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Rapid reversed-phase separation of proteins and peptides using optimized 'moulded' monolithic poly(styrene–*co*-divinylbenzene) columns

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Abstract

Monolithic macroporous poly(styrene–*co*-divinylbenzene) stationary phases have been prepared by free radical polymerization within the confines of 4.6-mm I.D. chromatographic columns. The optimized porous properties allow the mobile phase to flow through these columns at flow-rates of up to 10 ml/min. As opposed to the simultaneously tested columns packed with either silica or synthetic polymer beads, the monoliths exhibit only modest back pressure. The monolithic columns were able to separate mixtures of peptides and proteins in a very short time. Under the optimized conditions, the separation of five proteins can be easily achieved in less than 20 s.

Keywords: Stationary phases; LC; Poly(styrene–divinylbenzene) stationary phases; Proteins; Peptides

1. Introduction

Both preparative and analytical scale chromatography are an important part of all biotechnology processes such as the production of vaccines, drugs, growth factors, inhibitors, peptides, proteins, viruses, oligonucleotides, and many other biological products. Large preparative scale columns are often used for the separation and purification of intermediates and products, while analytical chromatography is an excellent tool for process monitoring and quality control. Rapid chromatographic separations are essential for achieving high productivity in all of these applications. Also, high throughput purification steps allow for a decrease in the number of the separation units without losing the overall productivity, thus improving the economy of down-stream processing. Fast process monitoring provides the almost immediate feedback required for running automated processes under optimal conditions. In addition, high-speed separations are mandated in other fields such as combinatorial chemistry. The rapid separation methods, such as chromatography and solid-phase extraction, are often an important part of the high throughput purification and screening.

Current chromatography is based on the use of columns packed with particulate separation media. Although the state-of-the-art columns have been developed to a high degree of perfection, HPLC of large molecules, such as peptides and proteins in columns packed with conventional beads, have some inherent limitations. The slow diffusional mass transfer of analytes within the stagnant mobile phase, which is located in the pores of the separation medium, is one of the most important constraints that limits the acceleration of the separation processes in packed columns. Several new approaches have been developed to reduce the effect of slow diffusion. For

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example, use of non-porous beads eliminates mass transfer within the pores completely. However, the very small area of these nonporous beads results in a dramatic reduction in column loading capacity [1]. Theoretical calculations have demonstrated that the enhancement of mass transport, and therefore the acceleration of chromatographic separations, can be achieved if the rate of mass transfer is augmented by convection of the mobile phase through the separation medium, rather than simple diffusion [2,3]. For example, a convective flow of only 2–5% of the mobile phase through the beads in perfusion chromatography is sufficient to substantially increase the rate of separation [4].

A further increase in the separation rate has been achieved by using monolithic separation media. These materials allow all the mobile phase to flow through their specifically designed porous structures. Typically, these monoliths include both large channels that enable unrestricted rapid flow-through of the mobile phase and a number of smaller pores that provide the surface required for the separation steps based on the analyte-stationary phase interactions. A series of excellent review articles published recently describes these approaches in detail [5–9].

At the beginning of the 1990s, we introduced rigid monolithic separation media for HPLC of large molecules that are prepared in a single step by a free-radical polymerization within a column acting as a mould [10,11]. The resulting highly porous polymer monolith is essentially the equivalent of a large, single-cylindrical particle. The initial 8-mm I.D. poly(styrene–*co*-divinylbenzene) monolithic columns have already proven to be very efficient for the fast HPLC separations of proteins, synthetic polymers, and mid-size molecules [12–14]. However, those columns required the pumping systems to be able to afford gradients at flow-rates as high as 25 ml/min. Since this high flow-rate is difficult to achieve with the typical HPLC systems, we have now prepared monolithic separation media in columns with an inner diameter of 4.6 mm that allow high flow velocities to be achieved even at moderate back pressure. Simultaneously, we have also optimized the porous properties of the monolithic columns in order to increase the loading capacity and resolution.

This communication reports our results achieved with this new generation of monolithic columns. The results demonstrate their excellent performance in the very fast reversed-phase chromatography of peptides and proteins.

2. Experimental

2.1. Chromatographic columns

Monolithic columns A (50×4.6 mm I.D.), A′ (150×4.6 mm I.D.), B (50×4.6 mm I.D.) and C (50×4.6 mm I.D.) used in this research were provided by ISCO (Lincoln, NE, USA). These columns were prepared by in situ polymerization of styrene and divinylbenzene in the presence of porogenic solvents using a modified procedure published elsewhere [12]. The loading capacities of the columns were increased from A to C. The Poros R2H column (50×4.6 mm I.D., PE, Framingham, MA, USA) was packed with 10-μm poly(styrene–*co*-divinylbenzene) beads (the manufacturer does not reveal the pore size), while the Hamilton PRP-3 and PRP-1 (150×4.6 mm I.D., Reno, NV, USA) columns contained 10-μm porous poly(styrene–*co*-divinylbenzene) beads with pore sizes of 300 and 90 Å, respectively. The macrosphere (Altech, Deerfield, IL, USA) 150×4.6 mm I.D. column was packed with 5-μm C_8 silica with a nominal pore size of 300 Å.

2.2. Chromatography

A computer-controlled ISCO HPLC system consisting of two pumps (Model 2350) and a UV detector (Model V4) was used to carry out all the chromatography. The protein recovery from the monolithic column was calculated as the percentage of the peak area of protein eluted from the column relative to the peak area of the same amount of the protein injected into a system without the column. This study was carried out using a 1-min gradient from 14% to 70% acetonitrile in water at a flow-rate of 10 ml/min. Dynamic loading capacities of the columns for bovine serum albumin were determined using a 0.1 mg/ml protein solution at a flow-rate of 10 ml/min and calculated as a value at 10% breakthrough. Protein and peptide standards were purchased from Sigma (St. Louis, MO, USA). Solvents

and other chemicals were purchased from Aldrich (Milwaukee, WI, USA).

3. Results and discussion

3.1. Hydrodynamic properties

Fig. 1 shows the back pressure in the system as a function of the flow-rate of 14% aqueous acetonitrile solution for five different HPLC columns. The two moulded monolithic columns, differing in their length, show the lowest flow resistance. The back pressure for the 50- and 150-mm columns is only 1.3 and 3.7 MPa, respectively, even at a very high flow-rate of 10 ml/min. The plots for these two monoliths are straight lines and document the stability of the material even under higher pressure. For comparison, Fig. 1 also shows the effect of flow-rate on back pressures for columns packed with commercial particulate stationary phases. Since all of the tested columns have the same diameter, their flow resistance depends only on the length of the columns, the size of the packing and its quality. Therefore, and as expected, the 150-mm long column packed with 5-μm silica beads exhibits the highest back pressure of over 20 MPa at a flow-rate of only 2 ml/min. The back pressure in the columns packed with 10-μm porous polymer beads is sufficiently lower for these columns to tolerate the flow-rate of 10 ml/min. However, the high flow-rate has an unexpected effect on the packing in the Hamilton column. The back pressure of this column does not increase linearly, as is typical for rigid materials, or exponentially, which would indicate compressible beads. Rather, the curve exhibits a distinct tendency to level off with the increase in the flow-rate. In contrast, the flow resistance of the Poros column appears to be rather high, approaching 18 MPa at 10 ml/min, despite only 50 mm of length.

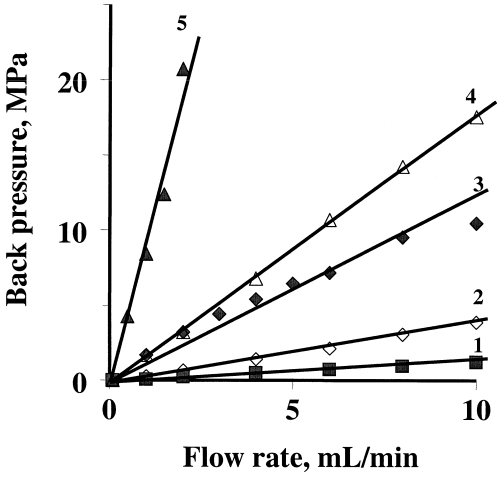

Fig. 1. Comparison of hydrodynamic properties of various columns using 14% acetonitrile solution in water. Columns: (1) Monolith A (50×4.6 mm I.D.), (2) Monolith A′ (150×4.6 mm I.D.), (3) Hamilton PRP-3 (150×4.6 mm I.D., 10 μm), (4) Poros R2H (50×4.6 mm I.D., 10 μm), (5) Alltech Macrosphere C_8 (150×4.6 mm I.D., 5 μm).

3.2. Chromatographic properties

3.2.1. 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. 2 shows breakthrough curves that were monitored for bovine serum albumin at a flow-rate of 10 ml/min. The curves are rather steep for all three of the monolithic columns. The highest dynamic binding capacity of 2.5 mg per ml of the column volume at 10% breakthrough was found for column C and

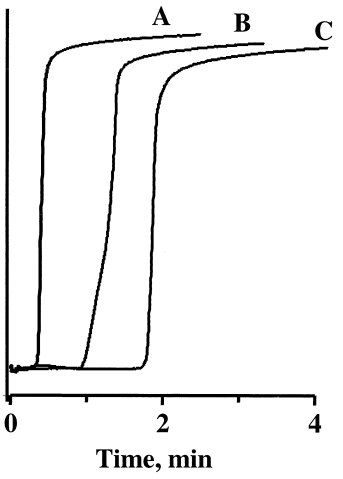

Fig. 2. Breakthrough curves of monolithic columns A, B, and C as determined with 0.1 mg/ml bovine serum albumin at a flow-rate of 10 ml/min.

results from its optimized porous properties. In contrast, a binding capacity of 0.9 mg/ml was determined for the column packed with PRP 3 beads.

3.2.2. Separation of peptides

A mixture of five peptides — bradykinine (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), leucine enkephaline (Tyr-Gly-Gly-Phe-Leu), methionine enkephaline (Tyr-Gly-Gly-Phe-Met), physalaemin (Glu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2), and substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) was used for testing the separation ability of the monolithic column. Fig. 3 shows this separation at a flow-rate of 2 ml/min and compares it with the separations achieved under comparable conditions on columns packed with C_8 silica and PRP-1 beads. The actual separation conditions used are not identical and were adjusted in order to achieve the best separation. The 5-μm silica-bead based column affords the baseline separation of all five peptides in about 3 min. In contrast, both the monolithic and PRP-1 columns do not separate the enkephalines completely. This result is most likely due to the insufficient selectivity of the monolith C and PRP-1 columns. The higher ef-

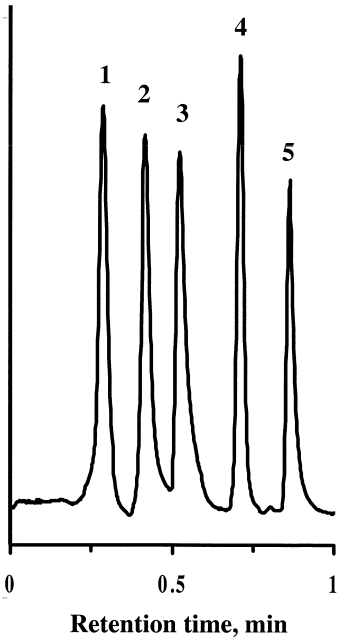

Fig. 4. Rapid separation of peptides at a flow-rate of 5 ml/min. Columns: Monolith B (50×4.6 mm I.D.); mobile phase gradient: 15–38% acetonitrile in 0.15% aqueous trifluoroacetic acid solution in 1.5 min. Peaks: bradykinin (1), leucine enkephalin (2), methionine enkephalin (3), physalaemin (4), substance P (5). Detection: UV 214 nm.

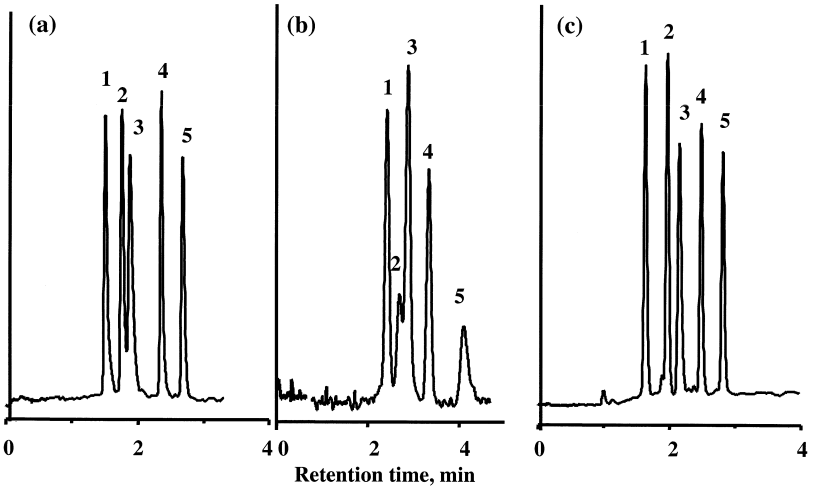

Fig. 3. Separation of peptides at a flow-rate of 2 ml/min. Columns: (a) Monolith C (50×4.6 mm I.D.); (b) Hamilton PRP-1 (10 μm, 150×4.6 mm I.D.); (c) Alltech Macrosphere C_8 Silica (5 μm, 150×4.6 mm I.D.). Mobile phase gradient: (a) 12–38% acetonitrile in 0.15% aqueous trifluoroacetic acid solution in 4.3 min; (b) 15–32% in 5.5 min; (c) 15–50% in 4 min. Peaks: bradykinin (1), leucine enkephalin (2), methionine enkephalin (3), physalaemin (4), substance P (5). Detection: UV 214 nm.

ficiency of the silica column also enhances the resolution of these closely related peptides.

The theory of gradient separation states that an equivalent separation can be achieved regardless of the variation in the flow-rate if the same gradient volume (a product of flow-rate and gradient time) is used. Since the monolithic column is characterized by a very low flow resistance, an acceleration of the separation should be possible by an increase in the flow-rate with a concomitant decrease in the gradient time. Indeed, Fig. 4 shows that a very good analytical separation of five peptides can be achieved in less than 50 s on the monolithic column at a flow-rate of 5 ml/min. This degree of acceleration is not possible with the C_8 silica column since the back pressure at a flow-rate of 5 ml/min would exceed the pressure limits typical of current HPLC instrumentation.

3.2.3. Separation of proteins

Fig. 5 shows the gradient elution of a protein mixture consisting of ribonuclease, cytochrome *c*, bovine serum albumin, carbonic anhydrase and chicken egg albumin. Using a 0.5-min gradient of acetonitrile in water and a flow-rate of 10 ml/min, corresponding to a flow velocity of 60 cm/min, all of the proteins are nearly baseline separated in less than 30 s by the monolithic columns. In addition, the peaks are quite symmetric. The separation of this mixture in the column packed with PRP-3 beads cannot be achieved under the same conditions. Only the use of a longer gradient time of 1.5 min, resulting in an increase in the overall analysis time, improves this separation. The resolution of individual peaks, however, is still worse than that observed for the separation on the monolith. This is presumably a result of mass transfer resistance of the small pore packing material. This assumption is supported by the better separation that is achieved with the column packed with Poros R2/H beads, which have much larger pores. Although separation conditions identical to those applied for the monolithic column (30 s gradient, 10 ml/min) were used, the separation by the Poros R2/H column is somewhat slower. This may be a result of an increased retention of the proteins on this stationary phase and/or a larger void volume of this type of column.

A steeper gradient compensated by a higher flow-rate (constant gradient volume) could be used for a further increase in the speed of this separation. Since

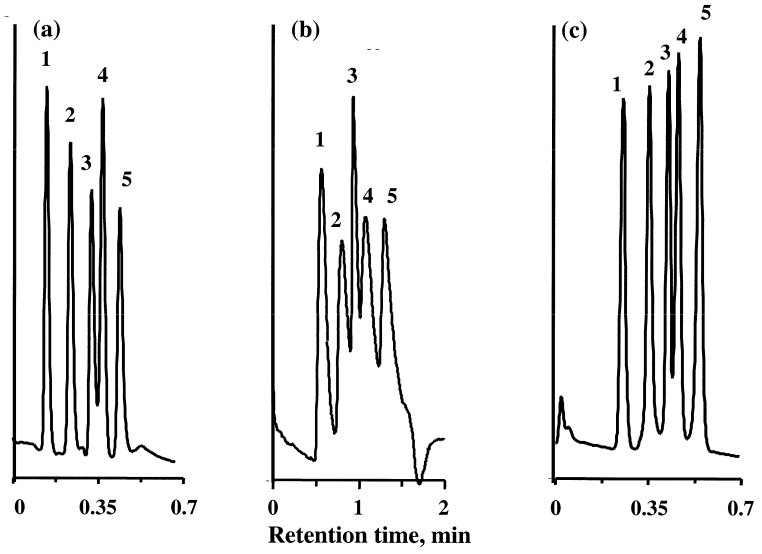

Fig. 5. Separation of proteins at a flow-rate of 10 ml/min. Columns: (a) Monolith A (50×4.6 mm I.D.); (b) Hamilton PRP-3 (10 μm, 150×4.6 mm I.D.); (c) Poros R2H (10 μm, 50×4.6 mm I.D.). Mobile phase gradient: columns (a) and (c) 30–70% acetonitrile in water with 0.15% trifluoroacetic acid for in 0.5 min; column (b) 20–60% in 1.5 min. Peaks: ribonuclease (1), cytochrome *c* (2), bovine serum albumin (3), carbonic anhydrase (4), chicken egg albumin (5). Detection: UV 280 nm.

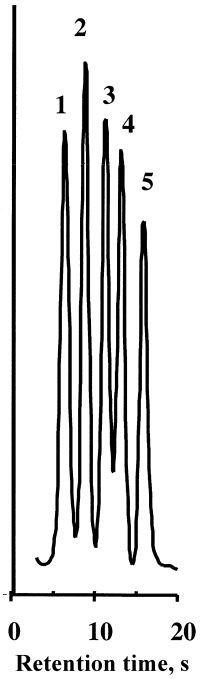

Fig. 6. Rapid reversed-phase separation of proteins at a flow-rate of 10 ml/min. Column: Monolith A (50×4.6 mm I.D.); mobile phase gradient: 42% to 90% acetonitrile in water with 0.15% trifluoroacetic acid in 0.35 min. The column was equilibrated for 1 min with 20% aqueous acetonitrile solution before each chromatographic run. Peaks: ribonuclease (1), cytochrome *c* (2), bovine serum albumin (3), carbonic anhydrase (4), chicken egg albumin (5). Detection: UV 280 nm.

the flow-rate of 10 ml/min represents the upper operational limit of this solvent delivery system, a shorter gradient time could not be achieved while the constant gradient volume was maintained. Despite this instrumentation limitation, a very good separation of five proteins, shown in Fig. 6, was achieved within 17 s at a flow-rate of 10 ml/min. It should be noted that this column was used over a period of more than 4 months and no change in either retention or recovery was observed, even after more than 250 injections.

4. Conclusion

We have demonstrated that the new generation of narrower 4.6-mm I.D. 'moulded' poly(styrene–*co*-divinylbenzene) monolithic columns can be used for rapid reversed-phase chromatography of peptides and proteins. The optimized monolithic media exhibit low back pressure at high flow velocities, steep breakthrough curves, and reasonably high loading capacities. Although much remains to be done in the development of monoliths with similar separation abilities for the preparative chromatography of biomolecules, the properties of the analytical scale columns shown here are very promising for the design and preparation of large-scale separation columns.

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