

Monolithic Stationary Phases for HPLC and Sample Preparation



Monoliths are separation media in the format that can be compared to a single large “particle” that does not contain interparticular voids. As a result, all the mobile phase must flow through the stationary phase. This convective flow greatly accelerates the rate of mass transfer. In contrast to diffusion, which is the typical driving force for mass transfer within the pores of particulate stationary phases during chromatographic processes, convective flow through the pores enables a substantial increase in the speed of the separation of large molecules such as proteins. A thorough theoretical treatment of the mass transfer within monolithic materials has been developed by Liapis (1) and Tallarek (2).

The first thoughts related to what we today call monoliths can be traced down to the works of the Nobel Prize winner Richard Syngé who in 1952 envisioned “a continuous block of porous gel structure” (3). However, materials like that were not available at his time (4). Real attempts to make “single-piece” stationary phase from highly swollen polymer gel (5) and open-pore polyurethane foams (6,7) during the late 1960s and the early 1970s were less successful. Interest in the monolithic formats only has been revived in the late 1980s as novel approaches towards both separation media with significantly decreased volume of interparticular voids such as stacked membranes and rolled woven matrices, and true monoliths such as compressed hydrophilic gels, macroporous polymer disks, columns, tubes, as well as silica rods, in which the void volume is completely eliminated, have been developed. Several monolithic stationary phases are now commercialized and some of their features are summarized in Table I. A very detailed account of these materials can be found in the monograph published recently (8) and several review articles (9–11).

Macroporous Monolithic Disks

The disk format is one of the first useful monolithic stationary phases originally designed for the rapid separation of proteins (12). In the mid 1980s, Belenkii and coworkers studied chromatography of proteins in gradient elution mode using stationary phases with a variety of chemistries

and column geometries and found that only a certain, often only a very short distance is required to achieve good separation (13). This finding resulted in the concept of short separation beds. Because it was very difficult to create such beds from particulate sorbents due to irregularities in packing density and excessive channeling, new monolithic stationary phase in disk format has been developed at the Institute of Macromolecular Chemistry in Prague (Czech Republic) that enabled very fast separation. Typically, the monolithic material is prepared in a flat or tubular mold, the sheet or cylinder removed from the mold, and the porous polymer punched or sliced to obtain up to 3-mm thin disks. These are then placed in a specifically designed cartridge. In the current commercial implementation called CIM-Disks (BIA Separations, Ljubljana, Slovenia), the disk is embedded in a polyolefin ring that forms an impermeable sidewall as shown in Figure 1a. Although the monoliths are sufficiently mechanically stable to be easily handled, this ring also serves to reinforce the disks and prevent fraying of their edges. Their color indicates specific chemistry of the disk. An additional benefit is that the flat face of the ring enables the disk to be firmly sealed between the bottom and top face of the cartridge without exercising excessive force on the porous polymer monolith. In contrast to the cartridge, the disks are disposable. The proprietary cartridge is designed to allow insertion of a single disk or several disks. This disk stack-

Frantisek Svec and Laurent Geiser

Department of Chemistry, University of California, Berkeley, California

Please direct correspondence to
Frantisek Svec at svec@berkeley.edu

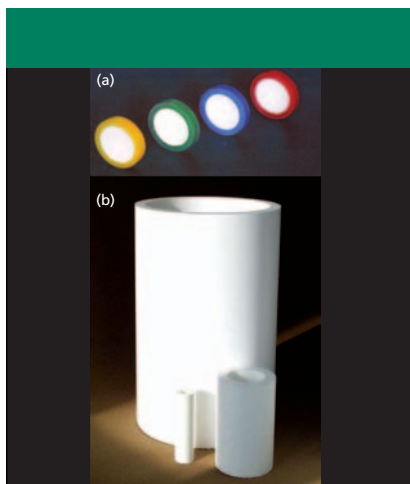


Figure 1: Picture of CIM (a) disks and (b) tubes (Reproduced by courtesy of BIA Separations.)

ing ability has led to the development of the multidimensional separation process called “conjoint liquid chromatography” in which disks with different chemistries are used in series simultaneously. This technique would be very difficult to implement with traditional columns. Figure 2 shows the rapid separation of two proteins and IgG in a single cartridge containing three disks provided with two different ligands, QE (strong anion exchange) and Protein G (affinity), respectively (14).

From the very beginning, monolithic disks exhibited an excellent performance in the rapid separations of proteins and nucleic acids using gradient elution (15). Most of the generic disks are prepared from reactive monomers such as glycidyl methacrylate that is subsequently modified to afford desired interacting functionalities. These modifications enable exten-

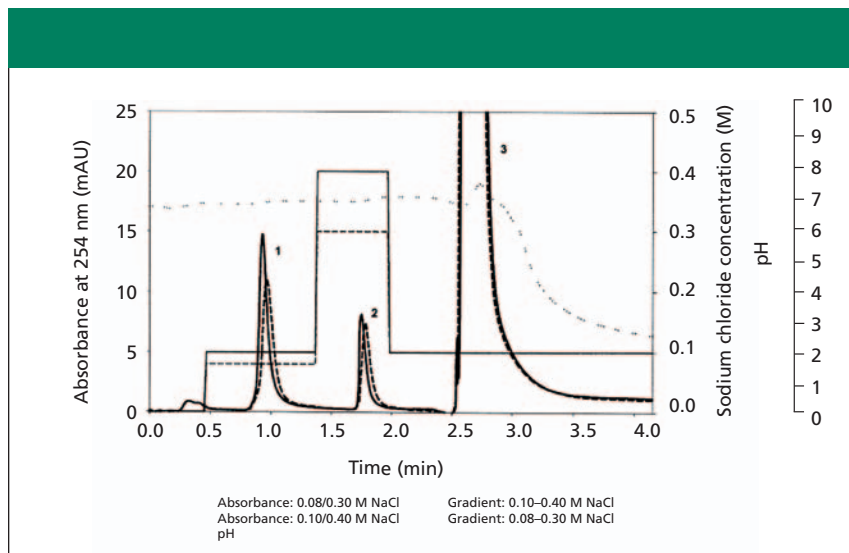


Figure 2: Superimposed chromatograms of two runs using different step gradients obtained by conjoint liquid chromatography separation of transferrin, albumin, and IgG using a stack of two CIM Protein G disks and one CIM QA disk. Separation media: 12 mm diameter \times 3 mm thick disks with a volume of 0.34 mL; binding buffer: 20 mM Tris-HCl (pH 7.0); elution buffer I: 20 mM Tris-HCl, 1 M sodium chloride (pH 7.0); elution buffer II: 0.1 M glycine-HCl, 0.1 M sodium chloride (pH 2.0); flow rate: 4 mL/min; IgG concentration in sample: 20 mg/mL; injection volume: 100 μ L; detection: UV absorbance at 280 nm. Peaks: 1 = transferrin eluted with 0.08 or 0.1 M sodium chloride, 2 = albumin eluted with 0.3 or 0.4 M sodium chloride, 3 = IgG eluted at low pH. (Reprinted with permission from reference 14. Copyright 2002 Elsevier.)

sion of the disk separations to a number of modes such as reversed-phase, ion-exchange, hydrophobic interaction, and bioaffinity chromatography.

Rigid macroporous polymer monolithic columns: In the early 1990s, we introduced new macroporous polymer monolithic columns formed by a very simple “molding” process (16). In contrast to disks portrayed previously, these monoliths are polymerized in situ within a tube such as chromatographic column or capillary in which they remain all the time after the preparation is

completed. Monolithic columns of this type are now available from Dionex (Sunnyvale, California) in numerous formats with functional groups suitable for reversed-phase and ion-exchange chromatography of proteins and polynucleotides.

In addition to original stainless steel analytical size columns, monolithic columns for high performance liquid chromatography (HPLC) also have been prepared in capillaries (17–23) LC Packings, part of Dionex Corporation, now sells these monolithic capillary columns made of

Table I: List of current commercial monolithic stationary phases for HPLC separations

Product	Shape	Producer	Chemistry	Separation modes
CIM Disk	Disk	BIA Separations	ORGANIC POLYMERS Functionalized polymethacrylate or polystyrene copolymers	Ion exchange, hydrophobic interaction, reversed phase, bioaffinity
SepraSorb	Disk	Sepragen	Functionalized cellulose	Ion exchange
CIM Tube	Radial flow column	BIA Separations	Functionalized polymethacrylate or polystyrene copolymers	Ion exchange, hydrophobic interaction, reversed phase, bioaffinity
UNO	Column	BioRad	Polyacrylamide based copolymers	Ion exchange
Monoliths	Capillary	LC Packings	Polystyrene copolymer	Reversed phase
	Column, capillary	Dionex	Functionalized polymethacrylate or polystyrene copolymers	Ion exchange, reversed phase
			SILICA	
Chromolith	Column, capillary	Merck	Bonded silica	Reversed phase
Onyx	Column, capillary	Phenomenex	Bonded silica	Reversed phase, normal phase
CB Silica Plate	Disk	Conchrom	Bonded silica	Reversed phase, normal phase

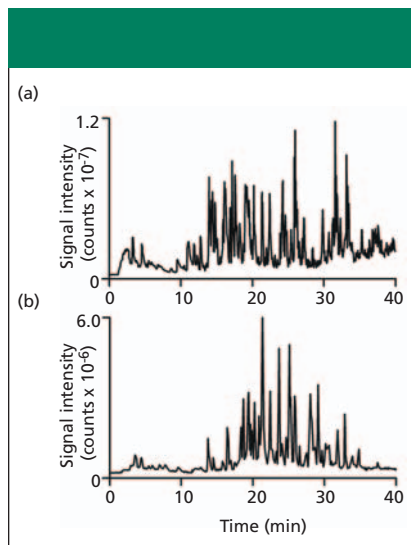


Figure 3: Reversed-phase HPLC–electrospray ionization (ESI)–MS–MS analyses of equal amounts of a complex peptide mixture using monolithic and microparticulate columns. Conditions: (a) 60 mm \times 0.1 mm poly(styrene-co-divinylbenzene) monolith and (b) 75 mm \times 0.075 mm Pepmap column packed with octadecyl silica particles; gradient: (a) 0–40%, (b) 5–60% acetonitrile in water in 5 min, in 0.050% trifluoroacetic acid; flow rate: (a) 0.1 μ L/min, (b) 0.5 μ L/min; temperature: 50 $^{\circ}$ C. Sample: tryptic digest of catalase, cytochrome C, myoglobin, α -lactalbumin, transferrin, carbonic anhydrase, β -lactoglobulin A, lysozyme, serum albumin, and ribonuclease A (0.65–270 fmol each). (Reproduced with permission from reference 24. Copyright 2005 Wiley VCH.)

styrene and divinylbenzene. This format is readily compatible with mass spectrometry (MS). Therefore, this format is becoming very popular because it is much simpler to polymerize liquid precursors and form the stationary phase in situ within a capillary than packing it efficiently with microparticles. Toll and colleagues (24) recently compared separations achieved using a capillary monolithic and packed column for the separation of a complex peptide mixture. Figure 3 clearly demonstrates the potential of the monolith.

Several methods are available for the preparation of monolithic stationary phases with desired surface chemistry. For example, directly polymerized poly(styrene-divinylbenzene) monoliths proved to be an excellent stationary phase for the very fast separation of peptides and proteins (25). Another option is the preparation of a monolith with reactive functionality and its subsequent modification to afford stationary phases for a variety of separation modes. In this technique, each

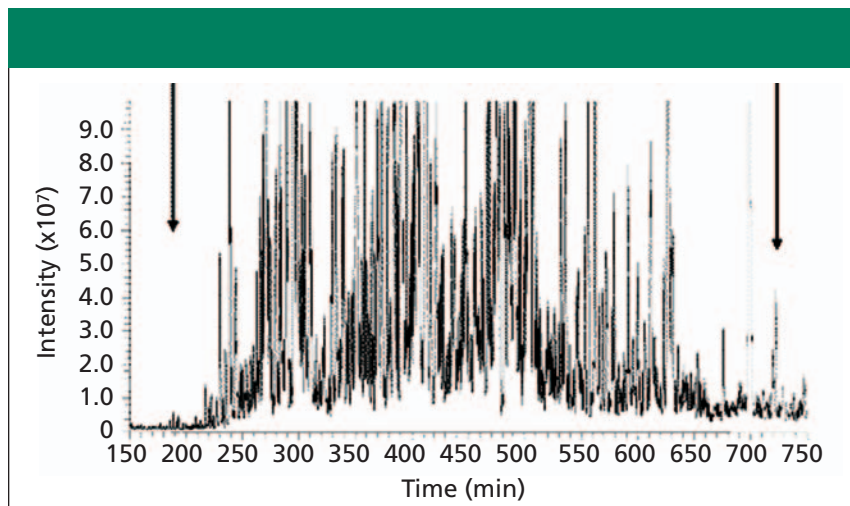


Figure 4: High-efficiency on-line microSPE–nanoLC–ESI–MS–MS analysis with extended LC gradient of for analysis of a 2.5 μ g *S. oneidensis* tryptic digest sample. Column: 70 cm \times 20 μ m silica-based monolithic; pressure: 34.5 MPa (5000 psi); mobile phase A: 0.2% acetic acid and 0.05% trifluoroacetic acid in water mobile phase B: 90% acetonitrile, 10% water, 0.1% trifluoroacetic acid, v/v; gradient: 0–75% B in 750 min. Data collection began at the start of the gradient. A 4 cm \times 50 μ m monolithic column was used as the on-line microSPE precolumn. A six-port positive-feedback switching valve with a sample loop of 10 μ L was used for sample introduction. (Reproduced with permission from reference 42. Copyright 2005 American Chemical Society.)

single reactive site generates one new functionality. Among others, glycidyl methacrylate-based monoliths mainly have been used in these applications (26). Yet another method that enables both preparation of monoliths and modulation of their surface chemistries relies on UV-light triggered processes. Using capillaries with UV-transparent PTFE coating, the polymerization can be initiated by irradiation with light and proceeds faster than its thermally initiated counterpart (27,28). The chemistries of these columns are dictated by the choice of monomers used for their preparation that have to be UV transparent (29). The desired surface chemistry is then obtained via grafting reactions initiated again by UV light (30). One of the major advantages of grafting is that it affords monoliths in which each surface site provides for numerous functionalities, thus, dramatically increasing the column loading capacity.

Tubular Monolithic Columns with Radial Flow

Large monolithic columns are desirable for the separations on preparative scale. However, free radical polymerization used for the preparation of porous polymer monoliths is an exothermic process that creates heat. While this does not appear as a problem in the preparation of analytical-scale monolithic columns, the accurate control of the polymerization temperature for larger

size monoliths is far more problematic (31). The unstirred nature of the polymerization within the confines of a mold leads to a decreased capacity to effectively dissipate the heat of polymerization. In addition to an overall deviation from the desired polymerization temperature, the temperature might also vary in magnitude across the contents of the mold in the radial direction. In light of the demonstrated effect of the polymerization temperature on the porosity of the resulting polymer, any significant variation in temperature within the mold leads to monoliths with heterogeneity in their pore structures and significantly reduced performance.

This problem was elegantly alleviated by the preparation of monoliths in an annular shape shown in Figure 1b (32). Because the walls of the annulus are thinner than those of a solid cylinder with an equal outer diameter, control of the temperature is facilitated. This approach also enables independent variation of both thickness of the tube wall and its inner diameter. Thus, while keeping the thickness of the wall constant, a significant increase in volume can be achieved easily. Another advantage of this approach includes the preparation of series of tubes that fit each in the other. This sort of “telescopic” format enables construction of rather large separation devices that would be very difficult to obtain in a single polymerization step. For example, tubular columns called CIM-Tubes (BIA Separation

tions) with a volume as high as 8 L operating in the radial direction at a flow rate as high as 10 L/min have been commercialized by BIA and used for the rapid preparative separations of 200 g of proteins in a single run. These tubes were also used in a good manufacturing practice (GMP) certified industrial purification of plasmid DNA for therapeutic applications. The largest unit enables recovery of 10–50 mg of a pure pDNA product in a single run.

Compressed polyacrylamide gel columns: One of the early approaches to monolithic columns developed by Hjertén consisted in the preparation of highly crosslinked gel by polymerization of aqueous solutions of *N,N'*-methylenebisacrylamide and acrylic acid in the presence of a salt, typically ammonium sulfate, followed by its compression using hydraulic pressure to a fraction of its original volume (33). Later, this technology has been refined and piperazine diacrylamide used as the crosslinker to produce commercial UNO columns (Bio-Rad Laboratories, Hercules, California). The interacting moieties required for separations in various HPLC modes are mostly introduced in the gel by copolymerization of monomers containing the desired functionalities. Because the polymerization is carried out in an aqueous solution, all of the components of the polymerization mixture should be water-soluble. This limits both the number of monomers available and separation modes that can be readily achieved. Therefore, most of the experimental work mostly concerned monoliths with ion exchange chemistries (34). Preparation of monolithic stationary phases for reverse phase using this approach is difficult because hydrophobic monomers are not soluble in water. As common to all monolithic stationary phases, these materials also have very favorable profiles of the van Deemter plots and high flow rates can be used to accelerate the separations. The intrinsically hydrophilic nature of these monoliths makes them suitable for the separations of proteins, oligonucleotides, and even intact adenoviruses (35). Although not commercially available, acrylamide-based monolithic columns were also prepared in capillary formats (36).

Silica-Based Monolithic Columns

Because porous inorganic materials are very popular supports widely used in chromatography, Tanaka developed monolithic

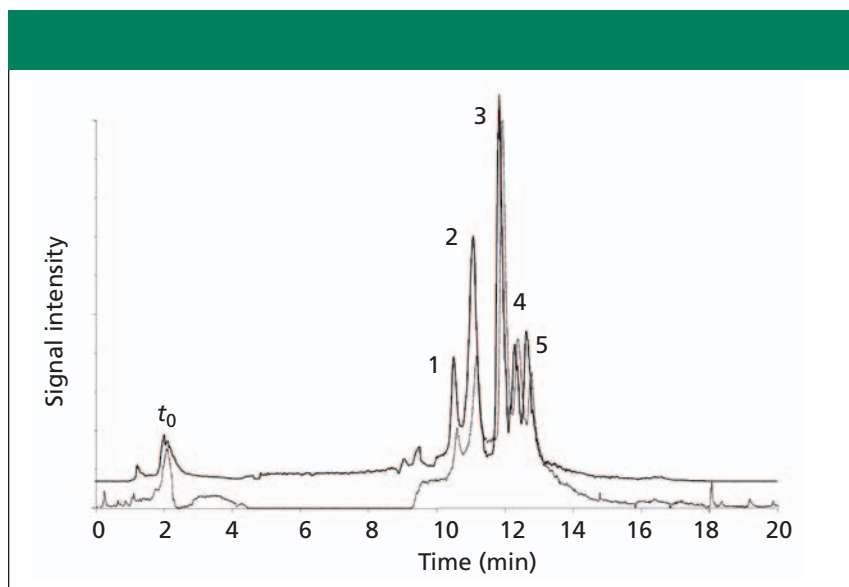


Figure 5: Repeatability of the HPLC-ESI-MS separation of five proteins. Column: 20 cm × 100 μm poly(butyl methacrylate-co-ethylene dimethacrylate) monolithic; flow rate: 0.5 μL/min; gradient: linear from 90% mobile phase A (1% formic acid in water) to 90% B (1% formic acid in acetonitrile) in 8 min. Injection number 400 (dashed line) and 800 (solid line). Peaks in order of elution: ribonuclease A, cytochrome C, myoglobin, hemoglobin, ovalbumin (0.5–5 pmol each).

columns prepared from silica (37,38). In contrast to columns made of organic polymers, the silica-based monolithic column in typical analytical sizes cannot be prepared in situ because of the significant shrinkage accompanying the solidification during hydrolytically initiated polycondensation of tetraalkoxysilane in the presence of poly(ethylene glycol) porogen. For example, rod with a diameter of 4.6 mm is obtained from 6-mm i.d. mold. Thus, the porous silica rod has to be prepared first, then removed from the mold, next encased with a PEEK tube, and finally bonded, typically with C18 chemistry to afford the chromatographic column for the separations in reverse phase mode. These columns are now marketed by Merck KGaA (Darmstadt, Germany) under the trade name Chromolith (39). Other chemistries, other than C18 and silica, and a variety of column sizes including narrow bore (1–2-mm i.d.) have yet to be demonstrated, although recently semiprep (100 mm × 10 mm) and prep (100 mm × 25 mm) columns have been introduced commercially. Due to the significant shrinkage, it is difficult to prepare straight rods longer than about 15 cm, which also limits the length of the final columns.

The family of Merck's silica-based monolithic columns was recently extended by introduction of 100-μm i.d. capillary columns (Chromolith CapRod). In contrast to their larger counterparts, the smaller diameter and attachment of the monolith to

the wall of the silica capillary tube via reaction of the silane enables the more reproducible in situ preparation of these narrow monolithic columns in any length (40). Colón (41) enriched the arsenal of metal oxides used for the preparation of monolith by introduction of columns prepared from zirconia and hafnia. Reaching the extremes, Luo et al. (42) reported the application of 70 cm × 20 μm monolithic silica columns combined with a quadrupole ion trap mass spectrometer for the analysis of a bacterial proteome. Using a very long gradient, they identified 2367 different peptides in the chromatogram shown in Figure 4 covering 855 distinct *Shewanella oneidensis* proteins using just 2.5 μg of tryptic digest.

Monolith For on-line Sample Preparation

Analytical processes including complex matrices such as biological fluids often require a sample preparation step before their separation and detection. While this step is frequently carried out off-line, on-line solid phase extraction (SPE) combined with HPLC-MS presents numerous advantages (43). It enables faster and fully automated analyses, reduces human contact with contaminated or hazardous samples, as well as a potentially increases sensitivity because the complete sample can be injected. Direct injection of biological fluids such as serum or plasma in columns packed with conventional chromatographic sup-

ports often leads to rapid deterioration of the performance. In contrast, monolithic supports are suitable for preconcentration of compounds from biological fluids. For example, commercial silica-based monoliths were used in both single column and column-switching configurations. In the former case, almost 300 direct injections of diluted plasma could be performed using Chromolith SpeedRod without significant deterioration in performance (44). A Chromolith Flash precolumn coupled to an analytical column via a six-port switching valve was used in the column switching configuration to monitor three drugs and their primary metabolites at nanogram-per-milliliter levels. More than 100 samples were treated in the same precolumn without a noticeable decrease in performance (45).

Similarly, polymer-based monoliths are also successfully applied to SPE. The first paper describing this entirely new approach was published in 1998 (46). The ability to prepare the monolith in a specific location via the photoinitiated polymerization, facilitates their broad applications as in-column preconcentration units in capillary electrophoresis (CEC), capillary electrophoresis (CE), and nanoHPLC. The current state of the art in this field is summarized in another review (47).

Silica or Polymer: That is the Question!

One of the common trends is to contrast silica and polymer-based monolithic columns against each other. Actually, this is not correct because these two types of monolithic columns are complementary. The macroporous structure of silica monoliths differs significantly from that of organic polymers (48). While the morphology of organic polymer consists of clusters of little organized microglobules with the large pores located among them, silica rods feature a well-ordered array of equally sized, about 1-mm through-pores and skeletons. In addition, the skeletons themselves are mesoporous and provide the monolith with a large surface area of as much as 300 m²/g, a feature particularly valued in the isocratic reversed-phase chromatographic separations. Thus, silica-based monoliths are excellent tools for the separation of small molecules thanks to their large surface area within their mesoporous structure (49). On the other hand, polymeric monoliths typically prepared not to contain any meso-

pores enable remarkably rapid separations of large molecules such as proteins, nucleic acids and even synthetic polymers. Therefore, use of monoliths made of polymers for the separation of small molecules and silica for proteins do not seem to be readily feasible using current technology.

Reproducibility and Longevity

With the introduction of this novel column format, the ever-lasting fundamental question of chromatography has surfaced again: How reproducible are these columns? As opposed to large homogeneous batches of silica, each of the monoliths is manufactured individually by either polymerization within the confines of the column or prepared in a mold and then placed in a holder. In addition, some monolithic columns require functionalization that is also carried out in situ. However, this technology avoids the packing of particles, which is the major source of column-to-column variability in the classical implementation. Obviously, the more steps used for the manufacture of the monolithic column, the broader the scatter in properties. Thus, poly(styrene-*co*-divinylbenzene) monolithic columns prepared in a single step are likely to be very reproducible. In contrast, silica-based monoliths that require a series of steps can exhibit more significant variations in their performance. However, an extensive study indicates that reproducibility of the Chromolith columns, whose manufacturing process includes multiple steps, is better or matches the values obtained with their packed counterparts (50). For example, RSD for relative retentions did not exceed 4% for most of the tested compounds.

To observe the effect of time on their performance, we used a single 50 mm × 4.6 mm poly(styrene-*co*-divinylbenzene) monolithic column for the separation of synthetic polymers in precipitation-dissolution mode over a period of about seven months with approximately 3000 injections (51). In each gradient run one component of the mobile phase was a good swelling agent for the material of the column while the other was a precipitant. Although the high level of crosslinking does not allow extensive swelling of the monolithic material, even small volumetric changes of the matrix constitute a periodic stress for the column. However, this repeated stress had no effect on long-term column performance. HPLC separations of

a mixture of eight polystyrene standards recorded more than two months and about 400 injections apart exhibited a very small difference lying within the experimental error of chromatographic measurements (51). Recently, we carried out similar experiment with a capillary column and a mixture of proteins. Although the overlaid chromatograms shown in Figure 5 were recorded 400 injections and three weeks apart, no significant changes in retention were observed. It is also worth mentioning that different mobile phases containing trifluoroacetic acid and higher flow rates affording a back pressure of as much as 25.0 MPa were used before and between these two runs. Two other monolithic columns of this kind were prepared later. They afforded identical chromatograms from the very beginning, thus, demonstrating good column-to-column repeatability.

Conclusion

Although monolithic columns are relatively new format of stationary phases for HPLC and much remains to be done, recent achievements open new avenues for the expansion of this entirely new class of columns. The large amount of experimental work that has been done so far and the commercial availability of various monolithic columns confirm the great potential of these new separation media (52). Their unique properties, in particular the ease of their preparation, the tolerance to high flow rates, and the rapid speed of chromatographic separations that can be achieved at acceptable back pressures, make the monolithic column format superior in some applications to the more common columns packed with beads.

Because monoliths, being in their teenage years, are rather "young," the number of different stationary phases, separation mechanisms, and methods developed with these media remains much smaller than that available for the much older packed columns. It is only a question of time when the range covered by the monolithic technology will be extended and successfully compete with all of the other well-established column technologies. What is currently preventing the monolithic columns from even wider acceptance? First of all, they compete with well established packed columns. The major "selling" feature, the high-speed of separations, might not be attractive for those who inject two

samples a day. In contrast, laboratories such as those in pharmaceutical or biotechnology industry where high-throughput is the indispensable must, can certainly appreciate the speed of monoliths. However, a significant problem arises from the limited number of companies producing monolithic columns. In fact, only Merck and Phenomenex (Torrance, California) are now independently marketing equivalent silica-based monolithic columns. Industrial laboratories, particularly pharmaceutical companies who are regulated for validated processes do not like sole sources. And last but not least, the current choice of sizes and chemistries of monolithic columns does not by far match that of the "classical" packed counterparts. In particular, longer 1–2 mm i.d. columns are not yet commercially available and the only really large-scale monolithic column is the 8-L tubular unit with axial flow produced by BIA Separations. LC–MS chemists have not adopted the standard size monoliths of 4.6-mm i.d. because the flow rate requirements are not optimized for electrospray ionization. They are awaiting the 1–2 mm i.d. monolith silica-based columns.

The extent of this short review only allowed describing monolithic columns for HPLC separations. However, a large promise of these flow-through materials can also be seen in other areas such as electrochromatography, microfluidics, gas chromatography, heterogeneous catalysis, and combinatorial chemistry (8).

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