Recent Applications of Organic Monoliths in Capillary Liquid Chromatographic Separation of Biomolecules

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Abstract

Monolithic columns are an attractive alternative to traditional particulate solid phases for capillary liquid chromatography. A monolith is a continuous interconnected skeleton with large through-pores. This structure reduces the diffusion path and provides high permeability, resulting in excellent separation efficiency. The integral structure enhances the mechanical strength, while the large through-pores (a few µm) have very low flow impedance. This combination allows smaller diameter monolithic columns to be operated at higher flow-rates, simultaneously increasing both sensitivity and throughput. Polymeric monoliths were first described back in the 1960s, but the first successful ones designed for protein separations appeared much later, in the late 1980s. Organic monoliths are based upon copolymerization of a monofunctional and a bifunctional (uncommonly trifunctional) organic precursor in the presence of a suitable initiator and porogenic solvents. During the last 15 years, a vast number of different monomers and crosslinkers have been introduced and copolymerized using different polymerization techniques and initiators. Various mechanisms, including thermally- and UV-initiated free radical polymerization, as well as ring opening metathesis copolymerizations, have been demonstrated for the preparation of monolithic columns. In this review, we summarize the recent application of different organic monoliths, including styrene-, acrylate-, methacrylate-, and acrylamide for the liquid separation of biomolecules (e.g., proteins, peptides, and oligonucleotides).

Introduction

Micro-liquid chromatography (µLC) has been increasingly recognized over the last few decades as a powerful separation technology, as it enables high performance, rapid and sensitive analysis, and low-cost operation and environmental compatibility. µLC has established itself as a complementary technique to conventional LC columns routinely used in high-performance liquid chromatography (HPLC). This is mainly due to the advantages found in an increasing number of µLC applications, especially where conventional LC falls short or cannot compete (1,2).

The increased mass sensitivity of the miniaturized columns is attractive for the detection of compounds present at low concentrations and/or in limited sample volumes (3). In addition, the flow rates used for µLC couple well with mass spectrometry (MS) and nuclear magnetic resonance with no need for post-

Abbreviations

PS-DVB: polystyrene-divinylbenzene
TFA: trifluoroacetic acid
TEA–HOAc: triethylamine-acetic acid
HFBA: heptafluorobutyric acid
PLOT: porous layer open tubular
SCX: strong cation exchange
POS–DVB: polyoctylstyrene–divinylbenzene
PS–OD–DVB: poly(styrene–octadecene–divinylbenzene)
Poly(MST-BVPE): methylstyrene and 1,2-bis(*p*-vinylphenyl)ethane
C4D: capacitively coupled contactless conductivity detection
AIBN: α,α^{1}-azoisobutyronitrile
BVBDMS: bis(p-vinylbenzyl)dimethylsilane
MST: methylstyrene
TMSiMS: trimethylsilyl-4-methylstyrene
(LME-co-EDMA): lauryl methacrylate-ethylene dimethacrylate
HIC: hydrophobic interaction chromatography
HILIC: hydrophilic interaction chromatography
poly(SPE-co-EDMA): *N,N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammonium-betaineandethylenedimethacrylate
DMSO: dimethysulfoxide
AMPS: 2-acrylamido-2-methyl-1-propanesulfonic acid
PEGDA: poly(ethylene glycol) diacrylate
THF: tetrahydrofuran
SPMA: 3-sulfopropyl methacrylate
PETA: pentaerythritol triacrylate
HMMAA: *N*-(hydroxymethyl) methacrylamide
TEAA: triethylamine acetate
FM: fluorescence microscopy
HEMA: 2-hydroxyethyl methacrylate
DATD: (+)-*N,N*^{1}-diallyltartardiamide
PDA: piperazine diacrylamide
GMA-co-EDMA: glycidyl methacrylate-co-ethylene glycol dimethacrylate
EGMP: ethylene glycol methacrylate phosphate
ROMP: ring-opening metathesis polymerisation
NBE: norborn-2-ene

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column splits. However, the packing of capillary columns is not easy and requires a tremendous amount of skill because of the small internal diameters of the columns and the small diameter of the particles used. Additional drawbacks associated with packed capillaries include the need for frits and high backpressure. Different approaches have been reported for the preparation of capillary columns that avoid the technical problems of frit fabrication, including siliceous monoliths prepared by polycondensation of alkoxysilanes within the capillary tubing followed by post-functionalization (4,5); packing the column with silica particles prior to initiating the sol-gel (6,7); consolidation of a packed bed by sintering the particles (8); or entrapment of the particles in an organic polymer monolith (9). An alternative approach is to use monolithic columns prepared by in-situ polymerization of functional monomers in the presence of pore-forming solvents. These columns exhibit several distinct advantages over their particulate counterparts.

Monoliths, in contrast to packed particle columns, are described as single large "particles" that do 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. The first thoughts related to what we today call monoliths can be traced back to the works of the Nobel Prize winner Richard Synge, who in 1952 envisioned "a continuous block of porous gel structure" (10). First attempts to make "single-piece" stationary phases from highly swollen polymer gel and open-pore polyurethane foam during the late 1960s and the early 1970s were not very successful (11–13). Hjerten was the first to develop continuous polymer beds based on polyacrylamide for the fast separation of biopolymers (14). Tennikova and Svec synthesized poly(glycidyl methacrylate-ethylene dimethacrylate) [poly(GMA-EDMA)] polymers as disks called macroporous polymer membranes (15).

During the last few decades, much attention has been paid to the development of macroporous monolithic separation media, representing the state-of-the-art stationary phases currently in use (16,17). The simplicity of their preparation, in combination with high axial permeability and internal porosity being responsible for the excellent chromatographic properties, and the wide spectrum of available surface chemistries, make monolithic column supports an attractive alternative to their particle-packed counterparts (18). Generally, monolithic stationary phases are favoured for designing separation columns in capillary format, which represents an important feature, because the hyphenation of micro- or even nano-scale chromatography to MS becomes more and more important for the separation and identification of complex sample mixtures, especially in the field of proteomic research (19). Monolithic materials are classified into two main groups: silica- (20,21) and organic polymer-based (22,23) monoliths, each with their own advantages and disadvantages. Organic polymer monoliths have several distinct advantages in life-science research, including wide pH stability, less irreversible adsorption, facile preparation, and modification. Organic monoliths are generally prepared by thermal- (24) or photo-initiated polymerization (25). In addition, microwave irradiation (26) and γ-radiation initiated polymerization (27) have been also employed.

Methods used to introduce active groups into the monolith include copolymerization of cross-linker with a functional monomer (28), grafting of pore surface (29,30), and a post-modification of reactive monoliths (31). Among the three methods described previously, copolymerization of cross-linker and functional monomer is the most straightforward strategy and allows the dynamic binding capacity of the column to be controlled by adjusting the amount of the functional monomer in the polymerization mixture.

At present, monolithic stationary phases are widely used in the processes of bioseparation (chromatography) and bioconversion (enzyme reactors) (32,33), as well as in other processes based on interphase mass distribution (e.g., solid-phase peptide and oligonucleotide synthesis) (34). Bioaffinity modes of suggested dynamic methods are very promising for their use in different analytical processes (immunological, ecological, medical, and other types of analytical monitoring), and in the preparative isolation of proteins such as myoglobin, hemoglobin, and immunoglobulins (35,36).

This review will focus on recent applications of organic polymeric monolith from 2004–2008 for the separation of biologically important molecules including proteins, peptides, and oligonucleotides for one- and multi-dimensional capillary LC.

Styrene-Based Monoliths

Monolithic capillary columns based on poly(styrene-divinylbenzene) (PS-DVB) are the most commonly used of all organic polymer-based monolithic capillary columns for ion-pair reversed-phase (IP-RP) LC for biomolecule analysis. PS-DVB-based capillary columns were applied for the separation of bioactive peptides and tryptic digests of various proteins under acidic and alkaline conditions by IP-RP-LC, using gradients of acetonitrile (ACN) in 0.050% aqueous trifluoroacetic acid (TFA), pH 2.1, or 1.0% triethylamine–acetic acid (TEA–HOAc), pH 10.6. Chromatographic performances with mobile phases of low and high pH were practically equivalent and facilitated the separation of more than 50 tryptic peptides of bovine serum albumin within 15–20 min with peak widths at half height between 4 and 10 s. Neither a significant change in retention nor efficiency of the monolithic column was observed during 17 days of operation at pH 10.6 and 50ºC. Upon separation by IP-RP-LC at high pH, peptide detectabilities in full-scan negative-ion electrospray ionization (ESI) MS were approximately two to three times lower as compared to IP-RP-LC at low-pH with positive (pos) ESI-MS detection. Tandem mass spectra obtained by fragmentation of deprotonated peptide ions in negative ion mode yielded interpretable sequence information only in a few cases of relatively short peptides. However, in order to obtain sequence information for peptides separated with alkaline mobile phases, tandem MS

could be performed in positive ion mode. The chromatographic selectivities were significantly different in separations performed with acidic and alkaline eluents, which facilitated the fractionation of a complex peptide mixture obtained by the tryptic digestion of 10 proteins utilizing off-line, two-dimensional IP-RP-LC at high pH × IP-RP-LC at low pH, and subsequent on-line identification by (pos) ESI-MS–MS (37).

This approach has been extended for the separation of phosphorylated and nonphosphorylated peptides. The separation properties are tested for synthetic phosphorylated peptides and their nonphosphorylated counterparts, and for the analysis of a tryptic digest of α-casein. As the choice of the ion pairing reagent in the mobile phase influences the selectivity of the separation as well as the total retention times of the peptides, separations at both low and high pH using TFA, heptafluorobutyric acid (HFBA), and TEA–HOAc as additives are compared. Between the two acidic ion pairing additives TFA and HFBA, the latter performed significantly better in terms of retentivity and selectivity, facilitating the retention and resolution of even very small and hydrophilic phosphorylated and nonphosphorylated peptides. The use of an alkaline TEA–HOAc eluent in conjunction with detection in negative ion mode leads to a drastic improvement in the detection limits for a multiphosphorylated peptide from α-casein digest. Furthermore, under these conditions, the detection of phosphospecific fragments (*m/z* 79, 97) was possible upon collisionally-induced decomposition. The parallel use of acidic eluent/positive ion detection mode and alkaline eluent/negative ion detection mode is shown to be a viable combination to optimize the results of protein identification and characterization (38).

High-speed, high-resolution LC separations, using a PS-DVB monolithic column, have been coupled to matrix-assisted laser desorption/ionization (MALDI) MS and MS–MS through an off-line continuous deposition interface. The system was applied for the analysis of complex proteomic samples using fast 10-min-long LC gradient and high-resolution monolithic LC columns. The separation was deposited at subatmospheric pressure on a MALDI plate, precoated with nitrocelluose, as a continuous serpentine streak with high uniformity in width and microcrystalline morphology (39).

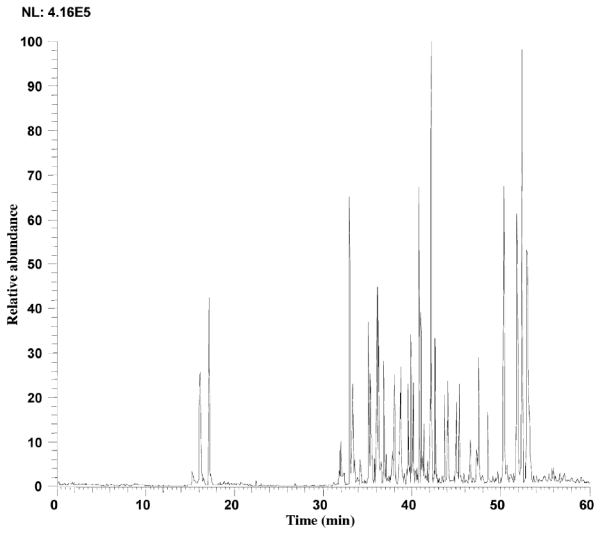

Figure 1. Gradient nanoLC–ESI-MS of a mixture of BSA tryptic digest and α-casein Lys-C digest using a 4.2 m × 10-µm-i.d. PSDVB PLOT column: an 800-amol amount of the mixture was loaded on the PLOT column; LC separation with direct loading and use of a microSPE column. Gradient: mobile phase A [0.1% (v/v) formic acid in water] to 40% B [0.1% (v/v) formic acid, 10% (v/v) water in ACN] in 45 min with data collection initiated at the start of the gradient; Flow rate: 20 nL/min at an inlet pressure of 2900 psi. Reproduced from Reference 40 with permission.

Karger and coworkers (40) explored the use of a 20 µm i.d. polymeric PS-DVB monolithic capillary column for the LC–ESI-MS analysis of a tryptic digest peptide mixture to improve the detection sensitivity. Efficiencies over 100,000 plates/m for peptide separation were achieved with optimized polymerization conditions and mobile phase composition, and high mass sensitivity (~10 amol of peptides) in the MS and MS–MS modes using an ion trap MS was observed, which was up to 20-fold improvement over 75 µm i.d. capillary columns. Recently, the same group demonstrated the performance of long, high-efficiency PS-DVB, 10-µm-i.d. porous layer open tubular (PLOT) capillary columns for ultratrace LC–MS proteomics analysis. Relatively high loading capacities, ~100 fmol for angiotensin I and ~50 fmol for insulin, were obtained with a 4.2 m × 10-µm-i.d. PLOT column. Low detection levels (attomole to sub-attomole) were achieved when the column was coupled on-line with a linear ion trap MS (LTQ). The PLOT column yielded a peak capacity of ~400 for the separation of a complex tryptic digest mixture when the sample preparation included a 50-µm-i.d. PS-DVB monolithic precolumn and ESI-MS detection (Figure 1). As an example of the power of the column, 3046 unique peptides covering 566 distinct *Methanosarcina acetivorans* proteins were identified from a 50 ng in-gel tryptic digest sample combining five cuts in a single LC–MS–MS analysis using the LTQ (41).

RP-HPLC–ESI-MS using PS-DVB monolithic capillary columns have been utilized for the investigation of membrane proteins at the intact molecule level. The analytical method was shown to be very flexible and enabled the identification of antenna proteins as well as most photosynthetic membrane proteins with few RP-HPLC–ESI-MS analyses with a gradient of ACN in 0.05% aqueous TFA. The membrane proteins, ranging in molecular mass (*M*r) from 4196 to more than 80,000, as well as isoforms, were identified on the basis of their intact *M*r and comparison with *M*r deduced from known DNA or protein sequences. High quality mass spectra enabled the identification and quantitation of the nonphosphorylated and phosphorylated reaction center subunits D1, D2, and CP43 of PSII, containing five to seven membrane-spanning α-helices. Because of its high flexibility and suitability for proteins having a very wide range of *M*r and hydrophobicities, the method is generally applicable to the analysis of complex mixtures of membrane proteins (42). Furthermore, Huber et al. integrate the protein separation by PS-DVB monolithic capillary to on-probe tryptic digestion for subsequent analyses by MALDI-time-of-flight (TOF) MS and MALDI-TOF–TOF MS. The method provides a means of directly interfacing separations to MALDI-MS, reducing the amount of time required for traditional procedures involving in-solution enzymatic

digestion and sample cleanup prior to MALDI-MS analysis. The use of monolithic capillary columns sufficiently resolved intact proteins so that peptide mass fingerprinting analysis by MALDI-TOF MS resulted in the identification of close to 40 unique proteins from 120 ng of sample obtained from pre-fractionated cell lysates from a human breast cancer cell line MCF10A at pH 6.34. The identifications of several of these proteins were also confirmed by intact molecular mass (M_r) and tandem mass spectrometric analysis. The reproducibility of this method has been demonstrated to be sufficient for the purpose of protein identifications. Experimental values of protein intact M_r are obtained and compared to that expected for each protein identified. The method is simple and robust and also effectively minimizes sample loss by avoiding sample transfers and additional sample cleanup procedures. Tandem MS can also be used with this approach to further confirm the identifications and for structural analysis. Nevertheless, the use of intact protein separation has distinct advantages over total protein digestion of the sample into peptides, in that the intact protein method provides protein M_r and improved sequence coverage for protein identification (43,44).

Monolithic columns based on PS-DVB were utilized both for preconcentration (in 10 mm × 0.20 mm i.d.) and analytical separation (in 60 mm × 0.20 and 0.10 mm i.d.) of peptides and proteins in column switching micro-scale HPLC. Trapping efficiency, especially for small and hydrophilic peptides, was optimized by using 0.10% HFBA instead of 0.050% TFA as solvent additive during sample loading. Long-chain perfluorinated carboxylic acids show the highest tendency to retain short and/or hydrophilic peptides, while long and very hydrophobic peptides can still be readily eluted with higher ACN concentrations (45). The applicability of the monolithic preconcentration column for multidimensional chromatography was tested by off-line two-dimensional separation, combining strong cation-exchange chromatography and IP-RP chromatography (46).

Huber and his coworkers (47) developed a two-dimensional separation scheme for shotgun proteome analysis employing high-pH RP-LC in the first and low-pH in the second dimension (IP-RP-LC) coupled to ESI-MS–MS. First-dimension separation by RP-HPLC at basic pH facilitates the fractionation of tryptic peptides generated from a bacterial proteome with higher efficiency and homogeneity as compared to classical strong cation exchange (SCX)-HPLC. This allows not only a significant increase both in the number of unique peptides and proteins identified but also a substantial decrease in total analysis time (i.e., 124 vs. 176 h for triplicate analysis of the collected fractions), because a smaller number of fractions need to be collected for an equivalent number of unique peptide identifications. Moreover, RP-LC offers some advantages in terms of practical handling and routine operation, such as the use of volatile hydro-organic mobile phases allowing easy evaporation of solvent in order to concentrate fractions. Contrarily, SCX-LC utilizes buffered, involatile salt solutions, which are more laborious to prepare and often a source of instrument corrosion and system clogging. Furthermore, the separation scheme was coupled offline to MALDI TOF–TOF-MS (48).

The determination of peptides and proteins by LC carried out on a monolithic capillary column with capacitively coupled contactless conductivity detection (C^4D) was demonstrated. The detection cell was positioned directly on the outlet tubing of the separation column, which ensured that the analyte zones were not dispersed; sharp peaks were detected, and no peak broadening was observed. Detection limits, which were lower by half an order of magnitude for C^4D compared to a UV-VIS detector, were achieved when these two detectors were connected in series for the same analytical run. Capillary columns in LC are often used in combination with an MS for detection, which of course has lower detection limits and delivers more information than contactless conductivity detection. It may, however, also be useful to employ the relatively inexpensive C^4D ahead of an MS to monitor in-line the eluent stream going to the spectrometer. It has already been demonstrated that C^4D can be used with non-transparent polymeric transfer tubings (49).

A poly(styrene–octadecene–divinylbenzene) (PS–OD–DVB) monolithic column was prepared in one step by introducing a C18 carbon chain as monomer. *N*,*N*-Dimethylformamide and decanol served as porogens to make a homogeneous polymerization mixture in a fused silica capillary (320 µm i.d.). The physical and chromatographic properties were compared with those of a PS–DVB monolithic column, fabricated by in-situ polymerization in a fused silica capillary with the same inner diameter. Six standard proteins were used to evaluate the columns and their potential application for the separation of human haemoglobin. It was shown that the PS–OD–DVB and PS–DVB monoliths appeared to have similar efficiency for rapid separation of six proteins within 3.5 min. The PS–OD–DVB monolith was found to have higher loading capacity and higher resolution for the separation of a and b chains of hemoglobin compared to the PS–DVB monoliths, and it shows great potential for the separation of bio-macromolecules (50).

IP-RP-LC has been evaluated as a method for the fractionation and desalting of ribonucleic acids prior to their characterization by ESI-MS. Monolithic, PS–DVB-based capillary columns allowed the rapid and highly efficient fractionation of both synthetic and biological ribonucleic acids. The common problem of gas-phase cation adduction that is particularly prevalent in the mass spectrometric analysis of ribonucleic acids was tackled through a combination of chromatographic purification and the addition of ethylenediaminetetraacetic acid to the sample at a concentration of 25 mmol/L shortly

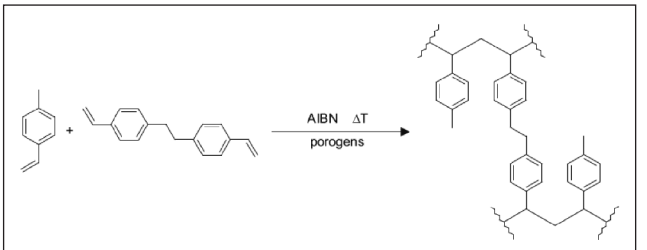

Figure 2. Scheme for the preparation of monolithic MST–BVPE polymer networks by thermally-initiated free radical copolymerization of MST and BVPE. Reproduced from Reference 53 with permission.

before on-line analysis. For RNA molecules ranging in size from 10 to 120 nucleotides, the mass accuracies were typically better than 0.02%, which allowed the characterization and identification of failure sequences and byproducts with high confidence. Following injection of a 500 nL sample onto a 60 × 0.2 mm column, the limit of detection for a 120-nucleotide ribosomal RNA transcript from *Escherichia coli* was in the 50–80 fmol range. The method was applied to the analysis of synthetic oligoribonucleotides, transfer RNAs, and ribosomal RNA. Finally, sequence information was derived for low picomole amounts of a 32-mer RNA upon chromatographic purification and tandem mass spectrometric investigation in an ion trap mass spectrometer. Complete series of fragment ions of the c- and y-types could be assigned in the tandem mass spectrum (51).

A poly(octylstyrene–divinylbenzene monolithic capillary column prepared by photoinitiated polymerization coupled with a carbon fiber emitter for capillary HPLC–ESI-MS has been developed and used for highly efficient separation and sensitive detection of tryptically digested proteins. The developed capillaries offered better chromatographic performances compared with a poly(lauryl methacrylate-ethylene dimethacrylate) [poly(LME-*co*-EDMA)] column, possibly due to superior surface morphology and smaller pore diameter. A carbon fiber emitter interfaced with a capillary monolithic column showed good long-term stability and high sensitivity. Results for the capillary HPLC–ESI-MS of tryptic peptides obtained using the monolithic column coupled with carbon fiber emitter demonstrate the feasibility of separation and highly sensitive detection of peptides for proteomic researches (52).

Trojer et al. (53) prepared a novel monolithic capillary support by polymerization of methylstyrene (MST) and 1,2-bis(*p*-vinylphenyl)ethane (BVPE) as a crosslinker in the presence of inert diluents (porogens). This polymeric MST–BVPE reversed-phase monolith showed excellent mechanical stability and minimized swelling in organic solvents. The overall polymer porosity is strongly influenced by the total monomer content as well as by the percentage and nature of the microporogen used (Figure 2). Changing these parameters offers the possibility of synthesising monoliths with a wide range of permeability. Increasing the monomer content from 35 to 39% (v/v) (MST–BVPE kept constant) resulted in a dramatic decrease in the mean pore diameter from 12.3 to 0.7 µm, which was characterized by a permeability that was 60 times higher for the more porous monolith. A mixture of five proteins was separated on three monoliths prepared with 35, 37, and 39% (v/v) total monomer concentration, the retention behaviour was shown to be similar for all three, indicating little influence of the polymer structure and porosity on the chromatographic behaviour for large biomolecules. On other hand, increasing the microporogen level produced the expected decrease in permeability of the product. Proteins were shown to be much less dependent on changes in porosity; they could be separated at very high flow-rates using either steep linear gradient

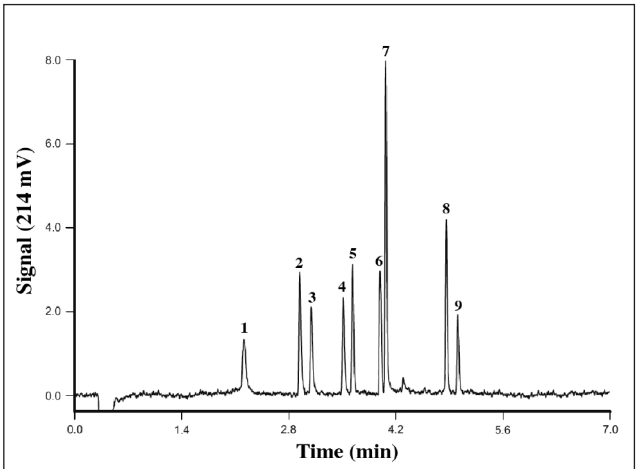

Figure 3. Separation of a mixture containing 9 peptides on a monolithic MST–BVPE capillary column (50 × 0.20 mm i.d.). Chromatographic conditions: mobile phase A, 0.1% TFA; B, 0.1% TFA in ACN; 0–30% B in 10 min, 4.0 µL/min, UV 214 nm; injection, 500 nL, 1 ng each. Peaks: bradykinin fragment 1–5, 1; vasopressin [arg8], 2; methionine enkephalin, 3; leucine enkephalin, 4; oxytocin, 5; bradykinin, 6; LHRH, 7; bombesin, 8; and substance P, 9. Reproduced from Reference 53 with permission.

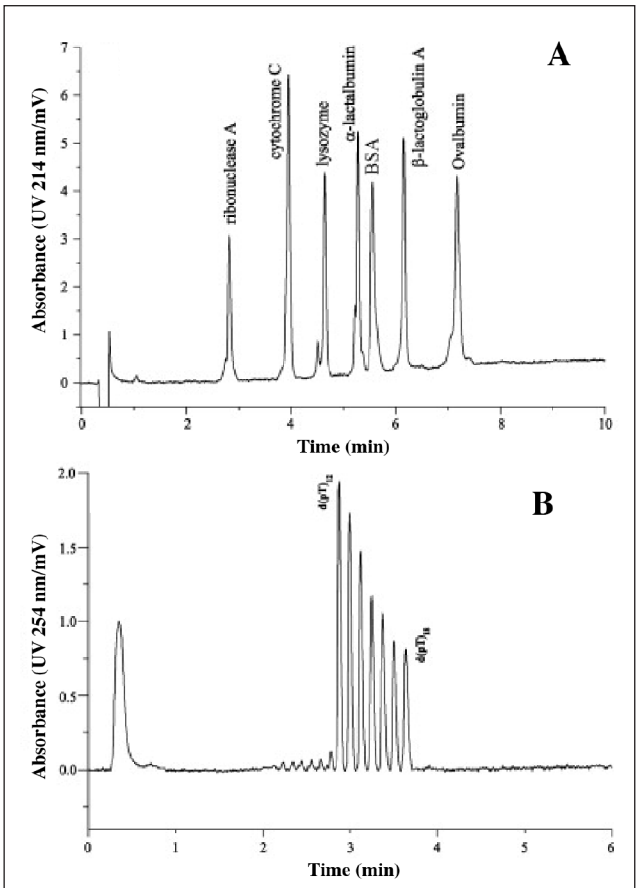

Figure 4. Separation of proteins (A) and oligonucleotides [$d(pT)_{12–18}$] (B) on MST–BVBDMS monolith. Chromatographic conditions: mobile phase A, 0.1% TFA; B, 0.1% TFA in ACN (A); mobile phase A, 100 mM TEAA, pH 7.0; B, 100 mM TEAA, pH 7.0, 25% ACN gradient (B); 15–60% B in 10 min; flow-rate, 5.6 µL/min; sample concentration, 20 µg/mL each; 500 nL loop; temperature, 60°C; UV 214 nm (A); gradient, 0–20% B in 1 min, 20–30% B in 9 min; flow-rate, 8.6 µL/min; sample concentration, 5 ng total; temperature, 60°C; UV 254 nm (B). Reproduced from Reference 54 with permission.

or single step gradient. The monolith prepared with 35% (v/v) total monomer was used to separate the five proteins using a flow rate of 150 µL/min in 35 s.

In contrast to large biopolymers like proteins, the separation of peptides is much more sensitive to monolith porosity in addition to the chromatographic conditions employed. Figure 3 presents the separation of a 9-peptide mixture on monolith, prepared with 37% v/v total monomer under optimized conditions. The test mixture (containing bradykinin fragment 1–5, vasopressin [arg^8], methionine enkephalin, leucine enkephalin, oxytocin, bradykinin, LHRH, bombesin, and substance P) covers a broad molecular mass range (556–1620 g/mol), and is a good criterion for the evaluation of a column.

Hydrophobic organo-silane-based monolithic capillary columns were prepared by thermally initiated free radical polymerization within the confines of 200 µm i.d. fused silica capillaries. A novel crosslinker, namely bis(*p*-vinylbenzyl) dimethylsilane (BVBDMS), was copolymerised with MST in the presence of 2-propanol and toluene, using α,α'-azoisobutyronitrile (AIBN) as initiator (54).

Figure 4A demonstrates baseline separation of 7 proteins [ribonuclease A, cytochrome c, α-lactalbumin, β-lactoglobulin A, ovalbumin, lysozyme, and bovine serum albumin (BSA)]. Oligonucleotides have been separated by IP-RP in less than 5 min with high peak sharpness and resolution (Figure 4B).

In another approach, Jackschitz et al. (55) described another silane-based monolithic copolymer for the rapid separation of proteins and oligonucleotide. The monolith was prepared by thermal initiated in situ copolymerisation of trimethylsilyl-4-methylstyrene (TMSiMS) and BVBDMS. Different ratios of monomer and crosslinker, as well as different ratios of micro- (toluene) and macro-porogen (2-propanol) were used for optimizing the physical properties of the stationary phase for biopolymer separations. Applying optimized conditions, a mixture comprised of five standard proteins ribonuclease A, cytochrome c, α-lactalbumin, myoglobin, and ovalbumin was separated within 1 min by IP-RPLC, obtaining half-height peak widths between 1.8 and 2.4 s (Figure 5). Baseline separation of oligonucleotides d(pT)12–18 was achieved within 1.8 min obtaining half-height peak widths between 3.6 and 5.4 s.

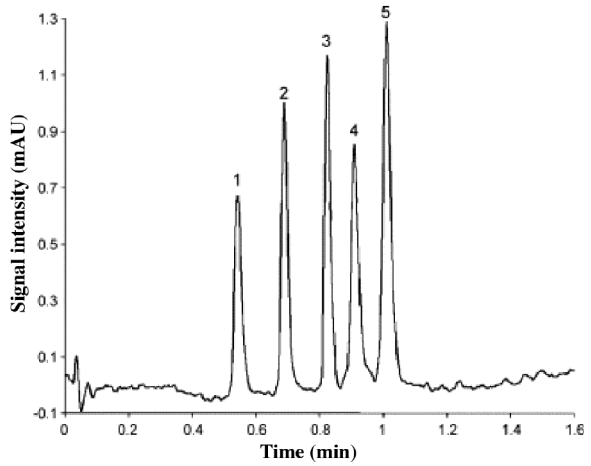

Figure 5. Separation of a protein mixture using a monolithic poly(TMSiMS-co-BVBDMS) column 50 × 0.2 mm i.d. Conditions: mobile phase A, 0.1% TFA; B, 0.1% TFA in ACN; gradient, 5–100% B within 1.5 min, 100% B 2 min; flow-rate, 28 µL/min; room temperature; injection volume, 500 nL, 2 µg/mL each; detection, UV, 214 nm. Peak assignment: ribonuclease A, 1; cytochrome c, 2; α-lactalbumin, 3; myoglobin, 4; ovalbumin, 5. Reproduced from Reference 55 with permission.

Acrylate-Based Monoliths

A large number and variety of methacrylate esters and the ease of their polymerization and modification contribute to the wide popularity of acrylate-based monoliths. These monoliths have been successfully used for various purposes in separation science including ion exchange, hydrophobic interaction, and hydrophilic interaction chromatography (HIC). This part of the review summarizes the recent applications of acrylate-based monoliths.

Proteins and peptides

Gu et al. (56) presented a non-adsorptive monolith for proteins. A poly(polyethylene glycol methyl ether acrylate-*co*-polyethylene glycol diacrylate) monolith was prepared by UV-initiated polymerization, using methanol and ethyl ether as porogens. The preparation of the monolith could be achieved within 10 min. The monolith had low flow resistance, and did not swell or shrink significantly in tetrahydrofuran (THF). The monolith had a total porosity of 75.4% and an internal porosity of 9.1%. Complete recovery of both acidic and basic proteins was achieved using 100 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl.

The porous structure as well as the polarity of methacrylate ester-based monolithic stationary phases has been optimized to achieve the separation of complex peptide mixtures. Three monovinyl methacrylate monomers differing in the length of their lateral alkyl chain (namely, butyl, lauryl, or octadecyl methacrylate) have been utilized to demonstrate the effect of polarity of the monolith on retention. In parallel, the optimization of porogenic solvent composition, due to its effect on the porous structure, is carried out to achieve an efficient separation of peptidic fragments obtained from cytochrome c tryptic digest. Of the three types of monoliths studied, poly(LMA-*co*-EDMA) columns featuring small pores and small microglobules provided the best results with sufficient retention and resolution. Raising the temperature from 25°C to 60°C enabled separations to be carried out at 40% higher flow-rates. Separations carried out at 60ºC with a steeper gradient proceeded without loss of performance in half the time required for a comparable separation at room temperature (57).

In another approach, monoliths prepared by thermal polymerization of butyl methacrylate with a series of crosslinkers including EDMA, diethylene glycol dimethacrylate, triethylene glycol dimethacrylate, and pentaerythritol tetraacrylate were investigated. For all crosslinkers, optimization of the porogenic mixture consisting of 1-propanol and 1,4-butanediol yielded monoliths with pore sizes greater than 1 µm, which were suitable for rapid separations at low backpressure. Very good separations were achieved for a protein mixture

consisting of ribonuclease A, cytochrome c, myoglobin, and ovalbumin with all tested columns (58).

Recently, Zou and his coworkers (59) presented an automated nano-LC–MS–MS platform without trap column, by using a 20 cm LMA-EDMA monolithic capillary column to allow preconcentration and separation of peptides. The monolithic column had the advantages of good permeability and low backpressure, resulting in higher flow-rates for capillary columns. Tryptic digests of bovine albumin and yeast protein extract were tested using the monolithic column system. High proteomic coverage using this approach was demonstrated. Furthermore, peptide samples extracted from mouse liver were separated by using the monolithic column system combined with size-exclusion chromatography prefractionation.

Monolithic capillary columns for HIC have been prepared by thermally initiated polymerization of mixtures of monovinyl monomers, including butyl methacrylate and/or 2-hydroxyethyl methacrylate (HEMA), with a divinyl crosslinker glycerol dimethacrylate or 1,4-butanediol dimethacrylate using two different porogen systems. Two porogenic solvent mixtures were used; one "hydrophilic," consisting of water, butanediol, and propanol; and one "hydrophobic," comprising dodecanol and cyclohexanol. The porous structures of the monoliths were characterized and their performance was demonstrated with a separation of a mixture of myoglobin, ribonuclease A, and lysozyme under conditions typical of HIC. The authors demonstrated that the retention in this HIC mode was not affected by the polarity of the porogenic solvent. This indicates that the pore surfaces of the monoliths exposed to solvent had the same hydrophobicity or, alternatively, that both stationary phases were sufficiently hydrophilic not to affect the retention to any significant extent. In contrast, the mechanical stability of the monolith is strongly affected by the choice of the porogenic solvents (60).

A porous zwitterionic monolith was prepared by thermal copolymerization of *N,N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammoniumbetaineandethylenedimethacrylate [poly-(SPE-*co*-EDMA)] inside a 100-µm i.d. capillary. The resulting monolith showed a typical hydrophilic liquid chromatography (HILIC) mechanism at high organic solvent content (ACN > 60%). The polar zwitterionic monolith provides an environment, not only capable of hydrophilic interaction with polar and charged analytes, but also offering the possibility of weak electrostatic interaction with analytes carrying either positive or negative charges. The effects of pH, salt concentration, and ACN content on the separation of acids showed that an HILIC/ion exchange chromatography mechanism on poly(SPE-*co*-EDMA) could be used to manipulate selectivity when optimizing methods of separation. The poly-(SPE-*co*-EDMA) monolith showed very good selectivity for neutral, basic, and acidic polar analytes. For charged analytes, both hydrophilic interactions and electrostatic interactions contributed to their retention. To demonstrate the special selectivity of the poly(SPE-*co*-EDMA) monolith, seven benzoic acid derivatives and a mixture of four pyrimidines or purines and three neutral compounds were used for further evaluation. Good separations were obtained with optimized chromatographic conditions (61).

A stable poly[2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS)-*co*-poly(ethylene glycol) diacrylate (PEGDA)] monolith containing as high as 40% AMPS was synthesized inside a 75-µm i.d. capillary by photoinitiated copolymerization with water, methanol, and ethyl ether as porogens. The resulting monolith was evaluated for SCX capillary LC of both synthetic and natural peptides. Although the monolith possessed relatively strong hydrophobicity due to the use of AMPS as one monomer, the monolith had a high dynamic binding capacity of 157 µequiv of peptide/mL, or 332 mg of cytochrome c/mL. Exceptionally high resolution was obtained, resulting in a peak capacity of 179 when using a shallow salt elution gradient. A unique structural feature of the new monolith is the use of PEGDA instead of the conventional EDMA cross-linker, which is believed to improve the resolution for peptide analysis (62). In another approach, two polymer monoliths were designed and synthesized with an attempt to decrease hydrophobicity for SCX chromatography. One was prepared from the copolymerization of sulfoethyl methacrylate and PEGDA, and the other was synthesized from vinylsulfonic acid and PEGDA. Both of the monoliths were synthesized inside 75-µm i.d. capillaries, by photopolymerization. The hydrophobicities of the two monoliths were systematically evaluated using standard synthetic undecapeptides under ion-exchange conditions and propyl paraben under RP conditions. The poly-(sulfoethyl methacrylate) monolith demonstrated similar hydrophobicity as a monolith prepared from copolymerization of AMPS acid and PEGDA, and 40% ACN was required to suppress any hydrophobic interactions with peptides under ion-exchange conditions. However, with the use of vinylsulfonic acid as the functional monomer, a monolith with very low hydrophobicity was obtained, making it suitable for SCX-LC of both peptides and proteins. It was found that monolith hydrophobicity could be adjusted by selection of monomers that differ in hydrocarbon content and type of vinyl group. Finally, excellent separations of model protein standards and high-density lipoproteins were achieved using the poly(vinyl-sulfonic acid) monolith. Five subclasses of high-density lipoproteins were resolved using a simple linear NaCl gradient (63). Fangjun et al. (64) synthesized an SCX stationary phase by in situ polymerization of ethylene glycol methacrylate phosphate (EGMP) and bisacrylamide in a ternary porogenic solvent consisting dimethylsulfoxide, dodecanol, and *N,N*-dimethylformamide.

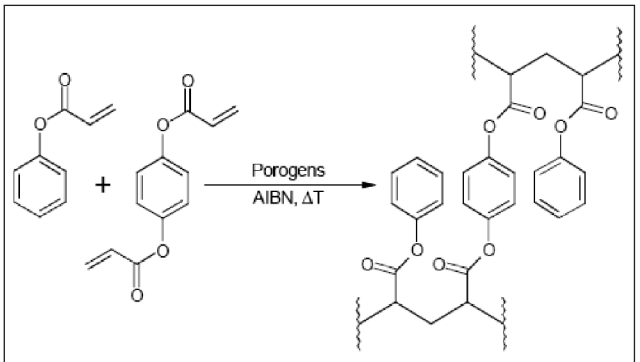

Figure 6. Reaction scheme, illustrating the copolymerization reaction of PA and PDA. Reproduced from Reference 65 with permission.

This phosphate monolithic column exhibits higher dynamic binding capacity, faster kinetic adsorption of peptides, and more than 10 times higher permeability than the column packed with commercially available SCX particles. It was applied as a trap column in a nanoflow LC–MS–MS system for automated sample injection and online multidimensional separation. It was observed that the sample could be loaded at a flow-rate as high as 40 µL/min with a backpressure of 1300 psi and without compromising the separation efficiency. Because of its good orthogonality to the RP separation mechanism, the phosphate monolithic trap column was coupled with an RP column for online multidimensional separation of 19 µg of the tryptic digest of yeast proteins. A total of 1522 distinct proteins were identified from 5608 unique peptides (total of 54,780 peptides) at the false positive rate of only 0.46%.

Although a large number of papers dealing with the preparation of monolithic separation media based on different (meth)acrylate chemistries can be found in literature, no aromatic, acrylate-based monolithic support material is reported up to the present. This was the inspiration of Bisjak et al. (65,66) for preparing novel monolithic capillary supports, based on aromatic precursors; namely phenyl acrylate (PA) and 1,4-phenylene diacrylate (PDA) (Figure 6). Because the surface of these monoliths exhibits hydrophobic character, the polymer was expected to be well-suited for application as RP stationary phase. These monoliths were produced in 200 µm i.d. capillaries using 2-propanol and THF as porogens. It was found that due to similarities in the structure of the monomer and cross-linker, monoliths were very homogenously cross-linked and as a result showed high mechanical stability, and they were consequently used to separate a mixture of proteins. The synthesized monoliths showed excellent mechanical stability and a low tendency to swell in organic solvents. Variations of the monomer-to-porogen ratio, the micro-

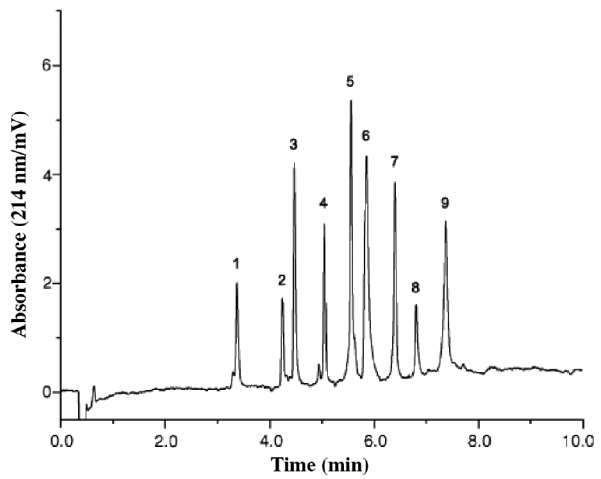

Figure 7. Separation of a mixture of 9 proteins on a monolithic poly PA–PDA capillary column (75 × 0.2 mm i.d.). Chromatographic conditions: mobile phase A, 0.1% TFA; B, 0.1% TFA in ACN; gradient, 10–75% B in 10 min; flow-rate, 9.4 µL/min; temperature, 50°C; detection, UV 214 nm. Peak identification: ribonuclease A, 1; insulin, 2; cytochrome c, 3; lysozyme, 4; α-lactalbumin, 5; BSA, 6; β-lactoglobulin B, 7; catalase, 8; ovalbumin, 9; injected sample amount, 80 fmol of each protein. Reproduced from Reference 66 with permission.

porogen nature, and the polymerization temperature were shown to significantly affect the porous structure and, as a direct consequence, the chromatographic performance of the novel monoliths. Systematic tailoring of the porous properties resulted in monolithic supports, which allowed high-resolution separations of a wide spectrum of biomacromolecules. The chromatographic performance was illustrated by the baseline separation of nine proteins using gradient elution on a 75 mm × 0.2 mm i.d. capillary at a flow rate of 9.4 µL/min (Figure 7).

Nucleotides

A porous monolithic column was prepared by in situ copolymerization of 3-sulfopropyl methacrylate (SPMA) and pentaerythritol triacrylate (PETA) in a binary porogenic solvent consisting of cyclohexanol/ethylene glycol. The monolith possessed bonded sulfonate groups and hydroxyl groups, and was evaluated for use in hydrophilic interaction and SCX chromatography. While the SPMA was introduced as multifunctional monomer, the PETA was used instead of ethylene glycol dimethacrylate as cross-linker due to its higher hydrophilicity. A typical HILIC mechanism was observed at a higher organic solvent content (ACN > 70%) for polar neutral analytes. For polar charged analytes, both hydrophilic interaction and electrostatic interaction contributed to their retention. Therefore, for charged analytes, selectivity can be readily manipulated by changing the composition of the mobile phase (e.g., pH, ionic strength, and organic modifier). With the optimized monolithic column, plate counts greater than 105,000 plates/m were easily obtained. The effects of ACN content, pH, and salt concentration on the separation of nucleotides showed that an HI-SCX chromatography mechanism on poly(SPMA-*co*-PETA) could be used to manipulate selectivity when optimizing methods for the separation of nucleic acid bases and nucleosides (67). Holdsvendova et al. (68) applied hydroxymethyl methacrylate-based monolithic columns for separation of oligonucleotides. *N*-(hydroxymethyl) methacrylamide (HMMAA) was copolymerized with EDMA, in the presence of the porogenic system composed of propane-1-ol, butane-1,4-

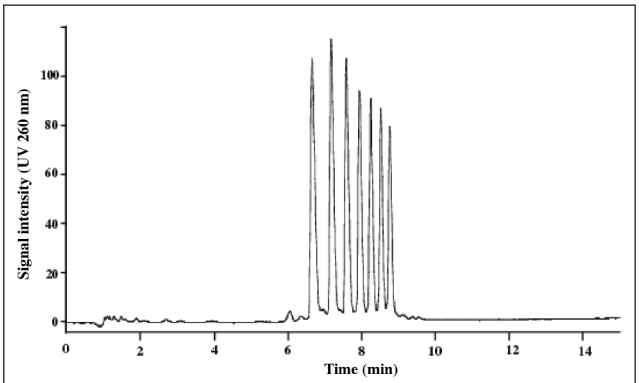

Figure 8. Column, poly(3-diethylamino-2-hydroxypropylmethacrylate-co-divinylbenzene), monolithic, 80 mm × 0.2 mm i.d. Mobile phase A: 20 mM KH_2PO_4, 20% ACN, pH 7.0; B: 20 mM KH_2PO_4, 20% ACN 1 M NaCl in A; gradient, 25–55% B in 2 min, 55–100% B in 7 min; flow-rate, 2.3 µL/min; room temperature; detection, UV, 260 nm; sample, $d(pT)_{12-18}$. Reproduced from Reference 68 with permission.

diol, and AIBN as initiator. Separations of oligonucleotides were performed in HILIC mode using 100 mM triethylamine acetate (TEAA) in ACN and in water as eluents. Oligonucleotides differing by only one base unit were separated under a gradient elution in 35 min. A high column-to-column reproducibility was found. Expressed as RSD of apparent retention factor and retention time, the value did not exceed 5%. Moreover, HMMAA monolithic stationary phases are advantageous due to their facile preparation because (*i*) silanization of inner wall of capillaries is not necessary and (*ii*) the porogenic system is simplified from a ternary mixture to a binary one (water is not added).

Bisjak et al. (69) demonstrated the separation of oligonucleotides using a weak anion exchange monolith. The capillaries were prepared by thermal-initiated copolymerization of GMA and DVB inside silanized 200 µm i.d. fused silica capillaries. Polymerization mixtures containing different amounts of porogen (1-decanol and THF) and different ratios of monomer and crosslinker were used for synthesis. After characterization, the produced GMA-DVB monoliths, which contain reactive epoxide groups, were modified by reaction with diethylamine to obtain a poly(3- diethylamino-2-hydroxypropyl methacrylate-*co*-divinylbenzene) ion-exchange monolithic stationary phase. The synthesized monoliths contain ionizable amino groups that are useful for anion-exchange chromatography. Poly(3-diethylamino-2-hydroxypropylmethacrylate-*co*-divinylbenzene) monolithic columns allowed a fast and highly efficient separation of a homologous series of phosphorylated oligothymidylic acids [d(pT)12-18] (Figure 8). Because durability is an important parameter of chromatographic column characterization, the separation performance for d(pT)12-18 in a freshly produced capillary column and on the same column after 100 chromatographic runs was compared. The developed monolith was further derivatized to obtain quaternary ammonium functionalities. From the van Deemter plot, the number of theoretical plates per meter was found to be 59324. The produced strong anion exchange monoliths turned out to be highly suitable for the separation of nucleotides and oligonucleotides (70).

Affinity chromatography

Acrylate-based monolithic supports have been frequently used for the immobilization of ligands, and their use in affinity chromatography is increasing. This part summarizes the affinity chromatography applications utilizing monolithic supports with attached ligands of different molecular sizes. Typically, the monolithic supports for affinity chromatography are polymerized in-situ and the ligand is then immobilized. Geiser et al. (71) investigated the use of two different monoliths located in capillaries for on-line protein digestion, preconcentration, and separation of peptides. The first monolith was prepared by in situ copolymerization of 2-vinyl-4,4-dimethylazlactone and EDMA and was used as support for covalent immobilization of pepsin. The second monolith was based on poly(LMA-*co*-EDMA) and provided a different porous structure for the preconcentration of peptides and their separation RP-LC mode. The top of the separation capillary was used as a preconcentrator, thus enabling the digestion of very dilute solutions of proteins in the bioreactor, and increasing the sensitivity of the mass spectrometric detection of the peptides using a TOF mass spectrometer with ESI. Myoglobin, albumin, and hemoglobin were digested to demonstrate feasibility of the concept of using the two monoliths in-line. Successive protein injections confirmed both the repeatability of the results and the ability to reuse the bioreactor for at least 20 digestions.

A nanoliter trypsin-based monolithic microreactor coupled with µRPLC-MS–MS was reported for shotgun proteome analysis. The proteins were rapidly digested by the microreactor, and the resulting protein digests were directly loaded onto a µRPLC column for separation followed with detection of the eluted peptides by tandem MS. The digestion efficiency and stability of the microreactor was demonstrated by using BSA as a model protein. Under these conditions, protein mixtures can be digested by the microreactor in several minutes as opposed to the 10 h required for conventional trypsin digestion. It was found that the digestion of proteins by the capillary microreactor is very efficient due to the presence of high concentrations of trypsin in the prepared microreactor, and the immobilized trypsin has higher tolerance to denaturing agents (e.g., urea). The efficiency of the digestion was further demonstrated by digestion of a real proteome sample, the yeast cell lysate of *Saccharomyces cerevisiae*, when compared to the digestion with free trypsin. The digestion time using the capillary microreactor was decreased by 960 times from 16 h to 1 min. Additionally, the number of identified proteins was only decreased by 13.3% from 624 to 541 when 590 ng yeast proteins were digested and analyzed. A total of 1578 unique peptides corresponding to 541 proteins were identified when 590 ng yeast protein was digested by the microreactor with an incubation time of only 1 min (72).

Monolithic capillary columns with surface-immobilized mannan have been introduced for affinity-based micro-column separations by nano-LC. Poly(GMA-*co*-EDMA) monoliths were prepared with immobilized mannan, that exhibited strong affinity toward mannose-binding proteins (MBP) such as the plant lectins concanavalin A (Con A) and *Lens culinaris* agglutinin (LCA) and a mammalian lectin (e.g., rabbit serum MBP). Due to its strong binding affinity, the monoliths with surface bound mannan allowed the isolation of MBP from rabbit serum in a single run with an amount sufficient to run both sodium dodecylsulfate-polyacrylamide gel electrophoresis and MALDI-MS, thus demonstrating their capability in "nano-proteomics" (73). The same monolith was applied for immobilization of two lectins, including Con A and wheat germ agglutinin. The monoliths with immobilized lectins exhibited strong affinity toward particular glycoproteins and their oligosaccharide chains (i.e., glycans) having sugar sequences recognizable by the lectin. Due to the strong binding affinity, the monoliths with surface bound lectins allowed the injection of relatively large volume (i.e., several column volumes) of dilute samples of glycoproteins and glycans, thus, allowing the concentration of the glycoconjugates and their subsequent isolation and detection at low levels (~10–8 M). To further exploit the lectin monoliths in the isolation of glycoconjugates, two-dimensional separation schemes involving lectin affinity chromatography in the first dimension and reversed-phase nano-LC in the second dimension were introduced (74,75).

Tetala et al. (76) prepared an affinity monolith for separation, enrichment, and binding studies of mannose-specific lectins employing HEMA in combination with (+)-*N*,*N*'-diallyltartardiamide (DATD) and piperazine diacrylamide (1,4-bisacryloylpiperazine) (PDA) as crosslinkers. After oxidation of DATD with periodate, α-mannose with amino terminated spacer was bound to the aldehyde groups of the polymeric skeleton via reductive amination. The affinity of the monolith was evaluated by frontal analysis and fluorescence microscopy (FM) using fluorescently labeled Con A. Frontal affinity chromatography (FAC) showed a specific interaction of two different lectins with the α-mannose-modified monolith. According to FM, the affinity sites were evenly distributed over the monolithic bed. In another approach, carbohydrates (β-galactose, β-glucose, and α-mannose) with an alkene terminated tetraethylene glycol spacer were used as functional monomers along the monomer HEMA. As crosslinkers, DATD and PDA were used. The specific interaction of the lectins Con A, LCA and *Arachis hypogaea* (PNA) with the carbohydrate stationary phase was studied by FAC. Con A and LCA were successfully eluted from the column using 0.1 M methyl-α- mannopyranoside and PNA with 0.1 M β-galactose. Dissociation constants for carbohydrate–lectin interactions were determined and compared with literature (77).

Zhao et al. (78) developed an affinity aptamer monolithic capillary chromatography technique for protein separation and detection, taking advantage of both monolithic columns and aptamers. Proteins were separated based on their different binding affinities to the immobilized aptamers. A biotinylated DNA aptamer targeting cytochrome c was successfully immobilized on a streptavidin-modified polymer monolithic capillary column. The aptamer, having a G-quartet structure, could bind to both cytochrome c and thrombin, enabling the separation of these proteins from each other and from the unretained proteins. Elution of strongly bound proteins was achieved by increasing the ionic strength of the mobile phase. Other proteins were also tested using the aptamer affinity monolithic columns: human immunoglobulin G, hemoglobin, transferrin, human serum albumin, cytochrome c, and thrombin. Determination of cytochrome c and thrombin spiked into dilute serum samples showed no interference from the serum matrix. The benefit of porous properties of the affinity monolithic column was demonstrated by selective capture and preconcentration of thrombin at low ionic strength and subsequent rapid elution at high ionic strength.

Potter et al. (79) reported the synthesis of boronate affinity monoliths and their applicability to retain ribonucleosides in µHPLC. Two approaches for the fabrication of porous polymer monoliths with boronate affinity ligands were compared. Base monoliths of poly(GMA-*co*-EDMA) had a median pore size of 1.19 µm and a surface area of 5.6 m^2/g. In one approach, the boronic acid functional group was attached through nucleophilic attack of the epoxide with *p*-hydroxyphenylboronic acid. In the second approach, the boronic acid functionality was attached via photografting a thin layer of poly(GMA) onto the surface of the poly(EDMA-*co*-GMA) monolith prior to reaction with the p-hydroxyphenylboronic acid. The performance of the boronic acid functionalized monolith was evaluated with ribonucleosides and their 2-deoxyribonucleoside counterparts as model analytes. The absence of a hydroxyl group prevents 2-deoxyribonucleosides from forming cyclic complexes with the boronate groups and thus reduces its retention, although they may still be retained by secondary interactions with the monolith (such as hydrophobic interaction). The separation of cytidine and 2-deoxycytidine using the boronic acid functionalized monoliths was obtained, and cytidine was significantly retained in both columns in relation to its 2-deoxy counterpart, indicating successful fabrication of a boronic acid monolith. Retention of adenosine over 2-deoxyadenosine was also observed, while guanosine and uridine showed significant interaction with the monolith with the peak being too low and broad to be readily detected.

Recently, a method to prepare a zirconium phosphate (ZrP)-modified monolithic capillary column for the highly specific capture of phosphopeptides was presented. In this method, the phosphate monolithic capillary column was prepared by direct copolymerization of the functional monomer containing phosphate group (EGMP) and cross-linker (bis-acrylamide) in a ternary porogenic solvent. Dimethysulfoxide (DMSO) was chosen as a good solvent so that all monomers could be dissolved completely. Dodecanol was chosen as poor solvent; in addition, *N,N* 9-dimethylformamide was added to improve the dissolution of EGMP. After Zr^{4+} was immobilized, the ZrP-modified monolithic capillary column was evaluated by the analysis of standard phosphoproteins, and the excellent selectivity of this approach was demonstrated by analyzing phosphopeptides in the digest mixture of β-casein and BSA with molar ratio of 1:200 (80).

Poly Norborn-2-Ene and Poly(Cyclooctene) Monoliths

In contrast to the most frequently employed free radical polymerization technique, Buchmeiser et al. introduced a novel class of monolithic polymer supports by employing ring-opening metathesis polymerization (ROMP) (81,82). This approach employed mixtures of norborn-2-ene (NBE) and 1,4,4a,5,8,8a-hexahydro-1,4,5,8-exo,endo-dimethanonaphtalene that were copolymerized in the presence of appropriate porogenic solvents, and a Grubbs type ruthenium catalyst as initiator. The resulting hydrophobic polymers showed surprisingly similar morphological characteristics to other organic polymer monoliths prepared by thermally- or photochemically-initiated free radical polymerization. ROMP-derived monolithic supports have been successfully applied to the separation of biopolymers in conventional columns as well as in capillary format (83,84). Using the monolithic capillary columns, different sets of analytes (i.e., standard systems) were used for the evaluation: (*i*) a protein standard consisting of six proteins in the range of 5000–66,000 g/mol, (*ii*) an insulin–albumin standard, and (*iii*) a peptide standard obtained from a tryptic digest of cytochrome c. With these three different standard systems, the reproducibility of synthesis in terms of separation performance proved to be 1–2% RSD in t_R. Variation of polymerization parameters had a significant influ-

ence on the monolithic morphology and, therefore, separation efficiency and backpressure. The maximum analytical loading capacity of ROMP-derived monolithic capillary columns for albumin was found to be 30–125 ng, depending on the monomer content. Long-term stability studies showed no alteration in separation performance (85). A 200-µm i.d. monolithic column demonstrated excellent separation behaviour for insulin and various insulin analogs, with equivalent separation performance to Vydac C4 and Zorbax C3-based stationary phases. Moreover, the high permeability of monoliths enabled chromatographic separations at higher flow-rates, which shortened analysis time to approximately one third. The capillary column also showed a good reproducibility in terms of retention times, with RSD of 1.9% for proteins and 2.2% for peptides (86).

Despite the high mechanical and thermal stability of these monoliths, a slow change of surface polarity of such monolithic columns can occur, due to oxidation of the tertiary allylic carbons located at the surface of the monolith. Buchmeiser and his coworkers tried to solve this problem by introducing a *cis*-cyclooctene and tris(cyclooct-4-enyl-1-oxy)methylsilane-based monolithic system. The poly(cyclooctene) polymer backbone is derived from secondary allylic carbons and therefore presents an attractive alternative to NBE-based systems. These monoliths (3 × 100 mm in size) were used for the separation of a mixture of five proteins (i.e., ribonuclease A, lysozyme, insulin, cytochrome c, and myoglobin). The obtained data of the new monolithic column are comparable to that based on NBE, and similar trends in polymerization kinetics and their effect on separation performance as well as on backpressure were observed. Importantly, long-term stability showed very good results over 1000 runs (87).

Acrylamide-Based Monoliths

Chiral separation of amino acids was achieved on particle-loaded phases following the separation principle of ligand-exchange. Three micrometer silica particles modified with l-4-hydroxyproline as a chiral selector were immobilized in a polymeric continuous bed. Particle-loaded monoliths containing a polymethacrylamide backbone were prepared by suspending a silica-based chiral phase in the mixture of the monomers, followed by in-situ polymerization in the capillary. As chiral selector, l-4-hydroxyproline chemically bonded to 3 µm silica particles was used following the separation principle of ligand-exchange (88).

Others

Husoya et al. (89) describes the development of novel wired chip devices for µ-LC analyses. The monolithic capillary column to be wired was prepared using a tri-functional epoxy monomer, tris(2,3-epoxypropyl)isocyanurate with a diamine, 4-[(4-aminocyclohexyl) methyl]cyclohexylamine. The prepared column was evaluated by scanning electron microscopy and µ-HPLC. In addition, the reproducibility in the preparation of long capillary columns having nearly 1 m length was extensively examined for applications of novel wired chip devices. The authors demonstrated that the monolithic structure of the prepared long capillary could be finely controlled under the strictly maintained operational conditions, and thus the RSD of the column properties such as the number of theoretical plates, retention factor, and permeability could be well controlled to become less than 10%. Furthermore, the wired chip device column showed that its high performance was kept even after chip preparation.

Conclusion

Due to their capability for very rapid separation of biomolecules, and their simple scaling up and scaling down, monoliths have a large potential for application in the separation of biomolecules. Considerable progress has been made in the fabrication of monolithic materials, and there is now a wealth of chemistries available that makes these materials available for a wide range of applications. The surface chemistry of the monolith may be tailored to a specific application, and thus opens up endless possibilities in terms of selectivity tuning. The applications listed here show broad compatibility of monoliths for µLC separation of biomolecules in different modes. Combinations of monolithic columns with different separation modes have been applied successfully for both enrichment and separation.

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Table I. Appendix: Summary of the Recent Application of Organic Monolith in Capillary Liquid Chromatography

Polymer	Characteristics	Analytes	Detection	Ref.
PS-DVB	IP-RP-LC	Peptides, proteins: BSA	+ESI-MS, –ESI-MS–MS	37
	IP-RP-LC	α-Casein digest (phosphorylated peptides)	–ESI-MS, CID	38
		Complex proteomic samples	MALDI-MS	39
	20 µm i.d. capillary	Tryptic peptides mixture	ESI-MS–MS	40
	10 µm i.d., PLOT	Methanosarcina acetivorans proteins	ESI-MS	41
	RP-LC multidimensional isoforms; phosphopeptides	Membrane antenna proteins,	ESI-MS, MALDI-MS	42
	In solution digestion	Human breast cancer cell line MCF10A	MALDI-MS, tandem-MS	43,44
	10 mm × 0.20 mm i.d.; pre-concentration, IP-LC, RP-LC	Peptides, proteins	ESI-MS, MALDI-MS	45,46
	IP-RP-LC, SCX-LC, multidimensional	Tryptic peptides from bacterial proteome	MALDI-MS–MS	47,48
		Peptides, proteins conductivity detection	Contactless	49
	Addition of EDTA	RNA from *Escherichia coli*	ESI-MS–MS	51
POS-DVB	Column coupled to carbon fiber emitter	Tryptic peptide digest	ESI-MS	52
PS-OD-DVB	High loading capacity	Six standard proteins, α-, β-chains of hemoglobin	ESI-MS	50
Poly(MST-BVPE)	Flexible adjustment of porosity	Standard proteins, peptides	UV, ESI-MS	53
Poly(MST-BVBDMS)	Flexible adjustment of porosity	Standard proteins, oligonucleotides	UV, ESI-MS	54
Poly(TMSiMS-bis(4-vinylbenzyl)dimethylsilane	Flexible adjustment of porosity	Standard proteins, oligonucleotides	UV, ESI-MS	55
Poly(ethylene glycol methyl ether acryltate-*co*-PEGDA)	Low flow resistance	Acidic and basic proteins	UV	56
Methacrylate ester-based	Small pores	Cytochrome C tryptic digest	UV	57
Butyl methacrylate with different crosslinkers	Low backpressure	Standard protein mixture	UV	58
Poly(LME-EDMA)	Automated nano-LC	Tryptic peptide digests	ESI-MS–MS	59
Monovinyl monomers polym. with acrylates	HIC	Standard proteins	UV, MS	60
Poly(SPE-*co*-EDMA)	HILIC and IEX mechanism,	Neutral, basic and acidic polar zwitterionic monolithanalytes, benzoic derivatives, pyrimidines, purines	UV	61
Poly(AMPS-*co*-PEGDA)	Strong cation IEX, narrow peaks, high peak	Cytochrome C peptides, capacityproteins, lipoproteins	UV	62,63
Poly(EGMP)	Strong cation IEX, high permeability, nano-LC	Peptides	ESI-MS	64
Phenyl acrylates polymerized with 1,4-phenyl acrylate	RP-LC, high-mechanical stability, low swelling	Peptides, proteins, oligonucleotides	UV, MS	65,66
SPMA polym. with PETA	Sulfonate and hydroxyl groups, HILIC, SCIEX	Nucleotides	UV	67
Hydroxymethyl methacrylaide monoliths polym. with EDMA	HILIC	Oligonucleotides	UV	68
Poly(GMA)	Weak anion exchange	Oligonucleotides	UV	69,70
2-vinyl-4,4-dimethylazlactone polym. with EDMA	Pre-concentration prior to RP-LC	Peptides	ESI-TOF-MS	71
Trypsine-based microreactor	Shotgun proteomics	Proteins	tandem MS	72
Poly(GMA-*co*-EDMA)	Affinity chromatography	MBP	MALDI-MS	73
HEMA, DATD, PDA	Affinity chromatography, α-mannose modification	Lectins	UV	75
Poly(GMA-*co*-EGDA)	Boronic acid functionalized	Nucleosides	UV	79
EGMP polym. with bis-acrylamide	ZrP-monolith	Phosphopeptides from β-casein and BSA	UV	80
NBE-based monoliths	High permeability, high reproducibility	Biopolymers	UV, MS	85,86