Options for Rapid Analysis of Peptides and Proteins, Using Wide-Pore, Superficially Porous, High-Performance Liquid Chromatography Particles with Unique Bonded-Phase Ligands

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Abstract

The large size and complexity of many proteins constrains the reversed-phase high-performance liquid chromatography packings that are useful for their separation. Wide-pore, superficially porous, silica-based packings with solid 4.5-µm cores and a 0.25-µm porous outer layer (Poroshell) demonstrate a variety of characteristics that are beneficial for the separation of proteins. A shorter diffusion distance allows separations of large molecules at high linear velocities. This benefit over totally porous particles is clearly shown using separations of a peptide-protein standard. The structure and reduced surface area (4.5 m^2/g) of these superficially porous particles simplifies interactions with its surface, resulting in improved peak shapes and resolution. Specialized bonding chemistries for low- and high-pH operation may be used to change band-spacing and achieve atypical separations. These rapid analysis options are demonstrated using protein standards and very high molecular weight glycosylated proteins including intact monoclonal antibodies, IgM, α2-macroglobulin, and glycophorin. In liquid chromatography–mass spectrometry analysis of a myoglobin peptide digest, bidentate-C18-bonded superficially porous packings achieve complete runs in 4 min and demonstrate an elution pattern that is unique from that of material bonded with sterically protected C18 ligands.

Introduction

The ability to more rapidly identify, isolate, and analyze molecules by high-performance liquid chromatography (HPLC), and its hyphenated techniques, continues to show increased benefit in the modern laboratory. Where biological research and drugs are concerned, this speed translates to faster discoveries and reduced time to market, as well as reduced cost of drug development. The use of high-throughput HPLC techniques for small molecules is widespread; these techniques use short HPLC columns (10–100 mm) having small-particle packings (1–3.5 µm) or monolithic structures (1–4). Mobile-phase linear velocities (relative flow rates) for small molecules may be as high as (1.5–6 mm/s). Recently, totally porous, sub-2-micron particles have become widely used. Columns packed with these small-particle materials retain narrow peak widths at even higher linear velocities, and are used to develop rapid and reliable methods for pharmaceutical analysis (5). In contrast, protein separations have not fully benefited from higher throughput at increased flow rates because of limitations to mass transfer. Diffusion coefficients for proteins (6) are an order of magnitude smaller than that for the small molecules that make up traditional pharmaceuticals (7). As a result, attempts to increase the flow rate beyond certain limits result in band broadening and reduced resolution. In addition to their slower diffusion rates, proteins are more complex than small molecules. Multiple folding structures and modifications for a protein can lead to broad distributions in molecular weight, charge, and size, causing complex interactions with the surface of an HPLC particle. The diversity of these interactions can cause band broadening even at lower linear velocities. Interactions of proteins with the particle surface have been studied in detail (refer to publications by C.T. Mant and R.S. Hodges) and detailed guidelines for protein separations established (8,9). Separations of proteins may be improved through decreased particle size, increased temperature, and solvent strength (10–12). Often, different bonded phases are compared to determine optimal separation conditions empirically (13).

New separation options arise from the characteristics of wide-pore superficially porous, silica-based HPLC particles (Poroshell) modified with a variety of specially designed bonded phases. These bonded phases fall into two general categories, one with enhanced stability at high pH (14) and one with enhanced stability at low pH (15,16). A family of low-pH-stable bonded phases provides additional selectivity options. Poroshell packings currently consist of a 4.5-µm solid-silica core covered with a 0.25-

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µm porous-silica shell. The particles were designed to provide high-speed reversed-phase HPLC analysis of macromolecules (e.g., proteins) at increased flow rates (17–19). While a macromolecule or protein may diffuse relatively slowly, the solid core of a superficially porous particle limits its diffusion path so that a molecule can get quickly into and out of the particle. This significantly reduces band broadening, helping to maintain peak width and resolution at higher linear velocities so that flow rate may be increased and higher throughput achieved. These superficially porous packings were recently used to achieve ultra-fast analysis of glutenin subunits (GS) in wheat (20). Two classes of GS could be separated in as little as 4 min without suffering from loss of resolution or inaccurate quantitation.

While rapid separations of peptides and proteins have been previously demonstrated on these wide-pore superficially porous particles, detailed comparisons to totally porous particles have not been made. The present work looks at peak width, resolution, and retention time for a set of peptide and protein standards analyzed at different flow rates on identical wide-pore columns packed with either superficially porous or totally porous particles of the same bonded phase. Similar comparisons are made using various bonded phases and column configurations to separate a wide variety of peptides and proteins (molecular weights from 1,673 to 950,000). Peak shape is investigated here for large, complex and heterogeneous proteins to demonstrate advantages of using wide-pore, superficially porous particles and certain bonded phases. Finally, superficially porous particles and pH change are used to achieve dramatic selectivity differences in a peptide digest. These experiments provide an empirically derived set of useful separation options.

Experimental

Column liquid chromatography

For HPLC and data collection, an Agilent Chemstation LC-3D and an Agilent 1100 HPLC were used (Agilent Technologies, Palo Alto, CA). Where indicated, the mixing column in the Agilent 1100 pump was replaced with a capillary (Agilent p/n: G1312-67301) to reduce dwell volume, for gradient elution. Except where noted, the diode-array detector was used with a 1.7 µL flow cell (6 mm path length) (Agilent p/n: G1315-60015) and with a 0.1 s response time for optimal data collection. Wavelength setting, column temperature, flow rate, and gradient times are indicated in the individual figures. For liquid chromatography–mass spectrometry (LC–MS) experiments, an Agilent 1100-MSD was used. Conditions are described in the figures.

All columns were obtained from Agilent Technologies. Superficially porous packings are commercially available as "Zorbax Poroshell"; columns 2.1 × 75 mm or 1.0 × 75 mm and contained 5 µm particles having a nominal surface area of 4.5 m^2/g and a nominal pore size of 300 Å. Bonded-phases included Poroshell 300SB-C18, 300SB-C8, 300SB-C3, and 300Extend-C18. The totally porous particles used in these experiments were Zorbax 300SB-C18 (2.1 × 75 mm, 5 µm custom and 1.0 × 50 mm, 3.5 µm) (with a surface area of 45 m^2/g, and a 300 Å pore-size) and Zorbax 300Extend-C18, 2.1 × 150 mm, 3.5 µm (with a surface area of 45 m^2/g and a 300 Å pore-size).

LC–MS

Chromatography for LC–MS was carried out using several different Zorbax HPLC columns listed in the figure legends. Flow rates varied from 0.2 and 1.0 mL/min, respectively. The mobile phases were as follows: A = 10mM NH_4OH–H_2O, B = 10mM NH_4OH–MeOH or A = 0.1% formic acid–H_2O, B = 0.1% formic acid–MeOH, and gradients are indicated in the figure legends. LC–MS was carried out on-line using an Agilent 1100 MSD in API-ES positive mode with a scan range of 200–1500 *m/z*, capillary voltage of 4500 V, drying gas flow of 13 L/min, 60 psi, and temperature of 350°C. The fragmentor was ramped from 70 V at 50 *m/z* to 120 V at 1500 *m/z*.

Chemicals

Proteins and peptides used in this study were purchased from Sigma Chemical (St. Louis. MO). Monoclonal antibodies were from Novartis Pharma, Biotechnology (Basel, Switzerland). Tryptic digests of myoglobin were obtained from Michrom Bioresources Inc., (Auburn, CA). Trifluoro-acetic acid (TFA) was purchased from Pierce Chemical Co. (Rockford, IL). Methanol, acetonitrile, acetic acid, and phosphoric acid were obtained from VWR Scientific (Bridgeport, NJ). Mobile phases were made using water from an Elix 10/Milli-Q water purification system (Millipore, Bedford, MA).

Sample preparation

Protein mixtures were typically prepared by weighing 1 mg of each into 1 mL of mobile-phase A (0.1% TFA, 0.1% formic acid, or 10mM NH_4OH) in separate vials. Equal volumes of each stock protein were added together to give the final standard mixture. In some cases, more or less of a single protein might be added, to compensate for its low absorption coefficient.

Results and Discussion

The essence of this work was to demonstrate the interesting and useful characteristics of wide-pore, superficially porous, silica-based HPLC particles in their ability to separate proteins having various molecular weights and modifications. The primary characteristic of these particles is the speed with which separations can be accomplished relative to that on totally porous particles. Other benefits arise from the thin porous layer and wide pores, such as reduced surface area, choice of bonded phase, and breadth of usable mobile-phase pH.

Structural comparison of totally porous and superficially porous silica particles

The general structure of totally porous (Zorbax) and superficially porous (Zorbax Poroshell) particles is depicted in Figure 1. This schematic diagram facilitates an understanding of differences between the two types of particles (the superficial layer is exaggerated to show detail). Both types of particle are 5 µm in overall diameter; however, the totally porous particle drawn here is made by agglomeration of silica-sol beads–much like melting

marbles together at the edges to make a porous bowling ball. The particle is of consistent porosity (having 300Å pores) and surface area (45 m^2/g) throughout the particle. In contrast, the superficially porous Poroshell particle starts with a 4.5 µm solid-silica core; the surface area may be assumed to be 1 m^2/g. Around this core is formed a 0.25 µm totally porous layer of silica-sol beads that makes the completed particle 5 µm in diameter and gives it a surface area of 4.5 m^2/g. The silica sol beads used to make this superficial layer are the same size as those used to create the 300 Å pores in the totally porous particle; both materials are wide-pore particles. This solid core, wide-pore, superficially porous structure was chosen so that slowly moving large molecules could move into and out of the particles quickly, reducing band broadening.

The diffusion constants for large proteins are typically 4 to 40-times smaller than those for small-molecule pharmaceuticals. For example, sucrose has a diffusion coefficient (D) of 52.1×10^{-7} at 25°C and that for glycine is 106.4×10^{-7} (6). In contrast, myoglobin (Mr = 12,400) has a D value near 13.0×10^{-7} and that for thyroglobulin (Mr = 669,000) is around 2.6×10^{-7} (7). Making the assumption that molecules move in a straight line into and out of these, an HPLC particle, at an angle 90° from the direction of flow, the diffusion distance for a molecule separated on a 5 µm totally porous packing should be 5 µm (2.5 µm in and 2.5 µm

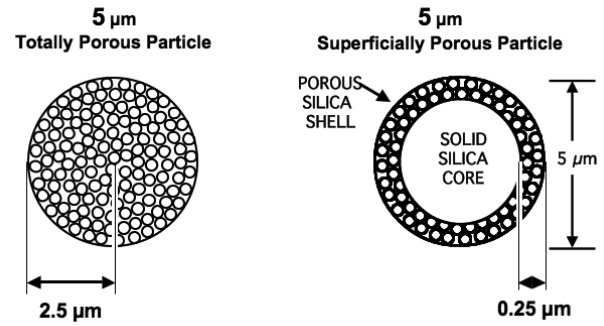

Figure 1. Schematic comparison of superficially porous particles and totally porous wide-pore particles. A drawing of a totally porous 5 µm particle is shown on the left, a superficially porous 5 µm particle (Poroshell) on the right. The figure is not drawn to scale. Agglomerated silica-sol beads form the totally porous particle, resulting in consistent porosity and surface area throughout; sample analytes may traverse the entire 5 µm particle. A 4.5 µm solid silica core makes up the much of the Poroshell particle. Silica-sol beads (the same diameter as those on the left) make up an outer shell that is only 0.25 µm thick; sample analytes may only traverse the outer 0.25 µm of particle.

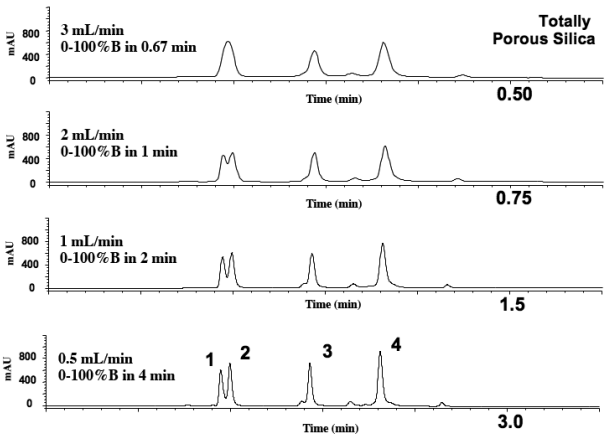

Figure 2. Comparison of chromatographic performance for a peptide/protein mixture using wide-pore, totally porous, HPLC particles at different flow rates. Chromatography was carried out on a custom Zorbax 300SB-C18 column in a 2.1 × 75 mm, 5-µm configuration at various flow rates using corresponding gradient times as listed in the figure. The chromatograms are normalized by gradient volume (flow rate) for comparison. The Agilent 1100 Binary HPLC system included a Well-Plate Sampler used in auto-delay volume reduction mode. The mixing column was removed and the piston stroke was set to 20 µL. The operating temperature was 70°C; detection was UV (215 nm). Mobile Phase: A = 95% H_2O, 5% ACN with 0.1%TFA; B = 5% H_2O, 95% ACN, with 0.07%TFA. The sample was a mixture of neurotensin (peak 1), RNase A (peak 2), lysozyme (peak 3), and myoglobin (peak 4) at approximately 0.25 mg/mL final concentration in mobile phase A.

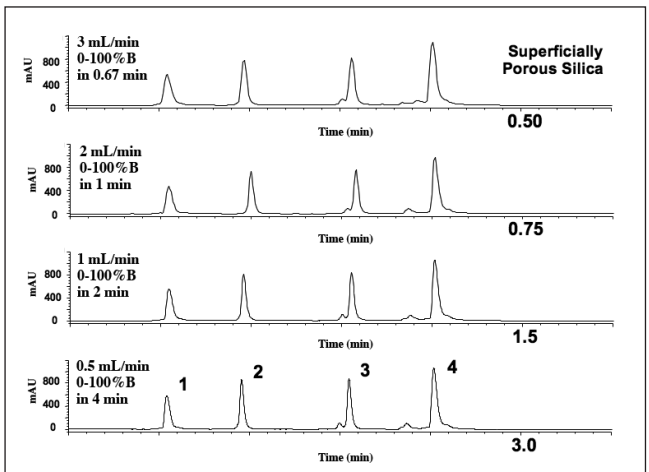

Figure 3. Comparison of chromatographic performance for a peptide/protein mixture using wide-pore, superficially porous, HPLC particles at different flow rates. Chromatography was carried out on a Zorbax Poroshell 300SB-C18 column in a 2.1 × 75 mm, 5-µm configuration at various flow rates using corresponding gradient times as listed in the figure. Other conditions are the same as those in Figure 2. The chromatograms are normalized by gradient volume (flow rate) for comparison.

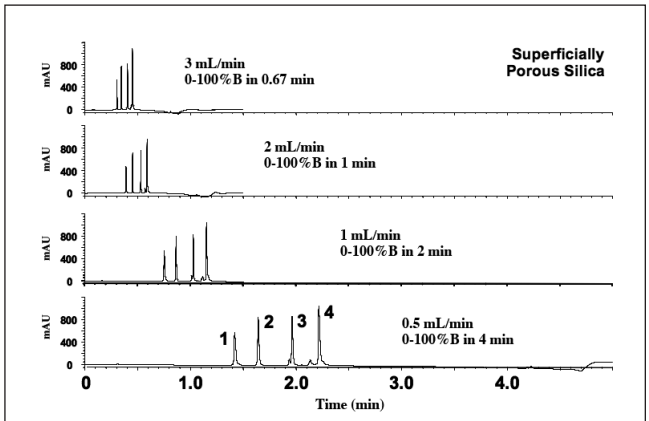

Figure 4. Comparison of chromatographic selectivity for a peptide/protein mixture using wide-pore, superficially porous, HPLC particles at different flow rates. Chromatograms from Figure 2 shown without normalization by gradient volume (flow rate).

back out). The diffusion distance for a molecule separated on a 5 µm superficially porous packing should be 0.5 µm (0.25 µm in and 0.25 µm out) because of the particle's solid inner core. In simple terms, the structure results in a 10-fold reduction in the time necessary for a molecule to traverse superficially porous particles. As a result, linear velocities (relative flow rates) may be increased well beyond typical levels, with reduced loss of resolution. This is demonstrated in the next set of experiments (Figure 2–4 and Table I).

Chromatographic performance of proteins using high-velocity chromatography

The chromatographic characteristics of macromolecules separated at increasing flow rate were evaluated (Figure 2–4 and Table I). A standard mixture of one peptide and three proteins (neurotensin, RNase A, lysozyme, and Myoglobin) was separated at different flow rates on a 2.1 × 75 mm column packed with wide-pore totally porous 5-µm particles (Figure 2); flow rates ranged from 0.5 to 3 mL/min. In this column configuration, these flow rates correspond to linear velocities of approximately 4.1 to 24 mm/s. Gradient times were adjusted for flow rate, and chromatograms were aligned by gradient volume, to demonstrate that the elution profile was kept constant. The effect of increasing linear velocity on peak width can be seen for peaks 1 through 4 by comparing the bottom chromatogram in Figure 2 with those above it. The effect is most noticeable for peaks 1 and 2, where peak widths are broadened to the point of complete overlap. Note that relative retention times stay constant as a result of gradient-volume adjustment; loss of resolution is only due to peak broadening.

The same peptide-protein mixture used previously was also used to test the chromatographic characteristics of macromolecules analyzed at increasing flow rates on wide-pore, superficially porous, HPLC particles (Figure 3 and 4). In Figure 3, as in the previous experiment, gradient time was adjusted inversely to flow rate to keep gradient volume and, therefore, relative elution profiles constant. Peak widths for the 4 macromolecules are nearly constant at flow rates of 0.5 to 2 mL/min. At 3 mL/min (the top chromatogram), a small increase in peak widths could be observed. Figure 4 shows chromatograms identical to those in Figure 3 with the exception that the time axis were not adjusted for differences in gradient volume. As these chromatograms are shown in absolute time, the dramatic decrease in run time is clearly seen.

Table I is a compilation of peak widths at half height (w_h) and resolution (R_s) for peaks 3 and 4 in the chromatograms of Figures 2–4. While peaks 1 and 2 show the most significant difference between totally porous and superficially porous wide-pore particles, they were not included in the Table because of overlap that occurred on the totally porous particles. The upper section of the Table shows the data for wide-pore superficially porous particles. The lower section of the table shows the data for wide-pore totally porous particles. To better show the dependency of band broadening on flow rate, values of w_h were first normalized to flow rate (w_h^F) and percent increase in w_h^F was calculated at each flow rate, relative to that at the lowest flow rate. Average percent increase in w_h^F for peaks 3 and 4 are shown to further demonstrate the trends in band broadening. At a flow rate of 3 mL/min, totally porous particles show a doubling of w_h^F (106%) while that for the superficially porous particles increased only 39%. The table further shows that sample molecules are broadened significantly on the totally porous packing even when flow is increased from 0.5 to 1 mL/min (a 28% increase in w_h^F); while, only a 7% increase is observed on the superficially porous material. Loss of R_s between peaks 3 and 4 closely parallels the increase in band broadening.

Selectivity options of sterically protected C18 and C3 ligands on wide-pore, superficially porous HPLC particles

The effect of two significantly different bonded-phase ligands on the ultra-fast separation of a protein mixture is shown in Figure 5. Wide-pore, superficially porous, silica particles bonded with either di-isobutyl-*n*-octadecyl-silane (SB-C18) or tri-isopropyl-silane (SB-C3) were used under the same chromatographic conditions to separate a mixture of 9 peptides and proteins. The separations are essentially the same, but have an

Table I. Chromatographic Performance of Analyses in Figures 2–4. Comparison of Linear Velocities, Peak Widths at Half Height (w_h), w_h Normalized to Flow Rate (w_h^F), and Resolution on Superficially Porous and Totally Porous HPLC Particles

Flow Rate (mL/min)	Linear Velocity (mm/s)	W_h (s)	W_h^F	Percent Increase	W_h (s)	W_h^F	Percent Increase	Mean Percent Increase	Resolution
		Peak 3			Peak 4			Peak 3 + 4	Peak 3 + 4
Superficially Porous Particle – Zorbax POROSHELL (2.1 × 75 mm, 5 µm)									
0.5	4.1	0.774	0.387	0%	1.026	0.513	0%	0%	10.0
1	8.3	0.450	0.450	16%	0.498	0.498	–3%	7%	10.1
2	16.5	0.222	0.444	15%	0.288	0.576	12%	14%	8.2
3	24.8	0.186	0.558	44%	0.228	0.684	33%	39%	7.0
Totally Porous Particle (2.1 × 75 mm, 5 µm)									
0.5	3.4	0.948	0.474	0%	1.128	0.5640	0%	0%	9.9
1	6.8	0.600	0.600	27%	0.726	0.7260	29%	28%	7.5
2	13.5	0.420	0.840	77%	0.48	0.9600	70%	74%	5.7
3	20.3	0.342	1.026	116%	0.366	1.0980	95%	106%	4.1

important difference. While peaks 5 and 6 coelute when separated on the C18-bonded material, they are nearly baseline resolved under these conditions on the C3-bonded material. This ability to pull out a co-eluting peak from a main peak can be invaluable if these are the critical pair of the separation. As would be expected, retention time for each of the analytes is slightly less when separated using the shorter ligand (SB-C3). This reduction in retention results in significantly increased peak width for peak 1 because it now elutes in the isocratic part of the gradient, before the gradient has reached the column.

Selectivity options in the separation of intact monoclonal antibodies by wide-pore, superficially porous HPLC particles

The ability to rapidly separate intact antibodies (Mr = 150,000) using wide-pore, superficially porous, HPLC particles is shown in Figure 6. These separations are effectively carried out in 10 min, with a turn-around time of 15 min. This was achieved at a flow rate of 1 mL/min; higher flow rates were not investigated for this analysis. Separations using wide-pore, superficially porous particles bonded with either di-isopropyl-*n*-octyl-silane (SB-C8, Figure 6A) or tri-isoproyl-silane (SB-C3, Figure 6B) were carried out under identical chromatographic conditions. The effect of bonded-phase ligand on selectivity of these 7 monoclonal antibodies (MAB's) is demonstrated by careful comparison of the two chromatograms. The chromatograms are, in general, similar; however, there are differences. The most important difference between the phases is in the selectivity observed for MAB's 4, 5, and 6. Use of SB-C8 packing allowed near-baseline separation of all MAB's except for the co-elution of 3 and 4. Changing only the bonded phase (SB-C8 to SB-C3), MAB's 5 and 6 coeluted; but all others, including 3 and 4, were separated. Separation of the doublet for MAB 2 is also improved by the switch to SB-C3. As in Figure 5, the SB-C3 phase shows slightly less retention for the analytes.

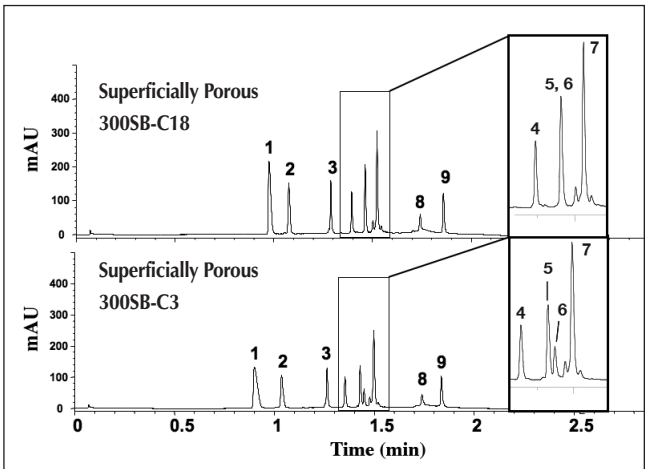

Figure 5. Comparison of chromatographic selectivity for a peptide/protein mixture using wide-pore, superficially porous, HPLC particles with SB-C18 or SB-C3 bonded phase. Chromatography was carried out on Zorbax Poroshell 300SB-C18 and 300SB-C3 columns in a 2.1 × 75 mm, 5-µm configuration at 0.5 mL/min. The operating temperature was 70°C with UV detection at 215 nm. Mobile phase A = 0.1% TFA/H_2O, B = 0.07% TFA–ACN in a gradient of 5–100% B in 3.0 min. The peptide–protein sample consisted of angiotensin II (peak 1), neurotensin (peak 2), RNase A (peak 3), insulin B Chain (peak 4), insulin (peak 5), cytochrome C (peak 6), lysozyme (peak 7), myoglobin (peak 8), and carbonic anhydrase (peak 9) at approximately 0.15 mg/mL final concentration in mobile phase A.

Separation advantages of wide-pore, superficially porous particles for the separation of large, heterogeneous proteins

The advantages of using wide-pore, superficially porous, HPLC particles for the chromatographic separation of large, heterogeneous and heavily glycosylated proteins are shown in Figure 7. The figure allows two comparisons. First, wide-pore, superficially porous particles having different bonded phases (SB-C18, C8, and C3) may be compared to each other in their chromatographic performance. Second, the wide-pore, superficially porous particles may be compared to wide-pore totally porous particles bonded with a sterically protected C18 ligand (SB-C18). The 1.0 × 75 mm, 5 µm version of this column was not available

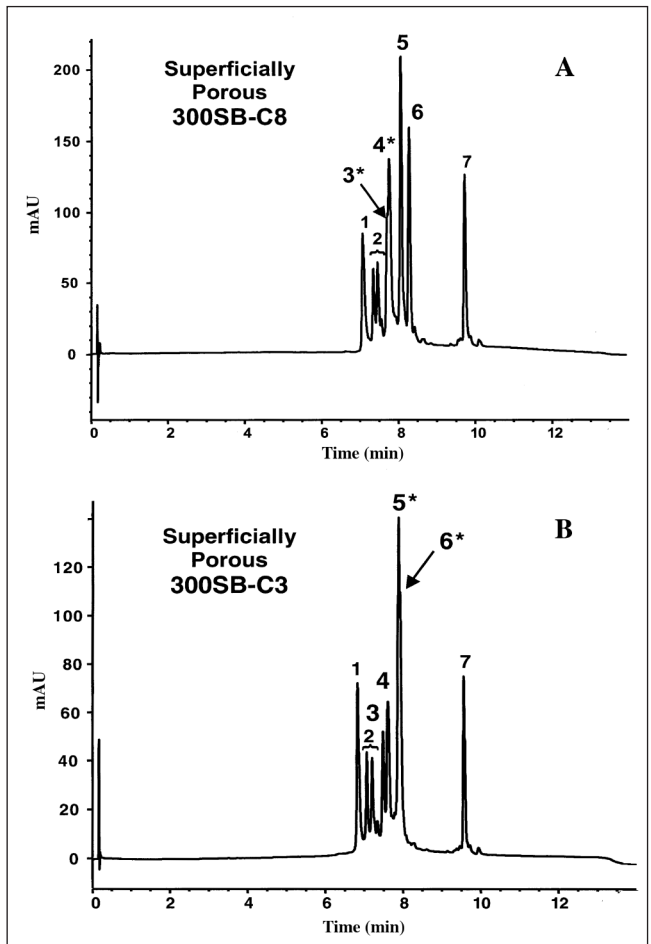

Figure 6. Comparison of chromatographic selectivity for intact monoclonal antibodies, using wide-pore, superficially porous, HPLC particles with SB-C8 or SB-C3 bonded phase. Chromatography was carried out on Zorbax Poroshell 300SB-C8 (Figure 6A) and 300SB-C3 (Figure 6B) columns (2.1 × 75 mm, 5 µm) at 1mL/min. The operating temperature was 70°C with UV detection at 210 nm. Mobile Phase A = 90% H_2O, 10% ACN with 0.1%TFA and 3 mL/L of PEG 300; B = 10% H_2O, 90% ACN, with 0.1%TFA and 3 mL/L of PEG 300. The multi-segment gradient was: 19% B at 0 min to 41% B by 12 min to 19% B by 12.1 min followed by a hold until 14 min. The sample consisted of 7 intact monoclonal antibodies. Antibody types are 1 through 3 and 5 through 7 = IgG1, 4 = IgG4.

so a 1.0 × 50 mm, 3.5 µm configuration was chosen to provide similar plates and linear velocity for the totally porous packing. Of the proteins used, IgM has a molecular weight of about 950,000 and monoclonal IgG about 150,000; it is common for both molecules to be glycosylated to varying extents. Human α2-macroglobulin has an approximate molecular weight of 720,000. Human glycophorin has a molecular weight around 50,000 and consists of 60% carbohydrate, in its normal state. Wide-pore, superficially porous particles (Figure 7B–7D), in general, provide superior peak shape and resolution of these large, heterogeneous proteins compared to the wide-pore totally porous, SB-C18-bonded packing (Figure 7A). Specifically, the peak shape for Macroglobulin (is superior on all of the wide-pore, superficially porous packings. This is also true for IgG; however, it cannot be easily seen in these chromatograms because of its overlap with IgM. Glycophorin demonstrates acceptable peak shape except on wide-pore, superficially porous particles bonded with SB-C18. Wide-pore, superficially porous particles bonded with SB-C3, overall, demonstrate the best peak shape and the shortest run time. However, there is somewhat reduced resolution under these gradient conditions. Note also the improved peak shape shown by increased peak heights in this chromatogram (the absorbance scale for the bottom chromatogram is 2-fold that for the two above it). These results are consistent with a reduced/simplified interaction of these complex proteins not only with wide-pore, superficially porous particles but additionally with the tri-isopropyl (SB-C3)-bonded packing. An alternative explanation for improved peak shape could be based upon the differences in organic strength at which these proteins elute. Under various organic conditions, proteins can undergo different degrees of folding resulting in altered secondary and tertiary structures that change interaction with other proteins and the bonded phase. The bonded phase itself may change at different organic concentrations. However, the behavior of peak 3, for instance, would contradict this explanation. Peak 3 elutes at nearly the same retention time (organic concentration) in the

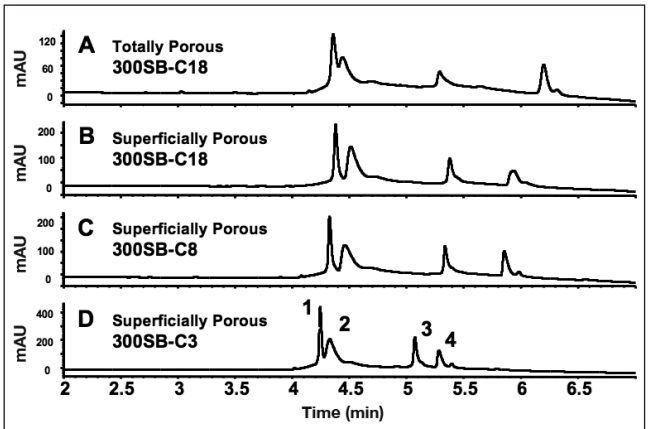

Figure 7. Comparison of totally porous and superficially porous, wide-pore, HPLC particles in their chromatographic separation of very large heterogeneous proteins. Chromatography was carried out on a totally porous Zorbax 300SB-C18 column (1.0 × 50 mm, 3.5 µm) (Figure 7A) and on Zorbax Poroshell 300SB-C18, 300SB-C8, and 300SB-C3 columns (1.0 × 75 mm, 5 µm) at 0.454 mL/min (Figure 7B–7D, respectively). The operating temperature was 70°C, with UV detection at 212 nm. Mobile phase A = 0.1% TFA–H_2O, B = 0.07% TFA–ACN, in a gradient of 5–100% B in 10.0 min. No mixer was used. The protein sample consisted of rabbit monoclonal IgG (peak 1), IgM (peak 2), α2 macroglobulin (peak 3), and glycophorin (peak 4) at a final concentration of approximately 0.25 mg/mL in mobile-phase A.

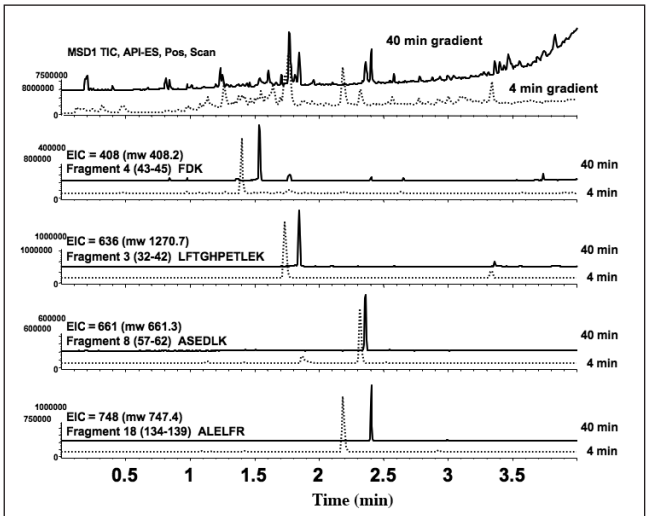

Figure 8. Comparison of bidentate-C18-bonded totally porous and superficially porous wide-pore HPLC particles in LC–MS analysis of myoglobin tryptic peptides. Chromatography was carried out on a totally porous Zorbax 300Extend-C18 column (2.1 × 150 mm, 3.5 µm) (solid line) and on a Zorbax Poroshell 300Extend-C18 (2.1 × 75 mm, 5 µm) (dashed line); flow rates were 0.2 and 1.0 mL/min, respectively. Mobile Phase was A = 10mM NH_4OH–H_2O, B = 10mM NH_4OH /MeOH, and the gradient was from 0-100% B in 40 or 4.0 min, respectively. The sample was 50 pmol of trypsin-digested myoglobin. LC–MS was carried out on-line in API-ES positive mode with a scan range of 200–1500 *m/z*, capillary voltage of 4500V, drying gas flow of 13 L/min, 60 psi, and temp of 350°C. The fragmentor was ramped from 70V @ 50 *m/z* to 120V @ 1500 *m/z*.

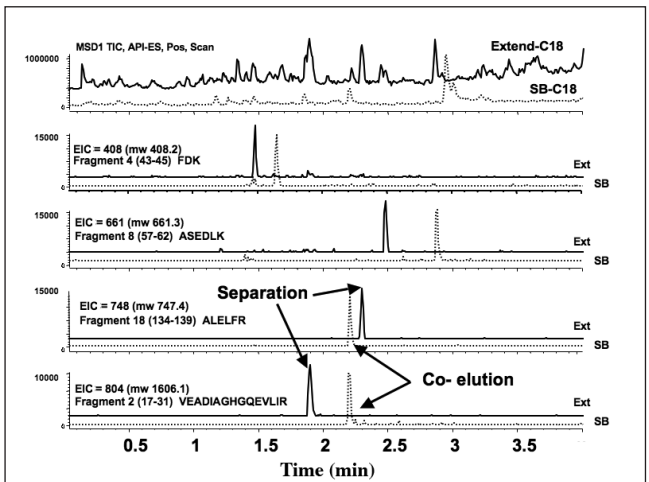

Figure 9. Comparison of wide-pore, superficially porous, HPLC particles bonded with bidentate-C18 or sterically protected-C18 ligands in LC–MS analysis of myoglobin tryptic peptides. Chromatography was carried out using either a Zorbax Poroshell 300Extend-C18column (solid line) or a Zorbax Poroshell 300SB-C18 column (dashed line) (both columns 2.1 × 75 mm, 5µm) at a flow rate of 1.0 mL/min. Mobile Phase was either A = 10mM NH_4OH–H_2O, B = 10mM NH_4OH–MeOH or A = 0.1% formic acid/H_2O, B = 0.1% formic acid/MeOH, respectively. Gradients were run from 0–100% B in 4.0 min. The sample was 50 pmol of trypsin-digested myoglobin. MS conditions were the same as those in Figure 8.

first and third chromatograms, yet has far superior peak shape on the wide-pore, superficially porous packing.

Separation options using bidentate-C18-bonded wide-pore, superficially porous particles in LC–MS

Experiments to this point have demonstrated only the advantages of using sterically protected bonded phases on wide-pore, superficially porous, HPLC packing materials. Sterically protected materials have significantly increased lifetime at low pH and ambient to high temperature (e.g., pH 2 and 20–80°C). A bidentate-C18 bonded phase was designed to provide extended lifetime when used at high pH (e.g., pH 10). Useful properties of wide-pore, superficially porous particles bonded with bidentate-C18 are shown in Figures 8 and 9. In both cases, tryptic digests of myoglobin were used as the analyte, and mass spectrometry was used for detection. This allowed tracking of multiple peaks, with positive identification.

Totally porous particles and superficially porous particles, both wide-pore materials and bonded with a bidentate-C18 ligand, were compared for their ability to separate peptides of a myoglobin tryptic digest. Column configurations and operating conditions are described in the figure and were chosen to represent typical conditions for peptide-digest separations on the two types of particles. The column configuration used for totally porous particles was 2.1 × 150 mm with 3.5 µm particles, and it was operated at a flow rate of 0.2 mL/min with a 40-min gradient time. The column configuration used for the superficially porous particles was 2.1 × 75 mm with 5 µm particles, and it was operated at a flow rate of 1 mL/min with a 4-min gradient time. The total-ion chromatogram (TIC) and several extracted-ion chromatograms (EIC's) from a separation on both columns are shown in Figure 8. Chromatograms are aligned with normalization for gradient volume. As can be seen in the TIC's, the elution pattern of peptides on the two packing materials is not identical, but retains similar features. In each case, comparison of EIC's from the chromatographic separation on wide-pore, superficially porous packing identifies a corresponding peptide from the separation on wide-pore, totally porous packing. In each case, the corresponding peptide elutes relatively earlier on the wide-pore, superficially porous packing. These peptides also tend to elute in a similar region of the chromatogram, while eluting 10-times faster in the wide-pore, superficially porous separation. For example, the extracted ion 636 (corresponding to the myoglobin 32–42 tryptic peptide) appears at approximately 18.5 min in the separation using wide-pore, totally porous packing and appears at 1.75 min in the separation using wide-pore, superficially porous packing. The ability to achieve fast LC–MS analyses on wide-pore, superficially porous particles at high pH is clearly demonstrated. Notably, peaks eluting from the wide-pore, superficially porous packing in 4 min at 1 mL/min are relatively broader than those eluting from the wide-pore, totally porous packing in 40 min at 0.2 mL/min. This is a result of the difference in particle size between the two columns; the superficially porous material 5 µm and the totally porous material 3.5 µm in diameter.

The benefit of different selectivity possible using wide-pore, superficially porous particles bonded with either bidentate-C18 or SB-C18 is demonstrated in Figure 9. The same conditions were used as in Figure 8 except that both column configurations were 2.1 × 75 mm with 5 µm wide-pore, superficially porous particles and were operated at a flow rate of 1 mL/min using a 4 min gradient time. Also, as described in the figure, wide-pore, superficially porous particles bonded with SB-C18 were used at low mobile-phase pH (0.1% formic acid) while those bonded with bidentate-C18 were used at high mobile-phase pH (10mM NH_4OH). EIC's at an *m/z* corresponding to many of the expected peptide fragments from myoglobin were compared for both analyses. From all of the EIC's, a subset was taken and shown in Figure 9. Comparison of these EIC's reveals an example of two peptides that overlap under one separation condition but are well separated under the other condition. The bottom two chromatograms in Figure 9 show that Fragment 18 (peptides 134–139) and Fragment 2 (peptides 17–31) overlap when analyzed using the bidentate-C18-bonded material at high pH (chromatogram second from bottom). These two peptides are separated by 12 s when analyzed using the SB-C18-bonded material at low pH (bottom chromatogram). Like in the other examples, this experiment demonstrates the ability to shift selectivity so that two analytes are well resolved. The implications of improved selectivity for analyte purification are obvious; but, improved selectivity in MS detection may result in reduced ion suppression or additional scan and fragmentation cycles.

Conclusions

The purpose of this paper was not only to demonstrate the extremely rapid analyses of proteins that may be achieved using wide-pore, superficially porous, silica particles but also to demonstrate, in a practical way, other beneficial properties of these materials with unique bonded phases. These beneficial properties include changes in selectivity and improvement in peak shape, and were shown here using a variety of different conditions and analytes. Analytes included peptides and proteins with molecular weights from 1,673 to 950,000. Proteins with known heterogeneity and up to 60% glycosylation, 7 intact monoclonal antibodies, and a protein digest were also studied. As expected, peptides and proteins show less peak broadening at high relative flow rates (linear velocity) on wide-pore, superficially porous particles as compared to that on totally porous particles. Significant selectivity changes can be made by switching from totally porous to superficially porous wide-pore particles. Changes to selectivity are also possible by switching from one bonded phase to another. Peak shape of the large and heterogeneous molecules can also be improved by switching from wide-pore, totally porous to wide-pore, superficially porous particles and is dependent upon the bonded phase. Finally, when stable bonded phases and mobile-phase pH are used together, significant changes in selectivity of peptide digests is achieved.

As new research focuses more and more on peptides and proteins, the rapid HPLC separation of these molecules becomes increasingly valuable. The selectivity and peak shape improvements shown here improve chances that the desired separation will be achieved. These options should be investigated when

developing separations of larger molecules; special attention should be given to molecules with post-translational modifications and heterogeneity. This is important for basic research but is especially important in the pharmaceutical industry where time to market is critical.

Dedication

This paper is dedicated in memory of Clifford B. Woodward III—for his wit and wisdom, for his scientific savvy, and for his fervent friendship.

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