Highly Sensitive Oligosaccharide Analysis in Capillary Electrophoresis Using Large-volume Sample Stacking with an Electroosmotic Flow Pump

Takayuki Kawai,[*] Masato Watanabe, Kenji Sueyoshi, Fumihiko Kitagawa, and Koji Otsuka

Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

*To whom correspondence should be addressed.

Tel.: +81-75-383-2449, FAX: +81-75-383-2450

E-mail: t.kawai@kt2.ecs.kyoto-u.ac.jp

<u>Abstract</u>

To obtain high sensitivity in capillary electrophoresis of oligosaccharide without reducing the high resolution with an easy experimental procedure, large-volume sample stacking with an electroosmotic flow pump (LVSEP) was investigated. As a fundamental study, effect of the conductivity of a sample solution in LVSEP was examined. It was revealed that LVSEP was successfully carried out even in using a sample solution with the ionic strength of 150 μM and the conductivity ratio of 20, indicating a good applicability of LVSEP to the analysis of real samples containing salts. When glucose oligomer was analyzed as a model sample in LVSEP-capillary zone electrophoresis (CZE), all peaks were well resolved with decreasing only 5% of the peak-to-peak distance, which suggested 95% of the whole capillary could be used for the effective separation. In the analysis of maltoheptaose, a good calibration line with correlation coefficient of 0.9995 was obtained. The limit of detection was estimated as 2 pM, which was 500-fold lower than that in the conventional CZE. *N*-linked glycans released from three glycoproteins, bovine ribonuclease B, bovine fetuin, and human α_1-acid glycoprotein were also analyzed by LVSEP-CZE. By the sample purification with a gel filtration column, further sample dilution to reduce the sample conductivity for LVSEP was not needed. All glycan samples were well concentrated and separated with up to a 770-fold sensitivity increase. The run-to-run repeatabilities of the migration time, peak height, and peak area were good with relative standard deviations of 0.1–1.3%, 1.2–1.7%, and 2.8–4.9%, respectively.

Introduction

Of many post-translation modifications of proteins, glycosylation plays important roles in living body, such as cell recognition, cell communication, cell proliferation, immune response, and differentiation [1–3]. The glycosylation has been examined by analyzing carbohydrates after releasing them chemically or enzymatically from glycoproteins. Some of the major analytical methods are based on chromatographic separation such as high performance liquid chromatograhy (HPLC) and anion-exchange chromatography [4–6]. Although they exhibit high resolution and high sensitivity, it is often difficult to separate closely-related carbohydrates. Capillary electrophoresis (CE) is also a powerful separation tool which provides rapid and high resolution analysis of oligosaccharide isomers with complicated molecular structures [4,7,8]. However, the concentration sensitivity is quite poor in CE due to the short optical path length and small injection volume, which has been preventing the real oligosaccharide analysis.

To overcome the drawback in CE, various online sample concentration techniques have been developed such as field-amplified sample stacking (FASS) [9], sweeping [10], isotachophoresis [11], and dynamic pH junction [12]. As for the carbohydrate analysis, several groups have reported the sensitivity enhancement by using these online concentration techniques. Quirino and Terabe reported sweeping of galactose and xylose with up to 40-fold sensitivity increase by using borate-diol interaction [13]. Kamoda et al. employed field-amplified sample injection (FASI) for analyzing *N*-linked glycan, succeeding in up to 360-fold sample concentration [14]. Auriola et al. reported up to 50-fold enhancement in sample loading by using transient isotachophoresis for the analysis of *O*-linked oligosaccharides [15]. Kazarian et al. combined FASS with dynamic pH junction for the analysis of mono-, di-, and trisaccharides, where tens-fold

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sensitivity improvement was achieved [16]. Although these techniques showed a good analytical performance, further improvements of the sensitivity, separation performance, complicated experimental procedure, and low repeatability are desired. Hence, we focused on the online sample concentration by large-volume sample stacking with an electroosmotic flow (EOF) pump (LVSEP) [17], which allows an efficient sample concentration without loss of the effective separation length in a simple experimental procedure, *i.e.*, the whole capillary is filled with the sample solution followed by only the application of a constant voltage. We have already reported the simple and sensitive analysis by microchip electrophoresis (MCE) using LVSEP in a single straight channel, resulting in up to a 2900-fold sensitivity increase in the oligosaccharide analysis [18]. To obtain further enhanced sensitivity and resolution, LVSEP was combined with capillary zone electrophoresis (CZE) in a long separation capillary, where increased amount of sample can be injected and longer effective separation length will be available. Although longer analysis time such as a few tens minutes will be required in the case of the long capillary, it will not increase the total analysis time for the oligosaccharide analysis, most of which is occupied for sample derivatization and pretreatment processes.

The aim of this study is to establish a simple, sensitive, and high resolution method for the LVSEP-CZE analysis of oligosaccharides, as well as to study LVSEP-CE as a versatile analytical method. To suppress the EOF and sample adsorption onto the capillary surface, a capillary coated with poly(vinyl alcohol) (PVA) was employed. As reported in our previous work [18], the EOF enhancement is important in LVSEP using an EOF-suppressed capillary, so we estimated the electroosmotic mobility to confirm the proper EOF change in a PVA-coated capillary

1 for the LVSEP process. Although many excellent applications of LVSEP-CZE have

2 been reported [19–26], no one has reported the limitation of sample conductivity,

3 sample inversion position, and correction of detection time. Hence, effect of the sample

4 conductivity was also evaluated by changing the electrolyte concentration in the sample

5 matrix (SM) in the LVSEP-CZE analysis of two fluorescent dyes. Glucose oligomer was

6 then analyzed as model carbohydrates both by conventional CZE and by LVSEP-CZE,

7 where sample inversion position, separation performance, and correction of detection

8 time were discussed. Finally, we performed the analysis of *N*-linked glycans by

9 LVSEP-CZE.

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11 **<u>Experimental Section</u>**

12 **Materials and Chemicals**

13 A fused silica capillary of 50 μm i.d. was purchased from Polymicro Technologies

14 (Phoenix, AZ, USA). Acetic acid, 2-[4-(2-hydroxyethyl)-1-pyperazinyl]ethylsulfonic

15 acid (HEPES), and maltoheptaose (G7), were purchased from Nacalai Tesque (Kyoto,

16 Japan). Sodium cyanoborohydride, 8-aminopyrene-1,3,6-trisulfonic acid (APTS),

17 tetrahydrofuran (THF), bovine ribonuclease (RNase) B, fetuin from fetal calf serum,

18 and human α_1-acid glycoprotein (AGP) were purchased from Sigma-Aldrich (St. Louis,

19 MO, USA), thiourea from Wako (Osaka, Japan), glucose oligomer from J-oil mills

20 (Tokyo, Japan), peptide-*N*-glycosidase F (PNGase F) from Prozyme (San Leandro, CA,

21 USA), fluorescein sodium salt from Tokyo Chemical Industry (Tokyo, Japan), Alexa

22 Fluor-488 carboxilic acid succinimidyl ester (Alexa) from Invitrogen (Carlsbad, CA,

23 USA), and PVA (M_w = 88 000, 99% hydrolyzed) from Japan VAM and POVAL (Tokyo,

24 Japan). All solutions were prepared with deionized water purified by using a Direct-Q

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System (Nihon Millipore, Japan), and filtered through a 0.45 μm pore membrane filter prior to use.

Capillary Coating

A fused silica capillary was coated with PVA in the same way as the previous papers [27,28]. Briefly, the capillary was activated and washed with 1 M NaOH and water, followed by the injection of a 5% PVA solution into the whole capillary. Both the capillary ends were immersed in the same PVA solution and left at room temperature for 15 min. The PVA solution was then removed out of the capillary and the capillary was heated at 140 °C for 18 h under a nitrogen gas flow. The capillary was filled with deionized water and stored at room temperature. Prior to use, the capillary was flushed with a back ground solution (BGS) for 15 min.

Apparatus

All CE experiments were performed on a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a diode-array UV detector or a laser-induced fluorescence (LIF) detector. The LIF detector consisted of a 488 nm argon ion laser module and photomultiplier detector with a 520 nm band pass filter. UV detection was performed at 200 nm.

Sample Preparation

Oligosaccharides were released from glycoproteins with PNGase F enzyme using the methods reported previously [18]. For fluorescence labeling, oligosaccharides released from 200 μg glycoprotein or 80 μg glucose oligomer were mixed with 5 μL of

1   0.1 M APTS in 15% acetic acid and 10 μL of 0.5 M NaCNBH$_3$ in THF. The mixture

2   was kept at 55 °C for 2 h, followed by dilution with water to 50 μL. In the analysis of

3   oligosaccharides from glycoproteins, the sample solution was desalted with a

4   Centri-Spin-10 column (Princeton separations, NJ, USA) to remove an excess APTS

5   and reagents used in the enzymatic reaction. These samples were diluted to the desired

6   concentration with deionized water and BGS in the LVSEP-CZE and the conventional

7   CZE analysis, respectively.

8

9   **Procedure**

10   The conductivity of the solution was measured by a conductivity meter B173

11   (Horiba, Kyoto, Japan). Prior to each run, the capillary with the total/effective lengths of

12   60/50 cm was conditioned with deionized water in LVSEP-CZE or with a 25 mM

13   HEPES buffer (pH 8.0) in conventional CZE for 3 min at 20 psi. Sample injection was

14   performed with a pressure of 20 psi for 90 s (whole capillary injection, 1.2 μL) in

15   LVSEP or of 0.3 psi for 3 s (injection volume, 1.7 nL) in conventional CZE. The applied

16   voltage and temperature were set at −30 kV and 25 °C, respectively.

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18   **Results and Discussion**

19   **Fundamental Study of LVSEP-CZE**

20   The concept of LVSEP using an EOF-suppressed microchannel has been discussed

21   in the previous paper [18]. The mechanism of the capillary-based LVSEP is the same as

22   that in the microchannel. Briefly, the EOF-suppressed capillary is filled with a low ionic

23   strength solution containing anionic analytes. After applying the voltage, anionic

24   analytes are concentrated at the sample matrix (SM)/BGS boundary by the difference in

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the electric field strength between the two zones. The focused analytes move toward the

cathode and the BGS is introduced into the capillary by the enhanced EOF in the low

ionic strength SM. As the analytes migrate to the cathode, the EOF velocity becomes

slower and the electric field strength in the BGS becomes higher. When almost all the

SM in the capillary is removed out from the cathodic end, the electrophoretic velocity of

the analytes exceeds the EOF rate, resulting in the inversion of the sample migration

direction. After the complete removal of the SM, the analytes are separated by CZE

during the anodic migration (See Supporting Information).

In LVSEP-CZE, therefore, the EOF in the capillary must be suppressed in the high

ionic strength BGS and be enhanced in the low ionic strength SM. Hence we

investigated the effect of the ionic strength of the BGS on the electroosmotic mobility

(μ_{EOF}) (see supporting information). As a typical result, μ_{EOF} in deionized water was

enhanced to be 5.0×10^{-4} cm^{2}V^{-1}s^{-1} and that in 25 mM HEPES buffer was suppressed

to be 3.0×10^{-5} cm^{2}V^{-1}s^{-1}, which gave a sufficient EOF change for the LVSEP process.

In employing FASS-based online concentration methods including LVSEP, the

conductivities (σ) of the sample solution and the BGS are important. To estimate the

conductivity limit of the sample solution in LVSEP-CZE, a HEPES buffer was used as

the SM, of which concentration ranged from 0 mM (deionized water, $\sigma = 0.055$ μS/cm)

to 1 mM ($\sigma = 53$ μS/cm). When 25 mM HEPES ($\sigma = 1090$ μS/cm) and a mixture of 100

pM fluorescein and 100 pM Alexa were employed as the BGS and analytes, respectively,

the LVSEP-CZE analyses were successfully performed under the SM concentration less

than 1 mM as shown in Figure 1. The first peak detected before 2 min was assigned to

the concentration boundary moving toward the cathode from anodic capillary end by the

enhanced EOF. Since the analytes are focused on the anodic-side SM/BGS boundary by

field-amplified sample stacking in LVSEP, the boundary can be detected as the sharp peak even in LIF detection. The detection time of this peak was gradually delayed as the SM concentration was increased, which supports that the EOF was increased with the decrease in the ionic strength as discussed previously. The gradual delay in the detection time of both Alexa and fluorescein was observed, which was also caused by the decrease in the EOF velocity. On the contrary, the peak-to-peak distance for the two analytes was not changed, indicating that the effective separation length in LVSEP was independent of the ionic strength of the SM. The peak width of Alexa was kept constant until the SM concentration reached 0.5 mM, whereas that in 1.0 mM SM became slightly broadened, probably because the insufficient conductivity difference between the 1.0 mM SM and 25 mM BGS reduced the stacking efficiency. The gradual broadening of the fluorescein peak could be explained in the same way. Theoretically, the slowly migrating fluorescein requires a long time to be completely concentrated, which might result in slightly broadened peak. We also observed that up to 2.0 mM HEPES buffer (100 μS/cm) could be applied to the SM in LVSEP, but the detection times were further delayed and peaks were more broadened. These results showed that a sample containing a small amount of salt can be analyzed by LVSEP-CZE. For example, the glucose ladder sample after the APTS labeling as described in the Experimental Section could be used in LVSEP without desalting since the conductivity in the sample was estimated to be around 100 μS/cm.

In the early work on the conductivity in FASS [29,30], the conductivity ratio γ of 10 is the best to obtain the highest peak, which conflicts with our data. In FASS, too large γ generates an EOF mismatch between the SM/BGS zones and/or decreases the electric field in the BGS (E_{BGS}), resulting in the band broadening. In LVSEP, of course, the EOF

mismatch occurs between the SM/BGS zones, resulting in the band distortion and broadening in the preconcentration stage. In the LVSEP-MCE analysis shown in the previous report [18], however, we had found that the concentrated band was further focused around the sample inversion timing, resulting in the sharp peak in spite of the whole capillary injection. Hence the effect of the boundary distortion is relatively small in LVSEP. The second effect by the decrease in E_{BGS} can also be neglected because E_{BGS} is recovered after the preconcentration in LVSEP. In LVSEP, the salt in the SM decrease the sample focusing efficiency, which directly broadens the focused band. The salts also decrease the EOF velocity in the SM zone. The slow SM removal causes the band broadening due to the longitudinal diffusion.

Performance of LVSEP-CZE in Oligosaccharide Analysis

To evaluate the performance of LVSEP-CZE for oligosaccharide analysis, a glucose oligomer was analyzed as a model sample. The PVA-modified capillary was employed as the separation column to suppress the sample adsorption onto the inner surface. Among several buffer systems (phosphate, acetate, Tris-HCl, HEPES, HEPPS, PIPES, MES, TES, BES and MOPS), we found that the 25 mM HEPES buffer was the optimal BGS in the LVSEP-CZE analysis of oligosaccharides. When the conductivity ratio between the sample solution and the BGS was high enough (*e.g.* >100), further improvement of the concentration efficiency was not attained by increasing the BGS concentration more than 25 mM. This result indicated that the obtained peak height was not determined by the concentrated band width immediately after the stacking process, but mainly by the peak broadening caused by the molecular diffusion during the anodic migration.

1   The APTS-labeled glucose oligomer was analyzed both by conventional CZE and

2   LVSEP-CZE. As shown in Figure 2, 32 ppt glucose oligomer was well concentrated and

3   separated without significant loss of resolution in the LVSEP-CZE analysis compared

4   with the result in conventional CZE. Since the separation of concentrated analytes in

5   LVSEP-CZE starts near the cathodic capillary end as in conventional CZE, the

6   electropherogram obtained in LVSEP-CZE were quite similar to that in conventional

7   CZE. All the peak-to-peak distances (d) in LVSEP-CZE (d_{LVSEP}) were 5% smaller than

8   those in conventional CZE (d_{CZE}). As shown in the following equation, d depends on the

9   effective separation length in the uniform electric field,

$$
\begin{aligned}
d_{\mathrm{CZE}} &= t_{\mathrm{M2,CZE}} - t_{\mathrm{M1,CZE}} \\
&= \frac{Ll_{\mathrm{CZE}}}{V\mu_{\mathrm{ep2}}} - \frac{Ll_{\mathrm{CZE}}}{V\mu_{\mathrm{ep1}}} \\
&= \left(\frac{1}{\mu_{\mathrm{ep2}}} - \frac{1}{\mu_{\mathrm{ep1}}}\right)\frac{L}{V}l_{\mathrm{CZE}}
\end{aligned} \tag{1}
$$

$$
\begin{aligned}
d_{\mathrm{LVSEP}} &= t_{\mathrm{M2,LVSEP}} - t_{\mathrm{M1,LVSEP}} \\
&= (t_{\mathrm{M2,LVSEP}} - t_{\mathrm{i}}) - (t_{\mathrm{M1,LVSEP}} - t_{\mathrm{i}}) \\
&= \frac{Ll_{\mathrm{LVSEP}}}{V\mu_{\mathrm{ep2}}} - \frac{Ll_{\mathrm{LVSEP}}}{V\mu_{\mathrm{ep1}}} \\
&= \left(\frac{1}{\mu_{\mathrm{ep2}}} - \frac{1}{\mu_{\mathrm{ep1}}}\right)\frac{L}{V}l_{\mathrm{LVSEP}}
\end{aligned} \tag{2}
$$

12   where t_{M}, t_{i}, V, L, and l are the detection time, starting time of the separation in

13   LVSEP-CZE, applied voltage, whole capillary length, and effective separation length,

14   respectively. Hence, the $d_{\mathrm{LVSEP}}/d_{\mathrm{CZE}}$ of 0.95 can be assumed as the ratio of the effective

15   separation lengths. Since 5% of the effective separation length of 30 cm or 1.5 cm can

16   be assumed as the position of the sample inversion in LVSEP-CZE, it was revealed that

17   at most 96% of the whole capillary length (38.5 cm) could be used for the effective

separation in the LVSEP-CZE analysis. This result shows the good agreement with the inversion position of 94% which was determined by the fluorescence imaging in LVSEP-MCE in the previous paper [18]. It also matched with the value of ($t_{M,LVSEP}$ − t_{cur})/$t_{M,CZE}$ (*e.g.*, for G7 peak, 97%), where t_{cur} is the time when current reaches the half of the stable current in the separation stage of LVSEP-CZE (see supporting information). Since the current is expected to be drastically increased around the complete removal of SM with the low conductivity, t_{cur} can be approximated as the time of SM removal, or the starting point of separation (t_i). Hence, it is reasonable that ($t_{M,LVSEP}$ − t_{cur})/$t_{M,CZE}$ is identical to the ratio of the effective separation lengths as shown in the following equations.

$$t_{M,CZE} = \frac{Ll_{CZE}}{V\mu_{ep}} \tag{3}$$

$$\begin{aligned} t_{M,LVSEP} - t_{cur} &= t_{M,LVSEP} - t_i \\ &= \frac{Ll_{LVSEP}}{V\mu_{ep}} \end{aligned} \tag{4}$$

$$\frac{t_{M,LVSEP} - t_{cur}}{t_{M,CZE}} = \frac{l_{LVSEP}}{l_{CZE}} \tag{5}$$

The ratio of the peak areas for oligosaccharides was also examined. Although the sample solution was injected by pressure in LVSEP, there was a possibility that some slowly migrating analytes might be lost out of the cathodic capillary end by the fast EOF [17]. Hence, we calculated the peak area ratio of G1, G3, G5, and G10, of which electrophoretic mobility ranged from 1.6×10^{-4} cm^{2}V^{-1}s^{-1} to 3.4×10^{-4} cm^{2}V^{-1}s^{-1}, whereas the enhanced μ_{EOF} in the LVSEP condition was estimated as 7.0×10^{-4} cm^{2}V^{-1}s^{-1} (see supporting information). As a result, the peak area ratio in the LVSEP analysis was estimated to be 1.00:0.52:0.42:0.22, which agreed with the ratio of

1.00:0.51:0.41:0.23 obtained with conventional CZE. This result implied that even slowly migrating analytes were not lost in LVSEP-CZE, which will be helpful for quantitative analysis.

The limit of detection (LOD) of G7 in LVSEP-CZE was evaluated. Since many *N*-linked glycans obtained from glycoproteins consist of more than six monosaccharides, it is reasonable to select G7 as a model analyte. In the LVSEP-CZE analysis, an obtained peak height was plotted against the molar concentration of the analyte to depict the calibration line. In the LVSEP-CZE analysis, the regression slope, intercept, and correlation coefficient (*R*) were calculated to be 3.37×10^{13} M^{-1}, 23.48, and 0.999, respectively, whereas those in conventional CZE were estimated as 6.36×10^{10} M^{-1}, 1.26, and 0.993, respectively (see supporting information). The LODs (S/N = 3) were estimated to be 1 nM and 2 pM in conventional CZE and LVSEP-CZE, respectively, indicating that a 500-fold sensitivity increase was achieved by LVSEP-CZE.

Analysis of *N*-linked Glycans Otained from Glycoproteins

Three glycoproteins, bovine RNase B, bovine fetuin, and human AGP were treated with peptide-*N*-glycocidase and acetic acid to obtain asialo *N*-linked glycans. Since peak assignments and characterizations of the molecular structure were difficult in the analysis of sialo glycans without MS detection, only asialo glycans were analyzed in this study, where the peak assignment was performed by comparing the results with those in the previous reports [14,31,32]. The obtained glycans were derivatized with APTS, followed by the purification with a gel filtration column [30]. This purification could reduce the concentration of unnecessary small ions in a few minutes without significant loss of glycans, resulting in the reduction in both the ionic strength and

conductivity (*e.g.* less than ~100 µS/cm) of the sample solution. Although the preconcentration by LVSEP without sample dilution using deionized water could be carried out, several-fold sample dilution was recommended to obtain good resolution.

The prepared glycan samples were analyzed by LVSEP-CZE. As shown in Figures 3–5, all glycan samples were successfully concentrated and separated in the LVSEP-CZE analyses without loss of the separation efficiency. Compared with the conventional CZE analyses, the sensitivity enhancement factors (SEFs) were estimated to be ranging from 400 to 770 as summarized in Table 1. As far as we know, these SEF values were the best compared to previous papers on the online preconcentration of oligosaccharides in CE. This is because LVSEP can stack the theoretically maximum amount, *i.e.*, whole capillary volume, of analytes in the case of the pressure injection. These results showed the high concentration performance of LVSEP-CZE and its compatibility with the oligosaccharide analysis. For the further concentration by LVSEP, the longer capillary should be used although the longer analysis time will be taken.

In the research on the glycoprotein activity, quantification of each structure of glycans including minor ones is very important [33]. In the conventional CZE analysis of glycans from human AGP, the peak intensity was so low that we could not detect more than 10 minor glycan peaks. To detect more minor glycan peaks, LVSEP was applied. As a typical result, at least 20 more peaks could be detected in the LVSEP-CZE analysis as shown in Figure 6 despite the sample concentration was 10-fold lower than that in conventional CZE. When the original sample solution was analyzed by LVSEP-CZE, the main glycan peaks became broader, which impaired the separation of minor peaks. For further better detectability of minor peaks, higher performance desalting column would be necessary. Although we could not confirm the glycan

structure of minor peaks without MS detection system in this study, LVSEP-CZE showed good potential for studying minor glycans.

Resolution for the first- and second-migrating peaks was also examined (Table 2). Although we could not estimate the correct resolution of oligosaccharides from fetuin due to the peak overlap, the resolution values obtained with LVSEP analyses of oligosaccharides released from other glycoproteins were as low as 74%−85% of those in conventional CZE. Since d_{LVSEP}/d_{CZE} ranged from 93% to 95% as shown in Table 2, the decrease in resolution was not caused only by the difference in the separation length, but mainly by the increase in the peak width. The peak width at the half height ($W_{1/2}$) was also shown in Table 2, where $W_{1/2}$ in the LVSEP-CZE analysis was as wide as 114%−125% of $W_{1/2}$ in the conventional CZE. This band broadening or the peak fronting is caused by the sample diffusion in the preconcentration step in LVSEP. Hence, the suppression of the molecular diffusion by an addition of some gel reagents and/or additional application of another preconcentration process to reduce the band broadening effect will be necessary to obtain the better resolution.

To evaluate the analytical reproducibility, relative standard deviations (RSDs) of t_M, the peak height, and peak area were calculated. As summarized in Table 3, the RSDs of t_M in the LVSEP-CZE analysis of M5 or AI from glycoproteins were higher than those in conventional CZE. It should be noted that the unstable EOF rate in LVSEP caused poor repeatability of the starting time of the separation or t_{cur}. As shown in the previous section, we can predict the starting time of the separation from the current change. Hence, when t_M of the analyte was corrected with subtraction by t_{cur} or by t_M of free APTS which was used as an internal standard, the RSD was improved to less than 0.1%. If the SM contains many salts which make the SM removal very slow, the

approximation of t_{cur} as t_i may be incorrect. This would make the correction of the detection time less efficient. On the other hand, the RSDs of the peak height in LVSEP-CZE were as good as 1.2%−1.7%, which were better than those in conventional CZE, 9.0%−19.6%. In conventional CZE, the sample solution was injected by a weak pressure of 0.3 psi for just 3.0 s to avoid the band broadening, resulting in less repeatable sample injection with poor RSD values. In contrast, the injected sample volume in LVSEP-CZE was constantly equal to the column volume, so that the repeatable injection with good RSD values could be performed. The RSD values of the peak area in LVSEP-CZE (2.8%−4.9%) were smaller than those in conventional CZE (4.1%−13.4%), which showed the good agreement with the discussion mentioned above.

The analytical performance of LVSEP-CZE in the oligosaccharide analysis was compared with that of the other online concentration methods [13−16]. The sensitivity enhancement factors (SEFs) more than 400 in LVSEP-CZE were higher than those ever reported. This is because the largest-volume sample could be stacked to a narrow band. Although Kamoda et al. reported the field-amplified sample injection method with up to a 360-fold sensitivity increase, the obtained repeatability was poor because the repetitive electrokinetic sample injection changes the balance of the ion components in the sample solution [24,25]. On the other hand, the repeatability in LVSEP-CZE was sufficient for the peak assignment. The separation performance of LVSEP-CZE was also comparable to or better than the other online sample concentration methods. This was because a long effective separation length can be utilized in LVSEP-CZE. The experimental procedure of LVSEP-CZE was quite simple: only the application of a constant voltage to the capillary entirely filled with the sample solution. Hence, no

optimization of the sample injection was required unlike in the other online concentration techniques. Therefore, LVSEP-CZE realized the quite high sensitivity improvement with good repeatability in a simple experimental procedure for the oligosaccharide analysis.

Conclusions

The effects of the EOF velocity and the conductivity of the sample matrix on the preconcentration and separation performance of LVSEP-CZE using the PVA-coated capillary were investigated. We found that the γ larger than 20 and the ionic strength of the sample less than 150 μM were needed to obtain the LVSEP effect. In the LVSEP-CZE analysis of oligosaccharides, up to a 2500-fold sensitivity improvement, good resolution utilizing long effective separation length with 95% of the total length, and good repeatability were achieved with a simple experimental procedure. Since LVSEP-CZE can be performed without diluting the sample solution, LVSEP should be widely applied to the CE analysis of various oligosaccharides. The high analytical performance will also contribute to the more practical analyses not only for oligosaccharides but also anionic biomolecules, *e.g.*, DNA, peptides, proteins, organic acids, metabolites, and so on.

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1   Science and Technology, Japan, administrated by JSPS.

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Figure Legends

2 **Figure 1.** Electropherograms obtained with the LVSEP-CZE analyses of 100 pM Alexa

3 and 100 pM fluorescein. BGS: 25 mM HEPES buffer (pH 8.0), SM: 0–1.0 mM HEPES
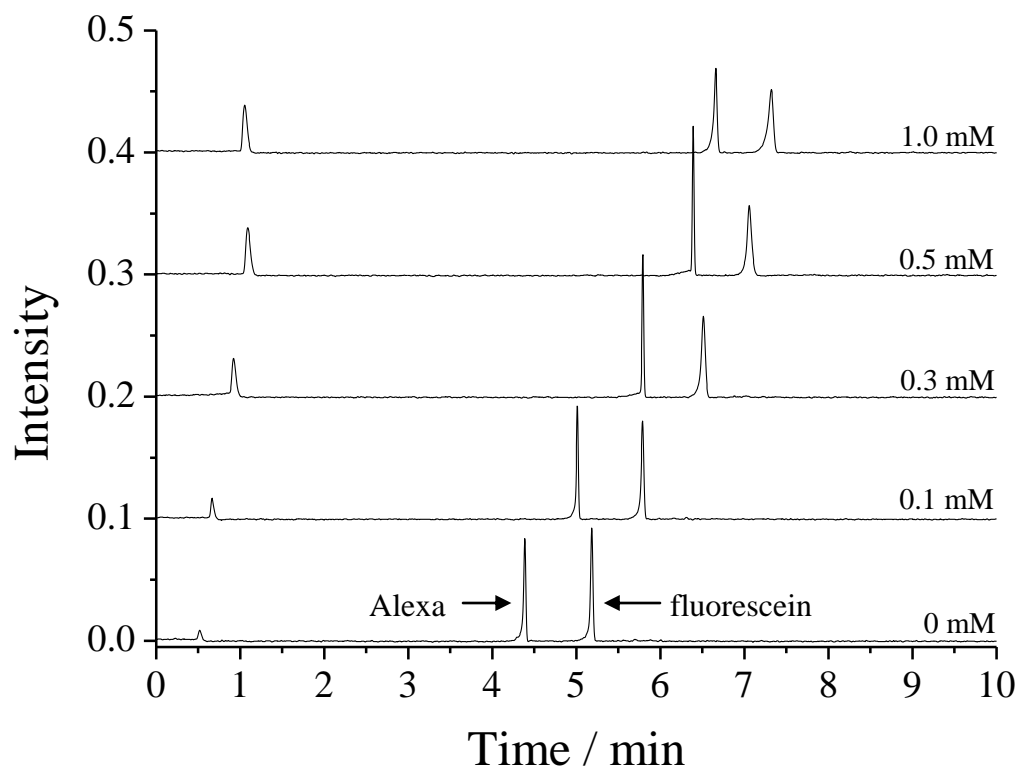
4 buffer (pH 8.0).

5

6 **Figure 2.** Electropherograms of glucose oligomer obtained with (a) conventional CZE

7 and (b) LVSEP–CZE. Sample concentration, (a) 16 ppb, (b) 32 ppt.

8

9 **Figure 3.** (a) Conventional CZE and (b) LVSEP–CZE analyses of oligosaccharides

10 released from ribonuclease B. Sample concentration in LVSEP–CZE was 500-fold
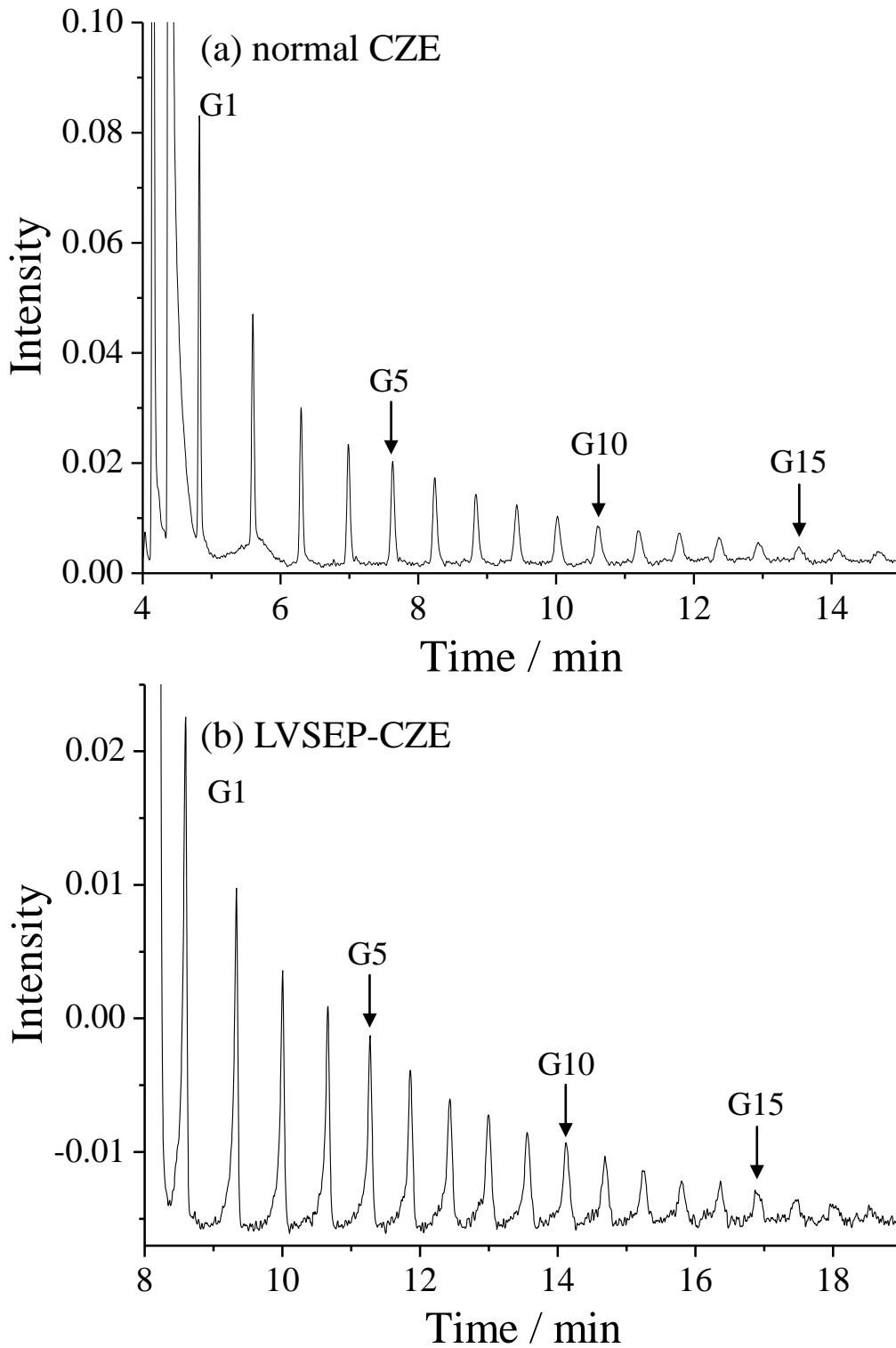
11 lower than that in normal CZE.

12

13 **Figure 4.** Electropherograms of fetuin glycans obtained in (a) conventional CZE and (b)

14 LVSEP–CZE. Sample concentration in LVSEP–CZE was 100-fold lower than that in

15 conventional CZE.

16

17 **Figure 5.** (a) Conventional CZE and (b) LVSEP–CZE analyses of glycans obtained

18 from AGP. Sample concentration in LVSEP–CZE was 400-fold lower than that in
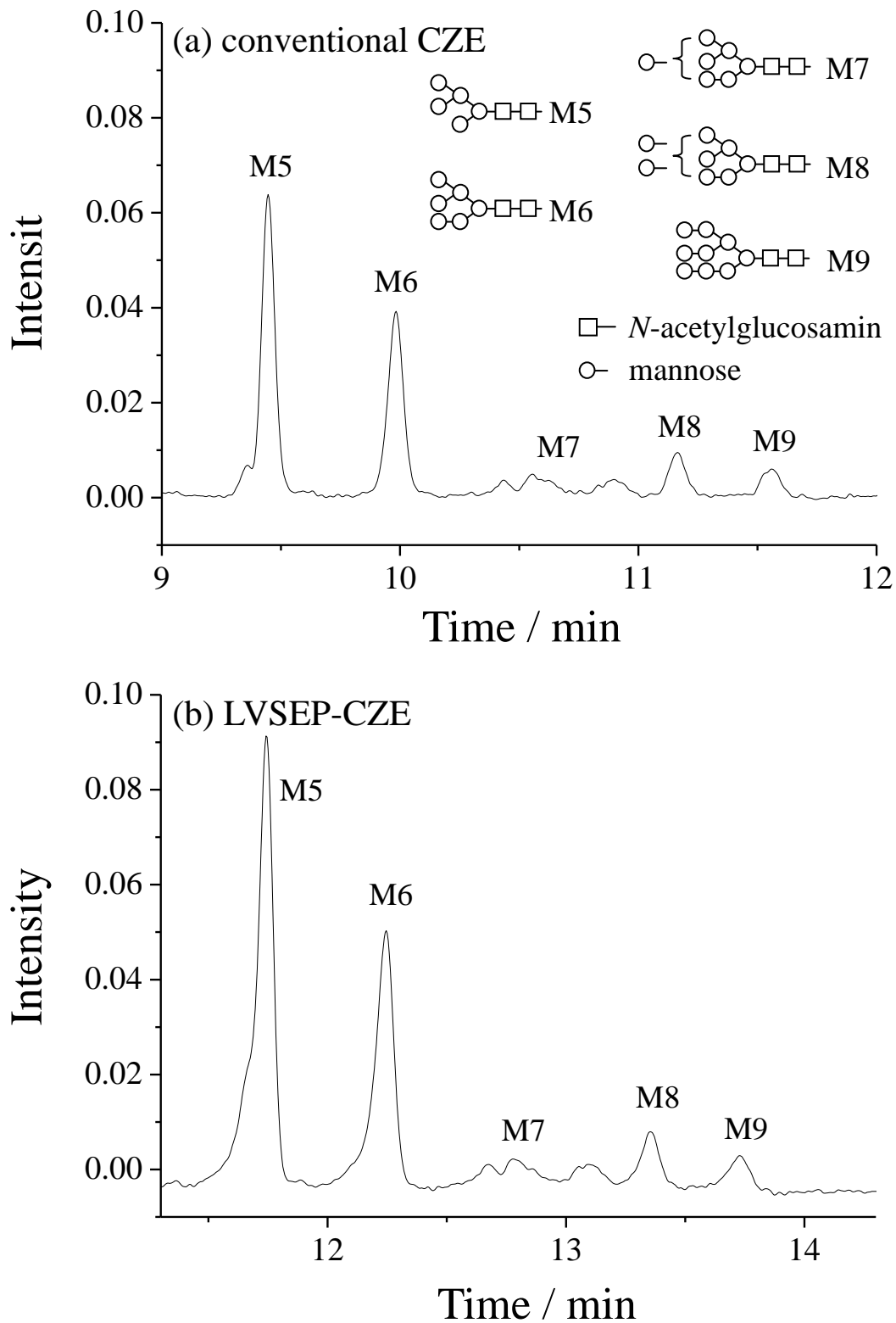
19 conventional CZE.

20

21 **Figure 6.** Detection of minor glycans obtained from AGP by LVSEP-CZE. 20 peaks

22 indicated with arrows could not be detected in conventional CZE.

23

Figure 1. Electropherograms obtained with the LVSEP-CZE analyses of 100 pM Alexa
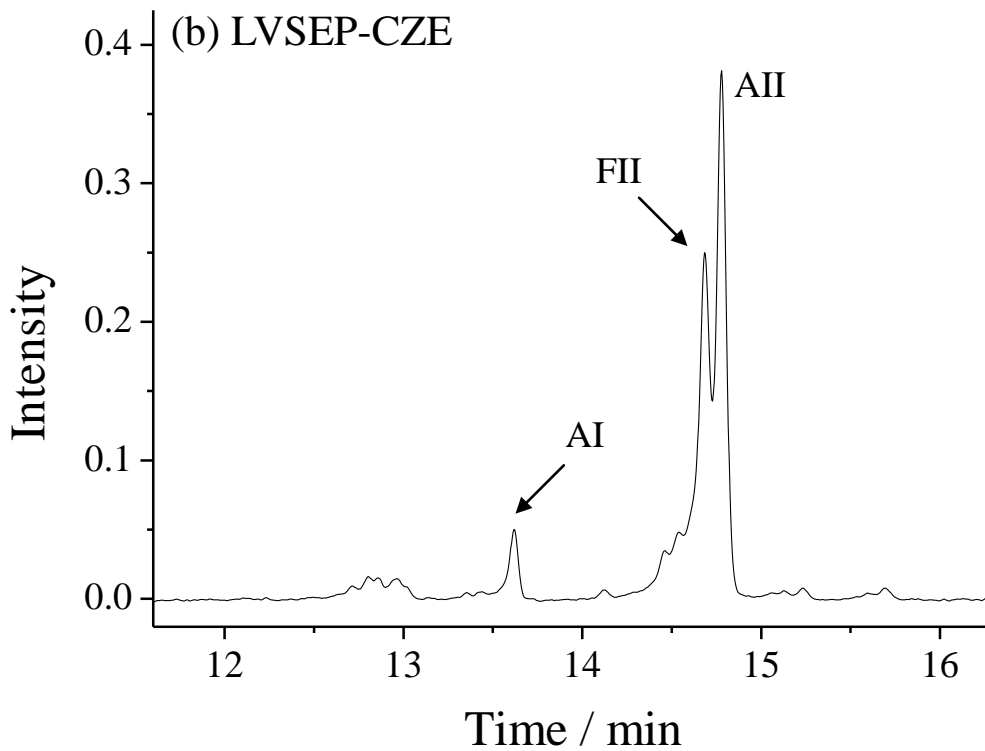and 100 pM fluorescein. BGS: 25 mM HEPES buffer (pH 8.0), SM: 0–1.0 mM HEPES
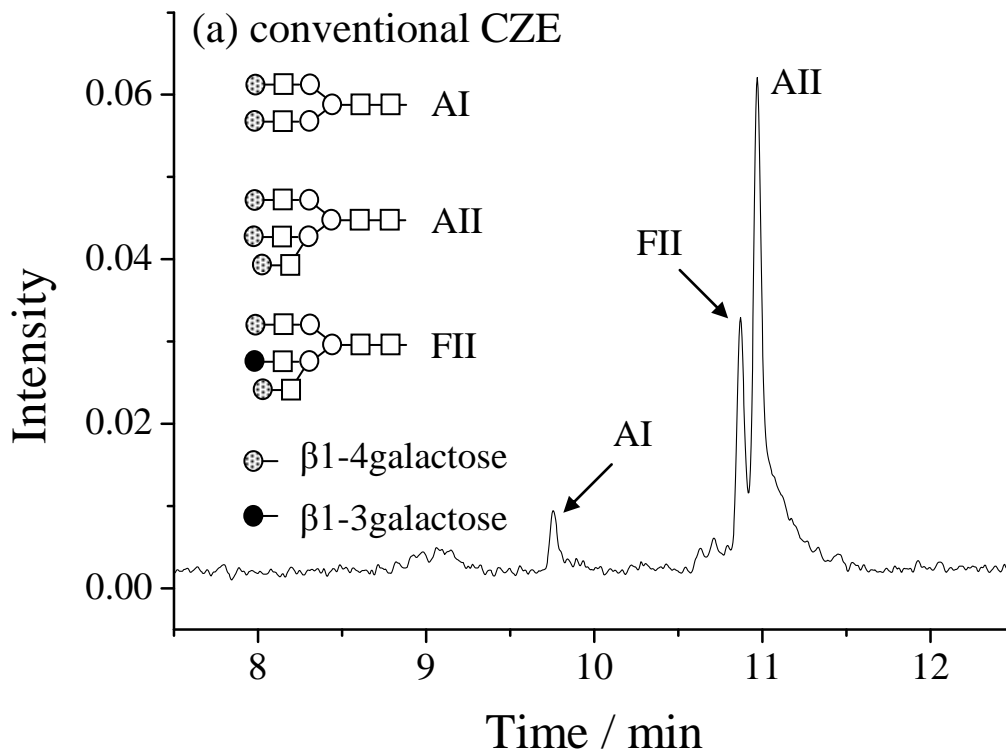buffer (pH 8.0).

Figure 2. Electropherograms of glucose oligomer obtained with (a) conventional CZE and (b) LVSEP–CZE. Sample concentration, (a) 16 ppb, (b) 32 ppt.

Figure 3. (a) Conventional CZE and (b) LVSEP–CZE analyses of oligosaccharides released from ribonuclease B. Sample concentration in LVSEP–CZE was 500-fold lower than that in normal CZE.

Figure 4. Electropherograms of fetuin glycans obtained in (a) conventional CZE and (b) LVSEP–CZE. Sample concentration in LVSEP–CZE was 100-fold lower than that in conventional CZE.

Figure 5. (a) Conventional CZE and (b) LVSEP–CZE analyses of glycans obtained from AGP. Sample concentration in LVSEP–CZE was 400-fold lower than that in conventional CZE.

Figure 6. Detection of minor glycans obtained from AGP by LVSEP-CZE. 20 peaks indicated with arrows could not be detected in conventional CZE.

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Table 1. SEFs of oligosaccharides obtained with the LVSEP-CZE analysis.

	M5	M9	AI	AV	FII
SEF	770	750	400	520	–

2

Table 2. Separation parameters in LVSEP-CZE and conventional CZE.

	$R\mathrm{s}^a$	d^a / min	$W_{1/2}{}^b$ /min
RNase B			
CZE	4.7	0.53	0.059
LVSEP-CZE	4.0	0.50 (94%)	0.069 (117%)
Fetuin			
CZE	–	1.11	0.058
LVSEP-CZE	–	1.06 (95%)	0.066 (114%)
AGP			
CZE	14.5	1.23	0.048
LVSEP-CZE	10.7	1.14 (93%)	0.060 (125%)

a. resolution or peak distance between the first two peaks, M5–M6, AI–FII, or AI‑AII.

b. width at half of the highest peak intensity for M5 or AI

3

Table 3. Repeatability in LVSEP-CZE and conventional CZE.

	%RSD of $t_M{}^a$	%RSD of peak height[a]	%RSD of peak area[a]
RNase B			
CZE	0.1	9.0	10.9
LVSEP-CZE	1.3	1.2	2.8
Fetuin			
CZE	0.1	19.6	4.1
LVSEP-CZE	0.1	1.7	4.9
AGP			
CZE	0.2	13.0	13.4
LVSEP-CZE	0.4	1.4	4.1

a. RSDs for the first peak, M5 or AI ($n = 3$).

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