Advances in HPLC Column Packing Design

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Even though HPLC column technology is considered to be somewhat mature, new developments continue. Improvements in packing-material design, bonded-phase chemistry, column construction and formats have occurred. Users now have a better understanding of the advantages and limitations of silica-based materials and do not attempt to use them under conditions that may shorten their lifetime or decrease their performance. In addition, new phases have extended operating pH ranges (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 favour 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 (\(d_p\) < 10 µm) came on the scene and appropriate packing methods were developed. 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 aim of HPLC packings early in the game was to achieve the best efficiency possible and consequently better chromatographic resolution. To better understand the various approaches employed to improve column efficiency, let’s briefly discuss the morphology of a porous packing material such as silica gel or alumina.

Diffusive pores dominate a typical porous packing (Figure 1(a)) and the major surface area of the particle is contained within these pores. A reduction in particle size improves both interparticle and 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 to interact with the stationary phase. Following this interaction, the solute molecules must diffuse out of the particle and continue their 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 particles 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 can now produce small diameter particles with fairly narrow particle-size distributions down to 1.5 µm average diameter, although 3–3.5 µm and 5 µm particles are still the norm.

Figure 1: Flow characteristics and design of packing particles in HPLC. (a) totally porous particle; (b) perfusion packing; (c) non-porous silica (NPS) or non-porous resin (NPR); (d) Poroshell particle.
However, congruent with this improvement in efficiency was a 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. Although pumps can provide the necessary increase in pressure output at normal flow-rates (e.g., 1–3 mL/min) with commonly used solvents (e.g., water, methanol, acetonitrile, hexane, etc.), users found that columns were not particularly stable when run near to pump pressure limits as high as 450 bar.

Column efficiency, H or HETP (height equivalent to a theoretical plate), is proportional to \( \frac{1}{d^2} \), where \( x \) is approximately 1.6–1.9. Resolution is proportional to \( \frac{1}{\sqrt{N}} \). 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 \( \mu \)m particles in 4.6 mm i.d. by 20–50 mm length), rather than longer columns with larger particles (5 \( \mu \)m particles in 4.6 mm i.d. \times 150–250 mm lengths). 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 (\( d_x = 5, 3.3, 1.8 \) \( \mu \)m) in shorter columns (\( L = 250, 100 \) and 30 \( \)mm, respectively), compared with more traditional HPLC analytical columns. The flow-rate on all columns is the same except for Figure 2(d) in which the flow is increased to 2 mL/min to illustrate a possible further decrease in separation time by increasing the flow-rate. Compared with the separation on a conventional 4.6 mm \( \times 250 \) mm column, the separation time was reduced 15-fold (a little over 2 min).

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 over 100,000 requires particles in long columns, 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 submicron 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 coworkers\(^4,5\) from the University of North Carolina involved small particles (down to 1 \( \mu \)m) with ultra high 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 less than an hour! The ultrahigh-pressure chromatograph can also be used for gradient elution. To direct users on how to employ these systems routinely, Milton Lee and coworkers\(^6\) from Brigham Young University in Provo, Utah, USA have devoted their attention to solving some of the practical concerns of ultrahigh-pressure systems. Particular attention has been paid to the designs of injection valves with respect to injection reproducibility, injection time, maximum operating pressure, sample amount injected and the valve’s impact on system efficiency. These workers have also used supercritical carbon dioxide as a packing solvent. Until commercial HPLC systems are available that can handle these ultrahigh-pressure columns, they will be mostly used in academic research laboratories.

**Perfusion Packings**

Perfusion packings were developed by Afeyan and co-workers\(^7-9\) and commercialized by Perseptive Biosystems (Cambridge, Massachusetts, USA, now part of Applied Biosystems) in the late 1980s that gave improved chromatographic performance, particularly for larger molecules. A simplified pictorial representation of a perfusion packing is shown in Figure 1(b). Compared with the porous packings, the perfusion packings consists of two different types of pores: diffusive pore 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 particles, a portion of the mobile phase flows through the particle 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 larger than those typically used in HPLC packings, with the smallest average particle size being \( \sim 12 \) \( \mu \)m. However, when compared...
with a porous packing of the same particle and pore size, the perfusion packings give better efficiency for large molecules.\(^9\)

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 may be used at higher flow-rates. At these higher flow-rates they maintain their sample capacity making them useful for preparative separations and purifications.

**Non-Porous and Superficially Porous Packings**

The use of non-porous packings represents another approach to improve the rates of mass transfer. There are two types of non-porous packings: non-porous silica (NPS) and non-porous resin (NPR). As depicted in Figure 1(c), the non-porous packings are very reminiscent of the older pellicular or porous-layer beads (PLBs) used in the early days of HPLC, but these materials are of much smaller particle sizes, typically in the 1.5–2.5 \(\mu\)m range.\(^\text{10}\) The thin porous 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 NPS and NPR unsuitable for preparative separations. In addition, because of their small particle size, the backpressures from NPS columns are generally much greater than those experienced with microparticulate HPLC porous packings of popular particle sizes (i.e., 5 and 3 \(\mu\)m). For more information on the use and advantages of NPS packings, consult reference 10. Such particles are finding less use in today’s chromatography laboratory.

Superficially porous packings (Figure 1(d)) are similar to NPS particles described above but the particle diameter is larger (~ 5 \(\mu\)m), providing a much lower pressure drop. These Poroshell particles (Agilent Technologies, Wilmington, Delaware, USA) are recommended for larger biomolecules that diffuse slowly into porous packings. When flow-rates are increased with porous packings, the biomolecule peaks broaden because of 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. Poroshell-type packings combine the advantages of rapid mass transfer (i.e., improved efficiency), a decent sample capacity and good recovery of biomolecules. Figure 3 shows the rapid gradient separation of several protein standards in less than 1 min on such a column.

**Monoliths**

Monoliths are columns that are cast as continuous homogeneous phases (just like concrete in a mould) rather than packed as individual particles. These types of columns have been reviewed earlier in \(\text{LC•GC}\)\(^\text{11}\) and in this present volume.\(^\text{12}\) There are several types of monolithic column:

- agglomerates of polycrylamide particles
- polymethacrylate block
- agglomeration of micron-size silica beads
- polystyrene-divinylbenzene block
- silica rods
- membranes of various types (made by many manufacturers).

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. The silica-based materials were developed by Tanaka and coworkers in Japan,\(^\text{13}\) introduced by Cabrera and coworkers\(^\text{14}\) at HPLC ’98 in St. Louis, Missouri, USA, and commercialized by E. Merck (Darmstadt, Germany) as its SilRod column. These columns are solid rods of silica monolith. Similar to the perfusion packings, they have both flow-through pores with macroporosity (1–2 \(\mu\)m in width) and diffusive pores (called mesopores). The silica rods can be modified using the same derivatization chemistries that are used for regular HPLC packings (e.g., C18 bonded phase). The SilRods, now encapsulated in PEEK, have been introduced as Chromolith.

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

The polymeric monolith columns have also made their mark on separation science. These columns consist of a continuous crosslinked, porous monolithic polymer usually poly(methacrylates or methacrylate copolymers. They can be fabricated into discs and tubes in convenient housings for easy connection to an HPLC system. Some examples of commercial products are the UNO from BioRad Laboratories (Richmond, California, USA), the CIM copolymers from BIA Separations (Ljubljana, Slovenia\(^\text{11}\)), and the Swift polystyrene-divinylbenzene monoliths from Isco (Lincoln, Nebraska, USA). To illustrate the use of a polymeric monolith, Figure 4 provides a 1 min separation of oligodeoxynucleotides (8- to 16-mers) on a CIM DEAE disc with a 3 mm thickness and a 16 mm diameter. This particular run was a fast gradient elution using 6 mL/min flow-rate that permits the use of a relatively thin disc of poly(glycidylmethacrylate-ethylene glycol) dimethacrylate copolymer. There are several polymeric monoliths.
commercially available with ion-exchange, hydrophobic-interaction, reversed-phase and affinity chromatography capability. 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, USA) has developed a unique approach for making a hybrid packing, especially useful for high-pH applications, in which silica gel has been less useful. Traditionally, when high-pH conditions were required to achieve greater retention of basic compounds or for compound stability reasons, polymeric packings, coated zirconia, alumina particles, or graphitized carbon materials were usually considered. For various reasons such as lower efficiency, swelling/shrinking problems, strong adsorption sites and other undesirable features, these materials have never 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 (called X Terra) has proved to be more stable in alkaline conditions than its typical silica-based packings.15 The company has also used ethylene-linked triethoxysilane (RO)3SiCH2CH2Si(OR)3 instead of MeSi(OR)3 to form a sol-gel. At high-pH conditions, the particle from this latter method has a 30% longer lifetime than the particle from the sol-gel of MeSi(OR)3.16 Another approach to make more alkaline stable silica bonded phases was used by Kirkland and coworkers17 in which 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 hydroxide ions.

Sol-Gel Silica
As silica gel is the most widely used base material for bonded phases in HPLC, there is an interest in expanding the pH operating range both on the acid and the basic sides of the pH scale. There are two types of silica particles used in commercial HPLC columns: sil-gel and sol-gel. Sil-gel particles, usually made by gelling soluble silicates or coalescing fumed silica, are characterized by higher porosities and irregular pore shapes with variable wall thicknesses. Sol-gel particles, which are made by aggregating silica sol particles, have lower porosities and more-regular pores with thicker walls defined by the surrounding solid silica-sol particles. Sol-gels are generally more mechanically stable than sil-gels. Both silica gel types can withstand typical mobile phase buffers that are used on the acidic end of the scale. At low-pH values, the unprotected siloxane bonded phases are the more vulnerable part of these bonded packings and may be subject to catalysed hydrolysis by the hydronium ion.18 Pertinent to the discussion of intermediate-to-high pH stability, because of their thinner pore walls sil-gel particles appear to dissolve more quickly than sol-gel particles.19,20 Accordingly, researchers anticipate developing more long-term stable methods at intermediate-to-high pH using columns made with sol-gel supports such as Hypersil (Thermo Hypersil Keystone, Bellefont, Pennsylvania, USA), Zorbax (Agilent Technologies) and Spherisorb (Waters) columns.

Other Packing Materials
Other non-silica–based packings have been introduced in the last few years; among them have been polystyrene-divinyl benzene (PS-DVB) co-polymers, other organic polymers, zirconia, graphitized carbon and hydroxyapatite. As graphitized carbon was recently the subject of a review,21 I will not elaborate on this packing material.

The PS-DVB polymers have been around for a long time. Users often turned to them when they needed a high-pH, reversed-phase alternative to bonded silica gel. These polymeric materials have a wide pH range, have high crosslinking, are pressure and temperature stable, and have no silanols to interact with basic compounds. However, they suffer from poorer efficiency than silica gel. Typically, a 5 μm polymeric column may exhibit about a third of the theoretical plates that commercially available with ion-exchange, hydrophobic-interaction, reversed-phase and affinity chromatography capability. Note that the functionalized membranes that have long been used in the isolation of biomolecules are also a form of monolith columns.

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a comparable silica gel bonded phase column. The rate of mass transfer in the bulk stationary phase appears to be slower than for a silica gel packing. Also, polymeric materials with a lower degree of crosslinking may swell and shrink as the mobile-phase composition is changed, as with gradient elution.

In the area of ion-exchange/ion chromatography and size exclusion chromatography (SEC), polymers have gained widespread acceptance. Silica gel–based ion exchangers do not display the ruggedness that polymers display, especially in strong buffers and with higher pH mobile phases. For ion chromatography, special polymers have been developed by Dionex (Sunnyvale, California, USA)\textsuperscript{22} and applied for separations of various anionic and cationic species. The company’s approach is to use a polymer bead consisting of ethylvinylbenzene 55% crosslinked with DVB as a base material and then to functionalize or graft it with various ionogenic groups. In some instances, the surface of the bead is pellicular-like and therefore the efficiency is better than the totally porous polymer materials.

Polymeric materials are generally recognized as the standard in gel permeation chromatography (GPC). Small particle (5 µm) GPC packings, compatible with organic or aqueous mobile phases display good efficiency and with various pore sizes available can separate small and large organic molecules. Silica gel packings with different pore sizes are available but have limited acceptance in GPC, presumably because of their surface activity. Silica gel packings with special deactivation have found use in the size separation of proteins.

Zirconia (ZrO$_2$) has many properties that make it attractive as an HPLC packing, particularly its stability. It can be produced in monodisperse, porous spherical particles and, for many separations, it can provide the efficiency of silica gel. Zirconia has excellent pH properties and can be used up to pH 14; it also has high-pressure and high-temperature stability. Unlike silica, the surface has no silanols for amine interactions.

However, its surface does possess hard Lewis acid sites and has a strong affinity for hard Lewis bases (e.g., hydroxyl, phosphate, fluoride and carboxylate). Therefore, one must add competing anions (40 mM concentration) for analytes containing these functional groups. Most successful separations on zirconia-based columns have been those achieved on polymer-coated phases. The most popular coated phase has been the polybutadiene (PBD). The column can be used for reversed-phase separations using water–acetonitrile or water–methanol mobile phases; its selectivity is different than C18-bonded silicas. Zirconia can also be covered with a thin film of pyrolytic carbon resulting in a surface that, in addition to possessing reversed-phase properties, has interesting selectivity for the separation of diastereomeric compounds and stereoisomers because of its high isomeric selectivity and for highly polar solutes for which retention on bonded phases is too low to be useful. The high-temperature capabilities of coated zirconia have spawned several applications using temperature (greater than 100 °C) as a variable in the optimization of LC separations. For a selection of applications of zirconia-based HPLC packings, consult reference.\textsuperscript{23}

In its low-pressure application, hydroxyapatite (a calcium phosphate material) has long been used for purification of a wide range of biomolecules including proteins and monoclonal antibodies. However, because of their fragility, these earlier materials were unsuitable for the higher pressure operation required in HPLC. A newer generation of materials, however, is more structurally rigid and show good recoveries (typically 90–100%) and provide high activity of sensitive biomolecules. Because hydroxyapatite is a pure material, there is no bonded organic phase that can strip to contaminate collected analytes. Columns show high capacity; a 1.0 × 10 cm column can handle 20.4 mg of lysozyme. A modern hydroxyapatite packing is depicted in Figure 5. As can be seen in the scanning electron micrograph, it has a unique porous structure. A typical gradient elution application of the separation of nucleotides showed a rapid separation and good pressure stability at a variety of flowrates.\textsuperscript{24}

**Figure 5:** Scanning electron micrographs of Hydroxyapatite particles (Courtesy of Wilhelmy Fine Particles, W. Leechburg, Pennsylvania, USA).

**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 alternatives, and ability to be functionalized will ensure their continued success. The trend towards the use of smaller particles (3 µm) packed into short columns will continue and, more than likely, a 3 µm packed column of 7.5–15 cm length by 0.46 cm internal diameter will replace the 5 µm column 25 cm length by 0.46 cm column that is the standard today. These shorter columns with smaller particles can provide the same resolution as a longer column with larger particles. The driving forces will be higher throughput requirements — quality assurance/quality control, LC/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 (<50 mm), extracolumn effects, dwell volumes, injection and detection volumes must match the narrow peak widths so that band spreading does not occur. For these same sized particles packed into long columns (25–50 cm), tremendous column...
efficiency can be realized for very difficult separations but ultrahigh-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 favour these miniature columns with small particle-sized packings. Detection by MS, especially tandem MS, provides increased sensitivity and structural information for tiny amounts of peptides in tryptic digests.

Monolithic columns should become further commercialized and cheap. The silica monoliths, currently only available in 4.6 mm i.d., require higher flow-rates than are desirable for LC-electrospray. 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. monolith column prepared in situ has been reported.24 Although bonded silica columns are now supplied with PEEK cladding, the silica rod cannot be easily replaced 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 into place without having to use any special configurations or tools. Both analytical- and preparative-size polymeric monoliths are already provided in housings and, in some instances, the bulk monolith can be removed and replaced.

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.25 New co-polymers that provide unique surface properties will continue to be developed, hopefully with better recovery, excellent efficiency, low cost and extraordinary stability.

The current studies on “lab-on-a-chip” will result in packing efficiencies of 100% for capillary electrophoresis (CEC) and solid-phase extraction. Sol-gel silicas have also been prepared in situ in chip channels28 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, may become a reality if taken to the dimensions of microchip flow channels.

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