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Review

Recent development of monolithic stationary phases with emphasis on microscale chromatographic separation

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Abstract

The column technologies play a crucial role in the development of new methods and technologies for the separation of biological samples containing hundreds to thousands compounds. This review focuses on the development of monolithic technology in micro-column formats for biological analysis, especially in capillary liquid chromatography, capillary electrochromatography and microfluidic devices in the past 5 years (2002–2007) since our last review in 2002 on monoliths for HPLC and CEC [2]. The fabrication and functionalization of monoliths were summarized and discussed, with the aim of presenting how monolithic technology has been playing as an attractive tool for improving the power of existing chromatographic separation processes. This review consists of two parts: (i) the recent development in fabrication of monolithic stationary phases from direct synthesis to post-functionalization of the polymer- and silica-based monoliths tailoring the physical/chemical properties of porous monoliths; (ii) the application of monolithic stationary phases for one- and multi-dimensional capillary liquid chromatography, fast separation in capillary electro-driven chromatography, and microfluidic devices.

Keywords: Review; Monoliths; Column technology; Chromatographic separation; Stationary phases

Contents

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1. Introduction

In the past decade, a comprehensive attention in the field of chromatographic separation has been paid to the novel state-of-the-art stationary phases, the porous monoliths, which have been extensively studied and gradually applied in microscale chromatographic separation, such as capillary liquid chromatography (CLC), capillary electrochromatography (CEC), and microfluidic devices. Monoliths can be described as the integrated continuous porous separation media without the interparticular voids and thus circumventing the preparation of end frits for microscale separation columns. The mobile phases are forced through the porous monolithic media, which results in the convective flow and consequently enhances the mass transfer rate. Also, monolith has the following advantages including easy of preparation, versatile surface modification, and higher permeability along with good peak capacity. These unique advantages have made the monolithic column as an attractive alternative to the packed and open-tubular columns in the field of microscale separation. Especially, as the rapid development of micro- and nanoscale chromatographic separation systems hyphenated to mass spectrometers, monoliths have been clarified to be the right choice for separation of complex biological samples, such as proteolytic digests in proteomics by providing the better stability, resolution and sensitivity compared to the regular scale separation systems.

Here, a bar chart on the number of publications relevant to the monolithic columns (or continuous beds) in chromatographic separation science during the period 1989–2007 is illustrated in Fig. 1 to visualize the overall trend of increased interests in this area. As can be seen, in the early stage (since 1989 till 1996), the number of published papers was less than 10 per year. Since the publication of a paper titled "New designs of macroporous polymers and supports: from separation to biocatalysis" at the *Science* magazine in 1996 by Svec and Frechet [1], there was a dramatic increase in the annual number of published papers on monolithic columns. The number of publications rises at a quasi-exponential increasing rate in the period of 1997 to 2006. Even in the first quarter of 2007, the total number of publications on monolithic columns has already reached 94.

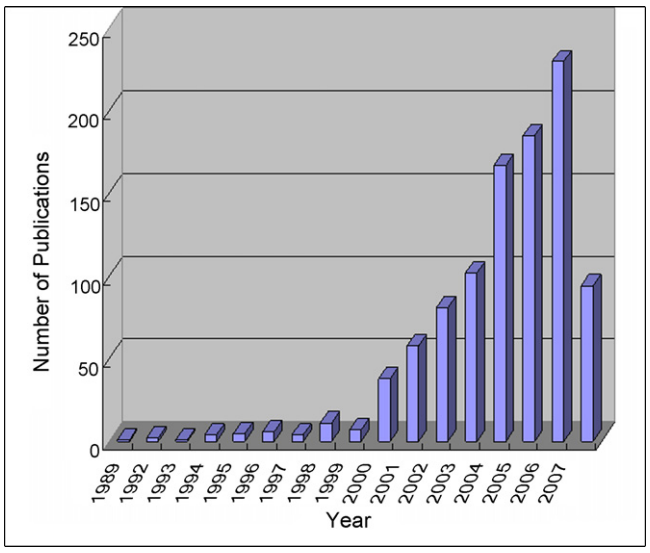

Fig. 1. The quasi-exponential rise in the number of publications in the field of monolithic columns in separation science since 1980s.

It is a challenge to write a review on the development of monolithic columns in the field of chromatographic separation science. Since our last review on monolithic columns in 2002 [2], there have been several tens of review papers published on its development oriented towards some specific research interests, such as the preparation methods as well as the applications in determination of biologically important compounds [3]; development of polymer-based monolithic columns for CEC with emphasis on the polymerization conditions, morphology, and surface chemistry [4], the preparation and application of polar stationary phases for CEC, including the monolithic columns in CEC [5], and an extensive description of monolithic columns specific to HPLC [6]. Others, such as Svec [7,8] presented the review papers to describe the uses of monoliths in sample preconcentrations, solid-phase extractions, and immobilized proteolytic enzyme reactors designed for studies in proteomics, and MIPs in CEC [9]. Besides, some review papers were focused on the development of monolithic stationary phases for the fast analysis of inorganic ions and other small molecules in ion chromatography (IC) and CEC [10], the microfluidic analytical components in microchips [11], the enantiomer-selective stationary phases in CEC [12], the analysis of compounds in plant metabolomics study [13], and the affinity monolith chromatography [14], etc. This review will focus on the last advances achieved over the past 5 years in the synthesis and further functionalization of the porous polymer- and silica-based monolithic stationary phases, and their application in microscale chromatographic separation systems emphasized on CLC, CEC and microfluidic devices.

2. Fabrication of monoliths in microscale format

The advantages of microscale liquid chromatography over conventional normal scale HPLC, and the high efficiency of microscale electro-driven chromatography have long been confirmed, which included the better separation resolution, lower solvent consumption and higher sensitivity as combined to concentration sensitive detection devices resulting from the reduced chromatographic dilution. However, packing a high-efficient capillary column needs skills as well as packing materials with suitable properties. In the last decade, the monolithic columns have been widely adopted in the microscale separation systems due to the laudatory features of easy preparation, fritless design, high permeability, low backpressure, fast analytes mass transfer, and versatile surface chemistry, etc. Technically, the preparation of monolithic columns in electro-driven microscale capillaries/channels is similar to that in pump-driven microscale liquid chromatography except the consideration of the generation of EOF for driving mobile phase, which requires the incorporation of charged moieties onto the surface of the monolith. Here, the emphases are given to the direct synthesis and post-modification of polymer- and silica-based monolithic capillary columns in CLC and CEC.

2.1. Polymer-based monolithic columns

2.1.1. Direct synthesis of polymer-based monoliths

Polymer-based monolithic columns have been quickly implemented in microscale separation systems because of the simplicity of *in situ* preparation and reliable chromatographic performance as well as good controllability over porous skeletons and surface chemistries. The preparation of a polymer-based monolithic column in a capillary and/or a microchannel is relatively simple and straightforward. Briefly, a mixture of monomers, cross-linker and initiator in the presence of porogenic solvents was introduced and sealed into a fused silica capillary, and then a polymerization was triggered by keeping at a certain temperature or exposing to UV light. After polymerization, the capillary column was attached to a pump and a solvent was delivered into the capillary to flush out the porogenic solvents and other reaction residuals from the polymerized monolithic matrix. In the past 5 years, a variety of new monolithic columns were prepared via direct copolymerization.

Reversed-phase (RP) stationary phases were most widely applied in CLC and CEC. The development of RP organic polymeric monolithic stationary phases via direct synthesis in capillaries has been well achieved before 2002, and already described in our pervious review [2]. Here we addressed the recent progress of the new monolithic polymeric stationary phases in CEC and CLC since 2002. As the most common polymeric monolithic column in capillary, poly(methacrylate), poly(acrylamide), poly(PS-DVB)-based monolithic columns were the good candidates to reversed-phase stationary phases in CLC and CEC. By using different monomers, a variety of polymeric monolithic columns have been developed and investigated, such as poly(methacrylate)-based monolithic stationary phases using cinnamic acid [15], butyl methacrylate [16], octyl methacrylate (MAOE) [17], hexyl-methacrylate (HMA) [18], lauryl methacrylate [19] as the monomers and EDMA as the cross-linker; C14 and C16 poly(acrylamide)-based monoliths [20,21]; poly(styrene-co-DVB-co-MAA) monolith [22] and cationic stearyl-acrylate monolithic columns [23]. Recently, Bisjak et al. [24] prepared a poly(PA-PDA) monolithic capillary column by *in situ* copolymerization of phenylacrylate (PA) and 1,4-phenylene diacrylate (PDA) in a capillary. The poly(PA-PDA) monolith showed high mechanical stability and ability to the separation of proteins and oligodeoxynucleotides. Li et al. [25] incorporated single-wall carbon nanotubes (SWNTs) into the preparation of polymeric monolithic columns via *in situ* copolymerization of vinylbenzyl chloride (VBC) and EDMA for CLC and CEC. The prepared poly(VBC-EDMA-SWNT) monolithic column was evaluated by separating a mixture of small organic molecules. It was observed that the incorporation of SWNTs indeed enhanced the retention of small neutral molecules on the monolithic column. Most recently, Jakschitz et al. [26] prepared a poly[(trimethylsilyl-4-methylstyrene)-co-bis(4-vinylbenzyl) dimethylsilane] monolithic stationary phases by thermally initiated *in situ* copolymerization of trimethylsilyl-4-methylstyrene (TMSiMS) and bis(4-vinylbenzyl)dimethylsilane (BVBDMSi) in a silanized fused silica column, and applied in the separation of proteins mixture in ion-pair RP-CLC. Trojer et al. [27,28] prepared a capillary poly(MS-co-BVPE) monolithic column via *in situ* copolymerization of methylstyrene (MS) and 1,2-bis(*p*-vinylphenyl)ethane (BVPE) in the presence of inert diluents. The prepared porous monolith of MS/BVPE showed excellent mechanical stability and minimum swelling in organic solvents. The permeability and column efficiency of this kind of porous monolithic column was found to highly depend on the ratio of total monomer versus porogen and can be controlled by changing or adjusting the composition of reaction reagent and porogens. As shown in Fig. 2, the conformation of the skeleton of MS/BVPE monoliths, the porosities, the shape and size of particles of the nodes of network have a significant differentiation. This kind of monolith probably will provide us a multiple selection of stationary phases for sample preparations and chromatographic applications. To form the gradient monolithic stationary phase, a series of different concentrations of hydrophobic monomer mixture solutions were prepared, and the gradient stationary phases were formed by consecutively drawing the different concentration of stationary phase precursors into a capillary. After copolymerization, the desired gradient stationary phase was polymerized *in situ*. Gradient format monolithic column have shown enhanced resolution compared to the capillary columns of averaged homogeneous composition. Interestingly, by controlling the compositions of polymerization mixture in a capillary, a gradient format reversed-phase monolithic stationary phase (shown in Fig. 3) could be prepared along the axis of a capillary [29], which also demonstrated the increased efficiency and resolution compared to the common (isotropic) stationary phases. Different from the above mentioned columns with one-piece porous monolithic rods inside the capillaries, a type of porous layer open-tubular (PLOT) columns were prepared, in which the layered porous monoliths of poly(BMA-co-EDMA) [30] and poly(PS-co-DVB) [31,32] were formed on the inner walls as the RP stationary phases for separation of separation of biological samples. By changing the conditions for *in situ* copolymerization, the thickness of the porous polymer layer could be controlled. This type of open-tubular capillary columns could provide good permeability as well as the enhanced peak capacity, thus could be prepared as the much longer capillary columns for CLC with low backpressure.

Ion-exchange was an important mode in CLC and CEC. For preparation of SCX monolithic column, Gu et al. [33] prepared SCX monolithic columns of poly(AMPS-co-PEGDA) and poly[2-acrylamido-2-methyl-1-propanesulfonic acid-co-poly(ethylene glycol) diacrylate] via photoinitiated copolymerization with water, methanol, and ethyl ether as porogens. The prepared monolithic column contained as high as 40% AMPS, and offered several features, such as high binding capacity, extraordinary high resolution and high peak capacity for separation of peptides. However, due to the use of AMPS, the monolithic column showed relatively strong hydrophobicity. Ion-exchange stationary phase with high degree of ionic interaction for CLC or CEC is always desirable. In CEC, Wu [34] developed a SCX monolithic stationary phase by *in situ* copolymerization of 2-(sulfooxy)ethyl methacrylate (SEMA) and ethylene dimethacrylate (EDMA). The sulfate group pro-

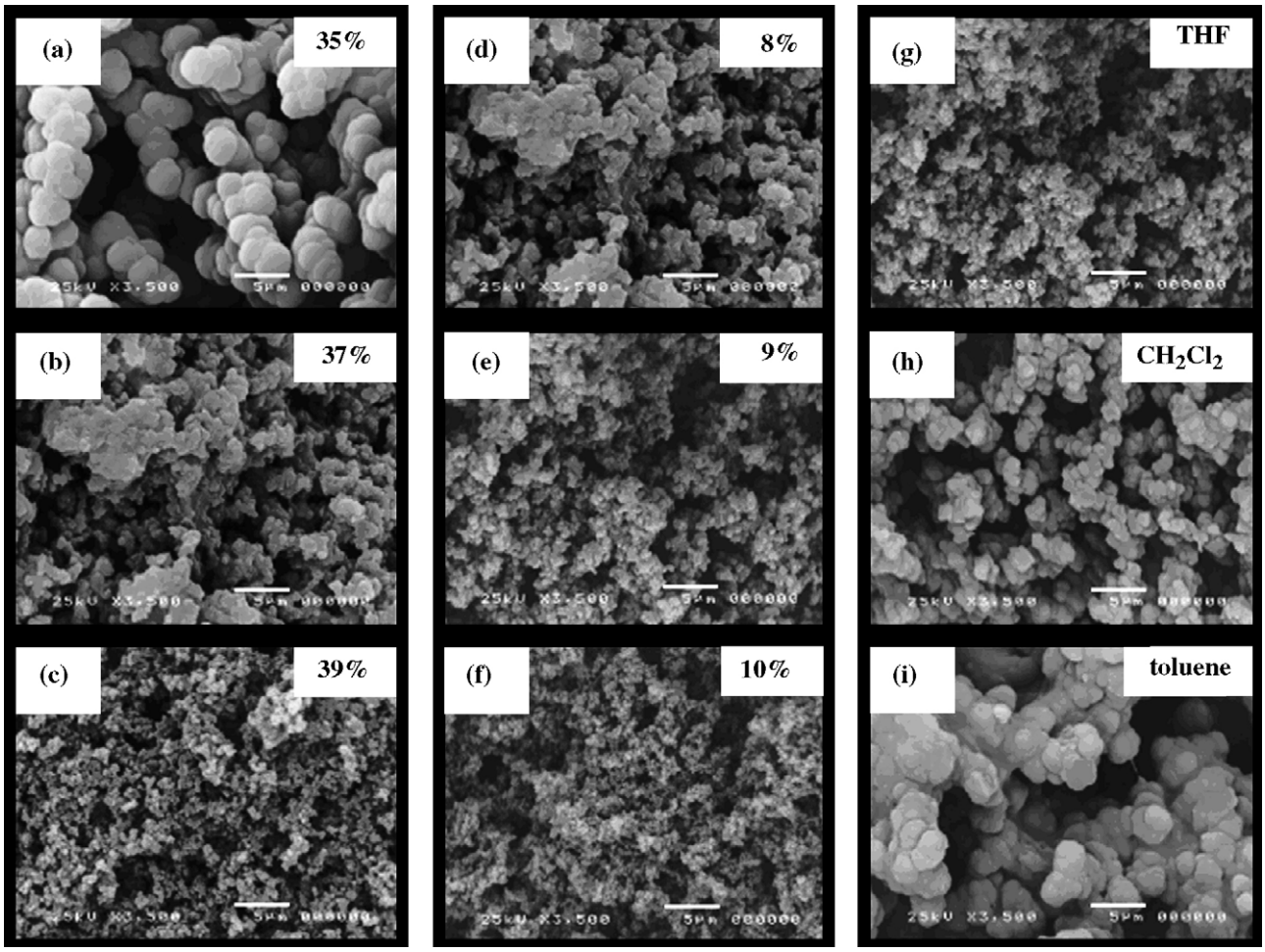

Fig. 2. Conformation of MS/BVPE monoliths with different composition. SEM micrographs: (a–c), effect of the total monomer content; (d–f), effect of the THF content; (g–i), effect of the microporogen nature, magnification: 3500 (reprinted from [27] with permission of Elsevier).

vided by the monomer SEMA on the monolithic bed was used for the generation of the electroosmotic flow (EOF) from the anode to the cathode, and these negatively charged groups also served as a SCX stationary phase. But, because of the hydrophobicity of the polymeric skeleton, the CEC separation of peptides could be explained under the mechanism of a RP/SCX mixed mode of CEC with both electrostatic interaction of SCX and hydrophobic interaction of RP. A RP/SAX mixed-mode monolithic column was developed by Fu et al. [35] via *in situ* polymerization of 2-(methacryloxy)ethyltrimethylammonium methyl sulfate (MEAMS) and EDMA. The ammonium groups on the surface of the stationary phase not only generated an electroosmotic flow (EOF) from cathode to anode for CEC, but also served as a SAX stationary phase. Meanwhile, the hydrophobic polymeric skeleton played as the RP stationary phase in CEC as well.

The amphiphilic and/or zwitterionic monolithic stationary phases had also received increased interest for preparing monolithic stationary phase in CEC and CLC [36]. Acrylamide-based monoliths containing hydrophilic and hydrophobic groups can be characterized as amphiphilic one. Their amphiphilic nature makes it possible to use them in both normal- and reversed-phase mode. Hoegger and Freitag [37] demonstrated a RP/hydrophilic monolithic stationary phase by preparing a *N*,*N*-dimethylacrylamide-piperazine diacrylamide-based monolithic stationary phases bearing sulfonic acid groups for the separation of charged amino acids and peptides in CEC. Differently, Wahl et al. [38] synthesized the amphiphilic stationary phases by free radical copolymerization of the bisacrylamide–cyclodextrin host–guest complexes with hydrophilic monomers and an additional hydrophilic cross-linker in aqueous solution. Al-Rimawi and Pyell [39]

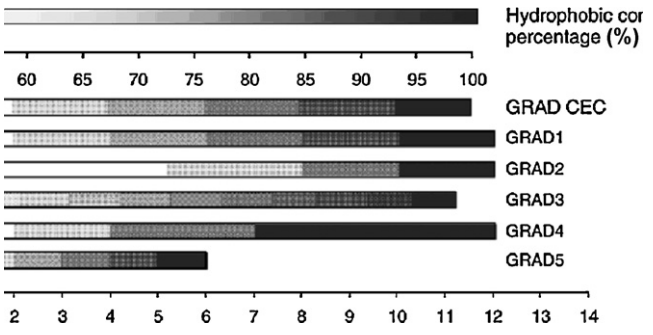

Fig. 3. Gradient monolithic stationary phase (reprinted from [29] with permission of Elsevier).

employed a CD-solubilized hydrophobic monomer, a water-soluble cross-linker (piperazinediacrylamide) and a charged monomer (vinylsulfonic acid) in aqueous solution, and an amphiphilic monolithic stationary phase for CEC and CLC were synthesized. Fu et al. [40] prepared a zwitterionic monolithic column by *in situ* polymerization of BMA, EDMA, MAA and 2-(dimethyl amino) ethyl methacrylate in the presence of porogens. The stationary phases possessed zwitterionic functional groups of both tertiary amine and acrylic acid groups. Jiang et al. [41] synthesized the zwitterionic poly(SPE-co-EDMA) monolithic column within a 100 μm i.d. capillary for the application in hydrophilic interaction liquid chromatography (HILIC). The prepared zwitterionic monolithic column showed very good selectivity for neutral, basic, and acidic polar analytes because of the coexistence of both hydrophilic interaction and electrostatic interaction contribution. Another type of HILIC monolithic stationary phase in CLC was prepared by Holdsvendova et al. [42], where the HILIC monolithic column was synthesized via the *in situ* polymerization of EDMA and the functional monomer of *N*-(hydroxymethyl) methacrylamide (HMMAA). And the separation of oligonucleotides was subsequently carried out in HILIC mode.

However, due to the difficulty of controlling of final structure and chromatographic properties of above mentioned free radical copolymerization of monomers/cross-linkers, such as styrene/divinylbenzene [43], acrylic acid/methylenebisacrylamide [44], glycidyl methacrylate/ethylene dimethacrylate, or acrylamide/methylenebisacrylamide [45] in the presence of porogenic solvents, a ring-opening methathesis polymerization (ROMP) approach for the preparation of monolithic stationary phase was proposed by Sinner and Buchmeiser [46] in 2000. Via the ring-opening metathesis polymerization method, the well-defined monolithic stationary phase could be synthesized under the strictly controlled conditions [47–49]. The synthesized columns were applied in the rapid and highly efficient separation of single- and double-stranded nucleic acids and proteins by liquid chromatography. The synthesis and properties of metathesis polymerization-derived supports for solid-phase extraction (SPE), liquid chromatography were reviewed by Buchmeiser [50] in 2004. Recently, Schlemmer et al. [51] prepared a monolithic column for CLC via ROMP using *cis*-cyclooctene (COE), tris(cyclooct-4-enyl-1-oxy)methylsilane (CL) as monomers, 2-propanol and toluene as porogens and $RuCl_2(Py)_2(IMesH_2)(CHC_6H_5)$ (Py = pyridine, $IMesH_2$ = 1,3-dimesityl-4,5-dihydroimidazolin-2-ylidene) as initiator within a 200 μm i.d. fused silica column. The column performance of the newly prepared monolithic column was evaluated by separation of a standard protein mixture consisting of six proteins in the molecular weight range of 5800–66000 Da, i.e. ribonuclease A, insulin, albumin, lysozyme, myoglobin and beta-lactoglobulin, and showed a good reproducibility of synthesis based on the determination of relative standard deviation of retention time of analytes. Further investigations on this monolithic column, demonstrated that the variations of monomer content and the ratio of COE to CL did not significantly change the properties of column, such as monolith morphology, backpressure and retention times. In addition, a long-term stability of the monolithic column was observed with no significant alteration in separation performance over 1000 consecutive runs at 50 °C. Besides the ROMP technique for the preparation of polymer-based monoliths under control, an interesting method for preparing the nicely controlled skeletal epoxy resin-based polymer monolith was adopted by Hosoya et al. [52] for CLC via the polycondensation reaction by utilizing a trifunctional epoxy monomer and a diamino compound in an appropriate porogenic solvent. The prepared monolithic matrix afforded a really homogeneous co-continuous monolith with a silica monolithic skeleton-like bimodal pore structure of submicrometer-size skeletons with micrometer-size through pores. It is of interest to pursue further investigation of this epoxy resin-based polymer monolith in capillary for CLC or CEC due to the controllability of the living polymerization [53].

2.1.2. Post-modification of polymer-based monoliths

Theoretically, through the direct copolymerization of the cross-linker and functional monomers, the versatile monolithic columns can be prepared by altering monomers, cross-linker, porogens, initiator, and the conditions of reaction, such as temperature, time and compositions, etc. However, due to the complexity of copolymerization these attempts to obtain monoliths with desired chromatographic properties, such as chromatographic retention, pore structure, and column efficiency are not always successful. For example, most of the functional groups of the monoliths prepared using the monomer containing functional groups are buried within the polymer mass rather than exposed on the surface of the stationary phases as the functional ligands to interact with analytes. Thus, the designed functional groups of the reactive monomer will mainly be inaccessible for analytes even incorporated in copolymerization. It is a real art to balance the pore structure and retention property for monolithic columns. So, the introduction of functional groups onto the polymerized monoliths via grafting and coating procedures surely represents a complementary and useful technique for the surface modification of porous polymeric monoliths, and provides an avenue to the preparation of monoliths by tailoring the versatile surface chemistries. The grafting approaches via epoxy [54], chloromethyl [55] and azlactone [56] reactive groups on polymer-based monolithic matrices had been reported before 2002. In recent years, the grafting approaches via ring opening of epoxy group of monomer GMA represented the most extensively applied route for post-modification of polymeric monolithic matrices, which included ion-exchange, affinity, pH gradient and reversed-phase stationary phases, etc. in CEC and CLC.

Due to the convenience of preparing hydrophobic polymeric monolithic columns via direct copolymerization, the preparation of monoliths for reversed-phase stationary phases via post-modification seemed not attracting much interests in this aspect. But motivated by the needs of suitable stationary phases for high resolution and high throughput protein separation in proteome research, Li et al. [57] developed a simple approach to prepare the monolithic column with octadecyl groups on the surface for CLC. The preparation of the surface-alkylated monolithic columns were achieved via the post-modification of poly(GMA-co-EDMA) monolith by grafting *n*-alkyl groups onto the porous

methacrylate-based monolith. It was observed that the alkylation of poly(GMA-EDMA) monolithic column with linear octadecyl groups appreciably improved the separation resolution for proteins over an octylated chromatographic surface. Another type of a lysine immobilized poly(GMA-co-EDMA) monolithic column was prepared by Dong et al. [58]. On this post-modified monolithic column, the hydrophobic interaction was responsible for separation of neutral analytes, and the separation of ionic or ionizable analytes on the monolithic capillary columns in CEC involved electrostatic interaction and electrophoretic migration in addition of hydrophobic interaction.

The ion-exchange stationary phases for CLC and/or CEC have also been prepared via post-modification upon synthesized polymeric monoliths. Bisjak et al. [59] grafted the ionizable amino groups onto the poly(GMA-co-DVB) monolith by the post-derivatization with diethylamine via the ring opening of epoxy groups to form an anion-exchange stationary phase. Wieder et al. [60] further extended this approach to prepare the strong anion-exchange stationary phase by a two-step derivatization of poly(GMA-co-DVB) monolith first with diethylamine and then with diethyl sulfate to form the designated quaternary ammonium groups via the ring opening of epoxy groups from GMA. Wei et al. [61] developed another derivatization approach via the ring opening of the epoxy group of poly(GMA-co-EDMA) monolith for the preparation of a weak cation-exchange (WCX) stationary phase. In the process of derivatization, the cation ion-exchange functional surface was obtained by an amination procedure. Namely, the monolithic column with epoxy groups on surface reacted with ethylenediamine at first step, and then treated by chloroacetic acid to obtain a new stationary phase for HPLC with carboxylic groups on the surface of the poly(GMA-co-EDMA) monolithic column. Preinerstorfer et al. [62] further developed an enantioselective SCX monolithic column for CEC by grafting (*S*)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutanephosphonic acid on the surface of monolith via radical addition. More directly, Savina et al. [63] grafted an anion-exchange polymer chains of poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA) and poly([2-(methacryloxy)ethyl]trimethylammonium chloride) (pMETA), and cation-exchange polymer chains of polyacrylate onto pAAm cryogels using potassium diperiodatocuprate as initiator. In this approach, the graft polymerization did not alter the macroporous structure of the pAAm cryogel, but the flow rate through the porous monolithic matrix decreased when the density of the grafted polymer increased due to the possible decrease of porosity of porous monolithic column.

Affinity chromatographic assays based on the capillary column with monolithic stationary phase have been reported by Pan et al. in 2002 [64]. In this report, the monolithic capillary column was prepared by *in situ* polymerization of GMA and TRIM, and then protein A was subsequently immobilized onto the monolith to form an affinity stationary phase for the microliter scale analysis of hIgG in CLC. Bedair and El Rassi [65] prepared a monolithic column via the *in situ* copolymerization of GMA and EDMA with a positively charged monomer [2-(methacryloxy)ethyl]trimethylammonium chloride (MAETA). Then, the prepared monoliths were further functionalized with yeast *Saccharomyces cerevisiae* mannan and/or lectins as the affinity stationary phase for separation of mannose-binding proteins and glycoconjugates, respectively. Tetala et al. [66] carried out a simple and different method for the preparation of an affinity monolithic capillary column, where 2-hydroxyethyl methacrylate (HOEMA) copolymerized with (+)-*N*,*N*′-diallyltartardiamide (DATD) and piperazine diacrylamide (PDA, 1,4-bisacryloyl-piperazine). After oxidation of DATD with periodate, α-mannose with spacer was bound to the aldehyde groups of the polymeric skeleton via reductive amination. The formed affinity monolithic column demonstrated good binding performance to the mannose-specific lectins. Recently, a tandem format affinity monolithic column was developed for the microscale depletion of the top eight most abundant proteins in human serum in a single run based on a poly(GMA-EDMA) monolith [67], which immobilized affinity ligands including protein A, protein G' and polyclonal antibodies. This tandem format could be extended to include additional affinity monolithic columns to deplete other proteins for which specific antibodies are available without running into high inlet pressure. Furthermore, the tandem affinity columns could be integrated with immobilized trypsin monolithic columns to achieve the simultaneous depletion and digestion of proteins.

CIEF, capillary isoelectric focusing, has been used in the separation of biomolecules, in which carrier ampholytes (CAs) were indispensable to establish a stable pH gradient. However, the decrease of sensitivity and the difficulty of coupling with mass spectrometry were the main disadvantages for using this technique. Thus, Yang et al. [68] realized the synthesis of the monolithic immobilized pH gradient (M-IPG) column via the *in situ* copolymerization of GMA and EDMA. Followed the copolymerization, the glutaraldehyde was introduced and linked to the monolithic matrix after a process of amination. Whereafter, an aqueous solution of commercial carrier ampholytes (CAs, Ampholine) was pumped in the monolithic matrix. By applied the voltage at the both ends of monolithic column, the CAs was focused along the column and the primary amino groups of CAs thus reacted with glutaraldehyde to form the IPG stationary phase along the axis of the capillary. This kind of column could be repeatedly used and even hyphenated with MS with good linearity and high resolution. This work was further developed, and a narrow immobilized pH gradient monolithic stationary phase was obtained [69].

Rather than above mentioned post-derivatization of GMA monoliths for specific chromatographic modes, Preinerstorfer et al. [70] prepared a universal reactive thiol-modified monolithic columns by transforming the pendent 2,3-epoxypropyl groups of poly(GMA-co-EDMA) monoliths into 3-mercapto-2-hydroxy-propyl residues via a nucleophilic substitution reaction, employing sodium-hydrogen sulfide as nucleophilic reagent. For giving an example, the *O*-9-tert-butylcarbamoylquinine (t-BuCQN) was attached to the monolith via a radical addition reaction with the reactive thiol groups onto the pre-prepared monolith for CEC enantiomer separation of (*R*,*S*)-DNB–Leu. This introduced reactive thiol groups onto the polymeric monolith surface can be utilized to further attach other chromatographic ligands in a second step by radical addition, graft

polymerization, nucleophilic substitution, disulfide formation or Michael addition reaction. However, it needs to be indicated that the post-functionalization of the polymer-based monoliths via these reactive groups is essentially decided by the primary coverage of these groups on the surface of porous monoliths, and which is not easy to be improved as any change of the composition of such reactive moieties for preparing the monoliths will result in the possible change in their structure.

Different from the afore post-modification methods via ring opening of reactive epoxide groups, the photografting approach of polymer-based substrates was also utilized for the surface functionalization of porous polymer-based monoliths with reactive monomers by using benzophenone to initiate the grafting reaction via abstracting the hydrogen from polymer substrates after the UV irradiation. Based on this surface grafting technique, Rohr et al. [71] demonstrated the surface functionalization of polymeric monoliths within the pores of a macroporous polymeric monolith in a fused silica capillary by introducing 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and 4,4-dimethyl-2-vinylazlactone onto the poly(BMA-co-EDMA) monolith for separation of peptides. Hilder et al. [72] carried out a modification approach by grafting two layers of different functional polymer chains onto the surface of a polymeric monolith. Namely, the synthesized polymeric monolith with optimized porous properties was first grafted an "interior" layer of ionizable monomer AMPS, and then coated by a "covering" layer of hydrophobic polymer chains. This two layers grafting technique could afford monolithic columns either the generation of EOF or hydrophobic property in CEC, while preventing ionized analytes from interacting with the charged surface functionalities. Pucci et al. [73] prepared a polymer-based monolithic column with a longitudinal gradient of functionalities by grafting ionizable polymer chains onto the surface of a porous polymeric monolithic matrix. In this procedure, a moving shutter and a neutral density filter were used to control the UV light exposure along the column for creating the longitudinal gradient of functionalities. The technique of preparing the polymer-based monolithic columns with the longitudinal gradient of functionalities may represent a new approach for controlling the surface chemistries of porous polymer-based monoliths. The UV-initiated photografting technique of the polymer-based monoliths was a simple and versatile approach that enabled the surface modification of synthetic polymers with a wide variety of chemistries and at precisely defined locations in an easy single step. Also, the grafted polymeric monoliths would provide a high loading of functional groups by circumventing the limitation of active sites on monoliths for further post-modification.

Simpler than grafting processes, the coating of latex-nanoparticles onto the polymer-based monoliths has also been applied for the functionalization of monolithic columns. Hilder et al. [74] prepared an anion-exchange monolithic column for separation of polysaccharides by coating 60 nm quaternary ammonium latex-nanoparticles on a poly(BMA-co-EDMA-co-AMPS) monolithic matrix via the electrostatic interaction. The SEM images before and after coating of latex particles on a monolith demonstrated that the macroporous structure of the monolith did not change but the microglobules of coated nanoparticles played as the anion-exchange stationary phase were found on the surface of the monolith. By applying this method, Hutchinson et al. [75] and Zakaria et al. [76] realized the separation of inorganic anions over a short separation period, and the elution order indicated the contribution of both ion-exchange and electrophoresis mechanisms on the latex-nanoparticles coated polymer-based monolithic column. However, because the electrostatic adsorption of quaternary ammonium latex onto the monolithic matrix was restricted by the amount of negatively charged groups on monolith, the increase of the density of surface active groups will accordingly improve the adsorption of latex-nanoparticles and consequently increase the ion-exchange capacity of the latex-coated monolithic stationary phase. Therefore, Hutchinson et al. [77] investigated the sulfonation of PS-DVB and poly(GMA-co-EDMA) monolithic columns with different methods for the preparation of high capacity agglomerated monolithic ion-exchangers for capillary ion chromatography. They found that the direct sulfonation of PS-DVB monoliths using concentrated sulfuric acid or chlorosulfonic acid was unsuccessful. In contrast, the chemical transformation of the poly(GMA-co-EDMA) monoliths containing glycidyl groups was used to increase the ion-exchange capacity (up to 14–29 μequiv/g). Three different sulfonation methods were considered, including reaction with 4-hydroxybenzenesulfonic acid under basic conditions; reaction with thiobenzoic acid followed by transformation to a reactive thiol and the subsequent oxidation to the sulfonic acid; and direct sulfonation with sodium sulfite. Of these, the reaction with sodium sulfite resulted in the most significant increase in the capacity and the best performance in separation of analytes.

2.2. Silica-based monolithic columns

Silica-based monolithic columns can provide good mechanical stability and specific meso- and macroporous structure as well as the variety of chemical modification for CLC and CEC. The aspirations of exploring the performance of silica monolithic columns in CLC and CEC have driven researchers to develop a variety of new methods for the preparation of silica-based monolithic columns in the last decade. Silica-based monolithic columns can be prepared by hydrolysis and polycondensation of alkoxysilanes catalyzed by acetic acid in the presence of a porogenic agent. After drying and heating treatments, the sol–gel network is derivatized by on-column silylation reaction. For modifying the surface feature for chromatographic separation, the functional groups should be introduced onto the silica monolithic matrix by post-modification or direct incorporation of desired functional monomers during the fabrication process in advance by means of the siloxane bond.

2.2.1. Direct synthesis of silica-based monoliths

By now, the methods for the direct synthesis of silica-based monolithic columns can be summarized into following approaches: (i) the earliest approach of preparing monolithic column described by Fields in 1996 [78]. In this method, a

silica xerogel was prepared from a potassium silicate solution. The steps of preparation involved the gelation, drying, and derivatization with octadecyl (ODS, C18) silane. However, the initial prepared xerogel silica monolithic column showed low efficiency; (ii) another early approach for silica monolithic column reported by Minakuchi et al. [79] in 1996. In this method, the monolithic column was prepared through an acid-catalyzed sol–gel procedure which involved hydrolysis of alkoxysilane, such as tetramethoxysilane (TMOS, reagent No. 1, Fig. 4) and tetraethoxysilane (TEOS, reagent No. 2, Fig. 4), condensation of the hydrated silica-tetrahedral forming ≡Si–O–Si≡ bonds, polycondensation of linkage of additional ≡Si–OH tetrahedra eventually resulting in a SiO_2 skeleton having silanol groups on the surface. Columns prepared from alkoxysilane via the sol–gel method showed high efficiency and permeability due to the existence of small-sized silica skeletons and large-sized through pores. This sol–gel method was further improved by Tanaka and his coworkers in recent years. Ishizuka [80,81], Motokawa et al. [82,83], Hara et al. [84] from Tanaka research group prepared a series of capillary silica gel monolithic columns with a various of pore structure by controlling the reaction conditions of condensation. After the derivatization of the intact silica skeletons to C18 phases, the columns were successfully applied in CLC and CEC.

A new type of the silica-based monolithic column, so called hybrid organic–inorganic silica-based monolithic column, has captured researchers' attentions in recent years. The hybrid monolithic columns with the stationary phase bonded through a surface silicon–carbon bond instead of a conventional siloxane bond. The structural property and selectivity of the sol–gel hybrid stationary phase can be tailored through changing the composition of original sol solution. In this method, a silane co-precursor containing a moiety of interest (e.g. octadecyltrimethoxysilane) was incorporated in the reaction mixture to form a hybrid support structure in a single step. Hayes et al. [85] prepared an organic–inorganic porous monolithic substrate inside fused silica capillaries for use as a separation bed in CEC. In this method, a sol–gel precursor, *N*-octadecyldimethyl [3-(trimethoxysilyl) propyl] ammonium chloride (Reagent No. 6, Fig. 4), was incorporated into the sol solution for the preparation of the organic–inorganic silica-based monolithic columns. Thus, the prepared silica-based monolith with C18 moiety could be directly applied in CEC, and the post-modification of the silica-based monolith was avoided. Constantin and Freitag [86] developed a similar one step sol–gel process for the preparation of a C8 monolithic, macroporous nanocomposite phase in fused silica capillary, which also did not require the additional derivatization.

Yan et al. [87,88] reported the preparation of the hybrid organic–inorganic silica-based monolithic columns functionalized with phenyl, octyl and amino moieties using phenyltriethoxysilane (PTES, reagent No. 3, Fig. 4), 3-aminopropyltriethoxysilane (APTES, reagent No. 4, Fig. 4) and C8-triethoxysilane (reagent No. 5, Fig. 4) as the functional monomers via the co-condensation of monomers by sol–gel process. These prepared monolithic columns showed high permeability and high column efficiency, and the structural morphology and chromatographic characteristics can be controlled by adjusting the composition of sol solution. Through the application in the separation of neutral, basic and acidic compounds, such columns showed advantages of high efficiency and satisfactory reproducibility. Recently, Ding et al. [89] carried out a stationary phases with mixed modes of RP and weak anion-exchange (WAX) on a hybrid monolithic column employing tetraethoxysilane (TEOS), aminopropyltriethoxysilane (APTES), and octyltriethoxysilane (C-8-TEOS) as the monomers in the reaction mixture via the sol–gel technique at mild temperature. Where the amino groups incorporated on the hybrid monolithic column dominated the charge on the surface and generated an EOF from cathode to anode at low pH. As designed, the monolithic column exhibited RP chromatographic behavior for neutral solutes. Fast and efficient separation of six aromatic acids was obtained using acidic mobile phase with column efficiency up to 160,000 plates/m by CEC. Symmetrical peaks can be obtained for aromatic amines because positively charged amino groups on the surface can effectively minimize the adsorption of positively charged analytes to the stationary phase.

Organic–inorganic hybrid silica-based monolithic capillary column was also demonstrated by Kanamori et al. [90]. In this hybrid monolithic column, the hydrophobic and hydrophilic moieties were simultaneously presented in-gel which prepared from an organic–inorganic hybrid siloxane gel derived from methyltrimethoxysilane (MTMS, reagent No. 7, Fig. 4). This hybrid silica-based monolithic column could be used in reversed-phase and normal-phase liquid chromatography due to its amphi-features of hydrophobic and hydrophilic property resulting from the methyl groups and silanol groups. In addition, the MTMS-derived monolithic column performed excellent formability, such as less shrinkage and cracks during preparation, particularly during aging and drying procedures. The approach for preparing the hybrid monolithic column is attractive for synthesizing the previous discussed organosilane monolith; however, it has not been commonly followed for the fabrication of silica-based monolithic columns for CEC yet due to the difficulty to control the hydrolysis and condensation reactions when some dissimilar precursors were used to produce the desired monolithic structure. Also, for synthetic conditions of hybrid monolith containing different functionalities the independent optimization process is needed correspondently. For this concern, Colon et al. [91] prepared a hybrid monolithic column with allyl groups on the monolith surface by mixing allyl-trimethoxysilane (allyl-TrMOS, reagent No. 8, Fig. 4) and TMOS in the sol–gel reaction mixture. The allyl groups allow the further derivatization to bind desired moieties on the monolithic column by Si–C bonds and the re-preparation and/or optimization of the monolithic column with the designed moieties can be avoided. However, no further work reported based on this allyl-hybrid monolithic column.

For hybrid silica-based monoliths, there is a weakness to undergo a further wide application in liquid chromatography due to its inherent non-porous property to form the internal pores like other silica-based media for HPLC. Recently, a new approach of silica-coating on macroporous MTMS-derived hybrid monolith

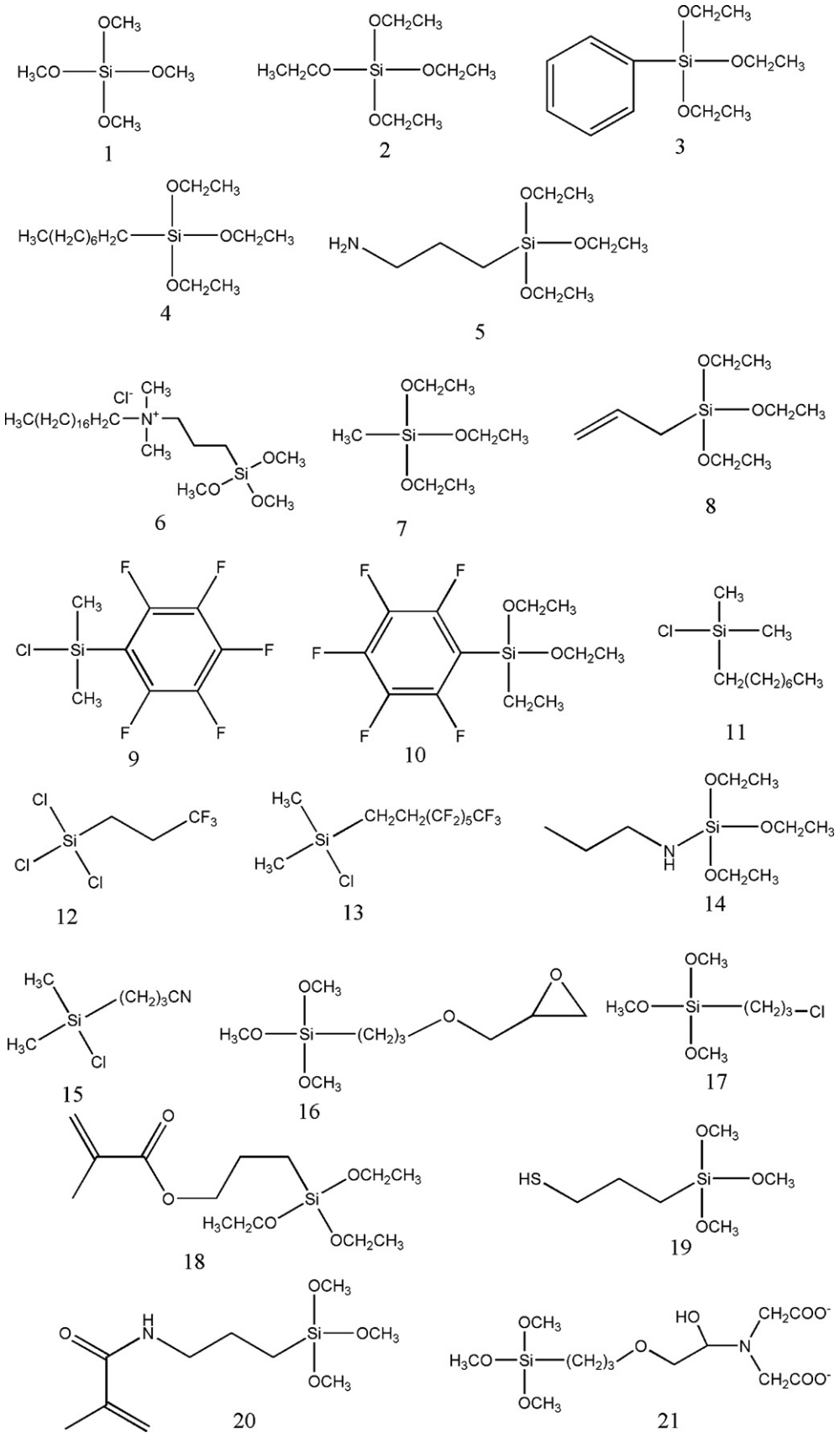

Fig. 4. List of silane reagents for silica-based monolithic stationary phases.

was developed in a small confine space to increase the mesopores of the hybrid monoliths [92]. After silica-coating, mesopore formation was found, and the significant increase of retention factor was exhibited by comparing to the non-coated MTMS monolith. However, the resultant mesopore volume was not very large and the pore size distribution was rather broad compared to those of typical silica beds. Additionally, via this coating technique, it is possible to coat a variety of small confined spaces with desirable chemical compositions, such as silica (SiO_2), organo-siloxane ($RSiO_{1.5}$) and organo-bridged siloxane ($O_{1.5}Si–R–SiO_{1.5}$), etc. where R denotes a (functional) organic moiety. Extended studies on controlling the coating thickness, mesopore formation, and surface modification are highly desirable in capillary scale hybrid monolithic column.

2.2.2. *Functionalization of silica-based monoliths*

Based on the silica-based monolithic columns prepared using above mentioned techniques, the study on the modification or functionalization of silica-based monolithic columns has been developing to fit the various needs for the separation of complex compounds in CLC and CEC. Via the post-modification, monolithic columns allow completely independent control of the porous properties and the chemical. Also, through post-modification, the re-optimization of the porous properties of a monolithic column can be avoided, rather than be prepared again each time when a change of chemical functionality is desired. Several pathways have been reported for the functionalization of porous silica-based monoliths as follows.

Using different silane reagents was the much straightforward approach to modify the silica-based monolithic columns. Dulay et al. [93] reported the chemical modification approach of sol–gel monoliths by silanizing the sol–gel surface with organochlorosilane or organoalkoxysilane coupling reagents, pentafluorophenyldimethylchlorosilane (PFPDM, reagent No. 9, Fig. 4), pentafluorophenyltriethoxysilane (PFP, reagent No. 10, Fig. 4), *n*-octadimethylchlorosilane (C8, reagent No. 11, Fig. 4), 3,3,3-trifluoropropyltrichlorosilane (C_3F_3 reagent No. 12, Fig. 4), (tridecafluoro-1,1,2,2-tetrahydrooctyl)dimethylchlorosilane (CF_{13}, reagent No. 13, Fig. 4) and *n*-propylaminotriethoxysilane (NH_2, reagent No. 14, Fig. 4), to form the stationary phases with the moieties of pentafluorophenylpropyldimethyl, pentafluorophenyl, 3,3,3-trifluoropropyl, *n*-octadimethyl, perfluorohexyl, and aminopropyl, respectively. After the derivatization, the modified monoliths had higher stability at pH values below 4 compared to the underivatized monolithic matrix. The separations of different mixtures containing nucleosides, positively charged peptides, and taxol derivatives were observed on these phase-bonded PSG columns in CEC. El Rassi and his coworkers [94–97] also developed a series of surface modification of silica-based monoliths (reagent No. 15–17, Fig. 4) to form the RP and polar stationary phases on silica-based monolithic columns for the different need of CEC and CLC. A silane reagent, glycidoxypropyltrimethoxysilane-iminodiacetate (GLYMO-IDA)-silane (reagent No. 21, Fig. 4), was lab-synthesized (reaction scheme, Fig. 5A) and used for the post-modification of silica-based monolithic column by Feng et al. [98], which avoided the use of an extreme pH condition (pH 10–12) for immobilizing IDA onto GLYMO pre-modified supports. The lab-made GLYMO-IDA-silane was continuously delivered through a pre-prepared silica-based monolithic capillary column to form an IDA modified silica-based monolithic capillary column. After coordinated by Fe^{3+} ions, the Fe^{3+}-IDA silica monolithic column was used for analysis of phosphopeptides with its high affinity to phosphoryl groups of phosphopeptides. The scheme for preparation of this IMAC column was illustrated in Fig. 5B.

Using mercapto silane reagent, Xie et al. [99] prepared the SCX silica-based monolithic column for SCX separation in CEC. In this method, the monolithic silica matrix from a sol–gel process was first chemically modified by (3-mercaptopropyl)-trimethoxysilane (reagent No. 19, Fig. 4), and then followed an *in situ* oxidation of thiol groups to sulfonic groups to produce the SCX stationary phase. The SCX stationary phase was characterized by its substantial and stable electroosmotic flow (EOF), and it was observed that the EOF value of the prepared column remained almost unchanged at different buffer pH values and slowly decreased with increasing phosphate concentration in the mobile phase. The monolithic silica column with SCX stationary phase was then employed in CEC for the separation of β-blockers and alkaloids, which were extracted from the traditional Chinese medicines (TCMs).

Introducing bifunctional methacryl-anchors on the surface of silica-based monolithic columns is a convenient and effective way to couple various functional groups on monoliths. *N*-(3-trimethoxysilylpropyl) methacrylamide [100,101] (reagent No. 20, Fig. 4), 3-methacryloxypropyltriethoxy silane [102] (reagent No. 18, Fig. 4), have been introduced onto the surface of silica monolith as the anchors. Following by the on-column copolymerization process, a variety of polymer films were thus grafted on the surface of silica monolith. In this way, the poly(acrylamide) [100], poly(acrylic acid) [101], and poly(methacrylates)-modified [102] monolithic silica columns were prepared for the separation of polar compounds, such as pyridylamino (PA)-sugars, peptides including a tryptic digest of BSA, proteins, nucleosides, nucleic acids and inorganic anions, running in anion-exchange, cation-exchange, and/or HILIC modes [100–102].

In 2004, Shi et al. [103] introduced a coating procedure for the modification of the silica monolith by forming a monolithic column with zirconia surface. In comparison to conventional silica monoliths, the zirconia surface increased the stability of the silica monolith and facilitated the separation of basic compounds. Furthermore, this zirconia surface could be easily modified further with alkylphosphonic acid and was employed as a hydrophobic stationary phase for separations of a variety of compounds. The coating with nanoparticles was also applied on the post-modification of silica monolithic columns. Hutchinson et al. [104] introduced the physical coating approach for ion-exchange CEC (IE-CEC) on a silica monolithic column by adsorbing 70 nm quaternary ammonium anion-exchange latex particles via electrostatic interaction. The resulting complex stationary phases were characterized in terms of their chromatographic performance and capacity. After the latex-coating

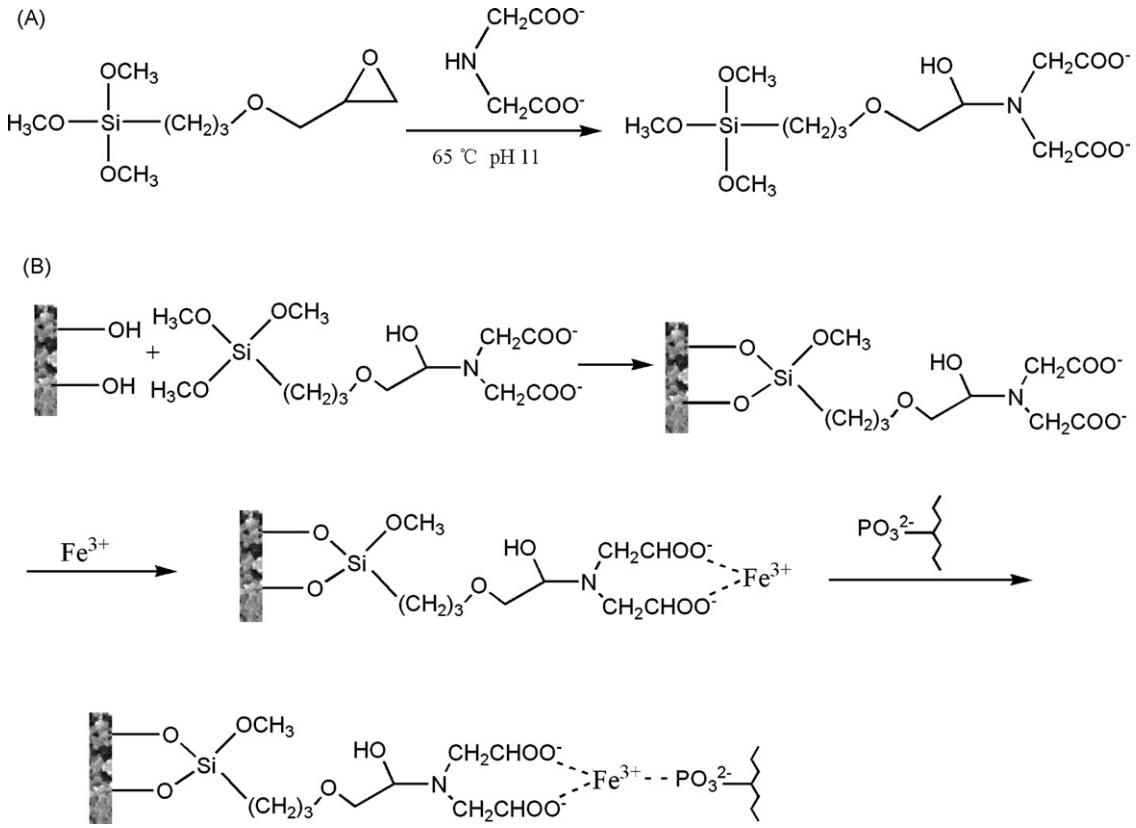

Fig. 5. Post-modification of a silica monolith for preparing GLYMO-IDA IMAC capillary column.

on the silica-based monolithic column, the electroosmotic flow (EOF) was reversed and the anionic analytes were separated in a co-EOF mode. In addition, there was a significant increase in the retention of analytes in CEC using latex-coated monolithic columns compared to their latex-coated open-tubular counterparts. However, this also required the use of higher ionic strength electrolytes in order to vary selectivity by manipulating the extent of ion-exchange interactions between the analytes and the stationary phase, which in turn led to problems with Joule heating effects and lower separation efficiencies for highly retained analytes in IE-CEC. For preparing the anion-exchange stationary phase for ion chromatography, Glenn et al. [105] also converted a silica monolithic column to an anion-exchange column by coating of positively charged latex-nanoparticles. After the coating, the latex-nanoparticles were tightly adsorbed on the silica monolithic matrix and thus without the drift of retention time for analytes. Although this work was done in a regular HPLC column, it indeed provided useful hint for the preparation of capillary silica monolithic column with stable anion-exchange stationary phase and superior efficiency.

For enantioseparation, as early as in 2001, Chen and Hobo [106,107] developed the ligand exchange for enantioseparation of dansyl amino acids with a L-phenylalaninamide/L-prolinamide-modified silica monolithic column. In this report, the native silica monolithic column was first chemically modified by a spacer of (3-glycidoxypropyl)trimethoxysilane (reagent No. 16, Fig. 4), and then attached chiral selectors. After being conditioned with Cu(II) aqueous solution, the positive charged ligand exchange-chiral stationary phase (LE-CSP) was obtained for the ligand exchange of chiral separation in CEC. This work was continued by Chen et al. and applied in CLC as well [108]. Later, Kang et al. [109], Lubda et al. [110] and Chen et al. [111] developed a series of methods for the preparation of chiral silica-based monolithic column using the sol–gel process in CEC by immobilizing β- or γ-cyclodextrin on the sol–gel monolith. Alternatively, covalently immobilized cellulose derivatives, such as cellulose tris(3,5-dimethylphenylcarbamate), 3,5-disubstituted phenylcarbamate derivatives of cellulose and amylase, amylose tris(3,5-dimethylphenylcarbamate), onto native silica monolithic columns for enantioseparation were also reported [112–114]. The covalent attachment of the cellulose derivatives enabled the columns to be used in combination with the mobile phases which were incompatible with coated-type polysaccharide columns due to the solubility of chiral selector in some organic solvents. In 2006, Chankvetadze et al. [113] carried out an *in situ* post-modification of a silica-based monolithic column by covalent binding of three different polysaccharide phenylcarbamate derivatives for the enantiomer separation in CLC. By using the mercapto silane reagent, Preinerstorfer et al. [115] prepared the mercapto silica monolithic column by derivatizing the native silica monolithic column with 3-mercaptopropyl trimethoxysilane. And then, a chiral selector of an amino phosphoric acid derivative was covalently bonded to the reactive monolithic stationary

phase via the radical addition reaction with these reactive thiol groups.

2.3. Particles-fixed monolithic columns

Particle-fixed monolithic column represented a different type of monolithic column in CEC/CLC, which could be prepared, respectively, by (i) sintering silica particles via hydrothermal treatment [116]; (ii) gluing silica particles with TEOS [117], TMOS [118], silicate solution [119]. Although the loading of particles in capillaries was still required, a variety of advantages of easy in packing, rich in commercial available materials, and avoid of frits did prove this technique a useful approach for column preparation in microscale chromatographic separations, especially in enantioseparation for CLC and/or CEC.

For achiral separation, recently, Qu et al. [120] prepared a particle-fixed monolithic column by fixing the particles in a capillary using potassium silicate–formamide instead of the silicate solution used by Chirica ad Remcho [119], which led a much less curing time (6 h) than that of Chirica's method (several days). After curing, the particle-fixed monolithic columns were octadecylated *in situ* by dimethyloctadecylchlorosilane. The prepared columns were quite stable and have been used for more than 1 month without loss in efficiency for the separation of aromatic compounds. Bakry et al. [121] prepared a new particle-fixed monolithic column by immobilizing particles in a polymer of poly(styrene-co-divinylbenzene) (PS/DVB). Compared to the traditional packed column, this type of hybrid monolithic columns could still offer high phase ratio and narrow pore size distribution, and led to high retention and separation efficiency. In addition, a separation of nucleotides on the anion-exchange particle-fixed monolithic column confirmed that the chromatographic properties of the silica particles packed in encapsulated in PS/DVB polymer were still active. Similarly, Jandera et al. [122] prepared a new hybrid inter-particle capillary monolithic column by *in situ* polymerization of BMA and EDMA in the presence of superficially porous particles. The prepared hybrid column showed improved hydrodynamic flow properties with respect to whole-volume monolithic capillary columns synthesized under the same polymerization condition due to the higher "equivalent permeability particle diameter" that enabled shorter separation times of proteins.

For chiral separation, Kato et al. [123] suspended 5 μm silica particles derivatized with a chiral selector in a solution of TEOS, and forced into a capillary to form a monolithic column. The advantage of this simple approach is the possibility of using the commercial available chiral stationary phase (CSP) without complicated packing and the need of frits. An alternative approach has also been presented by Schmid et al. [124]. There, silica particles containing a chiral selector were suspended in a monomer solution, which is drawn into the capillary followed by *in situ* polymerization. Thereby the silica-based particles containing the chiral selector were embedded in the achiral continuous bed. Most recently, Schmid et al. [125] prepared the particle-loaded monoliths with a polymethacrylamide backbone by suspending a silica-based chiral phase in the mixture of the monomers, followed by *in situ* polymerization in the capillaries. The capillaries were cut into desired length and ran both in LC and CEC mode, respectively. The results indicated that CEC mode was superior to LC mode in terms of retention times and resolution, although higher temperature resulted in faster separation. Differing from above described gluing approached for particle-fixed monolithic columns, Gatschelhofer et al. [126] developed a method for the preparation of the particle-fixed monolithic column via ring-opening metathesis polymerization (ROMP) within the confines of fused silica columns using norborn-2-ene (NBE), 1,4,4a,5,8,8a-hexahydro-1,4,5,8,exo,endodimethanonaphthalene (DMN-H6) as monomers, 2-propanol and toluene as porogens, $RuCl_2(PCy_3)_2(CHPh)$ as initiator, and chiral silica particles as the chiral selector. Particle-fixed ROMP monoliths showed good separation performance for glycyl-dipeptides in CEC.

2.4. Monolithic MIP columns

Monolithic molecular imprinted polymers (MIPs) are highly cross-linked polymers containing spatial and functionality memory for template molecules which can provide the specific selectivity when used as molecular selective stationary phases in chromatography. Molecular recognition and separation based on capillary monolithic MIPs are thought having following advantages, such as the rapid separation, high efficiency, the low consumption of templates and stationary phases. Capillary monolithic MIPs can be prepared either by *in situ* copolymerization of functional monomers and cross-linkers in the presence of template molecules or by post-grafting of MIPs on monolithic matrices. After removal of templates from the resulted monolithic MIPs, the highly selective materials with corresponding recognition cavities complementary to the templates in size, shape and chemical functionalities can be obtained.

Schweitz et al. [127] for the first time reported a monolithic MIP in CEC in 1997, where the polymer were prepared by *in situ* copolymerization using MAA as the monomer and TRIM as the cross-linker at −20 °C under a UV source. Later in 2001, Schweitz et al. [128] prepared a shorter monolithic MIP column for the rapid enantioseparation of (*R*)- and (*S*)-propranolol. The monolithic MIPs could be prepared by thermally initiated *in situ* polymerization using MAA as the functional monomer and EDMA as the cross-linker in the presence of designed templates, such as 4-aminopyridine [129], (*R*)-1,1′-bi-2,2′-naphthol [130] and 4-hydroxybenzoic acid [131], (*S*)-naproxen [132], caffeic acid [133], and (5*S*,11*S*)-(−)-Troger's base and l-tetrahydropalmatine [134].

Lin et al. [135] prepared a monolithic MIP column using an aromatic amino acid, L-phenylalanine, as the template for the selective recognition and separation of oligopeptides. In this method, the monolithic MIP was prepared by *in situ* copolymerization of MAA, 2-vinylpyridine (2-VPY), EDMA, and a Schiff base conjugate of L-phenylalanine, *o*-phthalaldehyde (OPA) and ally mercaptan. The scheme for preparation of this monolithic MIP was illustrated in Fig. 6. A baseline separation

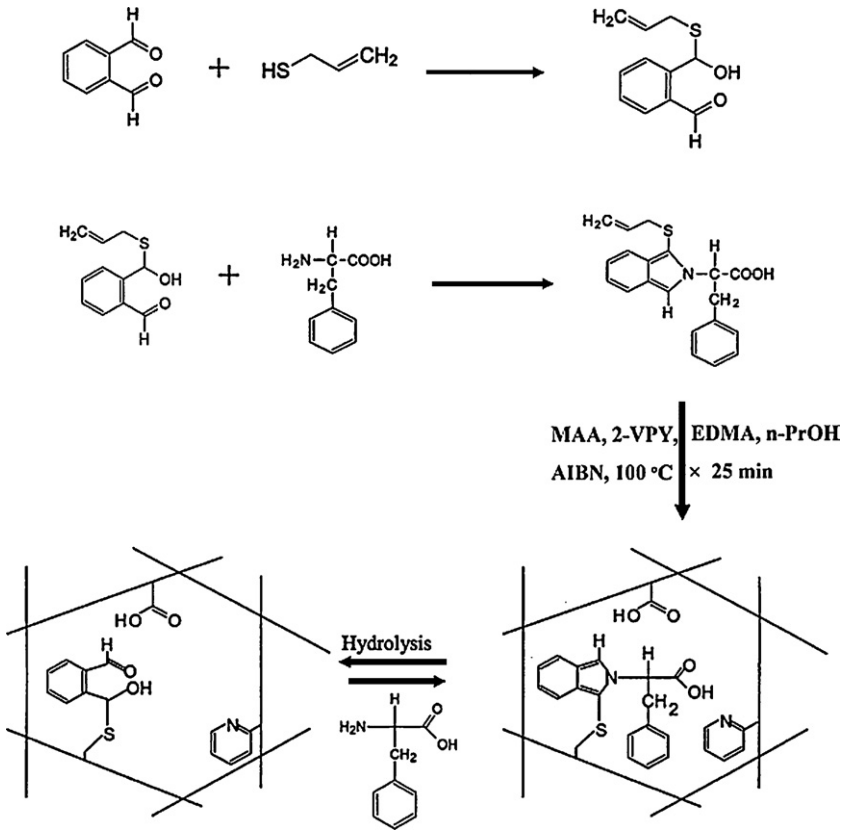

Fig. 6. Preparation of the o-phthalaldehyde basis of templated polymer (reprinted from [135] with permission of Elsevier).

of angiotensin I, angiotensin II, [Sar(1), Thr(8)] angiotensin, oxytocin, vasopressin, tocinoic acid, P-casomorphin bovine, P-casomorphin human, and FMRF amide were successfully carried out on this monolithic MIP column in CEC within 20 min. Although the analytes selected did not correspond to that of the template, the L-phenylalanine imprinted monolithic MIP column indeed was able to create the different type of selectivity for oligopeptides. Deng et al. [136] prepared a monolithic MIP column for the enantioseparation of ibuprofen in CEC by *in situ* copolymerization of 4-vinylpyridine and EDMA in the presence of (*S*)-ibuprofen as the template. The prepared 4-VPY monolithic MIP in CEC did yield better separation than that made with MAA. Most recently, a new method of preparing the monolithic MIP column, the MIP-derived silica monolithic capillary column, was developed for CEC by Ou et al. [137]. In this method, the vinyl groups were first introduced onto the silica monolith by immobilization of γ-MAPS. And then, the MIP film was coated onto the surface of the vinyl-modified silica monolith via an on-column copolymerization of MAA and EDMA in the present of templates and porogenic solvents. The baseline enantioseparations of Troger's base and tetrahydropalmatine were obtained within 4 min using the prepared silica monolithic MIP columns that molecularly imprinted with l-tetrahydropalmatine (l-THP) and (5*S*,11S)-(−)-Troger's base (*S*-TB), respectively. This method might provide a convenient and cost-efficient approach to develop a variety of new MIP columns for CEC by taking advantages of the merits of MIP and monolithic columns.

3. Recent application of monoliths in microscale separation

3.1. Monoliths for one-dimensional capillary liquid chromatography

Monoliths have the hierarchical mesoporous/macroporous structure of enhanced permeability, which leads to the fast mass transfer kinetics and lower backpressure during separation, and thus they can conduct separation fast and efficiently [138]. This property makes the monoliths an emerging choice to traditional packed columns for analysis of complex mixtures, such as drugs, metabolites and peptides. Since there have been lots of reviews on the use monoliths for pharmaceutical and biomedical analysis [3,13,139–142] here we mainly focused on the application of monoliths in proteome analysis, which greatly requires the high efficiency separation to resolve the extremely complex compositions of proteome.

Monolithic capillary columns based on PS-DVB are mostly used among all of the organic polymer-based monolithic capillary columns (both commercially available and homemade) in proteome analysis due to their facility of rapid and highly efficient separation performance of complex mixtures. Svec and Huber [143–148] have developed polymer-based columns and extended their use in proteome analysis [149]. As polymer-based monolithic columns could be applied over wider pH ranges, Huber and coworkers [150] used the monolithic PS-DVB capillary columns to analyze phosphorylated peptides

in alkaline eluent based on triethylamine-acetic acid (pH 9.2) in conjunction with detection in negative ion mode ESI-MS, which increased the detection limit greatly and 10 fmol detection limit was obtained for a phosphorylated peptide in β-casein digest. Due to the convenience of preparing a monolithic column within a slender capillary, Karger and coworkers [151] explored the use of a 20 μm i.d. polymeric PS-DVB monolithic capillary column for the LC-ESI-MS analysis of tryptic digest peptide mixture to improve the detection sensitivity. Efficiencies over 100,000 plates/m for peptide separation were achieved with optimized polymerization conditions and mobile phase composition, and high mass sensitivity (∼10 amol of peptides) in the MS and MS/MS modes using an ion trap MS was observed, which was up to 20 fold improvement over 75 μm i.d. capillary columns. The gradient nano-LC-ESI-MS 3-D overlay LC–MS chromatogram of a protein extract from a breast cancer tissue section digested with trypsin was illustrated in Fig. 7. Recently the same group also [32] developed ultratrace LC–MS proteomic analysis approach by using 4.2 m × 10 μm i.d. porous layer open-tubular PS-DVB capillary columns. 3046 unique peptides covering 566 distinct *Methanosarcina acetivorans* proteins were identified from a 50 ng in-gel tryptic digest sample.

Monolithic PS-DVB capillary columns are not only used as analytical columns, but also used as trap columns for sample injection with relatively large volume in nano-LC–MS/MS analysis due to its low backpressure at high flow rate, which increases the throughput of proteome analysis greatly [152,153]. However, many polymer-based SCX monolithic capillary columns are mixed modes with SCX and RP mechanisms because the polymeric monomers always have hydrophobic groups, which decreases the orthogonality in multidimensional separation with combination of SCX-RP. Sulfonate group is the most widely introduced in polymer-based SCX monolithic capillary columns but the sulfonate containing monolith is believed to swell excessively in aqueous buffer, which limits its usage for proteome

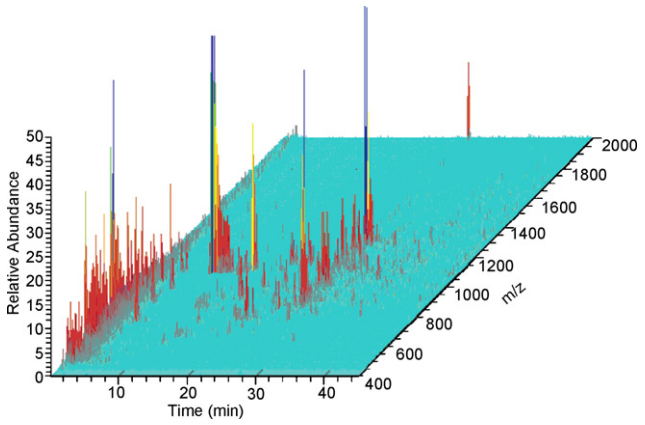

Fig. 7. Gradient nano-LC-ESI-MS 3-D overlay LC–MS chromatogram of a protein extract from a breast cancer tissue section digested with trypsin. 200 nL injected on the column; sample amount corresponding to an extract from ∼1000 cells. Mobile phase: (solvent A) 2% acetonitrile, 0.1% formic acid in water; (solvent B) 10% water, 5% 2-propanol, 0.1% formic acid in acetonitrile. Gradient: 0 min 5% B, 60 min 40% B; 65 min 90% (reprinted from [151] with permission of Elsevier).

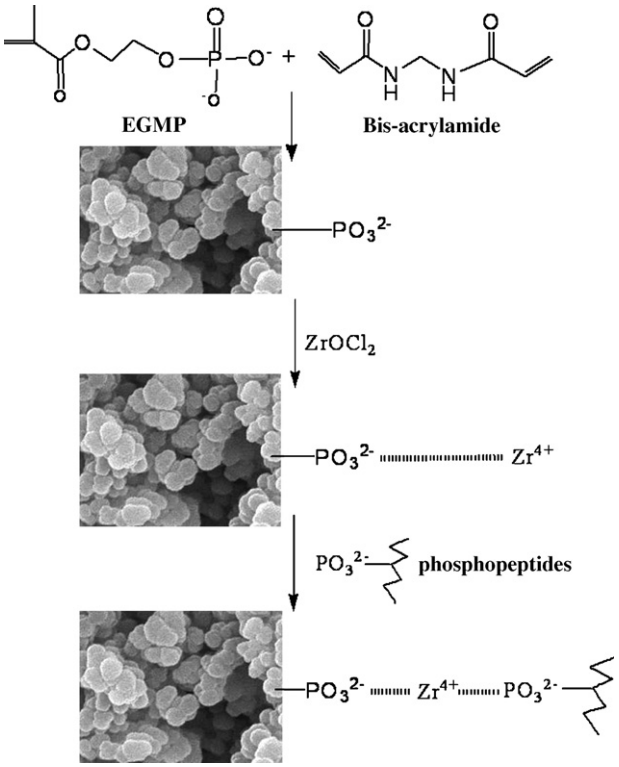

Fig. 8. Preparation of a phosphate monolithic column.

analysis greatly. Most recently, Dong et al. [154] prepared a phosphate monolithic capillary column via direct copolymerization of an ethylene glycol methacrylate phosphate (EGMP) and bisacrylamide in a trinary porogenic solvent consisting of dimethylsulfoxide, dodecanol, and *N*,*N*′-dimethylformamide, which showed less swelling and shrinking and was stable enough in different separation conditions for long time usage. Wang et al. used the phosphate monolithic capillary column as a trap column for automated injection in nanoLC systems [155]. The scheme for preparation of monolithic column and loading of peptides was illustrated in Fig. 8. This phosphate monolithic column showed higher dynamic binding capacity, faster kinetic adsorption rate with peptides, as well as great permeability than the columns packed with commercially available PolySulfoethyl A particles. It provided high resolution in the stepwise fractionation when applied to the online SCX-RP multidimensional separation, and a total of 1522 distinct proteins were positively identified from 5608 unique peptides at the false positive rate of only 0.46%. The stepwise chromatograms of an online multidimensional analysis of a tryptic digest were illustrated in Fig. 9. Due to the advantages of easy preparation and good performance in chromatographic separation, the phosphate monolithic capillary column may be widely applied in large-scale proteome analysis.

Although polymer-based monoliths have such advantages as good biocompatibility and wide application range of pH values, they also undergo shrinking or swelling in organic solvents and may contain domains of micropores negatively affecting the efficiency and peak symmetry. Silica monoliths

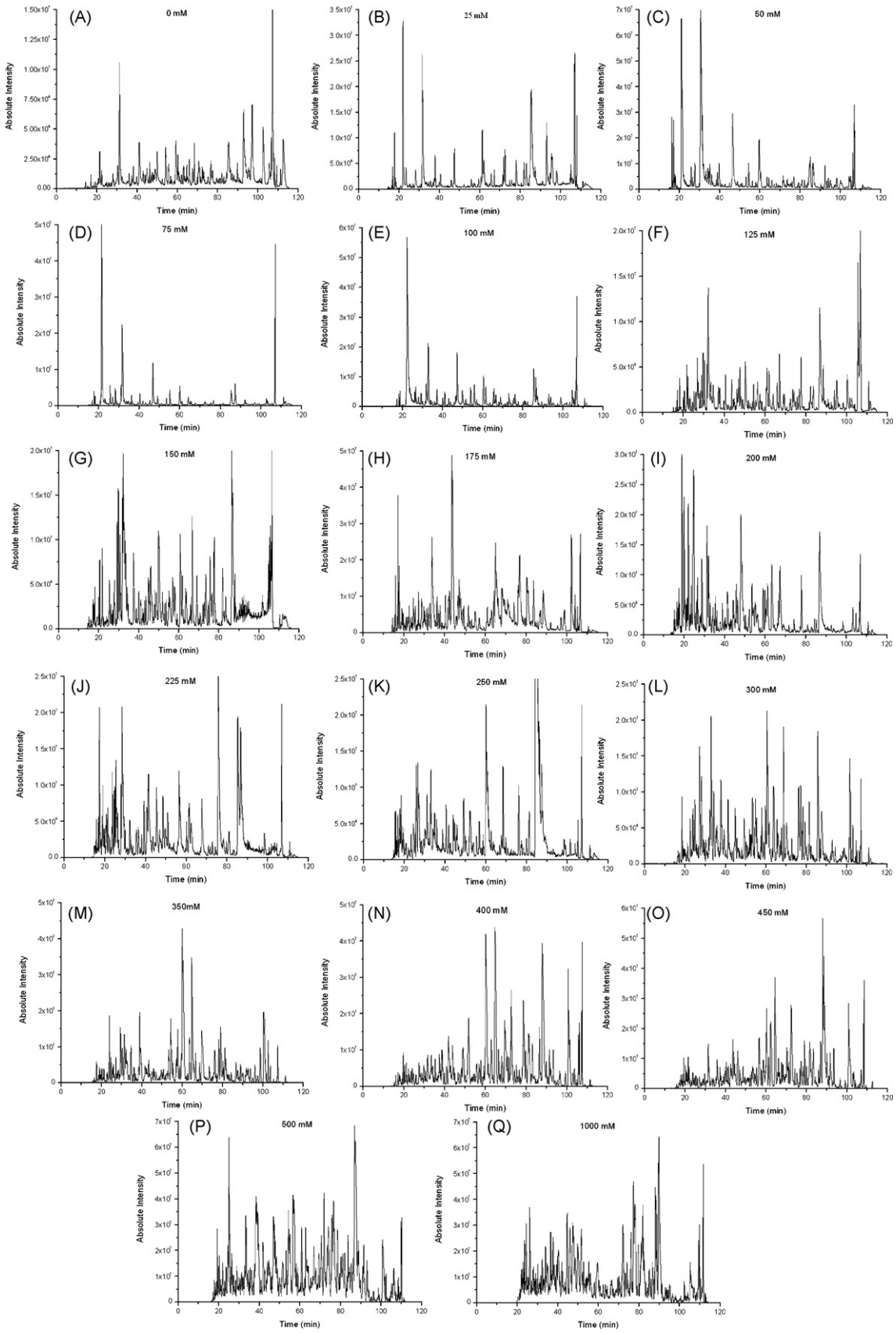

Fig. 9. Chromatograms of a 17-cycle online multidimensional analysis of a tryptic digest of yeast proteins.

are porous rods consisting of a silica skeleton with interconnecting macropores and have demonstrated high efficiencies and low backpressure as their bimodal pore structures can be controlled independently (through pores and mesopores). Xie et al. [156] constructed a silica-based monolithic column with an integrated emitter. Extremely sharp peaks were obtained for the separation of tryptic digest of BSA, indicating the high efficiency of the integrated column. The high separation efficiency of a 60 cm monolithic capillary column resulted in the identification of 1323 proteins through assignment of 5501 unique peptides from *Saccharomyces cerevisiae* in over 400 min gradient elution. Luo et al. [157] described the preparation and performance of 70 cm × 20 μm i.d. silica-based monolithic capillary LC columns. By connecting with a replaceable emitter, the monolithic capillary column could be readily interfaced with ESI-MS. The sensitivity and separation efficiency enabled the identification of 2367 different peptides covering 855 distinct *Shewanella oneidensis* proteins from a 2.5 μg tryptic digest sample in a single 10 h analysis. Since the monolithic columns can be prepared with smaller inner diameter fused capillary, a 10 μm i.d. silica-based monolithic capillary column with an integrated nanoESI emitter was therefore fabricated by Luo et al. [158], which allowed stable electrospray operation at a flow rate of 10 nL/min. 5510 unique peptides corresponding to 1443 distinct proteins were identified from a 300 ng tryptic digest of *Shewanella oneidensis* sample in a single 4 h nanoLC–MS/MS analysis, which showed the powerful separation efficiency and high detection sensitivity of monolithic columns with integrated monolithic ESI emitters.

Besides the improvement of MS detection sensitivity and separation efficiency by using smaller inner diameter monolithic columns in proteome analysis, the enhancement of selectivity for peptide separation has also been realized, such as in phosphor-proteome and phosphor-peptidome analysis, by utilizing the immobilized metal affinity monolithic capillary columns due to its high affinity for adsorption of phosphopeptides. Feng et al. [98] prepared a nanoscale IMAC column by immobilizing Fe^{3+} ions on an IDA-silica monolithic column and demonstrated the specific affinity to phosphopeptides from tryptic digest of α-casein in shotgun proteome analysis. Based on the database search and manual validation with conservative criteria, 29 mono-phosphorylated peptides were identified from the tryptic digest of a minute mouse liver lysate. Recently, Zhou et al. [159] observed that phosphopeptides had the strong and specific interaction with zirconium phosphonate-modified porous silicon. Based on this understanding, the zirconium phosphonate-modified poly(GMA-co-EDMA) beads were thus prepared for large-scale phosphoproteome analysis of mouse liver samples [160], and a phosphate monolithic capillary column was prepared by direct copolymerization of phosphate functional monomer (ethylene glycol methacrylate phosphate) and cross-linker (bis-acrylamide) in a trinary porogenic solvent [154] for analysis of protein phosphorylation in mouse liver lysate by identifying totally 118 unique phosphorylated peptides from a tryptic digest of only 10 μg mouse liver lysate.

3.2. Monoliths for multi-dimensional capillary liquid chromatography

Generally, for a comprehensive two-dimensional separation system, the separation in 2nd dimension requires to be finished as fast as possible because the most retained compound from 1st dimension must elute before the least retained compound of the next 2nd dimension separation. As having been described in afore part "Monoliths for fast separation", monolithic columns did have the potential to offer fast separation due to its macropore structure, which certainly matches the needs of fast separation in 2nd dimension for two-dimensional separation system. Additionally, the chemical flexibility of monolithic columns also makes monoliths the idea separation media to fit the orthogonal requirement of two-dimension separation. Thus, monolithic columns have been extensively used in two-dimension separation systems by coupled with different columns and operated in various separation modes, such as reversed-phase × reversed-phase, normal-phase × reversed-phase, ion-exchange × reversed-phase, affinity chromatography × reversed-phase, etc.

To achieve the best 2-D separation of PAHs from gasoline and gasoline exhaust, Murahashi [161] selected a pentabromobenzyl column as the 1st dimension and two commercial available C18 monolithic silica columns as the 2nd dimension with flow rate of 1 and 16 mL/min, respectively. In this combination, a factor of Sa/Sp, which represented the retention difference of alkanes/PAHs, was lowest for the 1st dimension and was very different from that on the C18 columns. The effluent from the 1st dimension was on-line injected into the 2nd dimension every 12 s. In this two-dimensional separation system, the orthogonality was from the difference of retention mechanism of polar and non-polar compounds on two different stationary phases. Meanwhile, the application of monolithic column in 2nd-D operated at high flow rate ensured the accomplishment of 2-D separation of PAHs from gasoline and gasoline exhaust. Similarly, Tanaka et al. [162] developed a comprehensive 2-D separation system by using C18 or (pentabromobenzyloxy)propylsilyl-bonded silica monolithic column as the 2nd dimensional column. Every fraction from the 1st dimensional column, packed with fluoroalkylsilyl-bonded silica particles, was subjected to the 2nd-D monolithic column. Monolithic silica columns in 2nd-D were operated at a high flow rate of up to 10 mL/min with the short separation time of 30 s that matched the elution of fractions every 15–30 s from the 1st-D run at a low flow rate of 0.4–0.8 mL/min. The peak capacity of ca.1000 in less than 60 min could be reached in that 2-D system. Interestingly, Ikegami et al. [163] developed a 2-D LC separation system by combined two chromatographic similar C18 monolithic columns in 1st and 2nd dimensions with a reasonable orthogonal property via applying gradient elution mode with different organic modifiers in mobile phases in two dimensions. Peak capacity of several hundred within 60 min was also successfully obtained on this 2-D LC system. While, an investigation on this platform also showed that the gradient elution could improve the resolution of polar samples, allowing more effective use of analysis time compared to isocratic sepa-

ration. The gradient operation at 2nd-D, even though the change in organic modifier concentration was much less than 1% during each run, seemed to be a convenient way to utilize the full capacity of 2-D LC system.

During 2004–2006, Dugo et al. [164–166] developed a normal-phase (NP) × reversed-phase (RP) 2-D separation system by coupling a microbore silica column as the 1st dimension run in NP mode and a C18 monolithic column as the 2nd dimension run in RP mode. This combination of NP and RP could be considered orthogonal and complemental in the separation of complex mixtures with different polarity and hydrophobicity. Advisably, the adoption of microbore column in 1st dimension permitted the small volume injection onto the 2nd dimensional column, making the transfer of incompatible solvents from the 1st to 2nd dimension possible. Also, due to the use of monolithic column in 2nd dimension, high flow rate was allowed in 2nd dimension to fast the analysis of analytes but without loss of resolution. This comprehensive NP × RP 2-D LC system was successfully applied in analysis of cold-pressed lemon oil.

Hata et al. [167] established a comprehensive 2-D LC system with a titania column as the 1st dimension and a monolithic column as the 2nd dimension for the on-line analysis of phosphopeptides. By using this 2-D HPLC system, phosphopeptides could be specifically isolated from non-phosphorylated peptides by the 1st-D titania column, and then could be further fast separated in the 2nd-D monolithic column. The separation of proteolytic digest of β-casein was finished within 30 min on this comprehensive 2-D LC system, and all phosphopeptides from β-casein were efficiently isolated and further off-line identified by MALDI-TOF-MS. Although this work was focused on the establishment of 2-D LC system by selecting two orthogonal separation columns with titania column as 1st dimension, the superior affinity of titania to phosphorylated peptides and the high throughput 2-D platform should promise a further extensive application in proteomics and peptidomics.

Related with proteomics, the "hottest" research topic in world, Kimura et al. [168] established a 2-D LC system by using a monolithic column as the 2nd dimension and a polymeric cation-exchange beads packed column as the 1st dimension in orthogonal requirement (ionic exchange × reversed-phase) for two-dimension analysis of tryptic digest of bovine serum albumin. Every fraction from the 1st-D column eluted at 50 μL/min with a salt gradient was subjected to the 2nd-D separation at 5.0 mL/min with an acetonitrile gradient in the presence of formic acid. Two-minute fractionation in the 1st-D, 118-s loading, and 2-s injection by the 2nd-D injector, allowed 1 min for gradient separation in the 2nd-D, resulting in a maximum peak capacity of ca. 700 within 40 min. Wienkoop et al. [169] applied the SCX × monolithic RP 2-D separation platform in proteomics for analysis of a leaf protein extract, and 1032 unique proteins were successfully identified in a single 4 mg total protein plant leaf tissue sample. The further application of this 2-D LC system was performed by Morisaka et al. [170] in the analysis of tryptic fragments from BSA by peptide mass fingerprinting too, and a good sequence coverage (81%) was obtained. By taking advantages of such high sensitivity with MS detection and the good permeability of a porous layer coated capillary monolithic column with small inner diameter, Luo et al. [31] developed a 2-D SCX/PLOT LC/MS platform by utilizing a 3.2 m × 10 μm i.d. porous layer PS-DVB monolithic capillary column as the 2nd-dimension for 2-D ultratrace proteomic analysis.

Hu et al. [171] improved the performance of our previously established 2-D HPLC system for the separation of traditional Chinese medicines (TCMs) by adopting a CN column as the 1st-D column and a silica monolithic column as the 2nd-D column. Based on the developed 2-D HPLC system, the separation performance for TCMs was greatly enhanced not only because of the decrease of analysis time resulting from the application of silica monolithic column as 2nd-D column with good permeability and high efficiency, but also the improvement of separation performance of TCMs on the 1st-D column. The improved 2-D LC system was successfully applied to the analysis of methanol extracts of two umbelliferae herbs Ligusticum chuanxiong Hort and Angelica sinensis (Oliv.) Diels. Based on the orthogonality between affinity and reversed-phase interactions, we developed a new 2-D LC system for the separation of TCMs [172] by using a HSA immobilized affinity column as 1st-D column to screen the biological active components of TCMs based on the affinity interaction between TCMs and HSA, and a monolithic ODS column as the 2nd-D column to further separate the fractions eluted from the 1st-D HSA column. This work opened an avenue for high throughput analysis of TCMs with the concept of biological fingerprinting analysis without purification or pretreatment. Similarly, another 2-D LC platform for analysis of glycol conjugates was described [173], where a lectin affinity column was applied as the 1st dimension and a RP monolithic column as the 2nd dimension. Recently, Su et al. [174] carried out a 2-D LC platform for the biological fingerprinting analysis of TCMs with a DNA immobilized column as the 1st dimension and a silica monolithic column as the 2nd dimension. It was found that seven compounds in Coptis chinensis Franch including berberine, palmatine and jatrorrhizine, and 14 compounds in *Rheum palmatum* L. including aloe-emodin, rhein, emodin, chrysophannol-8-*O*-glucophranoside and physionl-8-*O*-glucophranoside exhibited the activity to interact with the immobilized DNA.

Besides the applications of monolithic columns in comprehensive 2-D LC systems, another application of monolithic column in 2-D CE-CEC system was described by Zhang and El Rassi [175]. They developed a novel on-line 2-D CIEF-CEC separation scheme for proteomics by coupling of capillary isoelectric focusing (CIEF) and capillary electrochromatography (CEC) via a nano injector valve. The scheme was illustrated in Fig. 10. As can be seen in Fig. 10, the CIEF ran in the 1st dimension, while CEC orthogonally operated in the 2nd dimension using a neutral C17 monolithic column. The application of the RP monolithic column in the 2nd dimension ensured the rapid isocratic separation of proteins and peptides, rapid solvent change, column equilibration and avoided lengthy gradient elution. The solvent used in the 1st dimension (CIEF) was a weak eluent to the 2nd dimension (CEC), which allowed a smooth transferring of the focused fractions from CIEF to CEC without inducing band broadening but instead of generating a sharpened zone. The transferred protein fraction from CIEF column to the CEC monolithic column actually stayed tightly adsorbed

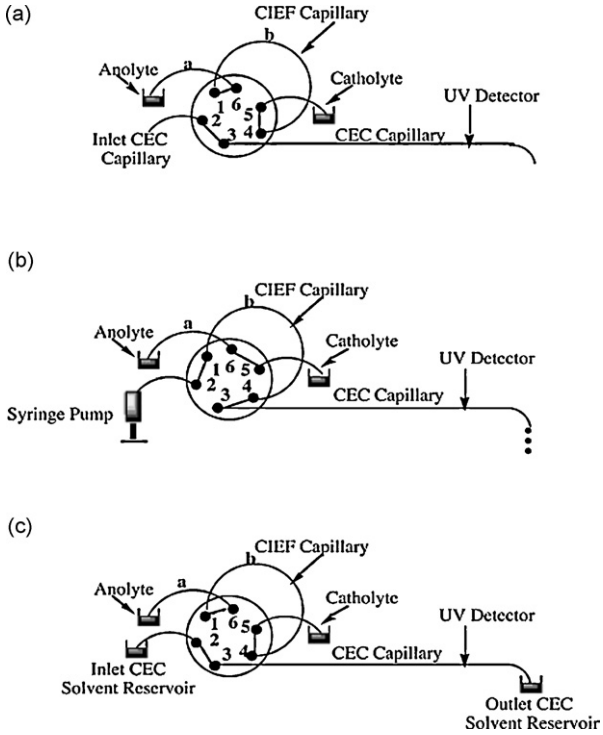

Fig. 10. Schematic illustration of the 2-D platform, which couples CIEF as the 1st dimension to CEC as the 2nd dimension using a nanoinjector valve with 25 nL port-to-port volumes. The effective CIEF capillary length (e.g. 38 cm) is the sum of segment a of 4 cm and segment b of 34 cm (reprinted from [175] with permission of ACS).

on the inlet beginning of the CEC column until it was eluted and separated into its protein components with a hydro-organic mobile phase. The estimated theoretical peak capacity of the 2-D CIEF × CEC platform could reach up to 54,320 by multiplying $n_{(CIEF)}$ (=560) and $n_{(CEC)}$ (=97), which was more than needed for proteomics analysis so far.

3.3. Monolith for fast separation in capillary electrochromatography

Capillary electrochromatography, which combines the features of liquid chromatography and capillary electrophoresis, permits the extremely high efficiency separation of ionic compounds and neutral compounds due to the plug profile flow of mobile phase driven by the electroosmotic flow (EOF) in a capillary column. In the last decade, the monolithic columns have been widely adopted in electro-driven microscale separation systems due to the unique features of easy preparation, fritless design, and versatile surface chemistries. Especially, attributed from the distinct merits of fast mass transfer, low backpressure and good permeability with inherent hierarchical mesoporous/macroporous structure, the monolithic columns have been thought to be the idea media for fast and high efficiency separation by combining the plug-like characteristic of EOF in CEC [128,176,177].

Van Deemter plot was commonly used to describe column performance by plotting the height equivalent to a theoretical plate (HETP or H) against the linear flow velocity (u) of mobile phases. The general form of the van Deemter equation is given by $H = A + B/u + Cu$, where A, B and C terms, respectively, represent the eddy diffusion, longitudinal diffusion and mass transfer between stationary and mobile phase as well as within the mobile phase. The minimum of H represents the maximum of column efficiency at an ideal flow velocity. The lower of the van Deemter curve, on the other hand, also represents the higher column efficiency even at higher flow rate of mobile phase, which leads the fast separation of analytes in CEC. So, to accomplish the fast analysis, meanwhile without the dramatic loss of column efficiency, the C term is the key but also the limitation to all chromatographic columns.

Monolithic columns with the macroporous structure, can provide smaller values of C terms or flatter curves of van Deemter plots for CEC separation. In Fig. 11A, a typical Van Deemter plot retrieved from our previous work [34] on a sulfate-polymer monolithic column has been demonstrated. As can be seen, the curves of van Deemter plots for three testing compounds remained almost flat when flow velocities were over 2.5 cm/min. And, for thiourea, the unretained compound, its estimated column efficiency of theoretical plate was up to 280,000 plates/m. Both benefits of flat feature of van Deemter curve and high

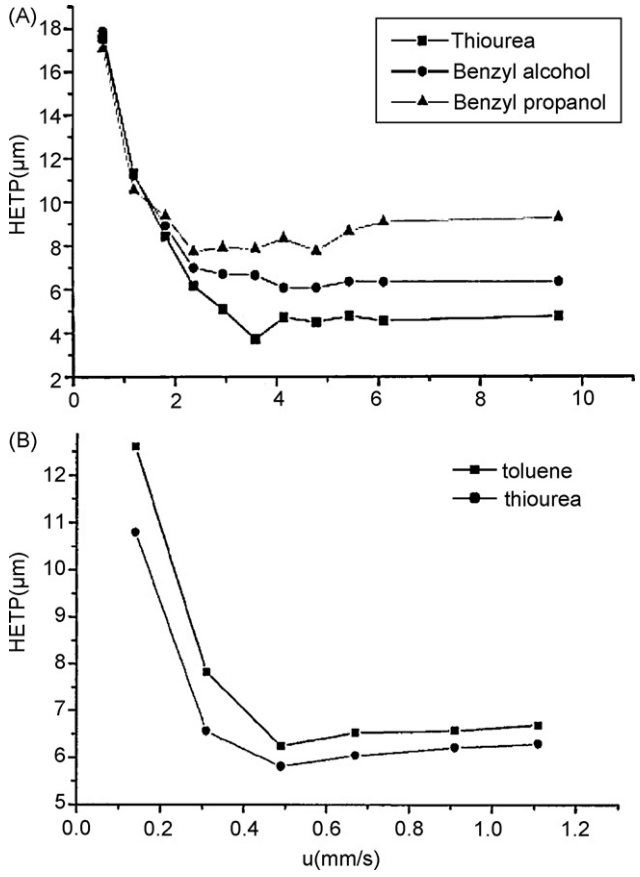

Fig. 11. The dependence of HETP vs linear velocity on polymer monolithic columns in CEC. (A) Experimental conditions: mobile phase, 2 mM phosphate buffer containing 40% ACN, pH 7.0; temperature, 25 °C; applied voltage, from 1 to 20 kV; injection, 1 kV for 1 s; detection wavelength, 214 nm. (B) Experimental conditions: mobile phase, 10 mM phosphate buffer (pH 4.7), 20% ACN; applied voltages, from 2 to 12 kV; detection wavelength, 214 nm; injection, 3 kV × 2 s (Fig. 10B, reprinted from [89] with permission of Wiley).

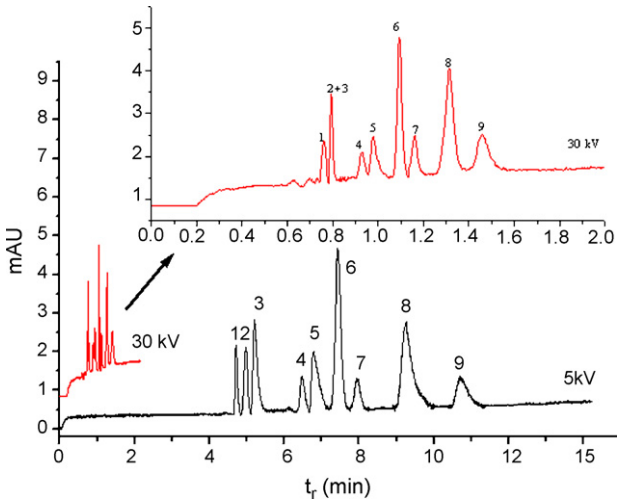

Fig. 12. The fast separation of basic compounds on RP/SCX monolithic column in CEC. Experimental condition: column, 10 cm (total length 30 cm) 100 μm i.d. × 375 μm o.d.; temperature, 25 °C; mobile phase, 16 mM phosphate buffer containing 60% acetonitrile, pH 3.0; detection wavelength, 214 nm; applied voltage, 5 kV; injection, 2 kV for 2 s. Peaks: 1. pyridine; 2. thiourea; 3. 3-methyl pyridine; 4. 3-amino pyridine; 5. 2,4,6-trimethyl pyridine; 6. 2,4-dinitro aniline; 7. 2,6-dinitro aniline; 8. 2,4-diamino toluene; 9. 2,6-dichlor-4-nitro aniline.

column efficiency at the high flow velocity range strongly support the fast separation of analytes upon monolithic columns in CEC. Thus, a fast separation of 9 basic compounds was achieved within 2 min, as shown in Fig. 12 (unpublished), based on this RP/SCX monolithic column in CEC. These results indicated that the absence of inter-particular volume in the monolithic column forced mobile phase flowing through the porous monolith medium, thus enhanced the mass transfer rate due to the convective interaction. However, because of the limitation of applying even higher voltages at the both ends of monolithic column as well as the impact of joule heat, the further enhancement of separation speed for CEC was not pursued. Recently, Ding et al. [89] prepared a organic–inorganic hybrid monolithic column. Compared to the plots in Fig. 11A, a very similar van Deemter curve for two compounds on this hybrid monolithic column was obtained and illustrated in Fig. 11B. It was clearly shown that no apparent loss of column performance was observed when the velocity was higher than 0.8 mm/s. Thus, the fast and efficient separation of six aromatic acids on this hybrid monolithic column was also obtained using acidic mobile phase with column efficiency up to 160,000 plates/m by CEC.

By taking advantage of monolithic column and CEC, Ping et al. [178] performed a separation of nucleosides on a methacrylate-based monolithic column in CEC. Through increasing the content of charged monomer in the reactant mixture for the preparation of the monolithic column, the high EOF associated fast separation for nucleoside was achieved. Schweitz et al. [128] realized a very rapid CEC mode enantioseparations of propranolol using short super-porous monolithic MIP columns, where the enantiomers of propranolol were resolved in less than 1 min. In 2002, Bedair and El Rassi [179] prepared a novel monolithic column with long alkyl chain ligands (C17) by *in situ* copolymerization of pentaerythritol diacrylate monostearate (PEDAS) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) in a ternary porogenic solvent consisting of cyclohexanol/ethylene-glycol/water. Here, AMPS was used to generate EOF, and PEDAS was introduced to provide the non-polar sites for chromatographic retention. By adjusting the content of AMPS in polymerization solution as well as the composition of porogenic solvent, monolithic columns with different EOF could be readily prepared. Up to ca. 3.4 mm/s of EOF velocity could be obtained when the content of AMPS increased to 2% (wt) of copolymerization mixture. An ultrafast separation on the time scale of seconds of 17 different charged and neutral pesticides and metabolites were carried out by using a short monolithic capillary column of 8.5 cm × 100 μm i.d. By applying CEC in microchip, Throckmorton et al. [180] realized a fast separation of peptides in 45 s based on photopatterned rigid polymer monoliths. Zhong and El Rassi [97] reported a novel polar monolithic capillary column for normal-phase CEC (NP-CEC) for the analysis of polar compounds including mono- and oligosaccharides, peptides, and basic drugs. The investigation demonstrated that a multistep-gradient elution in NP-CEC could bring about the rapid separation of a large number of polar species in a single run. Recently, Lu et al. [181] developed a pressurized capillary electrochromatography (pCEC) with monolithic column for the rapid separation and determination of five structurally related anthraquinones in Rhubarb. Due to the unique pore structure with high permeability and favorable mass transfer characteristics of the monolithic stationary phase, a rapid baseline separation of the five anthraquinones was obtained within 5 min with the separation voltage of −20 kV in 10 mmol/L phosphate buffer (pH 6.2) containing 65% acetonitrile. Most recently, Ou et al. [134] reported the enantioseparation of two chiral compounds, Troger's base and tetrahydropalmatine, on the (5*S*,11*S*)-(−)-Troger's base and l-tetrahydropalmatine imprinted monolithic capillary columns with CEC, respectively. The monoliths were prepared by *in situ* thermally initiated copolymerization of methacrylic acid (MAA) and ethylene dimethacrylate (EDMA). After optimizing the ratio of porogens (toluene and dodecanol), the obtained monolithic capillary columns show good flow-through property and enantioselectivity. Moreover, a fast separation was obtained within 4 min by applying higher voltage and assisting pressure of 6 bar.

3.4. Monoliths in microfluidic devices

In the last decade, considerable attention has been placed on microfluidics devices in life science, analytical chemistry, and biochemistry fields because of the advantages of smaller sample volume requirement, reduction of solvent consumption, shorter analysis time, and on-site analysis with portability. Various microfabricated components including channels, chambers, valves, pumps, mixers, filters, heaters, detectors can be integrated in a single chip substrate. To perform the small volume fluid manipulation, microchannels are the most widely incorporated ones in the sense of delivering sample fluids on-chip for separation. Silica, glass, and polymers were generally considered as on-chip channel structural materials. For chro-

matographic separation on microfluidic devices, however, the low surface-to-volume ratio of open microchannels and the difficult preparation of particulate packed channel both are the real limitations. Monoliths as stationary phase have been widely applied in liquid chromatography and capillary electrochromatography as afore described. The obvious merits of monoliths in separation processes include the fritless design, ease of preparation with adjustable porosity and pore diameter along with good permeability. Thus, the adoption of monoliths in microchip for improving the separation resolution, efficiency and capacity in such limited short channels is an inevitable selection. Because the reactants of monoliths can be easily introduced into the microchannels on microfluidic devices, the preparation of microchannel monolithic columns were thus convenient to be carried out.

The early work of using monolithic matrix as the separation stationary phase in microfluidic device was realized by Ericson et al. [182], who demonstrated the application of polyacrylamide-based monoliths in quartz microchips for electro- and pressure-driven chromatographic separation. In this work, the monolithic stationary phases were synthesized within sinuous microchannels, which were etched out from the quartz chip substrate with a size of ca. 30.6 cm length × 40 μm width × 20 μm depth and pretreated with 3-(methacryloxy)propyl trimethoxysilane, by *in situ* copolymerization of a mixture solution consisting of methylacrylamide (MAA) and piperazine diarcylamide (PDA, cross-linker) in the presence of isopropyl acrylamide (NIPAAm), vinylsulfonic acid (VSA) or diallyl dimethylammonium chloride (DDA) via a free radical polymerization initiated by ammonium peroxosulfate (APS)-tetramethylethylenediamine (TEMED). In this preparation, the anchoring of polymer monoliths on the pretreated channel surface and the controlling of the location of polymeric mixture in channel were necessary. The prepared monolithic stationary phase in microchip demonstrated a comparable good chromatographic performance to that in a fused silica capillary. A separation efficiency of 6300 plates (350,000 plates/m) was obtained for the unretained analyte uracil. A fast separation of uracil, phenol, and benzyl alcohol has been accomplished in less than 20 s.

Svec and his coworkers have also contributed broadly to the fabrication of microchannel monolithic stationary phases in microfluidic devices for chromatographic separation and sample solid-phase extraction [183–186]. They introduced the photoinitiated free radical polymerization for the preparation of the monolithic separation media within microchannels of quartz or glass microchips. Before the preparation of monoliths within microfluidic devices made from these inorganic materials, the inner walls of the microchannels were functionalized by using the well-known silane primer reagent 3-(trimethoxysilyl)propyl methacrylate to enable the covalent binding of monoliths onto the inorganic substrate via vinyl groups [180]. The use of functional methacrylate monomers, such as glycidyl methacrylate, 2-hydroxyethyl methacrylate, butyl methacrylate, and 2-acrylamido-2-methyl-1-propanesulfonic acid, 2-(methacryloxy)ethyltrimethylammonium chloride has been used for the preparation of monoliths for microfluidic devices with reactive, hydrophilic, hydrophobic, and ionizable functionalities, respectively. However, to avoid the high cost of the multi-step fabrication of inorganic microfluidic devices, the use of thermoplastic polymer materials together with inexpensive 'dry' techniques, such as injection molding or hot embossing demonstrated its superiority. Consequently, the attachment of monoliths within the microchannels of organic polymeric microchip was a big challenge for the development of monolithic microchannels on microfluidic devices due to the notoriously poor material compatibility of most polymeric materials with the native walls of plastic devices. For this challenge, Stachowiak et al. [187] successfully demonstrated a simple UV-initiated grafting method for the preparation of porous polymeric monolith within the microchannels of a cyclo olefin copolymer (COC) plastic microchip. Briefly, a thin layer of polymer with a multiplicity of pendent double bonds was photografted on the wall of plastic channel by using a mixture of ethylene diacrylate (EDA) and methyl methacrylate (MMA), and then followed by an *in situ* UV-initiated polymerization of a mixture consisting of butyl methacrylate (BuMA), EDMA, 1-decanol, and 2,2-dimethoxy-2-phenylacetophenone (DMPAP) to obtain the monoliths within the designed microchannel by using a photomask. As characterized by using scanning electron micrographs, the grafted polymer on wall played an important role in preventing the formation of voids at the monolith-channel interface that had usually encountered in bulk polymerization within untreated plastic channels due to the incompatibility between materials of monoliths and chip.

Recently, Ro et al. [188] and Liu et al. [189] functionalized the microchannel by using the photoinitiated graft polymerization method with a mixture of TMPTA, acetone and benzophenone to promote the bonding of the monoliths as described by Stachowiak et al. [187]. The surface functionalized channels were filled with a mixture of EHMA, EDMA, porogenic solvent, and DPA as an initiator to form the array of poly(ethylhexyl methacrylate-co-ethylene dimethacrylate) monolithic microchannel on a chip, which was then applied on the interface with a MALDI-TOF and an ESI mass spectrometry system for analysis of peptides mixtures. Bhattacharyya et al. [190] realized the solid-phase extraction of nucleic acids in a COC microfluidic device with a polymeric silica particle fixed monolithic matrix. The COC channel surface was first modified by using a benzophenone-initiated surface photopolymerization process with a mixture of EDA, MMA and benzophenone. Then the solid-phase bed was formed within a channel of the device by *in situ* photoinitiated polymerization of a mixture of methacrylate and dimethacrylate monomers along with silica particles. The solid-phase prepared by this method was applied for extraction and elution of nucleic acids in the microchip.

Besides using COC as the material of microfluidic devices, polydimethylsiloxane (PDMS) was another important material for fabrication of microfluidic devices. However, microdevices fabricated from PDMS generally exhibited the same inherent weaknesses of extreme hydrophobicity as COC, which made the microchannels difficultly to be filled with aqueous mobile phases and generate stable and controllable EOF. In terms of PDMS

microfluidic devices, Zeng et al. [191,192] developed a simple and feasible way to prepare the γ- or β-CD bonded monolithic column on a PDMS microfluidic device. The channel surface of PDMS microfluidic device was first pre-activated by UV light to induce silanol groups. And then, the PDMS microchannel was pretreated with 3-(trimethoxysilyl)-propyl methacrylate. After that, a mixture of acrylamide, AMPS, allyl-γ- or allyl-β-CD, APS, TEMED, and the cross-linker agent of Bis was sucked into the microchannel and polymerized within half an hour. Here, the incorporated allyl-γ- or allyl-β-CD was not only used as a multifunctional cross-linker in PAA gel to control the size of the pores, but also played as a chiral selector for enantioseparation. The performance of these microchannel monolithic columns was confirmed by the successful separation of fluorescein isothiocyanate (FITC)-labeled DNs-AAs.

Compared to organic continuous polymeric monoliths, silica-based monoliths prepared by sol–gel process can provide better mechanical strength and superior durability toward various solvents. The methods for preparation of silica-based monolithic columns by sol–gel process in capillaries through hydrolysis, condensation and phase separation of alkoxysilane solution could be transferred to microchannel in microfluidic devices. However, to obtain the desired monolith at a certain location in a microchannel was more difficult than that for obtaining a polymeric monolith at designed part of a channel by using the partial exposing technique with a photomask. Breadmore et al. [193] fabricated silica monoliths on a microchip by adapting the approach of silica monolithic columns in capillary developed by Tanaka and his coworkers [80]. In this method, the alkyl silane TMOS was mixed with a water-soluble poly(ethylene oxide) (PEO) under an acidic condition, and let it pre-react for about 30 min at 0 °C before introduced into the channel on a microchip by a syringe. Ishida et al. [194] introduced an octadecyl-modified monolithic silica microchip LC with a porous monolithic silica column prepared within a serpentine separation channel of a microchip. In this study, monolithic silica column was fabricated in a microchannel by using air pressure control technique to adjust the position of the silica sol in the channel.

4. Conclusions

The preparation and application of monolithic matrices in the field of chromatographic separation have gained increasing interest during the last 5 years (2002–2007) due to the distinct features of porous monolithic strategy, such as the facile preparation, versatile surface chemistry, fritless design, good permeability and fast mass transfer. Monolithic stationary phases have been widely used in fast and high efficiency one- and multi-dimensional separation systems, miniaturized devices, and hyphenated systems coupled with mass spectrometers. The developing technology for preparation of monolithic stationary phases is revolutionizing the column technology for the separation of complex biological samples, such as the complex mixtures for proteome, peptidome and metabolome analysis. Direct synthesis is a convenient and versatile route to prepare the monolithic columns for microscale separation. Whereas, obtaining desired monoliths with certain chromatographic properties is not easy to success because of the complexity of direct synthesis, for instance the dissolvability of long chain alkyl reagents suffered for preparing either organic polymer- or silica-based monoliths with enhanced hydrophobicity. On the other hand, the functional groups introduced into monoliths via direct synthesis are likely merged by the polymer mass rather than exposing on the monolith surface to fully play as the functional ligands or interacting sites with analytes. To well control the direct synthesis of monoliths to obtain the desired monolithic stationary phases with expected properties, the further and deep investigation are required yet through the principles to practical processes. Compared to the direct synthesis, the post-modification or post-functionalization of monoliths certainly represents a complementary and flexible technique for the preparation of monolithic stationary phases by tailoring the versatile surface chemistries. Post-modification of monolithic columns allows complete independent control of the porous properties and the chemical, thus the re-optimization of the porous properties of a monolithic column can be avoided rather than synthesize again each time when any change of chromatographic functionality is desired. However, the amount of the active sites on the synthesized monolithic matrices also restricts the post-modification of monoliths for obtaining stationary phases with high capacities at a certain extent. Either increasing the number of active sites without sacrificing the properties of monoliths prior to the post-functionalization in the steps of direct synthesis or developing/adopting post-modification approaches essentially bases on the presented sites/groups of polymer substrates, such as the photografting approach of polymers via hydrogen abstraction from the substrates. Monolithic columns with longer length, smaller inner diameter and specific selectivity to peptides or enantiomers will play the very important role in hyphenated systems for the requirement of throughput, efficiency, resolution, selectivity and sensitivity in the separation of complex biological samples, such as the complex mixtures of peptides for proteome analysis, and/or enantiomers. Since monolithic media can be easily prepared within channels of even very narrow dimensions by *in situ* direct synthesis, the monolith-based technology is obviously very suitable for the further development of miniaturized microfluidic devices. Even though the better understanding and controlling on the preparation of monolithic stationary phases yet require, there is no doubt that the development of monolithic stationary phases has opened the new avenue in chromatographic separation science and is in turn playing much more important roles in wide application area of CLC, CEC, and microfluidic devices.

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