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Superficially porous silica microspheres for fast high-performance liquid chromatography of macromolecules

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Abstract

Very fast reversed-phase separations of biomacromolecules are performed using columns made with superficially porous silica microsphere column packings (''Poroshell''). These column packings consist of ultra-pure ''biofriendly'' silica microspheres composed of solid cores and thin outer shells with uniform pores. The excellent kinetic properties of these new column packings allow stable, high-resolution gradient chromatography of polypeptides, proteins, nucleic acids, DNA fragments, etc. in a fraction of the time required for conventional separations. Contrasted with <2-μm non-porous particles, Poroshell packings can be used optimally with existing equipments and greater sample loading capacities, while retaining kinetic (and separation speed) advantages over conventional totally porous particles.

Keywords: Silica; Poroshell packings; Stationary phases, LC; Porous particles; Fast high-performance liquid chromatography; Proteins; Triton X-114; Nucleic acids

1. Introduction

Porous silica is used widely as a support in columns for high-performance liquid chromatography (HPLC) in both analytical and preparative applications [1].

Commercial silica supports typically have two conformations: (1) totally porous particles typically in the 3–10 μm particle size ranges, (2) porous and non-porous ≤2 μm particles. Totally porous particles in the 3–10 μm range form the basis for most of the columns now used in HPLC. The second particle group has only recently become commercial with limited applications to date.

Totally porous particles show useful properties for separating macromolecules such as proteins and nucleic acids, and now are widely used in important applications [2].

However, columns of totally porous particles can have constraints in separation speed because of stationary phase mass transfer limitations resulting from the relatively long diffusion times required for macromolecules to traverse the porous structure for interaction with the interior stationary phase [3]. Very small (<2 μm) totally porous particles have been proposed as a means of reducing these mass transfer limitations.

Optimum uses of columns with these materials may require special apparatus and technique because of the very fast, narrow peaks formed [4–7]. Use of these particles in conventional apparatus for isocratic separations has resulted in peaks that are broader than predicted by theory because of extra-column band-broadening effects.

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Non-porous <2 μm particles with a thin skin of stationary phases (so-called pellicular particles [8]) recently have been commercialized. Columns of such particles exhibit outstanding speed for separating macromolecules because of excellent mass transfer characteristics. Unfortunately, however, columns of these particles have the similar problems as described above for very small totally porous particles. Special apparatus and operating techniques may be required for optimum results in isocratic separations because of the very sharp, narrow peaks formed by these materials. Here, extra-column band broadening can be a special problem. However, recent fast gradient elution separations of proteins and peptides have been demonstrated with conventional chromatographic equipment [9]. These non-porous particles have limited sample loading capabilities because of the low surface area available for sample interaction [6].

We have prepared particles with another conformation that may represent a practical compromise of the advantages of existing particles: superficially-porous particles. Particles of this type are composed of an ultra-pure solid silica core with a thin porous shell (hereafter called ''Poroshell'' particles). Such a general particle conformation actually was used in the first high-speed support for HPLC, developed by one of the authors and made commercially by DuPont in the late 1960s: Zipax superficially-porous support for chromatography [10,11]. However, Zipax particles were 30 μm in an overall diameter with a 1-μm shell containing 100 nm pores, and were largely designed for separating small solutes and not macromolecules. Fig. 1 shows a cartoon of a typical Poroshell particle prepared during this study. Practical processes were developed for preparing ~3–6 μm particles with porous shells of 0.1–1 μm. Two methods were developed for preparing particles of different overall diameters, pore sizes and porous shell thicknesses. All operations were carried out to maintain the purity of the silica support and minimize contamination with undesirable impurities. The resulting Poroshell particles consist of an ultra-pure, less-acidic ''Type B'' silica which has been demonstrated to be most favorable for separating polar macromolecules, especially polypeptides and proteins [12–15].

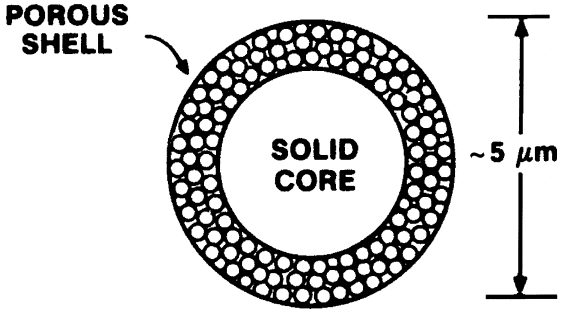

Fig. 1. Schematic of a Poroshell superficially porous particle.

2. Experimental

To create dense, ultra-pure cores of the desired size needed for Poroshell synthesis, Zorbax Rx-Sil particles (~50% porosity) [16] (Agilent Technologies, Wilmington, DE, USA) were sintered to totally dense particles by careful heating at high temperature. For example, totally porous particles of about 6.7 μm produced dense cores of about 5 μm. The Poroshell particles of this study were prepared either by a multilayering process (similar to that used to prepare Zipax superficially porous particles [11]) or by a coacervation method based on technology used to make Zorbax porous silica microspheres [17,18]. A spray-drying method previously used to prepare earlier Poroshell particles [19] was found to have the inherent disadvantage of forming some totally porous microspheres that could not be effectively separated from the desired superficially porous particles of the same size.

For particles created by the coacervate approach, the synthesis method required:

1. Forming the starting highly-purified dense silica microspheres (''cores'').
2. Coating the dense silica cores with a urea-formaldehyde/silica sol coacervate film.
3. Eliminating the urea–formaldehyde polymer from the outer coating by heating.
4. Sintering to increase particle strength and eliminate unwanted micropores.
5. Rehydrolyzing the surface of the superficially porous silica particles.
6. Size-classification by liquid elutriation.

7. Bonding of the porous shell with a suitable silane stationary phase.

The organic silane bonded stationary phase formed on these particles was in keeping with expected concentration levels. One Poroshell sample with a nitrogen surface area of 7.0 m^2/g showed a bonded sterically protected diisobutyl-C_{18} level of 0.41% carbon or a concentration of 1.9 $\mu mol/m^2$. This concentration is equivalent to results found for totally porous particles bonded with this bulky, sterically protected silane [20,21].

The thickness of the coacervate film was fixed by controlling the concentration of the silica sol used and the silica sol/urea-formaldehyde ratio. While temperature is a variable in this process, most reactions were performed at room temperature for convenience. Properly prepared particles then showed the relatively smooth surfaces illustrated in the Fig. 2 scanning electron micrograph.

The multilayered Poroshell particles were formed with a technique based on the approach pioneered by Iler [22]. Sintering, rehydrolysis, size classification and bonding were carried out in the same manner as for the coacervate-prepared particles.

For both synthesis methods, the size of the pores in the outer shell was determined by the size of the silica sols used. Particles made by both methods were made with highly purified, closely sized silica sols prepared by the controlled hydrolysis of tetraethyl-*o*-silicate with the Stöber method [23]. Small sol sizes were used to make particles with small pores, large sols for large pores. The pore size of the porous shell for the finished Poroshell particles was about one-half that of the size of the silica sols used to form the shells [24].

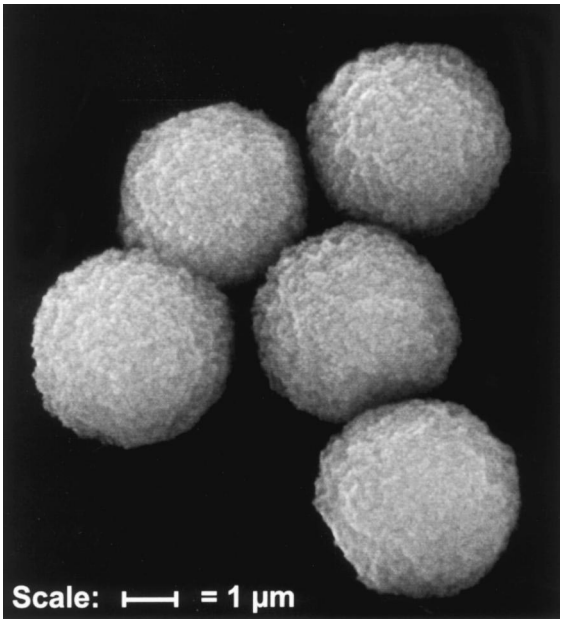

Fig. 2. Scanning electron micrograph of Poroshell particles.

To allow reaction of silicas with silanes to form stationary phases needed for separations, the surface of the sintered particles was rehydroxylated. In this study, the HF hydrolysis method generally was used, and this approach results in a fully hydroxylated surface of about 8 $\mu mol/m^2$ silanols [25].

During the processing of the Poroshell particles, some fines and aggregates can be formed. To eliminate these unwanted materials, the rehydroxylated Poroshell particles were liquid-elutriated with a procedure based on Stokes' law, similar to that described in Ref. [26].

The fully rehydroxylated and elutriated silica particles of this study were surface-modified with a monofunctional silane stationary phase. Sterically protecting silanes with the bulky side groups (e.g., diisobutyl-) largely were used to impart the silane-coated particles with unusual resistance to hydrolytic degradation at low pH — excellent column stability and separation reproducibility results [20,27]. The sterically-protected silane stationary phases also permit stable operation of columns at elevated temperatures (up to at least 90°C), for increased separation efficiency, potential changes in selectivity [28], and improved peak shapes with an increased column lifetime.

Separations were performed with Hewlett-Packard 1050 and 1100 liquid chromatographs without modification, except the use of a detector microcell and a sample injection valve with a 1 µl sample cavity. Data handling was with the Hewlett-Packard ChemStation using a peak width response time of 0.005. The very high strength of the Poroshell particles prepared by this laboratory allowed the use of conventional slurry-packing techniques for preparing efficient columns [1]. Solutes separated during this study were from Sigma (St. Louis, MO, USA) and used as received.

Particle sizes were determined with a Coulter Multisizer (Beckman-Coulter, Miami, FL, USA). Porous shell thicknesses were estimated by subtracting the mean particle diameter of finished particles from the mean particle diameter of the starting cores. Particle surface areas were determined with a FlowSorb II 2300 (Micromeritics, Norcross, GA, USA). Scanning electron micrographs were made by Micron, Inc. (Wilmington, DE, USA). Nominal particle pore diameters were estimated from the known relationship that the pore size of microspheres created by the coacervation reaction is about one-half the average diameter of the silica sols used in the reaction [24].

3. Results and discussion

3.1. Poroshell particles synthesized

Particles with a range of diameters, shell thicknesses, pore sizes and stationary phases were created during this study. Table 1 summarizes the characteristics of some of the particles produced, from 3.8 to 6.2 μm diameter, porous shells from 0.25 to 1.0 μm, pore sizes from 90 to 800 Å, and surface areas from 3.0 to 21 m^2/g.

3.2. Chromatographic properties

Columns of Poroshell particles prepared by conventional slurry packing techniques exhibit efficiency characteristics expected for such materials. The 7000–8000 plate numbers found for 100×3 mm columns of 6.2 μm Poroshell particles with 0.25-μm porous shells represent a column efficiency of about 3.5 particle diameters, which is reasonable since the packing process for these columns was not optimized. However, optimized 75×2.1 mm column packing methods for 5-μm particles with 0.25 shells of 300 Å pores produced plate heights of <2.5 particle diameters (~6200 plate number). These results suggest that the packing qualities of the very strong Poroshell particles are similar to those for typical totally porous silica microspheres in spite of the higher density of the particles with solid silica cores.

The two different synthesis methods produced particles with closely similar chromatographic properties, as illustrated in Fig. 3. Small differences in plate heights for these two preparations were due to differences in the size of the finished particles. Studies suggest that the coacervate method may be more desirable for making Poroshell particles with smaller pores, the multilayering method for particles with larger pores.

Basic insights [8] and previous experimental data [19] suggest that Poroshell particles may be better suited for rapidly separating macromolecules rather than small molecules. This is based on the fact that the diffusion of large molecules in and out of porous structures is very much slower than for small molecules. Therefore, the enhanced kinetic property of the thin porous shell for Poroshell particles is more

Table 1
Some poroshell silicas synthesized

Particle diameter (μm)	Shell thickness (μm)	Pore diameter (Å)	Surface area (m^2/g)	Preparation method[a]
4.8	1.0	90	21	C
6.2	0.5	90	10	C
5.5	0.25	90	8.4	C
3.8	0.2	90	6.9	C
5.5	0.4	200	6.3	C
5.3	0.5	300	7.0	C
3.9	0.25	300	4.1	M
6.0	0.25	300	3.8	M
4.8	0.25	300	3.0	M
4.0	0.6	800	5.3	C

[a] C=Coacervate method; M=Multilayer method.

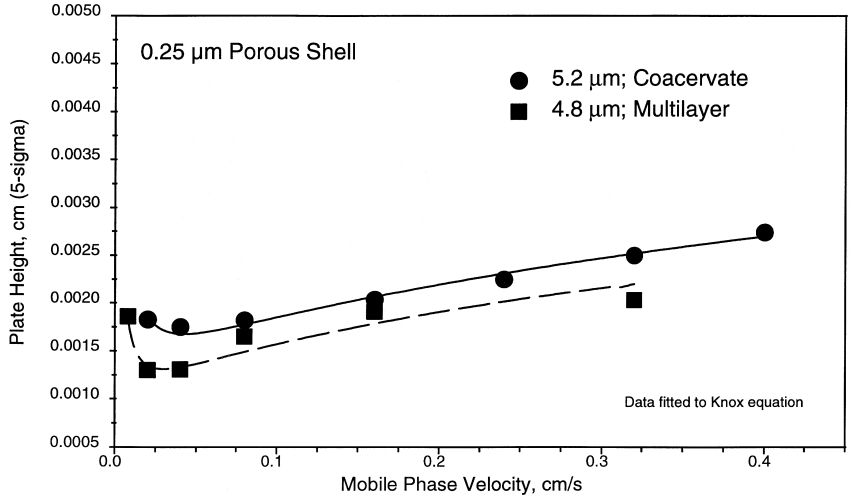

Fig. 3. Comparison of Poroshell particles from two synthetic methods: plate height versus mobile phase velocity. Columns: 75×2.1 mm, 5-µm Poroshell 300 SB-C_{18}; mobile phase: methanol–water (15:85); temperature: 23°C; solute: toluene.

advantageous for slowly diffusing large molecules than for more rapidly diffusing small molecules. The same reasoning suggests that particles with thin porous shells should show significant advantages over totally porous particles when operated at higher mobile phase velocities. This effect previously has been documented [19].

To obtain basic information on the chromatographic characteristics of Poroshell columns for macromolecules, we attempted isocratic studies with several proteins. We found that the retention factor *k* for insulin (molecular mass=5700) changes with mobile phase velocity, as shown by the plot in Fig. 4. Although the linearity of this plot may be for-

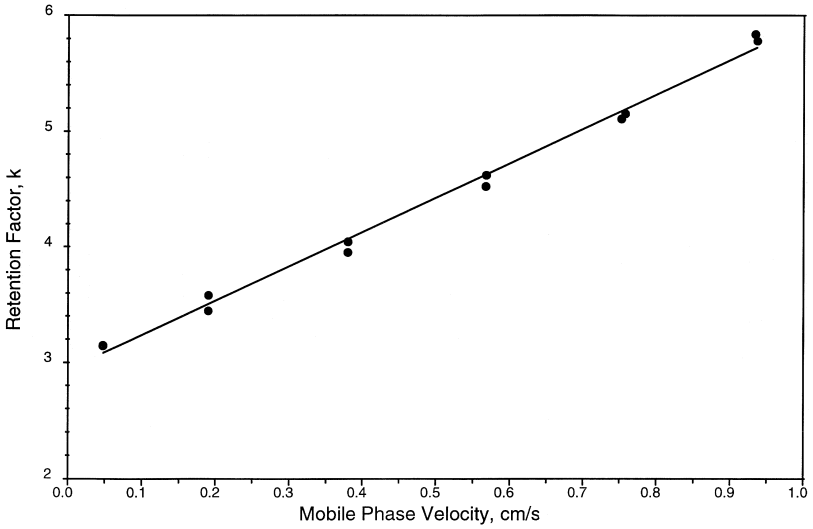

Fig. 4. Effect of mobile phase velocity on retention factor k for insulin. Column: same as for Fig. 3 except 100×3.0 mm; mobile phase: acetonitrile–aqueous 0.1% trifluoroacetic acid (28.1:72.8); temperature: 40°C; solute in 0.5% trifluoroacetic acid; UV detector: 214 nm.

tuitous, we speculate that this phenomenon is due to a conformational change of the protein with flow. With higher flow rates (and higher shear forces), the protein tends to ''unfold'', exposing more of a hydrophobic posture that interacts with the reversed-phase stationary phase for increased retention. While further tests would be needed to confirm this result, we have found similar results for carbonic anhydrase. The same effects have been noted previously by others [29].

Fig. 5 shows plate height versus mobile phase velocity data for two proteins on Poroshell columns containing 5-μm particles with 300 Å pores. The sterically-protecting diisobutyl-C_{18} stationary phase on these columns allowed stable operation at 60°C with a mobile phase containing 0.1% trifluoroacetic acid (about pH 2). Two different column conformations were tested for insulin with closely similar results. Note that the plate heights for the insulin plots show only modest increase with a large increase in mobile phase velocity. This effect is evidence of the favorable mass transfer properties of the Poroshell configuration. The result is that columns of these particles can be used with high mobile phase velocities for very fast separations of macromolecules without significant degradation in column resolution. The plot for urease (molecular mass= 83 000) is steeper and with a larger spread of experimental data, as might be predicted for the higher molecular weight solute with poorer diffusion properties.

The effect of porous shell thickness is illustrated by the protein separations in Fig. 6. With the same mobile phase, particles with the 0.25-μm porous shell (Fig. 5) show reduced peak width (and reduced retention) compared to similar particles with a 1-μm porous shell (Fig. 6). Such peak width and resolution differences would be greatly magnified with higher molecular weight proteins having poorer diffusion characteristics. The effect of temperature on plate height for insulin on a Poroshell column is shown in Fig. 7. As might be anticipated, increasing the column temperature decreases the plate height values and lessens the slope of the plate height versus mobile phase velocity plot. This effect likely is the result of improved kinetic (mass transfer) properties at higher temperatures: mobile phase viscosity is decreased and solute diffusion increased. Therefore, if macromolecular stability permits, improved separation and separation speed is anticipated at higher column operating temperatures.

Sample loading measurements were carried out on a 75×2.1 column of 3.0 m^2/g Poroshell particles with a 0.25-μm shell and 300-Å pores, using insulin

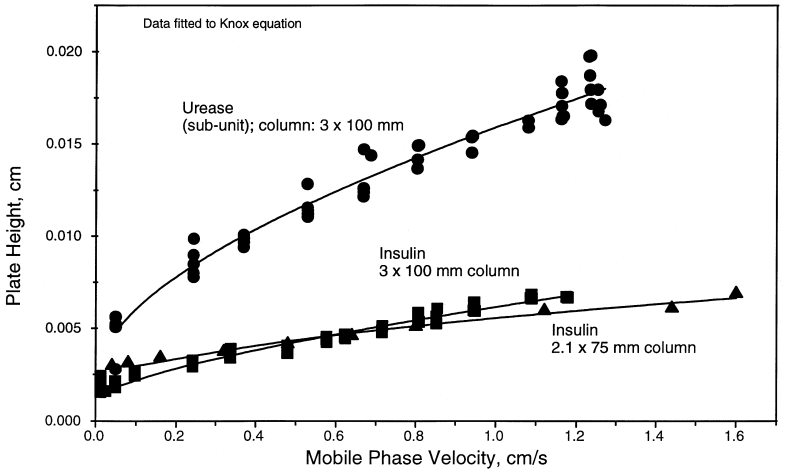

Fig. 5. Plate height versus mobile phase velocity plots for insulin and urease. Columns: 5-μm Poroshell 300 SB-C_{18}, 0.25-μm porous shell; mobile phase: urease (M_w 83 000): acetonitrile–0.1% aqueous trifluoroacetic acid (44.7:55.2); $insulin_1$ (M_w 5700): acetonitrile–0.1% aqueous trifluoroacetic acid (32.6:67.3); $insulin_2$ (M_w 5700): acetonitrile–0.1% aqueous trifluoroacetic acid (28:72); UV detector: 214 nm; samples: in 6 *M* guanidine, pH 6.8 phosphate buffer; temperature: 60°C.

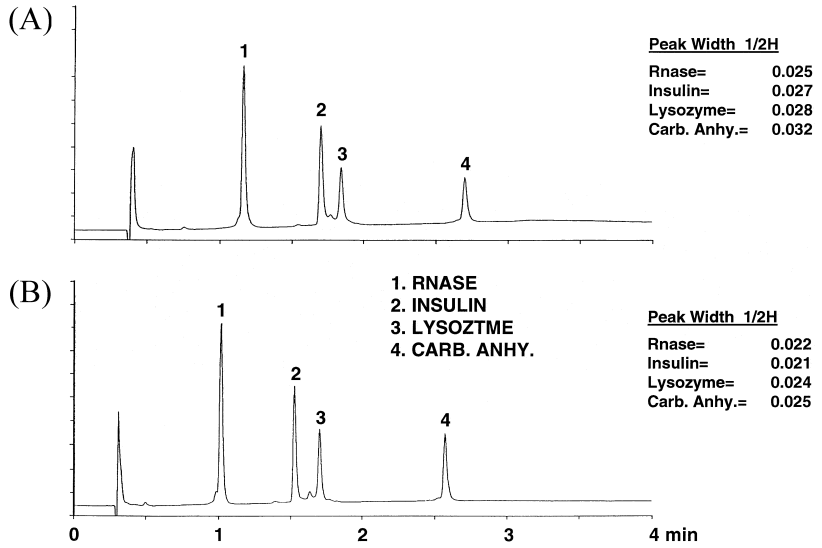

Fig. 6. Effect of porous shell thickness on protein separations. Columns: 150×4.6 mm, 5-μm Poroshell 300 SB-C_{18}; mobile phases: A=0.1% aqueous trifluoroacetic acid, B=0.09% aqueous trifluoroacetic acid; gradient: 23–53% B in 2.5 min; flow rate: 4.0 ml/min; UV detector: 215 nm; temperature: 60°C. Upper plot: 1-μm porous shell; lower plot: 0.25-μm porous shell.

as the test compound. The results given in Fig. 8 show that little change in retention or plate height occurred with up to about 0.1 μg of solute injected, with significant changes only taking place after injecting more than 0.5 μg. The improved sample loading capacity of these Poroshell particles relative

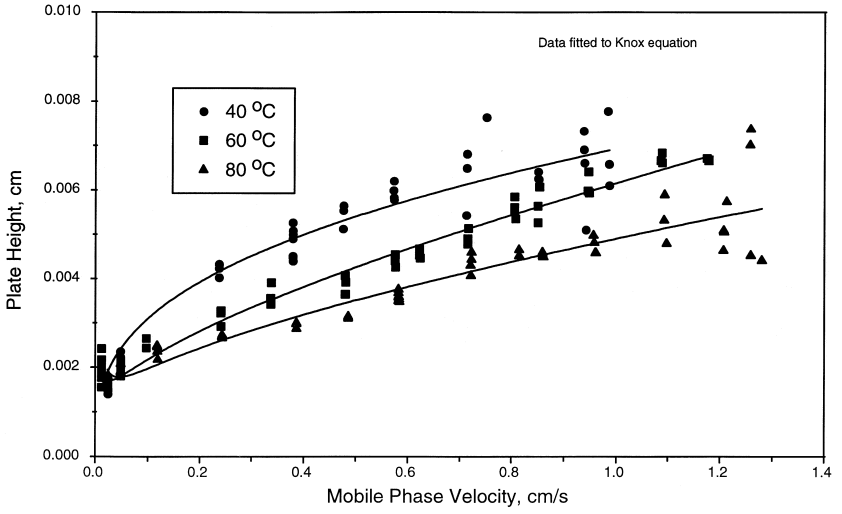

Fig. 7. Effect of temperature on Poroshell column performance. Column: 100×3.0 mm, 5-μm Poroshell 300 SB-C_{18}, 0.25-μm shell; mobile phase: 32.6% acetonitrile–aqueous 0.1% trifluoroacetic acid (32.6:67.4); UV detector: 214 nm; sample: 1.2 μl, 50 μg/ml insulin (M_w=5700) in 6 *M* guanidine with pH 6.8 phosphate buffer.

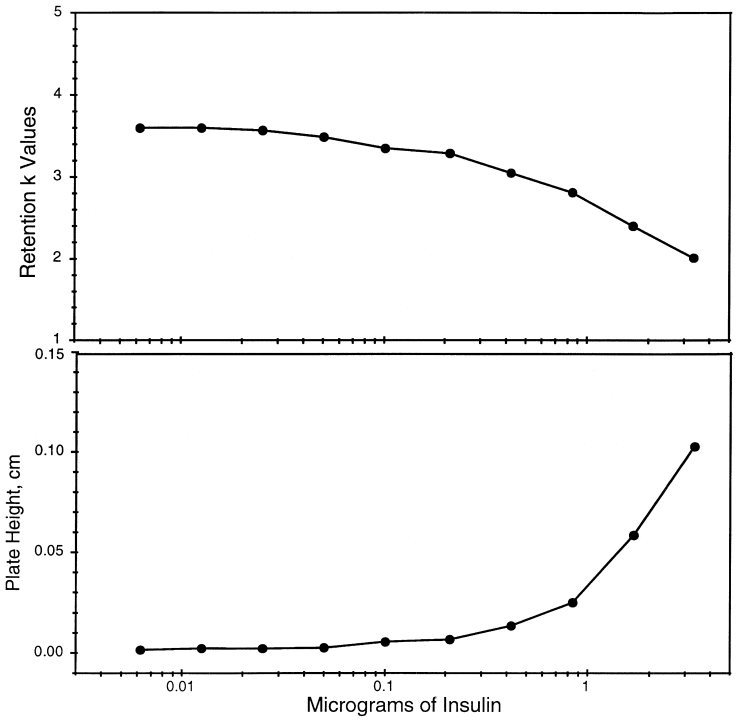

Fig. 8. Column sample loading study. Column: 75×2.1 mm, 5-μm Poroshell 300 SB-C_{18}, 0.25-μm porous shell (surface area: 3.0 m^2/g); isocratic mobile phase: acetonitrile–0.1% aqueous trifluoroacetic acid (25:75); flow rate: 0.2 ml/min; temperature: 60°C; detector: 215 nm.

to that for nonporous microparticles is a direct result of the higher surface area. Still higher loading capacities are found with Poroshell particles with higher surface areas. The higher loading capacity of Poroshell particles is a favorable property when isolations of purified fractions are required for other characterization studies.

4. Applications

Fig. 9 is an illustration of the speed and efficiency of a Poroshell column for separating protein mixtures. This synthetic mixture of nine proteins was gradient-separated in less than 2 min, with excellent peak shapes and resolutions exceeding 1.5 for all

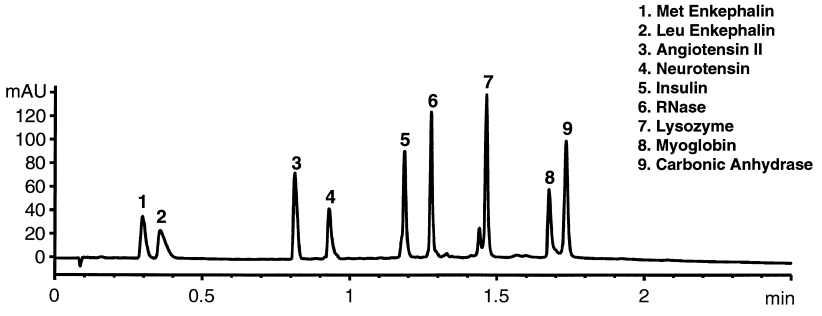

Fig. 9. Rapid separation of protein mixture. Column: same as for Fig. 8; mobile phases: A=acetonitrile–0.1% aqueous trifluoroacetic acid (5:95); B=acetonitrile–aqueous trifluoroacetic acid (95:5); gradient: 2% B to 65% B in 2.0 min; flow rate: 2.0 ml/min; temperature: 35°C.

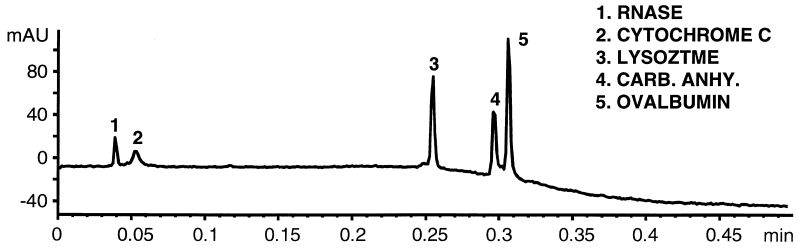

Fig. 10. Ultra-fast separation of proteins. Column: same as in Fig. 8; mobile phase: A=acetonitrile with 0.1% trifluoroacetic acid, B=0.1% trifluoroacetic acid; gradient: 25% acetonitrile to 65% acetonitrile in 0.1 min; flow rate: 4.0 ml/min; temperature: 80°C; pressure: 295 bar; detector: 215 nm.

peaks. Even faster separations of such compounds are possible at higher mobile phase velocities (higher flow rates) and elevated column temperatures because of the excellent kinetic properties of the Poroshell particles, as shown in Fig. 10. This separation of proteins was completed in about 20 s, again with good peak shapes. At this high mobile phase flow rate (4.0 ml/min for a 2.1 mm I.D. column; velocity ~3.2 cm/s), the pressure of the column was 295 bars, well within the operating range of modern pumping systems. The speed of this separation with the sharp, narrow (low volume) peaks actually challenged the integrity of the data handling system used, as noted by some of the asymmetrical peak shapes found. This separation is comparable in separation time and resolution to that reported for <2-μm non-porous particles [8]. Very small, non-porous particles have been claimed as the ultimate column packing for rapid HPLC separations. However, these materials have limited sample loading (because of very low surface area) and may require special HPLC apparatus for optimum performance in isocratic separations [6].

Rapid separations of more complicated mixtures also can be performed with Poroshell columns, as shown in Fig. 11. This separation of a pBr 322 Hae III digest with up to 587 base pairs was separated sufficiently in less than 14 min to identify all of the components in this mixture. It is not known whether the 300-Å pores in the Poroshell particles used actually is optimum for a separation of such components. Wider-pore particles may be better suited for resolving the higher-molecular mass components of this mixture.

Poroshell columns also can be effectively used for rapid separations of polymer oligomers and other macromolecules of this type. Fig. 12 shows a gradient chromatogram of Triton X-114 that was

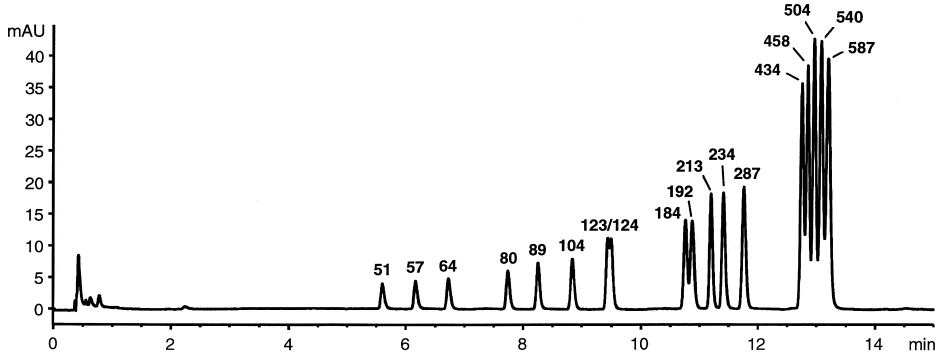

Fig. 11. Separation of pBr 322 Hae III digest with Poroshell column. Column: same as in Fig. 8; mobile phase: A=100 m*M*, pH 7, triethylamine acetate, 0.1 m*M* ethylenediaminetetracetic acid, B=A–acetonitrile (50:50); gradient: 17.5% B to 37.5% B in 15.0 min; flow rate: 0.4 ml/min; temperature: 50°C; UV detector: 214 nm.

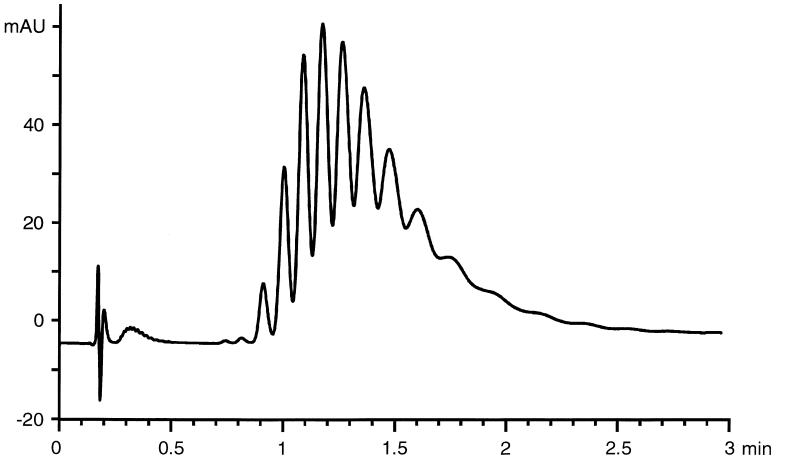

Fig. 12. Separation of Triton X-114 oligomers. Column: same as in Fig. 8; mobile phase: A=methanol–water (45:55), B=methanol; gradient: delay 1.0 min, then 0–5% B in 2.0 min; flow rate: 0.5 ml/min; temperature: 24°C; UV detector: 225 nm.

completed in less than 3 min with individual components clearly visible for identification.

5. Conclusions

Superficially porous silica particles (''Poroshell'') with solid cores and thin porous outer shells have been synthesized and evaluated as column packings for fast reversed-phase HPLC separations of macromolecules. Two different methods have been developed for preparing ultra-pure silica particles with different sizes (~ 3–6 μm), pore sizes (90–800 Å) and porous shell thicknesses (0.25–1.0 μm). These particles show excellent kinetic properties that make them especially attractive for the rapid, high efficiency separation of macromolecules. Because of the very small solute diffusion distances in the thin porous shell, very fast separations of both natural and synthetic macromolecules have been obtained by working at high mobile phase velocities.

Gradient separations of polypeptides, proteins, DNA fragments, etc. can be performed in a fraction of the time required for conventional separations. Contrasted with columns of non-porous <2-μm particles, columns of Poroshell particles have higher sample loading capacities and can be used in existing equipment using ordinary techniques. Poroshell columns appear useful for rapidly monitoring preparative separations of genetically engineered macromolecules, process control monitoring of fermentation broths or cell-reaction systems, quantifying polymerase chain reaction fragments, monitoring protein conjugation reactions, and similar applications where very fast, reproducible separations are required.

References

[1] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, 2nd ed, Wiley, New York, 1979, Ch. 5.
[2] C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins, CRC Press, Boca Raton, FL, 1991.
[3] W.W. Yau, J.J. Kirkland, D.D. Bly, Modern Size-Exclusion Liquid Chromatography, Wiley, New York, 1979, Ch. 2.
[4] N.D. Danielson, J.J. Kirkland, Anal. Chem. 59 (1987) 2501.
[5] K. Kalghatgi, Cs. Horvath, J. Chromatogr. 443 (1988) 343.
[6] H. Moriyama, M. Anegayama, K. Komiya, Y. Kato, J. Chromatogr. A 691 (1995) 81.
[7] E. Tanaka, M. Terada, T. Nakamura, S. Misawa, C. Wakasugi, J. Chromatogr. B 692 (1997) 405.
[8] Cs. Horvath, S.R. Lipsky, J. Chromatogr. Sci. 7 (1969) 109.
[9] T. Issaeva, A. Kourganov, K. Unger, J. Chromatogr. A 846 (1999) 13.
[10] J.J. Kirkland, Anal. Chem. 41 (1969) 218.
[11] J.J. Kirkland, US Pat. 3 505 785 (1970).
[12] J. Köhler, J.J. Kirkland, J. Chromatogr. 385 (1987) 125.
[13] J.L. Glajch, J.J. Kirkland, J. Köhler, J. Chromatogr. 384 (1987) 81.
[14] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd ed, Wiley, New York, 1997, Ch. 5.

[15] M.A. Stadalius, J.S. Berus, L.R. Snyder, LC·GC 6 (1988) 494.
[16] J.J. Kirkland, C.H. Dilks Jr., J.J. DeStefano, J. Chromatogr. 635 (1993) 19.
[17] J.J. Kirkland, F.A. Truszkowski, patent applied for (2000).
[18] J.J. Kirkland, J. Köhler, US Pat. 4 874 518 (1989).
[19] J.J. Kirkland, Anal. Chem. 64 (1992) 1239.
[20] J.J. Kirkland, J.L. Glajch, R.D. Farlee, Anal. Chem. 61 (1989) 2.
[21] J.J. Kirkland, J.W. Henderson, J. Chromatogr. Sci. 32 (1994) 473.22.
[22] R.K. Iler, US Pat. 3 485 658 (1969).
[23] W. Stöber, A. Fink, E. Bohn, J. Colloid Interface Sci. 26 (1968) 62.
[24] J.J. Kirkland, J. Chromatogr. 125 (1976) 231.
[25] J. Köhler, J.J. Kirkland, J. Chromatogr. 385 (1987) 125.
[26] C.D. Scott, Anal. Biochem. 24 (1968) 292.
[27] J.L. Glajch, J.J. Kirkland, US Pat. 4 705 725 (1984).
[28] B.E. Boyes, J.J. Kirkland, Peptide Res. 6 (1993) 249.
[29] M.A. Stadalius, H.S. Gold, L.R. Snyder, J. Chromatogr. 296 (1984) 31.