# Flow-Type Chemiluminescence Detection Cell Using an Optical Fiber for Capillary Electrophoresis

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A flow-type chemiluminescence detection cell using optical fiber was designed for capillary electrophoresis. The capillary was set up straight to the optical fiber with a certain distance in a Teflon® tube. Hydrogen peroxide was delivered through a three-way joint by a syringe pump and mixed with analytes at the capillary outlet. Luminol chemiluminescence was adapted for use with this detection cell. Analytical conditions, such as hydrogen peroxide flow rate, distance between the capillary and the fiber, and the reagent concentrations, were examined in detail with the cell. The present flow-type system provided a CL signal with high sensitivity, resolution, and reproducibility. The detection limit (S/N = 3) for luminol was $7.6\times10^{-9}$ M (28 amol; 3.7 nl injected) with theoretical plate numbers of 130000—160000 and a relative standard deviation of 3.3% for the peak height ($n$ = 10). A mixture sample of glycine, glycylglycine, and glycylglycylglycine, which were labeled with isoluminol isothiocyanate (2,3-dihydro-6-isothiocyanato-1,4-phthalazinedione), was also subjected to the present system. They were sensitively detected and represented with high resolution.

Chemiluminescence (CL) has been utilized as a sensitive detection scheme. Although CL reactions lack selectivity, the CL detection system combined with separation methods can offer excellent analytical selectivity and sensitivity.[1] The CL has already been verified to be a highly sensitive detection method in both flow-injection analysis (FIA) and high-performance liquid chromatography (HPLC).[2—4] Recently, the applicability of CL detection in capillary electrophoresis (CE) has been successfully demonstrated. Several CL reagents, such as luminol,[5—10] acridinium,[11] peroxyoxalate,[12—15] and Ru(II) complex,[16] have been utilized.

Compared with other detection modes widely incorporated in CE, CL detection is obviously an evolving technique. In the past few years, advances have focused on the development of new detectors that show more excellent performance than the existing systems.[1] Zhao et al.[6] proposed to use a sheath flow cuvette as a postcolumn reactor in CE. Daddo et al.[10] developed a CL detector interface that is implemented with CE by using an optical fiber. Gilman et al.[17] also utilized an optical fiber to easily transport a CL signal in CE. We reported on a batch-type cell using an optical fiber for CL detection in CE.[18]

In this study, a novel flow-type CL detection cell using an optical fiber was designed for CE. The capillary was set up straight to the optical fiber in a Teflon® tube. Hydrogen peroxide ($H_2O_2$) was constantly delivered by a pump to the CL reaction area and well mixed with CL reagents in front of the optical fiber. The CL light generated there was transported by the fiber to a photomultiplier tube (PMT). Luminol as an analyte gave a small detection limit. A mixture sample labeled with isoluminol isothiocyanate (ILITC) was also successfully separated and detected. The present flow-type system provided prominent performance in sensitivity, resolution, and reproducibility. The system is expected to become one of the most practical CE–CL detections.

## Experimental

**Reagents.** All reagents used were of commercially available and analytical grade. Ion-exchanged water was distilled for use. Microperoxidase (MP-11) was purchased from Nacalai Tesque. Luminol, glycine, and glycylglycylglycine were purchased from Sigma Co. Glycylglycine was purchased from Peptide Institute, Inc. ILITC was received from Tokyo Chemical Industry Co.

**Labeling Procedure.** Labeling using ILITC was carried out according to procedures described in earlier reports.[6,19] A definite amount of ILITC and amino acid or oligopeptide (micromolar order) was added in a microvessel and dissolved with a 100 μl of a mixture solution of water–triethylamine (95 : 5, v/v). The solution was subjected to ultrasonication for 1 min and then left in the dark for 20 min, while being vortex-mixed. The residue that was obtained by evaporation from the solution was redissolved in 10 mM phosphate buffer (pH 10.8, 1 M = 1 mol $dm^{-3}$) to give an ILITC-labeled sample.

**Chemiluminescence Detection Cell.** A schematic diagram of the CL detection cell is shown in Fig. 1. The capillary (50 cm length and 50 μm i.d.; GL Sciences Inc.) was positioned straight to the optical fiber (1 mm core diameter; PFU-FB1000, Toray Industries, Inc.) with 0.5 mm distance in 1.19 mm i.d. of the Teflon® tube. $H_2O_2$ was fed through a three-way joint by a syringe pump. A grounding electrode was also inserted into the end of the flow line by use of another three-way joint. When analytes emerged from the capillary, they reacted with $H_2O_2$ at the capillary outlet to produce

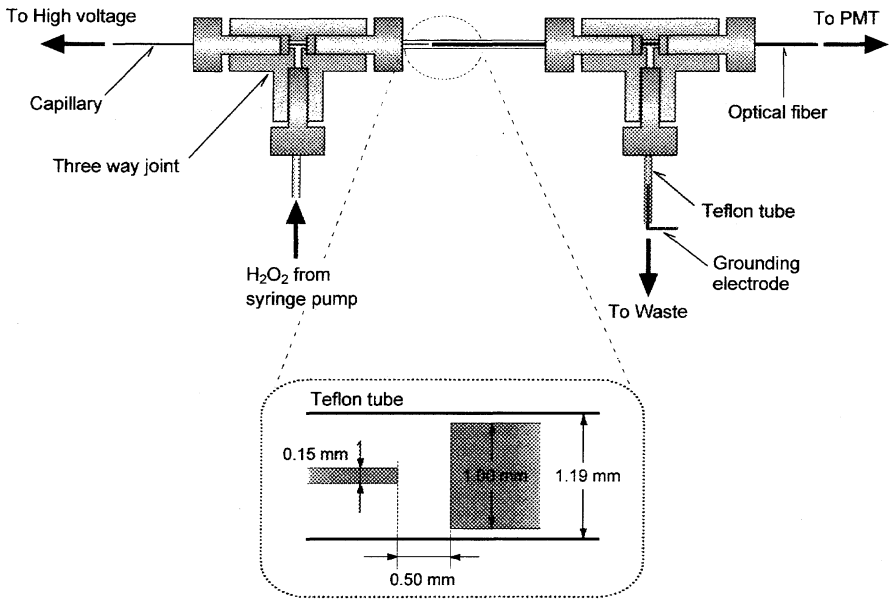


Fig. 1. Schematic diagram of the CL detection cell.

visible light. The CL light was captured by an optical fiber. The CL detection cell and PMT (Model R464, Hamamatsu) were enclosed in a dark box for safety.

**Capillary Electrophoresis.** A new capillary tube was treated with 1.0 M sodium hydroxide for 10 min and then washed with distilled water. A high voltage (12 kV) was applied to electrodes using a DC power supplier (Model HCZE-30PNO. 25, Matsusada Precision Devices Co., Ltd.). Luminol and $H_2O_2$ CL system was used together with microperoxidase as a catalyst. Luminol, ILITC, $H_2O_2$, and the catalyst were dissolved in a 10 mM phosphate buffer solution (pH 10.8), which was used as a migration buffer (an electrolyte solution). Stock solutions of the analytes (luminol and ILITC-labeled compounds) were also prepared in the phosphate buffer and diluted as needed. $H_2O_2$ was fed by a syringe pump at a flow rate of 8.33 $\mu l\,min^{-1}$. Sample injection was performed by an electrokinetic method for 4—8 s at 4 kV. A sample was migrated in the electrolyte solution toward the CL detection cell and then mixed with $H_2O_2$. The resulting CL light at the capillary outlet was transported by optical fiber to a PMT. The output from the PMT was fed to a photon counter (Model C1230, Hamamatsu) connected to an integrator (Chromatopac C-R6A, Shimadzu) to produce electropherograms.

## Results and Discussion

**Design of Flow-Type Chemiluminescence Detection Cell.** In the previous work,[18] we designed two batch-type CL detection cells using optical fibers for CE. One of them consisted of two optical fibers that were positioned at right angles to the inserted capillary. Another type consisted of one optical fiber that was set up straight to the capillary. The latter led to a higher sensitivity than the former. In the latter system, luminol emerged from the capillary and at once collided with the face of the optical fiber. The collision brought about an effective mixing to generate a large amount of CL light.

In this study, we proposed a flow-type CL detection cell using an optical fiber. The capillary was set up straight to the fiber, as shown in Fig. 1. The results in previous work[18] recommended their positioning. $H_2O_2$, which was fed by a pump to the CL reaction area, was mixed with analytes at the capillary outlet. The mixture immediately collided with the optical fiber to lead to good mixing under certain conditions. The constant and continuous supply of $H_2O_2$ by the pump and the effective mixing in front of the fiber must produce a CL signal with satisfactory sensitivity, resolution, and reproducibility.

The compact cell must be pressurized due to the flow of $H_2O_2$. The pressure difference between the detection and injection end induces flow in the capillary. This phenomenon was reported as an effect of sheath flow in an earlier report.[20] To investigate the effect of a pressurized flow cell on the migration of analyte in the capillary, the migration velocity of luminol was measured as a function of the applied potential (Fig. 2). $H_2O_2$ was fed to the cell at a relatively large flow rate (16.6 $\mu l\,min^{-1}$). A linear relationship ($r = 0.991$) having a slope of 0.119 $mm\,s^{-1}\,V^{-1}$ and an intercept of $-0.222$ $mm\,s^{-1}$ was obtained. The line in Fig. 2 was drawn by means of a least-squares fits of the plots. The pressure-induced flow velocity is given by this intercept. The negative sign arises because the flow is directed from the detection end to the injection end of the capillary. A parabolic flow profile of the pressure-induced flow seems to degrade the separation efficiency of CE. Therefore, most analytical data were taken at a $H_2O_2$ flow rate of less than 10 $\mu l\,min^{-1}$, which seems unable to produce a significant pressure rise in the system.

**Analytical Conditions for Chemiluminescence Detection.** It is difficult to degas $H_2O_2$ solutions, and bubble formation in the separation capillary is problematic. In addition, the presence of $H_2O_2$ with a relatively high concentration would perturb separations. We proposed the following usage of the reagents: $H_2O_2$ was fed by the pump to CL detection area and catalyst was added into the inlet reservoir. It was reported[6] that a relatively low concentration (micromolar

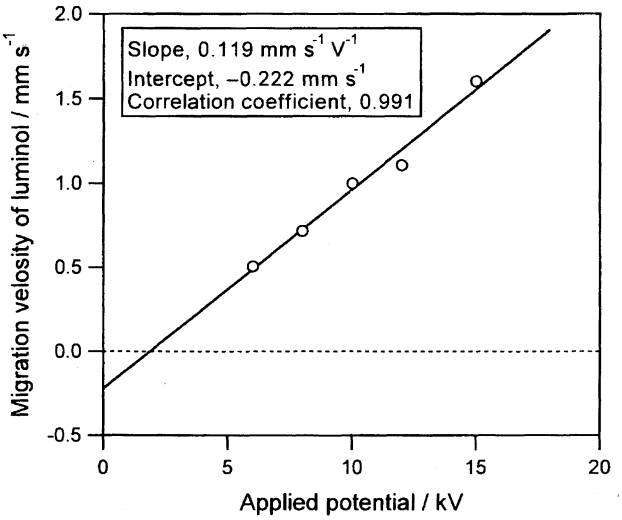


Fig. 2. Relationship between migration velocity of luminol and applied potential in CE. The capillary dimension was 50 cm length×50 μm i.d. 10 mM sodium phosphate solution (pH 10.8) was used as electrolyte solution. In addition, 4 μM microperoxidase was added to the inlet reservoir. 400 mM $H_2O_2$ was delivered at 16.6 μl min$^{-1}$. The distance between capillary and optical fiber was 0.50 mm. The voltage was 12 kV. Luminol of $3.0\times10^{-8}$ M was injected by electrokinetic method for 5 s at 4 kV.

order) of enzymes, such as microperoxidase in the capillary, did not influence the separation in CE. Here, the micromolar order of microperoxidase was used as a catalyst in the inlet, and migrated with the electrolyte solution in the capillary.

The effect of the $H_2O_2$ flow rate on the CL intensity of luminol was examined under the conditions of 0.5 mm distance between the ends of the capillary and fiber, 400 mM $H_2O_2$, and 4 μM microperoxidase (Fig. 3). The CL intensity clearly depended on the flow rate; a flow rate of 8.33 μl min$^{-1}$ gave the maximum CL intensity. At a flow rate of zero, that is, in the batch-type mode, the CL intensity was small. Namely, the presence of $H_2O_2$ flow at a certain flow

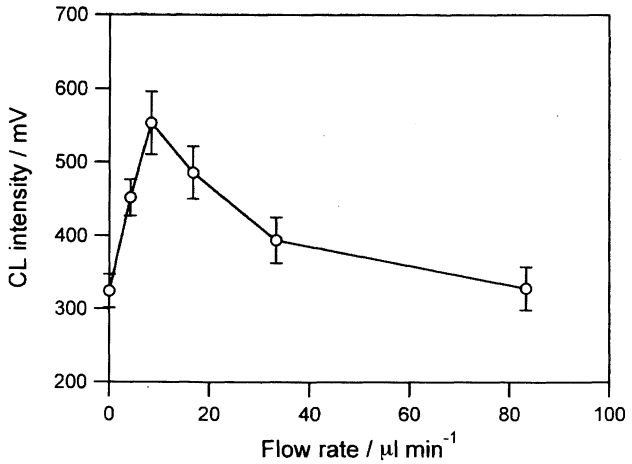


Fig. 3. Relationship between CL intensity and flow rate of $H_2O_2$. The experiments were carried out under the same conditions as those described in Fig. 2. Error bars represent one standard deviation.

rate surely increases the CL intensity. Around the optimal flow rate for the CL intensity, the effective mixing in front of the fiber must have occurred. At a higher flow rate, a large change in the reagent composition would disturb the generation of CL.

The CL intensity of luminol was examined against the distance between the capillary and the optical fiber under the conditions of a 8.33 μl min$^{-1}$ $H_2O_2$ flow rate, 400 mM $H_2O_2$, and 4 μM microperoxidase. The CL intensity also depended on the distance (Fig. 4); 0.5 mm distance gave the maximum CL intensity. The analytical conditions, such as the flow rate of $H_2O_2$, the distance between the ends of the capillary and the fiber, and the reagent concentrations are mutually influenced in the present flow-type system. The above flow rate and distance, which gave the maximum CL intensities, were adopted in the following experiments, although they may not really be the best conditions.

The reagent concentration is also a very important factor for CL detection in CE, as well as FIA and HPLC. The relationship between the microperoxidase concentration in the inlet reservoir and the CL intensity of luminol was examined under the conditions of 100 mM $H_2O_2$ concentration ((a) in Fig. 5). The maximum CL intensity appeared at around 1—2 μM microperoxidase concentration. The CL intensity decreased at a high concentration of microperoxidase. Microperoxidase at a high concentration must interact or adhere to luminol in a capillary to a large extent. The microperoxidase around luminol interfered with the contact between the luminol and oxidizing agent, so that the CL intensity might decrease at high concentration of microperoxidase.

Also, the relationship between the $H_2O_2$ concentration in the flow and CL intensity of luminol was examined at 2 μM microperoxidase in the inlet ((b) in Fig. 5). The maximum CL intensity was observed at over 100—200 mM $H_2O_2$. In previous batch-type work[18] at a high concentration of $H_2O_2$

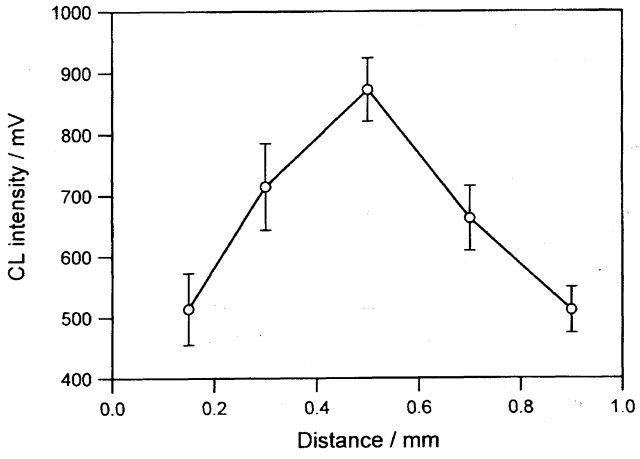


Fig. 4. Relationship between CL intensity and distance between the ends of capillary and optical fiber. The experiments were carried out under the same conditions as those described in Fig. 2 except for the following; the concentration of luminol was $5.0\times10^{-8}$ M. $H_2O_2$ was delivered at 8.33 μl min$^{-1}$. Error bars represent one standard deviation.

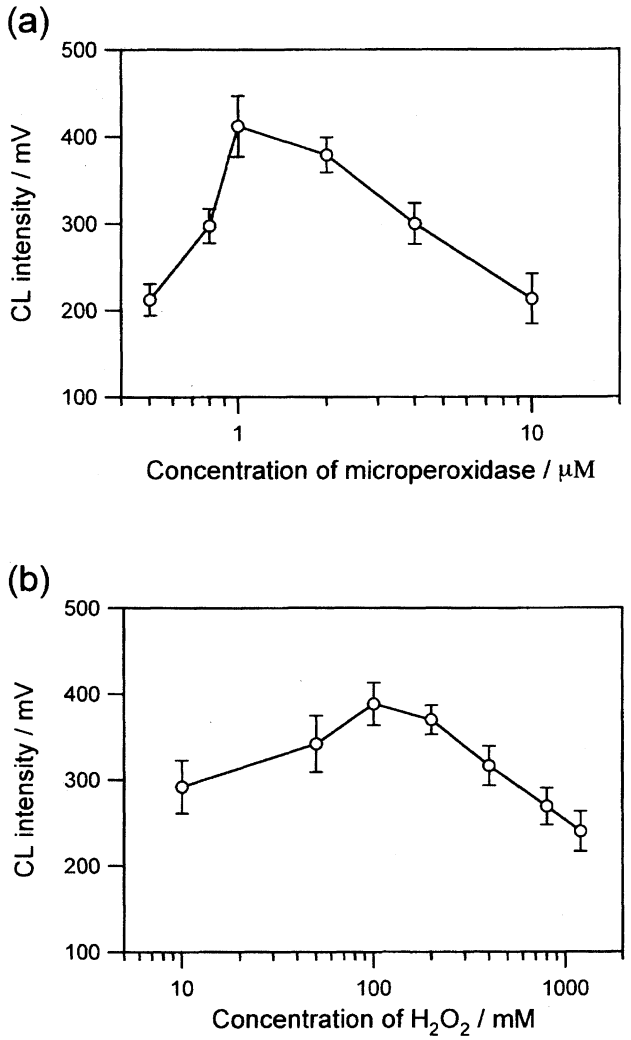


Fig. 5. Effect of (a) microperoxidase and (b) $H_2O_2$ concentration on CL intensity of luminol. The experiments were carried out under the same conditions as those described in Fig. 2 except for the following; the concentration of luminol was $2.0\times10^{-8}$ M. 2 μM microperoxidase was added to the inlet reservoir. 100 mM $H_2O_2$ was delivered at 8.33 μl $min^{-1}$. Error bars represent one standard deviations.

some bubbles appeared on the inner wall of the detection cell; the bubble formation brought about an increase of the deviation. However, in the present flow-type system, no bubble was observed at all in the detection cell, probably due to the flow of $H_2O_2$.

**Calibration Curve and Electropherogram of Luminol.** The calibration curve of luminol was examined under the recommended conditions described above; an 8.33 μl $min^{-1}$ flow rate of 200 mM $H_2O_2$, 0.5 mm distance between the capillary and the fiber, and 2 μM microperoxidase. The curve featured the determinable range, $7.6\times10^{-9}$—$9.0\times10^{-6}$ M ($r$ = 0.999, the linear relationship was observed in the range of more than 3 orders of magnitude); the detection limit (S/N = 3), $7.6\times10^{-9}$ M (28 amol, 3.7 nl injected); the relative standard deviation for peak height, 3.3% ($n$ = 10); and the theoretical plate numbers, 130000—160000. The electropherogram is also shown in Fig. 6. Daddo et al.[10] reported excellent design for CE–CL detector and derived the following data; the detection limit (S/N = 3) of luminol, $2.0\times10^{-8}$ M (500 amol, 25 nl injected) and the theoretical plate numbers, 10000—20000. We also reported in a previous paper that the detection limit (S/N = 3) of luminol, $1.7\times10^{-9}$ M (10 amol, 5.9 nl injected) and the theoretical plate numbers, 70000—80000.[18] It is difficult to exactly compare and discuss them because the sample injection volumes were different. However, the present system must represent one of the most performable results in CE–CL detection. At least, we consider that the obtained theoretical plate numbers are undoubtedly the best among any other systems reported so far.[5,6,10,17] In the present system, $H_2O_2$ was fed continuously and constantly by a pump and mixed with the eluate at the capillary outlet. The capillary faced straight to the optical fiber with a small space, so that the mixture instantly collided with the face of the fiber. The device structure must lead to a relatively small reaction/detection zone, effective mixing due to collisions with the fiber face, and the suppression of broadening of the analyte flow profile by the sheath flow of $H_2O_2$, bringing about extremely high resolution under certain conditions.

**Application for Isoluminol Isothiocyanate-Labeled Compounds.** Many luminol derivatives have been widely researched as labeling reagents.[6,19,21—23] ILITC[6,19] is a commercially available reagent for the labeling of amino groups. In this study, the ILITC was used for labeling of glycine, glycylglycine, and glycylglycylglycine. The optimum concentrations of microperoxidase and $H_2O_2$ for ILITC-labeled glycylglycine were investigated as follows: The relationship between the microperoxidase concentration in the inlet

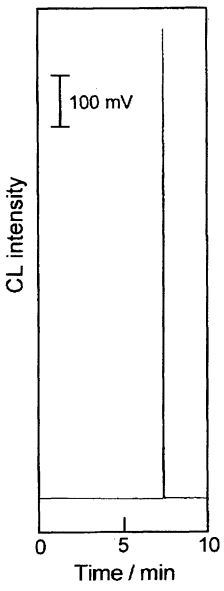


Fig. 6. Electropherogram of luminol. The experiments were carried out under the same conditions as those described in Fig. 5 except for the following; the concentration of luminol was $8.0\times10^{-8}$ M. Injection was performed by electrokinetic method for 8 s at 4 kV.

reservoir and the CL intensity was examined at constant $H_2O_2$ concentration (5 mM) in the flow ((a) in Fig. 7). The maximum CL intensity of ILITC-labeled glycylglycine was observed at around 20 μM microperoxidase concentration. The relationship between the $H_2O_2$ concentration and the CL intensity was also examined at the recommended microperoxidase concentration (20 μM) in the inlet reservoir ((b) in Fig. 7). The maximum CL intensity appeared at about 2—10 mM $H_2O_2$. As described above, the optimum concentrations of microperoxidase and $H_2O_2$ for ILITC-labeled glycylglycine indicated comparatively different regions from those for luminol. The labeling procedure would alter the CL quantum yield and rate kinetics.[6]

A mixed sample of glycine, glycylglycine, and glycylglycylglycine, which were labeled with ILITC, was subjected to the present CE–CL detection. The obtained electropherogram and calibration curves are shown in Figs. 8 and 9, respectively. They were sensitively determined and indicated complete base-line separation. The small peak at about 6 min would be due to a hydrolyzed ILITC. The reactivity of ILITC had been reported to be about 50% for every sample, judging from the calibration curves for ILITC.[18]

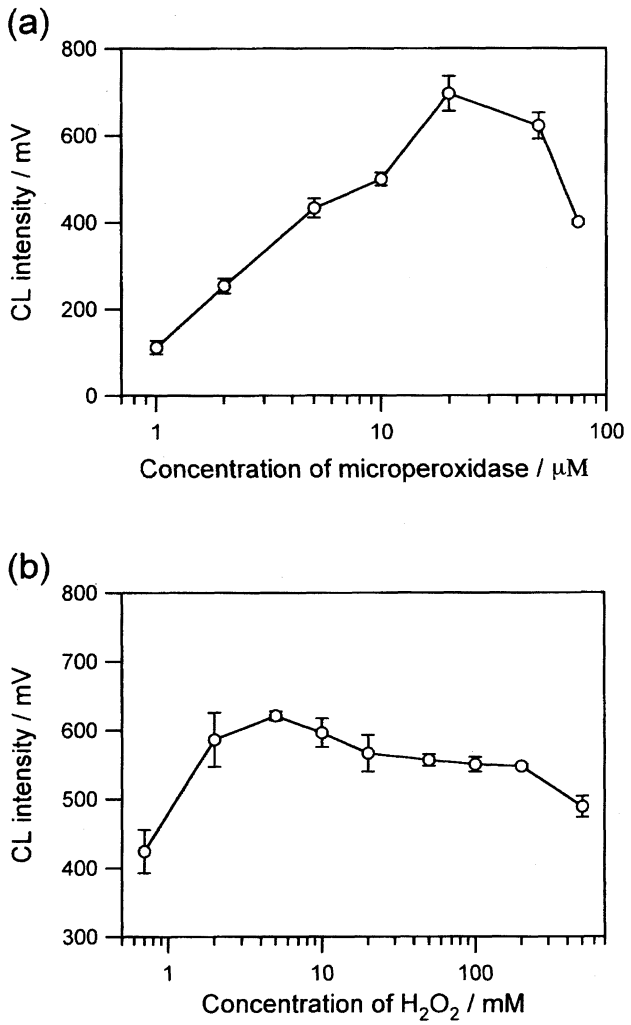


Fig. 7. Effect of (a) microperoxidase and (b) $H_2O_2$ concentrations on CL intensity of ILITC-labeled glycylglycine. The experiments were carried out under the same conditions as those described in Fig. 2 except for the following; $H_2O_2$ was delivered at 8.33 μl $min^{-1}$. The concentration of ILITC-labeled glycylglycine was $1.0\times10^{-5}$ M. Injection was performed by electrokinetic method for 4 s at 4 kV.

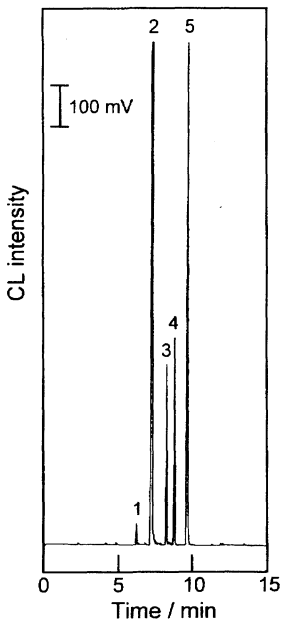


Fig. 8. Separation of ILITC-labeled compounds. The experiments were carried out under the same conditions as those described in Fig. 2 except for the following; the concentration of microperoxidase and $H_2O_2$ were respectively 20 μM and 5 mM. $H_2O_2$ was delivered at the flow rate of 8.33 μl $min^{-1}$. Injection was performed by electrokinetic method for 4 s at 4 kV. Peak identification: 1 hydrolyzed ILITC, 2 ILITC, 3 glycylglycylglycine, 4 glycylglycine, and 5 glycine.

The detection limit, the linear detection range, the correlation coefficient, and the theoretical plate numbers for the ILITC-labeled compounds are summarized in Table 1. For example, the ILITC-labeled glycine was determined over the linear detection range of $7.3\times10^{-7}$—$1.5\times10^{-5}$ M ($r$ = 0.995) with a detection limit (S/N = 3) of 1.6 fmol (2.2 nl injected). Still, a good separation efficiency was expressed by the theoretical plate numbers of 120000—150000. Table 2 gives a comparison of the peak resolutions. The present flow-type system indicated larger resolutions than the previous batch-type one. The high theoretical plate numbers must lead to a large peak resolution in the present system.

In conclusion, a flow-type CL detection cell was newly designed for CE. The cell could be combined with CE and operated for measurements. The luminol was eluted within 10 min with a detection limit (S/N = 3) of $7.6\times10^{-9}$ M (28 amol; 3.7 nl injected). The present system also featured higher theoretical plate numbers than any other CE–CL detectors reported so far. A mixture sample labeled with ILITC was separated with complete base-line separation and sensitively detected. The present system might be promising for numerous applications in the fields of environmental analysis and medicine.

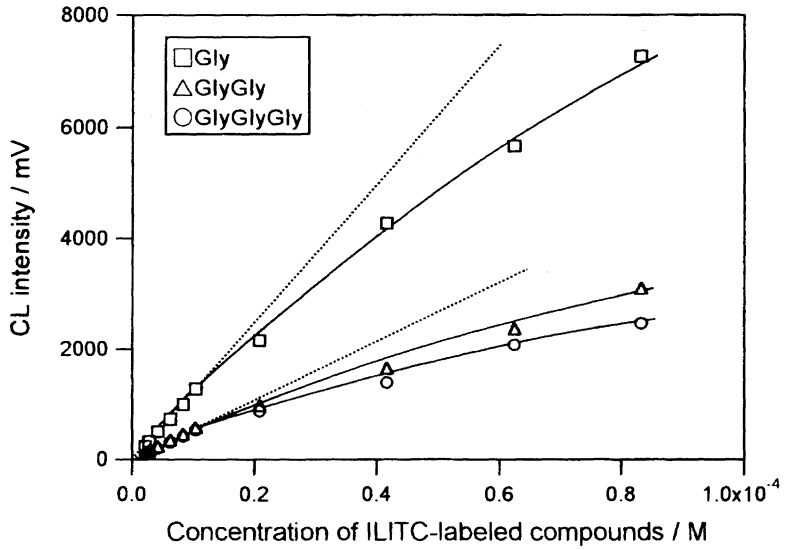


Fig. 9. Calibration curves of ILITC-labeled compounds. The experiments were carried out under the same conditions as those described in Fig. 8.

Table 1. Detection Limits, Linear Detection Ranges, Correlation Coefficients, and Theoretical Plate Numbers of ILITC-Labeled Compounds

| ILITC-labeled compound | Detection limit (S/N = 3) | | Linear detection range | Correlation coefficient | Theoretical plate number |
|---|---|---|---|---|---|
| | Concentration | Mass | | | |
| | M | fmol | M | | |
| GlyGlyGly | $1.5 \times 10^{-6}$ | 3.7 | $1.5 \times 10^{-6}$—$1.1 \times 10^{-5}$ | 0.994 | 80000—100000 |
| GlyGly | $1.5 \times 10^{-6}$ | 3.5 | $1.5 \times 10^{-6}$—$1.2 \times 10^{-5}$ | 0.995 | 110000—130000 |
| Gly | $7.3 \times 10^{-7}$ | 1.6 | $7.3 \times 10^{-7}$—$1.5 \times 10^{-5}$ | 0.995 | 120000—150000 |

Table 2. Comparison of Peak Resolutions

| Sample | Resolution[a)] | |
|---|---|---|
| | GlyGlyGly–GlyGly | GlyGly–Gly |
| Previous work[b)] (Batch-type) | 3.83 | 6.60 |
| Present work (Flow-type) | 5.05 | 7.95 |

a) Calculated from following equation: $Rs = 2(T_2 - T_1)/\{1.7\,(W_1 + W_2)\}$; $T_n$, migration time and $W_n$, half-width. Half-width was calculated from peak area and height assuming peak exhibited gaussian distribution. b) Refer to Ref. 18.

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