Monolithic Stationary Phases in Classic and Chiral Pharmaceutical Analysis with CEC and pCEC

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Abstract

New stationary phases play an important role in the evolution of separation methods. The use of monolithic phases in capillary columns has become rather widespread. The ease of their preparation, their versatility, and the abundance of available chemistries increase their attractiveness, even dethroning the particulate phases. This review paper overviews the different types of monoliths used in capillary electrochromatography and pressurized capillary electrochromatography for the enantiomeric separation of chiral molecules and the analysis of pharmaceutically relevant molecules. The diverse methods of monolith preparation as well as the different types of used materials are discussed, as well as the ways they can be modified to fulfill given analysis needs (e.g., selectivity, efficiency of separation, speed of analysis, and economical interest). In addition, some of the advantages and drawbacks of the different monolithic materials are mentioned.

Introduction

Capillary electrochromatography (CEC) is a technique derived from capillary electrophoresis (CE). In CEC, a capillary column containing a stationary phase is submitted to a high electric field, which causes an electro-osmotic flow (EOF) (1,2). This EOF is the driving force of the mobile phase and therefore also contributes to the mobility of the analyzed compounds. Because the capillary contains a stationary phase with which the analytes can interact, additional separation possibilities are provided compared to CE.

Pressure-assisted capillary electrochromatography (pCEC) can be described as the combination of capillary liquid chromatography (CLC) and CEC. The mobile phase is driven through the column by both voltage-powered EOF and a pressurized flow. The technique can either be executed on an LC system over which a voltage is applied, or on a CE instrument, where an additional pressure is provided at the inlet of the column.

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The advantages of these miniaturized techniques compared to the more conventional formats are the smaller sample and mobile-phase consumption (3). The flatter flow profile compared to pressure-driven flows moreover enhances the efficiency (4).

Monoliths are continuous stationary phase beds within the capillary. The use of retaining end frits which are required in particle-based columns, but which can lead to bubble formation or adsorption of analytes, is thus avoided (3,4). Moreover, preparing a monolithic capillary column is often a relatively more straightforward process compared to the fabrication of particle-based stationary phases (1,2,4). Packing and retaining the beads of the particulate stationary phases in the capillary is indeed a technical challenge (1,2,5). Monolithic materials are known to be highly permeable, to uniformly fill the capillary, to display a low backpressure, and to have a relatively high phase ratio and sample capacity, which result in high retentive capacity and selectivity (4–6). During the last decennia, monolithic stationary phases have become well-established in electrochromatography (2,4). Columns of various shapes and lengths can easily be synthesized (1). By controlling their chemical composition, which can easily be done, the properties of the columns can be tailored [e.g., the EOF strength, the separation functionalities, the total porosity or the pore structure (1,2)]. Two types of pores are usually distinguished in monolithic phases: the larger pores, or through pores, through which the mobile phase flows, and that are the reason for the typical low back pressure and high permeability; and the smaller pores, or mesopores, which determine the large surface available for interaction (4,7). Monoliths are, therefore, said to possess a bimodal pore structure (Figure 1).

Monolithic stationary phases are usually divided into four categories: the inorganic monoliths, the organic monoliths or polymers, the molecularly imprinted polymers, and the particle-fixed monoliths (Figure 1). The inorganic monoliths are usually based on silica and are prepared by a sol-gel process. They can be further subdivided in stationary phases containing only silica, and the so-called hybrid phases, which also include organic moieties. The organic or polymer-based monoliths are prepared via a simple in-situ polymerization reaction and can be further functionalized either within the polymerization

reaction or in a post-polymerization step. Molecularly imprinted polymers have a different way of being functionalized: their structure contains three-dimensional recognition sites, which are obtained by including templates during the synthesis of the monoliths, which are removed before analysis. Particle-fixed phases, finally, can be seen as an improvement of silica-based particle-packed columns because they do not require frits: the particles are fixed by embedding them in silica- or polymer-based networks. These four groups of monoliths as well as their properties will be discussed in this review. Furthermore, the possibilities of transforming the monolithic stationary phases to acquire different chromatographic properties are also described.

The focus of this review is the application of monolithic stationary phases in CEC and pCEC for the analysis of molecules with a pharmaceutical interest and the enantioseparation of chiral molecules. The literature considered is mainly limited to the period since 2005.

Chiral separations are important, because enantiomers can have different activities when exposed to a natural environment (5). Often only one enantiomer has the desired effect, whereas the other has no effect, or an unwanted or even toxic effect. Each enantiomer must thus be assayed individually. Enantiomers have the same physico-chemical properties and therefore can not be separated using classic non-chiral CEC methods. Enantiomer separation can either be carried out via direct or indirect methods. An indirect method consists of adding an enantiomeric pure derivatization reagent to the sample, which forms diastereomers with the enantiomers present in the sample (8). The diastereomers have different physicochemical properties and thus separation becomes possible on a classic non-chiral system. Because of a number of drawbacks, among which the high cost of the enantiopure reagent and the possible contamination of these derivatization reagents, resulting in modified reaction products, the indirect method is not preferred, certainly not in an industrial environment. The direct separation of enantiomers is performed by adding a chiral selector to either the stationary or the mobile phase. When adding the chiral selector to the mobile phase, the selector is lost with it after analysis. It is therefore more cost-effective to bind or coat chiral selectors to the stationary phase.

Analysis of pharmaceutical samples is relevant in several fields, like drug development, drug safety assessment, quality control, and toxicity studies amongst others. A miniaturized technique like CEC requires only small sample and mobile phase volumes, is fast, and might result in highly efficient analyses, which are quantitative, reproducible, and robust. All these assets make CEC a potentially valuable technique in the analysis of pharmaceutically relevant molecules.

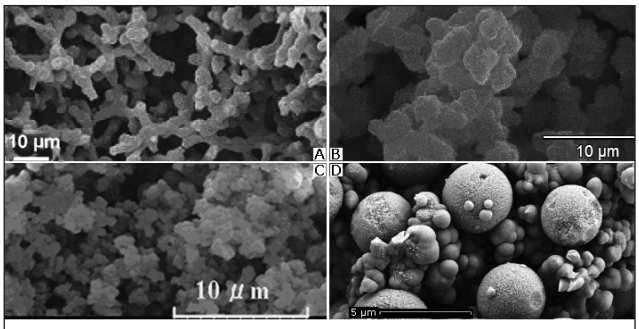

Figure 1. The four types of monolithic phases used in the analysis of drug molecules and chiral compounds. The bimodal pore structure typically present in monolithic phases is visible. A silica-based monolith post-functionalized to permit chiral analysis by covalently bonding a chiral selector. Adapted from Reference 18 (A). The methacrylate-based polymer monolith used for the analysis of drug molecules in Reference 24, prepared by polymerizing monomers in a one-pot reaction (B). An MIP based on polymer monolithic material. Adapted from Reference 31 (C). Particle-fixed monolithic columns: 3 µm silica particles bearing a chiral selector (teicoplanin aglycone) embedded within a norborene-naphtalene-based monolith (D). Reproduced with permission from Reference 26.

Inorganic Monoliths

Inorganic monoliths, like silica-based phases, are usually synthesized via a sol-gel reaction. The sol-gel reaction includes three consecutive steps, which are illustrated in Figure 2. The first step is the hydrolysis of a silica compound precursor into a hydroxy derivative. Secondly, two hydrolyzed silica groups are condensed to form a siloxane bond (Si-O-Si), and thirdly other silanol groups are polycondensated, forming three-dimensional networks (9).

The hydrolysis is either spontaneous, or activated by a catalyst which can be basic (e.g., ammonia) or acidic (e.g., hydrochloric acid). When an acidic catalysis is performed, the monolith network is randomly entangled because the velocity of the hydrolysis step is higher than that of the condensation step (9). Basic catalysis, on the other hand, results in a linear arrangement, because the condensation reaction is the fastest (9). After curing (i.e., drying and heating), the sol-gel network can be derivatized by an on-column silylation reaction to obtain the desired surface chemistries (4). Aging of the wet silica network during some days to weeks promotes further condensation and thus strengthens the network (9).

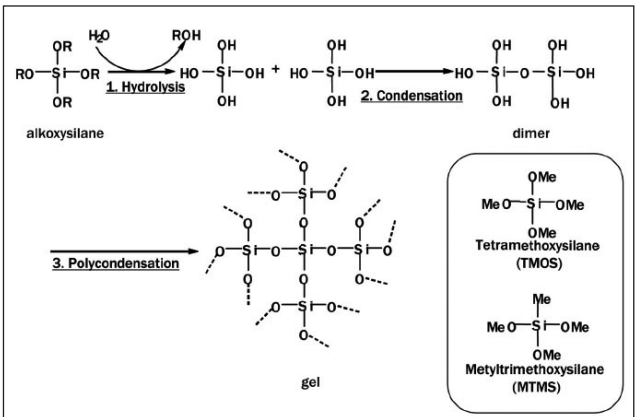

Figure 2. Synthesizing a silica-based monolith with the sol-gel reaction. TMOS and MTMS are commonly used precursors for silica monoliths. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Reference 9.

The properties of the sol-gel can be influenced by pH, temperature, reagent concentrations, reaction time, hydrolysis, condensation rates, nature of the catalyst, etc. (9). The choice of the alkoxysilanes or derivatives used as starting monomers also greatly influences the monolith's properties (9). The through- and mesopores can moreover be altered independently by post-treatment of the gelled monolith (e.g., using basic solutions) (2).

Silica-based monoliths are mechanically strong, display a high column efficiency, and are stable when using certain organic solvents, especially compared with the organic monoliths (7). However, during the heating process necessary in the formation of the silica monoliths, shrinkage and cracking can occur (10).

A drawback to the use of derivatized silica monoliths is that, due to the functionalization, the number of ionizable groups decreases, and therefore the EOF is reduced (2). This problem can be solved by functionalizing with charged groups (2).

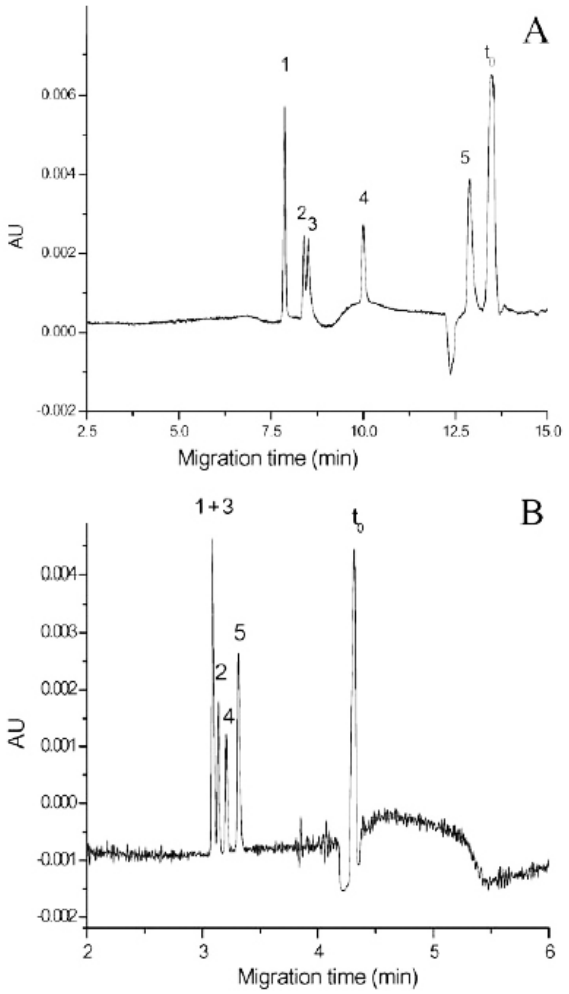

Figure 3. Separation of five β-blockers by CEC (A) and CZE (B). Conditions: capillary column, 40 cm (CEC) / 50.2 cm (CZE) × 50 mm i.d.; mobile phase, 10 mM TEAP buffer (pH 7.40) containing 60% acetonitrile; separation voltage: 20 kV; detection wavelength: 214 nm. Solutes: 1, alprenolol; 2, propranolol; 3, pindolol; 4, metoprolol; 5, atenolol. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Reference 12.

Another drawback, which is not only related to monoliths, is the reduction or loss of EOF at low pH values due to the suppression of the silanol ionization (11).

Besides the purely silica-based monoliths, where all bonds are made through siloxane bonds, a new type of hybrid phase has recently been developed (4). A silane precursor containing an organic moiety of interest can be added to the reaction mixture (4). The resulting hybrid organic-inorganic silica-based monoliths thus also have silicon–carbon bonds. A drawback of this technique, which still needs to be studied, is the difficulty of controlling the reactions, meaning that the final appearance of the created column can hardly be monitored (4).

Pharmaceutical applications on silica-based monoliths

Xie et al. (12) described the separation of five β-blockers (alprenolol, propranolol, pindolol, metoprolol, and atenolol) using CEC and capillary zone electrophoresis (CZE) (Figure 3). The monolith used in the CEC experiments was fabricated by mixing tetramethoxysilane and polyethylene glycol. The column lengths were 40 cm. The separation voltage was 20 kV and the mobile phase consisted of triethylamine phosphate (TEAP) buffer (pH 7.40)/acetonitrile (40/60 v/v). The five compounds were better separated in CEC, but the analysis time was higher. In an effort to improve the separation, the ionic strength of the mobile phase was varied. A decrease of ionic strength resulted in an increase of the retention factors of the analytes and an improved separation. Low ionic strengths (5 mM triethylamine phosphate) were thus preferred to improve the separation.

Tian et al. (13) fabricated an organic-inorganic hybrid silica-based monolithic column possessing vinyl ligands, which were chemically modified with a combined calyx[4] open-chain and crown ether functional group by free radical polymerization using α,α'-azobisisobutyronitrile (AIBN) as an initiator (see Figure 4). The advantage of this stationary phase is that the crown ether functional groups are covalently linked to the silica support through Si-C bonds, which are more hydrolytically stable at low pH values than the conventional Si-O-Si-C linkages which are hydrolytically unstable. The successful

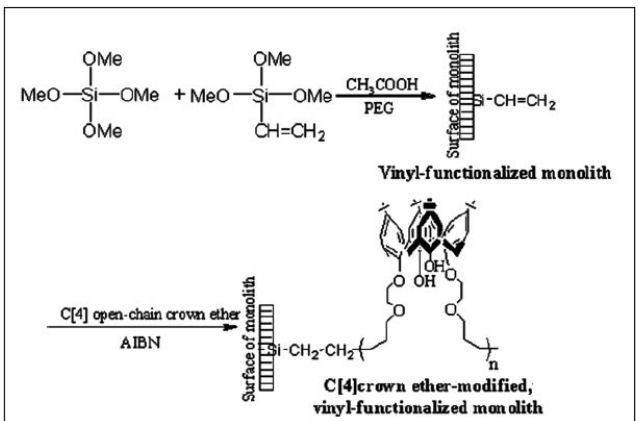

Figure 4. An organic-inorganic hybrid silica-based monolith containing vinyl groups, which can be functionalized with calyx[4] open-chain crown ether. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Reference 13.

binding of the calyx[4] open-chain crown ether to the monolith was demonstrated by infrared measurements. Besides neurotransmitters and nucleotides, 5 β-blockers (propranolol, pindolol, catenolol, metoprolol, and atenolol) with very similar pK_a values were baseline separated within 10 min with efficiencies between 80,000 and 100,000 plates/m. The columns had an internal diameter (i.d.) of 75 µm and an effective length of 30 cm, the mobile phase consisted of a 20 mM triethylamine phosphate buffer pH 7.0/ACN (80/20 v/v); a 15 kV voltage was applied, and the detection wavelength was 214 nm. The separation mechanism was based on the inclusion of the β-blockers into the cavity of both the calyx[4] and the crown ether parts of the functional moiety.

Zhang et al. (14) used a monolith based on two different silanes (tetramethoxysilane and methyltrimethoxysilane) for the separation of 11 basic, acidic, and neutral compounds (Figure 5). Using these so-called "crossbreed" phases, made from two different silanes, is claimed to minimize shrinkage of the monolith. The silica surface was chemically modified with octadecyldimethylchlorosilane by rinsing the monolithic bed. A fraction of the residual silanol groups of the stationary phase were endcapped to minimize peak tailing of the basic compounds. Hexamethyldisilazane was used for this purpose because it endcaps the column incompletely, in order to keep the EOF sufficiently high for analysis despite a reduction of the number of charged moieties. The obtained EOF was, however, not very high, and it was necessary to add pressure to elute the acids, because these solutes display their own mobility away from the detection end due to their charge. Performing the experiments in pCEC mode (i.e., the use of voltage combined with a stepwise pressure gradient) permitted the analysis of a diverse mixture of 11 molecules with different charges within 6 min (Figure 5). The columns had an effective length of 25 cm, a total length of 33.5 cm, and 100 µm i.d.

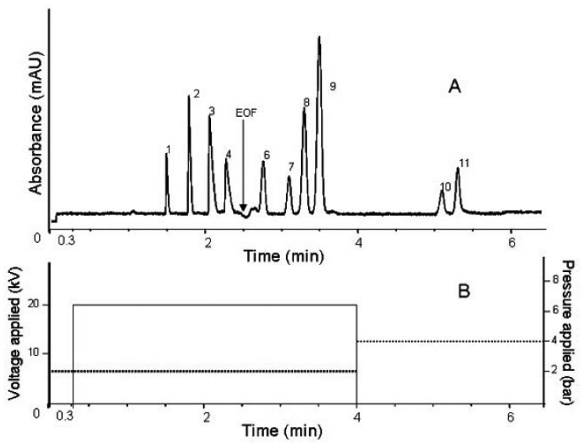

Figure 5. Simultaneous separation of acidic, basic, and neutral compounds by pCEC using a stepwise voltage/pressure gradient. Conditions: endcapped ODS silica-based capillary monolith; mobile phase: ACN–H_2O–50 mM ammonium buffer pH 8.5 (60:20:20 by volume); peak assignments: 1, benzylamine; 2, terbutaline; 3, nortriptyline; 4, remacemide; 6, benzamide; 7, anisole; 8, benzophenone; 9, biphenyl; 10, 4-hydroxymandelic acid; and 11, 4-methoxymandelic acid. Injection: 2 bar 3 s; detection: 210 nm (A). Voltage/pressure program; applied voltage: solid line; applied pressure: dotted line (B). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Reference 14.

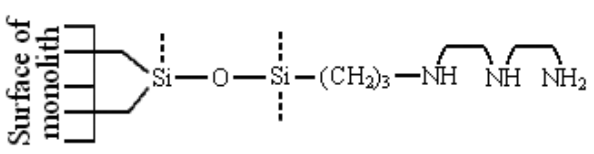

Figure 6. A silica column derivatized with DETAP ligands. The positive charge of the DETAP group ensures the EOF and minimizes peak tailing when analyzing basic molecules. Adapted from Reference 15.

A silica-based monolithic column with diethylenetriaminopropyl (DETAP) ligands (see Figure 6) for hydrophilic interactions was used by Ye et al. (15) for the analysis of some neutral and basic analytes with pressurized CEC. The stationary phase was prepared in two steps: the silica matrix was first formed by the sol-gel method, and subsequently a chemical bonding of the DETAP ligands was performed by an in situ reaction of a solution with the silica monolith. The triamino groups ensured hydrophilic interaction and generated a sustainable anodic EOF under acidic conditions. The total length of the column was 50 cm and the effective length was 25 cm, the mobile phase consisted of 10 mM citrate buffer (pH 5.0)/ACN (10/90 v/v); the detection wavelength was 270 nm; the applied voltage was 20 kV, and the added pressure was 100 psi. Under these experimental conditions, four tetracyclines (chlortetracycline, oxytetracycline, tetracycline, and doxycycline) could be separated within 5.5 min without observing peak tailing. The absence of tailing is explained by the shielding of the residual silanol groups by the amino groups of the DETAP monolithic stationary phase, thus preventing the adsorption of the tetracyclines on the negatively charged silanol groups.

In the analysis of drugs, monoliths can also have another function than being the stationary phase. Xu and Lee (16) used a hybrid sulfonic acid-functionalized silica monolith for an in-tube (250 µm i.d., total monolith length 15 cm) microextraction of four anesthetics and subsequently separated them with CE. They chose to include a mercapto-group in the monolith because it can easily be derivatized. The influence of different factors on the resulting monolith was tested. As a first factor, the use of water and/or methanol as solvent of the monolith precursors was considered. Both solvents were necessary, as both play a specific role in the sol-gel process. Secondly, the amount of polyethyleneglycol and the gelation temperature seemed to inversely influence the speed of the phase separation and the macropore size. Regarding the choice of the catalyst, an acidic catalyst (HCl) was found not to induce gelation, whereas a basic one (ammonia) resulted in a too slow hydrolyzation so that gelation occurred too early. Using two-step catalysis by first acidifying the mixture to achieve hydrolyzation and then raising the pH for gelation could be an option. Such a system is, however, complex and requires perfect timing, and therefore basic catalysis was finally preferred. A five-step extraction of four basic analytes (procaine, lidocaine, tetracaine, and bupivacaine) was performed successfully using the obtained monolithic phase. The first step was a

consecutive flushing with MeOH and water. In the second step, the sample was loaded by pumping the sample solution through the tube. Thirdly, the monolith was washed with water to remove impurities, and in a fourth step, the elution was performed with a 0.25% ammonia solution–methanol (20/80). The eluted material was collected in a vial for the subsequent CE separation. Finally, the monolith was washed with the elution solution. The relative recoveries (defined as the ratios of CE peak areas of the respective spiked urine sample extracts to spiked ultrapure water extracts) were 99.5%, 99.6%, 93.8%, and 92.3% for procaine, lidocaine, tetracaine, and bupivacaine, respectively.

Chiral separations

Chiral selectors can be incorporated in the silica-based monolithic stationary phases by in situ encapsulation or entrapment, physical absorption, or in-column derivatization (7). Different types of selectors have been used [e.g., cyclodextrins, antibiotics, chiral ion-exchangers, proteins, cellulose derivatives, and ligand-type phases (7)].

Dong et al. (7) used vancomycin as a chiral selector to baseline separate eight pairs of enantiomers (racemic mixtures) (thalidomide, benzoin, terbutaline, atenolol, metoprolol, alprenolol, pindolol, and propranolol) with CEC. After preparing a silica monolith, an in situ rehydroxylation process was performed in order to maximize the number of silanol groups on the monolithic silica. Epoxy (or ethylene oxide) groups were generated by reaction of a number of silanol groups on the stationary phase surface with 3-glycidoxypropyl trimethoxysilane. These epoxy groups were subsequently converted to aldehyde groups, onto which the vancomycin was immobilized by reductive amination. The remaining aldehydes were finally reduced back to hydroxyl groups. In this way, a high vancomycin loading could be obtained, which favored the enantioselectivity. The capillary columns were 16 cm long and had a 50 µm i.d. The applied voltage was 10 kV. High EOF values were obtained on these columns [1.24 and 1.38×10^{-4} $cm^2/V^{-1}/s^{-1}$ in non-aqueous (MeOH/ACN/HAc/triethylamine (TEA) (80/20/0.1/0.1 v/v)] and aqueous mobile phase [20% ACN in 10 mM TEA phosphate buffer at pH 6.5), respectively]. Repeatable dead-time marker injection values [0.82% and 0.75% relative standard deviation (RSD) in non-aqueous and aqueous mobile phase, respectively] were moreover achieved. The comparison between non-aqueous and aqueous mobile phases revealed the superiority of the polar organic solvent mobile phase. Due to the positive charge of the analyzed basic enantiomers, they eluted before the EOF, which resulted in short analysis times (less than 6 min). The highest enantiomeric resolution was obtained with a mobile phase containing 80/20/0.1/0.1 MeOH/ACN/HAc/TEA v/v. All eight pairs of enantiomers could be resolved. The repeatability of 5 consecutive injections on the same column was rather good, as RSD values approximately 0.75% for the retention time of the first enantiomer, 1.3% for resolution, and 2.5% for the efficiency of the first enantiomer peak were obtained. Column-to-column variability using 5 columns was acceptable (RSD values approximately 4.8% for the retention time of the first enantiomer, 6.7% for resolution, and 10.1% for the efficiency of the first enantiomer peak). No significant decline of resolution and efficiency, column bed shrinking, or swelling were observed after 50 injections, indicating a good stability and mechanical strength of the stationary phase.

Chen et al. (17) loaded Cu(II) ions on the surface of a silica monolith which was modified by 3-glycidoxypropyltrimethoxysilane and L-hydroxyproline. L-hydroxyproline and Cu(II) are both used in chiral separations because they form reversible diastereomeric metal complexes with some chiral compounds. No EOF could be measured on the resulting monolithic columns, probably due to their low charge. The addition of surfactants to the mobile phase did not improve the situation. Enantioseparations of amino acids could nevertheless be performed, thanks to their own mobility. Experiments for separations were performed in CEC and µ-LC modes and the latter was able to resolve all peaks, in a shorter time, but the peak shape quality seemed inferior compared with CEC. Columns with an effective length of 37 cm and an i.d. of 100 µm were used, with a mobile phase consisting of acetronitrile–0.50 mM NH_4Ac–50 mM $Cu(Ac)_2$ at pH 6.5 (70/30). The detection was performed at either 214 nm, or at 254 nm for dansyl amino acids and 208 nm for the other molecules. A voltage of 13.5 kV was applied. Conditions for µ-LC on the same systems were similar except the replacement of voltage by flow rates of 2 or 5 µL/min depending on the sample.

Kang et al. (10) coated a silica-based monolithic capillary column with Chirasil-β-Dex, which is a chiral polymer prepared by grafting permethyl-β-cyclodextrin to polymethylsiloxane with an octamethylene spacer (Figure 7). The tested compounds were mephobarbital, hexobarbital, carprofen, and benzoin. The grafting shielded some of the silanol groups of the monolith surface, but a sufficiently high EOF was still observed. Fused silica capillary columns with an effective length of 25 cm and an i.d. of 50 µm were used. UV detection was performed at 210 nm. Different mobile phases were used throughout the experiments, but all consisted of 2-morpholinoethanesulfonic acid buffer at pH 6 and methanol. The effect of the methanol concentration in the mobile phase was investigated by varying it from 10% to 40%. With increasing methanol content, the resolution between the two enantiomers decreased. A similar decrease was observed for the flow-rate. A low methanol (10% v/v) level was thus selected. Increasing the buffer concentration (between 20 and 100 mM) in the mobile phase slightly increased the retention factors, but efficiency, speed of analysis, and resolution decreased. A lower concentration is thus preferable. All tested chiral compounds could finally be separated. The precision of the retention times on these columns was evaluated in terms of run-to-run and day-to-day variability. The obtained %RSD values were approximately 0.9 and 7.3 (possibly due to temperature variations), respectively. The column-to-column variability was reported as poor.

Another type of chiral selector was used by Preinerstorfer et al. (18). They covalently bonded (S)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid to a silica monolith that was previously modified with 3-mercaptopropyl trimethoxysilane to obtain reactive thiol groups (Figures 1 and 8). The selector was bonded both to a monolithic and a particle-based stationary phase. The binding

of the chiral selector to the monolithic support was obtained by an in situ reaction, during 24 h at 60ºC, initiated by AIBN. The enantiomeric separation of several chiral basic drugs (acebutolol, celiprolol, clenbuterol, ephedrine, isopenbutolol, isoxsuprine, mefloquine, mefloquine-*t*-butyl-carbamate, nifenalol, salbutamol, sotalol, and talinolol) was performed in a nonaqueous medium, composed of ACN/MeOH (80/20 v/v), formic acid (25 mM for the monolith and 50 mM for the packed capillary), and 2-amino-1-butanol (12.5 mM for the monolith, 25 mM for the packed phase). The applied voltages were 8 kV and 15 kV for the monolith and the packed phase, respectively. The results obtained on the monolith were satisfying; however, compared to those obtained in a similar capillary packed with 3.5 µm silica particles, the efficiency and the retention factors of the solutes on the monolith were generally inferior. Resolutions between 1.4 and 4.0 were obtained for the twelve tested substances using the monolithic phase, and between 2.3 and 7.4 with the particulate phase. On the other hand, the monolithic columns were more robust (i.e., less subject to breaking or stationary phase displacement), and they kept a constant current throughout the studies.

A polysaccharide chiral selector [cellulose tris(3,5-dimethylphenylcarbamate), CDMPC] was covalently bonded onto a silica backbone monolith by Dong et al. (19) to separate chiral compounds (racemic benzoin, indapamide, praziquantel, Tröger's base, and pindolol) in CEC. A 50 µm i.d. capillary column with an effective length of 20 cm was used, combined with a mobile phase containing 4 mM of either triethylamine phosphate or phosphate buffer at pH 6.8/ACN (60/40). The applied voltage is 10 kV. The advantage of chemically binding the selector is that the resulting stationary phase is resistant to certain solvents, such as THF, which can improve enantioseparation. However, THF may damage coated chiral selectors, and chemically bonding is therefore required. In a first step of the column preparation, the silica monolith was formed via a sol-gel reaction and a maximization of the number of silanol groups was ensured by rinsing the monolith with HCl. The bonding of CDMPC was performed in a second step. When comparing the bonded and coated chiral phases, using the same mobile phase, the results were similar. However, better results were obtained on the bonded phase when using THF.

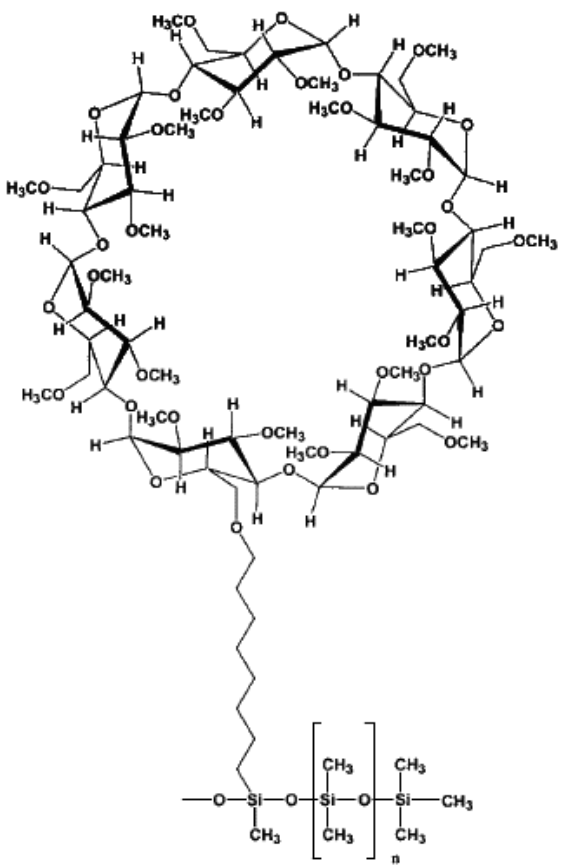

Figure 7. Silica-based monolithic capillary column coated with Chirasil-β-Dex. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Reference 10.

Organic monoliths

The first monoliths used in the capillary format were very loose swollen hydrophilic polyacrylamide gels, similar to those used in gel electrophoresis (1). These gels were not attached to the capillary wall but were rather stable anyway (1).

Nowadays, organic monoliths are prepared by a direct copolymerization from a liquid, called the polymerization mixture, that contains the precursors (1). The polymerization is a very simple in situ process (4). The polymerization mixture typically contains monomers (either bulk or functional monomers, with charge-bearing or cross-linking monomers as examples of the latter), pore-forming solvents, and an initiator, which is activated by UV light or heat (4). Most polymerization reactions are thermally induced. However, this thermal method limits the possibility to polymerize only in the desired areas. Using UV initiation is an interesting alternative (2). A mask is used to hide the areas where polymerization is not desired. Only the uncovered areas will be irradiated by the UV light, and therefore the polymerization reaction will only be initiated in these areas.

Depending on the monomers and additives used, a wide range of surface chemistries and monolith characteristics, such as different morphologies, hydrophobicities, and EOF values, can be obtained (1,20). Typically, monomers of the methacrylate or acrylate family are used.

By carefully controlling the composition of the polymeriza-

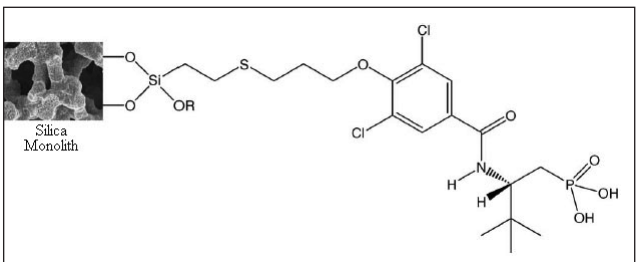

Figure 8. Structure of the (S)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutane phosphonic acid selector bound to the silica monolith via a 3-mercaptopropyl trimethoxysilane "bridge". Reproduced with permission from Reference 18.

tion mixture, it is even possible to prepare "gradient" stationary phases [i.e., stationary phases with gradually changing properties in consecutive parts of one capillary (21)]. This can be obtained by drawing the different polymerization mixtures in the capillary one after the other, so that a desired gradient stationary phase is polymerized in situ (4).

Because of the complexity of the copolymerization reactions, it is not always possible to obtain the desired properties in one polymerization step. Post-functionalization of an existing monolithic column is hence a frequent approach (4). Furthermore, functional groups included in the polymerization mixture could mainly be embedded within the monolithic structure instead of being located on the surface, where they can interact with the analyte. Therefore, post-functionalization of the surface with the desired functional groups through grafting or coating may be more useful. Post-functionalization moreover permits to graft non-permanent groups, thus enabling one column to consecutively have different properties (4). Post-functionalization is usually performed via photografting (i.e., through a reaction initiated by UV). Other methods can also be employed (e.g., the coating of latex-nanoparticles onto the monolith). This was performed by Hutchinson et al. (22) in the analysis of inorganic ions, but has not, to the best of our knowledge, been used for pharmaceutical or chiral compounds.

One main drawback of polymeric monoliths is that they are subject to swelling and shrinking under the influence of temperature and some organic solvents (16). This is inconvenient because the pore sizes can change and the monolith can crack or come loose from the wall.

Applications on methacrylate-based monoliths

Lin et al. (23) separated and determined five opium alkaloids (narcotine, papaverine, thebaine, codeine, and morphine) on methacrylate-based monolithic capillary columns using p-CEC (Figure 9). The stationary phase was prepared by polymerization of glycidyl methacrylate, 3-sulfopropyl methacrylate potassium salt, and ethylene dimethacrylate (EDMA). The epoxy groups on the surface of the monolith were hydrolyzed by hydrochloric acid to obtain diol groups. Both the diol groups and the sulfonic groups are responsible for the hydrophilic interaction (HI) between solute and stationary phase. The reversed-phase (RP) interactions are provided by the polymer skeleton, including the 4-carbon chains attached to the 3 carbons in the diol groups. The sulfonic groups are charged, which permits the EOF generation and provides cation-exchange interaction sites. Different conditions were tested by the authors. Fused-silica capillary columns with an i.d. of 100 µm and 30 cm effective monolith length were used. The applied voltage was 13 kV, an added pressure of 1000 psi was applied to the column inlet, and the flow rate of the pump was 0.05 mL/min during the separation. The detection wavelength was 224 nm. In the RP/cation-exchange mixed mode, high efficiencies could be obtained (up to 140000 plates/m) but not all compounds could be separated. HI/cation-exchange mixed mode resulted in an improved separation and a reduced analysis time (14 instead of 18 min), so the latter mode was preferred. Increasing the ACN content in the mobile phase between 83% and 92% increased the retention factors, which was attributed to the importance of the HI in the retention mechanism. Increasing the phosphate buffer concentration between 3 and 7 mmol/L resulted either in a decrease of the retention factors, or had no effect. This is explained by the weak protonation and the resulting weak cation-exchange properties of the stationary phase. Analysis time decreased as the pH of the mobile phase decreased from 6 to 4 due to the increasing positive charge of the alkaloids and hence their higher mobilities. At pH 3, however, not all compounds could be separated. The optimal mobile phase was found to consist of ACN/5 mmol/L phosphate buffer pH 4 (90/10 v/v) (Figure 9).

The effect of added pressure on the column inlet was also evaluated. Too low pressure (500 psi) led to long separation times. Too high pressures (1500 psi) decreased the analysis times but also the resolution. A pressure of 1000 psi was found to be most suitable.

At the optimal conditions, limits of detection of 1.5–6.0 µg/mL were obtained. % RSD values for the migration times (7 repetitions) were 1.94 (intra-day), 3.49 (inter-day), and 5.24 (column-to-column-variability), and for the peak areas 4.05 (intra-day), 6.48 (inter-day), and 8.21 (column-to-column-variability).

Adu et al. (11) separated nine therapeutic peptides [bradykinin, vasopressin, luteinising hormone releasing hormone (LHRH), substance P (4), bradykinin fragment 1–5, leucine enkephalin, methionine enkephalin, bombesin, and oxytocin] using CEC (Figure 10). The monolith is prepared from a polymerization mixture containing butyl methacrylate (BMA), EDMA, and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS). The EOF is supported by the sulfonic acid moiety of AMPS, whereas BMA provides the nonpolar interaction sites. To reduce the electrostatic interactions with the permanently negatively charged sulfonic acid moieties, it was ensured that the peptides were in a minimally protonated state by keeping the pH of the mobile phase high (9.5). The columns had an i.d. of 100 µm and an effective length of 25 cm. The mobile phase was an ACN/H_2O/50 mM sodium borate buffer pH 9.5 (70/10/20 v/v/v). The applied voltage was 10 kV, and to prevent bubble formation, a 6 bar pressure was applied on both in- and outlet vials. The detection was performed at 206 nm.

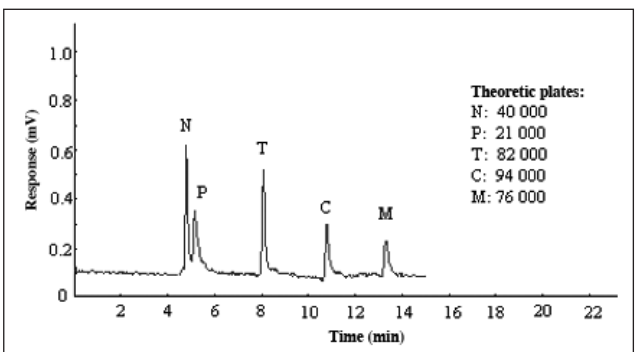

Figure 9. Separation of five opium alkaloids using pCEC and methacrylate-based monolithic columns. Mobile phase: 5 mmol/L phosphate buffer (pH 4.0)–ACN 10/90 v/v. Solutes: N, narcotine; P, papaverine; T, thebaine; C, codeine; M, morphine. Reproduced with permission from Reference 23.

The separation achieved on the monolith was compared with a CE separation on a bare fused silica column (see Figure 10), and the monolith provided superior results for resolution as well as analysis time. The stability of the stationary phase was also tested by repeated injections (50) of a mixture of the EOF marker and leucine enkephalin, followed by 10 injections of a four-component mixture of highly basic peptides [vasopressin, bombesin, substance P (4) and bradykinin fragment 1–5] and finally 40 injections of a mixture of thiourea and leucine enkephalin over 3 days. The reported RSD values for the retention time remained below 1.21%.

Our group evaluated the influence of changing the composition of the polymerization mixture on the analysis of drug molecules in CEC (24) and pCEC (25) (Figure 1). The analyzed molecules were warfarin, ketoprofen, praziquantel, paracetamol, metoprolol, pyrene, and oxazepam. The composition of a polymerization mixture, containing BMA, EDMA, and [2-(methacryloyloxy) ethyl] trimethylammoniumchloride as monomers, and a mixture of water, 1,4-butanediol, and 1-propanol as pore-forming solvents (PFS), was varied according to a central composite design. The total PFS fraction and the concentration of 1,4-butanediol within the PFS were thus varied. Two mobile phases were used, both containing ACN/buffer 50/50 (v/v). Two buffers were used: a 50 mM ammonium formate buffer at pH 3 and a 5 mM phosphate buffer at pH 11.5. The capillary columns had an i.d. of 100 µm and an effective length of 20 cm for pCEC and 21 cm for CEC experiments. Voltages of –5 (pCEC) or –15 kV (CEC) were applied to generate EOF. An external pressure of 4.8 bar was applied in CEC on both vials to prevent bubble formation. In pCEC, two pumps provided a total flow of 0.1 mL/min, which was split by means of a back-pressure regulator. Only a fraction was thus pumped over the column. Detection was carried out at 214 nm. In both CEC and pCEC, the composition of the pore-forming solvent mixture seemed to influence the chromatographic properties more than the total amount of pore-forming solvents in the polymerization mixture. A polymerization mixture generating columns with good values for the regarded responses (retention time, retention factor, peak asymmetry, and number of theoretical plates) was predicted both in pCEC and CEC, and testing it confirmed the predictions. When comparing pCEC and CEC experiments on the same monolithic columns, the added pressure showed no clear chromatographic benefit for the substances considered.

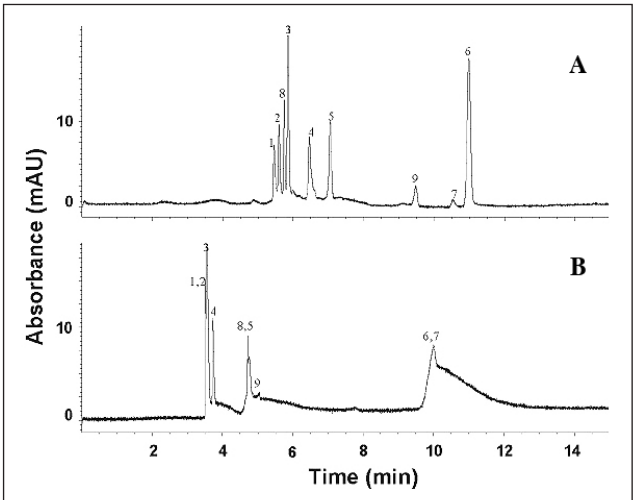

Figure 10. Separation of a nine peptide mixture by CEC on a buthylmethacrylate-based mixed-mode monolithic capillary (A) and by CE on a bare fused-silica capillary (B). Peaks: 1, bradykinin; 2, vasopressin; 3, LHRH; 4, substance P(4); 5, bradykinin fragment 1–5; 6, leucine enkephalin; 7, methionine enkephalin; 8, bombesin; 9, oxytocin. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from Reference 11.

Applications on acrylamide-based monoliths

Enlund et al. (20) studied the separation of three hydrophobic amines [i.e., the tricyclic antidepressants amitriptyline, nortriptyline, and a related ammonium compound (*N*-methylamitriptyline)] on acrylamide-based polymers. Capillary columns with an i.d. of 50 µm were used. The total column length is between 27 and 45 cm, because the authors simply cut the end of the capillary when a problem such as breaking, gas bubble formation, or dust particle gathering occurred there. The separation voltage was 20 kV and detection was performed at 211 nm. Different mobile phases were used when trying to improve the separation of the 3 amines. The polymerization mixture contained piperazine diacrylamide and methacrylamide. The monolith was derivatized with sulfonic acid groups, to generate an EOF and to provide electrostatic interactions with the analytes, and with the isopropyl groups responsible for weak hydrophobic interactions. The best columns generated efficiencies of up to 200,000 plates/meter. Some peak tailing was observed, but the addition of co-ions (e.g., *N,N*-dimethyloctylamine) to function as competition- or counter-ions to promote ion-pairing, did not have a significant influence. A limit of detection of 50 pg/mL could be obtained by dissolving the samples in 96% isopropanol. However, when the isopropanol plug reached the detector, a distortion of the baseline was observed due to a voltage drop. The effects of ionic strength (0.006–0.024 mM phosphate buffer) and organic modifier (50–80%ACN) content in the mobile phase on the resolution were also investigated. Organic modifier content seemed to have a negligible effect, but increasing the ionic strength was found to positively influence the efficiency and the resolution. Too high ionic strength resulted in increased retention times; therefore 0.012 mM was considered optimal. Separation of the three hydrophobic amines was thus achieved.

Chiral separations

Chiral analyses have also been performed on organic monoliths. The synthesis of chiral polymers is relatively easy, as the chiral selectors can be incorporated into the polymerization mixture and polymerize along with the other components (26). However, not all chiral selectors are suitable, (e.g., some are structurally not able to undergo allylation, while this is essential for radical polymerization). Moreover, the polymerization reaction is altered by the inclusion of a chiral selector, which results in a different monolith. Finally, the enantiomeric selectivity of the chiral selector can be modified due to the presence of the polymer.

Kornyšova et al. (27) describe the preparation of a mono-

lithic bed based on the polymerization of acrylic monomers. *N*-(hydroxymethyl)acrylamide and *N,N'*-diallyltartardiamide piperazine were used as bulk monomers and diacrylamide as the cross-linking monomer. The charge on the stationary phase, necessary for EOF generation, was obtained by adding vinyl sulfonic acid to the polymerization mixture. Allyl glycidyl ether supplied epoxy groups, which were converted to aldehyde groups to later allow the attachment of vancomycin via reductive amination. The conversion of epoxy groups to aldehydes was performed by a periodate treatment, which also resulted in an increase in the pore size due to cleavage of the cross-linker. The mobile phase contained ACN/0.15% TEAA at pH 4.6 (20/80 v/v); the voltage was 20 kV, and the column dimensions were an effective length of 25.2 cm and an i.d. of 75 µm. Detection was performed at 210 nm. The chiral drugs warfarin, thalidomide, and bupivacaine were baseline separated, and some others, like ketoprofen, felodipine, and atenolol, were partially separated, but optimization of the mobile phase might solve this problem. Run-to-run variability (% RSD) values were approximately 0.33 for the dead time and the retention times of both thalidomide enantiomers. The plate number repeatabilities were less good, with a %RSD of 2.5 for the dead time marker peak, and 7.24 and 10.17 for the first and second enantiomer, respectively. No column aging effects, in terms of theoretical plate number or resolution decrease, were observed over 100 runs. Plate numbers up to 12,000 plates per meter were obtained for all enantiomers.

Molecularly Imprinted Polymer-Based Monolithic Columns

Molecular imprinting is a technique to fabricate monolithic columns, where the polymer is synthesized in such a way that it contains spatial and functional recognition sites with high predetermined selectivity (1,4,5,28). This is typically obtained by copolymerizing monomers and cross-linkers with a template molecule. After polymerization, the template is extracted from the monolith, leaving an imprint with the shape and chemical arrangement of the template (1,5). Alternatively, the recognition sites can be post-grafted on monolithic matrices (4).

Initially, these molecularly imprinted polymers (MIPs) possessed pores too small to permit flow, so they had to be crushed and inserted in the capillaries as irregular particulate phases. Later, more porous phases were developed (1). Organic-polymer-based MIPs display pH stability, but shrinking or swelling can occur when they are exposed to different solvents (29), which deform the recognition sites and reduces the separation ability.

Xu et al. (30) used an (S)-naproxen-imprinted monolith, and found that its recognition selectivity depended mainly on the CEC conditions. (S)-Naproxen was added in the polymerization mixture together with the monomers methacrylic acid (MAA) and EDMA, the initiator AIBN, and the pore-forming solvents toluene and isooctane (4/1 v/v) to undergo polymerization in a one-pot reaction. After reaction (53ºC for 3 h), the column was extensively rinsed to remove the (S)-naproxen. Capillary columns with an i.d. of 100 µm and an effective length of 20 cm were used with an acetonitrile–acetate electrolyte (pH 3.0, 50 mM) (80/20 v/v). Different voltages were used and an additional pressure of 20 psi was applied over the column. The detection wavelength was set at 254 nm. Several CEC factors influence the chiral selectivity of the obtained MIP-monolith. Increasing the applied voltage (5–25 kV) led to a decreased resolution because two factors related to a shorter analysis time were affected (i.e., both the EOF and the temperature increased, resulting in a higher flow). Different organic modifiers (acetonitrile, methanol, ethanol, and dimethyl sulfoxide) were used, and the effect of their concentration was also investigated. ACN provided the fastest and best separations. At high ACN concentrations, the hydrogen bonding becomes stronger, which improves the chiral recognition between (S)-naproxen and the MIP monolith. A more concentrated buffer improved the chiral recognition and reduced the analysis time. The recognition selectivity of the MIPs was highest at a pH of the mobile phase close to the pK_a of the analyte. Finally, addition of surfactants, such as sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and Tween 20 showed potential to improve the chiral separation (Figure 11).

In addition to polymer MIP-columns, some groups have also tried to use silica-based MIPs. Silica-based MIPs do not deform under the influence of solvents, as their polymer-based analogues do, but they often crack or shrink during their preparation [i.e., during the high temperature curing and aging steps (30)]. Therefore, Wang et al. (30) proposed an alternative preparation process, called nonhydrolytic sol-gel, in which no curing or aging at high temperatures is required. They also used room temperature ionic liquid as a non-volatile drying liquid, which should further reduce shrinkage. The chiral separation of the zolmitriptan enantiomers could be

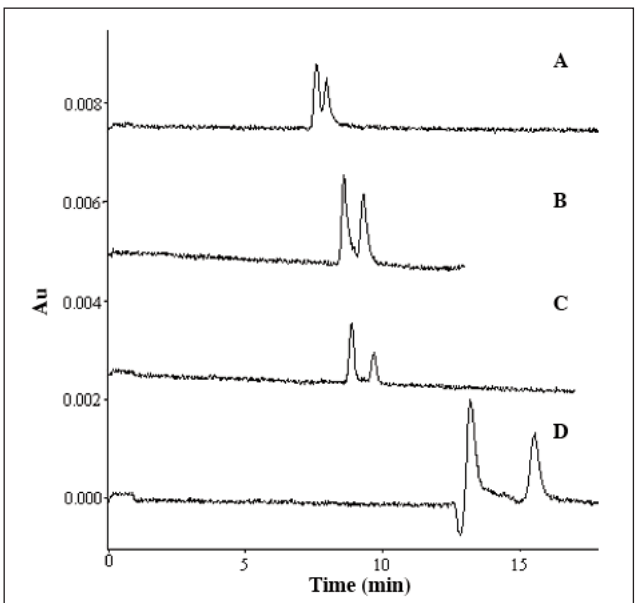

Figure 11. Chiral recognition of the racemic naproxen on the (S)-naproxen imprinted monolith with different surfactants in the electrolytes: no surfactant (A); CTAB (1 mM) (B); SDS (3 mM) (C); Tween 20 (40 mM) (D). Reproduced with permission from Reference 29.

achieved with the silica-based S-zolmitriptan imprinted columns they produced. These had an effective length of approximately 28 cm and an i.d. of 100 µm. The mobile phase used was a mixture of ACN and Tris-HCl (50 mM, pH 5.4) (70:30 v/v). The separations were performed at an applied voltage of 5 kV with an overpressure of 30 psi and the detection was done at 254 nm.

Two chiral compounds, Tröger's base and tetrahydropalmatine, were enantioseparated on molecular imprinted monolithic inorganic capillary columns with CEC (28). First, vinyl groups were grafted onto the silica monolith. Secondly, a MIP film was coated via copolymerization of MAA and EDMA in the presence of templates. (5S,011S)-(–)-Tröger's base and l-tetrahydropalmatine were used as templates. The columns had effective lengths of 24.5 cm and 75 µm i.d. The mobile phase was a 10 mM acetate buffer at pH 6.0–acetonitrile (15/85 v/v). A 10 kV voltage was applied and detections were performed at 214 nm. Both Tröger's base and tetrahydropalmatine could be baseline resolved, but the retention times were rather long. By increasing the voltage, the analysis time decreased, but the resolution decreased. By adding a pressure (6 bar) on the inlet vial, the analysis time could be further reduced (3 min instead of 15) while maintaining the resolution.

Particle-Fixed Monolithic Columns

Particle-fixed or particle-loaded monolithic columns can be prepared by sintering or gluing silica particles that have previously been packed into a capillary (3,4). Another method is to mix silica particles in a polymerization mixture, thus, embedding them in the formed monolith (3,4,26). The use of frits then becomes redundant, while the many commercially available particles can be employed, which makes these phases broadly applicable (3,4). The preparation of these particle-fixed monoliths is relatively straightforward. Particle-fixed monolithic columns can, therefore, be considered as a compromise between packed and monolithic phases.

Gatschelhofer et al. (26) performed the enantioseparation of glycyl-dipeptides by CEC using particle-loaded monoliths prepared by ring-opening metathesis polymerization. The capillaries had 200 µm i.d. and an effective length of 27.5 cm. Detection was performed at 208 nm. The mobile phase consisted of aqueous TEAA solution (0.2%, adjusted with acetic acid to pH 4.1)/ACN/MeOH in different proportions. The separations were powered by an applied electric field of 15 kV and a 12 bar pressure prevented bubble formation. The monomers were norborn-2-ene and 1,4,4a,5,8,8α-hexahydro-1,4,5,8,exo,endodimethanonaphthalene, while 3 µm silica-based particles containing the chiral selector teicoplanin aglycone were suspended into the polymerization mixture before polymerization. In this way, particle-based monoliths are easily prepared (see Figure 1). The polymerization method used by Gatschelhofer et al. is ring-opening metathesis polymerization, which is much faster than the polymerization of for instance methacrylamide, because the monoliths are formed within 30 min (26).

Conclusion

This review discusses the increased use of monolithic stationary phases as an alternative to packed columns in the analysis of pharmaceutical and chiral molecules with CEC and pCEC.

Four types of monolithic materials were discussed: organic and inorganic phases, MIPs, and particle-fixed monoliths. Each has advantages and drawbacks in terms of stability, ease of preparation, and functionality. However, none has yet proved to be significantly superior to the three others. It has been shown that functionalized monoliths are valuable in separation science. Combining the synthesis and the functionality of the monolith in a single step, which is mostly used for the polymeric monoliths, might be considered easier than post-synthesis modification, which is usually done for silica-based monolithic columns. However, the single-step method has the drawback that not all incorporated active groups are available on the surface to interact with the analytes. Post-synthesis derivatization ensures the availability of the reactive sites, but can be limited by the availability of derivatization sites within the monolith. Silica-based monoliths, moreover, restrict the pH range in the mobile phase due to their instability at high pH and lack of charged groups at low pH. Polymeric monoliths have the advantage that their morphology is finely tunable, but this process is challenging and demands great care during the preparation of the polymerization mixture. MIP phases are an interesting development, but their selectivity is also a drawback, as the analysis of structurally diverse molecules necessitates the production of different columns. The imprinting technology moreover needs to be improved. Particle-fixed columns seem an interesting alternative because many ready-made particles are commercially available. However, their use in pharmaceutical and chiral analysis is not yet common.

A few more topics still need further investigation to increase the applicability of the monolithic stationary phases. For example, the inclusion of fixed charges in the stationary phase to support the EOF should be examined, while the interference of these charged moieties with the separation process should be avoided. Secondly, the synthesis of the monolithic columns should become more reproducible.

In conclusion, while all these monolithic stationary phases have potential in CEC and pCEC, and some applications have already proven their practical use, further developments are required to overcome some difficulties.

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