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Comparison of enantioseparations using Cu(II) complexes with L-amino acid amides as chiral selectors or chiral stationary phases by capillary electrophoresis, capillary electrochromatography and micro liquid chromatography

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Abstract

In this paper we report that Cu(II) complexes with L-amino acid amides were used as chiral selectors for enantioseparation by capillary electrophoresis, capillary electrochromatography (CEC) and micro liquid chromatography using chemically modified monolithic columns. The enantioselectivity, enantiomer migration order, and the performance have been compared when different chiral selectors were used in these modes. L-Enantiomers showed longer retention times than D-forms in both CEC and LC modes. However, it has interestingly been observed that the migration order of Dns-DL-Ser showed an exception in CEC using L-prolinamide-modified column that Dns-L-Ser was eluted as the first peak. On the basis of proposed structures of complexes in the chiral recognition, differences in migration orders and recognition mechanism were discussed.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Chiral stationary phases, electrophoresis; Chiral stationary phases, electrochromatography; Monolithic columns; Metal complexes; Amino acids, Dns derivatives; Amino acid amides

1. Introduction

Without question, study of chirality and relative fields is receiving increasing attraction. The fact that the Nobel Prize for chemistry in 2001 was awarded to Dr. William Knowles, Dr. Ryoji Noyori, and Dr. Barry Sharpless for their distinguished contributions in the study on the development of asymmetric catalysis and the synthesis of chiral compounds tells us that scientists have paid great attention to the importance of chirality. It is to be expected that the study on chirality, such as asymmetric synthesis and chiral separation, will play a more and more important role in life sciences, the pharmaceutical industry and other fields in the future.

Amino acids are the basic units of proteins. Analysis of D- or L-amino acids is known to be important in exploring the life originality. With the development of analytical chemistry toward miniaturization, microcolumn and microchip-based separation methods such as capillary electrophoresis (CE), capillary electrochromatography (CEC) and nano or micro liquid chromatography (μLC) have

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become hot research fields in separation sciences. Many principles such as host–guest interaction, chiral–ligand exchange, have been used for the enantioseparations of amino acid enantiomers by these techniques [1,2].

Ligand exchange (LE), as a chiral recognition mode, was introduced to LC in the 1970s by Davankov and co-workers (see Ref. [3]) and to CE by Zare's group in the 1980s [4,5]. Since 1998 we first proposed to use ligand-exchange micellar electrokinetic chromatography (LE-MEKC) as a hybrid separation mode of LE and MEKC [6–12]. Schmid et al. published a paper on LE-CEC using polymer-based continuous beds in 2000 [13]; Chen and Hobo [14,15] first published papers on LE-CEC using silica-based monolithic columns in 2001 and μLC in 2002 [16]. The research and development of ligand-exchange chromatography in the 21st century, was discussed by one of the authors (Z.C.) with Dr. Davankov, a pioneer of ligand exchange chromatography, at the 13th International Symposium on Chiral Recognition (ISCD13 or Chirality 2001) in Orlando, FL, USA. They agreed that LE-CE and LE-CEC would become an important research topic in the 21st century. In recent years, we focused on LE-CE, LE-MEKC, LE-CEC and LE-LC using monolithic microcolumns. We recently reported that Cu(II) complexes with L-amino acylamides such as L-prolinamide, L-phenylalaninamide and L-alaninamide, were used as chiral selectors in CE [17], or monolithic chiral stationary phases (CSPs) for CEC [14,15] and μLC [16]. In this paper we focused our insights on the comparisons of enantioseparations from the points of view of enantioselectivity, performance and enantiomer migration orders among the modes of CE, CEC and μLC by using Cu(II) complexes with L-amino acylamides. Also, the mechanism and the difference in the elution orders will be discussed.

2. Experimental

2.1. Instrumentation

CE and CEC were carried out on an instrumental set-up, involving an HCZE-30PNO25-LD high-voltage power supply (Matsusada Precision Devices, Tokyo, Japan), a CE-1570 intelligent UV–Vis detector (Jasco, Tokyo, Japan) and a C-R7A plus Chromatopac integrator (Shimadzu, Kyoto, Japan). The μLC instrumental system was set up by a LC-10ADvp pump (Shimadzu), a CTO-10ACvp column oven (Shimadzu), an integrator (Chromatopac C-R7A plus, Shimadzu), and a Rheodyne 7520 injector with a 0.2-μl sample rotor (Supelco, Bellefonte, PA, USA) and a CE-1570 intelligent UV–Vis detector (Jasco). The detection wavelength was set at 254 nm.

2.2. Reagents

L-Prolinamide (L-ProA), L-phenylalaninamide (L-PheA), L-alaninamide (L-AlaA), dansyl (Dns) amino acids and hydroxy acids were obtained from Sigma (St. Louis, MO, USA). Acetonitrile for HPLC, copper acetate monohydrate and ammonium acetate were from Kanto (Tokyo, Japan). Fused-silica capillaries for making monolithic columns (0.375 mm O.D.×0.10 mm I.D.) and for CE (0.375 mm O.D.× 0.050 mm I.D.) were from GL Sciences (Tokyo, Japan).

3. Results and discussion

3.1. Comparisons of enantioselectivity using L-amino acid amides modified CSPs in LE-CEC

We developed monolithic sol–gel columns chemically modified with L-ProA, L-PheA and L-AlaA as LE-CSPs for CEC [14,15] and μLC [16]. It has been demonstrated that the monolithic LE-CSPs can well be used for enantioseparations of Dns-DL-amino acids and some hydroxy acids. Although we had reported the enantioseparations in previous works [14–16], we did not discuss the mechanism of chiral recognition deeply. The separation factors using these L-amino acylamides as CSPs are shown in Fig. 1. With regard to the differences in chemical structures of both selectors and selectands (analytes) and the mechanism of interaction between selectors and selectands, we focus our discussion on the differences in the enantioselectivities in this paper.

First, we discuss the enantioselectivity from the point of view of difference in the chemical structures

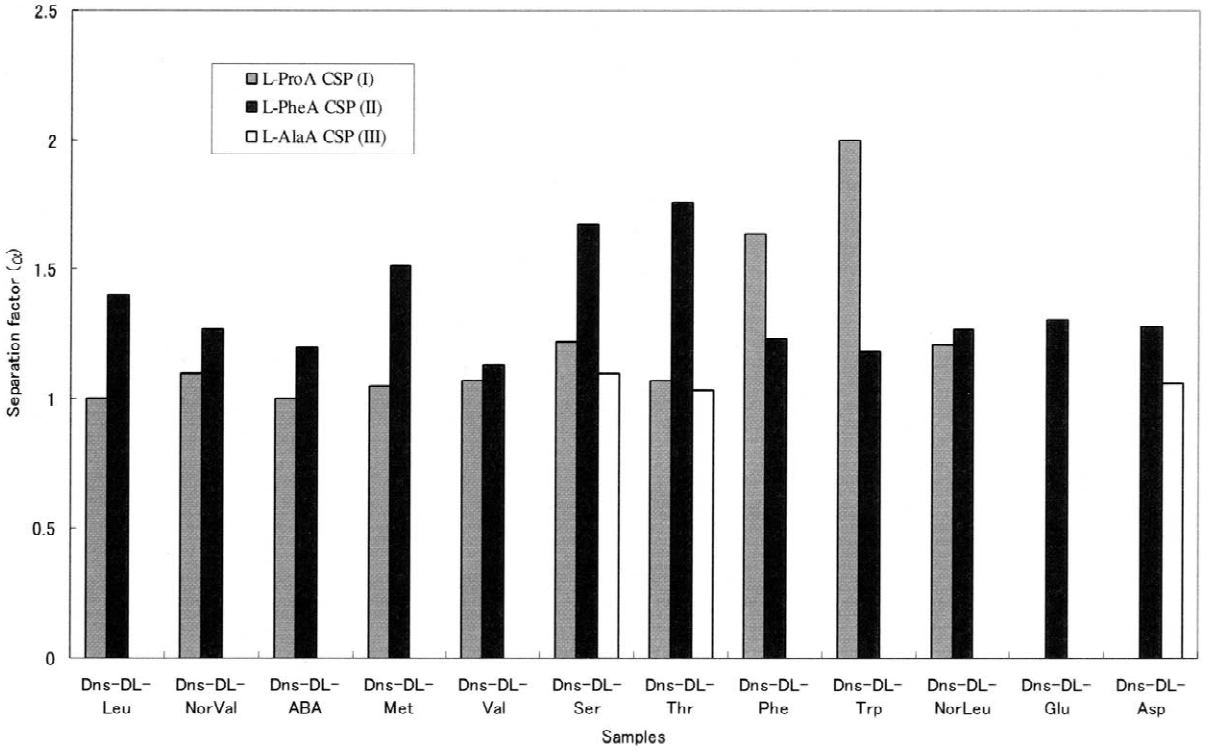

Fig. 1. Separation factors of Dns-DL-amino acids by CEC. (I) L-ProA CSP: mobile phase, pH 6.5, acetonitrile–[50 m*M* NH_4Ac–0.50 m*M* $Cu(Ac)_2$] (7:3); monolithic column, 34 cm (effective length 26 cm)×100 μm I.D.×375 μm O.D.; applied electric field strength −400 V/cm. (II) L-PheA CSP: mobile phase, pH 7.6, acetonitrile–[0.1 *M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$] (7:3); monolithic column, 37 cm (effective length)×28.5 cm)×100 μm I.D.×375 μm O.D.; applied electric field strength −315 V/cm. (III) L-AlaA CSP: pH5.5, acetonitrile–[50 m*M* NH_4Ac–0.50 m*M* $Cu(Ac)_2$] (7:3); monolithic column, 44 cm (effective length) 32 cm)×100 μm I.D.×375 μm O.D.; applied electric field strength −300 V/cm. $\alpha = t_2/t_1$.

among three chiral selectors. As shown in Fig. 1, a L-PheA-modified CSP shows a higher enantioselectivity for most dansyl amino acids than L-ProA- and L-AlaA-modified CSPs. An L-AlaA-modified CSP only shows enantioselectivity for Dns-DL-Ser, Dns-DL-Thr and Dns-DL-Asp. As compared with the enantioselectivities between CSPs modified with L-PheA and L-AlaA, we can draw the conclusion that the phenyl group in the chiral selector of L-PheA plays an important role in the chiral recognition for dansyl amino acids. As shown in Fig. 2I,II, the phenyl group in the L-PheA probably offers π–π and hydrophobic interaction between the phenyl and dansyl groups which benefits the chiral recognition of ligand exchange. Besides, we can notice that the L-prolinamide-modified CSP also shows high enantioselectivity for most dansyl amino acids and hydroxy acids. The reason probably is that the chiral Cu(II) complex with prolinamide on the monolithic column possesses a rigid pyrrolidine ring on the copper coordination plane, which benefits the chiral recognition due to the steric hindrance, as shown in Fig. 2III,IV. Unlike an L-ProA-modified CSP, there are no rigid rings included in the chiral Cu(II) complexes with L-PheA and L-AlaA, as shown in Fig. 2I,II. Thus, compared to the L-AlaA CSP, the phenyl group in the L-PheA, which offers π–π interaction and steric hindrance, is very important for chiral recognition.

On the other hand, the structures of selectands also have great influence on the enantioselectivity by using the same CSP, for example, L-prolinamide

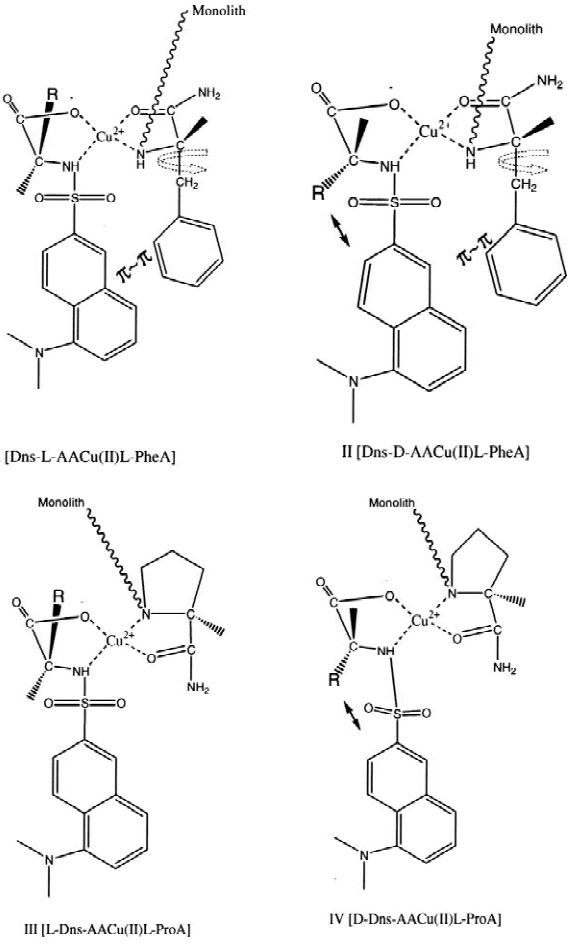

Fig. 2. Proposed chemical structures of ternary Cu(II) complexes on the monolithic CSPs.

CSP. As shown in Fig. 1, we can notice that a L-ProA-modified CSP shows higher enantioselectivity especially for the resolution of analytes which possess big substituents like phenyl or indole groups, such as Dns-DL-Phe and Dns-DL-Trp. For the chiral resolution of hydroxy acids [15,16], a similar trend was observed. Baseline separations of DL-*p*-hydroxyphenyllactic acid ($\alpha=1.21$) and DL-indole-3-lactic acid ($\alpha=1.23$), which possess big substituents of phenyl and indole, were achieved, whereas separation factors for other hydroxy acids were less than 1.10 on the same column by CEC. This suggests that the steric hindrance resulting from the big substituents plays an important role in the chiral recognition of ligand exchange.

3.2. Comparisons of enantioselectivity using different chiral selectors of L-amino acid amides in LE-CE

The success in LE-CEC using monolithic LE-CSPs modified with L-amino acylamides encouraged us to explore the possibility directly using Cu(II) complexes with L-amino acylamides as additives in electrolytes for enantioseparations by CE. It was demonstrated that Cu(II) complexes with L-PheA, L-ProA, and L-AlaA can be used as chiral selectors for enantioseparations of Dns-DL-amino acids by CE [17]. For three Cu(II) complexes with L-ProA, L-PheA and L-AlaA used as chiral selectors, separation factors of Dns-DL-amino acids are compared and shown in Fig. 3. As can be seen in Fig. 3, these complexes working as chiral selectors show quite different enantioselectivities for the resolution of Dns-DL-amino acids, as well as different enantiomer migration orders (EMOs). As in CEC, Cu(II) complexes with L-ProA show high enantioselectivity for all tested Dns-DL-amino acids, whereas Cu(II) complexes with L-AlaA only show enantioselectivities for quite a limited number of Dns-DL-amino acids. As discussed above, the difference among the chiral selectors probably results from the influence of the rigid pyrrolidine ring in Cu(II) complexes with prolinamide and the $\pi-\pi$ interaction offered by the phenyl group in phenylalaninamide. Comparing the separation behaviors in CEC and in CE, the enantioselectivity for the resolutions of dansyl amino acids almost showed a similar trend among three chiral selectors. However, the EMOs are different, as summarized in Table 1. In general, the difference in EMOs between CEC and CE resulted from the immobilization of chiral selectors. The chiral selectors in CEC were immobilized on the monolithic columns; however, in CE the chiral selectors added in the electrolyte solution can move freely by the influence of the electroosmotic flow (EOF) and the charge of themselves. Because many factors have influence on the EMOs, the explanation of EMOs in LE-CE is relatively difficult. Some further discussions can be seen in Section 3.4.

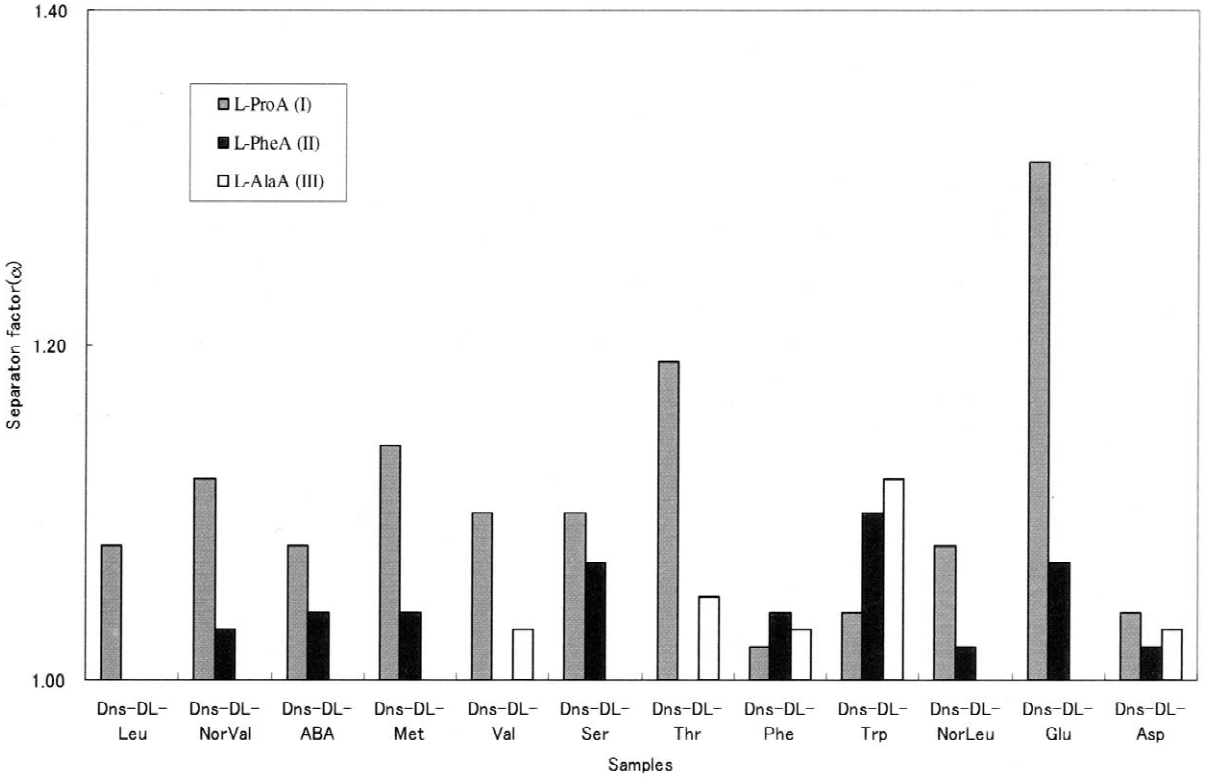

Fig. 3. Separation factors of Dns-DL-amino acids by CE. (I) Electrolyte: pH 5.0, 10 m*M* $Cu(Ac)_2$, 20 m*M* L-ProA and 20 m*M* NH_4Ac. Capillary: 50 cm (effective length 36.5 cm). Applied voltage: 13.6 kV. (II) Electrolyte: pH 5.0, 10 m*M* $Cu(Ac)_2$, 20 m*M* L-PheA and 20 m*M* NH_4Ac. Capillary: 55 cm (effective length 36.5 cm). Applied voltage: 15 kV. (III) Electrolyte: pH 5.0, 10 m*M* $Cu(Ac)_2$, 20 m*M* L-AlaA and 20 m*M* NH_4Ac. Capillary: 50 cm (effective length 36.5 cm). Applied voltage: 13.6 kV. $\alpha = t_2/t_1$.

3.3. Comparison of separation behaviors in μLC and CEC

After succeeding in CEC, we studied the enantioseparations by μLC using the monolithic LE-CSPs modified with L-amino acylamides [16]. As shown in Table 1, the EMOs of analytes in both CEC and CE are the same with the exception of Dns-DL-Ser. It is easy to understand the similar EMOs in these two modes, because the chiral selectors were immobilized on the monolithic columns. The unique difference is the driving force of mobile phase and analytes. The mobile phase is driven by the EOF in CEC, but by the pressure of pump in LC. As shown in Fig. 2, because L-enantiomers preferentially interacted with chiral stationary phases, they were eluted as the second peaks.

Table 1
Enantiomer migration orders in CE, CEC and μLC

Ligands	Enantiomer migration order (EMO)		
	CE	CEC	LC
Phenylalaninamide	L→D	D→L	D→L
Prolinamide	D→L	D→L[a]	D→L
Alaninamide	L→D	D→L	D→L

[a] Dns-DL-Ser: L→D.

The separation factors of Dns-DL-amino acids are shown in Fig. 4. A comparison between Figs. 1 and 4 shows that some samples can well be resolved in CEC, but not in μLC by using the same columns. The major reason is that the axial diffusion of analytes in the CEC mode is insignificant due to the

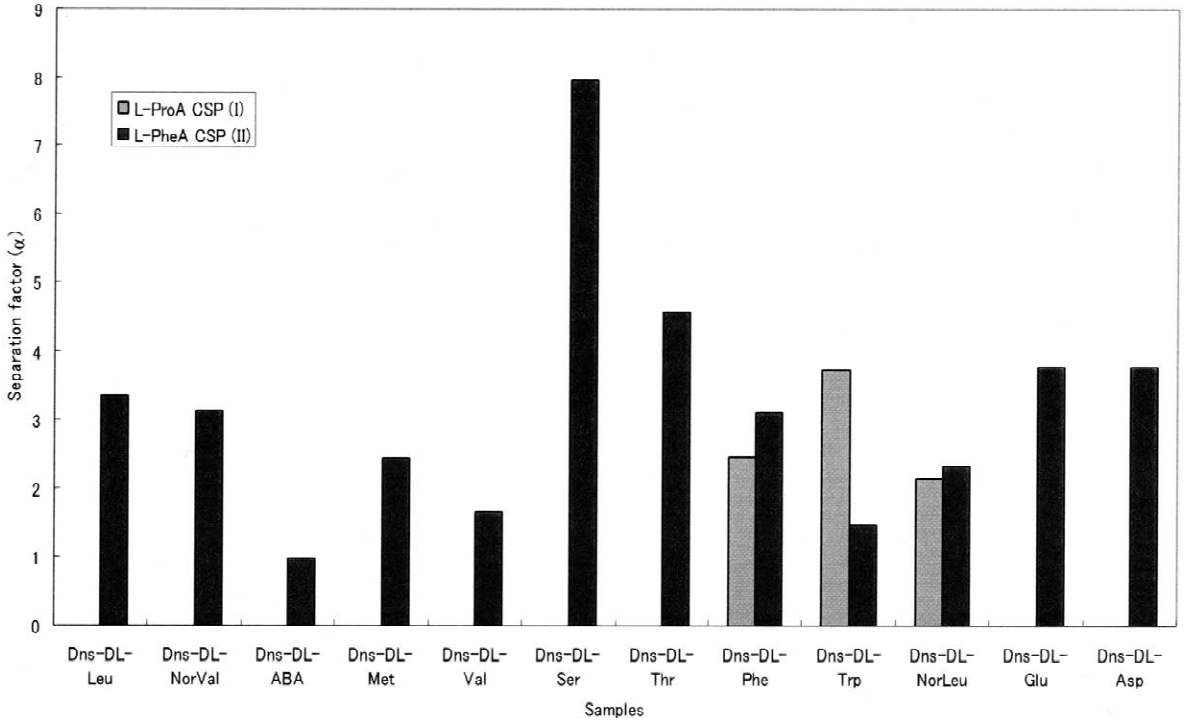

Fig. 4. Separation factors of Dns-DL-amino acids by LC. (I) L-ProA CSP: mobile phase, pH 6.5, acetonitrile–[50 m*M* NH_4Ac–0.50 m*M* $Cu(Ac)_2$] (7:3), flow-rate: 20 μm/min. (II) L-PheA CSP: 32 cm; mobile phase: pH 7.6, acetonitrile–[0.1 *M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$] (7:3); flow-rate: 20 μm/min, but 5 μm/min for Dns-Val, Dns-Phe and Dns-Trp. $\alpha = k'_2/k'_1$, $k'_i = (t_i - t_0)/t_0$.

merit of EOF driving in plug-like profile. The second reason is that the flow-rate of mobile phase in CEC is lower than that in μLC. Low flow-rate benefits frequent interaction between the CSPs and the analytes. The linear velocity of the EOF was controlled in the range of 0.05 to 0.45 mm/s, and most experiments were carried out at a linear velocity of near 0.25 mm/s (about 0.12 μl/min for the capillary column of 100 μm I.D.) in the CEC mode [14]. However, the lowest flow-rate at our μLC experiments was 1.0 μl/min, which is 10 times faster than in CEC. The analytes in μLC were eluted out when the chiral interaction between CSPs and analytes was not enough to achieve the enantioselective separation. The third reason is overloaded injection volume in the μLC, which was controlled by a sample rotor with a 0.2-μl volume. However, electrokinetically injected sample volumes in CEC are lower, and usually can be controlled at nano-scale.

We investigated the Van Deemter plots of monolithic columns in CEC [14] and LC [16]. A lowest theoretical plate height of 25 μm in CEC was obtained, but about 1 mm (about 40 times of CEC) in μLC. As discussed in previous works, the inherent reasons for a lower theoretical plate height in CEC are the plug-like profile of the EOF in CEC. On the other hand, the overload of injected samples and the fast flow-rate of mobile phase also result in the increase of the theoretical plate height in μLC.

3.4. Enantiomer migration orders in CE, LC and CEC

The EMO is an important aspect in chiral separations. It generally reflects the mechanistic information of chiral resolution. We investigated the EMOs using Cu(II) complexes with L-amino acylamide as chiral selectors in CE [17] or chemically modified CSPs in CEC [14,15] and μLC [16]. The results are summarized in Table 1. It was interestingly found that D-enantiomers migrated faster than L-ones when Cu(II) complex with L-ProA was used as chiral selector in CE, but L-enantiomers migrated faster than D-forms in the systems of Cu(II)-L-PheA

and Cu(II)-L-AlaA. To explain this phenomenon, continuous research works are carried out in our laboratory.

As shown in Table 1, the EMOs in CEC and LC have almost similar orders that L-enantiomers showed longer retention times than D-forms. With the help of Fig. 2, it is easy to understand the EMOs in CEC and LC. As shown in Fig. 2, compared to the ternary complexes of [L-PheA]Cu(II)[Dns-D-amino acid] (II) and [L-ProA]Cu(II)[Dns-D-amino acid] (IV), ternary complexes of [L-PheA]Cu(II)[Dns-L-amino acid] (I) and [L-ProA]Cu(II)[Dns-L-amino acid] (III) are more stable because the R and dansyl groups locate in different sides of the copper coordination plane. Therefore, L-enantiomers preferentially interact with CSPs and show stronger retention on the CSPs.

However, an exception of Dns-DL-Ser on L-ProA-modified CSP was observed in CEC, in which Dns-L-Ser migrated faster than the D-form. The electrochromatograms of Dns-DL-Ser in CEC using monolithic LE-CSPs modified with L-PheA and L-ProA are shown in Fig. 5. To explain the difference in the separation behaviors of Dns-DL-Ser, the possible chemical structures of the ternary complexes are proposed in Fig. 6. When an L-PheA-modified monolith column was used, the hydrophobic or π–π interactions between the phenyl group in L-PheA and the naphthylamino group of Dns-DL-Ser results in the ternary complexes present in the structures of Fig. 6I,II. Because Dns-L-Ser offers an apical interaction with the side-chain on Cu(II), it stabilizes the ternary complex, thus be more strongly retained. Since there are no such hydrophobic or π–π interactions between the pyrrolidine group in L-ProA and the

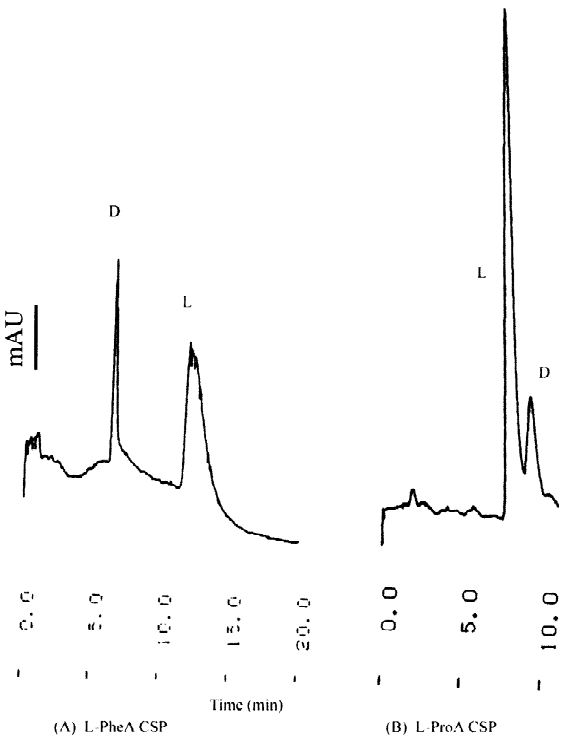

Fig. 5. Electrochromatograms of Dns-DL-Ser by CEC. (A) L-PheA CSP: 42 cm (effective length 32 cm); mobile phase: pH 6.5 acetonitrile–[50 m*M* NH_4Ac–0.50 m*M* $Cu(Ac)_2$] (7:3); applied voltage: −13.6 kV. (B) L-ProA CSP: 34 cm (effective length 26 cm); mobile phase: pH 6.5 acetonitrile–[50 m*M* NH_4Ac–0.50 m*M* $Cu(Ac)_2$] (7:3); applied voltage: −16.8 kV.

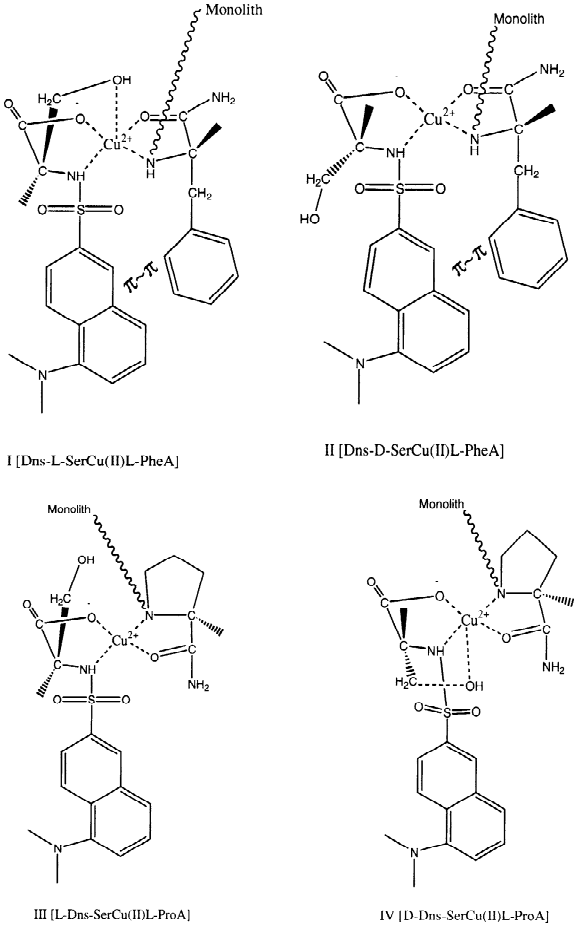

Fig. 6. Proposed chemical structures of ternary Cu(II) complexes with Dns-DL-Ser on monolithic CSPs.

naphthyl amino group of Dns-DL-Ser, as shown in Fig. 6III,IV, the complexes of III and IV take stable *cis* structures. Because of the steric hindrance and rigid structure provided by pyrrolidine ring in L-ProA, it is difficult for the side chain of Dns-L-Ser to offer an apical interaction with Cu(II). However, Dns-D-Ser can offer the apical interaction on the other side of pyrrolidine ring and stabilize the complex, as shown in Fig. 6IV, thus shows strong retention.

4. Conclusions

We have demonstrated that L-amino acylamides can successfully be used as the chiral selectors in electrolyte for enantioseparations by LE-CE, and as the chiral stationary phases chemically modified on monolithic columns for enantioseparations by LE-CEC and μLC. It has been observed that the separation behaviors such as enantioselectivities, enantiomer migration orders depend on the differences in both chemical structures of L-amino acylamides (chiral selectors) and the analytes (selectands). As the EOF has the merits of driving mobile phase in plug-like profile at low linear velocity and makes it possible to inject sample volume at nanoscale, CEC provides higher performance than LC. Based on the proposed structures of ternary complexes, the EMOs in the CEC and LC have been explained.

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