith (ε_{TG}) was determined from the elution volume of TG. Therefore, the porosity that represents pores that are useful for size separation of smaller proteins and peptides than TG (ε_{pp}) can be calculated as the difference between the total porosity and TG porosity. Using this technique, the relative porosities of the monoliths listed in Table 1 were systematically investigated. Monolithic columns M1, M2, M4

Table 1
Reagent compositions and properties of the poly(PEGMEA-co-PEGDA) monoliths.[a]

Monolith	1-Dodecanol	Hexanes	Brij 58P	ε_T	ε_{TG}	ε_{pp}	Permeability[c] ($\times10^{-15}$ $m^{2)}$)	Efficiency (plates/m)	Resolution (TG/BSA)
M0[b]	0.5	0.6	0	0.70	0.63	0.07	26.1	41,300	0.2
M1	0.8	0.3	*0.3*	0.62	0.45	0.17	7.7	35,100	1.0
M2	0.5	0.6	*0.3*	0.72	0.56	0.16	18.4	68,400	1.6
M3	*0.4*	*0.7*	*0.3*	0.76	0.67	0.09	24.2	43,400	0.4
M4	*0.4*	0.5	0.5	0.64	0.53	0.11	9.8	53,100	0.7
M5	*0.4*	0.3	0.7	0.59	0.45	0.12	5.9	32,400	0.8
M6	0.2	*0.7*	0.5	0.73	0.65	0.08	23.0	57,600	0.6
M7	0.5	*0.7*	0.2	0.71	0.62	0.09	19.4	54,300	0.6

Italicized values indicate weight fraction of one porogen which was kept constant while varying those of the other two porogens.

[a] The polymerization mixture contained 30% (W/W) monomers (PEGMEA and PEGDA, 0.15:0.45) and 70% porogenic solvent (Brij 58P, 1-dodecanol and hexanes), and the polymerization was carried out in 23 cm × 150 μm i.d. columns for 6 min.

[b] A reference polymer was prepared without Brij.

[c] Permeability data were calculated according to Darcy's law[16].

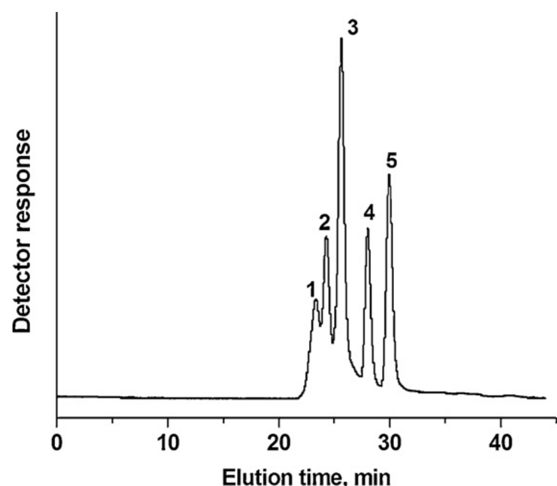

Fig. 3. SEC separation of a protein mixture using a poly(PEGMEA-co-PEGDA) monolith (M2, see Table 1). Conditions: 23 cm × 150 μm i.d. monolithic column; 20 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl, 0.15 μL/min; 60 nL injection volume; UV detection at 214 nm; Peak identifications: (1) TG, (2) CAT, (3) BSA, (4) LE, and (5) U.

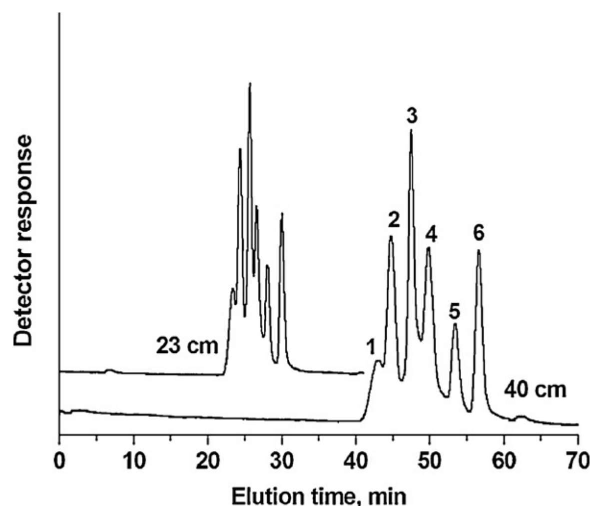

Fig. 4. Influence of column length on SEC resolution. Conditions as in Fig. 3. Peak identifications: (1) TG, (2) CAT, (3) BSA, (4) STI, (5) LE, and (6) U.

and M5 gave ε_{pp} values larger than 0.10, which could include micropores and small mesopores (smaller diameter than TG) that are useful for separation of peptides and proteins. It was also found that ε_{pp} did not appear to be directly related to low permeability. A low porosity combined with relatively small volumetric through-pore fraction yields monolithic columns with low permeability.

Fig. 2 shows SEM images of M0 and M2 monoliths prepared without Brij and with 0.3 g Brij in 1.4 g total porogens, respectively. The micrographs clearly show the effect of porogen composition on porosity. In M-0, globules are clumped together, however, individual globules are clearly visible. In M-2, the monolithic structure is more compact and the globules are fused together. The fused-globule morphology was found to be less permeable than the aggregated-globule morphology.

3.2. Chromatographic evaluation of the SEC monoliths

A combination of large mesopore volume and high efficiency would be optimal for SEC. Separation of BSA and TG was used for evaluation, since this pair represents an order of magnitude difference in MWs (i.e., 66,000 and 670,000). M2 gave the best resolution between TG and BSA due to a combination of large mesopore volume and high efficiency (68,400 plates/m). Other monolithic columns did not provide as good resolution of TG and BSA because of either low mesopore volume or low efficiency.

Fig. 3 demonstrates the separation of a mixture of three proteins and one peptide using a 23 cm long poly(PEGMEA-co-PEGDA) monolithic column. Both the column efficiency for this short SEC column and its selectivity were good. Considering the relatively low back-pressure experienced for this SEC separation, the column length can be easily extended for more efficient separations. As shown in Fig. 4, the separation of 4 proteins [i.e., (1) TG, (2) CAT, (3) BSA, and (4) STI] was observed despite limited resolution. The back pressures for 23 and 40 cm long columns were 363 and 652 psi, respectively. The resolution between TG and BSA increased from 1.5 to 1.9 when the column length increased from 23 cm to 40 cm. This obeys the proportional relationship between resolution and square root of column length. Of course, a longer column improves the separation mainly by increasing the column efficiency, but at the expense of longer analysis time. The preferred approach to improve resolution in relatively short time is to increase the mesopore volume of the monolith.

SEC calibration curves are similar to ISEC curves. The slope of the SEC calibration curve provides information related to the practical pore size distribution. Protein standards were used for calibration in this study because the biocompatible monolith developed was aimed at SEC separation of proteins. A calibration curve based on protein standards would provide the most relevant information, such as reliable estimation of molecular weight. Fig. 5 shows SEC calibration curves for poly(PEGMEA-co-PEGDA) monoliths prepared with and without Brij. The preparation of poly(PEGMEA-co-PEGDA) monoliths using low MW solvents (e.g., M0) yielded materials that were unable to separate proteins according to their molecular weights, due to the steep slope in the MW range between STI and TG. The SEC curve for M2 has a less steep slope in this range. This means that more mesopores suitable for protein separation were created due to the introduction of Brij into the progenic mixture. Additionally, the elution times of proteins on M2 (TG, CAT, BSA and STI) were nearly linearly related to the logarithms of their molecular weights. It should be emphasized, that the pore structure of a monolithic matrix determined by ISEC in this way assumes negligible interaction between the matrix and proteins or peptides in an aqueous buffer without organic additives. However, swelling or

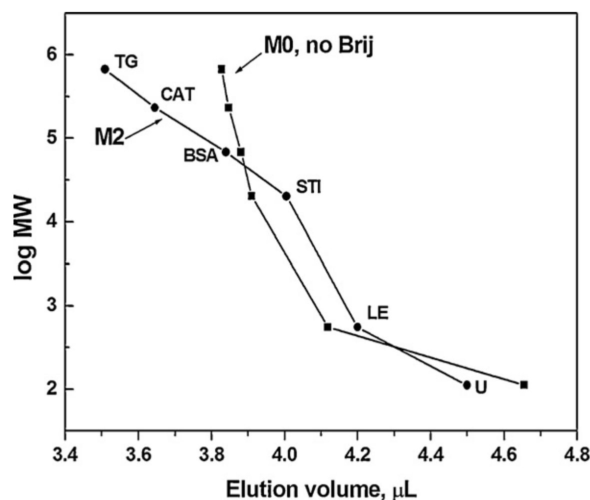

Fig. 5. SEC calibration curves for proteins and peptides using an M2 column (23 cm × 150 μm i.d.). A reference sample (M0) polymerized without Brij 58P is also included.

shrinking of the polymeric phase in any other media would alter its pore structure and change the pore size distribution.

4. Conclusions

Porous poly(PEGMEA-co-PEGDA) monoliths using a novel ternary porogenic solvent consisting of Brij 58P, 1-dodecanol and hexanes have been synthesized. The resulting monoliths were suitable for SEC separation of proteins. The newly developed porogenic system containing Brij produced pore properties in poly(PEGMEA-co-PEGDA) monoliths that are more appropriate for SEC separation of proteins, compared to monoliths produced by polymerization involving PEO–PPO–PEO porogens or low MW organic solvents. The improved resolution of proteins is attributed to a relatively large fraction of mesopores produced by the use of Brij 58P. A relatively low back-pressure and short analysis time are other significant advantages of this new SEC monolith. However, the relatively limited peak capacity still warrants future efforts to discover better mesoporogens or ways for maximizing the mesoporosity.

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