# Chiral Separation of Amino Acids by Ligand Exchange Microchip Electrophoresis

Hizuru Nakajima,* Hui Shen, Katsuhiko Kawata, Tatsuro Nakagama and Katsumi Uchiyama

*Department of Applied Chemistry, Graduate School of Engineering, Tokyo Metropolitan University,
1-1 Minamiohsawa, Hachioji, Tokyo 192-0397, Japan*

**Chiral separation of amino acid derivatives by ligand exchange electrophoresis in a microchannel chip was performed. Cu(II) complex with L-prolineamide was used as a chiral selector. The migration behaviors of eleven NBD-DL-amino acids were investigated by ligand exchange capillary electrophoresis (LE-CE). The enantiomer of five NBD-amino acids (Ser, Thr, Val, Phe and His) could be separated by LE-CE using 20mM ammonium acetate buffer containing 10mM copper acetate, 20mM L-prolineamide and 1 mM SDS. NBD-His was eluted in order, D-form and L-form, while the elution order of another enantiomers was, L-form and D-form. Under this condition, the enantioseparation of these five NBD-amino acids by ligand exchange microchip electrophoresis (LE-ME) was investigated using polydimethylsiloxane (PDMS) or glass microchip. The enantioseparation of NBD-Ser, -Thr and -His could be successfully accomplished by LE-ME using glass microchip. LE-ME was superior to LE-CE in terms of the short migration time and the good enantiomeric separation.**

## Introduction

Enantiomeric separation is one of the most important and challenging tasks in separation science. Chromatography has been a primary choice for this purpose, and has been successfully applied to the enantiomeric separation of various compounds. [1] Capillary electrophoresis (CE) including capillary electrochromatography (CEC) has recently been proven to be a powerful alternative for enatiomeric separation because of its high separation efficiency, short analysis time and small sample amount compared to liquid chromatography. [2, 3] We have developed ligand exchange capillary electrophoresis (LE-CE) using a Cu(II) complex with L-amino acid amide as a chiral selector for enatioseparation of dansyl amino acids. [4-14] In addition, we have developed monolithic silica columns chemically modified L-phenylalanine-amide, L-proline-amide and L-alanine-amide as a chiral selector in ligand exchange capillary electrochromatography (LE-CEC). [15-19] It has been demonstrated that the ligand exchange principle showed very excellent enantioseparation for dansyl amino acids and some hydroxyl acids.

Microanalysis based on micromachining technology has also been intensively investigated in recent years. [20-24] Common backgrounds of the studies on microanalysis are that the compact, light-weight and inexpensive analytical device leads to (1) on-site analysis required in the field of medical and environmental analysis, (2) reduction of consumption of sample, solvent and reagents, (3) high speed measurement. We have applied polymeric materials to microchip and high density surface moiety enabled open-channel electrochromatography with low cost and easy-to-make. [25-36] In addition, we have developed a micro-fluidic device with an integrated fluorescence detection system to miniaturize the whole of analytical system. [29, 40] Also, we have developed basic concept of micro chip enzyme sensor and micro reactor based on photochemical immobilization of protein on inner wall of microchannel. [37] From these studies, it was found that the effects of size and surface of the microchannel were extremely important in microanalysis. Microchip electrophoresis (ME) will promise high throughput analysis in the separation of enantiomer. However, very few attempts have been made at enantioseparation using microchips.

The purpose of this study is to develop a new chiral separation method using microchip. We applied ligand exchange to microchip electrophoresis and studied the enantioseparation of some amino acids by ligand exchange microchip electrophoresis (LE-ME). In liquid chromatography and CE separations, absorption spectroscopy was ordinarily used as a detection method. However, in microchip-based separation, the method does not provide enough sensibility because of the very shallow depth of microchannel and consequent very short optical path length. Therefore, we chose laser-induced fluorescence (LIF) as the highly sensitive and first choice detection method for microchip separation. [24] 4-Fluoro-7-nitrobenzofurazan (NBD-F) was used as a fluorescence derivatization reagent of amino acids. A Cu(II) complex with proline-amide was used as a chiral selector to separate the enantiomer of amino acids. We investigated the migration behaviors of some NBD-DL-amino acids in LE-CE and LE-ME. PDMS and glass microchip were used for LE-ME. We also discuss the mechanism of enantiomeric separation of NBD-amino acids

## Experimental

### Chemicals

The amino acids tested in this work were purchased from Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemicals (Tokyo, Japan), Tokyo Kasei Kogyo (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan). NBD-F was obtained from DOJINDO (Kumamoto, Japan). L-

proline-amide was from Aldrich (St. Louis, MO, U.S.A). Other chemicals used were of analytical reagent grade. Water was purified on a Milli-Q system (Nihon Millipore, Tokyo, Japan).

## Derivatization of amino acids

1.2 mL of 2.5 mM each amino acid in 50 mM borate buffer (pH 8.0) containing 10 mM EDTA and 0.6 mL of 5 mM NBD-F in acetonitrile were mixed and heated at 60 ˚C for 5 min to complete the reaction. After chilling the solution with ice, 1.2 mL of 20 mM hydrochloric acid was added to the solution to quench the excess of the reagent. The resulting solution was maintained a constant temperature of 4 ˚C.

## Apparatus

CE was performed with an $Ar^+$ laser (488 nm, 100 mW, LGK-7872-M, LASOS, Jena, Germany), a incident light fluorescence microscope (EFD-3, Nikon Tokyo, Japan), a photomultiplier tube (R535, Hamamatsu Photonics, Shizuoka, Japan), a laboratory-made amplifier, a chromatographic data processing system (CR-7A, Shimadzu, Kyoto, Japan), a high voltage power supply (HCZE-30PNO25-LD, Matsusada Tokyo, Japan) and a fused-silica capillary (0.100-mm-i.d., 0.375-mm-o.d., GL Science, Tokyo, Japan). The total length of the fused-silica capillary was 60 cm and the effective length was 30 cm. The fused-silica capillary was pretreated by flushing with 1 M sodium hydroxide, water, 1 M hydrochloric acid and water in this order. Analyte solutions were injected by electrokinetic method for 5 s at the same electric field strength applied for the separation. Electrophoresis separation was carried out by applying -8000 V to the one end of the capillary with the other end electrically grounded.

ME was carried out with a solid-blue laser (473 nm, 5 mW, SOLID STATE 473, Shimadzu, Kyoto, Japan), an incident light fluorescence microscopy (BX-40, OLYMPUS, Tokyo, Japan), a photomultiplier tube (R535, Hamamatsu Photonics, Shizuoka, Japan), a laboratory-made amplifier, a chromatographic data processing system (CDS, LA SOFT, Chiba, Japan), a laboratory-made high-voltage power supply equipped with changeover switch, which could manually change 'the injection mode' to 'separation mode' and a glass microchip (MC-BF4-SC, Micralyne, Edmonton, Canada). The microchip had a simple cross designed microchannel with 50 μm wide and 20 μm deep. The length of separation channel was 90 mm and that of injection channel was 10 mm. To clean the inner surface of microchannel, all microchannels were washed with 1 M sodium hydroxide, water, 1 M hydrochloric acid and water in this order before use. Analyte solutions were electrokinetically introduced into the channel intersection by applying -190 V to the sample waste reservoir with the other three reservoirs electrically grounded. Electrophoresis separation was carried out by applying -2080 V to the waste reservoir with the running buffer solution reservoir electrically grounded, while the voltages at the sample and sample waste reservoirs were kept at -190 V, respectively.

## CE and ME conditions

The running buffer solutions used were 20 mM ammonium acetate buffer (pH 9.0) containing 10 mM copper(II) acetate, 20 mM L-prolinamide and 1 mM SDS. Adjustment of pH was performed by addition of ammonia solution or acetic acid. The running buffer solutions without SDS were also used. All running buffer solutions were filtered through a 0.45μm membrane filter (JHWP02500, Nihon Millipore, Tokyo, Japan) and degassed with a vacuum pump before use.

Analyte solutions were prepared by diluting NBD-amino acid solutions with the running buffer solution to be used and were filtered through a 0.45μm HLC-DISK filter from Kanto Chemicals (Tokyo, Japan). Each migration on an analyte was carried out at least three times.

## Results and discussion

### Enantioseparation of NBD-amino acids by LE-CE

The enantioseparation of eleven NBD-amino acids by LE-CE was investigated. The principle of LE-CE is based on the formation of ternary complex between analyte, metal ion and chiral selector. [38,39] Ligands having mutually opposite configuration form diastereomeric complexes. The difference of the stability of these complexes makes the difference of migration time of amino acid enantiomers. It has been reported that Cu(II) complexed with amino acid with a stoichiometry of 1 : 2. [14] Thus, we used 20 mM ammonium acetate buffer (pH 9.0) containing 10 mM copper(II) acetate, 20 mM L-proline-amide and 1 mM SDS as a running buffer solution. The amino acids tested were Ser, Glu, Asp, Thr, Val, Trp, Phe, DOPA, PheGly, His and Pro. Five NBD-DL-amino acids, NBD-Ser, -Val, -Thr, -His and -Phe could be enantiomerically separated in this condition. Fig. 1 shows a typical electropherograms for the separation of NBD-DL-Ser, -Val, -Thr, -His and -Phe. Ascription of the peak was carried out by adding NBD-F or L-NBD-amino acid to DL-NBD-amino acid solution. The first peak was the peak of NBD-OH, the hydrolyzed derivative of NBD-F. The L-derivatives of Ser, Val, Thr and Phe migrated faster than the corresponding D-derivatives, while D-NBD-His migrated faster than L-NBD-His.

Fig. 2 shows the migration times and the separation factors of NBD-amino acids enantiomerically separated by LE-CE. Separation factor (α) was calculated from eq. (1)

$$\alpha = t_2/t_1 \qquad (1)$$

where $t_1$ is the migration time of enantiomer which eluted first and $t_2$ is that of enantiomer which eluted late.
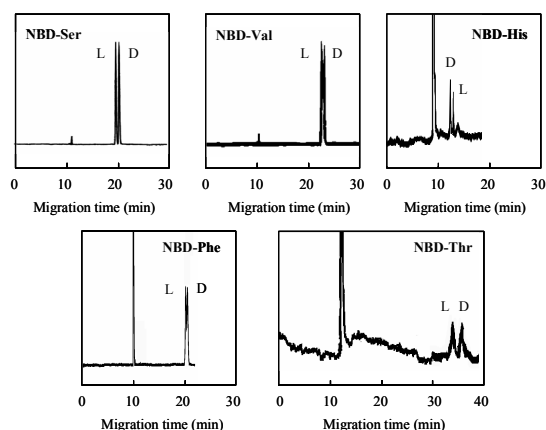


**Fig. 1** Electropherograms of NBD-DL-Ser, -Val, -Thr, -His and -Phe obtained by LE-CE.

Migration times shown in Fig. 2 correspond to the value of $t_1$. NBD-Ser, -Thr and –His were enantiomerically separated well, while imperfect base line separation of enantiomer of NBD-Val and -Phe was obtained. It seems that π-π electron interaction and steric hindrance between the NBD amino-acid and the chiral selector take part in the enantiomeric separation of NBD-amino acid derivatives. NBD-PheGly and NBD-Pro were not enantiomerically separated. As for our previous work, when Cu(II) complex with L-phenylalanine-amide was used as a chiral selector, the enantiomeric separation of dansyl-phenylalanine was not so good either. [13] Although the reason is not clear, it seems that the enantiomeric separation of amino-acid which is the same as the one used as the chiral selector is not so good in LE-CE. NBD-DL-Glu, -Asp, -Trp, and -DOPA were not eluted in this CE conditions. The reason probably is that the migration velocity of these amino acid derivatives itself, which was opposite to the electroosmotic flow (EOF), was larger than the EOF velocity so that the amino acids derivatives were not injected into the capillary.
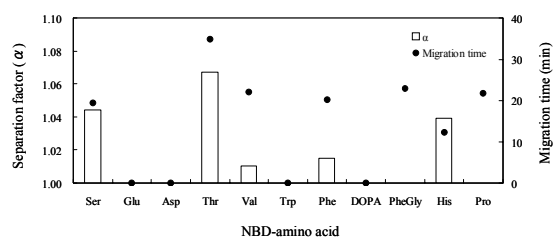


**Fig. 2** Migration times and separation factors of NBD-amino acids enantiomerically separated by LE-CE.

## Enantioseparation of NBD-amino acids by LE-ME

As shown above, ligand exchange using Cu(II) complex with L-proline amide could successfully apply to the enantioseparation of NBD-Ser, -Thr, -Val, -Phe and -His. Thus, enantioseparation of these five NBD-amino acids by ME was studied using PDMS microchip.

The design and the fabrication procedure of PDMS microchip were almost the same in the previous reports. [40, 41] Briefly, a master convex glass template was prepared through photolithography and wet-chemical etching procedure. [27, 28] The length of the separation channel was 140 mm and that of the injection channel was 20 mm. Both channels were 100 μm wide and 40 μm deep. Four Teflon tubes were placed on the grass template using water-soluble glue to define four buffer reservoirs. 10 g of PDMS base was mixed with 1 g of curing agent. The mixing solution was degassed with a vacuum pump and then 4 g of the mixture was poured onto the glass template with positive relief channel to duplicate the channel, while 4 g of the was used to make a cover plate with four reservoirs. After curing at 60 ˚C for 1 h, the two PDMS molds were peeled away and then exposed to plasma in a Sanyu Electron (Tokyo, Japan) Quick Coater SC-701 (etching mode, 3 mA for 30 s). The two pieces were immediately brought together and the mold was placed in an oven at 120 ˚C for 4 h to bond irreversibly.

When the running buffer solution without SDS was used, the migration of NBD-Ser on PDMS microchip was very slow and needed over 2 h for the separation. This would be due to the slow EOF of PDMS microchip. In our previous studies on electrochromatrography using polyester
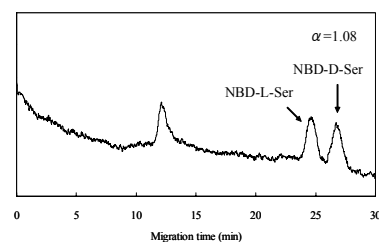


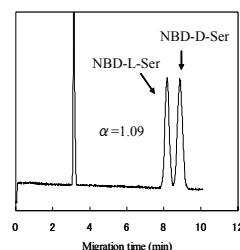**Fig. 3** Electropherogram of NBD-DL-Ser obtained on PDMS microchip.



**Fig. 4** Electropherogram of NBD-DL-Ser obtained on glass microchip.

microchip [35], it was found that when anionic surfactant was added to the running buffer solution, EOF velocity increased, while when cationic surfactant was added to the running buffer solution, the direction of EOF went across. These are phenomena not observed in fused-silica capillary. It might be inferred from these modifications of EOF that the surfactants adsorbed on inner wall of polyester microchannel so that the surface charge on inner wall of polyester microchannel changed. Therefore, we used the running buffer solution containing 1 mM SDS to increase the EOF of PDMS microchip. The use of the running buffer solution containing 1 mM SDS showed the increase of EOF so that NBD-Ser could be enantiomerically separated within 30 min. This result also means that SDS adsorbed on inner wall of PDMS microchannel. Fig. 3 shows the electropherogram of NBD-DL-Ser obtained on PDMS microchip. However, when PDMS microchip was used, the reproducible results were not obtained for the enantioseparation of the other NBD-amino acids. This is probably due to the meandering microchannel of PDMS microchip.

Therefore, commercially available glass microchip with simple cross microchannel was used for enantioseparation of NBD-amino acids by LE-ME. Fig. 4 shows the electropherogram of NBD-DL-Ser obtained on glass microchip. When the glass microchip was used, the migration time of NBD-DL-Ser dramatically decreased and the separation factor increased although the length of the separation channel is shorter than that of PDMS microchip. This is likely because the separation channel of glass microchip is straight, narrow and shallow. Fig.5 shows the dependence of the separation factor of NBD-DL-Ser on the length of separation channel. It can be seen that the separation factor increases with increase of distance from the cross section of the microchannel to the detection point. It was presumed that the chance of ligand exchange increases when the length of the channel becomes long.

Besides NBD-Ser, NBD-Thr and NBD-His could be enantiomerically separated using glass microchip. Table 1 shows the comparison of migration time and separation factor obtained by CE with that obtained by ME. The solvent composition used was the same in CE and ME. Although it is not possible to compare the results simply

because the apparatus and the electric field strength are different, the migration time obtained by ME was shorter than that obtained by CE. However, the separation factor is almost equal or increased. This is probably due to the size effect of microchip.
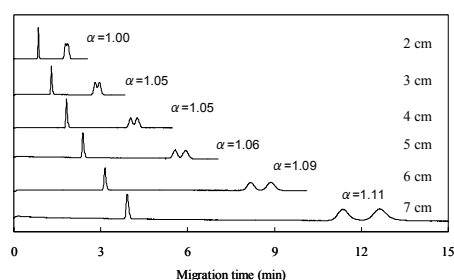


**Fig.5** Dependence of separation factor of NBD-DL-Ser on the length of separation channel. Right numerical values show the distance from the crossing point to the detection point. The symbol '$\alpha$' denotes the separation factor.

**Table 1** Comparison of migration time and separation factor obtained by CE with that obtained by ME

|  | Capillary Electrophoresis (CE) | | | Microchip Electrophoresis (ME) | | |
| --- | --- | --- | --- | --- | --- | --- |
|  | $t_1$ (min) | $t_2$ (min) | $\alpha$ | $t_1$ (min) | $t_2$ (min) | $\alpha$ |
| NBD-Ser | 19.43 | 20.29 | 1.04 | 8.18 | 8.88 | 1.09 |
| NBD-Thr | 34.88 | 37.22 | 1.07 | 4.29 | 4.56 | 1.06 |
| NBD-His | 12.18 | 12.66 | 1.04 | 8.48 | 9.16 | 1.08 |

## Conclusion

Chiral separation of NBD-amino acids by ligand exchange electrophoresis was studied using fused-silica capillary, PDMS and glass microchips. The running buffer solution used in this study was 20 mM ammonium acetate buffer (pH 9.0) containing 10 mM copper(II) acetate, 20 mM L-prolinamide and 1 mM SDS. NBD-Ser, -Val, -His, -Phe and Thr were enantiomerically separated by LE-CE. The enantiomeric separation of these NBD-amino acids was investigated by LE-ME. When glass microchip was used, NBD-Ser, -Thr and His could be enantiomerically separated. Although the reproducible results were not obtained when PDMS microchip was used, the EOF characteristic of the PDMS microchip could be manipulated by adding the surfactant to the running buffer solution. LE-ME was superior to LE-CE in terms of the short migration time and the good enantiomeric separation. It is necessary to study effects of pH and concentration of chiral selector or SDS. Enantiomeric separation of more amino acids would be achieved by optimization of running buffer solution.

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