

Review

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Recent advances in chiral separation principles in capillary electrophoresis and capillary electrochromatography

This review summarizes recent developments in chiral separation in capillary zone electrophoresis (CZE), electrokinetic chromatography (EKC), and capillary electrochromatography (CEC) covering literature published since the year 2000. New chiral selectors and innovative approaches for CE and CEC are introduced. Recent progress in column technology for CEC is highlighted and the development of new chiral stationary phases is discussed. This review is not dedicated to list applications but will focus on new developments.

Keywords: Capillary electrochromatography / Capillary electrophoresis / Chiral separation principles / Monolithic phases / Review
DOI 10.1002/elps.200406173

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Abbreviations: **CDmh**, histidine-modified β -cyclodextrin; **CSP**, chiral stationary phase; **EMO**, enantiomeric migration order; **HP- β -CD**, hydroxy propyl- β -cyclodextrin; **Leu-Leu**, leucyl-leucine; **M-CEC**, monolithic capillary electrochromatography; **MIP**, molecularly imprinted polymer; **NBD**, 7-nitrobenzo-2,1,3-oxadiazolyl; **OT-CEC**, open-tubular capillary electrochromatography; **P-CEC**, packed capillary electrochromatography

1 Introduction

Chiral separation techniques represent a very intensively worked-on field. Besides HPLC and GC, electromigration techniques attracted strongly increasing interest for enantioseparation in recent years. The main advantage of electromigration techniques is the high efficiency due to the plug-like flow profile caused by the EOF. Furthermore, there is a low solvent and selector consumption. A weak point still remains detection sensitivity. During the past four years no revolutionary new chiral separation principles have been discovered, however, some new chiral selectors have been introduced. Most progress has been done in CEC. A lot of chiral phases successfully applied in HPLC have been transferred to CEC and several new chiral stationary phases (CSPs) have been developed.

A recent trend is the development of monolithic phases for CEC since packing of capillaries is not easy and the preparation of frits by sintering a zone of the silica-based packing is a rather sophisticated procedure. Such frits are sources of air bubbles and often tend to break. Monolithic phases have been prepared on silica and organic polymer basis. Silica-based monoliths were prepared either by a sol-gel technique or by particle-fixed techniques. The latter techniques are based on sintering the silica-based phase after packing or fixing the particles in a packed capillary by drawing a solution of a silicate or silane through the column followed by heating. Other authors used a particle-loaded approach by suspending particles containing the chiral selector into a sol or polymerization mixture. Organic polymer monoliths were prepared on polyacrylate, polyacrylamide, and polystyrene

basis. An alternative to rigid polymers represent homogeneous gels, which are almost ideal chromatographic supports because they are highly porous and Eddy diffusion is negligible. However, their disadvantage is that they cannot be flushed hydrodynamically because of the lack of pressure stability. As can be seen, a remarkable number of new developments appeared in the last years, which are reported in the present review. This review covers the literature since a previous review published in 2000 [1]. A critical review discussing the advantages and disadvantages of different approaches appeared in 2001 [2].

2 Capillary zone electrophoresis

Cyclodextrins (CDs) still represent the most frequently used chiral selectors in CE. A remarkable number of new single isomers of cyclodextrin derivatives have been prepared, such as octakis (2,3-diacetyl-6-sulfato)- γ -CD [3], octakis-6-sulfato- γ -CD [4], heptakis (2-*N,N*-dimethylcarbamoyl)- β -CD [5], octakis (2,3-*O*-dimethyl-6-*O*-sulfo)- γ -CD [6], hexakis 2,3-di-*O*-acetyl-6-*O*-sulfo- α -CD [7], oktakis (2,3-dimethyl-6-*O*-sulfo)- γ -CD ([8]), heptakis (2-*O*-methyl-3,6-di-*O*-sulfo- β -CD [9], octa (6-*O*-sulfo)- γ -CD [10], and mono-6-*O*-phenylcarbamoyl- β -CD [11]. The applicability of these derivatives for chiral separation has been demonstrated by means of a broad spectrum of drugs.

A new class of CDs, hemispherodextrins, in which a trehalose capping moiety is bonded to β -CD, has been introduced by Cucinotta *et al.* [12–14] (Fig. 1). The authors applied these selectors to the chiral separation of phenoxy acid derivatives. They made the observation that binary mixtures of hemisphero-dextrins show complementary selectivity compared to single selectors in the BGE [14]. Chiari *et al.* [15] prepared a positively charged copolymer of allylamine and 2-hydroxyl-3-methacryloyl- β -CD. In this copolymer, the CD molecules are spaced from the backbone through a spacer arm which prevents sterical hindrance of the CD cavity. This selector was applied to the separation of 2,4-dinitrophenylamino acids. Matsunaga *et al.* [16] synthesized a methylated glucuronyl glucosyl- β -CD and demonstrated its applicability for chiral separation by means of 16 drugs. A new anionic CD derivative, (6-*O*-carboxymethyl-2,3-di-*O*-methyl)- β -CD has been prepared by Culha *et al.* [17] and compared with commercially available anionic CD-derivatives. The effect of different substituted positions of phenylcarbamoyl- β -CD on the enantioseparation of drugs has been studied by Zhang *et al.* [18]. They showed that the position of the phenylcarbamoyl group has a significant influence on chiral recognition. Eder *et al.* [19] evaluated various norbornene- β -CD-based monomers and oligomers as chiral selectors for nonaqueous CE. As model analytes the authors used dansyl amino acids.

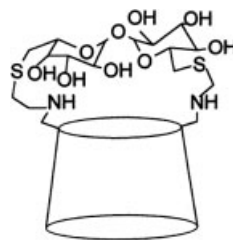


Figure 1. Schematic representation of the hemispherodextrin. Reprinted from [12], with permission.

Crown ethers have been shown to be powerful chiral selectors for compounds with primary amino groups [20]. Wang *et al.* [21] synthesized a new chiral crown ether, (*S,S*)-1,7-bis(4-benzyl-5-hydroxy-2-oxo-3-azapentyl)-1,7-diaza-12-crown-4), and demonstrated its applicability for chiral CE separation. Dual selector systems combining CDs and chiral and nonchiral crown ether derivatives have been shown to be useful alternatives for special separation problems, *e.g.*, [22, 23]. Such combinations can enhance or even enable chiral separation. A new class of crown ethers, tetraoxadiaza-crown ether derivatives, have been synthesized by Ivanyi *et al.* [24]. These selectors did not show any chiral recognition ability individually, however, they enhanced the enantioselective effect of different cyclodextrins in dual selector systems. The authors demonstrated the applicability of this approach by means of the chiral separation of some amino acid derivatives.

Billiot *et al.* [25] compared 18 chiral monomeric and polymeric amino acid-based surfactants for their chiral recognition ability for lorazepam, temazepam, propranolol, and 1,1'-bi-2-naphthol using the principle of micellar electrokinetic chromatography. Recently, the same group [26] investigated the effect of combining chiral polymeric surfactants with CDs. The authors concluded that the resolution and enantiomer migration order depend on the stereochemical configuration of the polymeric surfactant and the nature of the CD. Mwongela *et al.* [27] prepared a new polymeric amino acid-based surfactant, poly(sodium oleyl-L-leucylvalinate), and applied it to the chiral separation of various neutral, acidic, and basic analytes. Tran and Kang [28] investigated 1-*S*-octyl- β -D-thiogluco-pyranoside (OTG) as chiral selector for the resolution of dansyl amino acids. They made the observation that sodium dodecyl sulfate (SDS) and CDs exhibit a synergistic effect on separation. Mohanty and Dey [29] prepared a new chiral surfactant, *N*-[4-dodecyloxybenzoyl]-L-valinate, and observed by means of light microscopy the formation of giant bilayer vesicles. 1,1'-Bi-2-naphthol and 1,1'-2,2'-diylhydrogenphosphate were used as chiral model compounds. Polymeric alkenoxy amino acid-based surfactants have been developed by Rizvi *et al.* [30–32] (Fig. 2) and applied to the resolution of β -blockers [31], β -blockers with two stereogenic centers [30], and binaphthyl derivatives [32].

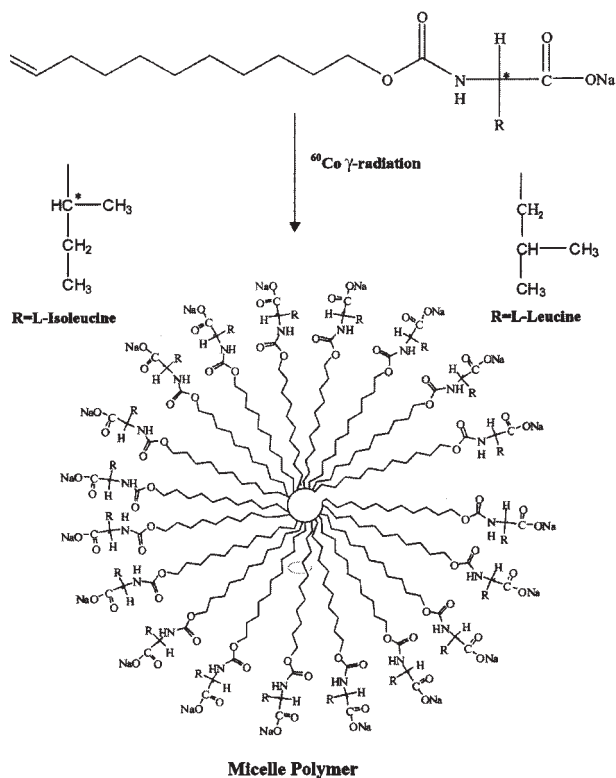


Figure 2. Structure of monomer and micelle polymer of alkenoxy surfactants. Reprinted from [30], with permission.

Kodama *et al.* used copper(II)-L-tartaric acid as a chiral selector for the chiral resolution of malic acid in apple juice [33] and copper(II)-D-quinic acid for the chiral separation of tartaric acid in food products [34]. Chen *et al.* [35] tested the copper(II) complexes of L-prolinamide, L-alaninamide, and L-phenylalaninamide for their ability to resolve dansyl amino acids. The authors made the observation that the enantiomer migration order depends on the nature of the chiral selector. Lu *et al.* [36] resolved the enantiomers of some aromatic amino acids using the copper(II) complex of L-lysine as chiral selector. Lecnik *et al.* [37] investigated the influence of structure, substitution pattern, and conformation of different *N*-alkyl derivatives of proline and hydroxyproline on the chiral recognition ability and enantiomer migration order (EMO) in ligand-exchange CE. Furthermore, the effect of surfactants, such as SDS and cetyltrimethylammonium bromide (CTAB) on EMO was investigated. Cucinotta *et al.* [38] used a histamine-modified β -CD (CDmh) in the presence of copper(II) ions for the chiral separation by the ligand-exchange mechanism (Fig. 3). Recently, the same group [39] reported on the investigation of the copper(II) complex of a new 3-amino derivative of β -CD for its ability of resolving amino acid enantiomers.

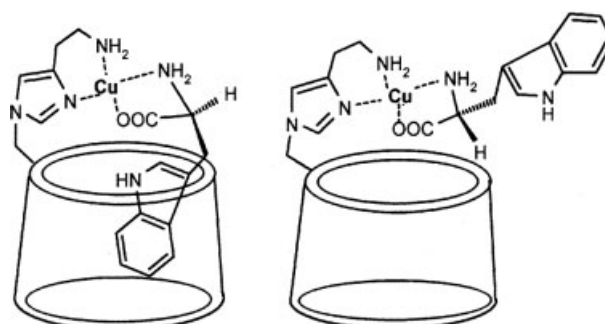


Figure 3. Copper(II)-CDmh ternary complexes with the tryptophan enantiomers. Reprinted from [38], with permission.

Carlsson *et al.* [40] used (–)-2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid as a chiral counterion in nonaqueous ion-pairing CE. With this approach, several β -blockers were resolved. Hedeland *et al.* [41] used *N*-benzoxycarbonylglycyl-L-proline as chiral counter-ion in nonaqueous medium for chiral separation of some pharmacologically active amines by the ion-pairing mechanism. For the chiral separation of tartaric acid (1*R*,2*R*)-(–)-1,2-diaminocyclohexane has been shown to be a useful ion-pairing reagent [42].

The influence of dimerization of selector molecules on the resolution power was tested by Piette *et al.* [43]. They synthesized six new dimeric forms of carbamoylated quinine and quinidine derivatives and checked with these selectors a series of amino acid derivatives. The authors found out that with these dimeric chiral selectors significantly higher resolutions were obtained compared to monomeric derivatives.

Pirkle-type chiral selectors have widely been used in HPLC but mainly in nonaqueous medium. Thormann *et al.* [44] showed that a Pirkle-type chiral selector, (*R*)-(–)-*N*-(3,5-dinitrobenzoyl)- α -phenylglycine, can be used in CE also with aqueous electrolytes. The authors applied this principle to the chiral separation of albendazole sulfoxide in human plasma.

Matsunaka and Haginaka [45] compared ovoglycoprotein (OGCHI) with deglycosylated OGCHI for its separation power for basic drugs. The deglycosylated OGCHI showed reduced chiral recognition ability compared to the intact OGCHI. Although it is believed that the chiral recognition sites exist on the protein moiety of OGCHI, it turned out that the sugars play an essential role in the chiral recognition mechanism.

Kilár and Visegrády [46] used iron-free human serum transferrin as chiral selector and studied the separation mechanism of tryptophan esters and several drugs by

molecular modeling. They showed that the different docking of the enantiomers to the transferrin is in good agreement with their migration behavior. The iron-binding sites of iron-free transferrin are believed to be responsible for the stereo-selective interactions. The authors show that in the case of tryptophan derivatives the *R*-enantiomer have a stronger complexation with transferrin compared to the *S*-enantiomers. The CE experiments match well with these findings since the *R*-enantiomers are always retained stronger.

Marinzi *et al.* [47] selected a series of cyclopeptides synthesized by combinatorial chemistry from a library of thousands of compounds for their chiral recognition ability. Selection was based on the resolution of a set of *N*- α -2,4-dinitro-phenyl amino acids by CE. Ye *et al.* [48] showed that even small amino acids can be used as chiral selectors in CE. With L-leucine, simply applied as an additive to the electrolyte, they succeeded in the separation of ephedrine enantiomers.

Nishi and Kuwahara [49] reported on the use of carboxymethyl (CM) derivatives of carbohydrates as chiral selectors in CE. CM-cellulose, CM-amylose, and CM-dextran showed an enhanced chiral recognition ability compared to the neutral polysaccharides. Two new amphiphilic aminosaccharide derivatives were investigated by Horimai *et al.* [50]. The compounds consisted of a glucoseamine backbone carrying three hydrocarbon chains as the hydrophobic part and three carboxylic groups as the hydrophilic part of the molecule (Fig. 4). These selectors were applied to the chiral separation of dansyl amino acids and new quinolone antibacterial agents.

Lee and Jung [51] introduced cyclosophoraoses, which are cyclic β -D-glucans produced by *Rhizobium meliloti* 2011, as new chiral selectors for CE. Using neutral or anionic cyclosophoraoses in the normal or reversed polarity mode, respectively, the authors succeeded in resolving terbutaline, amethopterin, thyroxine, and *N*-acetylphenylalanine.

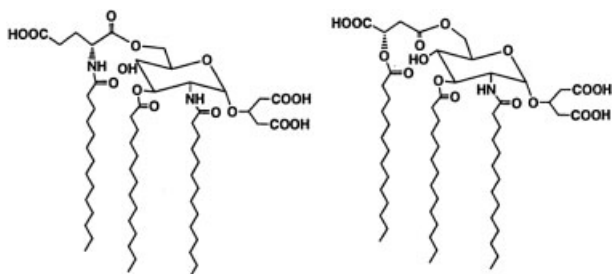


Figure 4. Chemical structures of aminosaccharide derivatives. Reprinted from [50], with permission.

Honzatko *et al.* [52] investigated different terguride derivatives (Fig. 5) as chiral selectors using dansyl amino acids as model analytes. 1-(5-Aminopentyl)-terguride showed the highest resolution power. Since the selectors are strongly UV-absorbing, a partial-filling method was applied. The chiral recognition mechanism is discussed based on X-ray studies.

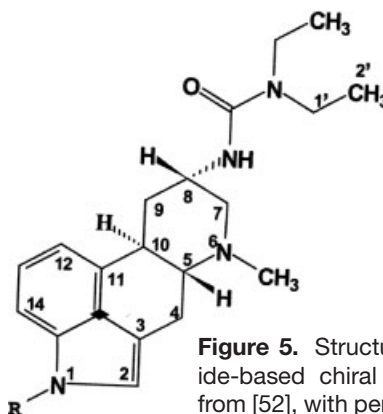


Figure 5. Structure formula of terguride-based chiral selectors. Reprinted from [52], with permission.

Macrocyclic antibiotics have been widely used as chiral selectors in CE. Hou *et al.* [53] first described the use of erythromycin as chiral selector in CE. The authors applied this selector to the chiral separation of four biphenyldimethylester derivatives with antihepatitis activity. Contrary to these findings, another group tested erythromycin and several erythromycin derivatives but did not observe any chiral recognition ability [54].

Tran *et al.* [55] used 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) as chiral selector for the separation of dansyl amino acids. The authors showed that the combination of CHAPS with SDS and CD results in significant enhancement in resolution. γ -CD was found to be superior to β -CD.

3 Capillary electrochromatography

Capillary electrochromatography (CEC) can be performed in open-tubular capillaries, (OT-CEC), packed capillaries (P-CEC) and monolithic columns (M-CEC). Several reviews appeared during the past years focused on this topic [56–58].

3.1 Open-tubular capillaries

A recent review gives an overview of techniques and applications of OT-CEC [59]. Liu *et al.* [60] prepared capillaries for OT-CEC by physical adsorption of avidin to the capillary wall. This simple approach was shown to be applicable to

the chiral resolution of 16 compounds. Wang *et al.* [61] prepared OT-CEC columns by a sol-gel process. The chiral selector 2,6-di-*n*-butyl- β -CD was first reacted with 3-(2-cyclooxypropyl) propyltrimethoxysilane and the resulting intermediate was then treated with tetraethoxysilane and hydrochloric acid to form a sol. The capillary was coated with this solution followed by thermal treatment. The applicability of this approach was demonstrated by means of the chiral separation of ibuprofen and binaphthol as model compounds. Lu and Ou [62] prepared an open-tubular capillary using a polysiloxane derivatized with allyl-permethyl- β -CD and vinylsulfonic acid. The product was coated to a capillary pretreated by sol-gel technology to increase the area of the inner wall. Recently, Wakita *et al.* [63] prepared an open-tubular capillary by copolymerization of a cellulose tris (3,5-dichlorophenylcarbamate) derivative with styrene in the presence of 2,2-azobisisobutyronitrile. The capillary was evaluated by means of *trans*-stilbeneoxide, laudanosine, etozolin, and piprozolin, whereby baseline resolution was obtained for the two latter compounds. Kapnissi *et al.* [64] developed a polyelectrolyte multilayer (PEM) coating procedure for OT-CEC by alternating rinses of positively and negatively charged polymers. Poly(diallyldimethylammonium chloride) was used as the cationic polymer and the surfactant poly (sodium *N*-undecanoyl-L-leucylvalinate) as the anionic chiral polymer. This approach was evaluated by means of the chiral separation of 1,1'-bi-2-naphthol, secobarbital, pentobarbital, and temazepam. Du *et al.* [65] reported on the preparation of a Polybrene/chondroitin double-coated capillary. The capillaries were coated with Polybrene (hexadimethrine bromide) layer to which in a second step chondroitin C was coated. Additionally, the chiral selector was added to the electrolyte, since the amount of chondroitin present in the coating was too small to achieve chiral separation. The main goal of the coating was to improve peak symmetry. The authors studied the influence of structure modification of chondroitin sulfate C. Chemical desulfation of chondroitin sulfate C did not show a significant decrease in enantioselectivity, whereas depolymerization with chondroitinase ABC resulted in complete loss of chiral recognition ability. Chondroitin sulfate A which differs from chondroitin sulfate C only by the position of the sulfate group, showed less enantioselectivity. These capillaries, which showed long lifetime and good chemical stability, were applied to the enantiomer separation of some basic drugs. Recently, the same group introduced a new chiral polysaccharide-based selector, colominic acid, which was also applied in a double-coated capillary in a similar way [66]. Primaquine, chloroquine, and tryptophan were baseline-resolved with this approach. The preparation of molecularly imprinted polymer coatings for OT-CEC by the use of a surface-coupled radical initiator will be discussed in another section [67].

3.2 Packed capillaries

An intensively worked-on type of chiral phases in CEC are polysaccharide phases on the basis of cellulose and amylose derivatives. Such phases which have been successfully used in commercially available HPLC columns are based on cellulose and amylose carbamates or esters which are adsorbed on wide-pore aminopropyl-derivatized silica. These phases were used in CEC with aqueous and nonaqueous mobile phases [68–75]. Mayer *et al.* [76] covalently immobilized a cellulose derivative to silica gel by photopolymerization for P-CEC. They demonstrated the applicability of these phases by the chiral separation of some selected drugs and other model compounds. Chen *et al.* [77] described a regioselective synthesis of a cellulose trisphenylcarbamate CSP by chemically bonding cellulose triphenylcarbamate to 3-aminopropylsilica via 4,4-diphenylmethane diisocyanate. The phase was tested with aqueous and nonaqueous mobile phases using *trans*-stilbene oxide, warfarin, praziquantel, bendroflumethiazide, and benzoin as model analytes. Later, the same group [78] prepared phases by chemically immobilizing cellulose biphenylcarbamate onto diethylenetriamine-propylated silica with tolylene-2,4-diisocyanate as a spacer. Due to the positively charged spacer an anodic EOF was observed in both aqueous and nonaqueous mobile phases. Compared to 3-aminopropyl silica a significantly enhanced EOF was observed. More recently, they prepared a positively charged cellulose derivative CSP by chemically immobilizing cellulose 3,5-dimethylphenylcarbamate onto methacryloyldiethylenetriaminopropylated silica via a radical copolymerization reaction [79]. Thereby a significant enhancement of the EOF was obtained. Interestingly, the authors used hexane-ethanol mixtures as mobile phases and resolved Tröger's base, β -blockers, and some other drugs.

Schurig's group [80] developed CD phases based on permethylated β -CD immobilized on (mercaptopropyl)methyl silica (Chirasil-Dex-silica). Phases on this basis containing β - or γ -CD were checked for the resolution of negatively charged analytes, such as dansyl amino acids under aqueous and nonaqueous conditions [81]. The authors demonstrated the possibility of inverting the EOF by adding 2-(*N*-morpholino)ethanesulfonic acid or triethylammonium acetate.

A drawback of using silica-based HPLC phases for CEC is the relatively low EOF due to the fact that most of the silanol groups are modified. This can be overcome by mixing the material with bare silica. Zhang and El Rassi [82] presented an interesting approach for enhancing EOF (Fig. 6). They prepared a stationary phase consisting of two different layers. A toplayer containing the chiral selector hydroxypropyl- β -CD was immobilized to a sub-

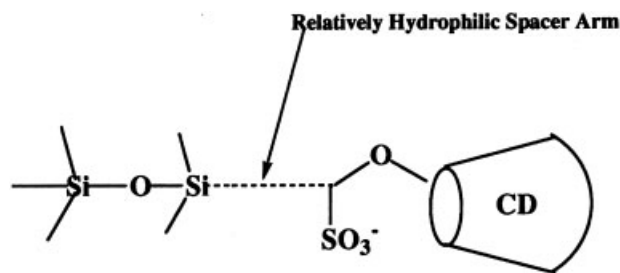


Figure 6. Schematic structure of the HP- β -CD-sulfonated silica stationary phase. Reprinted from [82], with permission.

layer consisting of a hydrophilic sulfonated material. This stationary phase was evaluated by means of the chiral separation of dansyl amino acids and phenoxy acid herbicides.

Gong and Lee [83] prepared crown ether-capped β -CD-bonded silica. This phase combined the chiral recognition sites of both CD and crown ether. The authors checked the chiral recognition ability of this phase by means of indipamide, nadolol, pindolol, and promethazine. The same group produced cyclam-capped β -CD-bonded silica phases [84, 85] which exhibited excellent enantioselectivity for a wide spectrum of compounds. If Ni(II) ions were added to the mobile phase, the selectivity was enhanced for some solutes.

Several groups used silica-based phases containing macrocyclic glycopeptide antibiotics, such as vancomycin [86–88], teicoplanin [89–91], and teicoplanin aglycone [92–94]. While the intact teicoplanin showed enantioselectivity preferentially for larger molecules, such as drugs [90, 91], the aglycone exhibited extremely high chiral recognition ability for amino acids and dipeptides [92–94] (Fig. 7). Berthod *et al.* [95] studied the different behavior of teicoplanin and teicoplanin aglycone by HPLC and concluded that the sugar chains are essential for the chiral recognition of drugs while docking of amino acids to the basket like cavity is favored if the sugar chains are removed. Recently, the group of Fanali [96, 97] studied a glycopeptide of the teicoplanin family, MDL 63,246 (Hepta-Tyr), for its ability of resolving hydroxy acids (Fig. 8). Since the hydroxy acids are negatively charged and move to the anode it is necessary to inverse the direction of the EOF. The authors solved this problem by mixing the Hepta-Tyr modified silica with aminopropylfunctional silica.

Wolf *et al.* [98] investigated a brush-type phase (3*R*,4*S*-Whelk-O CSP) bonded to 3 μ m silica for its chiral separation ability for a broad spectrum of analytes. π - π Interactions are the main interactions supposed to be respon-

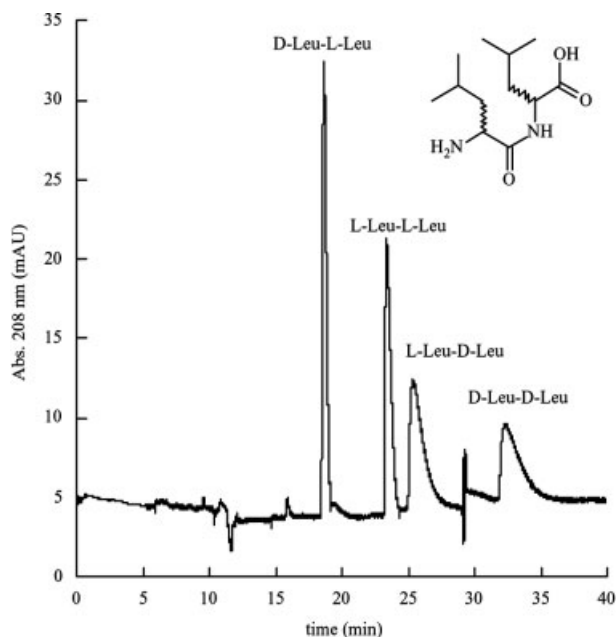


Figure 7. Chiral separation of Leu-Leu on teicoplanin aglycone bonded to 3 μ m silica by CEC. Reprinted from [93], with permission.

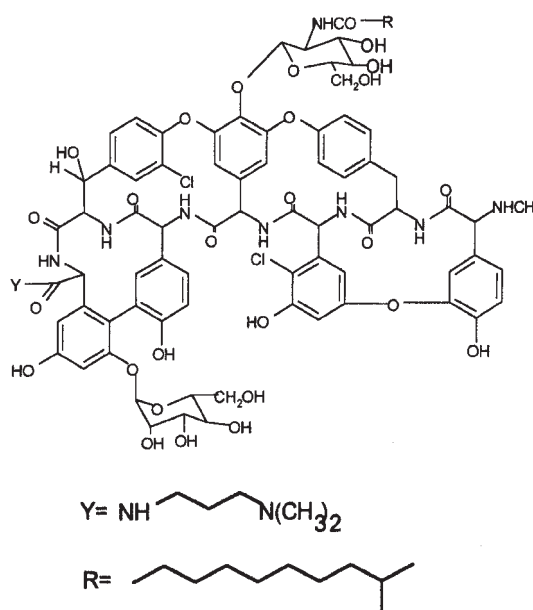


Figure 8. Chemical structure of MDL 63,246 (hepta-Tyr antibiotic). Reprinted from [97], with permission.

sible for chiral recognition. Honzatko *et al.* [99] packed capillaries with 3,5-dinitrobenzoyl-(*R*)-phenylglycine and 3,5-dinitrobenzoyl-(*R*)-naphthylglycine and applied these phases to the chiral separation of *N*-benzoyl-*p*-naphthylamide derivatives of amino acids.

Lindner's group [100, 101] investigated weak anion-exchangers based on *tert.*-butylcarbonyl quinine bonded to porous and nonporous silica particles for their ability to resolve the enantiomers of negatively charged analytes in aqueous and nonaqueous medium. In addition to ionic interactions, π - π interactions might be responsible for chiral recognition. The same group developed strong and weak cation-exchange-type CSPs based on 3,5-dichlorobenzoyl amino acid and phosphonic acid derivatives [102, 103] (Fig. 9). The phases, run in nonaqueous mode, showed enantioselectivity for a broad spectrum of basic drugs including β -blockers, local anesthetics, phenothiazines, and antihistamines. Constantin *et al.* [104] synthesized strong cation-exchangers based on cysteine derivatives with sulfonic acid groups which were bonded to thiol-modified silica particles. Recently, the same group developed several chiral cation-exchanger phases based on β -amino sulfonic acid-terminated dipeptide derivatives, amongst them a *N*-[*N*-(4-allyloxy-3,5-dichlorobenzoyl)-leucyl]-2-amino-3,3-dimethylbutane sulfonic acid was found to exhibit the highest enantioselectivity [105]. The phases were applied to the resolution of β -blockers, β -sympathomimetics, and other basic drugs using nonaqueous mobile phases.

Zhang and El Rassi [106] reported on a chiral CEC phase based on diol-silica dynamically coated with hydroxypropyl- β -CD (HP- β -CD). The chiral selector is added to the electrolyte and adsorbed by hydrogen bonds to the diol groups. The applicability of this approach was

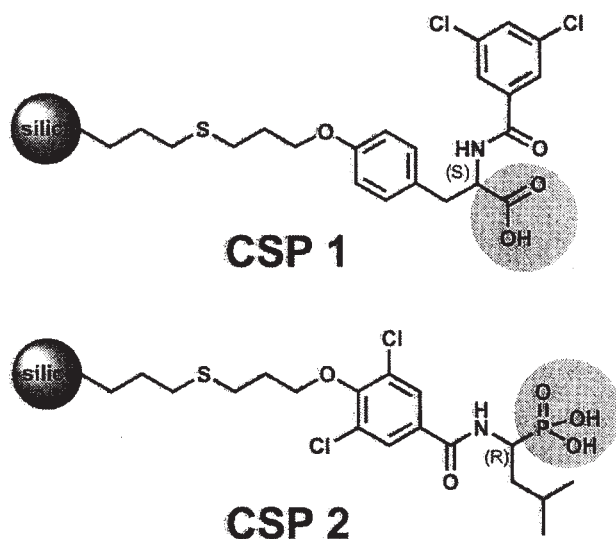


Figure 9. Structure of the CSPs investigated. CSP 1: weak chiral cation-exchanger based on *O*-allyl-*N*-(3,5-dichlorobenzoyl)-tyrosine; CSP 2: strong chiral cation-exchanger based on *N*-(4-allyloxy-3,5-dichlorobenzoyl)-1-amino-3-methylbutane phosphonic acid (phosphonic acid analog of leucine). Reprinted from [103], with permission.

demonstrated by means of the chiral separation of dansyl amino acids and organochlorine pesticides. Ye *et al.* [107] dynamically modified a strong anion-exchange stationary phase of a packed capillary by adding sulfated β -CD to the mobile phase. This approach was applied to the chiral separation of several drug enantiomers.

3.3 Monolithic phases

A recent trend is the preparation of monolithic phases (continuous beds). Thereby packing of capillaries and the preparation of frits by sintering a zone of the silica based packing is avoided. Such frits may break and are sources of air bubbles. Several approaches have been developed. Principally, monolithic phases can be divided into two main categories: monoliths on silica basis and organic polymer-based monoliths. General reviews give an overview on the application of monolithic phases in HPLC and CEC [108–110].

3.3.1 Silica-based monoliths

Siliceous monoliths have been prepared by a sol-gel process based on *in situ* polycondensation of alkoxy silanes [111, 112]. Chiral selectors can be attached by physical adsorption, encapsulating, and on-column derivatization. Liu and co-workers [113] reported on the preparation of a protein-based phase by simply physically adsorbing avidin to a monolithic silica column. This column showed higher separation power than a previously prepared open-tubular capillary containing avidin adsorbed to the capillary wall.

Monolithic phases for ligand-exchange CEC have been prepared by Hobo's group [114–116] using a sol-gel technique and subsequent derivatization with *L*-prolinamide, *L*-phenylalaninamide, and *L*-alaninamide using 3-glycidoxypropyl-trimethoxysilane as a spacer. The phases were loaded with Cu(II) ions and applied to the chiral separation of dansyl amino acids and hydroxy acids. Phases containing *L*-hydroxyproline as a chiral selector [117] showed chiral recognition ability for underivatized amino acids, dansyl amino acids, dipeptides, and hydroxy acids.

Kang *et al.* [118] prepared a CD-containing siliceous monolith capillary column by using the sol-gel process. After gelation, the monolith was hydrothermally treated at 100°C to prevent the sol-gel matrix from cracking. The monolith was coated with a chiral polymer consisting of permethyl- β -CD grafted to polymethylsiloxane by an octamethylene spacer (Fig. 10). The phase was tested by means of hexobarbital, mephobarbital, benzoin, and carprofen for its enantioselectivity. The column was found to be stable for hundreds of runs over a period of two months.

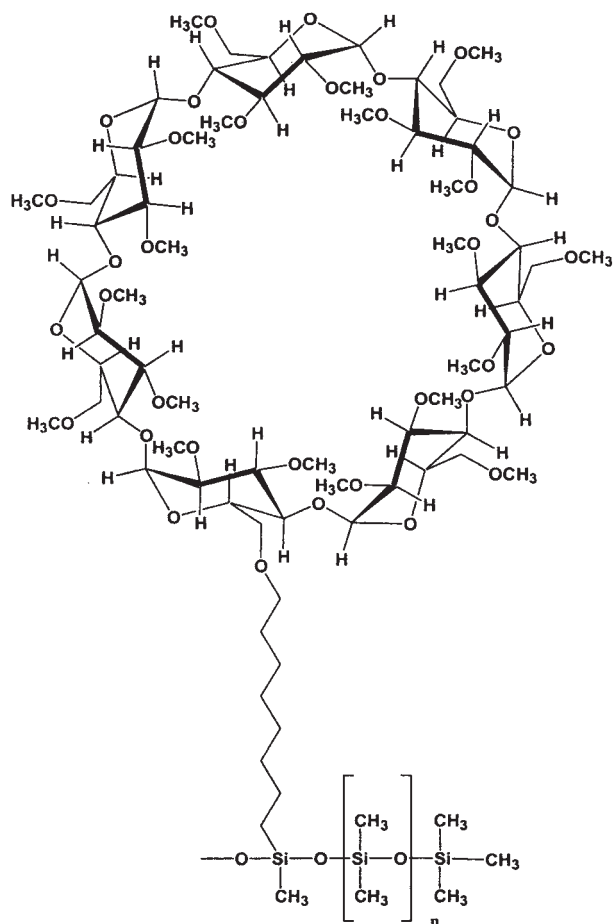


Figure 10. Structure of Chirasil-β-Dex. Reprinted from [118], with permission.

Chen *et al.* [119] described the preparation of chemically modified CD monoliths prepared by the sol-gel process. β- or γ-CD was bonded to the monolith by using 3-glycidoxypropyltrimethoxysilane as a spacer by on-column reaction. These phases were used for the chiral resolution of dansyl-amino acids and the separation of positional isomers of *o*-, *m*-, and *p*-cresols.

Kato *et al.* [120, 121] developed a protein-encapsulation technique for the preparation of monolithic columns for CEC (Fig. 11). Bovine serum albumine or ovomucoid is encapsulated in tetramethoxysilane-based hydrogel. Polycondensation takes place in the capillary which was pretreated with methacryloxypropyltrimethoxysilane. The chiral recognition ability of the capillary was demonstrated by means of tryptophan, benzoin, eperison, and chlorphenamine.

3.3.2 Polymeric monoliths

A different technique for preparing monolithic columns is the formation of organic polymeric continuous beds by *in situ* polymerization of monomers including a chiral selector directly in the capillary. Peters *et al.* [122] copolymerized a valine derivative with ethylene dimethylacrylate, 2-acrylamido-2-methyl-1-propansulfonic acid, and butyl or glycidyl methacrylate and resolved the enantiomers of *N*-(3,5-dinitrobenzoyl) leucine diallylamide as a model analyte on this phase. Pumera *et al.* [123] used a slightly modified approach for the preparation of neutral and negatively charged CD monoliths. They used either physically adsorbed *tert*-butyl-β-CD or copolymerized peracetyl-2'-*O*-β-CD as chiral selectors and tested the phases by means of the chiral separation of ephedrine and ibuprofen.

Schmid *et al.* [124] prepared a chiral continuous bed for ligand-exchange CEC by copolymerization of methacrylamide, piperazine diacrylamide as a cross-linker, vinylsulfonic acid as a comonomer for EOF generation, and *N*-(2-hydroxy-3-allyloxypropyl)-*L*-4-hydroxyproline as a chiral selector. The polymer is immobilized to the capillary wall by prior treatment with γ-methacryloxypropyltrimethoxysilane. This phase was applied to the chiral separation of amino acids [124] and hydroxy acids [125]. A decrease in retention of the negatively charged hydroxy acids was achieved by using diallyldimethylammonium chloride as a positive charge providing agent, thus inverting the EOF.

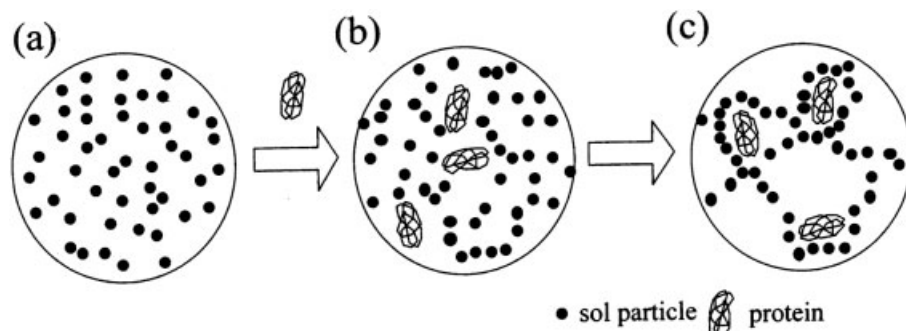


Figure 11. Scheme of protein encapsulation in the silicate matrix during sol-gel polymerization. (a) Formation of sol particles during hydrolysis and condensation. (b) Addition of protein into the sol. (c) The growing silicate network traps protein molecules. Reprinted from [121], with permission.

Kornysova *et al.* [126] prepared a continuous bed containing vancomycin as chiral selector by *in situ* copolymerization of *N*-(hydroxymethyl)acrylamide, piperazine diacrylamide, and allyl glycidyl ether. After conversion of the epoxy groups to aldehyde groups, vancomycin was grafted to the polymer by reductive amination. This phase was applied to the chiral separation of some acidic drugs. Lämmerhofer *et al.* [127, 128] prepared a monolithic chiral phase by copolymerizing *O*-[(2-methacryloxy)ethylcarbamoyl]-10,11-dihydroquinidine, ethylene dimethacrylate, glycidyl methacrylate, or 2-hydroxymethyl methacrylate in the presence of a mixture of cyclohexanol and 1-dodecanol as a porogenic solvent. Among others the authors investigated amino acid derivatives and achieved efficiencies up to 250 000 plates/m. Recently, the same group [129] used a new chiral monomer derived from cinchona alkaloid, *O*-9-(*tert*-butylcarbamoyl)-11-[2-(methacryloyloxy)ethylthio]-10,11-dihydroquinine together with 2-hydroxyethyl methacrylate and ethylene dimethacrylate in the presence of cyclohexanol and 1-dodecanol as porogens for the preparation of chiral monolithic phases. The performance of these phases was checked by means of 9-fluorenylmethoxycarbonyl (Fmoc), 5-dimethylamino naphthalene-1-sulfonyl (Dansyl), 7-nitrobenz-2,1,3-oxadiazolyl (NBD) and carbazole-9-carbonyl (CC) amino acids. Compared to the previous phases, these phases showed improved enantioselectivity, faster separations, and higher efficiency.

Machtejevas and Maruska [130] prepared continuous beds with immobilized human serum albumin as chiral selector. They added acetyl salicylic acid or *L*-tryptophan during the protein alkylation and polymerization steps for interacting with active sites of the protein which are responsible for chiral recognition, thus preventing them to be blocked during immobilization (Fig. 12). With *L*-tryptophan as a protecting compound higher enantioselectivity of the monolithic phase was observed.

Sinner and Buchmeiser [131, 132] made use of a metal-catalyzed ring-opening metathesis polymerization process (ROMP) using a norbornene derivative of β -CD for producing a monolithic column. Chiral separation was achieved for proglumide as a model compound. Kornysova *et al.* [133] presented a polyrotaxane approach for synthesis of continuous beds. The polyrotaxane continuous beds were formed by adding cationic or anionic β -CD derivatives to the solution of a neutral acrylic monomer and cross-linker prior to the initiation of the polymerization. The ability for chiral separation was demonstrated by means of metoprolol.

Koide and Ueno [134] developed a homogeneous polyacrylamide gel containing a chiral crown ether, which was applied to the chiral separation of primary amines. Vég-

vári *et al.* [135] prepared homogeneous gels on polyacrylamide basis by copolymerization of 2-hydroxy-3-allyloxy-propyl- β -CD, acrylamide, *N,N'*-methylenebisacrylamide and 2-acrylamido-2-methylpropane sulfonic acid as negative charge providing agent or dimethyl diallyl ammonium chloride for positively charged gels. The applicability of these phases was demonstrated by means of the chiral separation of a broad spectrum of neutral, acidic, and basic drugs.

3.3.3 Particle-fixed monoliths

A new generation of fritless monolithic columns represents the particle-fixed monolithic columns which can be classified into three principal types: particle-sintered monoliths, particle-entrapped monoliths, and particle-loaded monoliths. Particle-sintered monolithic columns are prepared by thermal treating after conventionally packing a capillary with silica-based materials. Recently, Wistuba and Schurig [136] prepared a chiral monolithic phase by sintering the bed at 380°C and subsequent coating with permethylated β -CD. As an alternative for immobilizing column packings the particle-entrapped approach has been developed by filling the packed capillary with a solution of a silicate sol or an alkoxy silane followed by curing the sol-gel [137, 138]. The latter group immobilized the bed by pumping a solution of methacrylate monomers through the column [139]. These approaches have not yet been applied to the preparation of chiral phases. All these techniques, however, require prior packing of the capillaries. An alternative for preparing fritless capillaries is the particle-loaded technique. Kato *et al.* [140] suspended 5 μ m silica particles modified with (*S*)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine or (*S*)-*N*-3,5-dinitrophenylaminocarbonyl valine in a mixture of tetraethylorthosilicate, ethanol, and aqueous hydrochloric acid which is injected into the capillary. The sol-gel matrix embeds the particles and immobilize the bed in the capillary. The authors demonstrated the applicability of this simple approach by means of the chiral separation of NBD-amino acids (Fig. 13). Lin *et al.* [141] prepared a chiral imprinted acrylate polymer using *L*-phenylalanine as a print molecule. After crushing and sieving, the polymer particles are suspended in an acrylamide gel and filled into the capillary. The remaining imprinted polymer was found to bind *L*-phenylalanine more strongly than the *D*-enantiomer.

Schmid *et al.* [142] prepared particle-loaded monoliths by suspending silica-based chiral phases containing macrocyclic antibiotics as chiral selectors in a mixture of methacrylamide, piperacyl diacrylamide, ammonium sulfate, and a charge-providing agent. This mixture is pulled into the capillary and polymerized *in situ*. A teicoplanin

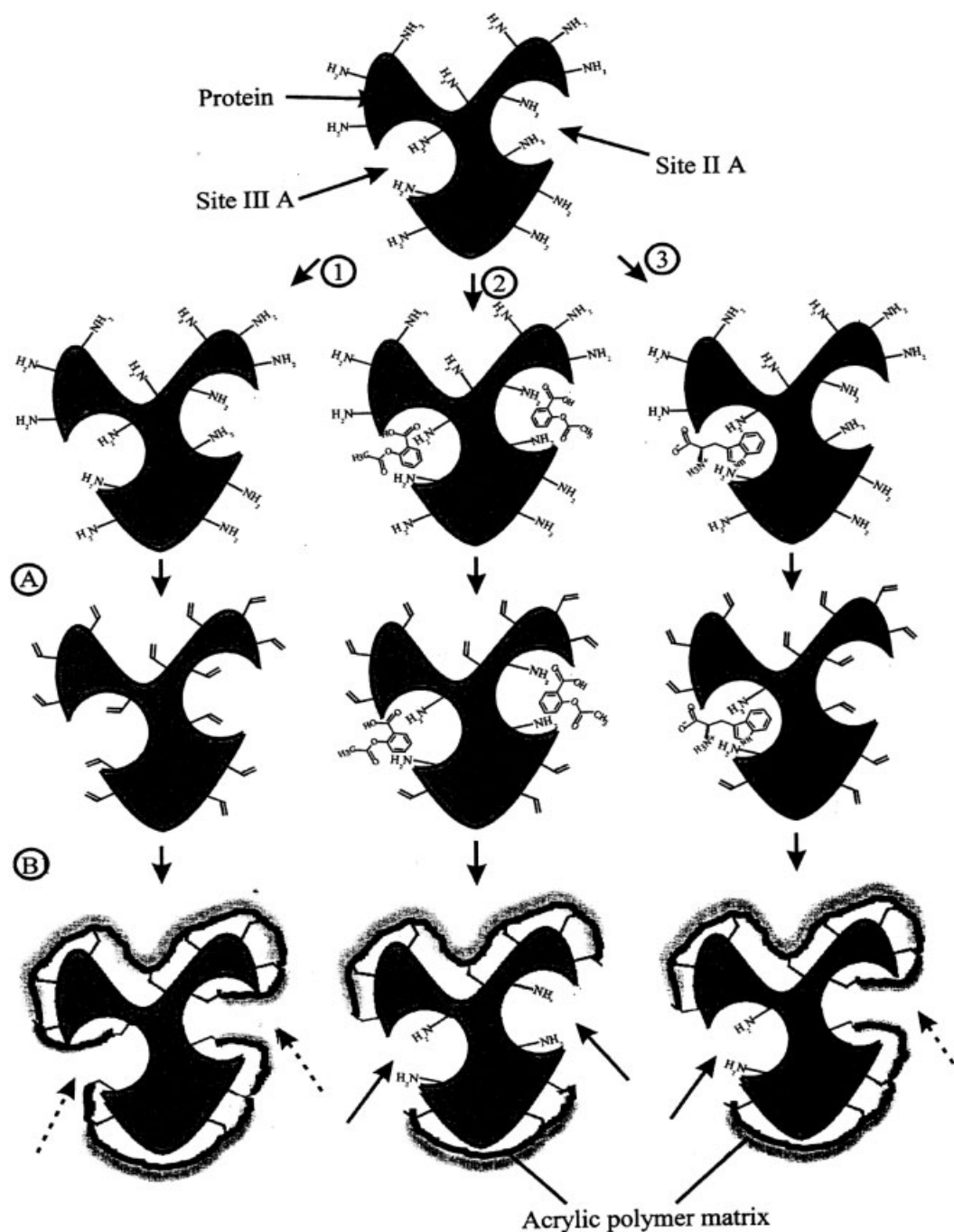


Figure 12. Schematic drawing of the HSA immobilization procedure without using an additive (branch 1), using acetylsalicylic acid (branch 2), and using L-tryptophan (branch 3) as an additive. Step A, allylation of the protein; step B, continuous bed synthesis. Free access to the active site for the analyte molecules (\rightarrow), limited access for the analyte molecules ($--\rightarrow$). Reprinted from [130], with permission.

aglycone phase was found to show a marked enantioselectivity for amino acids and dipeptides (Fig. 14) using vinylsulfonic acid as a charge providing agent whereas for the separation for hydroxy acids a ristocetin A phase in combination with diallyldimethylammonium chloride as

cationic agent for creating an anodic EOF was used. The advantages of this approach are the ease of preparation and the option of using any commercially available silica-based chiral phase, efficiency, however, is lower compared to packed capillaries.

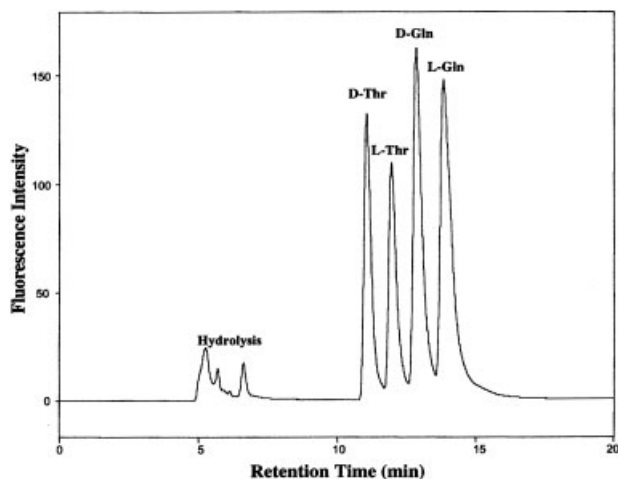


Figure 13. Electrochromatogram of DL-Gln and DL-Thr on a particle-loaded monolith. Reprinted from [140], with permission.

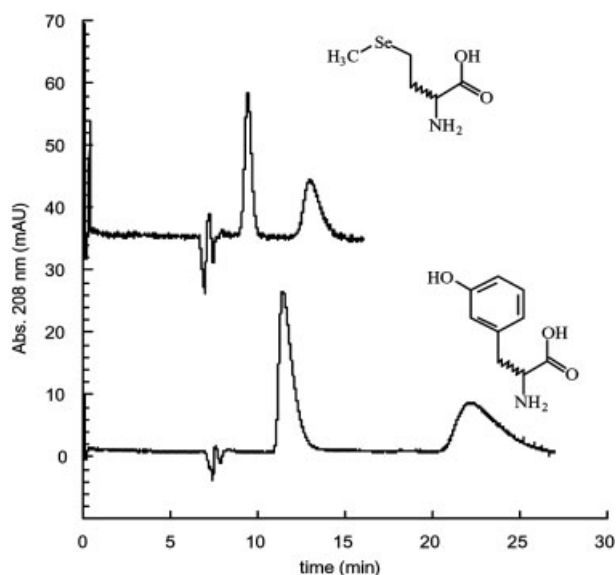


Figure 14. Enantiomeric separation of m-Tyr and seleno-Met using CEC on a 25% particle-loaded teicoplanin aglycone monolith. Reprinted from [142], with permission.

4 Molecularly imprinted chiral phases

Generally, molecularly imprinted polymers (MIPs) are prepared by polymerization of a mixture of monomers, a cross-linking agent, an initiator, and a chiral template. Polymerization can be initiated by heat or by UV-light. After polymerization the template is removed leaving an imprint which is able to recognize enantioselectively the original template molecule. This general approach has been utilized in different variations. Such phases were

prepared for OT-CEC, P-CEC, and M-CEC as well as HPLC. For more detailed information the reader is referred to specialized reviews [143–146].

Monolithic columns for different analytes were prepared by Nilssons' group [145] by *in situ* polymerization. The authors showed that the porosity of the polymer can be influenced by the choice of a nonpolar solvent as a porogen [147]. The same group demonstrated the possibility of rapid enantiomeric separation of propranolol by using short superporous MIP monoliths prepared by a photo-initiated polymerization reaction [148]. In a recent paper these authors discussed the use of multiple templates and studied the influence of surfactants as electrolyte additives on separation [149].

As an interesting alternative to monolithic phases, pseudostationary phases based on spherical microparticles have been prepared. Spherical microparticles on acrylate basis (0.2–0.5 μm) of imprinted (S)-propranolol suspended in a buffer were drawn into the capillary using a partial filling method [150, 151] (Fig. 15). Since the MIP particles contain carboxyl groups, they are negatively charged and tend to migrate to the anode, the analyte migrates to the cathode thus passing the MIP plug and reaching the detection window before the microparticles. Recently, the same group designed a multiple target approach using MIP nanoparticles [152] comparing two variations. In the first variation they mixed two singly templated MIP nanoparticles of different selectivity, while in the second approach they used two different templates during the preparation of the nanoparticles. As model templates (S)-propranolol and (S)-ropivacaine were used. A similar approach has been described by Boer *et al.* [153] using highly crosslinked microspheres imprinted with (+)-ephedrine. The imprinted microspheres showed chiral recognition ability for ephedrine and salbutamol.

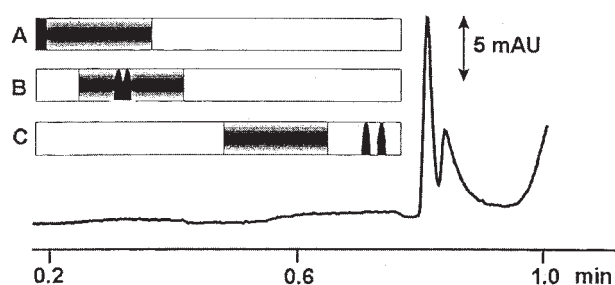


Figure 15. Electropherogram showing an enantiomer separation of propranolol achieved on MIP microparticles. The inset shows a schematic of the partial-filling technique. (A) MIP microparticles suspended in the electrolyte are injected prior to the sample. (B) As the electric field is applied, the sample starts to move through the MIP plug (due to its net positive charge compared to the negative charges of the microparticles) and (C) reaches the detection window prior to the MIP plug. Reprinted from [145], with permission.

Quaglia *et al.* [154, 155] developed a surface-initiated polymerization technique for the preparation of MIP-based CSPs (Fig. 16). Macroporous silica particles were modified with an azoinitiator and suspended in a mixture of methacrylic acid, ethyleneglycoldimethacrylate, and L-phenylalanine anilide as a template. The polymerization was initiated photochemically. After removing the template the phase was packed by conventional way into fused-silica capillaries over a length of 8 cm. Schweitz [67] described a similar technique for the preparation of open-tubular capillaries. A radical initiator was immobilized on the capillary wall. Then a mixture of methacrylic acid, trimethylolpropane trimethacrylate, and (S)-propranolol as a template was filled into the capillary and polymerization was initiated by UV-radiation. This technique requires exact timing of the polymerization to prevent filling the capillary com-

pletely with the polymer. After polymerization the capillary is flushed with acetonitrile/acetic acid to remove the template.

5 Microchip techniques

Microchip techniques attracted increasing interest in chiral CE in recent years. Schwarz and Hauser [156] resolved the enantiomers of adrenaline, noradrenaline, ephedrine, and pseudoephedrine on a microchip device using a new two-electrode amperometric detection technique. As chiral selector carboxymethyl- β -CD with or without addition of 18-crown-6 was used. Ludwig *et al.* [157] used simply UV detection in a commercial instrument. With highly sulfated CDs as chiral selectors the authors achieved baseline separation of 19 compounds in

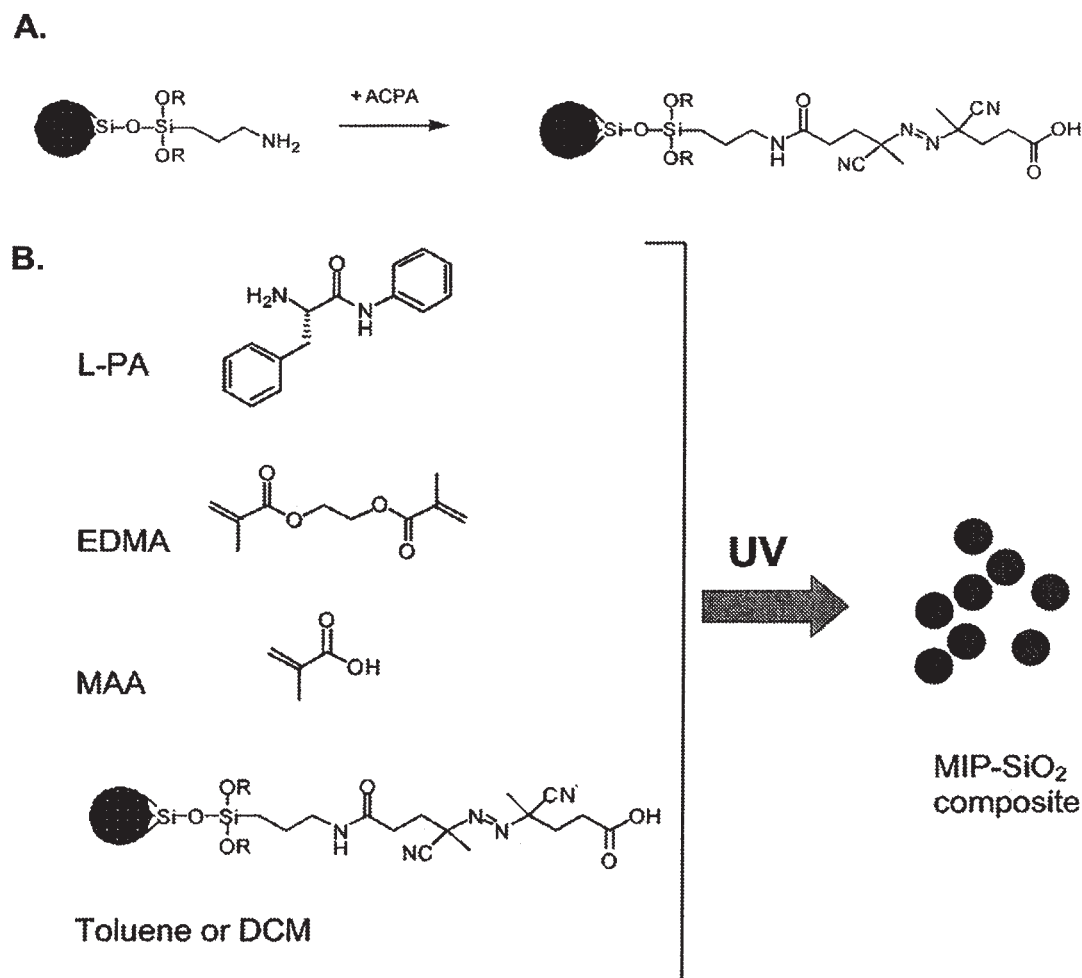


Figure 16. Synthesis of L-phenylalanine anilide (L-PA) MIP using surface immobilized initiators (EDMA, ethyleneglycoldimethacrylate; MAA, methacrylic acid; DCM, dichloromethane). Reprinted from [155], with permission.

less than 1 min. The fastest separation was obtained for norephedrine in 2.5 s. Wang *et al.* [158] resolved fluorescein-5-isothiocyanate (FITC)-labeled amino acids on glass chips equipped with a laser fluorescence (LIF) detector by electrokinetic chromatography using γ -CD and SDS as electrolyte additives. FITC-tagging was also used by other groups for chiral and nonchiral separations [159, 160]. Skelley and Mathies [161] investigated the enantiomers of fluorescamine-labeled amino acids in a microfabricated chip-capillary electrophoresis device using HP- β -CD as chiral selector and compared the results with FITC-labeled amino acids. As detector a confocal fluorescence microscope with a 404 nm blue diode laser excitation source was used. The authors showed that this technique has potential for determination of the enantiomeric ratio of amino acids for extraterrestrial life detection. Preliminary results on soil samples from the Atacama Desert indicated that fluorescamine labeling enables the detection of 100 ppb concentrations of amino acids.

Male and Luong [162] designed a CE system equipped with an array of microfabricated interdigitated platinum electrodes. The system was applied to the simultaneous chiral analysis of three neurotransmitters, epinephrine, norephedrine, and isoproterenol, using heptakis(2,6-di-O-methyl)- β -CD as chiral selector. The interdigitated electrode chip served as an amplification/detection system and consisted of an array of seven electrodes at oxidizing potential to oxidize the analytes and a detector electrode set at reducing potential. Thereby fouling of the detection electrode is avoided. Recently, Liu *et al.* [163] reported on a microchip CE system combined with on-chip chemiluminescence detection. Using HP- β -CD as chiral selector and the peroxy oxalate/hydrogen peroxide chemiluminescence system, dansyl amino acid enantiomers were resolved within 1 min and detected with high sensitivity. Ölvecka *et al.* [164] designed a microchip isotachopheresis (ITP) device with on-column conductivity detection using tryptophan enantiomers as a model compound. More informations can be found in a recent review by Belder and Ludwig [165].

6 Miscellaneous

Two new approaches for preparing frits have been presented by Zare's group [166, 167]. One approach is based on photopolymerization of glycidyl methacrylate and trimethylolpropane trimethacrylate. The second technique utilizes a sol-gel process by filling a capillary with a solution of 3-(trimethoxysilyl)propyl methacrylate, hydrochloric acid, water, and toluene as a porogen and a photoinitiator (Irgacure 1800) and exposing the section of the

capillary where the polyimide coating was removed by UV light. The unreacted products are removed by rinsing the capillary with ethanol. The capillary was packed with a slurry of silica particles modified with (S)-N-3,5-dinitrobenzoyl-1-naphthylglycine as chiral selector. The capillary was used for the chiral separation of NBD-amino acids.

On-line coupling of capillary ITP and CZE for enantiomer separation has been described by Fanali *et al.* [168]. The authors demonstrated the high potential of ITP for sample cleanup and preconcentration of analytes. As model analytes the enantiomers of tryptophan and 2,4-dinitrophenyl norleucine in different enantiomer ratios were applied and as chiral selector α -CD was used in both systems. In-column and post-column ITP sample cleanup techniques were compared. The authors showed that it is possible to detect traces of one enantiomer beside a high excess of the other enantiomer in spiked urine samples.

Zhong and Yeung [169] designed a capillary array system for combinatorial chiral separations. The system consisted of 96 capillaries, whereby the outlet ends of every eight capillaries were bundled together. Thereby in 12 bundles 8 compounds can be tested in the same run at 12 different separation conditions. Using neutral and sulfated CDs as chiral selectors with different buffers, for 49 out of 54 compounds tested, the optimal separation conditions could be found within short time.

Chankvetadze [170] gave an overview of factors influencing the EMO in CE and discussed possibilities for reversal of EMO. Reversal is of importance, for example, with enantiomer purity check of drugs. The enantiomer which has to be detected in trace amounts as an impurity in a sample of the biologically active enantiomer, should always appear as the first peak, otherwise it would be covered by the tailing of the major enantiomer. Briefly, the EMO can be reversed amongst others by using a selector of opposite chirality, changing from neutral to charged selectors, changing the pH, changing the mobility of the analyte or the selector and reversal of EOF. The EOF can be reversed by adding hydrophobic quaternary ammonium compounds to the electrolyte or by adding charged micelle forming surfactants.

A promising approach, microfluidic temperature gradient focusing, has been developed by Ross and Locascio [171]. With this approach, a 10 000fold concentration of the dilute sample can be achieved. This technique has high potential to be applied to chiral separation in biological fluids.

7 Conclusions

A lot of progress has been done in chiral CE and CEC during the past four years. Although no new chiral separation principles have been introduced, several new chiral selectors have been developed for CE. A lot of developments have been done in CEC. Several new chiral phases have been prepared for CEC. However, before becoming a routine method, CEC has certainly to undergo additional developments. Packing of a capillary with silica-based phases is not easy and the preparation of frits by sintering a zone of a packing still remains a sophisticated procedure. A lot of research has been performed in the field of monolithic fritless CEC phases reflecting the actuality of this recent trend. Miniaturization is also a future trend. The development of microchip based CE systems became a subject of intensive investigations. Sample pretreatment and preconcentration are also subjects to be investigated intensively. Coupling of CE with ITP or flow-injection analysis are approaches worth mentioning in this direction. Detection sensitivity is another problem in CE and CEC. LIF detection, chemiluminescence detection, and coupling electromigration techniques with MS have already been shown to be means for improving detection sensitivity.

Received July 14, 2004

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