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Photopolymerized monolithic capillary columns for rapid micro high-performance liquid chromatographic separation of proteins

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Abstract

The preparation of monolithic poly(butyl methacrylate–*co*-ethylene dimethacrylate) capillary columns using photoinitiated in situ polymerization within 200 μm i.d. capillaries and their application for μHPLC separations of proteins have been studied. The low resistance to flow characteristic of monolithic columns, enabled the use of very high flow rates of up to 100 μL/min representing a flow velocity of 87 mm/s. Very good separations of a model protein mixture consisting of ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin was achieved in less than 40 s using a very simple single step gradient of the mobile phase. Interestingly, no effect of the pore size on the separations of proteins was observed for these monolithic columns within the size range of 0.66–2.2 μm. The monolithic μHPLC columns are found very robust and no changes in the long term separation performance and back pressure were observed.

Keywords: Monolithic columns; Stationary phases, LC; Proteins

1. Introduction

Although HPLC in microcolumns packed with modified silica beads have been known for more than 20 years [1,2], it is only recently that both the chromatographic industry and the users have started to focus on this separation mode. Capillary μHPLC is currently the best way to clean up, separate, and transfer samples quickly and easily to a mass spectrometer, a feature valued most by researchers in the life sciences since it enables the direct coupling of HPLC with mass spectrometry (MS) and may not require flow splitting [3–6]. Monolithic capillary columns derived from their analytical size predecessors [7–14] represent a quantum leap in the current quest for highly efficient separation techniques with increased sensitivity that can analyze very small amounts of compounds in the complex matrices such as those typical of proteome research or found in the biopharmaceutical R&D environment. In contrast to the capillary columns packed with particulate stationary phases, monolithic columns for reversed-phase (RP) μHPLC of a variety of biological compounds provide for some significant advantages that have recently been demonstrated.

As of today, a few different approaches to monolithic capillary columns have been reported. Liao et al. [15] prepared compressed gel monolith with ion-exchange functionalities in 300 μm capillary. Tanaka and co-workers developed sol–gel process for the preparation of silica based monoliths in capillary format [16,17]. Ring-opening metathesis polymerization of norbornene derivatives has recently been added to the arsenal of methods affording monoliths in capillaries [18]. Most of the current monolithic capillary columns for μHPLC comprise porous polymers inspired by our early work [19–21]. For example, Moore et al. [22] used poly(styrene–*co*-divinylbenzene) monolith prepared in the nanoelectrospray needle for the separation of proteins and clearly demonstrated the superior performance of this column compared to its counterparts packed with beads. Huber and co-workers [23–27] used monoliths with the same chemistry prepared in 200 μm capillary under modified conditions and achieved excellent separations of nucleic acids, proteins, and peptides. Ivanov et al. [28] very recently reported monolithic poly(styrene–*co*-divinylbenzene) columns prepared in capillaries with an i.d. of only 20 μm and used them

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for low attomole electrospray ionization (ESI) MS and MS–MS analysis of protein tryptic digests with an exceptionally good resolution. Hydrophobicity of the monolithic poly(styrene–*co*-divinylbenzene) reversed phase columns was further increased by Friedel–Crafts alkylation with chlorooctadecane carried out in situ [29]. Monolithic poly(butyl methacrylate–*co*-ethylene dimethacrylate) capillary columns derived from those used previously for capillary electrochromatography [30,31] also proved efficient for the separations of small molecules in the RP-μHPLC mode [32,33].

The common feature unifying all of the above monolithic columns is their preparation using thermally initiated free radical polymerization. This technique is well suited for the preparation of larger monolithic structures that completely fill the entire volume of a hermetically closed mold such as a stainless steel or polyether ether ketone (PEEK) column for HPLC. This simple procedure also works well for capillaries that have easily adjustable length. However, one of our targets is to extend the use of μHPLC to microfluidic chips. Since the position of the channel on a chip-based device is fixed, the separation medium must be located only within the assigned space. Obviously, adjustments in the length and position of the monolith are hard to achieve after the polymerization process is complete. Therefore, the preparation method must ensure that the monolith is only prepared at the desired location within the channel. Such precise positioning would be difficult to accomplish using the thermally initiated polymerization process.

In contrast, UV light initiated polymerization is exceptionally well suited to achieve monolith formation within a specified space. Using a mask in a photopatterning process, the polymerization is restricted to the irradiated areas while monomers do not convert to polymer in those areas that are not irradiated. The same technology is being widely used for microelectronic patterning. We have demonstrated the suitability of UV initiated free radical polymerization for both the preparation and surface modification of porous monolithic materials in microdevices [34–37]. In addition to patterning, the photoinitiated polymerization approach is advantageous due to its speed and operation at room temperature [38]. Obviously, UV transparent “molds”, monomers, and porogens have to be used to achieve the desired photopolymerization.

Following our success with a variety of microdevices including immobilized protein reactors [39], solid phase preconcentrators [40], static micromixers [38], and capillary columns for CEC [41,42], we had anticipated that these monoliths would also be well-suited for the fabrication of μHPLC columns. In this communication, we report the photoinitiated polymerization process used for the preparation of porous poly(butyl methacrylate–*co*-ethylene dimethacrylate) monolithic columns within fused silica capillaries and their use for the rapid separation of proteins.

2. Experimental

2.1. Materials

Ethylene dimethacrylate (EDMA), butyl methacrylate (BuMA), 1-decanol, cyclohexanol, 2,2-dimethoxy-2-phenylacetophenone (DAP), and 3-(trimethoxysilyl)propyl methacrylate were purchased from Aldrich (Milwaukee, WI, USA). EDMA and BuMA were passed through a bed of basic alumina to remove inhibitors and then distilled under reduced pressure. Both 1-decanol and cyclohexanol, were dried over 4 Å molecular sieves and filtered through a 0.2 μm filter before use. HPLC grade water, acetonitrile, methanol, and acetone were purchased from Fisher Scientific (Pittsburgh, PA, USA). Basic alumina (Brockman activity I, 60-325 mesh), sodium hydroxide (NaOH), hydrochloric acid (HCl), trifluoroacetic acid (TFA), and acetic acid were also from Fisher Scientific. All proteins (ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin) were obtained from Sigma (St. Louis, MO, USA). PTFE-coated 200 μm i.d. capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Monolithic capillary columns

The internal wall surface of the PTFE-coated capillary was first vinylized to enable covalent attachment of the monolith to the walls using the method we have described in detail elsewhere [43]. Briefly, the capillary was rinsed with acetone and water, activated with a 0.2 mol/L sodium hydroxide for 30 min, washed with water followed by 0.2 mol/L HCl for 30 min, and finally rinsed with ethanol. A 20% solution of 3-(trimethoxysilyl)propyl methacrylate in 95% ethanol with its pH adjusted to 5 using acetic acid was then pumped through the capillary for 1 h using a syringe pump (kdScientific, New Hope, PA, USA). Following washing with ethanol and drying in a stream of nitrogen, the functionalized capillaries were left at room temperature for 24 h.

The modified capillaries masked using opaque electrical tape were filled with a polymerization mixture (Table 1) previously purged with nitrogen for 10 min to a length of 100 mm using capillary action. The capillaries were then placed in a box equipped with two 8 W UV tubes, which emit light with an overall intensity of 1150 $\mu W/cm^2$ (VWR Scientific Products, Plainfield, NJ, USA), and irradiated for 10 min. The monoliths were then washed with 200 μL of methanol using a syringe pump at a flow rate of 0.5 μL/min.

2.3. Porous properties

Since the amount of monoliths prepared in the capillaries is not sufficient for porosimetry measurement, the conditions prevailing in the capillary were mimicked using bulk polymerization in a larger volume mold. This mold consisted of

Table 1
Composition of the polymerization mixtures used for the preparation of monolithic columns and their porous properties

Column	EDMA[a] (%)	BuMA[b] (%)	1-Decanol (%)	Cyclohexanol (%)	DAP[c] (%)	D_p[d] (μm)	V_p[e] (mL/g)
I	16	24	60	0	1.0	2.24	1.99
II	16	24	40	20	1.0	0.66	1.94

[a] Ethylene dimethacrylate.
[b] Butyl methacrylate.
[c] Percentage of 2,2-dimethoxy-2-phenylacetophenone (DAP) with respect to the monomers.
[d] Pore size determined by mercury intrusion porosimetry.
[e] Pore volume determined by mercury intrusion porosimetry.

a circular PTFE plate and a quartz wafer (100 mm × 1.6 mm, Chemglass, Vineland, NJ, USA) separated by a 1 mm thick circular PTFE gasket (10 cm o.d. × 9 cm i.d.) sandwiched between an aluminum base plate and a top aluminum ring held together with 12 screws. The mold was filled with the polymerization mixtures used for the preparation of capillary columns and then polymerized under the same conditions as the capillary columns. After the polymerization was completed, the mold was opened, the solid polymer recovered, broken into smaller pieces, extracted in a Soxhlet apparatus with methanol for 12 h, and dried in vacuum at 60 °C for 12 h. The porous properties of these monolithic materials were determined using an Autopore III 9400 mercury intrusion porosimeter (Micromeritics, Norcross, GA, USA).

2.4. Chromatography

The HPLC system consisted of an Ultra Plus II pump, pump controller (both Micro-Tech Scientific, Vista, CA, USA), and a UV 200 detector (Linear, Thermo Electron, Whaltham, MA, USA). A T-union was used to connect a mixer, an injector, and an actuated six-port valve to the pump thus enabling splitting the flow and achieving the desired flow rates derived from the primary rate of 100 μL/min. The splitting ratio was adjusted through the length of a 50 μm i.d. capillary attached to the valve. The dwell volume between the splitter and the injector, which may affect the separations at different flow rates was minimized using tubing connection made of 50 μm i.d. capillary.

The front end of the monolithic capillary column was attached to the injector while the other end protruded through the alignment of the UV detector with the light passing through the capillary immediately after the end of the monolith to minimize band broadening. A 500 mm long × 100 μm i.d. capillary was attached to the loose end of the monolithic column to prevent bubble formation. The actual flow rate was determined from the weight of eluent collected during a certain period of time. Typically, the volume of solution injected (0.13 mg/mL of each protein) was 200 nL. The data collected was processed by ChromPerfect Spirit (Justice Laboratory, Denville, NJ, USA). All chromatograms were baseline subtracted to eliminate the effects of refractive index variations resulting from the use of gradient on UV detection.

3. Results and discussion

3.1. Column properties

Porous properties are important in all applications of molded polymer monoliths, since they are generally designed to operate in a flow-through mode. Two monolithic capillaries differing in their pore size were prepared from polymerization mixtures by varying the porogenic solvent (1-decanol and its mixtures with cyclohexanol) shown in Table 1. Since the total percentage of porogen in the polymerization mixture and consequently also the contents of the monomers remain constant, pore volumes are very similar and both monoliths have the same chemical composition. The pore size profiles for both columns tested are shown in Fig. 1. The monolith prepared in presence of decanol as the sole porogen (column I) exhibits a narrow and symmetrical pore size distribution with a maximum at 2.2 μm. In contrast, the distribution curve of the monolith obtained using a 2:1 mixture of decanol and cyclohexanol as the porogen (column II) has a peak at 0.61 μm with an average pore size of 0.66 μm due to the presence of a certain amount of larger pores in the monolith. Fig. 2 shows SEM micrographs of both columns I and II in which the well-developed macroporous structure with different levels of pore sizes is visible

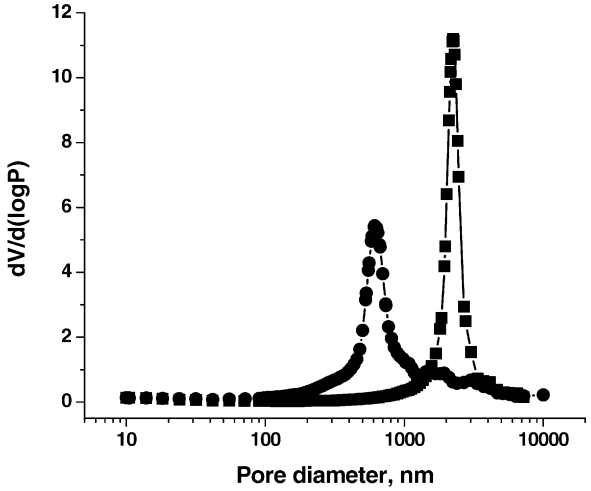

Fig. 1. Pore size distribution profiles for monolithic column I (■) and II (●) determined by mercury intrusion porosimetry.

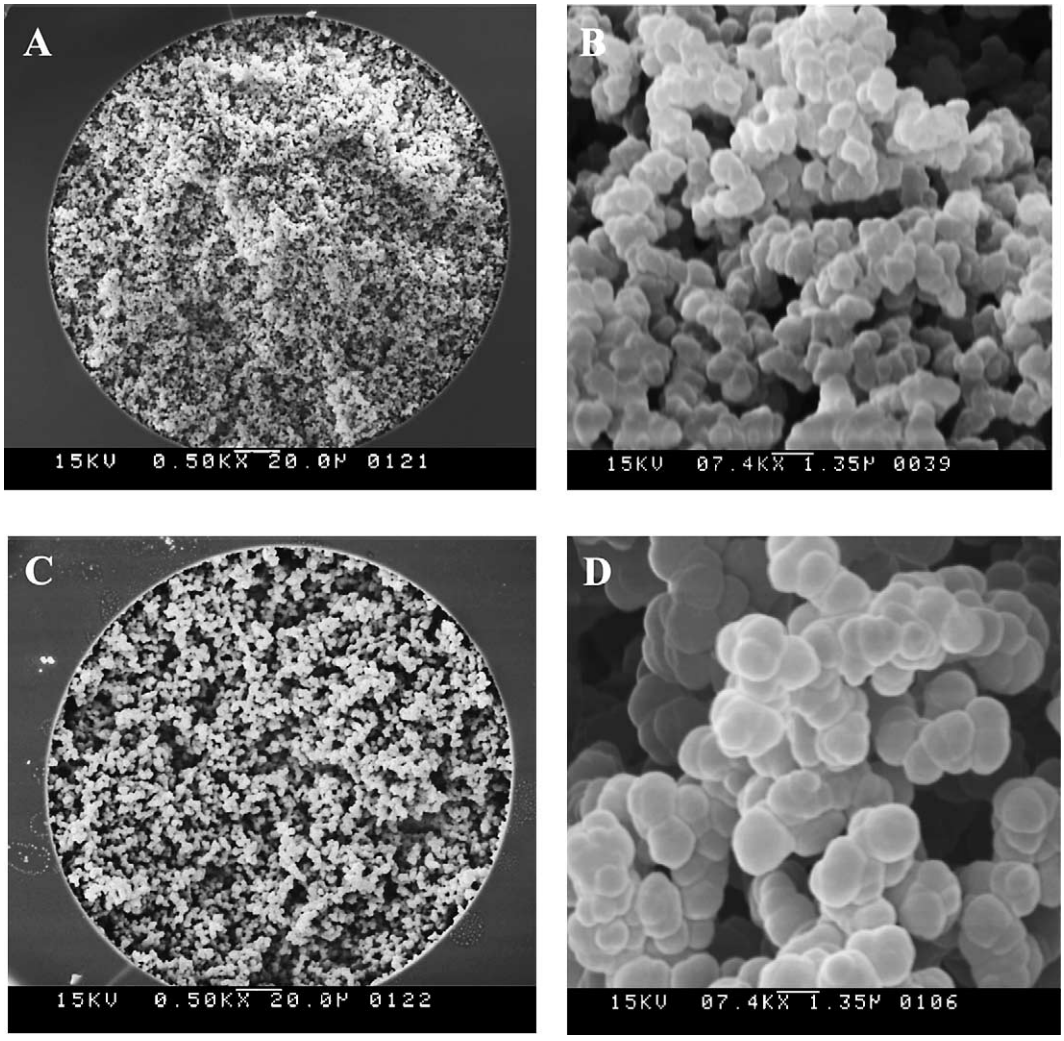

Fig. 2. Scanning electron micrographs of monoliths with a pore size of (A and B) 0.66 and (C and D) 2.24 μm. Magnifications: (A and C) 500× and (B and D) 7400×.

and no gap is seen between the monolith and the capillary walls confirming the efficiency of attachment.

The pore size of the monolithic columns also determines their flow resistance. This characteristic is easily discerned from plots of back pressure at different flow rates. Fig. 3 shows that these plots for both columns are linear within a wide range of flow velocities and confirm the structural rigidity and lack of incompressibility of the monoliths. As expected from its smaller pore size, the resistance to flow observed for column II is significantly higher than that for column I. It is worth noting that both columns withstand pressures of up to 25 MPa with no detectable damage.

3.2. Separation of proteins

The chromatographic performance of our monolithic capillary columns was tested using a mixture of four model proteins (ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin). Fig. 4 shows the separations obtained using gradient elution at a flow rate of 20 μL/min that corresponds to a linear flow velocity of 17 mm/s. Under these conditions, the back pressure is 3.8 and 13 MPa in column I and II, respectively.

At the same linear flow velocity, the retention times in HPLC are proportional to both the volume ratio of the stationary phase to the mobile phase (V_s/V_m), and the partition coefficient, which depends on the specific chemistry of the stationary phase [44]. Since the retention times in columns I and II for all four model proteins are very similar, both V_s/V_m and the chemistry of the capillary columns can be considered to be identical or equivalent. The chemistry of the stationary phases is determined by the monomers composition in the polymerization mixture, which has been intentionally kept fixed in both monoliths. The porosimetric data in Table 1 indicate that the pore volumes for both columns that define the values of V_m are almost identical. Since the pore volume automatically determines the volumes of both mobile and stationary phases for monolithic columns, the values of V_s/V_m must indeed be identical for both of our columns.

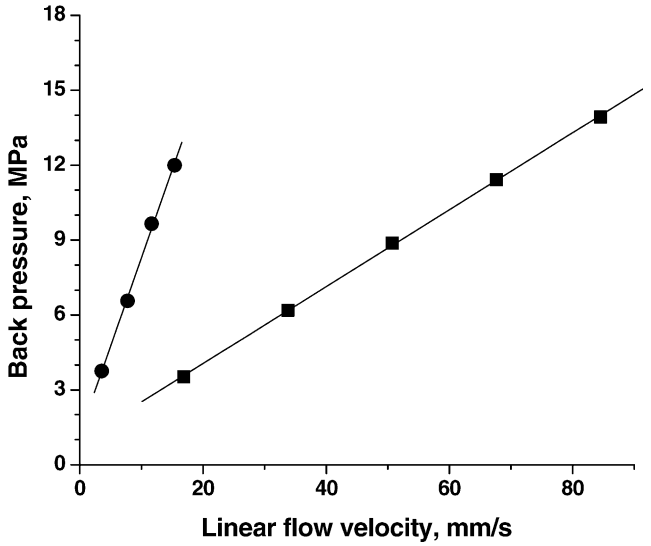

Fig. 3. Effect of linear flow velocity on back pressure in capillary columns varying in pore sizes. Conditions: column size 100 mm × 0.2 mm, pore size column I 2.24 (■), column II 0.66 μm (●). Mobile phase 0.1% TFA in water–acetonitrile (90:10, v/v).

3.3. Effect of flow rate and gradient profile

The high permeability of the monolithic columns demonstrated in Fig. 4 enables use of very high flow rates while running the separations. For example, Fig. 5 shows the separations of the protein mixture at flow rates of 4.2, 34, and even 100 μL/min. Note that the highest flow rate of 100 μL/min corresponds to a very high linear flow velocity of 85 mm/s. The use of such a high flow rate would not be feasible for a column of this size packed with HPLC grade particles due to the prohibitively high back pressure that would result. Fig. 6 shows separations achieved at a very high flow rate of 100 μL/min using three different gradient profiles. Application of a two-step gradient shown in Fig. 6c, in which the mobile phase composition increases from 5 to 40% of B in A immediately after injection and then steeply increases to 100% B in 1 min, significantly reduces the total analysis time compared to that using a continuous linear gradient (Fig. 6a).

The separation shown in Fig. 6b is obtained using a single step gradient with an increase to 50% B at time zero followed

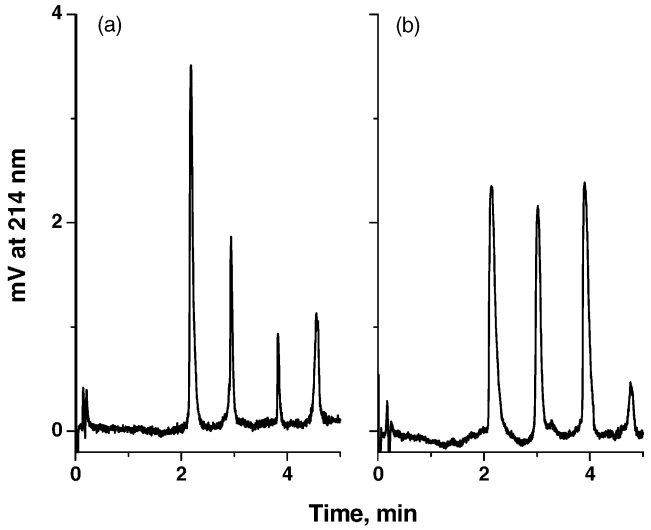

Fig. 4. Effect of pore size on the separation of a protein mixture. Conditions: column I (a) and II (b), 100 mm × 0.2 mm. Mobile phase A: 0.1% TFA in water–acetonitrile (90:10, v/v), mobile phase B: 0.1% TFA in water–acetonitrile (10:90, v/v). Gradient, 5–95% of mobile phase B in 8.5 min. Flow rate: 20 μL/min. Peaks: ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin (elution order).

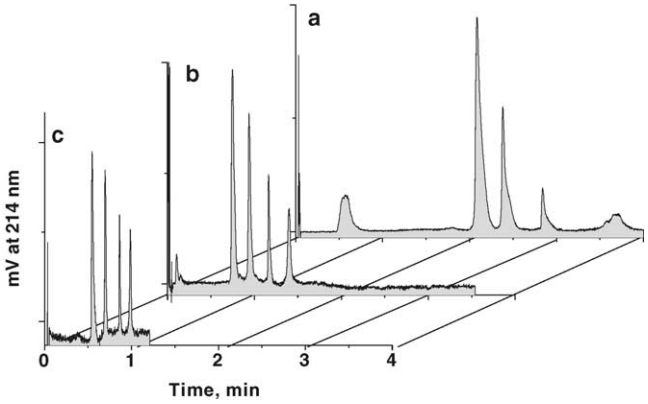

Fig. 5. Effect of flow rate on the separation of a model protein mixture using monolithic capillary column I. Conditions: column size 100 mm × 0.2 mm. Mobile phase A: 0.1% TFA in water–acetonitrile (90:10, v/v), mobile phase B: 0.1% TFA in water–acetonitrile (10:90, v/v). Flow rate (a) 4.2, (b) 34, and (c) 100 μL/min; gradients: 5–40% B in A at time 0 followed by linear increase from 40 to 100% B in A in (a) 28.7 min, (b) 3.5 min, and (c) 1.2 min. Peaks: ribonuclease A, cytochrome *c*, myoglobin, ovalbumin (elution order).

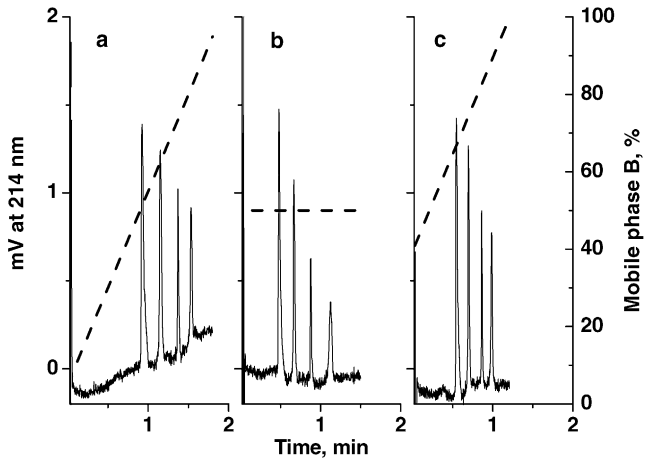

Fig. 6. Effect of gradient on separation of a protein mixture using monolithic column I at 100 μL/min. Conditions: column size 100 mm × 0.2 mm. Mobile phase A, 0.1% TFA in water–acetonitrile (90:10, v/v); mobile phase B, 0.1% TFA in water–acetonitrile (10:90, v/v). Gradient in (a) 5–95% B from 0 to 1.8 min; gradient in (b) 5–50–50% B from 0 to 0.01 to 1.5 min, gradient in (c) 5–40–100% B from 0 to 0.01 to 1.21 min. Peaks: ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin (elution order).

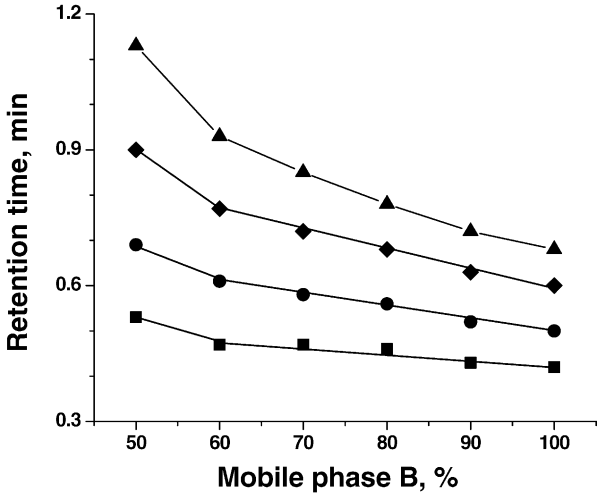

Fig. 7. Effect of the initial increases in percentage of solvent B in the mobile phase (step height at time 0) on retentions of ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin in monolithic column I.

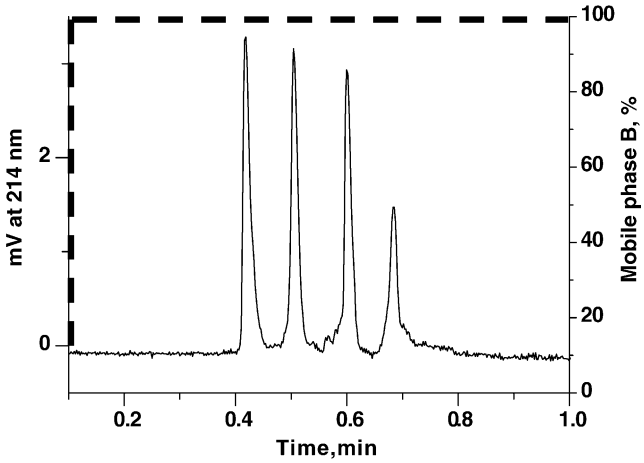

Fig. 8. Rapid separation of ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin using monolithic column I and a single step gradient at 100 μL/min. Conditions: column size 100 mm × 0.2 mm. Mobile phase A, 0.1% TFA in water–acetonitrile (90:10, v/v); mobile phase B, 0.1% TFA in water–acetonitrile (10:90, v/v); gradient profile that includes change from 100% mobile phase A to 100% B at time 0 is represented by the bold dashed line.

by an isocratic elution in this mobile phase. Obviously, the gradient that forms by turbulent flow in the mixer only at the interface of both mobile phases is sufficient to achieve a rather good separation. The clear benefit of this approach is a much better control of the composition of the mobile phase since difference in viscosity does not affect the gradient shape and both pumps can operate at their optimal flow rates. Therefore, we carried out a more thorough investigation of the separation of proteins using gradient mode.

Fig. 7 demonstrates the effect of the initial increase in the overall percentage of acetonitrile in the mobile phase on the retention of all four model proteins. Clearly, the higher the percentage of the strong solvent adjusted at time 0, the shorter the retention time. The retention profiles shown in Fig. 7 are similar for all of the proteins, and therefore, the separation should not deteriorate even at a simple switch from 100% A to 100% B. Indeed, Fig. 8 shows the excellent rapid separation of all four proteins using this approach achieved in the very fast time of less that 40 s.

3.4. Reproducibility and stability

A good column-to-column reproducibility is an important measure of the robustness of the process used to prepare the columns. Two additional columns (columns III and IV) were polymerized using newly prepared polymerization mixture and conditions matching those used for the preparation of the original column I. Fig. 9 presents an overlay of the separations of the model protein mixture using all three columns I, III, and IV. An excellent reproducibility characterized by relative standard deviations (R.S.D.s) for the retention times in the range of 0.005–0.01% was easily achieved. This excellent reproducibility is not surprising given that both the chemistry and the pore volume of the monoliths have been retained. Thus, only very small differences in the pore size could result from variations in the intensity of the UV irradiation and temperature used for the polymerization [38,45]. However, we have demonstrated above that the pore size of the monolith has little effect on the separations. Therefore, any small fluctuation in pore size that may exist would not negatively affect the column-to-column reproducibility of the separations. It is noteworthy to point out that the chromatogram shown in Fig. 9 represents 74th run with column I following a period of continuous use exceeding 1 week under a variety of conditions. Similarly, the separations shown for the other columns were obtained in the seventh and the

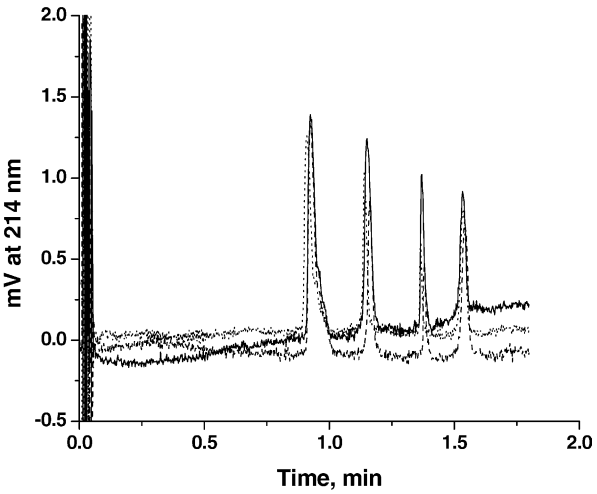

Fig. 9. Reproducibility of the protein separations by column I (solid line), column III (dashed line), and column IV (dotted line). Conditions: flow rate 100 μL/min. Mobile phase A 0.1% TFA in water–acetonitrile (90:10, v/v); mobile phase B 0.1% TFA in water–acetonitrile (10:90, v/v); gradient, 5% B to 95% B in 1.8 min. Peaks: ribonuclease A, cytochrome *c*, myoglobin, and ovalbumin (elution order).

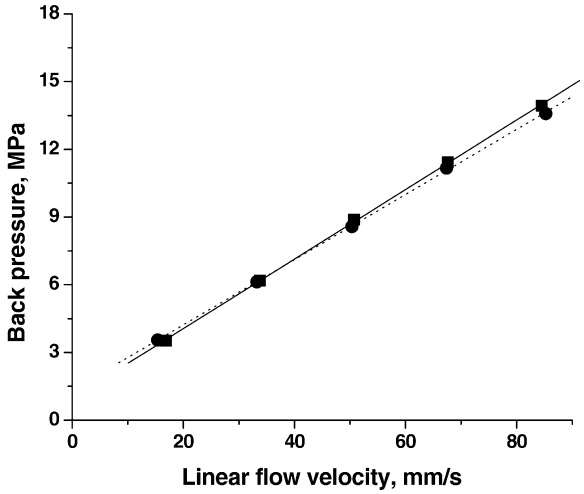

Fig. 10. Effect of linear flow velocity on back pressure for the new column I (■) and the same column after (●) 98 completed separations. Conditions: column size 100 mm × 0.2 mm. Mobile phase 0.1% TFA in water–acetonitrile (90:10, v/v).

ninth run, respectively. This also clearly confirms the robustness of the monolithic columns since their separation ability does not appear to deteriorate with either time or number of injections.

Fig. 10 compares the back pressure observed for column I immediately after preparation with that after numerous equilibrations and about 100 separation runs involving a wide variety of samples. Clearly, no difference exceeding the experimental error of the pressure sensor in our chromatographic system is detected thus demonstrating the durability of the monolithic column. Although a slow pressure build-up was observed under long term operation of the column, this was due to the accumulation of impurities from the samples in the inlet of the monolith, the original permeability was easily restored after flushing the column with the mobile phase at 100 μL/min in the direction opposite to the direction of flow used for the measurements.

4. Conclusion

The simplicity of the preparation via UV initiated in situ polymerization, the unique flow properties, and the enhanced mass transport ability of monolithic capillary columns makes them attractive as an alternative to particulate column packings for the rapid separation of proteins. Monolithic poly(butyl methacrylate–*co*-ethylene dimethacrylate) capillary columns that can be used for the efficient separation of proteins in RP-HPLC with a gradient elution constitute a viable, less expensive, and much faster alternative to packed columns. Although we report the separations of only a limited number of model proteins, the monolithic capillary columns are likely to be useful for the rapid separations of many biopolymers in several modes using optimized chemistries and gradients of suitable mobile phases. The excellent stability of these columns is also a promising feature for the highly reproducible separation of proteins and peptides within the framework of proteomic research.

Acknowledgements

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