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Atypical silica-based column packings for high-performance liquid chromatography

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Abstract

Column packings widely used for high-performance liquid chromatography (HPLC) mostly are based on porous silica microspheres with certain pore sizes and pore size distributions. Such materials have the most desirable compromise of properties that provide for effective and reproducible separations over a wide range of operating conditions. To provide desired separation characteristics, several manufacturers specially synthesize the silica particles for these packings. While such column packing materials have general utility for a wide range of needs, special silica-based particles have been synthesized with different physical conformations for special separation goals. This presentation describes some atypical types of silica-based particles with unique separation properties that enlarge the capabilities of HPLC methods.

Keywords: Silica-based column packings; Stationary phases, LC

1. Introduction

Currently, the most widely used column packings for high-performance liquid chromatography (HPLC) separations are based on 3–5-μm totally-porous silica microspheres with a single pore-size distribution [1]. This usage is based on almost 30 years of experience where it has been found that these materials have, on balance, the most desirable properties for conducting most separations. Small molecules such as drugs generally are separated with packings having pores in the 80–100 Å range, while larger molecules such as proteins often require particles with pores larger than 200 Å. Silica continues to be the favorite medium for HPLC column packings because of the combination of several important potential characteristics:

- Can be formed into a wide variety of shapes and sizes (etc., microspheres).
- Surface easily modified by certain covalent reactions.
- Can be created with desired pore sizes.
- Narrow pore size distributions can be formed with no ''ink-bottle'' pores.
- Compatible with water and all organic solvents.
- Can be made physically strong.
- Super-pure particles can be made with almost neutral pH surface.

To expand the utility of HPLC, several silica particle types other than the popular porous silica microspheres have been developed. These largely have been limited to applications where conventional totally porous silica microsphere column packings do not function well. For example, ≤2 μm non-porous

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silica microspheres have been made commercially available, and these have interesting properties for very rapid separations, especially for macromolecules having poor diffusional characteristics [2,3]. Another example is monolith columns of silica that include both large channels (which enable unrestricted rapid flow-through of the mobile phase) and smaller pores (which provide the surface required for separations) [4].

This report describes some other atypical silica structures that have been developed for special needs in HPLC applications. These materials were prepared in the author's laboratory during the last 30 years, suggesting the shift in separation needs and trends that have occurred during the development of HPLC as the most practiced analytical method available to scientists.

2. Experimental

Separations in this laboratory were performed with Model 1100 liquid chromatographs equipped with microsampling valves and micro (1.8 μl) detector cells (Agilent Technologies, Wilmington, DE, USA). Data handling was with the Agilent Technologies ChemStation using a detector response time of 0.005. Gradient separations were performed with a capillary bypassing the normal mobile phase mixer system to minimize gradient dwell volumes. Zorbax 300 SB-C18 and Poroshell 300 SB-C18 columns were from Agilent Technologies. Solutes were obtained from Sigma (St. Louis, MO, USA) and used as received. Scanning electron micrographs were made by Micron (Wilmington, DE, USA).

3. Results and discussion

3.1. Properties of original superficially porous particles

Early HPLC columns were composed of irregularly-shaped silica particles of the type used widely in packed gas chromatography columns of that time. However, in the late 1960s, superficially porous silica particles of approximately 40 μm were made available as the first material specifically synthesized for HPLC separations [5]. These particles, shown schematically in Fig. 1, were composed of a solid glass bead core and a 1-μm outer shell of silica with ~1000 Å pores. These Zipax superficially porous particles were commercialized by the DuPont Company in packed columns of 50 and 100 cm initially for liquid–liquid separations [6], but shortly afterwards for normal- and reversed-phase separations with covalently-attached silane stationary phases [7]. However, the advent of 5–10-μm porous silica microspheres in the early 1970s (e.g., Ref. [8]) quickly led to the widespread use of these materials as HPLC column supports because of superior efficiency and separation speed.

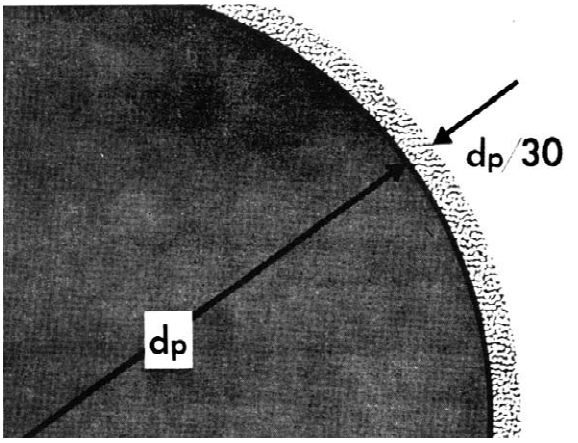

Fig. 1. Schematic of superficially porous particle.

3.2. Bimodal silica particles

Interest in the synthesis of new silica particle configurations continued so that during the mid-1970s, a new concept for size-exclusion liquid chromatography (SEC) led to another silica particle type. Prior to that time, SEC was performed with a collection of serial-connected columns with as many different pore sizes as possible (typically four or five different columns with increasing pore sizes). This collection of polymodal columns led to plots that only approximated linear retention volume versus log molecular mass calibrations that are desired for accurately calculating the molecular mass distributions of polymer samples. However, fundamental studies showed that truly linear retention volume

versus molecular mass calibrations could be obtained with the use of only two (bimodal) pore sizes, one small, one large, with about a decade of difference in pore size [9].

To demonstrate this concept, silica particles were synthesized with two different pores, again with about a decade difference in size. These particles, depicted in the schematic of Fig. 2A, showed the bimodal pore size distribution in Fig. 3. With columns of such particles, linear retention volume versus log molecular mass plots were obtained for a wide molecular mass range (over four decades of molecular mass), as compared to the inferior calibration plot available from a conventional polymodal SEC column set, as shown in Fig. 4.

Superficially porous particles with a bimodal pore size distribution also were synthesized, as shown in Fig. 2B [9]. Here, as shown on the left, solid microparticles were gathered as a shell around an impervious solid macroparticle core so that larger macropores were formed between the microparticles. Shown in the magnified schematic at the right of Fig. 2B, each microparticle then functioned as an impervious microcore on which was gathered a shell of ultramicroparticles to form micropores between these microparticles. The macropores and micropores formed between the spherical microparticles and ultramicroparticles then produced the bimodal pore size distribution of the total particle.

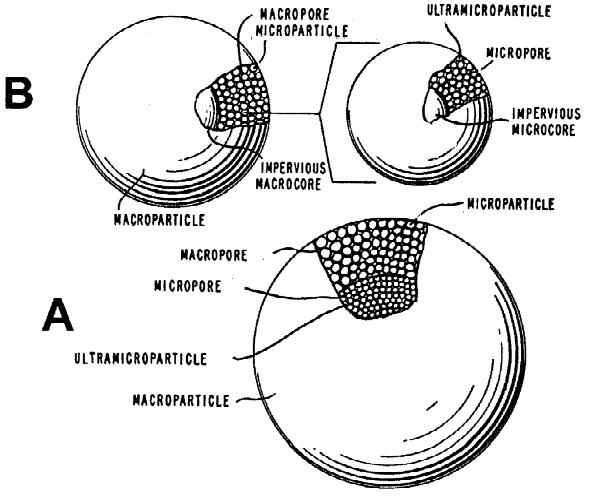

Fig. 2. Schematics of bimodal silica particles. (A) Totally porous particle. (B) Superficially porous particle.

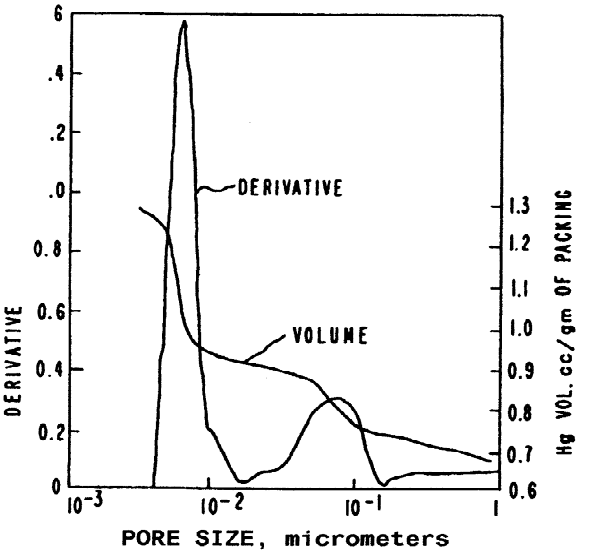

Fig. 3. Pore size distribution of bimodal totally porous silica particles by mercury intrusion.

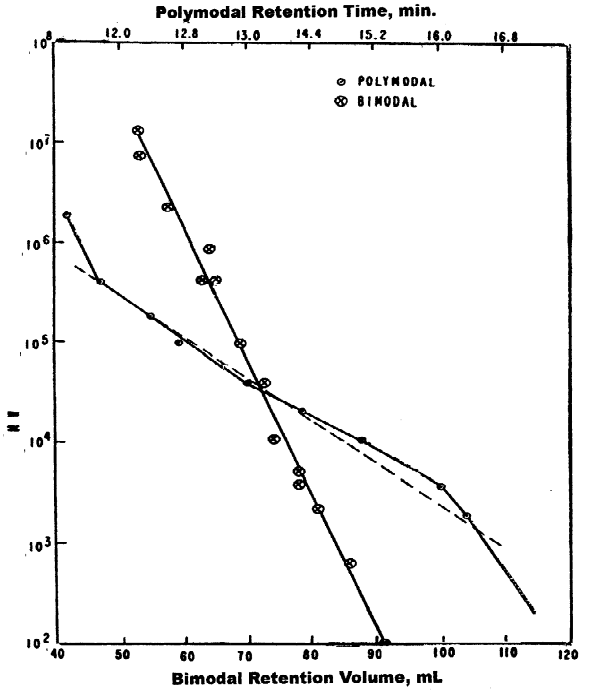

Fig. 4. Polystyrene calibration plots for silica-based size-exclusion columns.

The bimodal silica particles of Fig. 2 were not commercialized, however, since it was found more convenient (and less expensive) to connect two columns of single pore sizes with the required decade difference. Linear calibration columns now are widely available in which two columns with different appropriate pore sizes are connected, or in which particles with the two different pore sizes are intimately mixed in a single column. Trimodal pore size column assemblies can be prepared using an extension of this technology so that at least six decades of molecular mass calibration linearity can be obtained [10]. Other applications of bimodal pore size silicas now have been reported, for example, in perfusion chromatography [11]. Bimodal pore size distribution silica also has been synthesized for non-chromatographic purposes [12].

3.3. Gigaporous silica particles

Interest in separating very large molecules, especially macromolecules of biological interest, led to the development of gigaporous particles of silica. These ~20-μm particles, shown in Fig. 5, were prepared by a selective aggregation of a high-purity silica sol with a narrow pore size distribution. Note in Fig. 5 that the internal pores are the spaces between the aggregated individual 8000 Å silica sol that make up the total particles. The pore size distribution of these porous silica particles as measured by mercury intrusion is shown in Fig. 6. These particles have 4000 Å pores that permit very large molecules to enter the pore structure either for SEC separations or for separations by other mechanisms.

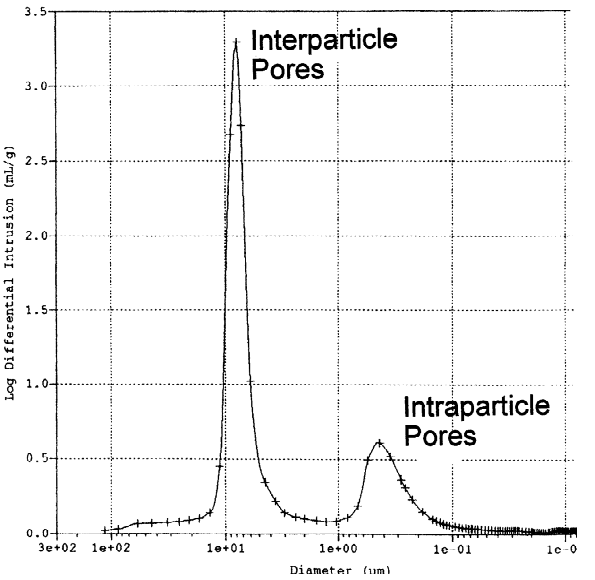

Fig. 6. Pore size distribution of gigaporous silica particles by mercury intrusion.

Fig. 7 shows a SEC log molecular mass versus retention volume calibration for these particles using narrow polystyrene standards. Note that the calibration shows that these particles still do not exclude a 25 000 000 molecular mass polystyrene standard,

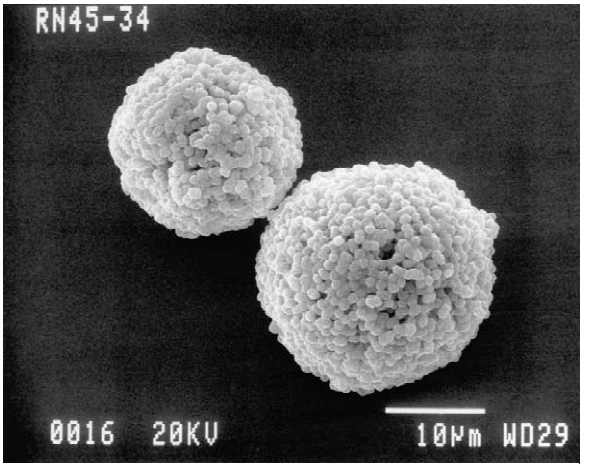

Fig. 5. Scanning electron micrograph of gigaporous silica particle.

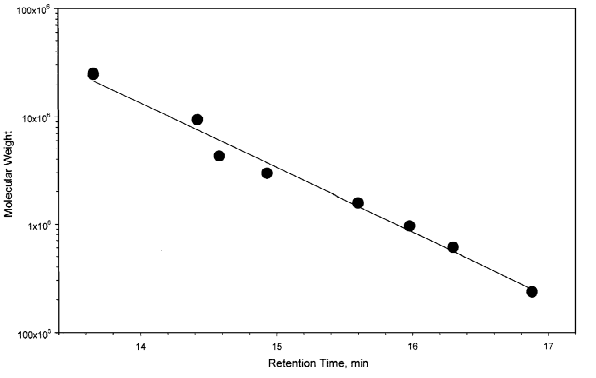

Fig. 7. Polystyrene standard size-exclusion calibration for gigaporous particles. Particles: 20 μm, 4000 Å pores, with diol stationary phase; column: 25×0.62 cm; mobile phase: tetrahydrofuran; flow-rate: 0.30 ml/min; temperature: ambient.

demonstrating their capability to admit very large molecules to the pore structure without hindrance. These same particles were covalently coated with a diol stationary phase and used to obtain a SEC chromatogram of λ-viral DNA, as shown in Fig. 8. The V_0 peak in this chromatogram represents strongly UV-absorbing, low-molecular-mass impurities that were present in this particular sample. Gigaporous particles such as these with a narrow pore size distribution present interesting new opportunities for rapidly separating very large molecules that are not well handled with existing materials. Other gigaporous silica particles also have been examined for protein separations [13].

3.4. Modern superficially porous particles

Superficially porous particles such as the Zipax material previously discussed have fundamental kinetic properties that make them attractive for ultrafast separations [3,14]. The thin porous shell of these particles allows very rapid access of large macromolecules to interactive surfaces within the porous structure. The result is that high mobile phase velocities can be used for very fast separations with good column efficiency. A range of small superficially porous silica microspheres now have been synthesized, and studies have shown that 5-μm superficially porous particles with 0.25-μm-thick shells and 300 Å pores (hereafter called Poroshell 300) allow ultra-fast separations of macromolecules such as polypeptides and proteins [3,14]. A schematic of such a particle is shown in Fig. 9. The synthesis of Poroshell silica particles has recently been described [14].

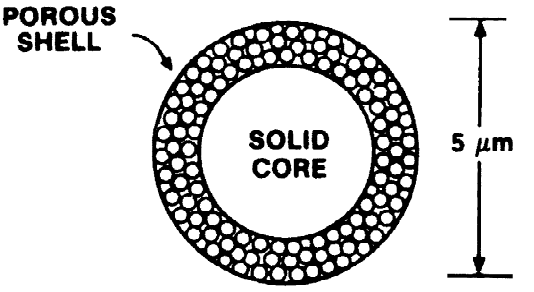

Fig. 9. Schematic of small superficially porous silica particle.

When compared to totally porous particles (Zorbax 300) of the same size with the same separating system, Poroshell columns show much lower loss of column efficiency with increasing mobile phase velocity, as shown in Fig. 10. For this solute of 13 600 molecular mass, the column efficiency of the Poroshell column is almost twice that of a column of totally porous particles at high mobile phase velocities. (As a frame of reference, a 12

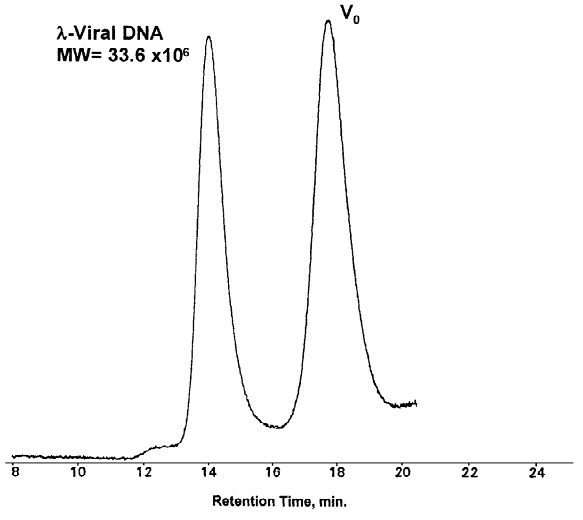

Fig. 8. Size-exclusion separation of λ-viral DNA with gigaporous particles. Conditions as in Fig. 7 except, mobile phase: 10 m*M* Tris, pH 7.5, 5 m*M* NaCl, 0.1 m*M* EDTA.

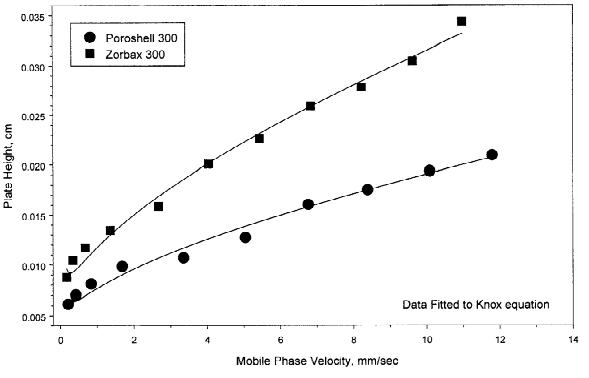

Fig. 10. Plate height vs. mobile phase velocity: totally porous vs. superficially porous particles. Columns: 75×2.1 mm Zorbax 300 SB-C18 and Poroshell 300 SB-C18, 5 μm particles; temperature: 50°C; solute: ribonuclease A, M_r=13 600; Zorbax 300 mobile phase: acetonitrile–0.1% trifluoroacetic acid (26:74); Poroshell 300 mobile phase: acetonitrile–0.1% trifluoroacetic acid (24:76).

mm/s mobile phase velocity is about eightfold greater than the mobile phase velocity for the plate height minimum with a typical small molecule.) This improved efficiency at higher mobile phase velocities is a direct result of the better kinetics associated with the thin porous shell of the Poroshell structure. Actually, the difference in relative column efficiency between Poroshell and totally porous particles at higher mobile phase velocities is greatly amplified as the molecular mass of the solute increases [14].

A practical difference between superficially porous and totally porous particles is illustrated in Fig. 11 for fast separations of a mixture of peptides and proteins. With the same particle size and operating conditions, the Poroshell column shows a much better separation for this <30 s run, illustrating the superior kinetics of the superficially porous structure. This effect also is shown in Fig. 12 which compares fast separations of a bovine serum albumin (BSA) digest with columns of totally porous 5-μm Zorbax 300 particles and superficially porous Poroshell 300 particles under the same operating conditions. About one-third more peaks are observed in the separation with the Poroshell column compared with the column of totally porous particles of the same size, using the same operating conditions. This effect largely is due to the superior kinetic properties of the superficially porous particles. A comparison of superficially porous, totally porous and nonporous particles has been given [3].

Poroshell packings necessarily have a lower surface area than totally porous particles, and this characteristic results in some interesting features. As expected, solute retention is less because of a lower amount of stationary phase for reversed-phase interactions. The effect of percent organic modifier (%B) on solute retention can be described by the relationship:

$$\log k = \log k_w - S\phi \quad (1)$$

where, k is the solute retention factor, k_w is the hypothetical retention factor with no organic modifier (0% B), S is the slope of the log k vs. %B plot, and ϕ is the volume fraction of organic modifier. Fig. 13 shows that the slope for superficially porous particles is significantly less steep ($S=5.8$) than for non-porous particles ($S=8.2$) and more like that for totally porous particles ($S=4.7$). Therefore, solute retention for Poroshell 300 particles is less affected by small changes in %B (and probably also changes in temperature and pH for ionizable solutes). These results suggest that columns of Poroshell particles might allow more reproducible and repeatable separations in routine operations than columns of nonporous particles for the same solutes.

As predicted by theory, however, The slope of log k versus %B plots are strongly dependent on the size of the solute. Fig. 14 shows plots for a small solute ($M_r=158$) and a protein ($M_r=13\,600$), with the latter showing a much steeper plot, even when

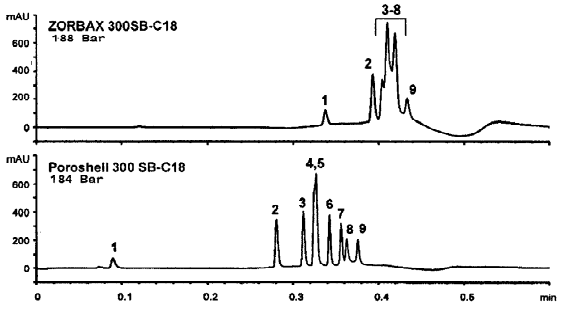

Fig. 11. Ultrafast separation of peptides and proteins: totally porous vs. superficially porous particles. Columns: 75×2.1 mm, 5 μm; gradient: 0–100% B in 0.25 min, A – 0.1% trifluoroacetic acid, B – 0.07% trifluoroacetic acid in acetonitrile; flow-rate: 4.0 ml/min; temperature: 70°C; UV detection at 215 nm; sample injected: 1 μl (125–500 μg/ml).

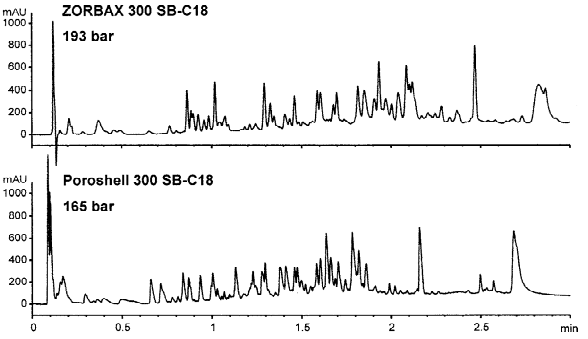

Fig. 12. Rapid separation of BSA tryptic digest; totally porous vs. superficially porous particles. Columns: 75×2.1 mm, 5 μm; gradient: 0–100% B in 6.0 min, A – 0.1% trifluoroacetic acid, B – 0.07% trifluoroacetic acid in acetonitrile; flow-rate: 2.0 ml/min; temperature: 70°C; UV detection at 215 nm; sample: 10 μl of 5 h incubation digest.

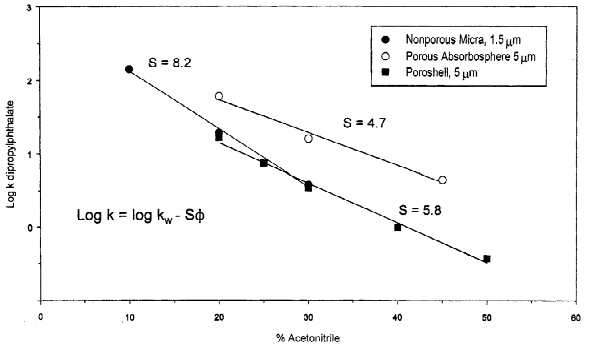

Fig. 13. Effect of mobile phase composition on elution. Columns: Micra C_{18}, 33×4.6 mm; Absorbosphere C_{18}, 150×4.6 mm, flow-rates: 1.0 ml/min; Poroshell 300 SB-C18, 75×2.1 mm, flow-rate, 0.25 ml/min; temperature: ambient.

chromatographed at a much higher temperature.

Because of the modest surface area of Poroshell 300 (~5 m^2/g), it was of interest to determine the effects of solute loading where this might be important, such as in isolations of small amounts of purified solutes for identification and other studies. As shown in Fig. 15, retention time values for ovalbumin (M_r = 45 000) did not vary (within experimental error) when up to 90 μg was injected with a 1.0 min gradient using a flow-rate of 1.0 ml/min (mobile phase velocity of ~4 mm/s). Peak

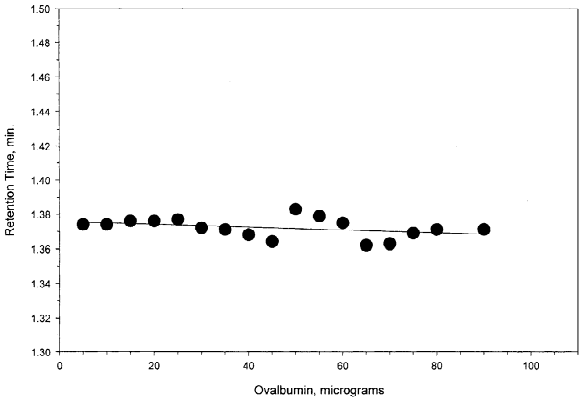

Fig. 15. Solute loading for superficially porous particles. Column: 75×2.1 mm Poroshell 300 SB-C18; gradient mobile phase: A – 0.1% trifluoroacetic acid in water, B – 0.07% trifluoroacetic acid in acetonitrile, 5–100% B in 1.0 min; flow-rate: 1.0 ml/min; temperature: 70°C; sample: 1.0 μl; UV detection at 240 nm.

width was constant up to about 50 μg of ovalbumin and increased only slightly at 100 μg, as shown in Fig. 16A. However, Fig. 16A also shows that angiotensin II (M_r = 1046) displays much increased peak widths at much smaller micrograms of solute loading. This effect appears strange at first, but is explained adequately by the plots in Fig. 16B where the amount of solute is described in nanomoles rather than micrograms. Here, the effect of band width is

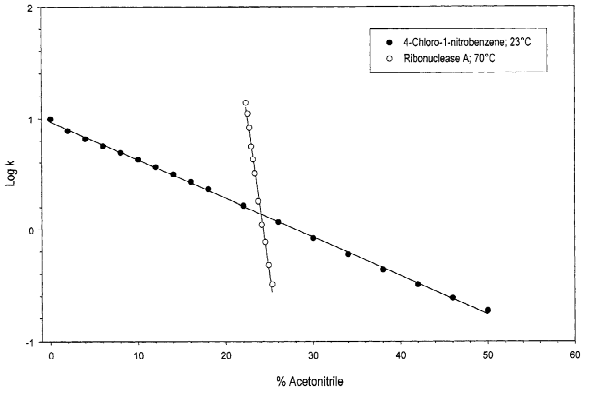

Fig. 14. Comparison of log *k* vs. %B plots for solutes with different molecular masses. Column: 75×2.1 mm Poroshell 300 SB-C18; 4-chloro-1-nitrobenzene mobile phase: acetonitrile in water; ribonuclease A mobile phase: 0.1% trifluoroacetic acid in acetonitrile, pH 2.

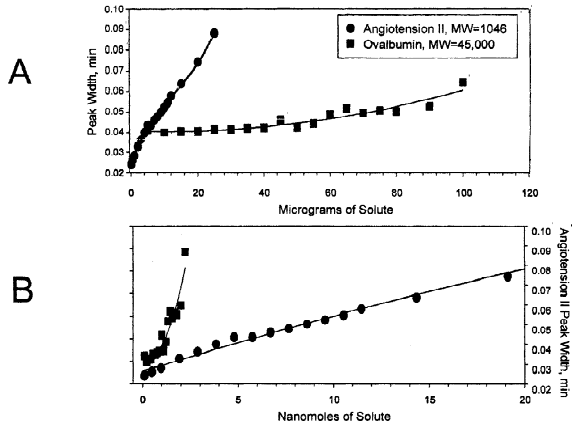

Fig. 16. Solute loading for superficially porous particles: effect of molecular mass. (A) Micrograms of solute. (B) Nanomoles of solute.

much more prominent for the higher molecular mass ovalbumin than for the lower molecular mass angiotensin II. One could speculate that band broadening to increased sample load is a function of the surface area occupied by the solute; large (high molecular mass) solutes occupying a larger surface area per molecule than a small solute. Therefore, on a molar basis, large molecules would occupy a larger surface area of the packing, making the column more susceptible to localized sample loading effects.

It is of particular interest to know whether peptides, proteins and other compounds that might be sensitive to unwanted interaction with silica supports are eluted unchanged from high-speed superficially porous Poroshell 300 columns. To test this effect, a series of such macromolecules were chromatographed with a Poroshell 300 column. The peak areas for these compounds then were compared with runs under identical conditions except that a short capillary was substituted for the column (no column). The results of these tests are summarized in Table 1, and show that, within experimental error, test compounds were eluted without loss. These results suggest the ''biofriendly'' aspects of the superficially porous silica used as a support for the Poroshell 300 SB-C18 column.

As predicted by theory, kinetics of the superficially porous structure for 5-μm particles should be less favorable for small molecules than for large molecules. (However, as indicated in Fig. 10, superficially porous particles have a distinct kinetic advantage for macromolecules at higher mobile phase velocities). Plate height versus mobile phase velocity plots are essentially identical for columns of 5-μm totally porous and superficially porous particles [3]. Diffusion rates for small molecules apparently are suffi-

Table 1
Poroshell 300 SB-C18 protein yield study

Protein	Peak area: no column	Peak area: with column	Average yield (%)
Angiotensin II	106	108	
	109	106	
	123	112	
	±8.3%	±3.1%	96
Ribonuclease A	53.0	67.7	
	64.4	74.7	
	56.6	59.1	
	±10.0%	±11.7%	116
Insulin	77.8	75.6	
	62.2	75.4	
	62.6	73.7	
	±13.0	±1.4%	111
Carbonic anhydrase	90.4	83.2	
	77.1	85.5	
	90.8	89.4	
	±7.8%	±3.6%	100
Average deviation/yield for all proteins (%)	10.1	4.9	106±9

Column: 75×2.1 mm Poroshell 300 SB-C18; mobile phase: A – 0.10% TFA–water, B – 0.07% TFA–ACN; gradient: 5–100% B in 1.0 min; flow-rate: 1.0 ml/min; temperature: 70°C; UV detection at 215 nm; samples: 0.1 mg/ml in 0.10% TFA–water; injection volume: 1.0 μl.

cient so that the mass transfer associated with such solutes is not seriously limiting when columns of these particles are used at optimum flow-rates.

Therefore, in terms of kinetics and column efficiency, it would appear that there is no advantage in using columns of superficially porous particles for separating small molecules. On the other hand, experiments suggest that there may be a practical advantage for using superficially porous particles in separating certain mixtures of small molecules. Fig. 17 illustrates this effect in the separation of a mixture of small peptides and some proteins. Peaks 1 and 2 (neurotensin and Rnase A) elute simultaneously with the totally porous Zorbax 300 column even though 3.5-μm particles were used. However, under the same separating conditions, these compounds are well separated with the Poroshell 300 column of 5-μm particles. This difference in selectivity likely is a thermodynamic effect resulting from the lower phase ratio (lower surface area) of the Poroshell column. Note that all compounds in this mixture elute earlier in the gradient, which means that a lower concentration of %B organic modifier (higher concentration of water) is required. Variations in selectivity with changes in %B are well documented [15]. Another potential advantage for superficially porous columns is for the separation of very strongly-retained, highly hydrophobic compounds. Here the lower phase ratio would allow elution with a lower concentration of organic modifier, which might be especially advantageous in certain LC–MS applications. The use of capillary columns of superficially Poroshell 300 porous particles has shown special promise in such studies [16].

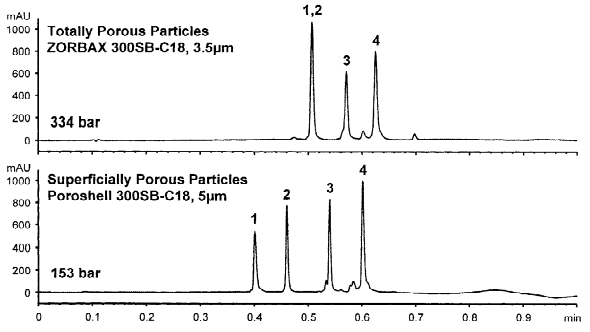

Fig. 17. Selectivity changes by phase ratio differences. Columns: 75×2.1 mm, 3.5-μm totally porous particles, 5-μm superficially porous particles; gradient 15–100% B in 1.0 min, A – 0.1% trifluoroacetic acid in water, B – 0.07% trifluoroacetic acid in acetonitrile; flow-rate: 2.0 ml/min; temperature: 70°C; UV detection at 215 nm; sample injection: 1.0 μl (125–500 μg/ml); solutes: 1, neurotensin; 2, Rnase A; 3, lysozyme; 4, myoglobin.

4. Conclusions

While column packings of conventional porous silicas have formed the basis for most current HPLC separations, atypical silica particles with different conformations potentially form the basis for important applications that are not well handled by conventional silica-based column packings. The ability to synthesize particles in a wide variety of sizes, configurations and pore structure is an inherent advantage of silica as a column packing material for HPLC. Silica likely will remain the basis for many HPLC column packings, for there are potentially many other forms (such as silica monoliths [4]) that will provide new and important ways of producing superior HPLC separations.

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