Highly Sensitive Analysis in Capillary Electrophoresis Using Large-volume Sample Stacking with an Electroosmotic Flow Pump Combined with Field-amplified Sample Injection

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To achieve highly sensitive analysis without labor-intensive experimental procedures in capillary electrophoresis (CE), large-volume sample stacking with an electroosmotic flow pump (LVSEP)–field-amplified sample injection (FASI) was combined with a dynamic coating technique. In this study, poly(vinyl pyrrolidone) (PVP) was employed for the dynamic coating additive. Since a standard fluorescent dye, fluorescein, was well concentrated in a conventional LVSEP, the PVP dynamically-coated capillaries can be also applied to the LVSEP-FASI analysis. In our home-made CE apparatus, however, current breakdown was often caused, especially at a longer electrokinetic injection time due to bubble formation. To avoid the interference of bubble formation, the distance between the tips of the electrode and the capillary in the vertical direction was changed from 0 to 2.5 cm under the magnetic stirring condition. This allowed for a long electrokinetic injection time of up to 20 min, resulting in a sensitive enhancement factor (SEF) of 34900 for fluorescein. The developed method was applied to the chiral analysis of amino acids in CE. As a result, leucine (Leu) was successfully separated in LVSEP-FASI with SEFs of 6420 and 4500 for the D- and L-Leu peaks, respectively.

Keywords: Capillary electrophoresis, LVSEP-FASI, dynamic coating, chiral separation

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Introduction

In capillary electrophoresis (CE), poor concentration sensitivity is a significant problem due to the short optical path length and the small sample injection volume. To overcome this drawback, various on-line sample preconcentration techniques have been developed.1–8 In our research group, on-line sample preconcentration by large-volume sample stacking with an electroosmotic flow pump (LVSEP) was applied to CE and microchip electrophoresis (MCE).9–16 LVSEP experiments can be performed with simple procedures, i.e., the whole capillary sample-injection and the application of a voltage without polarity switching. Furthermore, the enriched analytes zone is moved to the capillary inlet, and its migration direction is automatically reversed toward the outlet, which gives both high sensitivity and almost no-loss of resolution.

In our previous work, LVSEP was combined with field-amplified sample injection (FASI) to improve the preconcentration efficiency.9,11 In the LVSEP-FASI technique, capillaries are coated with neutral polymers to suppress the electroosmotic flow (EOF). Both the capillary and the inlet vial are filled with anionic analytes dissolved in deionized water, while a background solution (BGS) containing a high-concentration of buffer salts is in the outlet vial (Fig. 1(a)). In LVSEP-FASI, both the pressure from the inlet and the voltage is applied as shown in Fig. 1(b). Due to the field-amplified stacking effect, the analytes are concentrated on the sample/BGS boundary. Since the capillary is filled with the low-conductivity sample solution, a faster EOF is generated to move the stacked analytes toward the inlet (LVSEP step). Faster EOF introduces the BGS from the outlet vial, which decreases the sample zone length. When the stacked analyte zone reaches around the capillary inlet, the EOF velocity \( (v_{\text{EOF}}) \) is decelerated and balanced with the velocity of the pressure flow from the inlet \( (v_{\text{p}}) \), which keeps the concentration boundary inside the capillary during the FASI step. Due to amplification of the electric field strength in the remaining sample zone, efficient FASI occurs as shown in Fig. 1(c) (FASI step). After enrichment, the sample solution in the inlet vial is exchanged to the BGS (Fig. 1(d)). Finally, a voltage is applied to separate the focused analytes without pressure from the inlet (Fig. 1(e)). Both the LVSEP and FASI steps can be distinguished from the current monitoring, as shown in Fig. 1(f). During the LVSEP period \( (t_{\text{LVSEP}}) \), the current is almost zero, since the capillary is filled with a low-conductivity sample solution. When the stacked analytes move to the capillary inlet, the current is suddenly increased at the time of \( t_s \), which is defined as the starting time for FASI. After the FASI period \( (t_{\text{FASI}}) \), the inlet vial is exchanged from the sample to BGS at a time of \( t_e \) (vial exchange time). In a previous report, up to 110000-fold sensitivity increase for fluorescein was achieved at \( t_{\text{FASI}} \) of 15.0 min by LVSEP-FASI in CE.11

In the previous report,16 a fused silica capillary was almost permanently coated with poly(vinyl alcohol) (PVA) to obtain a...
specific EOF property, i.e., a faster and a slower EOF generated in the LVSEP and the separation steps, respectively. In the PVA coating, the capillary was filled with a 5% PVA solution and left for 15 min.\textsuperscript{17}\textsuperscript{-18} The PVA solution was then removed out, and the capillary was heated at 140°C for 18 h under a nitrogen gas flow. This thermal immobilization of PVA was labor-intensive and often caused clogging of the capillary, which reduced the success yield of the PVA coating. To overcome this limitation, in this study, a dynamic coating technique\textsuperscript{19}\textsuperscript{-22} was introduced for LVSEP-FASI. In the coating technique, only the use of BGS containing coating-polymers dynamically immobilize them onto the inner surface of the capillary in the conditioning of the capillaries and during the CE measurements. In the present study, furthermore, we investigated the effect of stirring and the electrode configuration in the inlet sample vial on the LVSEP-FASI efficiencies.

**Experimental**

**Chemicals**

A fused-silica capillary of 50 μm i.d. was purchased from Polymicro Technologies (Phoenix, AZ), poly(vinyl pyrrolidone) (PVP, M\textsubscript{w} 360000), (hydroxypropyl)methyl cellulose (HPMC, M\textsubscript{w} 220000) and polyethylene oxide (PEO, M\textsubscript{w} 8000000) from Sigma-Aldrich (Tokyo, Japan), fluorescein sodium salt and fluorescein isothiocyanate (FITC), thiourea, DL-leucine (Leu) and other reagents from Wako (Osaka, Japan). All solutions were prepared with deionized water purified by Auto-Still (WG510, Yamato, Japan) and filtered through a 0.45-μm pore membrane filter prior to use. For fluorescence labeling, 10 mL of 0.3 mg/mL racemetric amino acids in a 20 mM borate buffer (pH 9.2) was mixed with 0.25 mL of a 25 mM methanolic FITC solution. The mixture was kept for 24 h at room temperature. The obtained stock solution of FITC-labeled amino acids was stored at −20°C. For LVSEP-FASI and normal LVSEP analyses, fluorescent analytes were dissolved or diluted with deionized water, while in conventional capillary zone electrophoresis (CZE) and cyclodextrin-modified CZE (CDCZE) analysis with the background solution (BGS).

**Apparatus and procedures**

CE analyses were performed with a home-made apparatus, which consists of a fluorescence detector (FP-2020, JASCO, Tokyo, Japan) and a high-voltage power supply (HCZE-30PN0.25, Matsusada Precision, Shiga, Japan). The excitation and fluorescence wavelengths were 480 and 520 nm, respectively. Output signals were acquired by using a Chromatopac (C-R8A, Shimadzu, Kyoto, Japan). In the CE analyses, bare fused-silica capillaries with total/effective lengths of 69/48 cm were employed. They were conditioned with neutral polymer-containing BGSs to suppress the EOF at 7.5 mL/h for 30 min prior to each run. In the conventional CZE and CDCZE analyses, sample injections were performed hydrodynamically by lifting the inlet sample vial 15 cm (0.2 psi) above the outlet BGS vial for 30 s (injection length, 0.6 cm). The separation voltage was applied at −15 kV. In the normal LVSEP analyses, samples were injected by a syringe pump with 7.5 mL/h for 30 min (whole capillary injection). In LVSEP-FASI experiments, the whole capillary and the large inlet-vial (20 mL) were filled with the sample solution, whereas the small outlet-vial (2 mL) was filled with the BGS. Prior to the voltage application, deionized water was injected hydrodynamically by lifting the water vial to 15 cm above the outlet vial for 60 s so as to enhance the FASI efficiency.\textsuperscript{23}\textsuperscript{-24} To apply pressure during the LVSEP-FASI step, the inlet sample vial was lifted 7 cm (0.1 psi) above the outlet BGS vial. In the LVSEP-FASI enrichment, the inlet vial was stirred with a magnetic stirrer at 750 rpm and the injection voltage was applied at −10 kV. After preconcentration of the analytes for several minutes (2.0 – 20 min), the inlet end of the capillary was replaced from the sample to the BGS vial of which the liquid level was the same as that of the outlet BGS vial to stop the pressure application. A voltage of −15 kV was then applied to separate the concentrated analytes. The value of sensitive enhancement factor (SEF) was calculated by comparing the peak height obtained under the normal LVSEP or LVSEP-FASI condition with that in the conventional CZE or CDCZE experiment, while taking into account the dilution factor.

In determining the EOF mobility (μ\textsubscript{EOF}) in the dynamically polymer-coated capillary, the outlet vial was filled with the...
Table 1. $\mu_{eo}$ in dynamically neutral polymer-coated capillariesa

| Polymer       | $\mu_{eo,S}$ (cm²/V·s)a | $\mu_{eobgs}$ (cm²/V·s)b |
|---------------|--------------------------|---------------------------|
| 0.2% HPMC (M, 22000) | $+4.1 \times 10^{-4}$ | $+1.4 \times 10^{-4}$ |
| 0.01% PVP (M, 360000) | $+7.6 \times 10^{-4}$ | $+1.6 \times 10^{-4}$ |
| 0.01% PEO (M, 8000000) | $+8.0 \times 10^{-4}$ | $+2.3 \times 10^{-4}$ |

a. Measured in deionized water-filled capillary.
b. Measured in 40 mM HEPES buffer (pH 8.0)-filled capillary.

Results and Discussion

EOF properties of dynamically neutral polymer-coated capillaries for LVSEP

In this study, the dynamic coating technique was introduced to avoid the use of labor-intensive PVA-coating capillaries in LVSEP-FASI. Since PVA is rarely employed as the BGS additive in the dynamic coating,17 three typical neutral-polymer, HPMC, PVP and PEO,19–22 were tested. As mentioned in the Introduction section, it is important to obtain a faster and slower EOF in the LVSEP and the separation steps, respectively; $\mu_{eo}$ in a low-conductivity sample solution ($\mu_{eo,S}$) and in a high-conductivity BGS ($\mu_{eobgs}$) were measured, as summarized in Table 1. In evaluating the EOF properties of the dynamic coating capillaries, polymers with typical molecular-weight were employed with appropriate concentrations, which gave acceptable stability of the coatings with low-viscosity BGS during the CE measurements. As can be seen, the dynamic polymer coatings generated a higher $\mu_{eobgs}$ ($4.1 - 8.0 \times 10^{-4}$ cm²/V·s) and a lower $\mu_{eobgs}$ ($1.4 - 2.3 \times 10^{-4}$ cm²/V·s). In the dynamic coating capillaries, all values of $\mu_{eo,S}$ and $\mu_{eobgs}$ were higher than those in a thermally-immobilized PVA coating capillary ($\mu_{eo,S}$ of $5.0 \times 10^{-4}$ and $\mu_{eobgs}$ of $2.8 \times 10^{-4}$ cm²/V·s)14, except for $\mu_{eo,S}$ of $4.1 \times 10^{-4}$ cm²/V·s in HPMC. The obtained higher $\mu_{eo}$ in the dynamic coatings should be due to a lower polymer coverage of the silanol groups on the capillary surface. Among the three polymers, we considered that HPMC and PEO were not suitable for the LVSEP-FASI experiments. In the HPMC coating, $\mu_{eo,S}$ was lower than that in a permanent PVA coating capillary, which is disadvantageous for a fast transport of the stacked analytes in the LVSEP step. In the PEO coating, on the other hand, the highest $\mu_{eobgs}$ was observed among the three polymer coatings. A faster EOF generated in the separation step should reduce the enrichment efficiencies due to band broadening through a slower migration of the stacked analytes. Therefore, PVP was selected as the dynamic coating additive in the LVSEP and LVSEP-FASI experiments in the remaining sections.

Significant factors influencing electrokinetic injection in LVSEP-FASI

Prior to the LVSEP-FASI analysis, the dynamic coating with PVP was employed to the normal LVSEP. When the BGS containing PVP and the capillary conditioned with the PVP solution were used in the LVSEP analysis of an anionic standard fluorescein dye, a higher peak of the enriched analytes was observed relative to the normal CZE analysis, resulting in a SEF of 120 (data not shown). Thus, the PVP-coated capillary can be successfully applied to LVSEP analysis.

In applying the PVP-dynamic coating capillary to LVSEP-FASI, at first, the LVSEP-FASI experiments were carried out without magnetic stirring (stirring speed of 0 rpm in the left configuration of Fig. 2(a)). As a result, a further enhanced peak of fluorescein appeared under the no-stirring condition during the LVSEP-FASI step. However, a current breakdown was often caused especially at a longer $D_{e/c}$ due to bubble formation. Actually, many bubbles generated by water electrolysis were adhered onto the electrode surface after the LVSEP-FASI run. Furthermore, several shot signals were sometimes overlapped on the analyte peak. In our previous LVSEP-FASI study,11 such disturbances did not occur. This would be due to the difference...
in the apparatus. In the commercially-available P/ACE MDQ system employed in the previous report, the air pressure was directly applied to the sealing sample vial in LVSEP-FASI, which might eliminate the generated bubbles from the electrode surface to the head-space of the vial. On the other hand, in the present LVSEP-FASI system, the pressure to the sample vial was applied through the difference in the liquid level between inlet and outlet vials as shown in Fig. 1(b). As a result, the bubble formation problem was more significant in the present LVSEP-FASI experiments.

To reduce the influence of bubble formation during electrokinetic injection, magnetic stirring was introduced as shown in Fig. 2(a). As a result, $f_{EAS}$ could be extended upon increasing the stirring rate. At a stirring rate of 750 rpm, a sharp peak of the enriched fluorescein was observed at $t_{EAS}$ of 8.0 min, as shown in Fig. 2(b). By introducing magnetic stirring, acceptable experimental repeatabilities could be obtained at $t_{EAS}$ below 8.0 min, as summarized in Table 2. According to the FASI effect, SEF was improved from 120 (normal LVSEP) to 1130 (LVSEP-FASI). At $t_{EAS}$ above 8.0 min, however, bubble formation was still problematic to bring about a current breakdown and/or no-peak electropherogram. For a further elimination of the bubble-formation interferences, the distance between tips of the electrode and the capillary in the vertical direction ($D_{EC}$) was changed from 0 to 2.5 cm, as shown in the right configuration of Fig. 2(a). As a result, repeatable peaks could be obtained even at a $f_{EAS}$ of 8.0 – 20.0 min, as demonstrated in Figs. 2(c) and 2(d). Since the electrode was separated from the capillary inlet, the generated bubbles were less likely to enter the capillary, which allowed to extend $t_{EAS}$. Compared with Figs. 2(b) and 2(c), the peak height was increased upon increasing $D_{EC}$. Hirokawa’s group reported that a longer $D_{EC}$ increased the amount of analytes through an extension of the effective electric field during electrokinetic injection, as depicted in Fig. 2(a). As summarized in Table 2, SEF was improved from 1130 to 5120 by increasing $D_{EC}$ from 0 to 2.5 cm at $t_{EAS}$ of 8.0 min, even under the stirring condition. At a $t_{EAS}$ of 20.0 min, furthermore, SEF was increased to 34900. In spite of a 2.5-fold increase in $t_{EAS}$ from 8.0 to 20.0 min, almost a 7-fold increase in the SEF was obtained. The change in the pH in the sample vial was one conceivable reason. Actually, the pH in the sample vial was increased from 8.0 to 8.3 after the single LVSEP-FASI run. A higher pH might increase the injected amount of fluorescein during FASI, and sharpen the peak width at a $t_{EAS}$ of 20.0 min.

Unfortunately, the obtained highest SEF was lower than that obtained with LVSEP-FASI in the permanently PVA-immobilized capillary at a $t_{EAS}$ of 15.0 min. SEF of 110000. The reduced SEF in the present LVSEP-FASI would be due to the differences in several experimental factors. In the PVP dynamic coating capillary, $\mu_{BGS, BGS}$ (1.6 × 10$^{-4}$ cm$^2$/V·s) was higher than that in a previous study (2.8 × 10$^{-4}$ cm$^2$/V·s), which caused band broadening of the enriched analyte zone through a longer migration time in the separation step. Additionally, the pressure was changed from 0.5 to 0.1 psi, the electrokinetic injection voltage from 15 to 10 kV, and the detection window length from ~0.1 to ~5 mm according to the limitation of home-made apparatus. These differences would also deteriorate the SEF. Although the preconcentration efficiencies in the present system were decreased from the previous LVSEP-FASI, the obtained SEF was still higher above 10000. Hence, the application of the dynamic coating of PVP should be effective for the LVSEP-FASI experiments without labor-intensive capillary coating procedures.

**Application to chiral analysis**

To evaluate the separation performance in the present LVSEP-FASI system, FITC-labeled DL-Leu was analyzed by using a BGS containing β-CD. In the normal CDCZE analysis, completely separated DL-Leu peaks were observed, but the signal intensities were weak, as shown in Fig. 3(a). By applying the conventional LVSEP technique, the peak heights of the separated amino acids were comparable with those in CDCZE in spite of using 100-fold dilution sample (Fig. 3(b)). In LVSEP-FASI analysis, the racemic Leu were well enriched to give intense and sharp peaks at 14 – 15 min, as shown in Fig. 3(c), in spite of the 80-fold dilution sample compared to the normal LVSEP. The chiral resolution ($R_{S}$) in LVSEP-FASI was estimated to be 1.55, which was lower than that obtained in the normal CDCZE, as summarized in Table 3. Since the difference in the migration time ($t_{EAS}$) between the D- and L-Leu was not almost changed in the three systems, the obtained lower resolution would be due to the peak leadings in LVSEP and LVSEP-FASI. In spite of a slightly low separation ability, the racemic amino acids were completely separated in LVSEP-FASI. SEFs for D- and L-Leu peaks were 6420 and 4500, respectively. In the chiral analysis by CE, to our knowledge, the highest SEF of 13100 was attained by combining cation-selective exhaustive injection and sweeping. It is clear that, therefore, the SEF values in our experiments must be good. On the other hand, the RSD values for the peak height ($h$) were

| $D_{EC}$/cm | 0 | 2.5 | 2.5 |
|-------------|---|----|----|
| $t_{EAS}$/min | 10.9 | 10.6 | 10.8 |
| ($\%$RSD) | (1.3) | (9.6) | (5.6) |
| $h$/μV | 83 | 390 | 2660 |
| ($\%$RSD) | (16.0) | (7.1) | (20.0) |
| SEF | 1130 | 5120 | 34900 |

a. Conditions are as in Fig. 2.
apparently poor, ranging from 26.2 to 48.2%. For quantitation analysis, thus, the addition of internal standards is essential to improve the RSDs. To apply the proposed method to real samples, furthermore, sample pretreatment is essential to remove any excessive salts and contaminants; thus, the optimizations of clean-up conditions are also very important in LVSEP-FASI.

Conclusions

In this study, the LVSEP-FASI analysis was combined with the dynamic coating technique. Among three typical coating polymers, PVP gave the most suitable EOF characteristics for LVSEP enrichments. In the LVSEP-FASI analyses with the dynamically PVP-coated capillary, however, bubble formation due to the water electrolysis in the sample vial during electrokinetic injection was a serious problem, which caused a current breakdown. To suppress inference from bubble formation, the electrode was separated from the capillary inlet under the magnetic string condition. Since the generated bubbles were less likely to enter the capillary, the injection time could be extended to 20 min, resulting in SEF of 34900. In the chiral analysis, although the resolution of DL-Leu in LVSEP-FASI was lower than that obtained in the normal CDCZE, due to peak leading, the preconcentration efficiency was quite high with SEFs of 6420 and 4500 for D- and L-Leu, respectively. Since the developed method eliminated the complicated and labor-intensive procedures for the permanent polymer coating of the capillary, highly effective preconcentrations by LVSEP-FASI can be performed with simple experimental procedures, which is also applicable to various ionic analytes.

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References

1. M. C. Breadmore, W. Grochocki, U. Kalsoom, M. N. Alves, S. C. Phung, M. T. Rokh, J. M. Cabot, A. Ghiavand, F. Li, A. I. Shallan, A. S. Abdul Keyon, A. A. Alhusban, H. H. See, A. Wuethrich, M. Dawod, and J. P. Quirino, Electrophoresis, 2019, 40, 17.
2. A. Šlampová, Z. Malá, and P. Gebauer, Electrophoresis, 2019, 40, 40.
3. F. Kitagawa and K. Otsuka, Methods Mol. Biol., 2019, 1906, 65.
4. L. M. Fu, H. H. Hou, P. H. Chiu, and R. J. Yang, Electrophoresis, 2018, 39, 289.
5. T. Kawai, Chromatography, 2017, 38, 1.
6. F. Kitagawa, A. Matsuou, K. Sueyoshi, and K. Otsuka, Chromatography, 2017, 38, 38.
7. F. Kitagawa and K. Otsuka, J. Chromatogr. A, 2014, 1335, 43.
8. F. Kitagawa, T. Kawai, K. Sueyoshi, and K. Otsuka, Anal. Sci., 2012, 28, 85.
9. F. Kitagawa, T. Ishiguro, M. Tateyama, I. Nakatsuka, K. Sueyoshi, T. Kawai, and K. Otsuka, Electrophoresis, 2017, 38, 2075.
10. F. Kitagawa, S. Kinami, Y. Takegawa, I. Nakatsuka, K. Sueyoshi, T. Kawai, and K. Otsuka, Electrophoresis, 2017, 37, 380.
11. T. Kawai, M. Ueda, Y. Fukushima, K. Sueyoshi, F. Kitagawa, and K. Otsuka, Electrophoresis, 2013, 34, 2303.
12. T. Kawai, J. Ito, K. Sueyoshi, F. Kitagawa, and K. Otsuka, J. Chromatogr. A, 2012, 1267, 65.
13. T. Kawai, H. Koino, K. Sueyoshi, F. Kitagawa, and K. Otsuka, J. Chromatogr. A, 2012, 1246, 28.
14. T. Kawai, M. Watanabe, K. Sueyoshi, F. Kitagawa, and K. Otsuka, J. Chromatogr. A, 2012, 1232, 52.
15. T. Kawai, K. Sueyoshi, F. Kitagawa, and K. Otsuka, Anal. Chem., 2010, 82, 6504.
16. F. Kitagawa, T. Kawai, and K. Otsuka, Anal. Sci., 2013, 29, 1129.
17. M. Gilges, M. H. Kleemiss, and G. Schomburg, Anal. Chem., 1994, 66, 2038.
18. Y. Okamoto, F. Kitagawa, and K. Otsuka, Anal. Chem., 2007, 79, 3041.
19. P. G. Righetti, C. Gelli, B. Verzola, and L. Castelletti, Electrophoresis, 2001, 22, 603.
20. L. Hajba and A. Gutman, TrAC, Trends Anal. Chem., 2017, 90, 38.
21. J. Horvath and V. Dolník, Electrophoresis, 2001, 22, 644.
22. I. Rodrigueza and S. F. Y. Li, Anal. Chim. Acta, 1999, 383, 1.
23. N. Kaewchuay, K. Fukushi, A. Tsuboi, H. Okamura, K. Sueyoshi, and T. Kawai, J. Chromatogr. A, 2012, 1217, 1191.
24. X. L. Hou, D. L. Deng, X. Wua, Y. Lv, and J. Y. Zhang, J. Chromatogr. A, 2010, 1217, 5622.
25. Z. Xu, K. Nakamura, A. R. Timerbaev, and T. Hirokawa, Anal. Chem., 2011, 83, 598.
26. Z. Xu, K. Kawaihito, X. Ye, A. R. Timerbaev, and T. Hirokawa, Electrophoresis, 2011, 32, 1195.
27. T. Mikuma, Y. T. Iwata, H. Miyaguchi, K. Kuwayama, K. Tsujikawa, T. Kanamori, H. Kanazawa, and H. Inoue, Electrophoresis, 2016, 37, 2970.