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Journal of Chromatography A, 867 (2000) 271–279

JOURNAL OF CHROMATOGRAPHY A

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Microchip capillary electrophoresis using on-line chemiluminescence detection

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Received 27 July 1999; received in revised form 8 November 1999; accepted 8 November 1999

Abstract

Chemiluminescence detection was used in capillary electrophoresis integrated on a microchip. Quartz microchips have two main channels and four reservoirs. Dansyl-lysine and -glycine were separated and detected with bis[(2-(3,6,9-trioxadecanyloxycarbony)-4-nitrophenyl]oxalate as peroxyoxalate chemiluminescent reagent. These dansyl amino acids came into contact with the chemiluminescence reagent to produce visible light at the interface between the separation channel and chemiluminescence reagent-containing reservoir. The detection limit ($S/N=3$) for dansyl-lysine was $1\cdot10^{-5}$ *M*, which corresponded to the very small mass detection limit of ca. 0.4 fmol. However, the concentration sensitivity in the present system was approximately two orders of magnitude lower than that in the conventional capillary electrophoresis–chemiluminescence detection system. The relative standard deviations of migration time and peak height for dansyl-lysine were 4.2 and 4.5%, respectively. A channel conditioning before every run and an appropriate control of voltages were needed for the reproducible results. The present system had advantages in rapid separation time (within 40 s), small (several 10 pl) and accurate sample injection method using a cross-shaped injector, and simplification and miniaturization of the detection device.

Keywords: Microchip capillary electrophoresis; Capillary electrophoresis, microchip; Chemiluminescence detection; Detection, electrophoresis; Amino acids, Dns derivatives

1. Introduction

Recently, the micro total analysis system (μ-TAS) has received much attention. Since Harrison et al. demonstrated the feasibility of a chemical analysis system on a small glass substrate using electrokinetic phenomena [1,2], capillary electrophoresis (CE) integrated on microchip (microchip CE) has been much studied. Laser-induced fluorescence (LIF) detection has been the most commonly used in microchip CE [3–6]. However, LIF detection essentially necessitates a large laser light source and spectroscope. The requirement not only leads the instrumentation to expensiveness but also reduces benefits of miniaturization.

Chemiluminescence (CL) does not need any light source, so that CL could be one of the most attractive detection methods in μ-TAS. A number of

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)01169-3

researchers have explored its use for detection in CE [7–13]. We have also demonstrated that the self-constructed CE with CL detection system was applicable to the separation and determination of small amounts of metal ions, metal complexes, dyestuffs, proteins, alkaloids, and oligopeptides [14–17]. Several CL reagents, such as luminol [15], peroxyoxalate [14,17], and ruthenium(II) complex [16], were used for their detection. Mangru et al. reported CL detection using luminol CL reaction for microchip-based capillary electrophoresis [18]. Post-column derivatization manifolds to make fluorescent products were utilized as a tool for a mixing CL reagent and analytes in the study. Although such manifolds are easy to fabricate onto a glass substrate with essentially zero dead volume, a requirement of the manifolds for chemical reaction makes the construction of the devices intricate [2,19–21].

We will report here microchip CE–CL detection using the peroxyoxalate CL reaction. The preliminary results of this investigation have been briefly reported in a recent communication [22]. The present system features the simplest device construction in principle; only two main channels, four reservoirs, cross-shaped injector, and absence of the mixing junction for CL reaction. Bis[(2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO) reaction was used with H_2O_2, and dansyl amino acids were separated and detected in the microfabricated device. We describe the basic properties of the analytical system such as separation efficiency, detection sensitivity, and reproducibility, as well as some interesting findings.

2. Experimental

2.1. Chemicals

All chemicals were analytical grade and were used without further purification. Ion-exchanged water was distilled before use. TDPO was purchased from Wako. Dansyl (Dns-) amino acids were from Sigma. Other reagents were received from Nacalai Tesque. For preparing a 2 m*M* stock sample solution, dansyl amino acids were dissolved in 0.1 *M* Tris–H_3BO_3 buffer (pH 7.0) as migration electrolyte, and the stock solution was diluted with the buffer for use. The CL reagent was a mixture of 1 ml of 4 m*M* TDPO in acetonitrile and 21 μl of 30% (w/w) H_2O_2 aqueous solution. Sample and migration electrolyte solutions were filtered using 0.45 μm pore disposable filters (Kurabo, Osaka, Japan).

2.2. Microchips

Schematic layout of the microchips (a) (28.0×11.0 mm) and (b) (34.0×12.5 mm) are illustrated in Fig. 1. Microchips (a) were used unless otherwise noted. These microchips made of quartz were fabricated at the Technology Research Laboratory of Shimadzu (Kyoto, Japan). Each microchip consists of a bottom and cover plate. The bottom plate (1.0 mm thickness) has sample load and separation microchannels of 20 μm deep and 50 μm wide except for the large part of 460 μm wide in (b), while the cover plate (0.5 mm thickness) has ca. 1 mm diameter holes drilled into it, to facilitate access to the microchannels and to serve as reservoirs (R1–R4) in Fig. 1. After the surface of the bottom plate was mediated with 1% (w/v) HF, the cover plate was bonded to the bottom plate maintaining a high pressure of 0.16 MPa at room temperature for 24 h. The channel lengths from intersection to R4 (which corresponds to effective separation length) are (a) 21.0 mm and (b) 33.0 mm, respectively. Four silicone rubber [poly(dimethylsiloxane)] tubes (I.D. 2.0 mm, length ~5 mm; 125490, Tokyo Rikakikai, Tokyo, Japan) were attached to the cover plate to make reservoir volumes larger for the electrolyte solution.

2.3. Procedures

After the channels were filled with migration electrolyte using a disposable syringe, the sample was placed in R1, buffer in R2 and R3, and CL reagent in R4, respectively. Platinum wires as electrodes were inserted into these reservoirs. A program written in Labview (National Instruments, Austin, TX, USA) was used to control the power supplies (Model HCZE-30PNO. 25, Matsusada Precision Devices, Shiga, Japan) and relay device used.

The CE procedure was schematically shown with applied voltages and electric fields (E_1–E_4, V/cm) in Fig. 2. The applied voltages described below were values used typically in the present experiments.

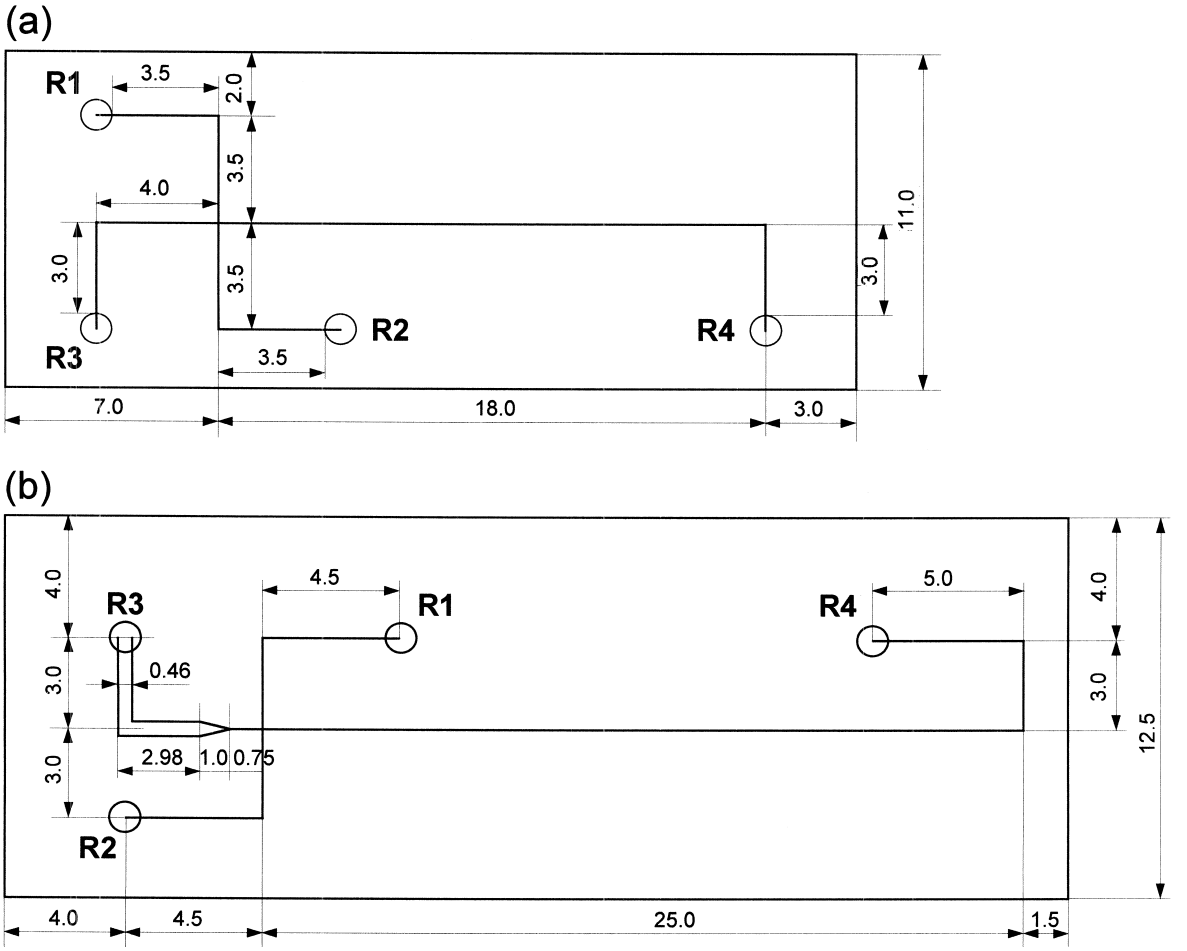

Fig. 1. Schematic layout and dimensions of microchips (a) and (b). Dimensions are given in millimeters. R1–R4 indicate reservoirs. Solutions distribution to the respective reservoirs: R1, sample; R2, buffer; R3, buffer; R4, CL reagent.

Sample plug formation was simply achieved by use of a cross-shaped injector as follows. The sample was electrokinetically delivered from R1 toward R2 by applying 600 V for 20 s to R1, with R2 held at ground. At this process, the analyte stream was pinched with 400 V applied to R3 and R4 to prevent the sample spreading out to the separation channel. It is considered that the CL reagent in R4 slightly leaks to the separation channel due to electroosmotic flow. After the intersection was fully filled with the sample, 500 V was applied to R3, with R4 grounded, allowing the sample to inject and migrate down the separation channel. Also, 360 V was applied to R1 and R2 during separation to hinder the sample in the sample load channel leaking to the separation channel. The CL reagent, which leaked to the separation channel during sample load, can easily return to R4 during separation (note the direction of E_4). This injection technique made it possible to introduce a definite and small sample aliquot (several 10 pl).

The sample, which migrated toward R4, came into contact with the CL reagent to produce visible light at the interface between the separation channel and R4. The light was detected with the photosensor module (Model H5783, Hamamatsu Photonics, Shizuoka, Japan) which was located under R4. The output from the module (operated at 900 mV) was fed to an amplifier (Model EN-21, Kimoto Electric, Osaka, Japan) connected to an integrator (C-R6A, Shimadzu) to produce electropherograms.

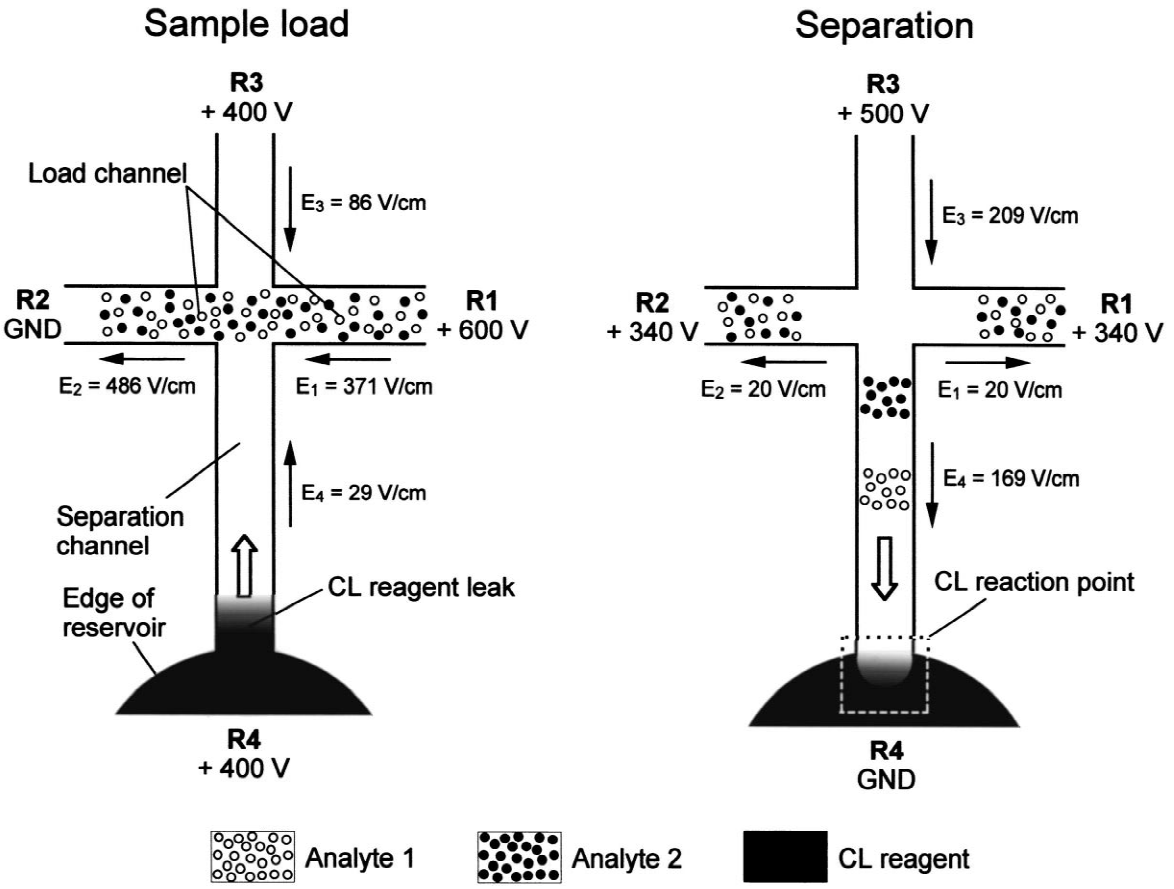

Fig. 2. Schematic layout of CE procedure, with the potentials and electric fields. Arrows depict direction of flow. Potentials and electric field indicated are the typical values.

3. Results and discussion

3.1. Separation of dansyl amino acids

The present injection is able to introduce a short-length sample plug compared with double-T injection [4,18,21,23], allowing separation with high efficiency. We performed here separations of dansyl amino acids. Electropherograms (A) and (B) were obtained by use of microchips (a) and (b), respectively, as shown in Fig. 3. Dns-Lys and -Gly were successfully separated and detected within 50 s. The very short run times could be obtained due to extremely short separation length on the microchips, in spite of applying the comparatively small electric field (170 V/cm) to the separation channel. Peak tailings in the electropherograms are probably attributed to the long lifetime of the present CL reaction and interaction of analytes with the channel wall.

Migration and separation data for the electropherograms (A) and (B) were represented in Table 1. Electrophoretic mobility (μ_{ep}) showed a good agreement between (A) and (B) since the solutions and channel conditions were the same. Longer separation channel of the microchip (b) increased the number of theoretical plates (N) to approximately twice that of the microchip (a). The separation efficiencies obtained here appear to be lower than those obtained in conventional CE. The lowering of the efficiencies is considered to be caused by short

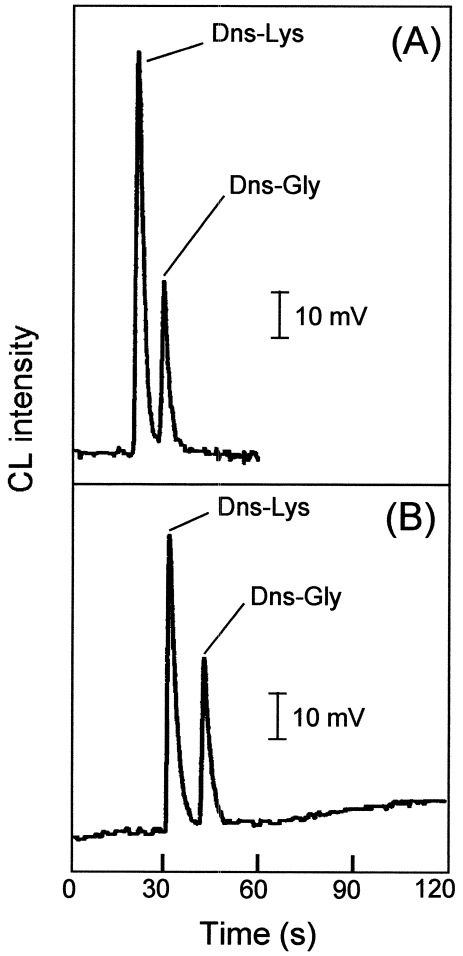

Fig. 3. Separation of Dns-Lys and -Gly using microchips (a) and (b). Microchips (a) and (b) were used for electropherograms (A) and (B), respectively. Concentrations of each dansyl amino acid were $4 \cdot 10^{-4}$ *M*. Applied voltages for (A) were the same as indicated in Fig. 2. Voltages for (B) were as follows: during sample load, 600 V to R1, 500 V to R3 and R4, with R2 grounded, while during run, 500 V to R1 and R2, 600 V to R3, with R4 grounded.

separation length of several cm, slow CL reaction, and large detection area.

We attempted the separation of Dns-Lys and -Gly, together with other dansyl amino acids such as Dns-Try, -Leu, -Val, -Ser, -Glu, -Phe, -Met, and -Ala. However, the separation failed because electrophoretic mobilities of these Dns-amino acids are comparatively close to that of Dns-Gly. The separation of these compounds was not achieved in the present work even if higher electric fields were applied to the separation channel.

Table 1
Retention and efficiency data for electropherograms in Fig. 3

	Dns-Lys	Dns-Gly
Fig. 3A		
t_m (s)[a]	20.4	29.0
μ_{ep} ($cm^2/V/s$)[b]	6.11×10^{-4}	4.30×10^{-4}
N[c]	300	650
R_s[d]	1.92	
Fig. 3B		
t_m (s)[a]	31.2	42.4
μ_{ep} ($cm^2/V/s$)[b]	6.11×10^{-4}	4.53×10^{-4}
N[c]	590	1300
R_s[d]	2.32	

[a] t_m, migration time.
[b] μ_{ep}, electrophoretic mobility.
[c] N, number of theoretical plates.
[d] R_s, resolution.

3.2. Repetitive measurements

Under the same voltage configurations as in Fig. 3, we carried out repeated runs of mixture samples of Dns-Lys and -Gly without exchange of the solutions in each reservoir between runs. Table 2 represents results for migration time, peak height, area, theoretical plates, and resolution concerning seven repeated runs. Migration times increased with increasing run number. The CL reagent in R4 leaked to the separation channel during sample load by applying the voltages described in the Experimental section, and the leaked CL reagent readily returned to R4 again during separation. The repetition of leak and return of the CL reagent could vary the electrophoretic condition, so that migration times gradually became larger.

Peak heights showed constant values within approximately four runs, and then they were decreasing by degrees. Peak areas were increasing with increasing run number. Naturally peaks became broad, meaning a decrease of theoretical plates. The followings seem to bring about these changes. During the separation, the composition of the solution in R4 should keep on changing with the introduction of the

Table 2
Variation of migration times, peak areas, peak heights, theoretical plates, and resolutions for repetitive runs

Run number	Migration times (s)		Peak area (mV·s)		Peak height (mV)		Theoretical plates		R_s
	Dns-Lys	Dns-Gly	Dns-Lys	Dns-Gly	Dns-Lys	Dns-Gly	Dns-Lys	Dns-Gly	
1	20.5	29.1	414.9	171.6	120.1	51.4	220	480	2.69
2	21.2	30.1	472.1	184.5	120.1	50.5	180	430	2.51
3	21.4	30.5	517.4	221.2	121.2	52.5	160	330	2.27
4	21.9	31.1	530.7	234.4	117.2	51.5	150	290	2.15
5	21.9	31.6	534.9	236.8	111.5	49.3	130	270	2.15
6	22.3	32.0	532.1	265.1	102.3	47.3	120	200	1.90
7	22.4	32.3	512.1	267.8	95.9	45.6	110	190	1.88

buffer to R4 through the separation channel. The buffer constituents introduced to the CL reagent slows the present CL reaction and decreases the CL intensity.

3.3. Channel conditioning

As shown in Table 1, repetitive runs brought about gradual changes in respective variables. We found out that respective variables readily returned to the initial values by rinsing of the channel with the buffer using a disposable syringe for 1–2 min and the re-distribution of a fresh solution to each reservoir. It was better for reproducible analysis to perform the rinse and the re-distribution at every run.

After several tens of runs, however, migration times began to increase and never went back to the initial times even if the channels were rinsed with the buffer at each run. The delay of migration times was due to the adsorption of the analytes onto the channel wall. The electropherogram is shown in Fig. 4. It was interesting that the channel submitted the adsorption increased the resolution (R_s=4.0) between Dns-Lys and -Gly (compare with Fig. 3A). The finding suggests that some modifications on the wall give a possibility to improve the separation efficiency. However, the migration times would not stop increasing and reproducible results could not be obtained.

Then, channel re-conditioning was examined. The channel was flushed with water for 10 min, 1 *M* HCl for 20 min, 1 *M* NaOH for 20 min, and again with water for 10 min. The electropherogram obtained after the procedure showed good agreement with that of the beginning. Therefore, we performed the HCl to NaOH treatment every ten runs as well as the rinsing of the channel every run in order to get reproducible results.

3.4. Calibration curves and reproducibility of determination

Calibration curves of Dns-Gly and -Lys are shown in Fig. 5. Linear correlation could be obtained between concentration and peak height for both the compounds. The above-mentioned channel condi-

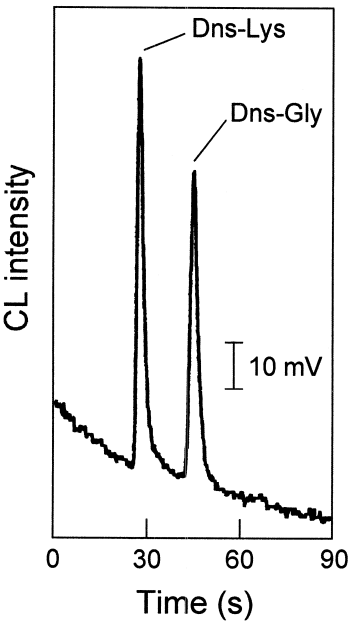

Fig. 4. Electropherograms of Dns-Lys and -Gly using the microchip of analytes-adsorbed onto the channel surface. The experiments were performed using microchip (a) under the same conditions as described in Fig. 3.

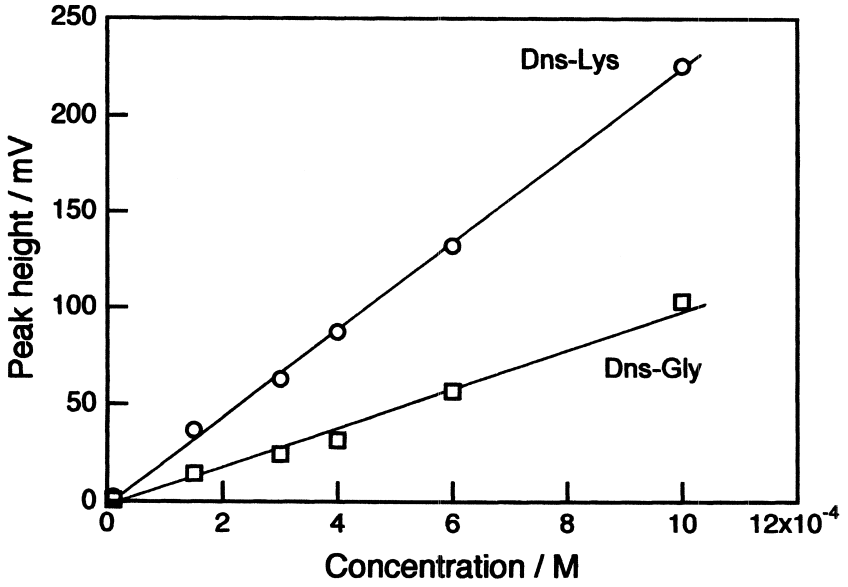

Fig. 5. Calibration curves of Dns-Lys and -Gly. The experiments were performed using microchip (a) under the same conditions as described in Fig. 3.

tions led to a good reproducibility. The relative standard deviations in migration time and peak height for Dns-Lys were 4.2 and 4.5% ($n=6$), respectively. The mass detection limit ($S/N=3$) for Dns-Lys showed a very small value of ca. 0.4 fmol. However, the concentration detection limit for it in the present work was $1\cdot10^{-5}$ *M*, which was ca. two orders of magnitude higher than those obtained in the conventional CE with CL detection system. Several nanolitres of sample volume was commonly injected in conventional CE while several 10 pl of sample volume was injected in microchip CE. CL intensities are significantly dependent on reagent volumes. The extremely small injection volumes in microchip CE are considered to be the reason for less concentration sensitivity.

3.5. Effect of sample volume on detection sensitivity

Low voltages were applied to R3 and R4 during sample load, which can provide larger sample volume, in order to examine the effect of sample volume on detection sensitivity. Separations of Dns-Lys and -Gly were performed under the conditions of applying voltages of (a) 400, (b) 200, and (c) 180 V to R3 and R4, which corresponded to the electric fields of 86, −86, and −103 V/cm for E_3 and 29, −29, and −34 V/cm for E_4, respectively. The directions of E_3 and E_4 in voltages of (b) and (c) were from the intersection to each reservoir, while that in the condition of (a) was the opposite. In other words, in (b) and (c), the analytes migrated to R3 and R4 as well as to R2 during sample load. Although we do not know the exact sample volumes injected, the sample volumes in the voltages of (a), (b), and (c) increased in this order. Fig. 6 shows the electropherograms (A), (B), and (C) obtained under the voltage configurations (a), (b), and (c). The smaller the applied voltages to R3 and R4, or the larger the sample volume, the larger the signals could be observed. The signal-to-noise ratio of Dns-Lys at 180 V increased ca. 40-fold compared with that at 400 V. However, reproducibility significantly decreased under the conditions of (b) and (c), which means that controlling the volume of sample spreading out to the separation channel reproducibly is difficult in the present system. It is necessary for

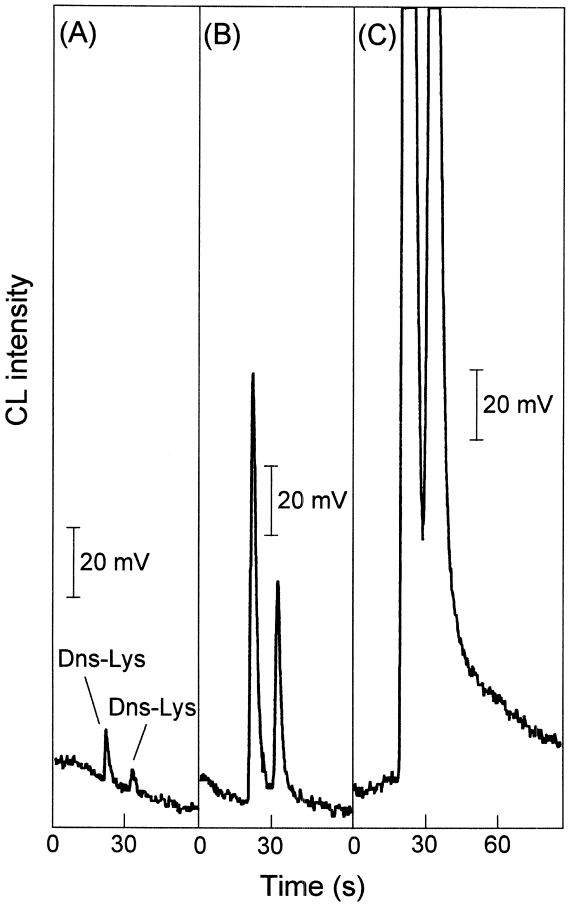

Fig. 6. Effect of applied voltage to R3 and R4 during sample load on extents of CL signals. Applied voltages to R3 and R4 during sample load were (a) 400 V, (b) 200 V, and (c) 180 V, respectively. The concentrations of each dansyl amino acid was $2.5 \cdot 10^{-4}$ *M*. Other conditions were the same as in Fig. 3.

reproducible determination to pinch the sample stream from the opposite sides of the sample load channel.

4. Conclusion

Microchip CE–CL detection was constructed by use of peroxyoxalate CL reaction. The separation and detection system was simple and miniature since CL detection needs neither light source nor complex spectroscopes. The size of the detection device matches with that of microfabricated separation and reaction devices. As compared with an ordinary CE system, the present microchip CE–CL detection system has advantages as follows: rapid separation time; small sample volume necessary for analysis; accurate injection volume; and simplification of detection device. However, the small sample volume compromised the concentration sensitivity. More studies are needed in order to improve the concentration sensitivity of the current system by means of some useful technique such as on-line sample stacking.

Acknowledgements

This work was supported by a grant to RCAST at Doshisha University from the Ministry of Education, Japan. This was also supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, as well as the Aid of Doshisha University's Research Promotion Fund.

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