Porous Monoliths—Stationary Phases of Choice for High Performance Liquid Chromatography in Various Formats

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Abstract—Modern porous monoliths have been conceived as a new class of stationary phases for high performance liquid chromatography (HPLC) in classical columns in the early 1990s and later extended to the capillary format. These monolithic materials are prepared using simple processes carried out in an external mold inorganic monoliths or within the confines of the column organic monoliths and all capillary columns. These methods afford macroporous materials with large through-pores that enable applications in a rapid flow-through mode. Since all the mobile phase must flow through the monolith the convection considerably accelerates mass transport within the monolithic separation medium and improves the separations. As a result the monolithic columns perform well even at very high flow rates. The applications of monolithic capillary columns are demonstrated on numerous separations in the HPLC mode.

Key words—high performance liquid chromatography, porous monoliths, stationary phases, synthetic polymers, silica

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Monoliths are a new format of porous polymers that vastly simplified can be compared to a single large particle in a device that in contrast to packed columns does not contain interparticular voids. As a result all the mobile phase must flow through the monolith. 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 materials mostly beads convective flow through the large pores enables a substantial increase in the speed of the separation of large molecules such as proteins or nucleic acids and also enhances activity of immobilized biocatalysts. Liapis et al. and Tallarek et al. have recently developed a thorough theoretical treatment of the mass transfer within monolithic materials.

The first attempts to make single-piece stationary phase from highly swollen polymer hydrogel and open-pore polyurethane foams during the late 1960s and the early 1970s were less successful. Interest in these new formats has only 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 as well as true monoliths such as compressed hydrophilic gels macroporous polymer discs columns tubes as well as silica rods in which the void volume is completely eliminated have been developed. A very detailed account of these materials can be found in the recently published monograph. Some aspects of applications of monolithic columns were also summarized in excellent reviews. This review focuses on the preparation and applications of monolithic materials fabricated both from silica and synthetic polymers mostly in micro- and nano-HPLC and also presents some new trends in this field.

1 Silica-based monolithic columns

Since porous inorganic materials are very popular supports widely used in chromatography no wonder that they attracted attention of scientists active in the monolithic field. Tanaka adopted macroporous silica originally developed by Nakahishi et al. and constructed first silica based monolithic columns. In contrast to columns made of organic polymers the analytical and semipreparative silica-based monolithic column in typical analytical sizes cannot be pre-
pared directly in situ because of the significant shrinkage accompanying the solidification during hydrolytically initiated polycondensation of tetraalkoxysilane in the presence of polyethylene glycol porogen. For example, a rod with a diameter of 4.6 mm is obtained from 6 mm i.d. mold. Thus, the porous silica rod is prepared first removed from the mold encased with a PEEK tube and provided with the bonded typically C18 chemistry to afford the chromatographic column for the separations in reverse phase mode. The family of silica based monolithic columns was recently extended by introduction of capillary columns. 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 with wall of fused silica enables the in situ preparation of these narrow monolithic columns in any length. A variety of chemistries are currently under development. The state-of-the-art has been summarized in recent reviews.

As shown in Fig. 1 the macroporous structure of silica monoliths differs from that of organic polymers. While the morphology of organic polymer consists of clusters of little organized microglobules with the large pores located among them silica monolith feature a well-ordered array of equally sized about 1 μm large through-pores and skeletons. In addition, the skeletons themselves are mesoporous and provide the monolith with a large surface area of up to 300 m2/g a feature particularly valued in the isocratic reversed phase separations. This also makes monolithic silica-based columns suitable for rapid separations of small molecules. Very low back pressure typical of all monolithic columns allows these separations to be carried out in 100 mm × 4.6 mm i.d. column at a flow rate of 9 mL/min it is at a flow velocity which is unprecedented for typical HPLC column of this size and type of separation yet reaching a back pressure of only 7.2 MPa. In contrast to the polymer-based monolithic stationary phases that are particularly well suited for the separations of large molecules such as proteins and nucleic acids most of the research concerning silica-based monolith focuses on the separations of small and mid size molecules. This feature makes them rather unique in the field of monolithic columns.

Tanaka et al recently demonstrated that the high speed makes the monoliths excellent choice for the second dimension column in comprehensive two-dimensional 2D separations of hydrocarbons and benzene derivatives. The first dimension 150 mm × 4.6 mm i.d. column was packed with fluoroalkylsilyl-bonded FR silica particles while the second was one or two 30 mm × 4.6 mm i.d. octadecysilylated C18 monolithic silica columns. These columns were eluted at a flow rate of up to 10 mL/min which enabled to reduce the separation time to 30 s and allowed to separate 15–30 s fractions from the first dimension. They presented three different scenarios. In the simplest scheme, the eluent from the 1st-D was directly loaded into an injector loop of 2nd-D HPLC for 28 s and 2 s were allowed for injection. Two six-port valves each having a sample loop were used to collect the eluent from the 1st-D alternately for 30 s for one 2nd-D column to effect comprehensive 2D-HPLC with a complete sampling of the eluent from the 1st dimension. The most sophisticated setup included a switching valve two monolithic silica columns for 2nd-D

Fig. 1 SEM micrographs of silica-based a and poly styrene-co-divinylbenzene b monolithic capillary columns
and two chromatographic instruments. This system permitted separation of each fraction from the first dimension alternately in one of the two 2nd-D columns. Although not completely orthogonal reversed phase mode was used in both dimensions this simple system afforded a peak capacity of about 1 000 in 30 min.

Having available monolithic capillary columns Tanaka’s group modified their original 2-D procedure described above and used a combination of an analytical scale column packed with cation exchange polymer beads and a 100 mm × 0.1 mm i. d. monolithic C18 capillary [26]. In contrast to their previous paper [19] they separated a very complex mixture of peptides prepared by tryptic digestion of bovine serum albumin. The eluent leaving the microbore 1st-D column 2.1 mm i. d. [collected at a flow rate of 50 µL/min was directed in the loop of the 2nd-D injector for about 3.93 min. Then the valve was turned to enable injection in the 2nd-D at a split ratio of 3/2000 for 7 s 300 nL injection volume and returned back to the collection mode while the peptides were separated in the reversed phase capillary columns in a gradient of acetonitrile in the mobile phase. The use of a capillary column in the 2nd-D led to less solvent consumption and better compatibility with mass spectrometer detector compared to an analytical size column. These features make this approach desirable for the proteomic studies since it affords a peak capacity of about 700 within 40 min. Fig. 2 shows the peptide map trace together with mass spectra of specific spots on the trace.

Luo et al. [21] demonstrated the enormous potential of monolithic silica-based capillary columns. They prepared a 70 cm long 20 µm i. d. silica-based monolithic capillary column that afforded a flow rate of 40 nL/min or a linear velocity of 0.24 cm/s at a pressure drop of only about 35 MPa. This column used in a single dimension provided a peak capacity of 420. Fig. 3 shows the 10 h long separation of tryptic digest of a microorganism Shewanella oneidensis with a 15 amol detection limit. The nanoelectrospray ionization mass spectrometer used for detection de facto represented second dimension separation and enabled the identification of 2 367 different peptides covering 855 distinct proteins.

Fig. 2 Two-dimensional separation of tryptic digest of bovine serum albumin in simple 2D-HPLC mode
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Conditions: First dimension 50 mm × 2.1 mm i. d. column packed with 5 µm weak cation exchange polymer beads mobile phase [A] aqueous 5 mmol/L ammonium formate pH 3.1 [B] B 500 mmol/L ammonium chloride in 5 mmol/L ammonium formate buffer pH 3.1 gradient from 100% A to 85% B in 50 min flow rate 50 µL/min. Second dimension 100 mm × 0.1 mm i. d. monolithic C18 silica column mobile phase A 0.1% formic acid in water B 0.1% formic acid in acetonitrile gradient from 100% A 30 s 100% A to 50% B in 170 s from 50% B to 100% B in 12 s then back to 100% A and held for 30 s for re-equilibration.

2 Compressed polyacrylamide gel columns

One of the early approaches to monolithic columns developed by Hjerten 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 [22]. Later this technology has been refined and piperezine diacrylamide used as the crosslinker to produce commercial columns. This column enabled high-resolution separations of complex samples such as pre-processed extract from Xenopus laevis oocytes containing two elongation factor complexes p30-p47 and p30-p36-p47 shown in Fig. 4. This separation was achieved at a flow rate of 4.5 mL/min [23].

The interacting moieties required for separations in various HPLC modes are mostly introduced in the gel by copolymerization of monomers containing the desired functionalities. Since 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...
Fig. 3 Base peak chromatogram from the high-efficiency on-line microSPE-nano-LC-ESI-MS/MS analysis of a 2.5 μg tryptic digest S. oneidensis protein

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Conditions: microSPE 4 cm × 50 μm i.d. monolithic column nano-LC 70 cm × 20 μm i.d. silica-based monolithic column 35 MPa. Mobile phase A: 0.2% acetic acid and 0.05% trifluoroacetic acid in water B: 0.1% trifluoroacetic acid in 90:10 acetonitrile-water gradient from 100% A to 75% B in 750 min.

Fig. 4 Separation of Xenopus laevis oocytes extract using UNO Q1 column

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Conditions: Load: Xenopus laevis oocytes 40% - 50% ammonium sulfate fraction 6 mg protein buffer A: 50 mmol/L Tris-2 mmol/L EDTA-1 mol/L dithiothreitol pH 7.4 buffer B: 1 mol/L NaCl in buffer A gradient 0 - 60% B in 50 mL flow rate 4.5 mL/min UV detection at 280 nm.

modes that can be readily achieved. Therefore most of the experimental work mostly concerned monoliths with ion exchange chemistries. Preparation of monolithic stationary phases for reverse phase using this approach is difficult since hydrophobic monomers are not soluble in water. As 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. Although not commercially available acrylamide-based monolithic columns were also prepared in capillary formats. Unfortunately nothing has been reported on these monolithic columns lately.

3 Macroporous monolithic discs

The disc format is one of the first useful monolithic stationary phases originally designed for the rapid separation of proteins. In the mid
1980s Belenki 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. This finding resulted in the concept of short separation beds. Since 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 disc format has been developed that enabled very fast separation. An outstanding review describing these materials has been published recently.

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 discs. These are then placed in a specifically designed cartridge. As shown in Fig. 5 the CIM discs are embedded in a polyolefin ring that forms an impermeable sidewall. Although the monoliths are sufficiently mechanically stable to be easily handled this ring also serves to reinforce the discs and prevent fraying of their edges. Its color indicates specific chemistry of the disc. An additional benefit is that the flat face of the ring enables the disc 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 discs are disposable. The cartridge is designed to allow insertion of a single disc or several discs thus forming the CIM disc monolithic column. This disc stacking ability also enabled the development of the multidimensional separation process called "Conjoint Liquid Chromatography" in which discs with different chemistries are used simultaneously. This technique would be very difficult to implement with traditional columns. Example in Fig. 6 demonstrates that using a stack of two Protein G disks and one quaternary ammonium QA disk this approach enables a complete separation of immunoglobulins albumin and transferrin could be achieved in a single step in less than 5 min. From the very beginning monolithic discs exhibited an excellent performance in the rapid separations of proteins and nucleic acids using gradient elution. One of the most successful applications is the purification of plasmid and genomic DNA featuring high loading capacity that can be achieved at a high flow rate. This resulted from the careful optimization of the properties of the separation medium. As an example Fig. 7 shows the effect of pore size on the shape of the breakthrough profiles for genomic DNA.

![Picture of CIM discs](https://example.com/cim_discs.png)

The color of the ring indicates specific chemistry of the disc.

![Optimized separation of transferrin albumin and IgG using monolithic column consisting of two CIM Protein G and one CIM QA disks](https://example.com/optimized_separation.png)

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Chromatograms of two different runs using different step gradients are superimposed. Conditions: Binding buffer [20 mmol/L Tris-HCl pH 7, 0.15 M elution buffer [1] 20 mmol/L Tris-HCl [2] 1 mol/L NaCl pH 7, 0.15 M elution buffer II] 0.1 mol/L Glycine-HCl [0.1 mol/L NaCl pH 2, 0.1 flow rate 4 mL/min sample concentration IgG 2 mg/100 μL.

Peak 1 indicates the transferrin peak eluted with 0.08 or 0.1 mol/L NaCl. Peak 2 indicates the albumin peak eluted with 0.3 or 0.4 mol/L NaCl. Peak 3 indicates the IgG peak eluted at low pH.

Most of the generic discs are prepared by copolymerization of reactive monomer glycidyl methacrylate that is subsequently modified to afford desired interacting functionalities. These modifications enable extension of the disc separations to a number of modes such as reversed phase ion exchange hydrophobic interaction and bioaffinity chromatography.
4 Tubular monolithic columns with radial flow

Large monolithic columns are desirable for the separations on preparative scale. However\[ free radical polymerization that is 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\[. 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 may 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 Fig. 8\[. Since 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.

Thus the tubular column with a volume of up to 8 L operating in the radial direction at a flow rate of up to 10 L/min has been manufactured. The most important feature of all these tubular columns is their identical porous properties allowing for a simple transfer of methods between tubes of different sizes as demonstrated in Fig. 9.

These tubular columns were used for the rapid preparative separations of 200 g of proteins in a single run. These tubes were also used in an GMP certified industrial process for purification of plasmid DNA for therapeutic applications. The largest unit enables isolation of 10 – 50 mg of a pure pDNA product in a single run\[.

5 Rigid macroporous polymer monolithic columns

This new category of macroporous polymer monolithic columns formed by a very simple “molding” process was introduced in the early 1990s\[. In contrast to discs portrayed above\[ 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. Typically the mold is filled with a mixture of monomers one of them must be a crosslinker free radical initiator and porogenic solvent sealed and the polymerization is carried out under carefully controlled thermal conditions. The seals are then replaced with fittings and the column attached to a pump. Using a suitable solvent such as methanol or tetrahydrofuran the porogens and other unreacted components are removed from the pores and the column is ready for the chromatographic operation. Fig. 1 shows the morphology of this type of monolith.

In addition to typical stainless steel and glass monolithic columns for HPLC have also been prepared in capillaries. With the advent of proteomics this format readily compatible with both electrospray ionization ESI and matrix assisted laser desorption ionization MALDI mass spectrometry starts to be very popular since it is much simpler to polymerize liquid precursors and form the stationary phase in situ within a capillary than packing it efficiently with microparticles. Karger recently demonstrated the potential of a monolith prepared in fused silica capillaries for the very efficient separations of tryptic digests. An example of this separation using matrix assisted laser desorption ionization-time-of-flight mass spectrometry MALDI-TOF-MS is shown in Fig. 10.

The arsenal of the classical polymerization techniques relying mostly on thermally initiated reaction was recently extended by development of UV-light triggered processes. Using capillaries with UV transparent teflon coating the polymerization may be initiated by irradiation with light
and proceeds faster than its thermally initiated counterpart \cite{51,52}. The chemistries of these columns are dictated by the choice of monomers used for their preparation that have to be UV transparent. Fig. 11 shows rapid separation of proteins using pol\text{[N]} butyl methacrylate-co-ethylene dimethacrylate [capillary column] \cite{53].

![Fig. 11 Rapid separation of ribonuclease A\text{[N]} 1, cytochrome c\text{[N]} 2, myoglobin\text{[N]} 3, and ovalbumin\text{[N]} 4 using pol\text{[N]} butyl methacrylate-co-ethylene dimethacrylate\text{[N]} monolithic capillary column and a single step gradient at 100 \mu L/min](image)

Conditions\text{[N]} Column size\text{[N]} 10 cm \times 200 \mu m i. d. Mobile phase\text{[N]} A-0.1 \% trifluoroacetic acid in 90:10 \% v/v water/acetoniitre B-0.1 \% trifluoroacetic acid in 10:90 water/acetoniitre\text{[N]} Gradient profile that includes change from 100\% A to 100\% B at time 0 is represented by the bold dashed line.

Diverting from the typical free radical polymerization\text{[N]} Buchmeiser recently reported successful use of ruthenium catalyzed ring-opening metathesis polymerization of selected norbornene-based monomers for the preparation of monolithic columns suitable for the separation of peptides\text{[N]} proteins and nucleic acids\text{[N]} 34-36\text{[N]}.

Several methods are available for the preparation of monolithic stationary phases with desired surface chemistry. For example\text{[N]} directly polymerized poly styrene-co-divinylbenzene monoliters proved to be an excellent stationary phase for the very fast separation of peptides\text{[N]} proteins\text{[N]} and nucleic acids\text{[N]} 57-60\text{[N]}). Another option is the preparation of a monolith with a reactive functionality and its subsequent modification to afford stationary phases for a variety of separation modes. In this technique schematically shown in Fig. 12 each single reactive site affords one new functionality. Among others\text{[N]} glycidyl methacrylate-based monoliths have been widely used in these applications\text{[N]} 61\text{[N]}). Yet another method that enables modulation of surface chemistries relies on photochemical reactions. The desired pore surface chemistry is obtained via grafting reactions initiated by UV light\text{[N]} 62\text{[N]}). 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.

6 Conclusion

Although monolithic columns are relatively new format of stationary phases for HPLC and much remains to be done\text{[N]} recent achievements opened new vistas for the preparation of entirely new class of columns\text{[N]} also called “ stationary phases of fourth generation” with exactly tailored properties\text{[N]} 42\text{[N]}). Guiochon claimed a short time ago\text{[N]} 44\text{[N]} that the invention and development of monolithic columns is a major technological change in column technology\text{[N]} indeed the first original breakthrough to have occurred in this area since Tswett invented chromatography a century ago.” Large extent 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\text{[N]} 43\text{[N]}). Their unique properties\text{[N]} in particular the ease of their preparation\text{[N]} the tolerance to high flow rates\text{[N]} and the rapid speed of chromatographic separations that can be achieved at acceptable back pressures\text{[N]} make the monolithic column format superior in some applications to the more common columns packed with beads. Since monoliths are rather “ young”\text{[N]} the number of different stationary phases\text{[N]} separation mechanisms\text{[N]} and methods developed with these media remains much smaller than that available for the packed columns. It is only question of time when the range covered by the monolithic technology will be extended and will successfully compete with all of the other well-established separation technologies.

The extent of this communication only al-
lowed describing applications of monolithic columns in HPLC. However, a large promise of these flow-through materials can also be seen in other areas such as electrophromatography microfluidics gas chromatography heterogeneous catalysis and combinatorial chemistry.

References

Biographical Sketch

SVEC Frantisek received both B.S. in chemistry and Ph. D. degree in polymer chemistry from the Institute of Chemical Technology Prague Czech Republic in 1965 and 1969 respectively. In 1976 he joined the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences where he was promoted through the ranks to the Head of Department and the Scientific Secretary of the Institute. He accepted an offer and joined faculty at the Cornell University in 1992. Since 1997 he is appointed at the University of California Berkeley. He is also visiting professor of analytical chemistry at the University of Innsbruck Austria and staff scientist in the Lawrence Berkeley National Laboratory. Dr. Svec is the author or co-author of over 320 scientific publications he edited 2 books and authored 75 patents. He is the Co-Editor in Chief of the Journal of Separation Science and member of editorial boards of a number of renowned journals including Journal of Chromatography A, Electrophoresis, Applied Macromolecular Chemistry and Engineering and Chinese Journal of Chromatography. In 2003 he was elected the President of the California Separation Science Society. In 2005 he was awarded with M. J. E. Golay Medal in Chromatography and EAS Award for Achievements in Separation Science. He is best known for his research in the area of macroporous polymers in different shapes such as beads, flat sheets and in particular monoliths. His studies involve use of these materials in numerous applications including liquid chromatography, electrochromatography, supports for solid phase chemistry, enzyme immobilization and microfluidics.