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Rigid porous polyacrylamide-based monolithic columns containing butyl methacrylate as a separation medium for the rapid hydrophobic interaction chromatography of proteins

Shaofeng Xie[a], Frantisek Svec[b], Jean M.J. Fréchet[b,*]

[a]Cornell University, Department of Chemistry, Baker Laboratory, Ithaca, NY 14853, USA
[b]Department of Chemistry, University of California, Berkeley, CA 94720-1460, USA

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Abstract

Macroporous poly(acrylamide-co-butyl methacrylate-co-N,N′-methylenebisacrylamide) monoliths containing up to 15% butyl methacrylate units have been prepared by direct polymerization within the confines of HPLC columns. The hydrodynamic and chromatographic properties of these 50 mm×8 mm I.D. columns – such as back pressure at different flow-rates, effect of percentage of hydrophobic component in the polymerization mixture, effect of salt concentration on the retention of proteins, dynamic loading capacity, and recovery – were determined under conditions typical of hydrophobic interaction chromatography. Using the monolithic column, five proteins were easily separated within only 3 min. © 1997 Elsevier Science B.V.

Keywords: Monolithic columns; Stationary phases, LC; Proteins; Butyl methacrylate; Acrylamide; Methylenebisacrylamide

1. Introduction

Hydrophobic interaction chromatography (HIC) was pioneered by Porath et al. [1] and Hjertén [2] more than two decades ago. This gentle separation technique is very useful for the separation and purification of proteins and it complements the other chromatographic modes such as ion-exchange, reversed-phase, affinity, and size-exclusion chromatography [3–5]. The concept of HIC is based on interaction of hydrophobic patches located on proteins with hydrophobic ligands located on the separation medium in an environment, e.g. aqueous salt solutions, that promotes these interactions. The column-bound ligands are typically short alkyl chains or phenyl groups. The strength of the interaction depends on many factors such as the intrinsic hydrophobicity of the protein, the type of ligands, their density, the separation temperature and the salt concentration. Typically, the separation is achieved by decreasing the salt concentration in the mobile phase, causing the less hydrophobic molecules to elute first. In contrast to highly hydrophobic reversed-phase chromatographic media that require elution with organic solvents, the column surface incorporates a much lower density of ligands interspersed within a highly hydrophilic surface, allowing elution with entirely aqueous eluents. The original

*Corresponding author.

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column packings for hydrophobic interaction chromatography were derivatives of polysaccharide gels. Currently, a wide variety of hydrophobic media based on both inorganic and organic polymer beads [5] is available for HIC.

Recently, Hjertén has developed a process for the preparation of continuous chromatographic columns obtained from swollen crosslinked polyacrylamide gels. The resulting gel columns are useful for the separation of proteins and peptides in several chromatographic modes including HIC [6–13]. Almost simultaneously, we introduced novel separation media, rigid monoliths that incorporate an ideal array of pores, both flow through and separative, prepared directly in a chromatographic column used as a mold [14,15]. Monolithic poly(styrene-co-divinylbenzene) columns have been used directly for reversed-phase chromatography [16,17] while poly(glycidyl methacrylate-co-ethylene dimethacrylate) columns modified in situ have been used for the separation of proteins by ion-exchange chromatography [18,19]. In contrast to the typical suspension processes used to prepare beads, the polymerization mixture for the in situ preparation of monolithic media consists of only one phase. This increases significantly the number of monomers that may be used directly in the preparation of these porous materials and even water soluble monomers can be polymerized easily. For example, we have demonstrated the preparation of hydrophilic poly(acrylamide-co-methylenebis-acrylamide) monoliths [20]. We have found that the porous properties of these hydrophilic monoliths can be controlled through system variables such as the type of porogen, the polymerization temperature, and the percentage of both initiator and crosslinking monomer. Optimization of the reaction conditions afforded highly crosslinked rigid acrylamide monoliths with large pores that can serve as a platform for the preparation of specific separation media for chromatographic modes such as hydrophobic interaction and affinity chromatography, both of which require hydrophilic surfaces endowed with ligands or functionalities.

This report extends the concept of our original porous hydrophilic platform to the preparation and use of monolithic separation media for hydrophobic interaction chromatography of proteins.

2. Experimental

2.1. Materials

Acrylamide (electrophoresis grade), N,N′-methylenebisacrylamide, benzoyl peroxide, butyl methacrylate, dimethyl sulfoxide and all of the 1-substituted aliphatic alcohols were obtained from Aldrich. Cytochrome *c*, ribonuclease, carbonic anhydrase, lysozyme and α-chymotrypsinogen were purchased from Sigma.

2.2. Preparation of monoliths

N,N′-Methylenebisacrylamide (1.68 g), acrylamide and butyl methacrylate (total 1.12 g) were dissolved in a mixture of 3.38 ml of dimethylsulfoxide and 1.82 ml of an aliphatic alcohol (Table 1) at a temperature of 75°C. After addition of benzoyl

Table 1
Porous properties of poly(acrylamide-co-butyl methacrylate-co-N,N-methylenebisacryl-amide) monoliths[a]

	BuMA (%)[b]			
	0	5	10	15
Porogen alcohol	$C_{12}H_{25}OH$	$C_{14}H_{27}OH$	$C_{16}H_{27}OH$	$C_{18}H_{29}OH$
$D_{p,med}$ (nm)[c]	550	648	940	887
V_p (ml g^{-1})[d]	1.49	1.44	1.51	1.43

[a] Polymerization conditions: reaction mixture: monomers 35% (methylenebisacrylamide 60%, acrylamide+butyl methacrylate 40%), benzoyl peroxide 1% with respect to monomers, dimethylsulfoxide 45.5%, aliphatic alcohol 19.5%; temperature 75°C; polymerization time 48 h.

[b] Content of butyl methacrylate in the monomer mixture.

[c] Pore diameter at the highest peak in the pore size distribution profile.

[d] Total pore volume.

peroxide (1 wt.% with respect to monomers), 50 mm×8 mm I.D. stainless steel columns sealed at one end were filled with this solution and then sealed at the other end. The polymerizations were allowed to proceed at 75°C for 24 h. The seals were removed, the column provided with end fittings and attached to the chromatographic system. Isopropanol (20 ml), methanol (100 ml) and water (overnight) were pumped through the column at a flow-rate of 1 ml min^{-1} to remove the porogenic solvents and other soluble compounds present in the polymer rod after the polymerization was completed.

2.3. Characterization of porous properties

The pore size distribution in the dry state was determined by mercury porosimetry using an automated custom-made combined BET-Sorptometer and mercury porosimeter (Porous Materials, Ithaca, NY, USA).

2.4. Chromatography

A computer controlled Hewlett–Packard HPLC 1050 system consisting of quaternary pump, autoinjector, and UV diode array detector was used to carry out all the chromatography. The protein recovery from the monolithic column was measured under standard chromatographic conditions and calculated as the percentage of the peak area of protein eluted from the column with respect to the peak area of the same amount of the protein injected into a system from which the column was removed and the inlet and outlet capillaries were connected.

3. Results and discussion

3.1. Preparation and characterization of molded monolithic columns

In contrast to the preparation of monoliths from styrenic and methacrylate ester monomers, the use of acrylamide and bisacrylamide requires substantial modifications of the process developed earlier [14] because of the limited solubility of the solid crystalline monomers in the porogenic mixture consisting of dimethylsulfoxide and higher aliphatic alcohols. Thus, a sufficiently concentrated monomer solution can be obtained only at a temperature that is close or equal to that at which the polymerization occurs. Therefore, all of the components of the polymerization mixture are first dissolved at a higher temperature and the free radical initiator is only added to this mixture immediately before the column tubes are filled. Due to the inhibition effect of oxygen that is intentionally left in the polymerization mixture, the polymerization does not start immediately and allows enough time to effect the transfer into the column tubes. No appreciable polymerization is observed during handling and the polymerization mixture remains transparent.

The hydrophobicity of the original crosslinked poly(acrylamide-co-methylene-bisacrylamide) platform is not sufficient for a good separation of proteins in hydrophobic interaction mode. Therefore, a more hydrophobic monomer, butyl methacrylate, was added in an amount of 5–15% to the polymerization mixture used for the preparation of monolithic columns. Unfortunately, this simple replacement of a part of the acrylamide with butyl methacrylate leads to a sharp decrease in the pore size. For example, an average pore diameter of 550 nm was found for the acrylamide–bisacrylamide monolith prepared in the presence of dodecanol as a coporogen while the substitution of 15% of the acrylamide with butyl methacrylate affords a rod with 48 nm pores. This monolith is obviously unsuitable as it does not allow liquid flow at reasonable back pressures. However, the target pore size in the range 500–1000 nm required for liquid flow [21] may be restored using alcohols higher than dodecanol as porogens (Table 1) [20].

Inversed size exclusion chromatography (ISEC) is often used for the determination of pores smaller than about 50 nm (mesopores). In contrast to mercury porosimetry and nitrogen adsorption–desorption (BET) data that has been shown for these materials elsewhere [20], the ISEC measurements are particularly well-suited to determine the porous properties in the swollen state because they are carried out with monoliths swollen with the mobile phase. Fig. 1 shows the calibration curve obtained by ISEC for one of the present monoliths using poly(ethylene glycol) standards in water as the mobile phase. The

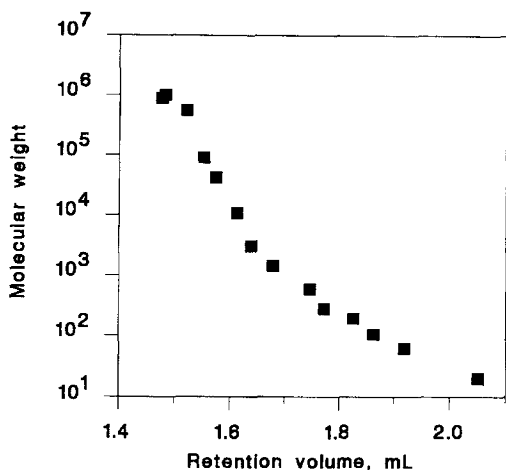

Fig. 1. Size exclusion chromatography calibration curve of the molded poly(acrylamide-co-butyl methacrylate-co-N,N′-methylenebisacrylamide) monolithic column. Conditions: column 50×8 mm I.D., 10% butyl methacrylate, mobile phase water, flow-rate 1 ml min^{-1}, analytes poly(ethylene glycol) standards, RI detection.

curve shows that more than 30% of the available pore volume is located within the mesopores.

3.2. Hydrodynamic properties

Fig. 2 shows the back pressure as a function of flow-rate of water through the 50 mm×8 mm I.D. molded monolithic columns. The highest back pressure was observed for the most hydrophilic acrylamide–methylenebisacrylamide copolymer that also has the smallest pores (550 nm). In contrast, monoliths with 10 and 15% butyl methacrylate and ~900 nm pores have a much lower resistance to flow. The plots for these two monoliths are almost straight lines at flow-rates up to 3 ml min^{-1} corresponding to linear flow velocities as high as 480 cm h^{-1}. These plots document the excellent behavior of the material even under higher pressure.

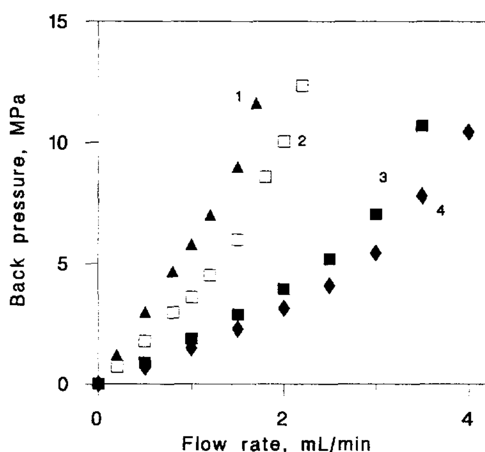

Fig. 2. Effect of flow-rate of water on back pressure in the molded poly(acrylamide-co-butyl methacrylate-co-N,N′-methylenebisacrylamide) 50×8 mm I.D. monolithic columns. Content of butyl methacrylate 0 (curve 1), 5 (2), 10 (3) and 15% (4).

3.3. Chromatographic properties

Hydrophobic interaction chromatography is a separation process driven by changes in entropy [3–5]. The retention of proteins depends both on the hydrophobicity of the protein surfaces, and the density and type of hydrophobic ligands attached to the surface of the medium. While the hydrophobicity of proteins is an intrinsic property that cannot be changed in chromatography, the polarity of the separation medium can be adjusted during its preparation. As a rule, the higher the ligand density the stronger the retention of proteins. Eq. (1) relates retention to the composition of the mobile phase [22]:

$$\log k' = \log k'_w + S m_s \quad (1)$$

Here, k' is the retention factor, k'_w is the retention factor at the concentration of the antichaotropic salt $m_s = 0$ (pure water), S is the slope and m_s is the concentration of the salt in the eluent. The plots k' vs. ammonium sulfate concentration shown in Fig. 3 for ribonuclease and monoliths with various hydrophobicity are almost linear. Their coordinates clearly depend on the polarity of the separation medium. Fig. 4 presents these plots for cytochrome c, ribonuclease, carbonic anhydrase and lysozyme using a monolithic column containing 10% of butyl methacrylate. The coordinates of these plots in the graph reflect the hydrophobicity of the individual proteins. Obviously, the lines of both Figs. 3 and 4 have shapes typical of the gradient separations [22]. Their steeply increasing, almost linear parts fit well Eq. (1) and confirm the hydrophobic nature of the interactions. Table 2 summarizes the experimental S values

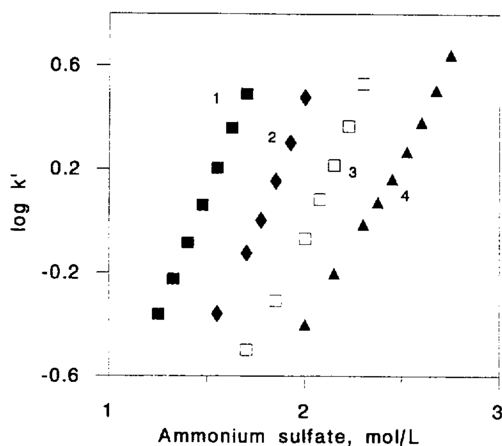

Fig. 3. Effect of the mobile phase composition and the column hydrophobicity on retention factor k' of ribonuclease. Conditions: columns 50×8 mm I.D., 0–3 M ammonium sulfate in 0.01 M sodium phosphate (pH 7), flow-rate 1 ml min^{-1}. Content of butyl methacrylate in columns 0 (curve 4), 5 (3), 10 (2), and 15% (1).

(a measure of hydrophobicity) for all of the proteins and monolithic columns.

3.3.1. Dynamic binding capacity

Breakthrough curves provide valuable information for the evaluation of the dynamic binding capacity of the separation medium. This is a very important characteristic for large scale separations. Fig. 5 shows two almost identical breakthrough curves that were monitored for lysozyme at flow-rates of 1 and 3 ml min^{-1}. The curves are rather steep and do not depend on the flow-rate. The highest dynamic binding capacity of 6.5 mg ml^{-1} for lysozyme was found for columns prepared with 10% butyl methacrylate while capacities of 5.2 and 4.2 g ml^{-1} were achieved for columns made with 5 and 15% of hydrophobic monomer, respectively. These capacities compare favorably to those of common rigid polymer-based beads for HIC [23,24]. The highest capacity observed for the 10% butyl methacrylate monolith is the result of an optimal combination of its hydrophobicity and porous properties.

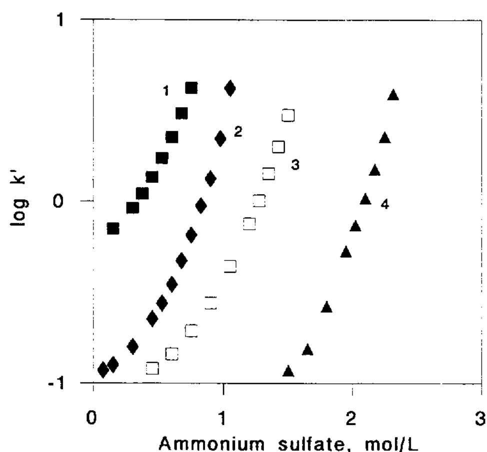

Fig. 4. Effect of the mobile phase composition on retention factor k' of lysozyme (curve 1) carbonic anhydrase (2), ribonuclease (3), and cytochrome c (4). Conditions: column 50×8 mm I.D., 10% butyl methacrylate, 0–3 M ammonium sulfate in 0.01 M sodium phosphate (pH 7), flow-rate 1 ml min^{-1}.

3.3.2. Effect of loading

Variables such as selectivity and retention factor k' that characterize a separation under non-overload conditions are normally independent of the sample size [25]. Fig. 6 shows the separation of cytochrome c, ribonuclease, and lysozyme in an ammonium sulfate gradient. The loading was increased by more than one order of magnitude from 0.36 to 1.46 to 5.84 mg. Despite the broad loading range and high flow-rate, both retention times and selectivities are only slightly affected. The invariability of chromatographic characteristics with loading, already observed for monolithic columns made from other monomers [16], seems to be a general feature of this novel format of separation media.

3.3.3. Recovery

High yields in the separation of proteins is an essential requirement for industrial downstream processing. Table 3 shows recoveries of individual proteins measured as the amount of protein eluted after adsorption for one of the columns tested in this study. Obviously, the best recovery is achieved with the least hydrophobic protein, cytochrome c. Recoveries always exceed 90% with the highest values achieved with the column prepared with 10% butyl methacrylate. Once again this is assumed to result from the ideal combination of chemical and structural features of this monolith.

3.3.4. Chromatographic separation

Data summarized in Table 2 and shown in Figs. 1 and 2 indicate the suitability of the monolithic

Table 2
Effect of butylmethacrylate content in the monolithic column on the retention of model proteins[a]

BuMA (%)[b]	S^c			
	Cytochrome *c*	Ribonuclease	Carbonic anhydrase	Lysozyme
0	1.42	1.36	1.41	0.67
5	1.60	1.65	1.70	1.14
10	2.06	1.72	2.00	1.14
15	2.34	1.91	2.53	1.15

[a] Conditions: column, 50 mm×8 mm I.D.; ammonium sulfate solution in 0.01 *M* phosphate buffer (pH 7); flow-rate 1 ml min^{-1}.
[b] Content of butyl methacrylate in the monomer mixture.
[c] Slope of the straight lines k' vs. ammonium sulfate concentration in the mobile phase.

columns for the separation of protein mixtures. Indeed, Fig. 7a shows that a very good analytical separation of five proteins can be achieved within 10 min on the 10% butyl methacrylate monolithic column in a gradient of ammonium sulfate at a flow-rate of 1 ml min^{-1}. Since the breakthrough curves shown did not change with an increase in the flow-rate, the separation can be accelerated using a higher flow-rate of 3 ml min^{-1} and a steeper mobile phase gradient. This approach shown in Fig. 7b allows the reduction of the separation time to less than 3 min without deterioration in resolution. This speed compares favorably to that obtained recently by Hjertén with an analogous column of crosslinked poly(acrylamide-co-piperazinebisacrylamide-co-N-isopropyl-acrylamide) [13].

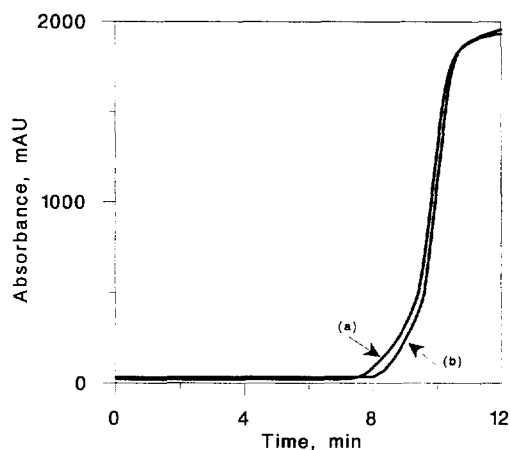

Fig. 5. Breakthrough curves for lysozyme at a flow-rate of (a) 3 ml min^{-1} and (b) 1 ml min^{-1}. Conditions: column 50×8 mm I.D., 10% butyl methacrylate, lysozyme solution 1.5 mg ml^{-1}, 1.5 *M* ammonium sulfate in 0.01 *M* sodium phosphate buffer (pH 7).

4. Conclusion

We have demonstrated that 'molded' poly(acrylamide-co-butyl methacrylate-co-N,N′-methylenebisacrylamide) monolithic columns can be used for rapid HIC of proteins. The hydrophobicity of the interacting surface can be easily controlled by the percentage of butyl methacrylate in the polymerization mixture. The optimized monolithic media exhibit low back pressure even at high flow-rates and provide for steep breakthrough curves and reason-

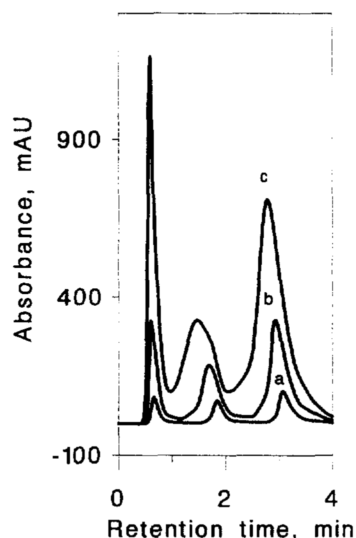

Fig. 6. Effect of column loading on the separation of cytochrome *c*, ribonuclease and lysozyme (order of elution). Conditions: column 50×8 mm I.D., 10% butyl methacrylate, mobile phase gradient from 1.65 to 0.1 *M* ammonium sulfate in 0.01 *M* sodium phosphate buffer (pH 7) in 3 min. Protein loading: (a) 0.36, (b) 1.46 and (c) 5.84 mg.

Table 3
Recovery of model proteins from monolithic column under conditions of hydrophobic interaction chromatography[a]

Protein	Cytochrome *c*	Ribonuclease	Carbonic anhydrase	Lysozyme
M_s^b	2.5	2.0	1.5	1.2
Recovery (%)	96	92	90	98

[a] Conditions: column, 50 mm×8 mm I.D., poly(acrylamide-co-10% butyl methacrylate-co-N,N′-methylenebisacrylamide), elution in 0.01 *M* phosphate buffer (pH 7), flow-rate 1 ml min^{-1}.
[b] Concentration of ammonium sulfate used for adsorption of the protein.

ably high loading capacities. Although much remains to be done in the development of monoliths with even better resolution and decreased peak tailing for the preparative chromatography of proteins, the properties of the analytical scale columns shown here are very promising for the design and preparation of large-scale separation columns.

Acknowledgments

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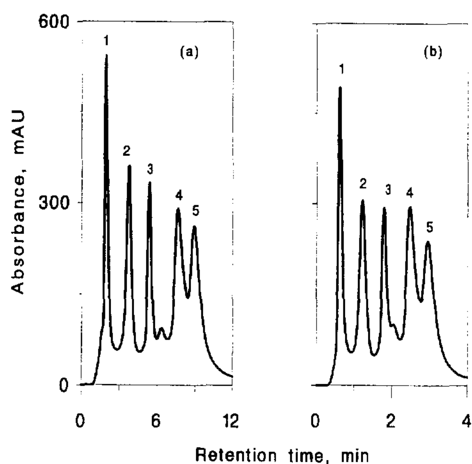

Fig. 7. Separation of cytochrome *c* (1), ribonuclease (2), carbonic anhydrase (3), lysozyme (4), and chymotrypsinogen (5) by hydrophobic interaction chromatography on molded poly(acrylamide-co-butyl methacrylate-co-N,N′-methylenebisacrylamide) monolithic column. Conditions: column 50×8 mm I.D., 10% butyl methacrylate, mobile phase gradient from 1.5 to 0.1 *M* ammonium sulfate in 0.01 *M* sodium phosphate buffer (pH 7) in 3 min. Flow-rate 1 ml min^{-1}, gradient time 10 min (a) and 3 ml min^{-1} and 3.3 min (b).

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