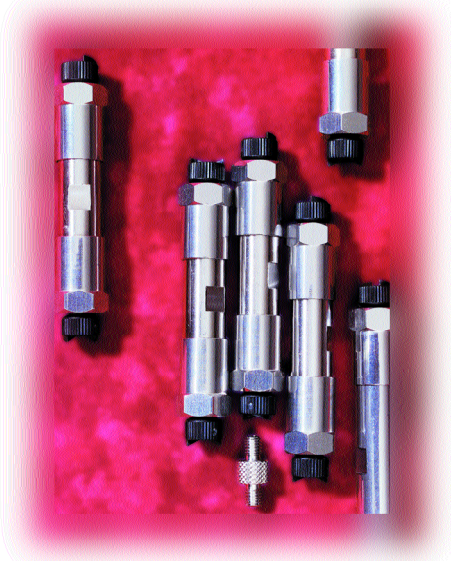
# Advances in HPLC Column Packing Design



**Column packing materials continue to evolve as user needs for high-throughput, high-resolution, and high-sensitivity HPLC analyses drive further developments. In this introductory article to the Special Supplement, the author covers basic column packing morphology and particle design and compares and contrasts modern HPLC columns. Future directions in packing developments are predicted.**

Even though high performace liquid chromatography (HPLC) column technology is considered to be somewhat mature, new devdopments continue. Improvements have occurred in packing material design, bonded-phase chemistry, column construction, and formats. In addition, new phases have extended the pH range (high and low), providing more versatility. In this article, I will update developments in packing morphology and particle design. Instead of trying to cover the entire domain of HPLC column development, I will focus on a few key areas.

## Improvements in Porous Packings

Porous packings have been in favor throughout the history of HPLC. The transition from large porous particles and pellicular materials to small porous particles occurred in the early 1970s, when microparticulate silica gel (< 10-µm $d_p$) came on the scene and appropriate packing methods were devdoped. Irregularly shaped microparticulate packings were in vogue throughout the 1970s until spherical materials were developed and perfected. The spherical packings could be packed more homogeneously than their irregular predecessors, gave better efficiencies, and could be manufactured in higher purity. Indeed, the so-called Type B silica that was low in trace-metal content became the standard in the early 1990s and now most commercial silica-based analytical HPLC packing materials are of this higher level of purity. Trace metals in silica gel cause interactions with certain compounds and can affect the acidity of residual silanols (1). One goal for the optimum use of HPLC packings early in the game was to achieve the best efficiency possible, thereby leading to better overall chromatographic resolution. To better understand the various approaches to improve column efficiency, let us briefly discuss the morphology of a porous packing material such as silica gel or alumina.

Diffusivepores dominate a typical porous packing (Figure 1a), and the major surface area of the particle is contained within these pores. A reduction in particle size improves both the interparticle mass transfer and the intraparticle mass transfer. In a porous particle, solutes transfer from the moving mobile phase outside of the particles into the stagnant mobile phase within the pores in order to interact with the stationary phase. Following this interaction, the solute molecule must diffuse out of the particle and continue its journey down the column. Such a mass transfer occurs many thousands or even millions of times as the differential separation process proceeds and the solute is eluted from the column. While the solute spends its time in the diffusive pores, the mobile phase in which it was located originally moves down the column ahead of the solute. This slow rate of mass transfer into and out of the porous particle is a major source of band broadening in HPLC. The use of smaller particles shortens the path length of this diffusion process, improves mass transfer, and provides better efficiency. Manufacturers now can produce small-diameter particles with fairly narrow particle size distributions down to 1.5-µm average diameter, although 3–3.5- and 5-µm particles are still the norm.

However, congruent with the improvement in efficiency was the decrease in column permeability; that is, an increase in column backpressure. The increase in pressure is proportional to the inverse of the particle diameter squared. Thus, halving particle diameter will increase the column head pressure by a factor of four.

Column efficiency, $H$ or height equivalent to a theoretical plate (HETP) is proportional

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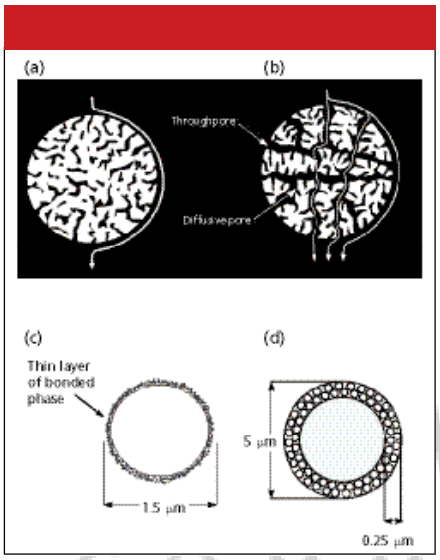


**Figure 1:** Schematics of various particle types, incl. (a) totally porous, (b) perfusion, (c) nonporous, and (d) superficially porous particles.

to $d_p^x$, where $x$ is approximately 1.6–1.9. Resolution is proportional to $N^{1/2}$. Thus, if one uses smaller particles packed into shorter columns of the same internal diameter, the loss in resolution does not fall off as rapidly as the efficiency improves. A current trend in HPLC for high-throughput separations is to use shorter columns with smaller particles (3- or 3.5-µm particles in a 50 or 20 mm × 4.6 mm column) rather than longer columns with larger particles (5-µm particles in a 150–250 mm × 4.6 mm column). Because separation time is proportional to length, shortening the column results in faster separations. Figure 2 provides an example of the time saved when using smaller particles (1.8 µm and 3.5 µm) in shorter columns (100 mm and 30 mm) compared with the more traditional HPLC analytical columns. The flow rate on all columns is the same (1 mL/min) except for the bottom figure where the flow was increased to 2 mL/min to illustrate a possible further decrease the separation time by increasing the flow rate. Compared with the separation on a conventional 250 mm × 4.6 mm column, the separation time was reduced 15-fold (a little over 2 min). There was a slight decrease in resolution as the column length was reduced, but as depicted in Figure 2, even the shortest column provided more than adequate resolution for the low $k$ peaks.

Although short columns with small particles provide rapid separations, the column plate number (efficiency) is not increased. Thus, complex multicomponent samples cannot be separated on these columns. To increase plate count, the smallest available particles combined with longer columns will generate more than 100,000 theoretical plates but at the expense of greatly increased column pressure. The first demonstration of ultrahigh pressure HPLC separations was by Bidlingmeyer and co-workers (2,3) in 1969 with sub-micrometer particles packed into long, thick columns. However, the quality of the packings was not equivalent to today's materials, and more recent studies by Jorgenson and colleagues (4,5) from the University of North Carolina (Chapel Hill, North Carolina) have used particles as small as 1 µm $d_p$ with ultrahigh pressure. Conventional pumps cannot handle these columns, so special high-pressure pumps capable of pressures in excess of 5000 bar (75,000 psi) are required. However, such small-particle columns have the capability of generating a quarter of a million plates in < 1h. The ultrahigh-pressure chromatograph also can be used for gradient elution.

## Perfusion Packings

Perfusion packings developed by Afeyan and co-workers (6–8) and commercialized by Perseptive Biosystems (Cambridge, Massachusetts, now part of Applied Biosystems) in the 1980s gave improved chromatographic performance, particularly for larger molecules. A simplified pictorial representation of a perfusion packing is shown in Figure 1b. Compared with the porous packing, the perfusion packing consists of two types of pores: diffusive pores and through pores. The diffusive pores are the same type present in the porous particles and provide the sorption capacity. The through-pores allow mobile phase to pass through the packing itself, thereby increasing the rate of mass transfer in the mobile phase. Instead of predominantly flowing around the particle, a portion of the mobile phase flows through the particle, thereby allowing the solute to spend less time undergoing the mass transfer process and giving narrower peaks. The process is actually a combination of diffusion and convection.

Commercial perfusion packings are polymeric particles of larger particle size than typically used in HPLC packings, with the smallest average particle size being around 12 µm. However, when compared with a porous packing of the same particle and pore size, the perfusion packings give better efficiency for large molecules (8). In addition, compared with the older soft, organic porous packings used for biomolecules such as fast-flow agarose or polydextrans, which tend to collapse at higher linear velocities, the perfusion packings can be used at higher flow rates. At these higher flow rates, they maintain their sample capacity, making them useful for preparative separations and purifications.

## Nonporous and Superficially Porous Packings

The use of nonporous packings represents another approach to improve the rates of mass transfer. There are two types of nonporous packings: nonporous silica and nonporous resin. As depicted in Figure 1c, the nonporous packings are very reminiscent of the older pellicular or porous-layer beads used in the early days of HPLC, but these materials are of much smaller particle sizes, typically in the 1.5–2.5 µm range (9). The nonporous layer allows much faster rates of mass transfer and separations of only a few minutes can be achieved for both large and small molecules. Unfortunately, the thin layer of stationary phase also limits the capacity of the packing, making the nonporous silica and nonporous resin unsuitable for preparative separations. In addition, due to their small particle size, the backpressure from the nonporous silica columns are generally much greater than those experienced with microparticulate HPLC porous packings of popular particle sizes (that is, 5- and 3-µm). For more information on the use and advantages of nonporous silica packings, consult reference 10. Such particles are finding less use in today's chromatography labs.

Superficially porous packings, depicted in Figure 1d, are similar to the nonporous silica particles described earlier, but the particle size is larger, around 5 µm in diameter, providing a much lower pressure drop. In addition, the surface area is larger (4–6 $m^2/g$) than the nonporous silicas, providing increased sample capacity. These Poroshell particles (Agilent Technologies, Wilmington, Delaware) are recommended for larger biomolecules that diffuse slowly into porous packings. When flow rates are increased with porous packings, the biomolecule peaks broaden due to slow diffusion into and out of the pores. The thin layer of stationary phase is derivatized with alkyl bonded moieties such as C3, C8, and C18, providing rapid separations of proteins by reversed-phase chromatography. These Poroshell type packings combine the advantages of rapid mass transfer (that is, improved efficiency), a decent sample capacity, and provide good recovery of biomolecules. Figure 3 shows the rapid separation of several protein standards in less than a minute on a Poroshell Stablebond C18 column.

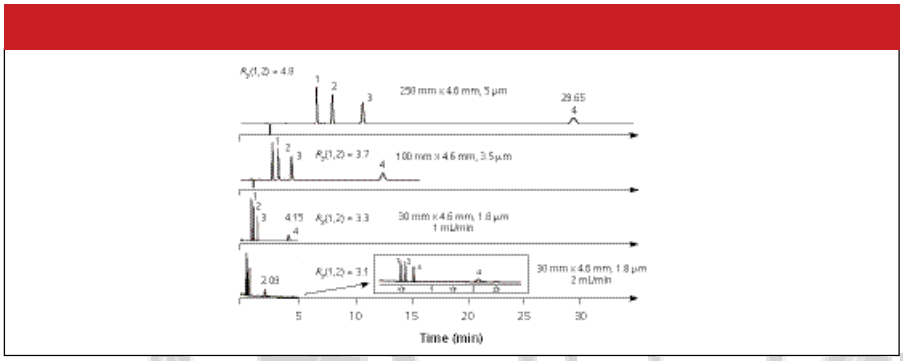


**Figure 2:** Separations obtained using 250, 100, and 30 mm × 4.6 mm columns packed with 5-, 3.5-, and 1.5-µm particles, respectively. Columns: Zorbax SB-C18; mobile phase: 50% 20 mM monobasic sodium phosphate (pH 2.8), 50% acetonitrile; flow rate: 1.0 mL/min (except for the bottom chromatogram, for which the flow rate was 2.0 mL/min); temperature: ambient; detection: UV absorbance at 230 nm. Peaks: 1 = estradiol, 2 = ethynylestradiol, 3 = dienestrol, 4 = norethindrone. (Courtesy of Agilent Technologies.)

## Monoliths

Monoliths are columns that are cast as continuous homogeneous phases (just like concrete in a mold) rather than packed as individual particles. These types of columns have been reviewed in *LCGC North America* (10) and in the present volume (11). Since the latter reference details the synthesis and features of monolithic columns, I will just highlight these advances for completeness.

Monolithic columns have great potential in offering a stable, easily replaced column for both analytical and preparative separations. Both silica-based and polymer-based monoliths have been extensively studied.

There are two important characteristics for current silica monolith columns—they have the efficiency equivalent to about a 3- to 5-µm silica particle and their pressure drop is about 30–40% lower than a 5-µm silica particle. Thus, columns can be coupled in a serial manner, thereby generating higher plate counts for more difficult separations.

The polymeric monolith columns also have made their mark on separation science. These columns consist of a continuous cross-linked, porous monolithic polymer, usually polymethacrylates, methyacrylate copolymerizates, or polystyrene-divinylbenzene. They can be fabricated into disks and tubes in convenient housings for easy connection to an HPLC system. The polymeric monoliths seem to be favored for the separation of larger biomolecules. Note that the functionalized membranes that have long been used in the isolation of biomolecules are also a form of monolith columns.

## Inorganic–Organic Hybrids

Waters (Milford, Massachusetts) has developed a unique approach for making a hybrid packing, especially useful for high pH applications, where silica gel has been in disfavor. Traditionally, when high-pH conditions were required to achieve greater retention of basic compounds or for compound stability reasons, polymeric packings, coated zirconia or alumina particles, or graphitized carbon materials usually were considered. For various reasons, such as lower efficiency, swelling–shrinking problems, strong adsorption sites, and other undesirable features, these materials never have achieved the popularity of silica gel as a base material. Waters has attempted to combine the advantages of silica with those of organic polymers.

Most modern silica gels used in HPLC are produced by the polymerization of tetrachloro- or tetraethoxy-silane monomers, eventually resulting in a silica-gel polymer with siloxane bonds (Si-O-Si) and various types of terminal silanols (-Si-OH) at their surface. In its synthesis process, Waters starts with a silane monomer that contains both a methyl group and three ethoxy groups, thereby incorporating a methyl group into the silica-based final packing material. The column constructed from this material is called XTerra and has proved to be more stable in alkaline conditions than their typical silica-based packings (12). They also have used ethylene-linked triethoxysilane $(RO)_3SiCH_2CH_2Si(OR)_3$ instead of $CH_3Si(OR)_3$ to form a sol gel. At high pH, the particle from this later method has a 30% longer lifetime than the particle from the sol gel of $CH_3Si(OR)_3$ (13). The reversed-phase material, available in 1.7-µm particle diameter, appears to have good high-pressure stability and is now a key part of the Acquity UPLC system. Figure 5 provides an application example of this new hybrid material packed into a 100 mm × 2.1 mm column run at 0.3 mL/min. A 5-µL injection of a rather complex ginger root extract was separated into a number of peaks in just under 6 min using gradient elution.

Another approach to make more alkaline stable silica bonded phase was used by Kirkland and colleagues (14) where a special bidentate bonded phase anchored at two adjacent silanols combined with a high degree of endcapping protected the underlying silica backbone from attack by the hydroxide ion.

## Future Directions in Packing Development

Silica gel with chemically bonded phases will be around for a long time. Even though many new materials have surfaced, silica-based packings retain their dominance in most laboratories. Their excellent efficiency, rigidity, lower cost than the alternatives, and ability to be functionalized to fit just about any HPLC mode will ensure their continued success. For simple sample mixtures encountered, the trend toward the use of smaller porous particles (now as small as 1.7 µm) packed into short columns will continue. However, more than likely, a 7.5–15 cm × 0.46 cm, 3–3.5 µm packed column will replace the 25 cm × 0.46 cm, 5-µm column as the standard workhorse column. These shorter columns with the smaller particles can provide the same resolution of a longer column with larger particles. The driving forces for short, fast columns will be high throughput requirements such as quality assurance/quality control, LC–mass spectrometry (MS) and LC–MS-MS, and combinatorial chemistry needs, with solvent savings and increased sensitivity as secondary benefits.

It remains to be seen if particles in the 1–2 µm range become mainstream. For optimized results with short columns (less than 50 mm), extracolumn effects, dwell volumes, and injection and detection volumes must match the narrow peak widths that are encountered so that band spreading does not occur. For these same sized particles packed into long columns (> 50 cm), tremendous column efficiency can be realized for very difficult separations, but ultra-high-pressure instruments must become available to permit their use.

Columns of smaller dimensions with internal diameters of less than 100 µm with small particle packings have become interesting to those studying proteomics. The small samples and low concentrations of analyte strongly favor these miniature columns with small-particle packings.

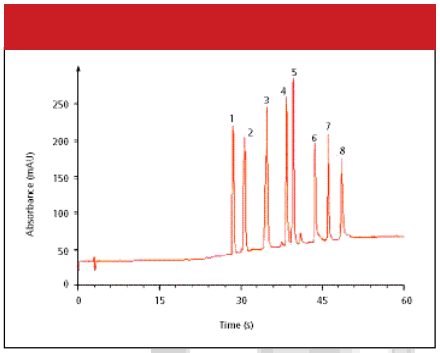


**Figure 3:** Fast, high-resolution separation of peptides and proteins. Column: 75 mm × 2.1 mm Poroshell 300SB-C18; mobile phase A: 0.1% trifluoroacetic acid; mobile phase B: 0.07% trifluoroacetic acid in acetonitrile; gradient: 5–100% B in 1.0 min; flow rate: 3.0 mL/min; temperature: 70 °C; pressure: 260 bar; detection: UV absorbance at 215 nm. Peaks: 1 = angiotensin II, 2 = neurotensin, 3 = RNase, 4 = insulin, 5 = lysozyme, 6 = myoglobin, 7 = carbonic anhydrase, 8 = ovalbumin. (Courtesy of Agilent Technologies.)

Detection by MS, especially tandem MS, provides increased sensitivity and structural information for tiny amounts of peptides in tryptic digests.

The monolithic columns should become further commercialized and of lower cost. For mainstream separations, the silica monoliths, currently only available in 4.6-mm i.d., require higher flow rates than are desirable for LC-electrospray MS. A 2.1-mm column would have optimized flow rates in a desirable range but these are not yet commercially available. Recently, a 100-µm i.d. silica monolith column prepared in situ has been introduced by Merck and a 100-µm PS-DVB monolith by LC Packings Dionex (Sunnyvale, California). Although bonded-silica columns are now supplied with PEEK cladding, the silica rod cannot be replaced easily because the cladding is integral to the column. If a bonded-silica monolith or silica-rod column could be placed into a holder or housing and provide the efficiency and lifetime that clad columns have shown, they would be an ideal and easily replaced column. A dead column could be removed easily and a new rod slid in place without having to use any special configurations or tools. Both analytical- and preparative-sized polymeric monoliths are already provided in housings and, in some cases, the bulk monolith can be removed and replaced. Silica monoliths are covered by patents, so their widespread development might be hindered because the technology is not widely available.

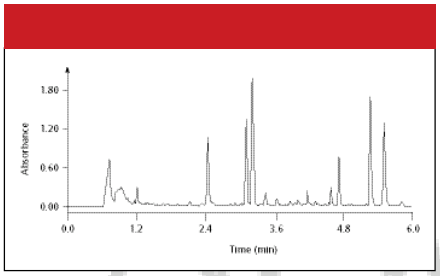


**Figure 4:** LC analysis of ginger root extract. Column: 100 mm × 2.1 mm, 1.7-µm $d_p$ Acquity UPLC; mobile phase A: water; mobile phase B: acetonitrile; gradient: 50% B for 1.36 min, 50–100% Bover 2.31 min; flow rate: 0.3 mL/min; injection volume: 5 µL; temperature: 30 °C; detection: UV absorbance at 230 nm. (Courtesy of Waters Corporation.)

Polymeric monoliths, although also covered by various patents, seem to have many possible synthetic approaches with a wide number of manufacturers now investigating and commercializing this technology. Both capillary and large-scale preparation devices have appeared.

New types of particles constructed from newer materials will undoubtedly be developed as chromatographers and manufacturers look for the ideal packing that will provide high recovery, excellent efficiency, low cost, and extraordinary stability. For example, titania is being studied as a base material for bonded phases and as a "bare" material for ion-exchange separations (15). New copolymers that provide unique surface properties will continue to be developed, hopefully with better efficiency. One example is the hydrophilic polymers provided as SEC packings that can be used with either organic or aqueous mobile phases and converted from one solvent system to another. Similarly, chiral columns that are useful in both aqueous and organic solvent systems also have become available. These two types of columns now provide a lower cost alternative to chromatographers who do samples in both types of solvent systems because they only have to buy a single size exclusion or chiral column. Bimodal SEC phases, in which multiple pore sizes are available in one bead or in one column, might give polymer chemists a packed bed that can cover a wide molecular weight range in one column. Hybrid inorganic–organic materials might offer a better compromise to solve stability problems for observed silica-based materials at higher pH, and more of them will be studied.

The current studies on "lab-on-a-chip" will result in chromatography columns that will be fabricated (rather than packed) on the inner walls of the tiny capillaries. The movement of liquids through these tiny columns can be by electroosmotic flow rather than conventional hydraulic means, although pumping systems and nano valves can be integrated onto the chip. Methacrylate monoliths have been prepared inside of capillary tubes (16,17) for capillary electrochromatography and solid-phase extraction. Sol-gel silicas also have been prepared in situ in chip channels (28) and used for the separation and amplification of DNA. Many laboratories are investigating these technologies. In fact, open-tubular liquid chromatography, the ultimate in column performance, might become a reality if taken to the dimensions of the microchip flow channels.

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