Focusing Review

Development of Chiral Separation Systems for Capillary Electrophoresis, Electrochromatography and Liquid Chromatography

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Abstract

This review paper summarizes the author's research work on the development of chiral separation systems undertaken since 1997 during which time he has studied and worked at Tokyo Metropolitan University. The main topics include ligand−exchange capillary electrophoresis, ligand−exchange micellar electrokinetic chromatography, chiral monolithic silica columns and their applications in chiral capillary electrochromatography and microcolumn−liquid chromatography, electrokinetic capillary chromatography using 18−crown−6 tetracarboxylic acid as a chiral selector and/or using the surfactant of sodium alkyl polyoxyethylene sulfate as a pseudo−stationary phase in the electrolyte, and the diamide−type chiral stationary phase for liquid chromatography.

Keywords: Chiral separation; Chiral stationary phase; Ligand exchange; Monolithic column; Capillary electrochromatography; Capillary electrophoresis; Micro−liquid chromatography; Micellar electrokinetic chromatography; Amino acid; Hydroxy acid; Amino acylamide; Cyclodextrin.

1. Introduction

Since the turn of the twentieth century, it has been recognized that many drugs, agrochemicals, food additives and fragrances are chiral compounds and their chirality may considerably affect their bioactive behavior [1, 2]. The demand has been increasing for new analytical methods with high selectivity and efficiency. Chiral separation has become an attractive and important field in the separation sciences. Chromatographic techniques, such as thin–layer chromatography (TLC), gas chromatography (GC), supercritical fluid chromatography (SFC) and high performance liquid chromatography (HPLC), have often been used [3,4], and are conventional techniques for chiral separation.

In recent decades great attention has been paid to new separation techniques, such as capillary electrophoresis (CE), capillary electrochromatography (CEC) and microcolumn–liquid chromatography (μ–LC). CE is an analytical technique with a wide range of applications in, for example, the pharmaceutical, biological, and environmental analysis fields. The diverse separation modes make the CE technique a very important tool for chiral analysis. The most frequently applied CE modes for chiral separation are capillary zone electrophoresis (CZE), with the addition of a chiral selector to the background electrolyte (BGE), electrokinetic capillary chromatography (EKC), using chiral pseudo–stationary phases or charged chiral selectors, capillary gel electrophoresis (CGE), with chiral selectors incorporated in gels, and CEC, which employs capillary columns coated with chiral stationary phase (CSP) on the inner wall of capillary, and either packed with CSP or fabricated by monolithic methods. The development of new column technologies, separation modes and molecular recognition materials promotes the development of the chromatographic sciences.

This paper provides a brief review of recent advances in the development of chiral separation techniques achieved in the author's group. The topics include ligand exchange–capillary electrophoresis (LE–CE), ligand exchange–micellar electrokinetic chromatography (LE–MEKC), chiral monolithic silica columns and their applications in CEC and μ–LC, EKC using 18–crown–6–

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tetracarboxylic acid ($18C6H_4$) as a chiral selector and the surfactant of sodium alkyl polyoxyethylene sulfate as a pseudo−stationary phase in a BGE, and diamide−type CSP for LC.

2. LE−CE and LE−MEKC

2.1 Background of chiral LE and proposed LE−MEKC

Ligand exchange chromatography (LEC), a technique suggested by Helfferich in 1961 [5], was further developed by Davankov et al. [6] into a powerful chiral chromatographic method in the early 1970 s. These original LEC phases were based on L−proline residues bonded to chloromethylated polystyrene, using Cu(II) or other metal ions as complexing agents for resolving amino acids (AA) with remarkable enantioselectivity. Gübitz et al. developed the chiral LEC phases on silica gel [7, 8]. Subsequently, a number of papers reported chiral separation by LEC [9, 10]. Cu(II) complexes of L−/D−histidine or aspartame used previously as a chiral additive in HPLC [11] were first introduced into CE by Zare's group in 1985 [12] and 1987 [13] as chiral selectors for resolving dansyl amino acids (Dns−AA). Subsequently, several groups reported the application of LE−CE for chiral separation. Recent review papers have summarized the advances made in LE−CE [14−16].

The principle of LE is based on the formation of diastereomeric ternary mixed metal complexes between the chiral selector ligand and the analytes. The chiral resolution results from the difference between the complex stability constants of two mixed complexes with analyte enantiomers. Figure 1 A shows a schematic representation of LE−CE. The following equilibrium is used to express the LE mechanism, where Sel indicates a selector and AA stands for amino acid.

$$Cu(L\text{-}Sel)_2 + DL\text{-}AA$$

$$Cu(L\text{-}Sel)\,(D\text{-}AA) + Cu(L\text{-}Sel)\,(L\text{-}AA) \qquad (1)$$

LE showed enantioselectivity for resolving a variety of enantiomers, however, in many cases the selectivity for analytes and the enantioselectivity for enantiomers is insufficient. MEKC introduced by Terabe et al. in 1984 [17] employs micelles as a pseudo−stationary phase for resolving uncharged or charged analytes with excellent selectivity depending on the partition coefficient of the analyte inside the micelle. To improve both the enantioselectivity between enantiomers and the selectivity among the analytes, we proposed a hybrid mode, called LE−MEKC [18, 19]. The LE−MEKC model is shown in Figure 1 B. The Cu(II) complex with L−hydroxyproline, (L−OH−Pro), functions as the chiral selector for enantioselective recognition, and the micelles function as the pseudo−stationary phases for manipulating the selectivity among the analytes and between the enantiomers. The synergistic interaction of LE and MEKC improves both the enantioselectivity between the enantiomers and the selectivity among the analytes. Therefore, LE−MEKC integrates advantages of high enantioselectivity in LE and excellent selectivity in MEKC, and greatly expands the LE application range.

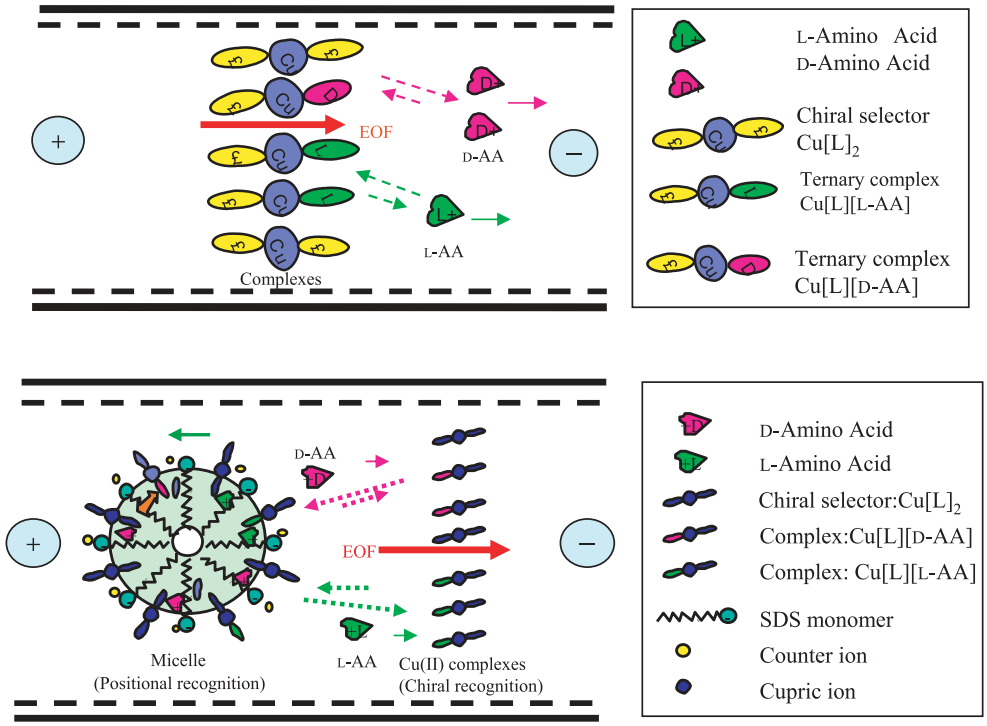


Figure 1. Schematic representation of proposed models of LE−CE (A, top) and LE−MEKC (B, bottom). (From Ref. [19]).

2.2 Simultaneous separation of positional and optical isomers by LE−MEKC

A successful application of LE−MEKC was the simultaneous separation of positional and optical isomers. As shown in Figure 2, the simultaneous separation of 12 positional enantiomers of *o*−, *m*−, *p*−DL−tyrosine and *o*−, *m*−, *p*−fluoro−DL−phenylalanine was achieved by using an electrolyte containing 25 mM Cu(II)−50 mM L−OH−Pro and 10 mM sodium dodecyl sulfate (SDS) at pH 4.0 [20]. The pH and the SDS concentration both played important roles in the simultaneous separation. pH was used to control the velocity of electroosmotic flow (EOF) , and the SDS concentration was employed to manipulate the selectivity amnong the positional isomers and enantiomers. Furthermore, by using an electrolyte containing 25 mM Cu(II), 50 mM L−OH−Pro and 10 mM SDS at pH 5.25, we also achieved the simultaneous separation of 16 positional enantiomers of tryptophan derivatives, as shown in Figure 3 [21].

In LE−MEKC, micelles work as the pseudo−stationary phase and improve the separation selectivity especially for positional isomers, and Cu(II) complexes with L−OH−Pro as the chiral selectors for chiral recognition. A baseline simultaneous separation can be

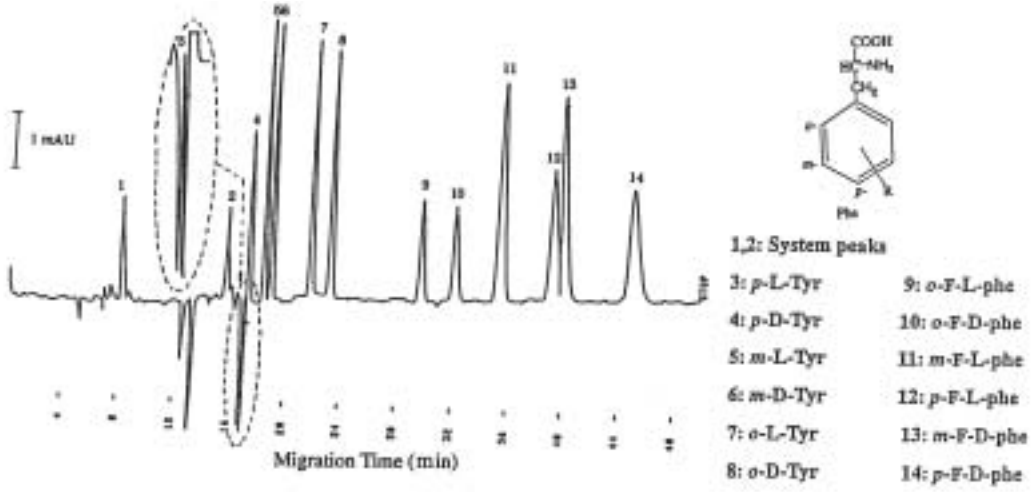


Figure 2. Simultaneous separation of 12 *o*−, *m*−, *p*−DL−tyrosine and *o*−, *m*−, and *p*−fluoro−DL−phenylalanine. Electrolyte: 25 mM Cu(II), 50 mM L−hydroxyproline and 10 mM SDS at pH 4.0. Applied voltage: 10 kV. Fused silica capillary: 40 cm effective length, 55 cm total length, 50 ìm i.d. and 375 ìm o.d., UV detection: 208 nm. (From Ref. [20]).

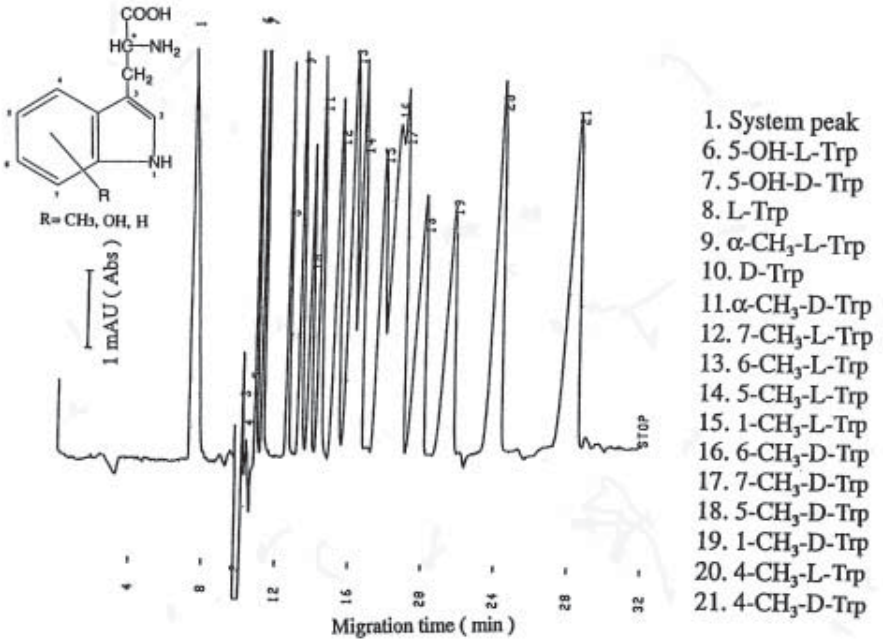


Figure 3. Simultaneous separation of 16 positional enantiomers of tryptophan derivatives. Electrolyte: 25 mM Cu(II), 50 mM L−hydroxyproline and 10 mM SDS at pH 5.2. Other conditions are the same as in Figure 2. (From Ref. [21]).

achieved because the analytes interact concurrently with both the micellar phase of SDS and the chiral selector of the Cu(II) complex with L-OH-Pro, as shown in Figure 1 B. In addition, we demonstrated that the positional isomers were resolved by MEKC using the SDS micellar phase. This result suggests that the micellar phase can be used for recognizing positional isomers. We also noticed that the migration times of analytes in LE-MEKC were correlated with log P_{ow} (the logarithm of the octanol-water partition coefficient). Therefore, log P_{ow} can be used to predict the migration orders of analytes in LE-MEKC.

2.3 Reversal of enantiomer migration order in LE-MEKC

To explore the chiral recognition mechanism of ligand exchange in LE-MEKC, we have systematically investigated the separation behavior, particularly that of the enantiomer migration order (EMO) [22, 23]. Interestingly, we found that the EMOs in LE-MEKC were reversed not only by the introduction of the micellar phases of anionic surfactants [23, 24], but also by the change in the chirality of the selector ligand and/or the *cis*/*trans* conformation of the 4-hydroxy group in 4-hydroxyproline [22]. Figures 4 and 5 show the EMO reversal that resulted from the SDS micellar phase, and from the *cis*/*trans* conformation in the presence of the micellar phase, respectively. Moreover, the free amino acid enantiomers and hydroxy acids behaved differently in the presence of the micellar phases, in other words, the micellar phases can cause the EMO reversal of amino acids, but not of hydroxy acids. The EMO behavior of amino acids and hydroxy acids is summarized in Table 1.

The EMO reversal caused by the change in the chirality of the ligand and/or *cis*/*trans* conformation could be explained by the difference in the complex stability, as well as in terms of the influence of the tridentate ligand. As shown in Figure 1 A, under experimental conditions, analytes (DL-amino acids: DL-AAs) possess positive charges and migrate toward the cathode at a velocity faster than EOF. Cu(II) complexes such as the chiral selector of Cu(II)(L-Sel)$_2$ (for example: L-Sel = L-proline) and ternary complexes of (L-Sel) Cu(II) (L-AA) and (L-Sel) Cu(II) (D-AA) that are formed after exchanging ligands have a neutral charge and migrate with the same velocity as EOF. L-amino acid interacts preferentially with the chiral selector of Cu(II) (L-Sel)$_2$, and so, unlike D-AA, it forms a more stable ternary complex, (L-Sel) Cu(II) (L-AA), than D-AA, i. e. $K_{L^*-L} > K_{L^*-D}$. Thus, L-AA has a slower migration time. In the same way, if D-proline is used as a chiral selector, D-amino acids interact preferentially with the chiral selectors, resulting in a slower migration time than L-amino acids. In this case, the chiral selectors work as the CSP in LC. Based on the complex formation constants estimated in our work [25], ternary complexes of (L-proline) Cu (II) (L-AA) and (D-proline) Cu(II) (D-AA) are more stable than those of (L-proline) Cu(II) (D-AA) and (D-proline) Cu(II) (L-AA).

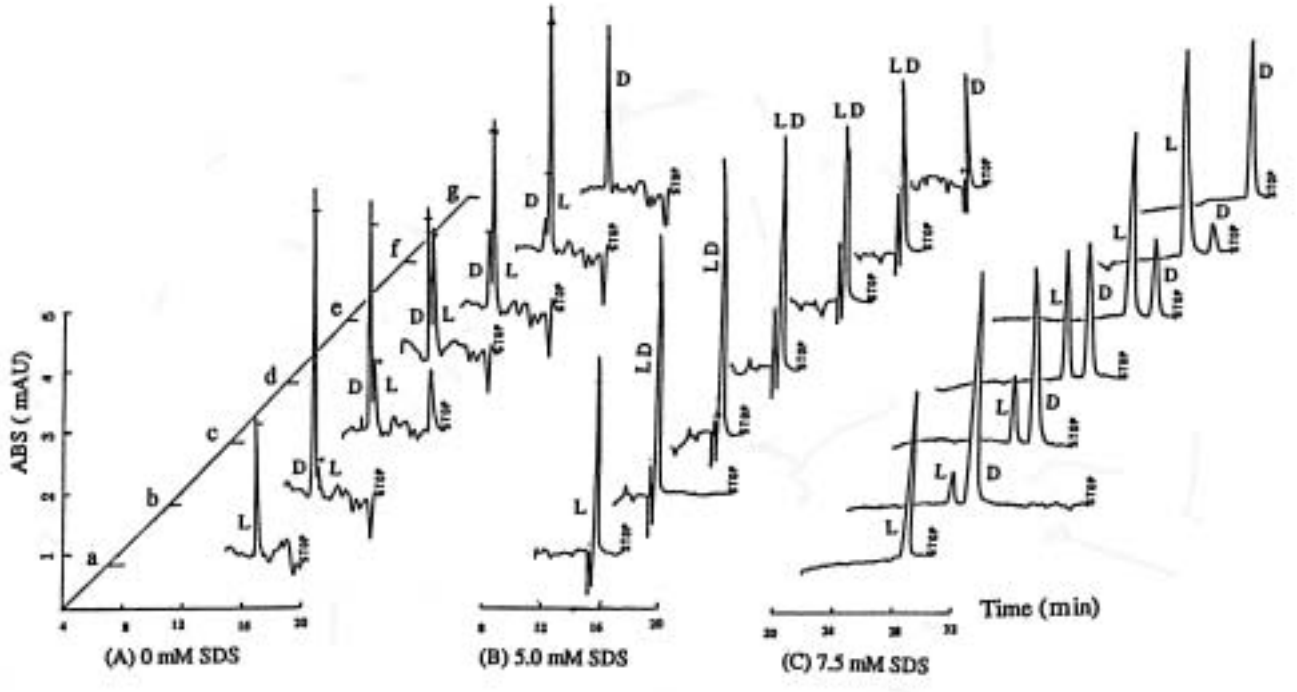


Figure 4. EMO reversal of DL-Phe in different ratios of D- to L-enantiomers resulting from the introduction of SDS micelles. Electrolyte: 25 mM Cu(II), 50 mM L-hydroxyproline and different concentrations of SDS at pH 4.0. (a) L (5×10^{-4} M), (b) D/L= 9:1 ($\times10^{-4}$ M), (c) D/L=7.5:2.5 ($\times10^{-4}$ M), (d) D/L=5:5 ($\times10^{-4}$ M), (e) D/L=2.5:7.5 ($\times10^{-4}$ M), (f) D/L=1:9 ($\times10^{-4}$ M) and (g) D (5×10^{-4} M). Other conditions are the same as in Figure 2. (From Ref. [23]).

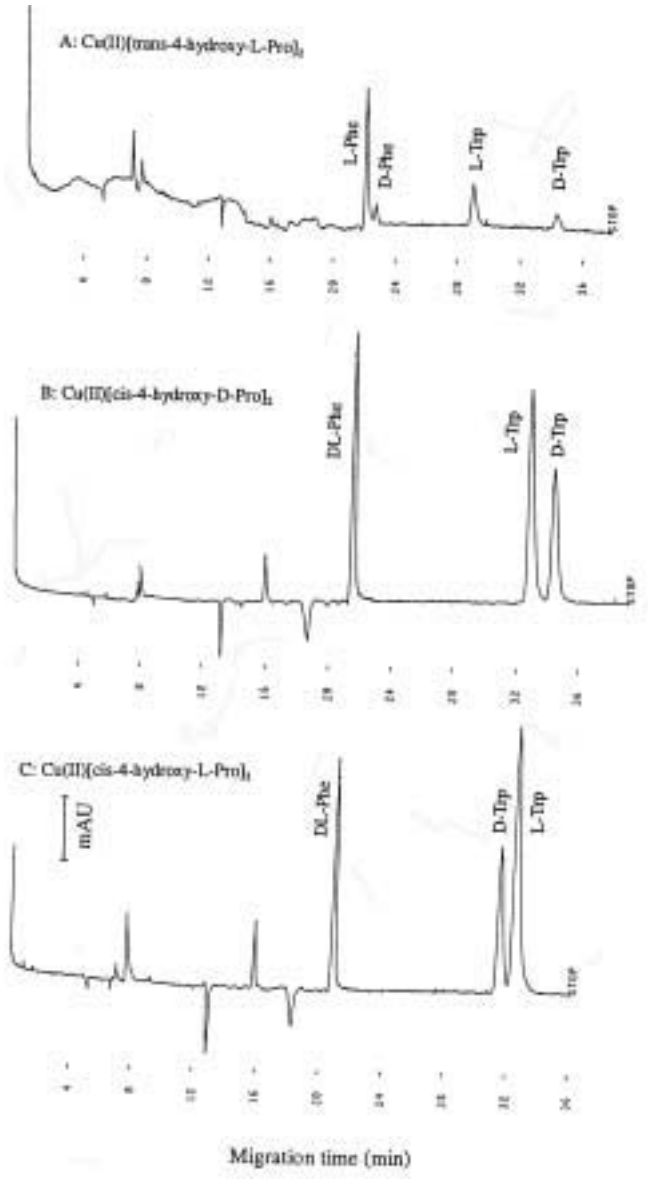


Figure 5. Reversal of EMO of DL−Phe and DL−Trp resulting from the change of chirality and *trans*−/*cis*−conformation. Electrolyte: pH 4.0, 6 mM SDS, 20 mM $CuSO_4$, and 40 mM *trans*−4−hydroxy−L−proline (A), *cis*−4−hydroxy−D−proline (B), and *cis*−4−hydroxy−L−proline, respectively. Other conditions are the same as in Figure 2. (From Ref. [22]).

The stability of the complexes can be explained by their molecular models, as shown in Figure 6. In Figure 6, DL−Phe is used as an example of a solute with which to form ternary complexes. When D−proline is used as the ligand, as shown in Figures 6 A and B, a complex with D−Phe offers a more stable conformation than that obtained with L−Phe. As shown in Figure 6 A, although the phenyl ring in phenylalanine rotates freely, it can adopt a *trans* lowest energy conformation with a proline ring around the copper coordination plane. However, in Figure 6 B the free rotation of

Table 1. Effect of ligand chirality, 4−hydroxy *trans*/*cis* conformation and anionic surfactants on EMO of amino acids and hydroxy acids

Ligands	EMO Without SDS		EMO With SDS	
	amino acide	hydroxy acids	amino acide	hydroxy acids
D−proline	L→D	D→L	D→L	D→L
L−proline	D→L	L→D	L→D	L→D
DL−prolineL	L=D	D=L	L=D	D=L
cis−4−hydroxy−D−proline	D→L	L→D	L→D	L→D
cis−4−hydroxy−L−proline	L→D	D→L	D→L	D→L
trans−4−hydroxy−L−proline	D→L	L→D	L→D	L→D

substituent phenyl makes either a *cis* conformation between the phenyl ring and the proline ring around the copper coordination plane or induces a sterically hindered interaction between the phenyl ring and the axial water molecule; both result in a complex with an unstable structure. This stereo−conformation suggests that ternary copper(II) complex (D−Pro) Cu(II) (L−Phe) has less stability than (D−Pro) Cu(II) (D−Phe). Consequently, the EMO showed that the L−form is faster than the D−form. In the same way, when L−proline is used as a ligand, proposed stereo−conformations of ternary complexes are formed, as shown in Figures 6 C and D. It can be seen that the complex has a more stable conformation with L−Phe than with D−Phe. The different stereo−conformations suggest that the D−enantiomer migrates faster. The EMO reversal caused by the *cis*−/*trans*− conformation is probably because *cis*−hydroxy−L−proline represents a tridentate ligand, as shown in Figures 6 E and F.

In the presence of anionic micellar phases, as shown in Figure 1 B, we must take account of two concurrent interactions where analytes exchange ligands with the chiral selector Cu(II) $(Sel)_2$ (L−Sel = L−proline) to form ternary complexes and a partition occurs between the micellar and electrolyte phases. When L−proline is used as the chiral selector, L−AA interacts preferentially with the chiral selector to form a more stable ternary complex (L−Sel) Cu(II) (L−AA), i.e. $K_{L^*-L} > K_{L^*-D}$. It leads to the result that the partition of L−AA inside the micellar phases migrating toward the anode is more difficult than that of D−AA, i.e. $P_{L-Micelle} < P_{D-Micellle}$. Consequently, these two concurrent interactions (partition and complexation) lead to the L−AAs having a faster migration time than D−AAs in LE−MEKC.

We also investigated the effects of several surfactants and organic modifiers on the EMO [21, 23]. We found that anionic surfactants, such as SDS, sodium n−decyl sulfate (SdeS), sodium n−tetradecyl sulfate (STS), linear alkylbenzesulfonates (LAS) including LAS−C_8, LAS−C_{10} and LAS−C_{12} caused the EMO reversal of amino acids in the Cu(II)−L−OH−Pro system. Nonionic surfactants such as Tween−20 improved the resolution at the expense of the re-

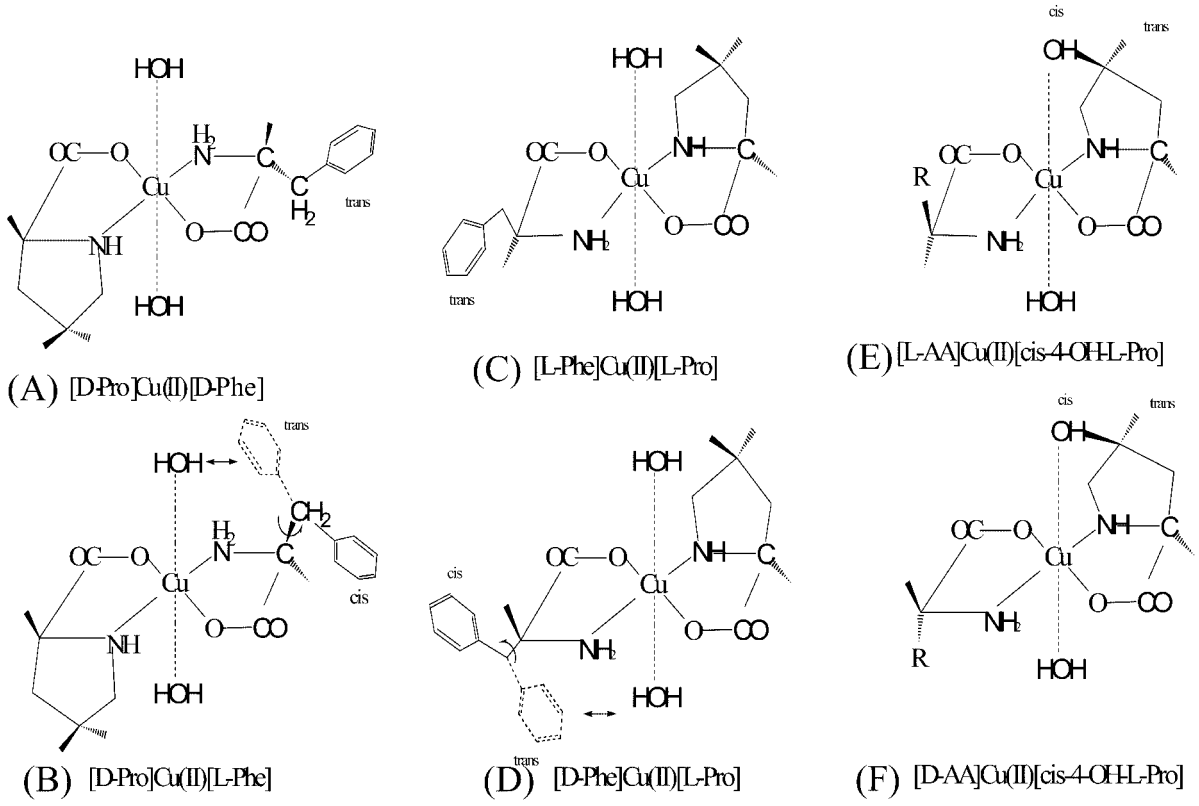


Figure 6. Proposed chemical structures of ternary Cu(II) complexes with different proline ligands. (From Ref. [22]).

tention time. The addition of N−cetyl−N, N, N−trimethyl−ammonium bromide (CTAB) reversed the direction of the EOF, but did not improve the resolution (R_s). Organic modifiers did not inverse the EMO, but caused an R_s decrease.

The separation and EMO behavior of hydroxy acid enantiomers were investigated by using LE−CE. Hydroxy acids were separated by LE−CE by using Cu(II)−L−OH−Pro complex as the chiral selector, however, their migration times were very long since the EOF and hydroxy acid migration directions were opposite. As listed in Table 1, their EMOs were not reversed by the introduction of anionic surfactants [26], probably because hydroxy acids possess negative charges and cannot partition inside anionic micellar phases. However, a reversal was obtained when the cationic surfactant CTAB was used, due to a reversal of EOF.

2.4 Estimation of CMC of anionic surfactants by LE−MEKC

Another application of LE−MEKC is the determination of the critical micelle concentration (CMC) of anionic surfactants [27]. As described in section 2.3, the EMO was reversed by the introduction of anionic micelles. When investigating the effect of anionic surfactant concentration on the R_s of amino acids, we found that the R_s of enantiomers decreased with an increase of surfactant at concentrations lower than CMC. At the CMC concentration, the peaks of a pair of enantiomers overlapped, and R_s became zero. With further increases in the surfactant concentration above that of CMC, the EMO reversed and the R_s increased linearly. This phenomenon implied that the point of reversal of the EMO was correlated with the CMC of the surfactant. Thus, the CMC can be determined by calculating the concentration at $R_s = 0$ in the equation that expresses the relationship between R_s and surfactant concentration. With this method, the CMCs of several anionic surfactants, namely SDS, SDeS and STS, were determined and the results coincided well with the data determined by CE [28]. In addition, we also investigated the effect of organic modifiers on the CMC by LE−MEKC by using acetonitrile as a test sample. The results showed that the CMCs of anionic surfactants increased with the addition of acetonitrile.

We also used LE−MEKC to determine the CMCs of several surfactants used in the makeup and oil industries, such as sodium alkyl polyoxyethylene sulfate (AES−Na) and sodium α−sulfonated fatty acid methyl esters (α−SFNa) [29]. Furthermore, we investigated the possibility of using AES−Na as a pseudo−stationary phase in MEKC. AES−Na provided better separation selectivity for resolving aromatic derivatives than SDS. Besides, as with SDS, either AES−Na or α−SF−Na added to the electrolyte containing Cu (II) complexes with L−proline or L−OH−Pro improved the selectivity for resolving *o*−, *m*−, and *p*−fluoro−DL−phenylalanine enantiomers in LE−MEKC. The EMOs of analytes in the presence of

either AES−Na or α−SF−Na micellar phases were of a similar order to those in the SDS micellar phase.

2.5 Estimation of complex formation constant by LE−CE

In recent years, CE has been known not only as a separation technique, but also as a tool for determining chemical constants. In this work we established a method for estimating the formation constants of Cu(II) complexes with mixed amino acid enantiomers [25] by LE−CE. The concept for the estimation of complex constants is as follows. The chiral separation of enantiomers by LE−CE is based on the difference in the stability of two ternary Cu(II) complexes. Therefore, the electropherograms provide information on the complex formation constants. From the LE equilibrium in the electrolyte, theoretical equations for the estimation of the formation constants of Cu(II) complexes with mixed amino acid enantiomers can be expressed as follows [25],

$$\frac{\mu_0-\mu_d}{\mu_d} = K_{L^*\text{-}D}\,[Cu(II)(L^*)] + (K_{2D}K_{1D}\,[Cu(II)]\,[D\text{-}AA] + K_{DL}\,[Cu(II)]\,[L\text{-}AA]) \quad (2)$$

$$\frac{\mu_0-\mu_l}{\mu_d} = K_{L^*\text{-}L}\,[Cu(II)(L^*)] + (K_{2L}K_{1L}\,[Cu(II)]\,[L\text{-}AA] + K_{DL}\,[Cu(II)]\,[D\text{-}AA]) \quad (3)$$

where μ_l, μ_d are the observed mobilities of L-AA and D-AA; μ_0 is the mobility of D- or L-AA in the absence of a chiral selector. L* indicates the ligand of a chiral selector such as L-hydroxyproline. D-AA and L-AA represent the D- and L-amino acids of the analytes. $K_{L^*\text{-}D}$ and $K_{L^*\text{-}L}$ are the formation constants of ternary Cu(II) complexes: (L*) Cu(II) (L-AA) and (D*) Cu(II) (L-AA). K_{DL}, K_{1D}, K_{2D}, K_{1L} and K_{2L} are the formation constants of Cu(II) complexes: (D-AA) Cu(II) (L-AA), Cu(II) (D-AA), Cu(II) $(D\text{-}AA)_2$, Cu (II) (L-AA), and Cu(II) $(L\text{-}AA)_2$, respectively.

When $(\mu_0-\mu_d)/\mu_d$ and $(\mu_0-\mu_l)/\mu_l$ are plotted as a function of [Cu(II) (L*)], the slopes provide the formation constants of ternary Cu(II) complexes: $K_{L^*\text{-}D}$ and $K_{L^*\text{-}L}$, and the intercepts the items of ($K_{2D}K_{1D}$ [Cu(II)] [D-AA] + K_{DL} [Cu(II)] [L-AA]) and ($K_{1L}K_{2L}$ [Cu (II)] [L-AA] + K_{DL} [Cu(II)] [D-AA]). In terms of equations 2 and 3, the formation constants of four complexes, [(L-OH−Pro) Cu (D-Phe)], [(L-OH−Pro) Cu (L-Phe)], [(L-OH−Pro) Cu (D-Trp)], and [(L-OH−Pro) Cu (L-Trp)], were found to be 21.3, 28.3, 34.3, and 45.1 M^{-1}, respectively. These estimated formation constants provide evidence that helps us to understand the ligand exchange mechanism.

2.6 Chiral resolution of Dns−DL-AAs by LE−CE using Cu(II) complexes with amino acylamides as chiral selectors

Few Cu(II) complexes with L-amino acids or derivatives have been reported as chiral selectors in LE−CE [14−16]. In order to develop new LE systems for resolving amino acids with different enantioselectivity, we explored the possibility of using Cu(II) complexes with amino acylamides as chiral selectors added to the electrolytes [30, 31]. It is known that Cu(II) complexes with L-amino acylamides are present in several species in an electrolyte and the distribution depends on the pH. Therefore, the form of chiral selector can be manipulated by changing the pH for chiral recognition with a different enantioselectivity. Three Cu(II) complexes with L-prolinamide (ProA), L-phenylalaninamide (PheA) and L-alaninamide (AlaA) were employed as chiral selectors. We compared the separation behavior of Dns−AAs by using Cu(II)−L-amino acylamide complexes as chiral selectors [32]. It is known that, when working as chiral selectors, these complexes exhibit quite different enantioselectivities for resolving Dns−DL−AAs. Cu(II) complexes with L-ProA showed almost the highest enantioselectivity for all tested Dns−DL−AAs, Cu(II) complex with L-PheA showed enantioselectivity for most Dns−DL−AAs, whereas, Cu(II) complexes with L-AlaA only showed enantioselectivity for a very limited number of Dns−DL−AAs. As discussed in our paper [32], the difference between the chiral selectors probably results from the influence of the rigid pyrrolidine ring in the Cu(II)−ProA system and the π−π interaction offered by the phenyl group in the Cu (II)−PheA system. In addition, the EMOs of Dns−DL−AAs in Cu (II)−ProA system showed that D-enantiomers were faster than L-enantiomers, but the reverse was true for the Cu(II)−PheA and Cu (II)−AlaA systems where the L-enantiomers migrated as the first peak.

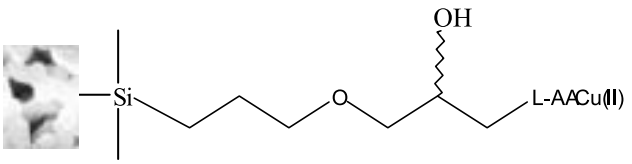
3. Chiral monolithic silica column and its application in CEC and μ−LC

Since the 1990 s, CEC, a separation mode integrating features of both HPLC and CE, has generated great interest among those working in the chromatographic sciences. Open tubular and packed columns are used as conventional columns in CEC. Recently, attention has been focused on the development of monolithic columns. This is because of their many advantages, including in−capillary preparation of stationary phases, fritless design and the absence of bubbles during CEC operation. We have already studied the development of 'chiral' monolithic silica columns for CEC and μ−LC. We succeeded in fabricating the columns by using the sol−gel process and chemical modification. Two categories of chiral CSPs, LE−CSPs [33−37] and cyclodextrin (CD)−modified CSPs [38, 39], have been successfully developed to resolve a variety of important chiral compounds related to the life sciences, namely amino acids, hydroxyl acids, dipeptides, positional isomers and natural chiral compounds by CEC and μ−LC.

3.1 Chiral monolithic LE−CSPs and their application in CEC and μ−LC

As described in the above sections, LE has been used for chiral separation in the LE−LC, LE−CE and LE−MEKC modes. The first paper on LE−CEC was not published until 2000 when Schmid et al. [40] reported a polymer−based continuous bed column for resolving amino acid enantiomers by LE−CEC. The LE−CSP was prepared by the copolymerizing of methacrylamide as a monomer, piperazine diacrylamide as a crosslinker, vinylsulfonic acid as a charge providing agent, and N−(2−hydroxy−3−allyloxypropyl)−L−4−hydroxyproline as a chiral selector. It was said that in some cases polymer−based monolithic columns suffered from the so−called swelling/shrinking phenomena. Monolithic silica columns are highly stable as regards organic solvents in mobile phases, and can be modified with the desired CSPs through the reactions between silanol groups and a spacer, and between the spacer and a chiral selector. Therefore, the monolithic silica column seems to have great potential for use in CEC. The originality of our work focused on the development of 'chiral' monolithic silica columns. In 2001, we first reported our preparation of a chiral monolithic silica column by the sol−gel process and chemical modification and its use for chiral separation by LC−CEC [33].

We fabricated the chiral monolithic columns modified with LE−CSPs in 3 steps: (1) the preparation of a monolithic silica column, (2) the chemical modification of a spacer and a chiral selector on the surface of the monolithic column, and (3) the loading of LE−CSP with Cu(II) ions. L−PheA [33], L−ProA [34], L−AlaA [33], L−lysinamide [35] and L−OH−Pro [36] have been employed as the CSPs. The chemical structures of the LE−CSPs are shown in Figure 7. These LE−CSPs with L−amino acylamides generated a reversed EOF with the direction from the cathode to the anode under an external electric field. The EOF was strong enough to perform the separation by CEC, and dependent on the pH and the compositions of the mobile phases. CSPs modified with L−amino acylamides generated a relatively stronger EOF, because positively charged Cu(II) complexes with L−amino acylamides were formed on the surface of the monolithic columns. The EOF on L−lysinamide CSP was about twice as strong as that on PheA CSP, possibly because there are 3 amino groups in a molecule of lysinamide [35]. However, the EOF generated on L−OH−Pro CSP was too weak to be determined by acetone as a neutral marker [36], probably because the Cu(II) complexes with L−OH−Pro were neutral. The scanning electron micrographs (SEM) in Figure 8 show that the monolithic bed was well bonded with the capillary inner wall and had morphology with a continuous skeleton and a large through−pore.



AA: amino acylamide or amino acid

LE-CSP1: AA=L-phenylalaninamide
LE-CSP2: AA=L-alaninamide
LE-CSP3: AA=L-prolinamide
LE-CSP4: AA =L-lysinamide
LE-CSP5: AA= L-hydroxyproline

Figure 7. Chemical structures of monolithic LE−CSPs.

Chiral separation has been achieved for a variety of compounds on these monolithic LE−CSPs by CEC and μ−LC with different enantioselectivities. An L−PheA−modified monolithic column was used for resolving 12 dansyl amino acids by CEC [33]. Figure 9 shows the electrochromatograms obtained when resolving a mixture containing six enantiomers of three pairs of Dns−DL−

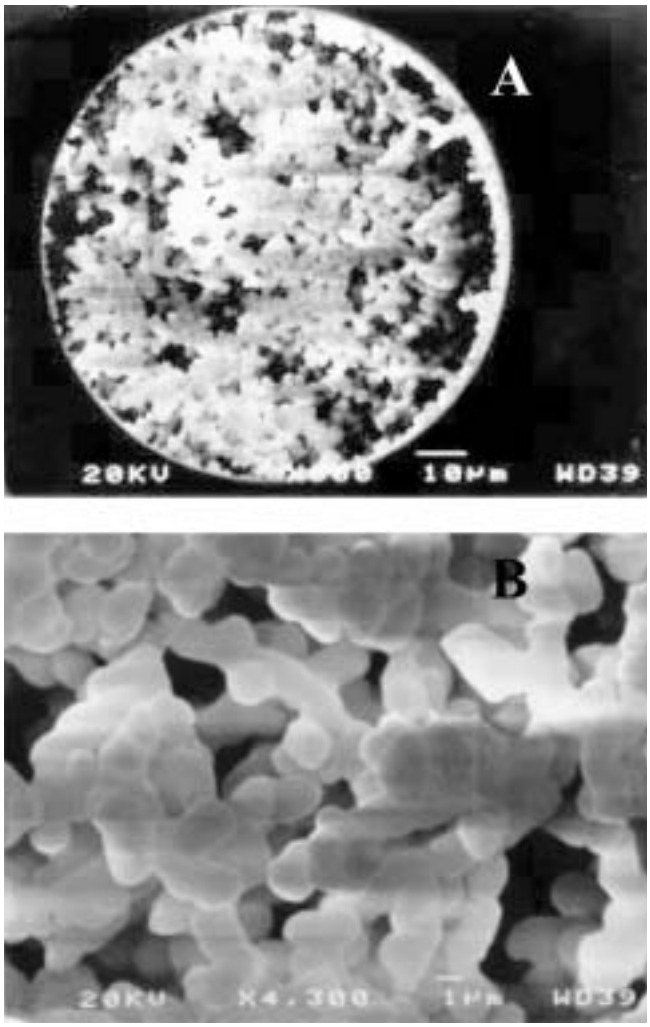


Figure 8. SEM photographs of sol−gel monolithic column modified with L−Phenylalaninamide: 20 kV, WD 39, and magnification (A) 800×, (B) 4300×. (From Ref. [33]).

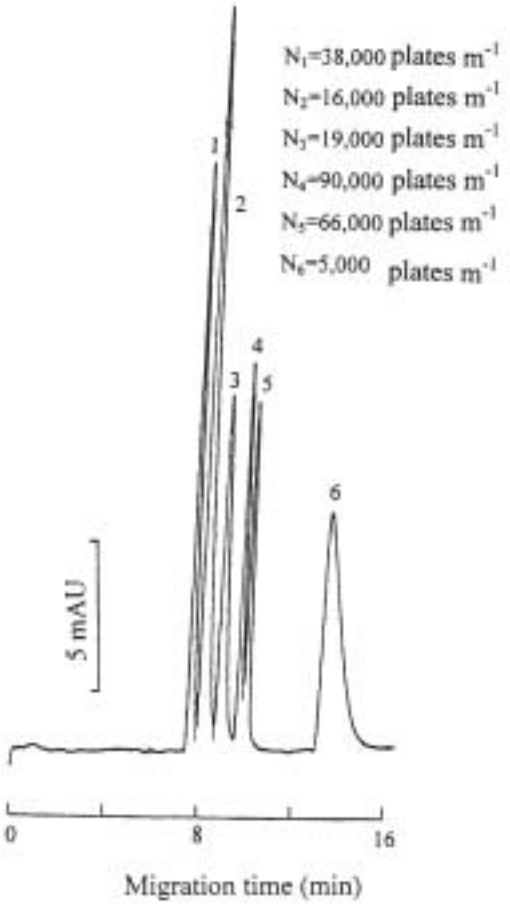


Figure 9. Electrochromatograms obtained when resolving a mixture containing six enantiomers of three pairs of dansyl amino acids. Peak identification: 1, Dns−D−Thr; 2, Dns−D−Ser; 3, Dns−L−Thr; 4, Dns−D−Leu; 5, Dns−L−Leu; 6, Dns−L−Ser. Column: L−PheA−modified monolithic column (TL 35 cm, EL 26.5 cm; i.d. 100 μm, o.d. 375 μm). Mobile phase: pH 5.5 acetonitrile/0.50 mM $Cu(Ac)_2$−50 mM NH_4Ac (7:3). Applied electric field strength: −300 V/cm; UV detection 254 nm; electrokinetic injection 3−5 s. (From Ref. [33]).

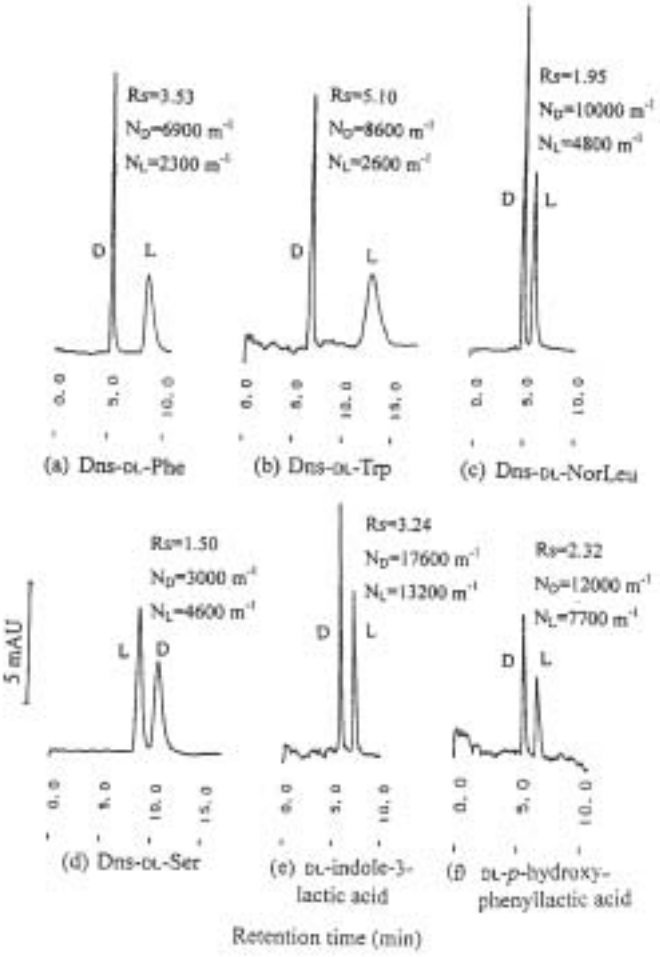


Figure 10. Representative electrochromatograms of Dns−DL−AAs and DL−hydroxy acids. Separation conditions: monolithic L−ProA CSP, EL 26 cm, TL 34 cm, i.d. 100 μm, o.d. 375 μm; mobile phase: acetonitrile: 0.50 mM $Cu(Ac)_2$−50 mM NH_4Ac (7:3), pH 6.5; applied voltage: −13.6 kV, electric current 37 μA. (From Ref. [34]).

AAs. The maximum theoretical plate number up to 90,000 m^{-1} was obtained for Dns−D−Leu. This efficiency is believed to be the highest achieved in the approximately 30−year history of LEC. Both the dansyl amino acids and several hydroxy acid enantiomers were resolved on an L−ProA−modified monolithic column by CEC and μ−LC [34]. Figure 10 shows representative electrochromatograms of several Dns−DL−AAs and DL−hydroxy acids. As shown in Figure 10, the LE−CEC on L−ProA CSP was much better in terms of resolving hydroxyl acids than LE−CE. When Cu(II)−hydroxyproline complex was used as a chiral selector for the separation of hydroxy acids by LE−CE, the migration times were over 30 minutes [26]. However, the separation times in CEC on L−ProA CSP were reduced to less than 10 minutes. It is because negatively charged hydroxy acids migrated toward the anode, as the same direction as the anodic EOF generated by L−ProA−CSP. It was shown that ProA CSP exhibited high enantioselectivity, especially for resolving analytes with large substituent groups such as phenyl and indole rings at the chiral α−carbon of dansyl amino acids and hydroxyl acids [34]. When we compared the enantioselectivities of L−AlaA and L−PheA CSPs, we found that higher separation factors were obtained on L−PheA CSP than on L−AlaA CSP. This fact suggests that the phenyl group in the L−PheA played an important role in chiral recognition [33], as found with CE [30]. Although the EOF was extremely weak on monolithic L−OH−Pro CSP, the enantioseparation for dansyl amino acids, hydroxy acids and dipeptides was achieved by CEC [36], since the analytes migrated through the monolithic bed as a result of their electrophoretic mobility. The retention times on monolithic L−OH−Pro CSP were longer than on

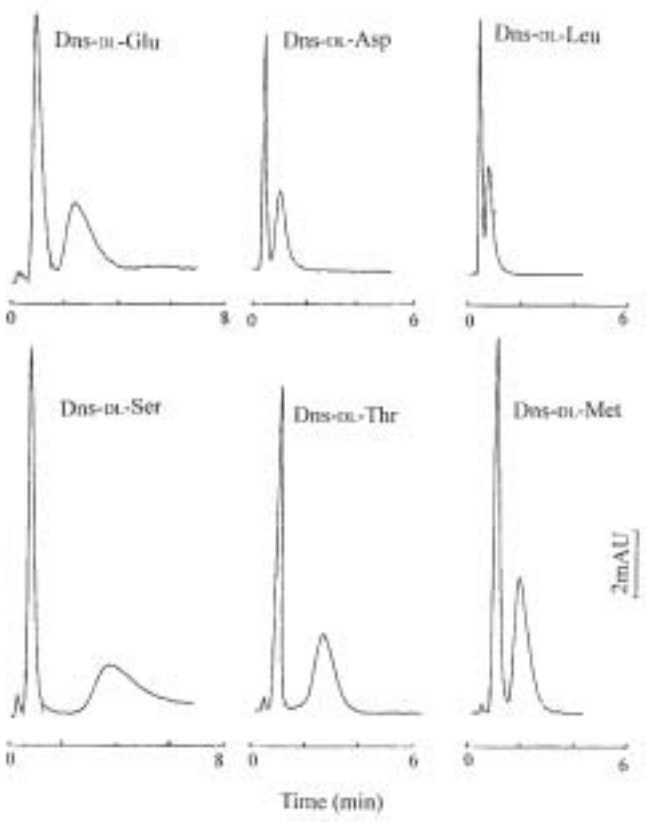


Figure 11. Representative chromatograms for resolving Dns−DL−AAs on monolithic L-PheA CSP by LC. Monolithic CSP: 32 cm; mobile phase: pH 7.6, acetonitrile−0.1 M NH_4Ac−0.25 mM $Cu(Ac)_2$ (7:3); flow rate: 20 μl/min. injection volume 0.2 μl. (From Ref. [37]).

monolithic L-ProA CSP, because the EOF on L-OH−Pro CSP was weaker than that on L-ProA CSP.

These monolithic columns modified with LE−CSPs have also been employed for chiral separation in the μ−LC mode [37]. Figure 11 shows representative chromatograms obtained when resolving Dns−DL−AAs on monolithic L-PheA CSP by μ−LC. The EMOs in both μ−LC and CEC were of the same order in that D-enantiomers were eluted as the first peak. However, there was an exception with CEC in that Dns−L-Ser migrated faster than Dns−D-Ser [32]. The EMOs are summarized in Table 2, where L-amino acylamides were used as the chiral selectors in CE or CSPs in CEC and μ−LC. The chromatographic efficiency in CEC was much higher than in μ−LC. The lowest theoretical plate height of 25 (m on monolithic L-PheA CSP in CEC was obtained, but only about 1 mm (about 40 times the CEC value) was obtained in μ−LC. The high efficiency in CEC results inherently from the plug−like flow profile of EOF, which drives the mobile phase. In addition, we regarded the nanoliter sample volume realized by electrokinetic injection as an additional reason for this high efficiency. In contrast, we regarded the parabolic flow profile driven by pressure, the overload of injected

Table 2. EMOs in CE, CEC and μ−LC using Cu(II) complexes with L-amino acylamides and L-hydroxyproline as chiral selectors

Ligands	Enantiomer migration order (EMO)		
	CE	CEC	LC
Phenylalaninamide	L→D	D→L	D→L
Prolinamide	D→L	D→L*	D→L
Alaninamide	D→L	D→L	D→L
Lysimamide	−	D→L	D→L
Hydroxyproline	D→L	D→L*	D→L

* Dns−DL−Ser :L→D

samples, and the fast flow rate of the mobile phase in μ−LC as reasons for the low chromatographic efficiency and separation selectivity in μ−LC. Consequently, some samples were well resolved by CEC, but not in μ−LC. Therefore, CEC can be considered a very promising chromatographic technique with high efficiency and selectivity. However, in some cases, CEC loses its power, because it depends on the EOF and the mobility of the analytes. When the EOF and analyte migration directions are opposite and the analyte mobility is stronger than that of EOF, the analytes cannot be driven through the column bed. In this case, enantioseparation cannot be achieved by CEC, but it is possible with μ−LC. For example, free amino acids (DL−Phe and DL−Trp) were not resolved on L-OH−Pro CSP by CEC, but well resolved by μ−LC [36]. Therefore, it is also very important to develop the μ−LC technique for chiral separation.

3.2 CD−modified monolithic CSP and its application in CEC and μ−LC

The success in the development of monolithic columns modified with LE−CSPs encouraged us to develop monolithic columns modified with other CSPs such as β− and γ−CDs [38, 39]. The monolithic silica column was fabricated by the sol−gel process, and then chemically immobilized using β− or γ−CDs as CSP with a spacer of 3−glycidoxypropyltrimethoxysilane by in−column reactions. Figure 12 shows the chemical structure of γ−CD−modified monolithic column. A γ−CD−modified monolithic column has been successfully employed for resolving dansyl amino acid enantiomers, and a β−CD−modified monolithic column has been used for resolving positional isomers of *o*−, *m*− and *p*−cresols, the racemic enantiomers of benzoin, and several dansyl amino acids by CEC. Figures 13 and 14 show representative electrochromatograms of several dansyl amino acids on γ−CD CSP, and the electrochromatograms of positional isomers of cresols and benzoin enantiomers on β−CD CSP by CEC. The separation efficiency of 5.0×10^4 theoretical plates/m for dansyl−L-threonine was obtained on γ−CD CSP at an electric field strength of −300 V/cm in the mobile phase

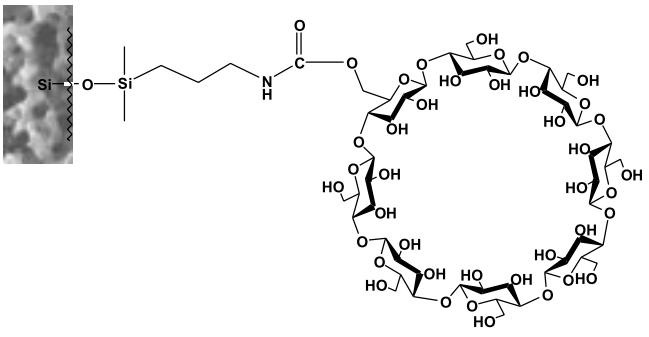


Figure 12. Chemical structure of monolithic γ−CD CSP. (From Ref. [38]).

of 50 mM MES−Tris/methanol (70/30) buffer at pH 7.0. L−Enantiomers were eluted as the first peak.

We investigated the effects of the separation conditions, such as pH, buffer composition, organic modifier and temperature, on the retention behavior of dansyl amino acids in CEC. It is known that ionic strength has a significant influence on the retention behavior, since it affects not only the chromatographic interaction as in HPLC, but also the EOF and mobility of analytes [38]. We were able to manipulate the enantioselectivity by the addition of organic solvents, such as methanol, acetonitrile and tetrahydrofuran in the mobile phases. The effect of temperature on the separation behavior showed that a relatively low temperature under enthalpic control benefited the enantioselectivity. Furthermore, we investigated the correlation between the separation factors and the hydrophobicity expressed by log $P_{o/w}$. This investigation indicated that the hydrophobic interaction between CD and the analytes contributed to the retention on the CD−CSP. In addition, the stereo−selectivity of the analyte molecules in the CD cavity was considered to be another main factor as regards the chiral recognition on CD−CSPs in CEC [39].

4. Chiral separation by EKC using 18C6H$_4$ as chiral selector

18−crown−6−tetracarboxylic acid (18C6H$_4$) is a well−known chiral selector with high selectivity for resolving solutes bearing the primary amine functional group. The pKa values of 18C6H$_4$ are 2.13, 2.84, 4.29 and 4.88. When the pH of an electrolyte is lower than 3.0, it dissociates its two protons and possesses two negative charges. The negatively charged 18C6H$_4$ works as a pseudo−stationary phase and a chiral selector in EKC and migrates toward the anode. Positively charged amino acids migrate toward the cathode. The countercurrent migrations of 18C6H$_4$ and amino acids enhance the resolution. The enantioselective recognition on 18C6H$_4$ is based on the host−guest interactions between selector and selectand, mainly involving the ion−dipole interaction between the cation of the primary amine and the oxygen donor atoms in the

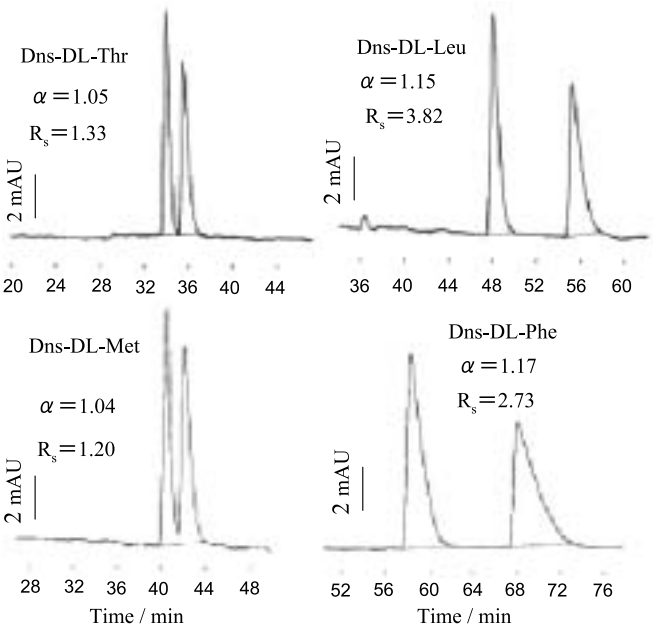


Figure 13. Representative electrochromatograms of several dansyl amino acids on monolithic γ−CD CSP. Monolithic γ−CD CSP, 45 cm EL, 55 cm TL; mobile phases, 50 mM MES−Tris buffer/methanol, at 80:20, pH 7.0 for Dns−DL−Thr, at 70:30, pH 7.0 for Dns−DL−Leu and Dns−DL−Met, and 60:40, pH 8.0 for Dns−DL−Phe. Applied voltage, −18 kV, but −15 kV for Dns−DL−Phe. (From Ref. [38]).

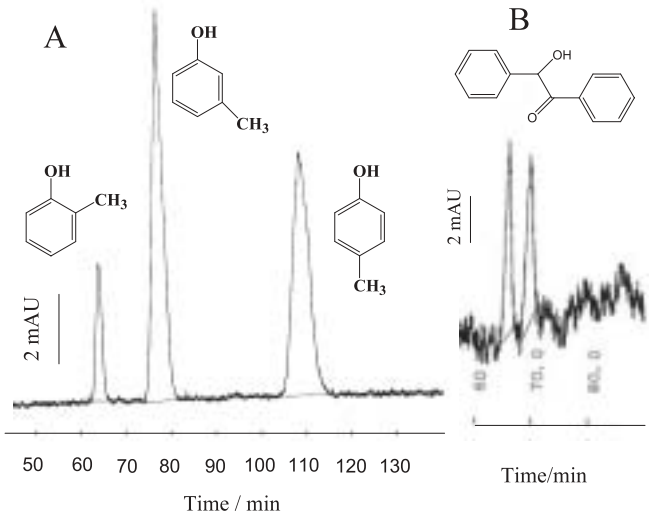


Figure 14. Separation of positional isomers of *o*−, *m*−, and *p*−cresols (A) and benzoin racemate (B) on monolithic β−CD CSP (45 cm EL, 57 cm TL). Mobile phase, 50 mM phosphate buffer (pH 7.5)/methanol (80/20); applied voltage, 12 kV; current, 80 μA. (From Ref. [38]).

ring structure of the cyclic polyether, and stereo−selective interaction between the substituents of the selectand and the carboxylic groups in 18C6H4.

We succeeded in employing 18C6H4 as a chiral selector for the simultaneous separation of positional enantiomers of methyl−DL−tryptophan derivatives [41], and *o*−, *m*−, *p*−enantiomers of the phenylalanine family by EKC [42], which were resolved by LE−MEKC. Figure 15 shows the simultaneous separation of 12 enantiomers of α−, 1, 4−, 5−, 6, 7−methyl−DL−tryptophan derivatives. The resolution depended on the separation conditions, namely pH, 18C6H4 concentration and applied voltage.

To explore the chiral recognition mechanism, we investigated the interaction between 18C6H4 and analytes by observing the R_s and migration orders. We found that the introduction of α−CH_3 in the analytes dramatically reduced both R_s and the migration times, which suggests that there is considerable steric hindrance in the host−guest complex. Furthermore, we found that the position of the substituents had a considerable influence on the migration times and R_s for the methyl−DL−tryptophan derivatives. The EMO was as follows: α−CH_3−L−Trp, α−CH_3−D−Trp, 6−CH_3−L−Trp, 5−CH_3−L−Trp, 7−CH_3−L−Trp, 1−CH_3−L−Trp, 4−CH_3−L−Trp, 6−CH_3−D−Trp, 5−CH_3−D−Trp, 7−CH_3−D−Trp, 1−CH_3−D−Trp, 4−CH_3−D−Trp. The L−enantiomers were always faster than the D−enantiomers. 6−CH_3−DL−Trp, in which methyl group was separated from the asymmetric carbon with a longest distance of 6 C−C bonds, had the shortest migration time, 4−CH_3−DL−Trp with a distance of 4 C−C bonds had the longest migration time. In addition, we were interested to observe that the R_s depended on the distance from the substituents to the asymmetric carbon, which suggested that the enantioselectivity is mainly based on the steric barrier effect. 6−CH_3−DL−Trp with the longest distance of 6 C−C bonds from the asymmetric carbon has the lowest R_s. 5−CH_3−DL−Trp and 7−CH_3−DL−Trp a with a similar distance of 5 C−C bonds had a similar R_s level. 1−CH_3−DL−Trp and 4−CH_3−DL−Trp with a distance of 4 C−C bonds also had a similar R_s level. Therefore, we could conclude that the steric barrier effect between 18C6H4 and the positional substituents of the analytes plays a very important role in chiral recognition [41].

5. Diamide−type CSP for LC and others

As described in the above sections, in our work we employed chiral ligand exchange using Cu(II) complexes with L−amino acylamides and L−amino acids, and host−guest inclusion using CDs and chiral 18C6H4 as chiral selectors for chiral separation. The chiral recognition on diamide−type CSP is based on a three−point

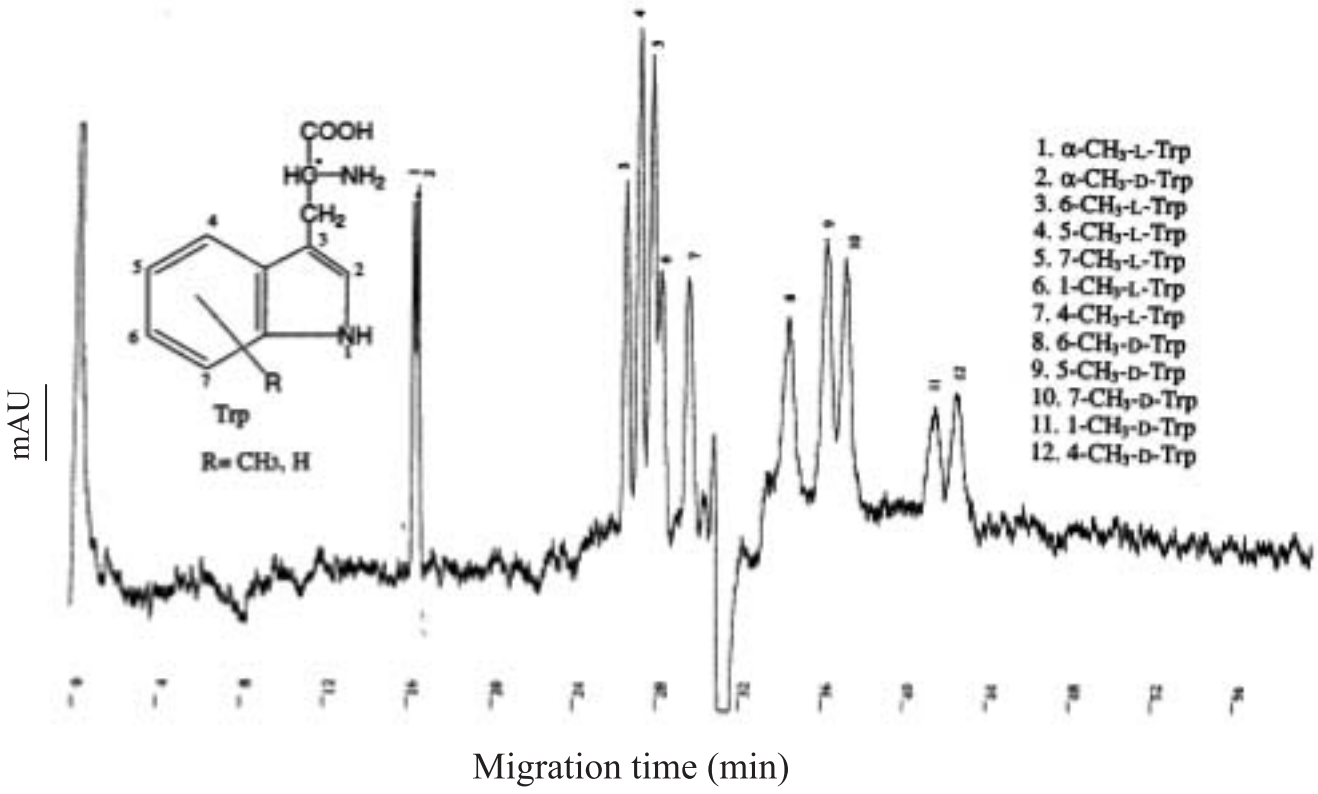


Figure 15. Simultaneous separation of 12 enantiomers of α−, 1, 4−, 5−, 6, 7−methyl−DL−tryptophans by using 18C6H4 as a chiral selector in the electrolyte. Electrolyte solution, 10 mM Tris−H_3PO_4, 20 mM 18C6H4, pH 3.0; 20 kV. Capillary: 56 cm TL, 40 cm EL, 50 μm i.d. and 375 μm o.d. (From Ref. [41]).

interaction including hydrogen association, aromatic π−π interaction and the steric effect of the stereogentic center [43].

We reported a diamide−type CSP chemically bonded with N−stearoyl−L−leucine on aminopropylsilica for resolving amino acid derivatives by both normal and reversed phase LC [44]. Furthermore, in order to examine the effect of spacers on the retention behavior of benzoyl−DL−amine and benzoyl−DL−amino acid isopropyl esters, we synthesized a group of diamide−type CSPs immobilized with N−(3, 5−dimethylbenzoyl) phenylglycine on aminopropylsilica or silica bonded with different spacers. We found that a long spacer in CSPs generally improves the enantioselectivity. The secondary amine group in the spacers causes a decline in the separation factors. Moreover, we investigated the effect of organic modifiers on the retention behavior. Interestingly, we observed that the organic modifiers had a 'masking effect' on the silanol groups in CSPs. The addition of a suitable amount of organic modifier can improve the enantioselectivity, and enantioselectivity dependent on the cross−sectional area of the organic modifier in the mobile phase [45].

In addition to developing new chiral separation systems for CE, CEC, and μ−LC as described above, we also studied the intermolecular interaction between the stationary phase and analytes by thermodynamic analysis [46]. We examined the intermolecular interactions between four stationary phases chemically bonded with octadecyl (ODS), phenyl (Ph), pyrenyl (PYE) and ß−cyclodextin bromide (ß−CD), and 53 solutes including 27 compounds of *p*−substituted alkylbenzenes (PSABs), 14 compounds of polyaromatic hydrocarbons (PAHs) and 26 compounds of substituted benzenes by using both methanol−water and acetonitrile−water as mobile phases. We observed that there are obvious differences in the π−π intermolecular interactions among the stationary phases of ODS, Ph and PYE by plotting *ln k'* with enthalpy (ΔH). Based on a thermodynamic analysis of the interaction between the ß−CD column and solutes, we found that solutes which have similar molecular lengths to ß−CD cause large enthalpy changes in the host−guest interaction. Moreover, we discussed the retention mechanism in terms of thermodynamic analysis. We showed that both hydrophobic and host−guest interactions contributed to the retention on the ß−CD stationary phase; and the solute structures had a critical influence on the retention.

6. Conclusion

This paper reviewed recent advances in the development of new chiral separation systems for CE, CEC and μ−LC achieved by the author's group at Tokyo Metropolitan University. LE−MEKC was developed as a powerful method for simultaneous separation of positional and optical isomers, and as measurement tools for estimating the CMCs of surfactants and the formation constants of Cu(II) complexes with mixed amino acid enantiomers. We found that both the introduction of anionic micellar phases in electrolytes containing Cu(II)−L−OH−Pro and/or L−Pro complex and changes in the chirality and *cis*/*trans*−conformation of the 4−hydroxy group in OH−Pro resulted in the reversal of amino acid EMOs. The mechanism of LE−CE and LE−MEKC was investigated, based on the EMO, complex stability and enantioselectivity.

Furthermore, two categories of monolithic silica columns modified with LE−CSPs and β− and γ−CDs−CSPs were successfully developed for resolving various important chiral compounds related to the life sciences, namely amino acids, hydroxyl acids, dipeptides, positional isomers and natural chiral compounds by CEC and μ−LC. The combination of the sol−gel process and chemical modification provides a new promising approach to the development of column technology for CEC and μ−LC. Further chiral monolithic columns modified with other new CSPs are expected to be developed by following this method.

Moreover, $18C6H_4$ was also used for the simultaneous separation of positional and optical isomers of some amino acid derivatives. The mechanism of the host−guest interaction between $18C6H_4$ and a selectand was discussed by investigating the EMO and enantioselectivity. We found that the steric barrier effect between the substituent groups of the selectand and the carboxylic groups of $18C6H_4$ plays a very important role in chiral recognition. Finally, diamide−type CSPs were developed for resolving amino acid and amine derivatives by conventional HPLC. We expect them to be used in CE, CEC and μ−LC in the future.

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