ELSEVIER

Journal of Chromatography A, 954 (2002) 5–32

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Monolithic stationary phases for liquid chromatography and capillary electrochromatography

Hanfa Zou*, Xiaodong Huang, Mingliang Ye, Quanzhou Luo

National Chromatographic Research and Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, China

Received 10 October 2001; received in revised form 15 January 2002; accepted 15 January 2002

Abstract

A monolithic stationary phase is the continuous unitary porous structure prepared by in situ polymerization or consolidation inside the column tubing and, if necessary, the surface is functionalized to convert it into a sorbent with the desired chromatographic binding properties [J. Chromatogr. A 855 (1999) 273]. Monolithic stationary phases have attracted considerable attention in liquid chromatography and capillary electrochromatography in recent years due to their simple preparation procedure, unique properties and excellent performance, especially for separation of biopolymers. This review summarizes the preparation, characterization and applications of the monolithic stationary phases. In addition, the disadvantages and limitations of the monolithic stationary phases are also briefly discussed.

Keywords: Reviews; Stationary phases, LC; Stationary phases, electrochromatography; Monolithic columns

Contents

*Corresponding author. Tel.: +86-411-369-3409; fax: +86-411-369-3407.
E-mail address: zouhfa@mail.dlptt.ln.cn (H. Zou).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00072-9

1. Introduction

In the past years, comprehensive attention was paid to liquid chromatography and capillary electrochromatography due to the need for rapid development in life sciences, environmental sciences and pharmaceutical industry. However, conventional commercial columns packed with beads in HPLC possess some inherent limitations such as the slow diffusional mass transfer and the large void volume between the packed particles, which lead to the efficiency of conventional commercial columns lingering in the range of 10 000 to 30 000 plates/column in the past two decades [1,2]. Although some new stationary phases such as the non-porous beads [3,4] and perfusion chromatography packings [5,6] are designed to resolve these problems, these limitations cannot be overcome in essence. In recent years a novel type, the monolithic stationary phase, has attracted increasing attention in liquid chromatography because of its easy preparation, excellent properties and high performance compared to conventional columns packed with particles for the separation of biopolymers. A monolithic stationary phase is the continuous unitary porous structure prepared by in situ polymerization or consolidation inside the column tubing and, if necessary, the surface is functionalized to convert it into a sorbent with the desired chromatographic binding properties [7]. The pioneer preparations of this type of ''single-piece'' separation medium can be dated back to the late 1960s and early 1970s. Kubin et al. [8], prepared a swollen poly(2-hydroxyethyl methacrylate) polymer for size-exclusion chromatography to separate proteins under low pressure in 1967, but permeability was too low to be used in practice. Ross and co-workers [9,10] also prepared another monolithic open-pore polyurethane foam for both high-performance liquid and gas chromatography. Despite the excellent permeability, they were found to suffer from excessive swelling and softening in some solvents, which prevented their further use. Other approaches towards continuous media emerged in the late 1980s, including stacked membranes [11], rolled cellulose sheets [12], and rolled woven matrices [13], compressed soft poly(acrylamide) gels [14], and macroporous disks [15]. The compressed soft gels called ''continuous beds'' developed by Hjertén et al. [14] in 1989 are the onset when continuous media were successfully used in chromatographic separation. In the early 1990s, Svec and Fréchet [16] introduced an entirely new class of continuous media based on rigid macroporous polymer monoliths produced by a very simple ''molding'' process. Their unique properties allow these materials to be used in a broad variety of applications and gradually became the mainstream of the research of continuous media family. Inorganic silica-based monoliths were later reported by several groups starting in 1996 [17,18].

Capillary electrochromatography (CEC) is a novel microcolumn separation technique that combines features of both HPLC and CZE. In CEC, electroosmotic flow (EOF) is used to drive mobile phase instead of hydrodynamic flow. Because of the flat plug-like profile of the EOF, CEC offers greatly enhanced separation efficiencies relative to HPLC. The predominant class of column in CEC is the packed column. According to the structures of the

packed materials, packed CEC column can also be classified into two categories: (1) columns packed with particulate material, and (2) monolithic columns. By far, the most often used columns in CEC are of the first type—columns packed with suitable chromatographic particulates. Two frits need to be fabricated to retain the particle material in place in this type of column. There are several drawbacks to using frits in CEC. These include the extreme difficulty in their preparation, their lack of reproducibility, their tendency to act as a catalyst for bubbles, their unpredictable influence upon electroosmotic flow and band-spreading [19,20]. Recently, monolithic columns containing a wall-supported continuous porous bed have shown great potential for CEC due to being fritless, thereby eliminating the drawbacks listed above. Various columns with organic and inorganic polymer monoliths, and particle-fixed monoliths, have been reported [20].

Actually, the monolithic stationary phases have become a rapidly burgeoning field in preparation of chromatographic stationary phases in recent years and even some of these have been commercialized [21]. Some chromatography researchers consider them as the fourth-generation chromatography sorbents [22]. This review summarizes the preparation, characterization and applications of the monolithic stationary phases, and the disadvantages and limitations of monolithic stationary phases are also discussed.

2. Preparation of monolithic stationary phases

2.1. Preparation of monolithic stationary phases in HPLC

2.1.1. Preparation of silica monoliths

Although the continuous silica supports from silicate-surfactant solutions had been already reported at the end of the 1970s [23], useful silica monoliths for chromatographic applications had only emerged in 1996. So far, two methods have been developed for preparation of the silica monoliths. One of the methods was introduced by Fields in 1996 [17], which was similar to that used to cast column end frits in fused-silica tubing for packing capillary HPLC. A fused-silica column was filled with a potassium silicate solution and heated at 100 °C for 1 h. The column was then washed and dried with helium for 24 h at 120 °C and then filled with a 10% solution of dimethyloctadecylchlorosilane (ODS) in dry toluene and heated at 70 °C for 5 h. After the reaction was finished, the column was washed and was used for reversed-phase HPLC. Although the method can produce a continuous silica xerogel with mean pore diameter on the order of 2 μm, the morphology of the material was heterogeneous.

Another method, i.e. the sol–gel approach, was introduced by Minakuchi et al. [18] in the same year, by which a more uniform structure of monolith could be constructed. In this method, a porous silica rod was prepared by hydrolytic polycondensation of alkoxysilane accompanied by phase separation in the presence of water-soluble organic polymers. The process of gelation, ageing and drying was involved in the preparation procedure. In a typical procedure, tetramethoxysilane (TMOS) was added to a solution of poly(ethylene oxide) (PEO) in water and a suitable catalyst, such as acetic acid, was also added. The mixture was stirred at 0 °C for 30 min. The resultant homogeneous solution was poured into a cylindrical polycarbonate mould and allowed to react at 40 °C. The gelation occurred within 2 h and the gelled sample was subsequently aged at the same temperature for 1 day. The formed wet silica rod was washed with distilled water and then immersed in an aqueous ammonium hydroxide solution in order to tailor the mesopore structure. Evaporation drying and heat treatment were successively performed, which led to the decomposition of organic constituents and stabilization of the surface of the hydrophilic silica gel. Since the gel shrunk in the ageing and drying, the resultant silica gel had to be encased in heat-shrinking poly(tetrafluoroethylene) (PTFE) tubing and compressed with external pressure to ensure that there was absolutely no void space between the silica rod and tube. The rod could be subsequently octadecylsilyated to C_{18} phase by an on-column reaction. With this method, silica rods with 1–2 μm through-pore size and 5–25 μm mesopore size could be prepared and the surface area of silica rods reached 300–400 m^2/g [24,25].

2.1.2. Preparation of organic polymer monoliths

The preparation of a polymer monolithic rod is relatively simple and straightforward compared to that of a silica rod. A mixture consisting of the monomers, cross-linker and initiator in the presence of at least one, usually two porogenic solvents was poured into a mold, typically a tube, which was sealed at one end, and then sealed at the other end. The polymerization was then triggered frequently by heating in a bath at a temperature of 55–80 °C or by UV light. The seals were replaced with the fittings and attached to a chromatographic pump, and a solvent was pumped through the column to remove the porogens and other soluble compounds that remained in the polymer rod after the polymerization was completed. After the monolithic support was prepared, the column could be subsequently derivatized for diverse chromatographic modes. So far, the monoliths based on polystyrenes, polymethacrylates and polyacrylamides are the most commonly reported.

2.1.2.1. Monolithic rods based on polystyrenes

The monomers usually used to prepare the polystyrene monolithic rod are styrene and 4-(chloromethyl)styrene and the cross-linker is frequently divinylbenzene (DVB). Since the styrene monomer is hydrophobic, the poly(styrene–co-divinylbenzene) monolithic rod can be directly used in reversed-phase and precipitation–redissolution chromatography [26–28]. The monomer of 4-(chloromethyl)styrene can afford reactive groups relative to styrene for further derivatization. A hydrophilic monolithic rod had been prepared by two-step modification on the poly(chloromethyl)styrene monoliths, firstly with ethylenediamine then with γ-gluconolactone, which introduces a highly hydrophilic surface on a hydrophobic polymeric skeleton [29]. It can also react with ethylenediamine followed with chloroacetic acid for a weak cation-exchange column [30] or activated by grafting 4-vinyl-2,2-dimethylazactone to its pore surface for scavenging of excess amines from reaction mixtures [31]. Some chromatographic modes such as affinity, ion-exchange, chirality, adsorption, etc., rely on intimate contact with a surface that supports the active sites. To obtain a large surface area, a large number of smaller pores should be incorporated into the polymer. A monolith with a specific surface area as high as 400 m^2/g yet still permeable to liquids at a reasonable back pressure had been prepared by Svec and co-workers [32,33] using high-grade divinylbenzene and suitable porogenic solvent. Furthermore, a novel TEMPO (2,2,6,6-tetramethylpiperidyl-1-oxy)-mediated ''living'' radical polymerization was also developed for preparing monoliths with high specific surface areas (>300 m^2/g), in which a significantly larger proportion of pores smaller than 50 nm could be obtained [33–35].

2.1.2.2. Monolithic rods based on polymethacrylates

In the preparation of monolithic rods, poly(glycidyl methacrylate–co-ethylene dimethacrylate) is the most frequently used support which affords the reactive epoxide groups for easy derivatization. Svec and Fréchet [36,37] introduced the amino group on the poly(glycidyl methacrylate–co-ethylene dimethacrylate) monoliths by reaction with diethylamine for weak anion-exchange chromatography. It can also react with ethylenediamine followed with chloroacetic acid or directly with iminodiacetic acid (IDA) for a weak cation-exchange column [38,39]. Viklund et al. [40] also introduced the sulfonic acid group for strong cation-exchange chromatography by grafting poly(2-acrylamido-2-methyl-1-propanesulfonic acid) onto the internal surface of hydrolyzed poly(glycidyl methacrylate–co-ethylene dimethacrylate) monoliths. Furthermore, Peters et al. [41] prepared thermally responsive polymer monoliths for temperature-controlled hydrophobic interaction chromatography by a two-step grafting procedure, which involved the vinylization of the pore surface by reaction with allylamine and a subsequent in situ radical polymerization of *N*-isopropylacrylamide (NIPAAm) within these pores. The properties of this polymer can change in response to external temperature. In addition, it can also be used to immobilize enzymes for bioreactors by reaction with ethylenediamine and glutaraldehyde [42].

Although the preparation of polymer monoliths on a small scale is easy, the preparation of large size monoliths is quite difficult. The unstirred nature of the polymerization within the confines of a mold leads to a decreased capacity to effectively dissipate

the heat of polymerization and the occurrence of exotherms and radial temperature gradient across the contents of the mold also result in the creation of inhomogeneities in the pore structure. This is the reason why most of the work reported to date has been focused on the development of small size monoliths. Two methods for the preparation of large size poly(glycidyl methacrylate–co-ethylene dimethacrylate) monoliths by decreasing the rate of polymerization and a slow gradual addition of the polymerization mixture to the reaction vessel had been reported by Peters et al. [43] for the control of the evolution of the heat of polymerization. The latter approach proved especially powerful; a homogeneously structurally large cylindrical macroporous polymer monolith with internal diameters of up to 50 mm and with length of 200 mm was obtained. Recently, Podgornik et al. [44] analyzed the heat release during the polymerization and derived a mathematical model for prediction of the maximal thickness of the monolithic annulus having a uniform structure. On the basis of the calculation, a large volume of poly(glycidyl methacrylate–co-ethylene dimethacrylate) monolith was prepared for radial chromatography and the monolith exhibited flow-independent separation efficiency and dynamic binding capacity up to flow-rates higher than 100 ml/min.

2.1.2.3. Monolithic rods based on polyacrylamides

The monomer of acrylamide is very hydrophilic and is usually used in water phase polymerization for gel electrophoresis. The preparation of continuous beds introduced by Hjertén and co-workers [45,46] is also based on this class of monomer. However, they are rarely used in preparation of monolithic rods. Xie et al. [47] prepared a rigid porous poly(acrylamide–co-butyl methacrylate–co-*N,N′*-methylenebisacrylamide) monolithic column for hydrophobic interaction chromatography of proteins. The hydrophobicity of the monolithic column can be easily controlled by the percentage of hydrophobic monomer of butyl methacrylate in the polymerization mixture. They also prepared a poly(acrylamide–co-*N,N′*-methylenebisacrylamide) monolithic rod for studying the effect of the polymerization conditions on the porous properties of monolithic rods [48].

2.1.2.4. Monolithic molecularly imprinted polymers

Molecular imprinting is a technique for preparing tailor-made artificial synthetic affinity media with high selectivity toward the substrate molecules (imprinted molecules). In this method, complexes between print molecule (template) and functional monomers are firstly allowed to pre-organise or self-assemble in solution via covalent or non-covalent interactions, and subsequently, the three-dimensional architecture of these complexes is arrested by polymerization with a high concentration of cross-linker. Then the imprinted species are extracted from the resultant polymer, sites are left in the polymer complementary in shape and functionality to the imprinted molecule, and these sites can be allowed to rebind the print molecule. The resultant molecularly imprinted polymers (MIPs) have been applied in numerous fields, such as chromatographic resolution of racemates, artificial antibodies, chemosensors, selective catalysts, etc. [49–54]. The conventional preparation approach is to synthesize by bulk polymerization [55], crush, grind the resulting polymer block into particles and sieve the particles into desired size ranges. Although the process of bulk polymerization is simple, the following steps are tedious and time-consuming. In addition, the resulting particles are polydisperse both in shape and size, which limits column efficiency and resolution. Matsui et al. [56] firstly employed the in-situ polymerization technique for preparation of the molecularly imprinted monoliths. A template compound and initiator are dissolved in a mixture of a functional monomer [methacrylic acid or 2-(trifluoromethyl)-acrylic acid], a cross-linker (ethylene glycol dimethacrylate) and porogenic solvents (cyclohexanol and 1-dodecanol), then the mixture is degassed and poured into a stainless steel column. Polymerization is carried out in situ in a water bath. After the polymerization has finished, the template and porogenic solvents are removed by exhaustive washing with methanol–acetic acid. The method is very simple with respect to the conventional protocol and its macroporous structure offers excellent hydrodynamic properties. The capability of the material for molecular recognition in the separations of positional isomers of diaminonaphthalene and phenylalanine anilide enantiomers has been demonstrated [56]. After that, a series of the materials has also

been prepared for recognizing the cinchona alkaloid [57,58], xanthine derivatives [59] and nicotine [60].

2.1.2.5. Preparation conditions affecting the porous properties of monolithic rods

The prepared monolithic rods possess a bimodal pore size distribution consisting of both large micrometer-sized through-pores and much smaller pores in the 10-nm size range. The large pores allow liquid to flow through these materials under low pressures even at high flow-rates, and the small pores are the most substantial contributions to the overall surface area. Although a number of parameters in the preparation of the monolithic rod affect the porous properties, some key variables such as temperature, composition of the porogenic agents and content of the cross-linker are frequently used to tune the average pore size within a broad range spanning at least two orders of magnitude from tens to thousands of nanometers. The effects of these key polymerization conditions on the porous properties of poly(styrene–co-divinylbenzene), poly(glycidyl methacrylate–co-ethylene dimethacrylate) and poly(acrylamide–co-*N*,*N*′-methylenebisacrylamide) monolithic rods have been described in detail [48,61–63]. The effect of polymerization conditions on the porous properties of these monolithic rods is essentially similar. The polymerization temperature, through its effects on the kinetics of polymerization, is a particularly effective means of control, allowing the preparation of macroporous polymers with different pore size distributions from a single composition of the polymerization mixture. As a rule, the higher the polymerization temperature, the smaller the pores, which can readily be explained by considering the number of polymer nuclei formed at different temperatures at constant initiator concentration as well as the rate of their formation. The choice and composition of porogenic solvents is another tool that may be used to control porous properties without changing the chemical composition of the final polymer. In general, larger pores are obtained if poorer solvents are used because of an earlier onset of polymer phase separation. In addition to the high aliphatic alcohols usually used as the poor solvents for forming the large pores, linear polymers [48], supercritical carbon dioxide [64] and solid granules [65] can also be used to obtain the larger pores. In contrast, increasing the proportion of the cross-linker in the monomer mixture affects the chemical composition of the final monoliths, which also leads to a decrease in average pore size as a result of early formation of highly cross-linked globules with a reduced tendency to coalesce. In addition to the divinyl cross-linking monomers described above, the trivinyl monomer trimethylolpropane trimethacrylate (TRIM) is frequently used to provide a higher degree of cross-linking [66]. The polymerization time affects the conversion of monomers and the use of shorter reaction times than required for complete monomer conversion leads to porous objects with larger flow through channels [62]. The effect of the concentration of initiator on the porous properties of the monoliths has also been reported by Xie et al. [48]. A higher concentration of initiator leads to a higher number of radicals being formed in the system and translates into a larger number of nuclei that result in the smaller pores.

2.2. Preparation of monolithic columns in capillary electrochromatography

Various CEC columns with monolithic stationary phases have been reported. Generally, they can be classified into porous polymer monolithic columns and particle-fixed monolithic columns. The preparation of porous polymer monolithic columns is similar to that in HPLC as addressed previously. Monomeric precursors are introduced into the capillary and allowed to polymerize in situ, forming a rod-like structure. However, charged groups should be coupled to the surface of polymer monoliths used in CEC to generate EOF since EOF is the driving force for the mobile phase. Accordingly, the composition of the polymerization mixture and the procedure for the preparation may be different from that in HPLC. The polymer monolithic column is prepared in a capillary without frits which typically require the attachment of polymer to the capillary wall, but such an attachment is not required in HPLC. The experience acquired earlier with monolithic columns for HPLC may not be directly transferable to the preparation of capillary columns for CEC. Consequently, we will focus on the difference in the preparation of polymer monolithic columns in CEC

from that in HPLC in this section. The second type of monolithic columns, particle-fixed monolithic columns, is unique in CEC. Several methods were developed to prepare this kind of column by fixing conventional spherical packing materials in a capillary in situ to form a continuous monolith.

2.2.1. Polymer monolithic columns

Both organic and inorganic polymer-based monoliths were prepared for CEC columns. The organic porous polymer columns can be prepared by polymerizing organic monomers in the presence of a porogen. Svec and co-workers [67–69] have published several excellent reviews on the in situ polymerization of synthetic organic polymers as monolithic stationary phases for CEC. Up to now, three types of polymers, i.e. polyacrylamides [70–80], poly(methacrylate esters) [81–89] and polystyrenes [90,91], have been used for the preparation of organic monolithic CEC columns. Whatever type of polymer is adopted, the procedure for the preparation is similar. Fig. 1 illustrates schematics for the preparation of organic monolithic capillary column. The preparation procedure consists of three major steps: pretreatment of capillary inner surface, preparation of porous monolithic matrix in capillary and functionalization of the matrix. The preparation of the matrix and coupling of chromatographic ligands may be similar to that in HPLC. However, it can be seen from Fig. 1 that there are many differences between the preparation of monoliths in CEC columns and that in HPLC columns. The pretreatment of the empty capillary, partial filling of the gel in the capillary and coupling of charged groups to the matrix are necessary for preparation of CEC monoliths, but they are not required in HPLC.

In HPLC, the monolith is blocked by two frits at the two ends, therefore, chemical attachment of polymer to the inner wall of the column is not necessary. However, the monolith polymerized in capillary column are of no frits, chemical attachment is required in order to prevent the movement of monolith during the wash and separation procedure. Only Svec and co-workers [83,85–88,92] reported preparation of CEC columns directly in untreated capillary columns; in order to obtain good physical stability, most columns are polymerized in silanized columns. The silanization of the inner wall of the capillary is similar to that in preparation of coating a capillary for capillary zone electrophoresis [7,90]. The capillary column was firstly washed with strong basic solution so that the siloxane groups at the inner surface of raw fused-silica capillaries is hydrolyzed, and thereby increase the density of the silanol groups serving as anchors for the subsequent silanization. Then the capillary column is filled with a bifunctional reagent solution, typically γ-methacryloyloxypropyltrimethoxysilane in acetone, and allowed to be reacted for a period of time. After such a treatment, Si–O–Si–C bonds are formed between the capillary wall and the reactive methacryloyl groups, which are available for subsequent attachment of the monolith to the wall during the polymerization reaction.

The polymerization mixture for the preparation of a monolithic matrix is similar to that in HPLC. However, the filling of the mixture into the column is different from that in HPLC. In HPLC, the column is fully filled with the polymerization mixture and the synthesized monolith occupies all the space of the column. In CEC, on-column detection is conducted, which require transparency of the section of the detection window. Only a polyacrylamide gel with low cross-linker ratio is transparent in UV light, and the detection can be carried out on gel [70,71]. Accordingly, the entire capillary column can be filled with the polymerization mixture. But other polymers are all opaque; in order to perform on-column detection, the detection window section should be without the polymer. This is simply accomplished by filling the monomer solution into the capillary and stopping the flow after reaching a certain length. After polymerization, part of the capillary column is filled with the monolith and part of the capillary is just empty. The detection window is made in the open tubular section at the end of the monolith and detection in free solution is achieved. An alternative way to prepare a detection window in a capillary fully filled with opaque polymer is reported by Ericson et al. [81]. The window was made by burning off a 1–2 mm section of the polyimide coating with the aid of an electrically heated tungsten wire while pumping cold water continuously through the column. The heat from the wire breaks covalent bonds at the inner wall and creates a small water-filled gap. The detection window prepared in this

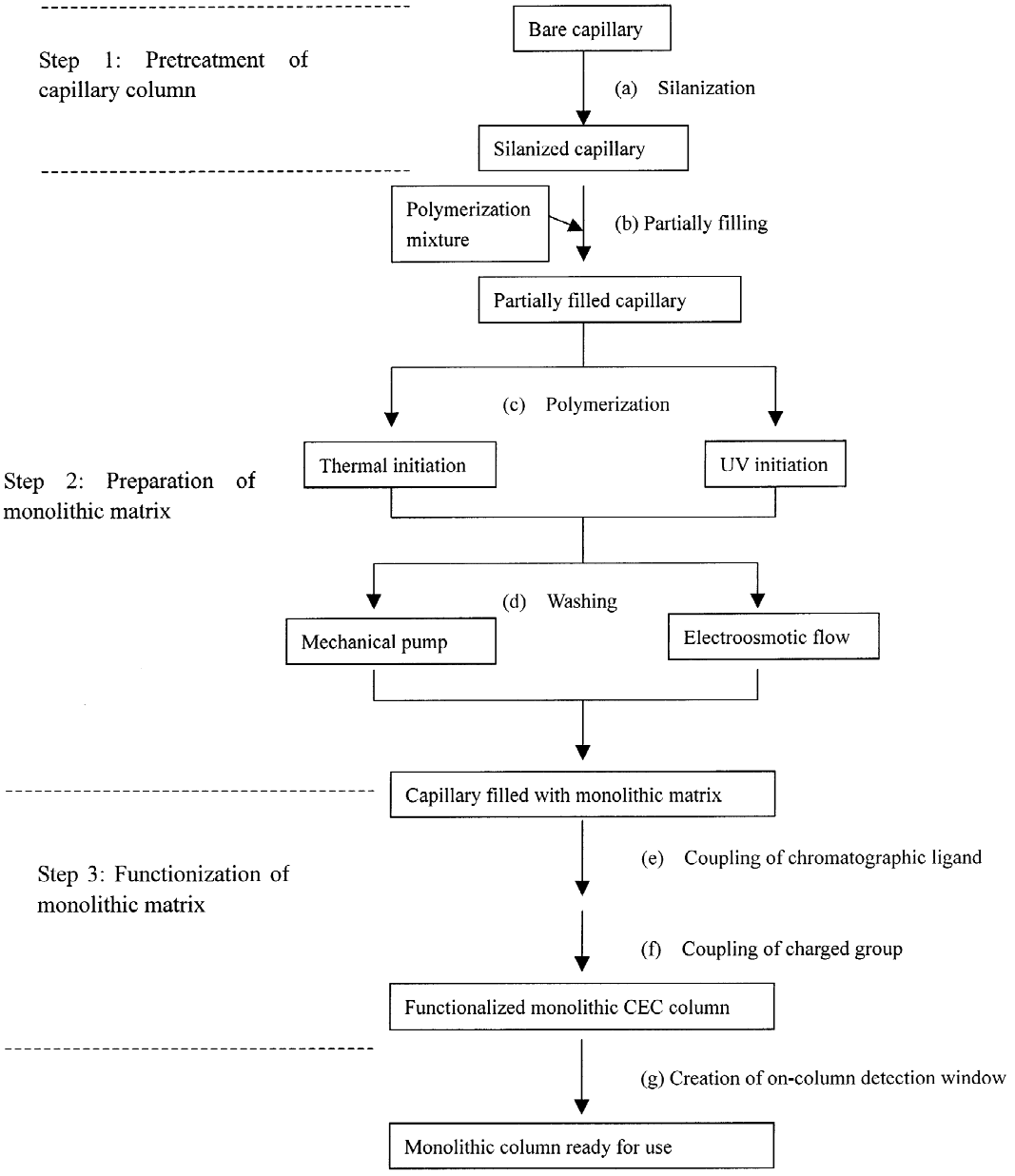

Fig. 1. Schematics for the preparation of monolithic CEC column.

way was found to have about the same UV transmission as a window in empty capillary.

After the polymerization, the porogenic solvent and other agents in the capillary need to be washed out. The washing procedure is typically achieved by a mechanical pump as in the preparation of HPLC monolithic columns. The disadvantage for washing columns by pressure is that some soft gels may be destroyed. Preparation of a long column in this way may also be difficult because ultra high pressure is required. The strong back pressure generated for washing a long column may also destroy even some

rigid gels. Therefore, preparation of a monolithic column with soft gel or long length in this way is not desirable. In fact, the washing procedure can also be carried out by electroosmotic flow in CEC [70–72,75,77–79]. This can be simply accomplished by placing the two ends of the capillary into buffer solution after polymerization and applying voltage for a period of time. The residue monomers, uncross-linked polymers, initiator and catalyst will be removed by EOF as well as electrophoresis. After the current stabilized, the prepared capillary was ready for use. The preparation of CEC monolithic column without pressure and driven of mobile phase by EOF render some very loose and swollen soft gel could be used in CEC, while this is discouraged in HPLC.

After the monolithic matrix is prepared, the surface of the matrix need to be functionalized to convert it into a surface suitable to be used as a stationary phase in CEC. As in HPLC, a chromatographic functional groups such as hydrophobic groups in reversed-phase chromatography should be coupled to the matrix. In addition, charged groups should also be coupled to the matrix in order to generate EOF in CEC. Ericson et al. [81] have presented a way with three steps to prepare a monolithic CEC column. A rigid matrix was first prepared, then dextran sulfate was immobilized, and finally C_{18} was covalent linked to the continuous bed. Some authors also reported the preparation of CEC monolithic columns with two steps [1,84]. Preparation of column with multiple steps is time-consuming and also difficult to control over the entire process. In fact, the preparation and functionization of the matrix can typically be combined in a single step. The polymerization solution is prepared by mixing the two functional monomers, one for generation of the chromatographic surface and another for coupling of charged groups, with the matrix monomers, then this solution is filled into the capillary and allowed to be polymerized in situ. After polymerization, a monolith with chromatographic surface and charged groups will be formed. For example, a monolithic column used in reversed-phase CEC was prepared in a single step by co-polymerization of acrylamide, *N*,*N*′-methylenebisacrylamide, lauryl acrylate and vinylsulfonic acid [74]. After polymerization, the incorporated lauryl groups will retain the solutes by hydrophobic interaction and the incorporated sulfonic acid groups will generate a strong EOF. The preparation of monolithic columns for chiral separations can be achieved in the same way. For example, a monomer with a chiral selective group, (+)-tetraallyl 18-crown-6 carboxylate, was copolymerized with acrylamide, *N*,*N*′-methylenebisacrylamide and 2-acrylamido-2-methylpropane sulfonic acid, and the prepared monolithic column was applied to the enantiomer separation of some primary amino compounds [75]. The polymerization solution can either be prepared by water or organic solvent. Perhaps the greatest limitation for the aqueous-based polymerization is the poor solubility of a number of polymerizable monomers in water, i.e. the nonpolar monomers required to achieve the necessary hydrophobicity for a reversed-phase CEC bed are typically insoluble in water. These hydrophobic monomers can be soluble in the aqueous monomer mixture by addition of a surfactant. For example, stearyl methacrylate used for the introduction of hydrophobic ligands was rendered water-soluble by incorporation into Triton X-100 micelles [82]. In contrast to the fixed solubilizing properties of water, the wealth of organic solvents possessing polarities ranging from highly nonpolar to extremely polar allows the formulation of mixtures with solvating capabilities that may be tailored over a very broad range. Therefore, many organic monolithic columns were prepared from polymerization solution with organic solvents.

The inorganic porous polymer columns, typically silica-based monolithic columns, are prepared using a sol–gel process. The procedure is similar to that in the preparation of organic monolithic column. The pretreatment of the silica capillary is also necessary for the sake of effective attachment of silica skeletons to the wall. The capillary could be treated with sodium hydroxide [93], hydrochloric acid [94] or water [95] at a relatively high temperature. After pretreatment, sol–gel solution is introduced into the capillary column, and a three-dimensional network will be created when hydrolysis and polycondensation reactions of the solution are completed. The prepared porous monolithic matrix was then derivatized to a reversed-phase by on-column reaction with silane reagent. The coupling of other charged groups onto the matrix is not always necessary since a negatively charged surface will be generated due to

the ionization of silanol groups on the silica monolith, which results in a cathodic EOF. For example, Tanaka and co-workers [93,96,97] have fabricated monolithic silica columns in this way. The macroporous silica gel network was prepared by in situ hydrolysis and polymerization of tetramethoxysilane and poly(ethylene oxide) inside a 100 μm I.D. capillary column, then allowed to react overnight. The monolithic silica thus formed was washed with water and then treated with aqueous ammonium hydroxide solution followed by a wash with ethanol. The column was dried in an oven for 24 h at 330 °C and then reacted with octadecyldimethyl-*N*,*N*-diethylaminosilane to form reversed-phase chromatographic surface. Suzuki et al. [98] and Fujimoto [94] have also followed a similar procedure to fabricate monolithic columns for CEC. Hayes and Malik [95] have presented a sol–gel approach to prepare silica monolith with surface-bonded ligands for chromatographic interaction in a single step, thus reducing the time and labor associated with column fabrication. In the presented approach, *N*-octadecyldimethyl-[3(trimethoxysilyl)propyl]ammonium chloride (C_{18}-TMS) and tetramethoxysilane (TMOS) were used as the two sol–gel active precursors. The chemical structure of C_{18}-TMS is unique and specifically suitable to prepare CEC monolithic column. The structural design of this precursor contains three important features: (1) the octadecyl moiety capable of providing chromatographic interactions with the analytes. (2) Three methoxy groups attached to the silicon atom that can undergo hydrolysis, followed by condensation, thereby facilitating the in situ creation of a chemically bonded monolithic matrix throughout the entire solution-filled inner capillary volume. (3) The positively charged quaternary ammonium moiety that can provide a positive surface charge within the matrix to support the essential EOF in CEC. Separation efficiencies of up to 1.75×10^5 plates/m were achieved on a 50 cm×50 μm I.D. column using polycyclic aromatic hydrocarbons and aromatic aldehydes and ketones as the test solutes.

2.2.2. Particle-fixed monolithic columns

In a conventional HPLC column, the packed bed is typically kept in place by terminating sieves or frits, that are porous to the liquid but too narrow for the particles to move through. In a CEC column the packed bed can also be kept in place by sintered frits, however frits meet many problems. In order to inherit the versatility of very well-developed packing materials and to prepare a CEC column without frits, a new generation of CEC monolithic columns, namely particle-fixed monolithic columns, has been developed. They are prepared by fixing conventional spherical packing materials in a capillary in situ using various methods to form a monolithic structure. This type of monolithic column is unique in CEC. According to the different preparation methods, they can be classified into three categories: (1) particle-sintered monolithic column [99–101], (2) particle-entrapped monolithic column [102–110], and (3) particle-loaded monolithic column [19,111–113].

Particle-sintered monolithic columns are prepared by immobilization of microparticulate silica-based packing materials by a thermal treatment. As described by Asiaie et al. [99], ODS silica particles were first packed into a fused-silica capillary using a solvent slurry method, then the packed capillary was rinsed with 0.1 *M* $NaHCO_3$ followed by water and acetone. Then, the column was heated at 120 °C, followed by 360 °C for a period of time. After sintering, the monolithic packing was reoctadecylated in situ with dimethyloctadecylchlorosilane. The prepared column was mechanically strong and had efficiency similar to the packed columns with the same particles. Due to the harsh experimental conditions used in the sintering process, the stationary phase was destroyed and, thus, post deactivation and functionalization of the sintered bed is necessary. Wistuba and Schurig [101] have reported the preparation of a chiral monolithic stationary phase by packing a capillary with bare porous silica and sintering the silica bed at high temperature, then the resulting silica monolith was polymer-coated with Chirasil-Dex, a permethylated β-cyclodextrin covalently linked via an octamethylene spacer to dimethylpolysiloxane. The multistep procedure used to fabricate the column is a drawback of this approach. Adam et al. [100] have presented a method to prepare particle sintered monolithic columns in a single step by a hydrothermal treatment using water for the immobilization process. By carefully controlling the immobilization

temperature and other parameters, the column can be prepared without damage to the surface of the reversed-phase silica, and thereby it can be used directly for separation after sintering. Under optimal conditions, no remarkable difference in the retention time and the efficiency was observed before and after the immobilization.

Particle-entrapped monolithic columns were prepared by introducing the entrapping solution after the column had been packed. The method developed by Chirica and Remcho [102] makes use of silicate sol solutions to fill the pressure packed capillaries with subsequent heating. After the capillary was packed with packing materials, it was filled with silicate sol solutions and subsequently heated for several days. Then, the column was cured by flushing it with 0.1 *M* NH_4OH followed by drying in an oven. Both silica-based reversed-phase packing material and molecular imprinted polymeric packing were entrapped by silicate. The reported efficiency of this column is similar to that of a packed column. But the retentive characteristics were significantly reduced due to a combination of masking of the stationary phase with the entrapping silicate and hydrolysis of the stationary phase during the NH_4OH rinse. The prepared columns were silanol groups active, which may lead to peak tailing. An additional drawback of the preparation procedures is that they are time consuming.

Lee's group [103–106] reported the development of sol–gel bonded monolithic columns for CEC in which packing materials were first packed into a fused-silica capillary using a CO_2 supercritical packing technique. Then the packed column was filled with a specially prepared dilute sol solution formed by hydrolysis and polycondensation of tetramethoxysilane and ethyltrimethoxylsilane precursors. Then the column was dried using supercritical CO_2 at high temperature after the column was gelled and aged. The column inertness was improved significantly using an inert sol–gel bonding matrix and supercritical CO_2 drying. The sol–gel bonded monolithic columns exhibited almost the same properties as conventional packed columns, but without the need for end-frits.

However, the surface of the silica-based entrapment matrices renders separation media prone to nonspecific adsorptive interactions. Chirica and Remcho [107] have presented the immobilization of reversed-phase sorbents within the walls of fused-silica capillary tubes using an organic-based entrapment matrix. A mixture of porogenic solvents and methacrylate-based monomers is pumped through a packed column to provide, following a polymerization step, an organic matrix capable of holding the sorbent particles in place, thus rendering the end frits unnecessary. The new columns demonstrated excellent chromatographic performance with reduced plate height varied from 1.1 to 1.5 in CEC, while minimizing secondary interactions encountered when silica-based entrapment matrices are employed. In addition to delivering mechanically robust columns, the methacrylate matrix provides a mechanism for fine tuning of the EOF velocity when 2-acrylamido-2-methyl-1-propanesulfonic acid is incorporated into the polymerization mixture.

The last type of particle-fixed monolithic column is the particle-loaded one. In fact, particle-loaded monolithic columns belong to a type of particle-entrapped columns, however preparation of these columns combines packing the stationary phase particles and entrapping these particles in a single step. In this approach [19,111–113], a sol–gel solution, typically a mixture containing alkoxysilanes, ethanol and hydrochloric acid, is prepared and the particles are added to form a suspension containing the particles. A capillary is then filled with the suspension. The sol–gel matrix embeds the particles after drying, and prevents them from exiting the column during separation. However, the reported separation efficiencies of columns prepared by this approach are not as high as those of traditionally packed columns. The reason may be the presence of inhomogeneities in their columns and some inaccessibility of analytes to the particles due to steric blocking by the sol–gel elements.

Besides being used directly as the stationary phase in CEC, monolithic media can also be fabricated as the frits in CEC columns packed with particulates. Recently, Zare and co-workers [114,115] reported two types of monolithic frits by photopolymerization based on the preparation methods described above. The preparation of CEC column frits is simple, rapid and reproducible and bubble formation was not observed to occur during any of the chromatographic runs.

3. Characterization

Several methods are usually employed for measuring the porous properties of the monoliths. The porosity data and the pore size distribution profiles of the monoliths in dry state are usually obtained by means of mercury intrusion porosimetry and the range of pore size determined is from 10 nm to 150 μm. This technique is well suited for the determination of large pores, but its accuracy for the measurement of smaller pores is limited due to the compressibility of the polymeric matrix itself. Therefore, inverse size-exclusion chromatography (ISEC) often used for the determination of pores smaller than about 50 nm. The concept of ISEC is based on the measurement of pore volumes that are accessible to polymer standards of well-defined molecular sizes [116]. In addition, the specific surface area of monoliths can be obtained from nitrogen adsorption/desorption isotherms.

For practical uses, the back pressure of a column should be as low as possible. The first concern of a monolithic column is the permeability to mobile phase, which depends fully on the macropores of the medium. Many applications of porous material are usually dependent on its surface area. To obtain a large surface area, a large number of micropores, with diameters smaller than 2 nm, and mesopores ranging from 2 to 50 nm should be incorporated into the polymer. And large macropores with diameters over 50 nm make only an insignificant contribution to the overall surface area. Therefore, a balance must be found between the requirements of low flow resistance and large surface area. The pore size distributions of the molded monoliths can be carefully controlled through the optimization of the polymerization conditions as described above. An example of pore size distribution of a molded monolith is shown in Fig. 2 [36]. According to mercury intrusion porosimetry, pores with diameters smaller than 100 μm and larger than 5 μm take about 10 and 15% of the total pore volume, respectively. The pores with diameter in the range of 100–5000 nm take more than 70% of the total pore volume [117].

The morphology of the monolithic rod is closely related to porous structure, and is also a direct consequence of the polymerization conditions. The scanning electron micrograph shown in Fig. 3 reveals the details of the structure of a molded monolith of poly(glycidyl methacrylate–co-ethylene dimethacrylate). The polymer forms a dense network of polymer bundles that are highly convoluted. ''Molded'' monoliths provide broad channels for liquid flowing through, so very low flow resistance could be achieved. The back pressure of the continuous rod with I.D. of 4.0 mm and length of 5 cm is only about 6.5 MPa when the flow-rate reached 9.0 ml/min. In addition, the dependence of the back pressure on flow-rate is directly proportional to the flow-rate of the mobile phase in the range from 1.0 to 9.0 ml/min [117].

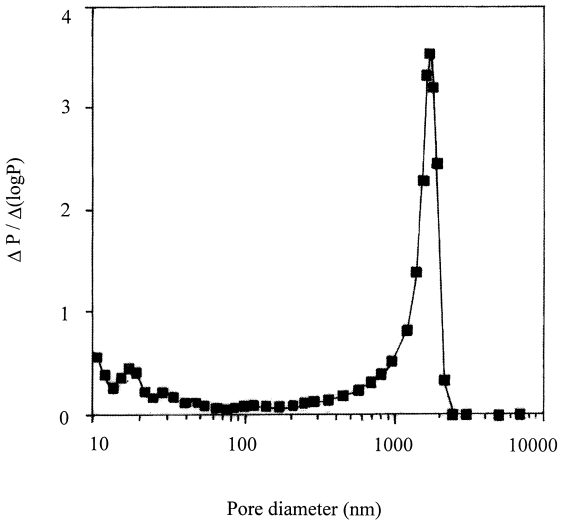

Fig. 2. Pore size distribution curve of the modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) rod measured by mercury intrusion porosimetry. Reprinted with permission from Ref. [36].

Conventional porous material has the disadvantage that the mass transfer between the surface and bulk liquid phase is restricted by hindered diffusion through pores. In particular, in the case of macromolecules, the slow rate of diffusional mass transfer limits the overall rate in chromatography. As presented in the Van Deemter equation the efficiency of the HPLC separation deteriorates rapidly as the flow-rate increases (when mass transfer becomes dominant). In contrast to diffusion, for which the concentration gradient is the driving force, convection uses flow to dramatically accelerate the mass transfer of solutes. However, pore sizes for most convention-

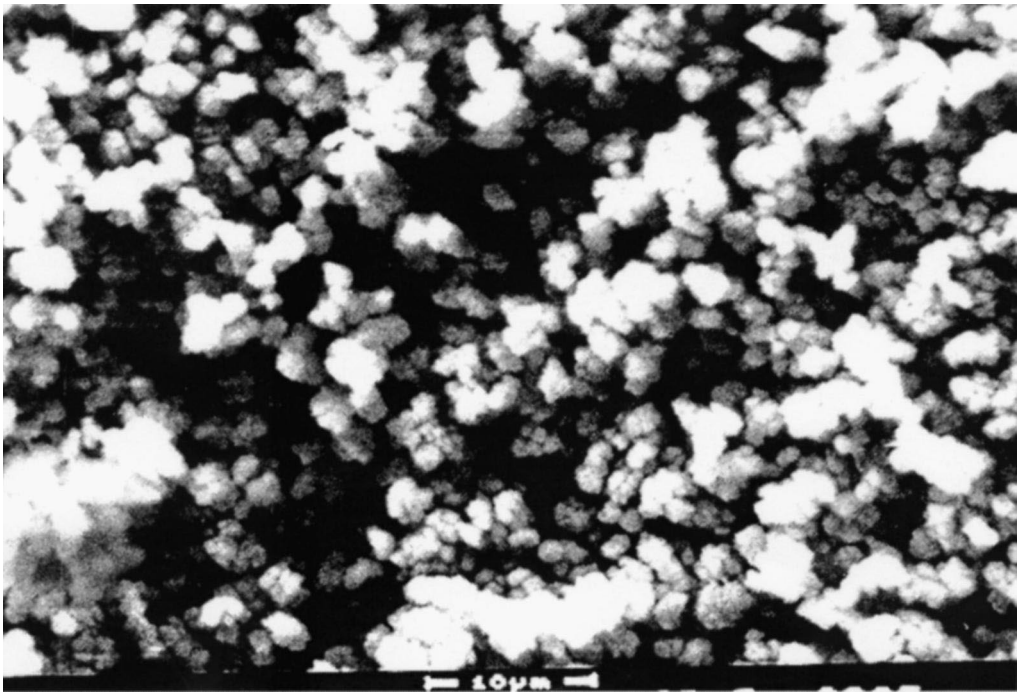

Fig. 3. Scanning electron micrograph of the inner part of the modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) monolith. Reprinted with permission from Ref. [117].

al porous materials are too small to allow convection. A theoretical analysis of conditions affecting the efficiency of a column in the separation of macromolecules with molecular mass exceeding 10^5 revealed that the optimum size of pores in the packing medium is about 1 μm [118]. When this size is reached, the slow diffusion of solutes within the pores does not restrict the separation quality. Perfusion chromatography, which is based on the use of packings with very large pores of up to 1 μm, was used to improve the kinetics of the separation process. Though these macropores allow part of the mobile phase to flow through the beads, the convective flow through the pores accounts for less than 5% of the total [118]. As a result of the convective flow of the solution through the pores of monolithic column, the mass transfer resistance is tremendously reduced. Fig. 4 shows the effect of flow-rate on the efficiency of a poly(styrene–co-divinylbenzene) rod for tetrahydrofuran. The plot of plate height against flow-rate remains relatively flat on a monolithic column with dimension of 50×8 mm I.D. through

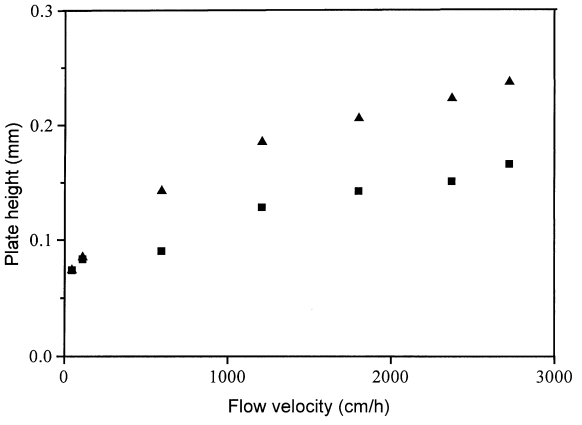

Fig. 4. Effect of flow velocity on efficiency of the continuous poly(styrene–co-divinylbenzene) rod column for benzene (▲) and bradykinin (■). Experimental conditions: column, 50×8 mm I.D.; mobile phases, tetrahydrofuran for benzene and acetonitrile–water (50:50) for bradykinin; temperature, 30 °C. Reprinted with permission from Ref. [122].

the linear velocity range of 200–1000 cm/h. Frontal analysis, which provides the breakthrough curve, serves as a useful tool for the evaluation of mass transport kinetics. In an ideal case, the breakthrough curve, that shows the solute concentration at the column outlet versus the total volume passed through the column, is almost vertical. If the saturation rate of the separation medium with the solute is slower than the flow-rate, the curve is less steep. A breakthrough curve of chicken egg albumin on the amine-modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) monolithic column was measured, and the obtained breakthrough curve is very sharp, which confirms the fast mass transport kinetics of the monolith [36].

Because of the multiple steps involved in the preparation of a conventional chromatographic column, some batch-to-batch variation in column performance is expected. Continuous rods are prepared in situ within the confines of a chromatographic column or a capillary tube, which leads to very high column reproducibility. This is a significant advantage as the efficiency of packed bead columns is known to be affected by packing conditions, including the skills of the operator, as well as other variables [119]. Liao [120] compared the performance of 23 different batches of the UNO Q column. The retention time of four solutes including adenosine, AMP, ADP, and ATP were determined under the same gradient conditions, and the results showed that the coefficient of variation of the tested solutes was less than 1.3%.

The physical and chromatographic characteristics of three monoliths including CIM-QA disks (BIA Separation), UNO-Q (Bio-Rad), Sartobind-Q (Sartorius, Göttingen, Germany), and a monodisperse chromatography material, Mono-Q (Pharmacia-Amersham, Uppsala, Sweden) were compared by Iberer et al. [121]. Two objective parameters, the porosity and the shape of the breakthrough curve at different chromatographic linear velocities, were selected as the evaluation of above four media. The porosities are approximately 0.7 for the UNO-Q column, 0.5 for the CIM-QA disk, and 0.4 for the Sartobind column, which is significantly higher than that measured with conventional chromatographic columns—usually between 0.2 and 0.3. Because axial dispersion caused by restricted diffusion into pores is excluded, the breakthrough curves for the CIM-QA disks and UNO-Q columns at different velocities exhibit almost identical shape and position when normalized in respect to the elution volume. Additional band broadening with increasing velocity was observed on Sartobind Q because of the high extra-column volume with increased mixing, lateral diffusion in the grafted surface, and imperfect stacking of the membrane sheets. The shape of the breakthrough curve for Mono-Q column changes with velocity and the onset of the breakthrough commences earlier and becomes flatter with higher velocity [121]. Due to the advantages of simple handling and high-speed operation with high efficiency, these monolithic chromatographic adsorbents are very attractive for bioanalytical, biochemical, and biotechnological applications.

4. Applications

4.1. LC applications

4.1.1. Reversed-phase chromatography

Svec and co-workers [26,122,123] prepared poly(styrene–co-divinylbenzene) continuous rod columns for the reversed-phase separation of proteins, alkylbenzenes, and peptides. The isocratic and gradient separation of alkylbenzenes on continuous poly(styrene–co-divinylbenzene) rod column exhibits about 13 500 plates/m at a low flow velocity, which is much lower than that of a typical column packed with small beads. The interactions between the hydrophobic surface of the molded poly(styrene–co-divinylbenzene) monolith and alkylbenzenes do not differ from those observed with beads under similar chromatographic conditions. The average separation factor of alkylbenzene homologues $\alpha(CH_2)=1.42$, which reflects the contribution of one methylene group to the overall retention of a solute, is close to that reported in the literature for polystyrene-based beads [124,125].

Peptides are very important compounds produced by biotechnology processes. Chromatography is a valuable tool for both in-process control and downstream processing. Five peptides, bradykinine, leucine enkephaline, methionine enkephaline, physalaemin, and substance P were used for testing

the separation ability of the monolithic column [123]. It was observed that the silica-based column affords the baseline separation of all five peptides in about 3 min, while both the monolithic and Hamilton PRP-1 columns do not separate the enkephalines completely. However, a very good separation of those five peptides was achieved in less than 50 s on the monolithic column at a flow-rate of 5 ml/min (Fig. 5). This is not possible with the C_8 silica column since the back pressure at a flow-rate of 5 ml/min would exceed the pressure limits [123].

Gradient elution with a monolithic column has been applied to the separation of ribonuclease, cytochrome *c*, bovine serum albumin, carbonic anhydrase and chicken egg albumin and the comparative

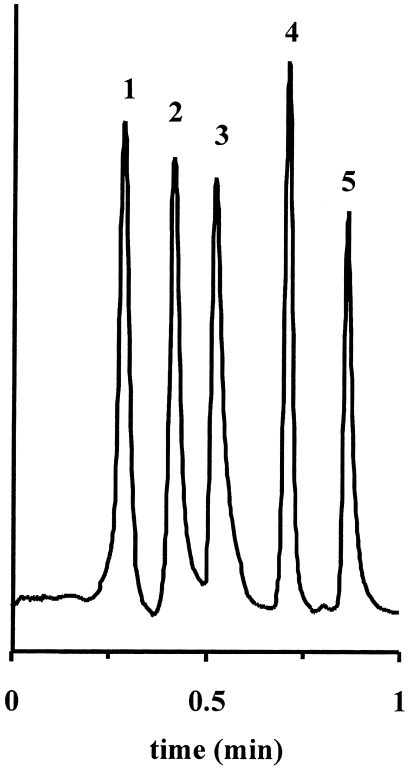

Fig. 5. Rapid separation of peptides on a poly(styrene–co-divinylbenzene) monolithic column. Experimental conditions: column, 50×4.6 mm I.D.; mobile phases, gradient elution from 15 to 38% acetonitrile in 0.15% aqueous trifluoroacetic acid solution in 1.5 min; flow-rate, 5 ml/min; detection, UV 214 nm. Peaks: 1=bradykinin; 2=enkephalin; 3=methionine enkephalin; 4=physalaemin; 5=substance P. Reprinted with permission from Ref. [123].

separations on the columns packed with Poros R2H and Hamilton PRP-3 beads were carried out, and the results obtained are shown in Fig. 6. It can be seen that the monolithic column and Porous R2H beads packed column have similar separation ability, which is much higher than that of the column packed with Hamilton PRP-3 beads [123].

An early study of the separation of styrene oligomers by HPLC on reversed-phase octadecyl silica columns under gradient elution showed that the retention depended on both the composition of the mobile phase and the number of repeat units in the oligomer [126]. Smaller oligomers are eluted prior to larger ones, quite unlike SEC for which the large molecules elute first. Svec and co-workers [27,127] used molded poly(styrene–co-divinylbenzene) rod columns in a gradient elution HPLC mode for the separation of styrene oligomers and polymers. A comparable separation of a commercial sample of polystyrene oligomers with average molecular mass of 630 was achieved in a high-performance SEC column and the molded column. Both of the chromatograms exhibit a number of peaks that can be assigned to the individual styrene oligomers and these two chromatograms are almost mirror images [27]. The separation of a mixture of narrow polystyrene standards with molecular masses ranging from 519 to 2.95×10^6 in the rod column was also achieved. All eight standards are well separated in a 50-mm-long molded rod column at different flow-rates. The molecular mass distribution profiles of a polystyrene sample obtained by SEC and molded rod column were determined and the curves matched quite well. Molded rod column allows use of high flow-rates and higher sample loads, which makes it a valuable and fast alternative to SEC. Recently, Janco et al. [128,129] also reported excellent results for separation of polystyrenes, poly(methyl methacrylates), poly(vinyl acetates), and polybutadienes on a poly(styrene–co-divinylbenzene) monolithic column by means of precipitation/redissolution.

4.1.2. Ion-exchange chromatography

Ion-exchange chromatography (IEC) is probably the most widely used chromatographic method for the separation of proteins. Due to the mild elution conditions used in ion-exchange chromatography, higher yields of structurally and functionally intact

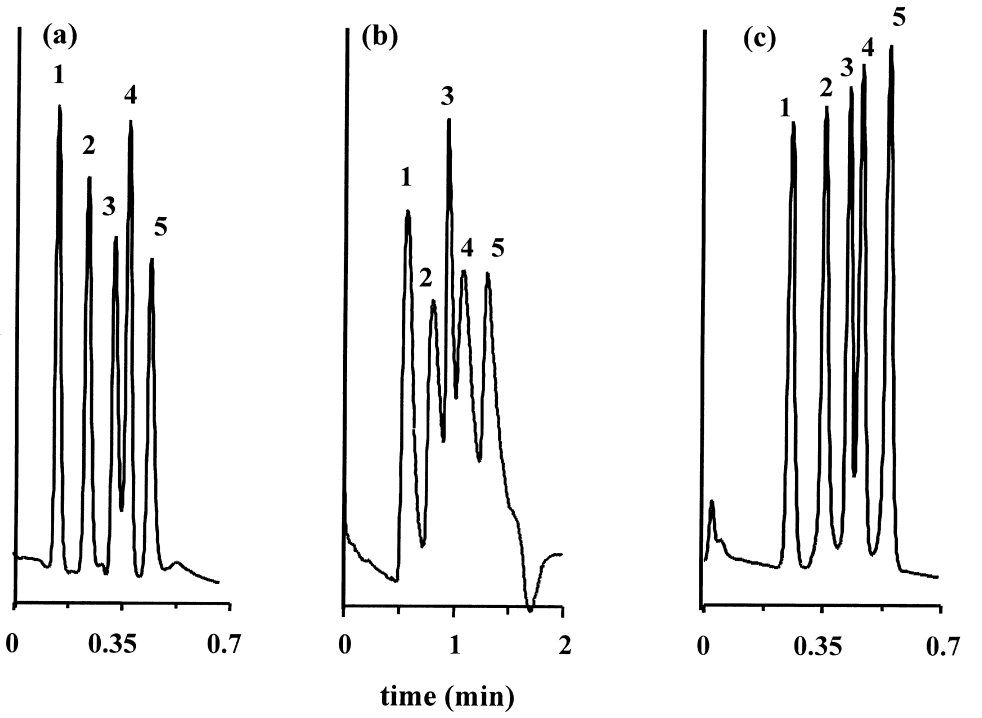

Fig. 6. Separation of proteins on different columns. Experimental conditions: (a) monolithic column based on poly(styrene–co-divinylbenzene) (50×4.6 mm I.D.), (b) Hamilton PRP-3 (10 μm, 150×4.6 mm I.D.); (c) Porous R2H (10 μm, 50×4.6 mm I.D.). Mobile phase, gradient elution from 30 to 70% acetonitrile in 0.15% aqueous trifluoroacetic acid solution in 0.5 min for columns (a) and (c) and that from 20 to 60% acetonitrile in 1.5 min. Peaks: 1=ribonuclease; 2=cytochrome *c*; 3=bovine serum albumin; 4=carbonic anhydrase; 5=chicken egg albumin. Detection: UV 280 nm. Reprinted with permission from Ref. [123].

proteins could be obtained compared with other chromatographic techniques. The epoxide groups of the molded poly(glycidyl methacrylate–co-ethylene dimethacrylate) readily react with many compounds to form ion exchangers. The amine-modified monolithic column prepared by Svec and Fréchet [36] was used even for the preparative-scale separation of proteins [37]. The reaction with diethylamine affords the rod columns ionizable functionalities required for the ion-exchange chromatographic mode. The loadings cover more than two orders of magnitude from 0.33 to 62.5 mg while the retention times are almost identical. A protein mixture consisting of lysozyme, soybean trypsin inhibitor and conalbumin was separated on the ion-exchange column. All of the proteins are baseline separated and the symmetry of the peaks is very good. Sykora et al. [130] also successfully separated the oligonucleotides using this type of monolithic column. The reaction with iminodiacetic acid (IDA) and chloroacetic acid provides the molded poly(glycidyl methacrylate–co-ethylene dimethacrylate) rod columns carboxymethyl groups required for cation-exchange chromatography [38,39]. Four proteins consisting of bovine serum albumin (BSA) (isoelectric point, p*I* 4.98), α-chymotrypsinogen A (p*I* 9.5), cytochrome *c* (p*I* 10.6), and lysozyme (p*I* 11.0) were chromatographed on the naked IDA column (as shown in Fig. 7). Good separation of these four proteins was achieved within 10 min. The naked IDA continuous rod column was also used for the separation of lysozyme from egg white in a single step process [39]. Grafting can also be used to provide the ion-exchange groups for the monolithic columns. A monolithic column grafted with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) chains on poly(glycidyl methacrylate–co-ethylene dimethacrylate) had been used for the fast ion-exchange chromatography of proteins [40]. Three

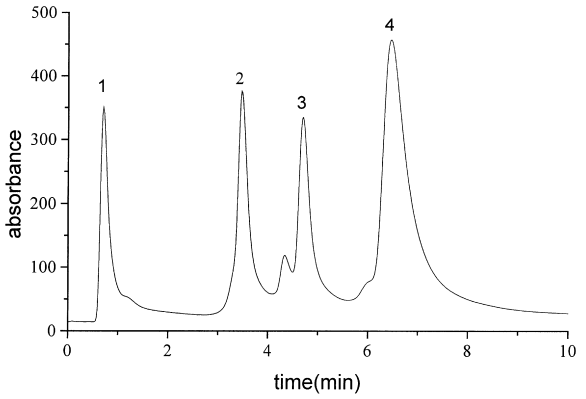

Fig. 7. Separation of model proteins on a naked IDA monolithic column. Experimental conditions: column, 50×4 mm I.D. with monolithic rod of poly(glycidyl methacrylate–co-ethylene dimethacrylate) modified by IDA; mobile phase, gradient elution from 0 to 0.5 mol/l NaCl in 20 mmol/l phosphate buffer (pH 7.0) in 6 min; flow-rate, 1.0 ml/min; UV detection at 280 nm. Peaks: 1=bovine serum albumin; 2=trypsin; 3=cytochrome *c*; 4=lysozyme. Reprinted with permission from Ref. [39].

proteins were successfully separated in less than 1.5 min.

One of the major perceived limitations of methacrylate-based separation media is the limited stability of their ester bonds to the alkaline conditions. To overcome this drawback, molded poly [4-(chloromethyl)styrene–co-divinylbenzene] continuous rod columns were prepared [30] and a weak cation-exchange column was obtained after a two-step modification process involving reaction with ethylenediamine, then with chloroacetic acid. Good separation of five standard proteins was achieved on this monolithic column in a salt gradient elution. And the hydrophilicity of the rod surface was greatly increased after the chemical modification.

In-situ modification of the rod column is time-consuming and the amount of functional groups attached on the rod is limited. Li et al. [131] prepared a microcolumn for cation-exchange chromatography simply by polymerization of an aqueous solution of appropriate monomers, including the desired ligand, directly in a fused-silica tube. To avoid the ''wall effect'', the inner wall of the fused-silica was activated with γ-methacryloyloxypropyltrimethoxysilane. Methoxy groups react with silanol groups at the surface of the tube wall and free methacryloyl groups were used for subsequent attachment of the continuous bed to the wall of the tube by polymerization. Baseline separation of four standard proteins (Mh, myoglobin from horse; Mw, myoglobin from whale; cytochrome *c*; lysozyme) was achieved on a 100×0.025 mm I.D. column. A macroporous poly(glycidyl methacrylate–divinylbenzene–triallyisocyanurate) rod was also recently prepared for direct use in anion-exchange chromatography [132].

4.1.3. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) complements the other chromatographic modes such as ion-exchange, reversed-phase, affinity, and size-exclusion chromatography for separation of proteins. Zeng et al. [46] prepared a continuous HIC column using piperazine diacrylamide, methacrylamide, and isopropylacrylamide as monomers in a single step. The retention of proteins depends both on the hydrophobicity of the protein surfaces, and the density and type of hydrophobic ligands attached to the surface of the medium. While the hydrophobicity of proteins is an intrinsic property that cannot be changed, the polarity of the separation medium can be adjusted during its preparation. In their study, the hydrophobicity of the interacting surface is controlled by the percentage of isopropyl groups in the polymerization mixture. Xie et al. [47] prepared a macroporous poly(acrylamide–co-butyl methacrylate–co-*N*,*N*′-methylenebisacrylamide) monolithic column for hydrophobic interaction chromatography of proteins. Fig. 8 shows a separation of five proteins on a 10% butyl methacrylate monolithic column within 10 min. The retention time and selectivities are only slightly affected while the loading increased from 0.36 to 5.84 mg and the recoveries always exceed 90%. In addition, a porous poly(glycidyl methacrylate–co-ethylene dimethacrylate) monolith grafted with poly(*N*-isopropylacrylamide–co-methylenebisacrylamide) was also used in hydrophobic interaction chromatography [41]. The separation of proteins can be achieved at constant salt concentration using the hydrophobic–hydrophilic transition of the grafted chains that occurred in response to changes in temperature. Carbonic anhydrase and soybean trypsin inhibitor were successfully separated on the monolithic column.

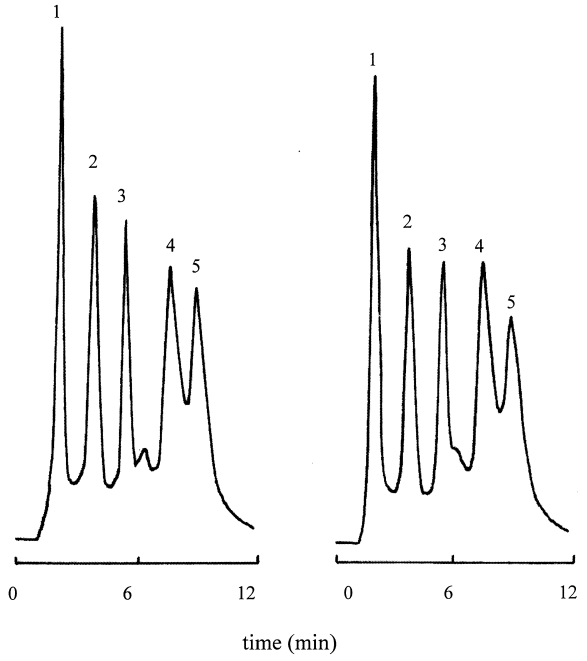

Fig. 8. Separation of proteins by hydrophobic interaction chromatography on a molded monolithic column. Experimental conditions: column, 50×8 mm I.D. with monolithic rod of poly(acrylamide–co-butyl methacrylate–co-*N*,*N*′-methylenebisacrylamide); Mobile phase, gradient elution from 1.5 to 0.1 mol/l ammonium sulfate in 0.01 mol/l sodium phosphate buffer, pH 7.0 in (a) 10 min at a flow-rate of 1.0 ml/min and (b) 3.3 min at a flow-rate of 3.0 ml/min (b). Peaks: 1=cytochrome *c*; 2=ribonuclease; 3=carbonic anhydrase; 4=lysozyme; 5=chymotrypsinogen. Reprinted with permission from Ref. [47].

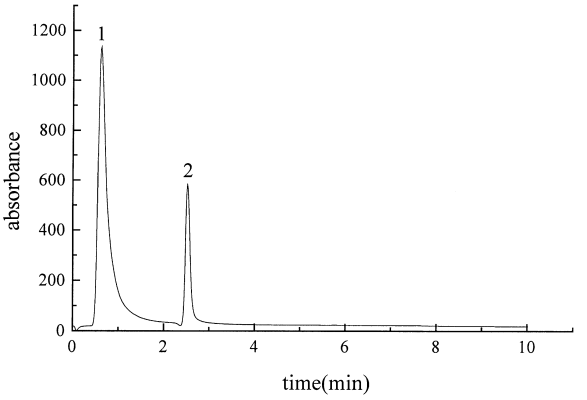

Fig. 9. Chromatographic analysis of human serum on protein A monolithic rod column. Experimental conditions: column, 50×4 mm I.D. with immobilization of protein A on a monolithic rod of poly(glycidyl methacrylate–co-ethylene dimethacrylate); 20 μl of the sample solution was injected with loading buffer, and 2 min after injection of sample solution, the elution buffer was used as the eluent; flow-rate, 1.0 ml/min; UV detection, 280 nm. Peaks: 1=nonretained solutes; 2=human IgG. Reprinted with permission from Ref. [117].

4.1.4. Affinity chromatography

Affinity chromatography is a unique method in separation technology as it is the only technique that permits the purification of biomolecules based on biological functions rather than individual physical or chemical properties. Protein A is a cell wall protein of *Staphylococcus aureus* of molecular mass 42 000 with a strong, specific affinity for the Fc region of immunoglobulins [133,134]. Monolithic columns with immobilization of protein A on the continuous rods of poly(glycidyl methacrylate–co-ethylene dimethacrylate) without or through a spacer arm were prepared for the fast analysis of human immunoglobulin G (IgG) recently [117,120]. The adsorption capacity of human IgG on the latter column is higher than that of the former one because of the steric hindrance occurring between the immobilized ligands and solutes. The protein A column was used for the determination of human IgG in human serum. Fig. 9 shows a typical chromatogram of human serum on the protein A monolithic column. The analysis could be finished within 3 min at a flow-rate of 1.0 ml/min, and the peak width at half height for human IgG is only 0.1 min. However, protein A is expensive and unstable in chromatographic systems. To overcome this drawback, a pseudo-biospecific ligand, histidine, was used as affinity ligand for the purification of IgG [135]. A standard human IgG sample was separated on the histidine monolithic column in Tris–HCl and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffers. Almost all human IgG was retained on the column in the Hepes buffer, whereas, only a small part of human IgG was retained in the Tris–HCl buffer [135].

Immobilized metal ion chelating affinity chromatography (IMAC) is a powerful tool for the separation of proteins and peptides at both the analytical and the preparative scale. It takes advantage of the selective interaction between immobilized metal ions and the functional groups of amino acids located at the surface of biomolecules, such as the imidazole

group of histidine, the thiol group of cysteine, and the indolyl group of tryptophan. IDA type adsorbent was prepared by covalent coupling of IDA to the monolithic continuous rods of macroporous poly-(glycidyl methacrylate–co-ethylene dimethacrylate) [39]. The reaction conditions for coupling of IDA group to the rods were optimized. Different metal ions, including Cu^{2+}, Zn^{2+} and Ni^{2+}, were immobilized on the columns and used for the separation of proteins. Fig. 10 shows the separation of mixed proteins on Cu^{2+}–IDA continuous rod columns, respectively. The four proteins were separated within 10 min. The effect of pH of the equilibration buffer on the adsorption capacity of BSA on a Cu^{2+}–IDA rod column was investigated and the optimized pH is 7.5. In addition, dye–ligand affinity chromatography on continuous beds had also been used for chromatographic purification of dehydrogenases from yeast enzyme concentrate by Mohammed et al. [136].

4.1.5. Separation of chiral compounds

So far, chiral stationary phases based on proteins, cyclodextrins, cellulose derivatives and macrocyclic antibiotics are widely used in the separation of chiral compounds by liquid chromatography. These chiral ligands are usually immobilized on silica material. Unfortunately, similar chiral stationary phases based on monolithic rods have not been reported so far. The only example of the separation of chiral compounds is in monolithic molecularly imprinted polymers. Enantiomers of phenylalene anilide were resolved on the monolithic molecularly imprinted polymers with the L-form or D-form as template [56]. The L-form imprinted polymer showed a higher separation factor (1.7) than the D-form (1.4). After that, the same group prepared a cinchona alkaloid molecular imprinted polymer rod with methacrylic acid or 2-(trifluoromethyl)acrylic acid as monomer [58]. A 5.3 stereoseparation factor was obtained with the 2-(trifluoromethyl)acrylic acid monomer [58]. The diastereomers of cinchonine and cinchonidine, quinine and quinidine was successfully separated on a cinchonine imprinted monolithic column by adopting stepwise or linear gradient elution [137] (as shown in Fig. 11), and the peak shape of the later-eluted compound was greatly improved under the gradient elution.

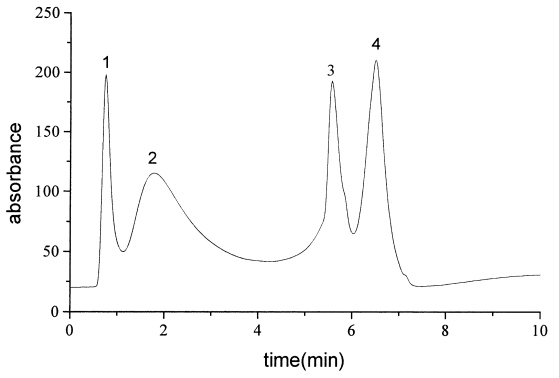

Fig. 10. Separation of proteins by IMAC on Cu^{2+}–IDA monolithic column. Experimental conditions: column, 50×4 mm I.D. with Cu^{2+}–IDA immobilized on a monolithic rod of poly(glycidyl methacrylate–co-ethylene dimethacrylate); gradient elution from 0 to 50% B in 6 min; eluent A, 20 mmol/l phosphate buffer containing 1.0 mol/l NaCl (pH 7.0); eluent B, eluent A containing 100 mmol/l imdazole (pH 7.0). Peaks: 1=trypsin; 2=lysozyme; 3=bovine serum albumin; 4=myoglobin. Reprinted with permission from Ref. [39].

4.1.6. High-throughput bioreactors

The immobilization of enzymes onto solid supports used as bioreactors is beneficial for biocatalytic processes because the supports can be repeatedly used and the products can be easily isolated from the supported enzyme. However, the supports often exhibit significantly lower apparent activities than their soluble native counterparts due to the slow transport of large substrate molecules to the active sites. The monoliths allow total convection of mobile phase through their pores and mass transfer is dramatically increased, which provides an advantageous performance as supports for the immobilization of enzymes. This has been demonstrated in comparative studies by immobilization of trypsin onto the macroporous GMA–EDMA beads and onto the GMA–EDMA monolith [42] and poly(2-vinyl-4,4-dimethylazlactone–co-acrylamide–co-ethylene dimethacrylate) monoliths [138,139]. It was observed that the enzymatic activity of trypsin immobilized on the monoliths was always higher than that of the enzyme immobilized on beads, though a relatively small size (10 μm) of beads had been used to minimize the diffusional path length. The activity of the enzyme immobilized on the monolithic 20×1.0 mm I.D. supports was nearly twice as high as that of the beads at a linear flow-rate of 25 cm/min; at

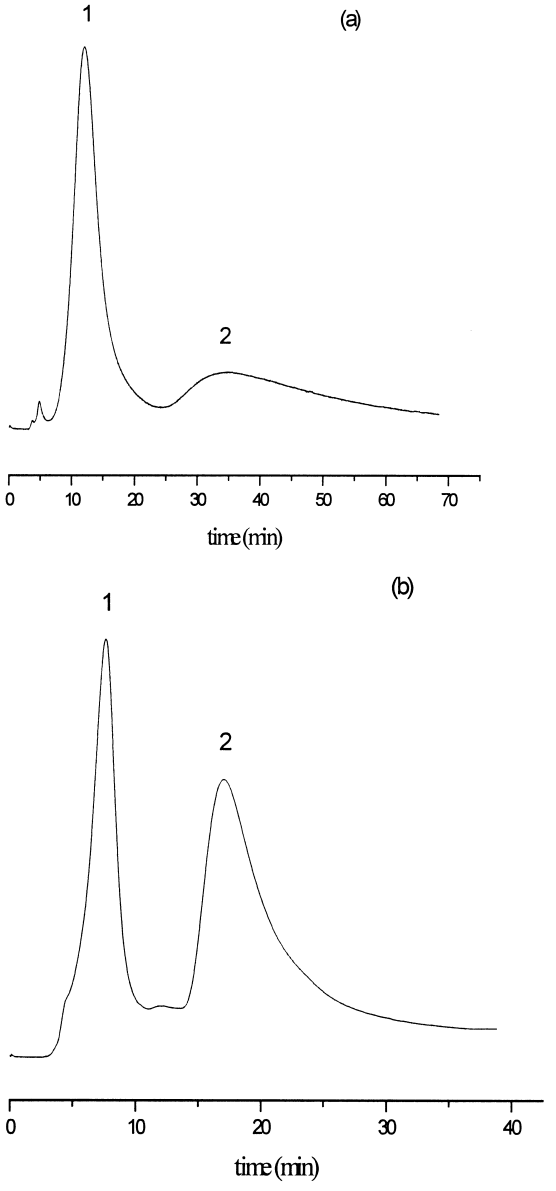

Fig. 11. Chromatogram for separation of cinchonidine and cinchonine on imprinted monolithic column. Experimental conditions: column, 150×4 mm I.D. with a monolithic rod of poly(methacrylic acid–co-ethylene dimethacrylate); (a) mobile phase, acetonitrile–acetic acid (98:2, v/v); flow-rate, 0.5 ml/min; (b) linear gradient elution from acetonitrile–acetic acid (99:1, v/v) to acetonitrile–acetic acid (90:10, v/v) within 15 min and lasted 20 min with acetonitrile–acetic acid (90:10, v/v); flow-rate, 0.5 ml/min. An eluent of acetonitrile–acetic acid (97:3, v/v) was used to equilibrate the column before the gradient elution. Peaks: 1=cinchonidine; 2=cinchonine. Reprinted with permission from Ref. [137].

higher flow-rates (40 cm/min) the monolithic bioreactor could still maintain its activity, while the beads packed bioreactor could not be used due to the steep increases in column back pressure with increasing flow-rate. The low back pressure together with the high activity of immobilized trypsin at high flow-rates contributed to very high throughput. The superior performance of trypsin in the digestion of cytochrome *c* had been demonstrated in studies. The trypsin/cytochrome *c* ratio used for digestion in the column packed with beads was 2250 times higher than that in a monolithic column because the digestion rate is limited by diffusion of the large protein molecules into the pores of beads. Poly(2-vinyl-4,4-dimethylazlactone–co-acrylamide–co-ethylene dimethacrylate) monoliths are more hydrophilic than methacrylate-based material and the enzyme can be immobilized in a single step, therefore these monoliths are better suited for enzyme-based bioreactors. A high activity in the hydrolysis of both low- and high-molecular mass substrates such as L-benzoyl arginine ethyl ester and casein was achieved and the catalytic activity of the monolithic bioreactor was maintained even at a flow-rate of 180 cm/min. The highest apparent activity of 810 μmol/min per ml was observed for the immobilized trypsin at a flow-rate of 102 cm/min and a temperature of 75 °C, which suggested that a very-high-throughput bioreactor could be designed using the monolithic supports.

4.2. Capillary electrochromatography applications

In most applications to date, CEC with monolithic columns has been used for the separation of small, neutral organic molecules under conditions of reversed-phase chromatography. Similar to conventional CEC, aromatic neutral compounds are often used as test solutes to evaluate column performance. For example, efficiencies up to 398 000 plates/m were obtained for the separation of a mixture of alkylphenones in an acrylamide-based monolithic column [72]. Other small neutral analytes, like herbicides [100], aldehydes and ketones [71,95], retinyl esters [108], steroids [74], and saccharides [72] were also successfully separated by CEC with monolithic columns. In addition to the ''classical'' use of monolithic capillary columns for the sepa-

ration of small molecules in the reversed-phase mode, larger styrene oligomers were separated under isocratic elution conditions [83]. Due to the involvement of an electrophoretic mechanism, the separation of ionic compounds is more complex than that in HPLC. The application of RP-CEC to the separation of ionic compounds will be addressed in more detail.

Cathodic EOF is generated in conventional RP-CEC with columns packed with HPLC silica-based packings due to the ionization of residual silanols. Acidic compounds tend to migrate against the EOF and require ion suppression for a successful separation [140]. The disadvantage of this method is the long analysis time because of the poor EOF by suppression of the ionization of silanols at low pH.

Separation of acidic compounds was also conducted in CEC with monolithic columns [67]. Fig. 12a shows the separation of aromatic acids at pH 2.4 on a monolithic capillary column. The sulfonic acid functionalities of the monolithic polymer remain dissociated under low pH, creating a flow velocity sufficient to achieve separation in a short period of time.

Peak tailing of basic compounds typically occurs in conventional RP-CEC due to the interaction of these compounds with the silanol groups. In order to improve the peak symmetry, a competing base was added to the mobile phase to compete with the analyte. Negatively charged groups like sulfonic acid groups and carboxyl groups were often coupled to the monolith to generate a strong EOF. Poor peak shape for basic compounds may also occur in CEC with monolithic columns due to the electrostatic interaction of analytes with the charged groups. Peak tailing was found in the separation of some basic analytes, the anilines, in CEC with monolithic poly-(styrene–co-divinylbenzene–co-methacrylic acid) as the stationary phase [90]. Separation of basic compounds on a monolithic CEC column with addition of competing base was not reported. However, they can also be separated with good peak shape in the ion-suppressed mode due to the excellent stability of the polymer-based stationary medium over a wide range of pH values. Fig. 12b shows the separation of anilines at pH 12 on a polymethacrylate-based monolithic capillary column [67]. The ionization of the anilines is suppressed at high pH, and they are in neutral form. Accordingly, there are no electrostatic interactions between them and the monolithic sur-

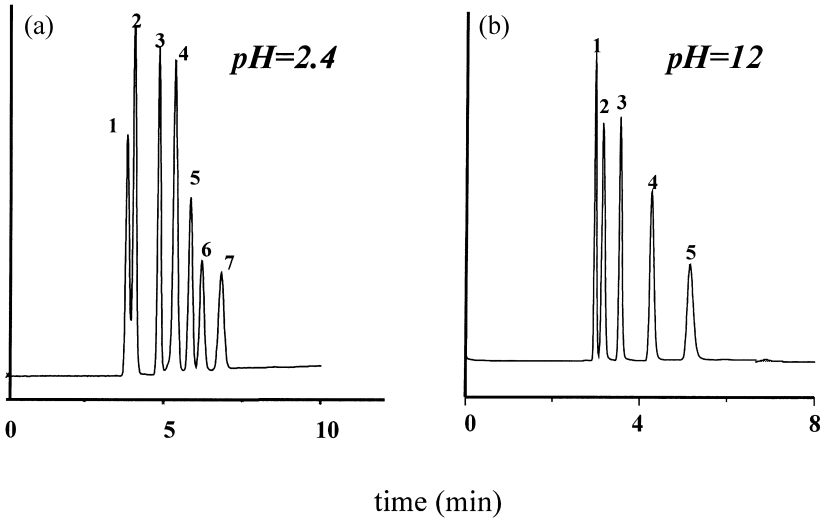

Fig. 12. Electrochromatographic separation of (a) aromatic acids and (b) anilines on monolithic capillary columns. Experimental conditions: capillary column, 30 cm (25 cm effective length)×100 μm I.D. with a monolithic rod of poly(butyl methacrylate–ethylene dimethacrylate containing 0.3% (w/w) 2-acrylamido-2-methyl-1-propanesulfonic acid); (a) mobile phase, acetonitrile–5 mmol/l phosphate buffer (pH 2.4) (60:40, v/v). Peaks: 1=3,5 dihydroxybenzoic acid; 2=4-hydroxybenzoic acid; 3=benzoic acid; 4=2-toluic acid; 5=4-chlorobenzoic acid; 6=4-bromobenzoic acid; 7=4-iodobenzoic acid. (b) Mobile phase, acetonitrile–10 mmol/l NaOH (pH 12) (80:20, v/v). Peaks: 1=2-aminopyridine; 2=1,3,5-collidine; 3=aniline; 4=*N*-ethylaniline; 5=*N*-butylaniline. Reprinted with permission from Ref. [67].

face, and therefore, symmetric peaks were obtained. It should be noted that such extreme pH conditions could not be adopted on typical silica-based packings.

The disadvantage of a CEC column by coupling charged groups onto the monolithic matrix is that the charge density and sign of the charge cannot be conveniently changed during the separation. Wu et al. [141] have reported that cathodic EOF or anodic EOF can be generated by dynamical adsorption of sodium laurylsulfate (SDS) or cetyltrimethylammonium bromide (CTAB) onto a neutral monolithic rod. It is very convenient to change the magnitude and direction of the EOF through changing the concentration of cationic or anionic surfactants in the mobile phase. Fig. 13 shows the simultaneous separation of acidic, basic and neutral compounds on CEC with a monolithic column modified by SDS and CTAB. In the first case, a low-pH mobile phase (pH 2.5) is applied, and baseline separation of 12 compounds including acidic, basic and neutral compounds is achieved. Acidic compounds are suppressed and separated based on the reversed-phase partition mechanism. However, the basic compounds of pyridine and quinoline as well as the dipeptides of Gly–Ala and Tyr–Trp are eluted before the void time of thiourea, which means that the separation of those compounds is mainly based on the mechanism of electrophoresis. In the case of CTAB as the modifier agent and with an eluent of pH 7, the EOF is reversed from cathode to anode. Under such separation conditions the basic compounds are not ionized, so basic and neutral compounds were separated based on the reversed-phase partition mechanism. Whereas the acidic compounds are ionized under these conditions and eluted before the void time of thiourea, their separation is mainly based on electrophorectic migration. Peak symmetry is very good for all ionic compounds at different separation conditions, which means that the irreversible adsorption of basic compounds on the capillary wall and monolithic stationary phase can be neglected.

Almost all CEC separations were conducted with a mobile phase driven by EOF. Charged groups on the packing surface were necessary to generate EOF, however, electrostatic interactions will inevitably take place. In fact, the migration of ionic compounds in CEC can be driven with electrophoretic mobility.

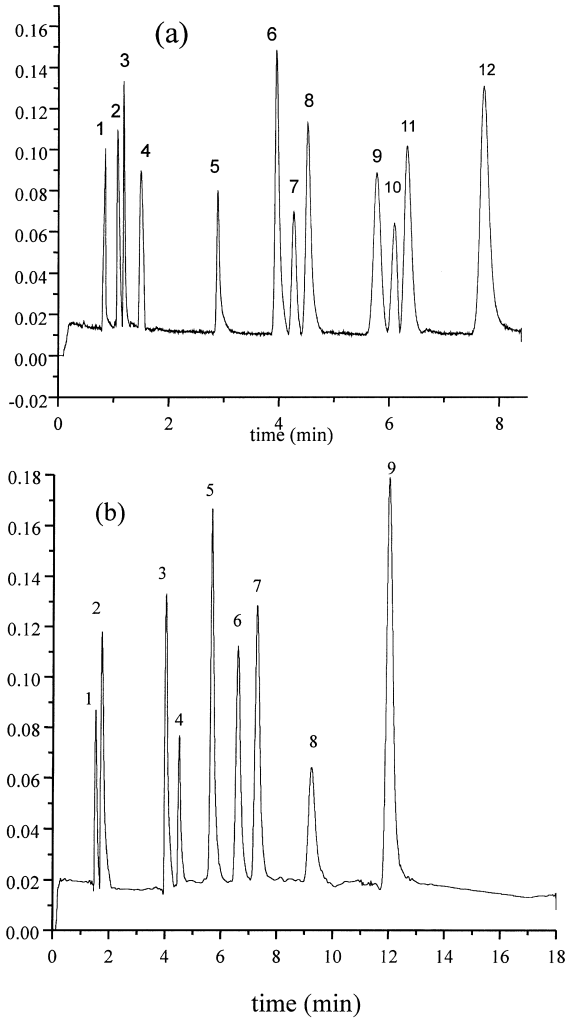

Fig. 13. Electrochromatogram for separation of basic, acidic and neutral compounds on a monolithic column in CEC modified with (a) SDS and (b) CTAB. Experimental conditions: capillary column, 27 cm (effective length 6.5 cm)×100 μm I.D. with a monolithic rod of poly(butyl methacrylate–co-ethylene dimethacrylate); (a) mobile phase, 35% acetonitrile in 5 mmol/l phosphate buffer (pH 2.5) containing 2.5 mmol/l SDS; Peaks: 1=pyridine; 2=quinoline; 3=Gly–Ala; 4=Tyr–Trp; 5=thiourea; 6=benzyl alcohol; 7=phenylacetic acid; 8=benzyl ethanol; 9=benzyl propanol; 10=*o*-toluic acid; 11=*p*-cresol; 12=benzonitrile. (b) Mobile phase, 35% acetonitrile in 5 mmol/l phosphate buffer (pH 7.0) containing 2.5 mmol/l CTAB. Peaks: 1=*o*-toluic acid; 2=phenylacetic acid; 3=thiourea; 4=pyridine; 5=benzyl alcohol; 6=benzyl ethanol; 7=quinoline; 8=benzyl propanol; 9=benzonitrile. Reprinted with permission from Ref. [141].

Fujimoto et al. [70] have reported the separation of dansylated amino acids on a polyacrylamide column without EOF. Separation of 12 dansylated amino acids was successfully achieved and all peaks are relatively symmetric. However, the hydrophobicity of this column is poor, the separation is based on the size selectivity of polyacrylamide column and the differences in electrophoretic mobilities. Recently, the separation of ionic compounds driven by electrophoretic mobility on a neutrally hydrophobic monolithic column was reported [91]. The monolithic column was prepared from the in situ copolymerization of lauryl methacrylate and ethylene dimethacrylate to form a C_{12} hydrophobic stationary phase. The peptides at acidic buffer were separated based on their differences in electrophoretic mobility and hydrophobic interaction with the stationary phase. The baseline separation of a peptide mixture was obtained, and all of the basic peptides containing amino acids of His and Lys showed very good peak symmetry.

Peptides and proteins are typically separated at acidic pH by reversed-phase HPLC. Under these conditions, they are positively charged and will migrate electrophoretically to the cathode in CEC. It seems that fast separation of peptides and proteins could be obtained in CEC with a cathodic EOF. However, a negatively charged surface is required to generate a cathodic EOF, therefore, strong electrostatic interaction will inevitably take place between the positively charged analytes and the negatively charged stationary phase surface, which results in long elution time or even no peak being detected. Such an interaction can be suppressed by adding salt to the mobile phase. However, the use of a mobile phase with high ionic strength will result in serious Joule heating and bubble formation. In fact, in order to eliminate the strong electrostatic attraction of the charged groups on the organic polymer monolith, separation of peptides and proteins was typically performed in CEC with ''counterdirectional mode'' [76,84]. In this CEC system, the peptides and proteins migrate electrophoretically in a direction opposite to that of the EOF. For example, the column filled with the methacrylic monolith bearing tertiary amino functions and butyl chains was used for the separation of angiotensin-type peptides at pH 2.5 [84]. Under these conditions, peptides are positively charged and they tend to electrophoretically migrate to the cathode. The surface of the monolith is also positively charged, which results in an anodic EOF. Because the EOF velocity was greater than the electrophoretic velocity, these peptides will be eluted to the anode by the EOF. Since both the peptides and the surface of the monolith are positively charged, electrostatic interaction between analytes and the stationary phase is eliminated. This system was also successfully applied to the separation of a protein mixture of ribonuclease A, insulin, α-lactalbumin and myoglobin.

Ericson and Hjertén [76] also reported the separation of positively charged proteins on a column filled with a continuous bed derivatized with C_{18} groups and ammonium groups. Both the proteins and the EOF generating ligands thus had positive charges to eliminate electrostatic interactions. Gradient elution generated by an HPLC instrument was adopted for the separation of proteins. The gradient and the sample were introduced at the same end of the capillary as in conventional (electro)chromatography or in a new approach, at different ends. In the former mode (normal-flow gradient), the electroosmotic velocity must be higher whereas in the latter mode (counterflow gradient), it must be lower than the electrophoretic velocity. A protein mixture of ribonuclease A, cytochrome *c*, lysozyme and α-chymotrypsinogen was successfully separated by either normal-flow or counterflow gradient elution.

There were only few papers published on the separation of compounds on the monolithic columns with other modes of CEC. The size-exclusion chromatography separation of polystyrenes in the CEC mode using a methacrylate-based monolithic capillary column was reported [83]. Successful separation of polystyrene standards with molecular masses up to 10^6 has been achieved [83]. Taurine conjugates, being strong anions, could not migrate under the cathodic EOF conditions with a RP-CEC monolithic column. A normal-phase (amino phase) monolithic column with a reversed EOF (from cathode to anode) was prepared to separate these anionic solutes [73]. It was observed that the retention increases with an increasing number of hydroxyl groups on the steroid backbone. The elution order indicates the normal-phase separation mechanism. The apparent efficiencies of this separation are very high, up to ~610 000

theoretical plates per meter. This type of CEC column provided an outstanding separation of a mixture of the relatively hydrophilic glycine and taurine conjugates, but did not separate adequately the more hydrophobic free bile acids in the same run.

However, CEC separation of chiral compounds on molecular imprinted monolithic capillary columns has attracted much attention [142–147]. The primary advantage of this approach is that a customized phase with predetermined selectivity for the analyte of interest can be prepared. Enantiomeric separations by CEC with charged polyacrylamide gels incorporating chiral selectors such as cyclodextrin and crown ether were reported by Koide and Ueno [75,77–79]. Resolution of various enantiomers, especially amino acids, was successfully achieved in this system. The CEC method with a crown ether-bonded negatively charged polyacrylamide gel-filled capillary was evaluated for use as an optical purity test [75]. Fig. 14 shows an electrochromatogram of L-alanine-2-naphthylamide; 0.1% of the minor enantiomer (D-form) is detectable. Svec and co-workers [85,86,92] also reported the preparation of a poly(methacrylate ester)-based monolithic column for

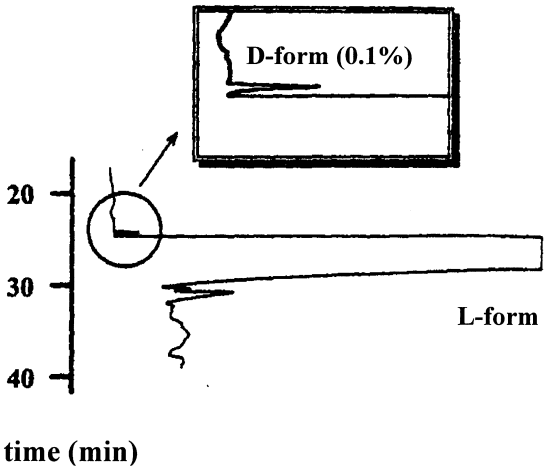

Fig. 14. Optical purity test of L-alanine-2-naphthylamide with a (+)-tetraallyl 18-crown-6 carboxylate-bonded negatively charged polyacrylamide gel-filled capillary. Experimental conditions: capillary column, 80 cm (effective length 35 cm)×75 μm I.D.; mobile phase, [200 mmol/l triethanolamine–300 mmol/l boric acid buffer (pH 6.0)]–acetonitrile (80:20, v/v). Sample: L-alanine-2-naphthylamide spiked with ca. 0.2% of DL-alanine-2-naphthylamide. Reprinted with permission from Ref. [75].

CEC chiral separation. Chiral selective groups, such as *N*-L-valine-3,5-dimethylanilide and quinidine, were coupled to the matrix in a single step by a simple copolymerization of mixtures of functional monomers and matrix monomers. Good enantioselectivity and excellent efficiencies were observed for a number of derivatized amino acids. Recently, a chiral ligand-exchange phase for CEC based on a monolithic column was reported [80]. Good enantioselectivity for a series of test amino acids with reasonable separation times was achieved.

Particle-fixed monolithic columns were also applied to chiral separation. Enantiomeric separation of amino acids and non-protein amino acids was achieved using a particle-loaded monolithic column [112]. The silica particles were modified with a chiral selector, (*S*)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine or (*S*)-*N*-3,5-dinitrophenylaminocarbonylvaline. On the basis of resolution the enantiomeric separation obtained by CEC is judged to be superior to that obtained previously with HPLC. Wistuba and Schurig [101] have developed a particle-sintered monolithic column modified with cyclodextrin for chiral separation in CEC. Various racemates, such as barbituric acids, benzoin, carprofen and so on, were successfully resolved. About two to three times higher efficiency was found in the CEC mode compared to the LC mode.

5. Disadvantages and limitations

Although the monolithic stationary phases possess a number of unique properties compared to traditional stationary phases, some disadvantages and limitations are inevitable. For the polymeric monolithic stationary phases, most of them are known to swell in organic solvents. This frequently leads to a lack of stability. Furthermore, the preparation of polymeric monoliths usually leads to micropores, which negatively affect the efficiency and peak symmetry of the column. Therefore, it is not easy to obtain high efficiency for small molecules. The low column capacity may be another significant disadvantage for their applications, which may be attributed to their low specific surface area compared to traditional packings. Although some attempts have been made to increase the specific surface area [32–35], most of

the resulting polymeric monoliths in previous reports only showed relatively low specific surface areas. The reason was mainly due to the high proportion of large pores in the total pore volume. Although several new methods have been developed [43,44], the preparation of preparative-scale polymeric monoliths was still difficult. The silica monoliths can provide either micrometer-size through-pores or high specific surface areas and can be well suited for both small molecules and biopolymers, but as with other silica packings, they cannot be used under too high and low pH.

Monolithic columns with wall-supported continuous porous beds have shown great potential as alternatives to packed columns in CEC because of their inherent advantages such as the absence of end-frits and the stability of the column bed. However, these columns also have their disadvantages and limitations. The disadvantage for polymer monolithic column is similar to that in HPLC, swelling of organic polymer in solvent and inadequate stability of silica-based monolithic column under extreme pH conditions. For particle-fixed monolithic columns, some disadvantage is obvious, such as damage of the surface of the reversed-phase silica for particle-sintered monolithic columns [99], reduced retentive characteristics for some particle-entrapped monolithic columns [102] and poor efficiency for particle-loaded monolithic columns [111–113].

6. Conclusion

The preparation of monolithic supports in HPLC and CEC has gained significant interest during the last few years due to a number of unique properties. Their ease of preparation, high reproducibility, versatile surface chemistry and fast mass transport can be used to specific advantage in a variety of applications. A great advantage in the high-speed separation of biological and synthetic molecules has been exhibited. Today, some commercial products have come into the market. Although they are unlikely to totally replace particulate supports, they can serve as an effective complementary option. A number of studies done so far have confirmed their great potential as chromatographic stationary phase. The various chromatographic modes have been developed in either HPLC or CEC, and high-throughput bioreactors and enzyme immobilized affinity chromatography have also been used. Although the performance of monolithic materials in CEC is encouraging, the true potential of this technique lies in its possible application in future miniaturized separation systems. The technique can be contribute to more complex chip-based systems with multiple applications. Based on their ease of preparation and high throughput, these materials can also be designed as smaller units for sensors for faster diagnostics. The preparative-scale monolithic column may be applied in future to industrial separation and purification of drugs and proteins. The monolithic molecular imprinted column for chiral separations may be another important aspect in future development.

Acknowledgements

The financial support from the Chinese Academy of Sciences (KCX2-3-6) and Knowledge Innovation Program of DICP to H.Z. is gratefully acknowledged. H.Z. is the recipient of the excellent young scientist award from the National Natural Science Foundation of China (No. 29725512).

References

[1] F. Svec, J.M.J. Frechét, Ind. Eng. Chem. Res. 38 (1999) 34.
[2] E.C. Peters, F. Svec, J.M.J. Frechét, Adv. Mater. 11 (1999) 1169.
[3] D. Lee, J. Chromatogr. 443 (1988) 143.
[4] M. Hanson, K.K. Unger, LC–GC 15 (1997) 364.
[5] F.E. Regnier, Nature 350 (1991) 634.
[6] N.B. Afeyan, S.P. Fulton, F.E. Regnier, J. Chromatogr. 544 (1991) 267.
[7] I. Gusev, X. Huang, Cs. Horváth, J. Chromatogr. A 855 (1999) 273.
[8] M. Kubin, P. Spacek, R. Chromecek, Collect. Czech. Chem. Commun. 32 (1967) 3881.
[9] W.D. Ross, R.T. Jefferson, J. Chromatogr. Sci. 8 (1970) 386.
[10] F.D. Hileman, R.E. Sievers, G.G. Hess, W.D. Ross, Anal. Chem. 45 (1973) 1126.
[11] D.K. Roper, E.N. Lightfoot, J. Chromatogr. A 702 (1995) 3.
[12] J.F. Kennedy, M. Paterson, Polym. Int. 32 (1993) 71.
[13] Y. Yang, A. Velayudhan, C.M. Ladish, M.R. Ladish, J. Chromatogr. 598 (1992) 169.
[14] S. Hjertén, J.L. Liao, R. Zhang, J. Chromatogr. 473 (1989) 273.

[15] T.B. Tennikova, F. Svec, B.G. Belenkii, J. Liq. Chromatogr. 13 (1990) 63.
[16] F. Svec, J.M.J. Fréchet, Anal. Chem. 64 (1992) 820.
[17] S.M. Fields, Anal. Chem. 68 (1996) 2709.
[18] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal. Chem. 68 (1996) 3498.
[19] C.K. Ratnayake, C.S. Oh, M.P. Henry, J. Chromatogr. A 887 (2000) 277.
[20] L.A. Colón, G. Burgos, T.D. Maloney, J.M. Cintron, R.L. Rodriguez, Electrophoresis 21 (2000) 3965.
[21] R.E. Majors, LC–GC 18 (2000) 586.
[22] G. Iberer, R. Hahn, A. Jungbauer, LC–GC 17 (1999) 1001.
[23] V. Pretorius, J.C. Davidtz, D.H. Desty, J. High Resolut. Chromatogr. Chromatogr. Commun. 2 (1979) 583.
[24] K. Cabrera, G. Wieland, D. Lubda, K. Nakanishi, N. Soga, H. Minakuchi, K.K. Unger, Trends Anal. Chem. 17 (1998) 50.
[25] F. Rabel, K. Cabrera, D. Lubda, Int. Lab. (2000) 23.
[26] Q.C. Wang, F. Svec, J.M.J. Fréchet, Anal. Chem. 65 (1993) 2243.
[27] M. Petro, F. Svec, I. Gitsov, J.M.J. Fréchet, Anal. Chem. 68 (1996) 315.
[28] M. Petro, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 752 (1996) 59.
[29] Q.C. Wang, F. Svec, J.M.J. Fréchet, Anal. Chem. 67 (1995) 670.
[30] Q. Luo, Y. Wei, T. Liu, G. Lei, X. Geng, Chin. Chem. Lett. 10 (1999) 215.
[31] J.A. Tripp, J.A. Stein, F. Svec, J.M.J. Fréchet, Org. Lett. 2 (2000) 195.
[32] S.F. Xie, F. Svec, J.M.J. Fréchet, Chem. Mater. 10 (1998) 4072.
[33] F. Svec, J.M.J. Fréchet, Polym. Mater. Sci. Eng. 81 (1999) 544.
[34] E.C. Peters, F. Svec, J.M.J. Fréchet, Macromolecules 32 (1999) 6377.
[35] U. Mayer, F. Svec, J.M.J. Fréchet, C.J. Hawker, K. Irgum, Macromolecules 33 (2000) 7769.
[36] F. Svec, J.M.J. Fréchet, J. Chromatogr. A 702 (1995) 89.
[37] F. Svec, J.M.J. Fréchet, Biotech. Bioeng. 48 (1995) 476.
[38] Y. Wei, X. Huang, Q. Chen, X. Geng, Chin. J. Anal. Chem. 28 (2000) 1194.
[39] Q. Luo, H. Zou, Q. Zhang, X. Xiao, Z. Guo, L. Kong, X. Mao, J. Chromatogr. A 926 (2001) 255.
[40] C. Viklund, F. Svec, J.M.J. Fréchet, Biotech. Prog. 13 (1997) 597.
[41] E.C. Peters, F. Svec, J.M.J. Fréchet, Adv. Mater. 9 (1997) 630.
[42] M. Petro, F. Svec, J.M.J. Fréchet, Biotech. Bioeng. 49 (1996) 355.
[43] E.C. Peters, F. Svec, J.M.J. Fréchet, Chem. Mater. 9 (1997) 1898.
[44] A. Podgornik, M. Barut, A. Strancar, D. Josic, T. Koloini, Anal. Chem. 72 (2000) 5693.
[45] J. Liao, R. Zhang, S. Hjertén, J. Chromatogr. 586 (1991) 21.
[46] C. Zeng, J. Liao, K. Nakazato, S. Hjertén, J. Chromatogr. A 753 (1996) 227.
[47] S.F. Xie, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 775 (1997) 65.
[48] S.F. Xie, F. Svec, J.M.J. Fréchet, J. Polym. Sci. A 35 (1997) 1013.
[49] G. Wulff, Angew. Chem. Int. Ed. Engl. 34 (1995) 1812.
[50] K. Mosbach, Trends Biochem. Sci. 19 (1994) 9.
[51] B. Sellergren, Trends Anal. Chem. 16 (1997) 310.
[52] R.J. Ansell, O. Ramström, K. Mosbach, Clin. Chem. 42 (1996) 1506.
[53] G. Vlatakis, L.I. Andersson, R. Muller, K. Mosbach, Nature 361 (1993) 654.
[54] V.T. Remcho, Z.J. Tan, Anal. Chem. 71 (1999) A248.
[55] L.I. Andersson, K. Mosbach, J. Chromatogr. 516 (1990) 313.
[56] J. Matsui, T. Kato, T. Takeuchi, M. Suzuki, K. Yokoyama, E. Tamiya, I. Karube, Anal. Chem. 65 (1993) 2223.
[57] T. Takeuchi, J. Matsui, J. High Resolut. Chromatogr. 23 (2000) 44.
[58] J. Matsui, I.A. Nicholls, T. Takeuchi, Anal. Chim. Acta 365 (1998) 89.
[59] J. Matsui, Y. Miyoshi, R. Matsui, T. Takeuchi, Anal. Sci. 11 (1995) 1017.
[60] J. Matsui, T. Takeuchi, Anal. Commun. 34 (1997) 199.
[61] C. Viklund, F. Svec, J.M.J. Fréchet, Chem. Mater. 8 (1996) 744.
[62] F. Svec, J.M.J. Fréchet, Chem. Mater. 7 (1995) 707.
[63] F. Svec, J.M.J. Fréchet, Macromolecules 28 (1995) 7580.
[64] A.I. Cooper, A.B. Holmes, Adv. Mater. 11 (1999) 1270.
[65] M. Zhang, Y. Sun, J. Chromatogr. A 922 (2001) 77.
[66] C. Viklund, E. Pontén, B. Glad, K. Irgum, P. Hörstedt, F. Svec, Chem. Mater. 9 (1997) 463.
[67] F. Svec, E.C. Peters, D. Sykora, J.M.J. Fréchet, J. Chromatogr. A 887 (2000) 3.
[68] F. Svec, E.C. Peters, D. Sykora, J.M.J. Fréchet, J. High Resolut. Chromatogr. 23 (2000) 3.
[69] F. Svec, in: Z. Deyl, F. Svec (Eds.), Capillary Electrochromatography (Journal of Chromatography Library, Vol. 62), 2001, Ch. 6.
[70] C. Fujimoto, J. Kino, H. Sawada, J. Chromatogr. A 716 (1995) 107.
[71] C. Fujimoto, Y. Fujise, Anal. Chem. 68 (1996) 2753.
[72] A. Palm, M.V. Novotny, Anal. Chem. 69 (1997) 4499.
[73] A.H. Que, T. Konse, A.G. Baker, M.V. Novotny, Anal. Chem. 72 (2000) 2703.
[74] A.H. Que, A. Palm, A.G. Baker, M.V. Novotny, J. Chromatogr. A 887 (2000) 379.
[75] T. Koide, K. Ueno, J. Chromatogr. A 909 (2001) 305.
[76] C. Ericson, S. Hjertén, Anal. Chem. 71 (1999) 1621.
[77] T. Koide, K. Ueno, J. Chromatogr. A 893 (2000) 177.
[78] T. Koide, K. Ueno, Anal. Sci. 16 (2000) 1065.
[79] T. Koide, K. Ueno, J. High Resolut. Chromatogr. 23 (2000) 59.
[80] M.G. Schmid, N. Grobuschek, C. Tuscher, G. Gubitz, A. Vegvari, E. Machtejevas, A. Maruska, S. Hjertén, Electrophoresis 21 (2000) 3141.
[81] C. Ericson, J. Liao, K. Nakazato, S. Hjertén, J. Chromatogr. A 767 (1997) 33.
[82] J. Liao, N. Chen, C. Ericson, S. Hjertén, Anal. Chem. 68 (1996) 3468.

[83] E.C. Peters, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Chem. 70 (1998) 2296.
[84] S. Zhang, X. Huang, J. Zhang, C. Horvath, J. Chromatogr. A 887 (2000) 465.
[85] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M.J. Fréchet, W. Lindner, Anal. Chem. 72 (2000) 4614.
[86] M. Lämmerhofer, F. Svec, J.M.J. Fréchet, Anal. Chem. 72 (2000) 4623.
[87] E.C. Peters, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Chem. 70 (1998) 2288.
[88] E.C. Peters, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Chem. 69 (1997) 3646.
[89] C. Yu, F. Svec, J.M.J. Fréchet, Electrophoresis 21 (2000) 120.
[90] B. Xiong, L. Zhang, Y. Zhang, H. Zou, J. Wang, J. High Resolut. Chromatogr. 23 (2000) 67.
[91] R. Wu, H. Zou, M. Ye, Z. Lei, J. Ni, Anal. Chem. 73 (2001) 4918.
[92] E.C. Peters, K. Lewandowski, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Commun. 35 (1998) 83.
[93] N. Ishizuka, H. Minakuchi, K. Nakanishi, N. Soga, H. Nagayama, K. Hosoya, N. Tanaka, Anal. Chem. 72 (2000) 1275.
[94] C. Fujimoto, J. High Resolut. Chromatogr. 23 (2000) 89.
[95] J.D. Hayes, A. Malik, Anal. Chem. 72 (2000) 4090.
[96] N. Tanaka, H. Nagayama, H. Kobayashi, T. Ikegami, K. Hosoya, N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Cabrera, D. Lubda, J. High Resolut. Chromatogr. 23 (2000) 111.
[97] N. Tanaka, H. Kobayashi, in: Z. Deyl, F. Svec (Eds.), Capillary Electrochromatography (Journal of Chromatography Library, Vol. 62), Elsevier, Amsterdam, 2001, Ch. 5.
[98] S. Suzuki, Y. Kuwahara, K. Makiura, S. Honda, J. Chromatogr. A 873 (2000) 247.
[99] R. Asiaie, X. Huang, D. Farnan, C. Horvath, J. Chromatogr. A 806 (1998) 251.
[100] T. Adam, K.K. Unger, M.M. Dittmann, G.P. Rozing, J. Chromatogr. A 887 (2000) 327.
[101] D. Wistuba, V. Schurig, Electrophoresis 21 (2000) 3152.
[102] G. Chirica, V.T. Remcho, Electrophoresis 20 (1999) 50.
[103] Q. Tang, M.L. Lee, J. High Resolut. Chromatogr. 23 (2000) 73.
[104] Q. Tang, N. Wu, M.L. Lee, J. Microcol. Sep. 11 (1999) 550.
[105] Q. Tang, B. Xin, M.L. Lee, J. Chromatogr. A 837 (1999) 35.
[106] Q. Tang, M.L. Lee, J. Chromatogr. A 887 (2000) 265.
[107] G.S. Chirica, V.T. Remcho, Anal. Chem. 72 (2000) 3605.
[108] L. Roed, E. Lundanes, T. Greibrokk, J. Chromatogr. A 890 (2000) 347.
[109] L. Roed, E. Lundanes, T. Greibrokk, J. Microcol. Sep. 12 (2000) 561.
[110] G.S. Chirica, V.T. Remcho, Electrophoresis 21 (2000) 3093.
[111] M.T. Dulay, R.P. Kulkarni, R.N. Zare, Anal. Chem. 70 (1998) 5103.
[112] M. Kato, M.T. Dulay, B.D. Bennett, J. Chen, R.N. Zare, Electrophoresis 21 (2000) 3145.
[113] C.K. Ratnayake, C.S. Oh, M.P. Henry, J. High Resolut. Chromatogr. 23 (2000) 81.
[114] J. Chen, M.T. Dulay, R.N. Zare, F. Svec, E. Peters, Anal. Chem. 72 (2000) 1224.
[115] M. Kato, M.T. Dulay, B.D. Bennett, J.P. Quirino, R.N. Zare, J. Chromatogr. A 924 (2001) 187.
[116] J.H. Knox, H.P. Scott, J. Chromatogr. 316 (1984) 311.
[117] Q. Luo, H. Zou, Q. Zhang, X. Xiao, J. Ni, Chin. J. Anal. Chem. 29 (2001) 497.
[118] N.B. Afeyan, N.F. Gordon, I. Mazsaroff, L. Varady, S.P. Fulton, Y.B. Yang, F.E. Regnier, J. Chromatogr. 519 (1990) 1.
[119] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 1979, p. 202.
[120] J. Liao, Adv. Chromatogr. 40 (2000) 467.
[121] G. Iberer, R. Hahn, A. Jungbauer, LC–GC 17 (1999) 998.
[122] Q.C. Wang, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 669 (1994) 230.
[123] S.F. Xie, R.W. Allington, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 865 (1999) 169.
[124] R.M. Smith, D.R. Garside, J. Chromatogr. 407 (1987) 19.
[125] N. Tanaka, T. Ebata, K. Hashizume, K. Hosoya, M. Araki, J. Chromatogr. 475 (1989) 195.
[126] J.P. Larmann, J.J. DeStefano, A.P. Goldberg, R.W. Stout, L.R. Snyder, M.A. Stadalius, J. Chromatogr. 255 (1983) 163.
[127] F. Svec, J.M.J. Fréchet, M. Petro, Polym. Mater. Sci. Eng. 77 (1997) 42.
[128] M. Janco, D. Sykora, F. Svec, J.M.J. Fréchet, J. Schweer, R. Holin, J. Polym. Sci. A 38 (2000) 2767.
[129] M. Janco, D. Sykora, F. Svec, J.M.J. Fréchet, Polym. Prep. 41 (2000) 373.
[130] D. Sykora, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 852 (1999) 297.
[131] Y. Li, J. Liao, K. Nakazato, J. Mohammad, L. Terenius, S. Hjertén, Anal. Biochem. 223 (1994) 153.
[132] M. Zhang, Y. Sun, J. Chromatogr. A 912 (2001) 31.
[133] T.M. Phillips, Analytical Techniques in Immunochemistry, Marcel Dekker, New York, 1992, p. 80.
[134] A. Denizli, A.Y. Rad, E. Piskin, J. Chromatogr. B 668 (1995) 13.
[135] Q. Luo, H. Zou, Z. Guo, X. Mao, L. Kong, X. Huang, J. Chromatogr. B, submitted for publication.
[136] J. Mohammad, A. Zeerak, S. Hjertén, Biomed. Chromatogr. 9 (1995) 80.
[137] X. Huang, H. Zou, X. Xiao, X. Chen, Q. Luo, X. Mao, Biomed. Chromatogr., submitted for publication.
[138] S.F. Xie, F. Svec, J.M.J. Fréchet, Biotech. Bioeng. 62 (1999) 30.
[139] S.F. Xie, F. Svec, J.M.J. Fréchet, Polym. Prep. 38 (1997) 211.
[140] M.G. Cikalo, K.D. Bartle, M.M. Robson, P. Myers, M.R. Euerby, Analyst 123 (1998) 87R.
[141] R. Wu, H. Zou, M. Ye, Z. Lei, J. Ni, Electrophoresis 22 (2001) 544.
[142] L. Schweitz, L.I. Andersson, S. Nilsson, Chromatographia 49 (1999) S93.

[143] L. Schweitz, L.I. Andersson, S. Nilsson, J. Chromatogr. A 817 (1998) 5.
[144] L. Schweitz, L.I. Andersson, S. Nilsson, J. Chromatogr. A 792 (1997) 401.
[145] L. Schweitz, L.I. Andersson, S. Nilsson, Anal. Chem. 69 (1997) 1179.
[146] J.M. Lin, T. Nakagama, K. Uchiyama, T. Hobo, Chromatographia 43 (1996) 585.
[147] J.M. Lin, T. Nakagama, X.Z. Wu, K. Uchiyama, T. Hobo, Fresenius J. Anal. Chem. 357 (1997) 130.