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Capillary electrophoretic system incorporating an UV/CL dual detector

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Abstract

We developed a capillary electrophoretic system incorporating an ultra-violet absorption (UV)/chemiluminescence (CL) dual detector, taking advantage of the CL reaction of luminol-hydrogen peroxide and the batch-type CL detection cell. UV detection was carried out using the on-capillary method while CL detection was performed using the end-capillary method. Examination of isoluminol isothiocyanate (ILITC) as a model sample revealed two main peaks with UV detection and one main peak with CL detection. The first peak in the UV detection data corresponded to the main peak in the CL detection data. We then determined that the ILITC sample included natural ILITC as well as an impurity that had absorption behavior but did not have CL properties and labeling ability. Furthermore, the components of a mixture containing glycine, glycylglycine and glycylglycylglycine, all labeled with ILITC, were well separated and detected using the present system. The present system easily, rapidly, and simultaneously produces useful information due to the presence of both UV and CL detectors.

Keywords: Capillary electrophoresis; Chemiluminescence detector; Dual detector; Luminol

1. Introduction

The capillary electrophoretic (CE) system has received much attention as a powerful separation instrument in the fields of not only analytical chemistry but also pharmaceutical chemistry and medicine. Absorption and fluorescence phenomena are the most basic principles of the detection technique in instrumental analysis. They have also been commonly adopted as a detection technique in CE systems, due to the very small dimensions of the capillary into which a very small amount of sample migrates. These detection techniques use an on-capillary.

On the other hand, chemiluminescence (CL), which has a profound relationship to the above detection principles, has been found to be a useful detection technique in FIA, HPLC, and CE systems [1–4]. CL detection is highly sensitive, determinable over a wide range, easy to operate, and requires inexpensive apparatus and reagents. Recently, CL detection was estimated to be one of the best matched detection methods to the micro-total analysis system (μ-TAS), as CL does not require any light source or spectroscopes [5,6]. Naturally, CL detection in the CE system is brought about with an end-capillary (post column reaction), since a sample eluted from the capillary must be mixed with CL reagent at the tip of capillary to induce CL. From another viewpoint, end-capillary detection possesses an interesting "micro-space area" for reaction/detection at the tip of the capillary. The "micro-space area" enables simultaneous analysis of plural samples in the CE system with the CL detector [7]. To date, various types of CL detection cells for the CE system, including batch- and flow-types, have been developed [8].

In the present study, we developed a CE system that incorporates an ultra-violet absorption (UV)/CL dual detector. The sample was first analyzed by UV detection with an on-capillary, followed by CL detection with an end-capillary. An illustration of the UV/CL dual detection system is shown in Fig. 1. We have briefly described the preliminary results of this investigation in a previous communication [9].

Conditioning of the capillary inner wall is one of the most important and difficult steps in the CE system. The difficulty in controlling the state of the inner wall often leads to poor reproducibility in the CE analysis. Thus, comparing data obtained using different CE systems with

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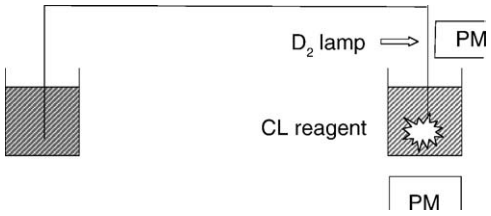

Fig. 1. Illustration of the UV/CL dual detector in the CE system.

their respective capillaries can be difficult. However, in the present system, the same sample is subjected to one capillary electrophoretic procedure, and consecutively detected with both the UV and CL detectors. The UV and CL data were simultaneously obtained for the same sample, and easily and precisely compared by considering the migration times. We successfully demonstrated that the present CE system incorporating a UV/CL dual detector provided interesting and useful information in analyses of isoluminol isothiocyanate (ILITC) and ILITC-labeled compounds.

2. Experimental

2.1. Reagents

All reagents used were of commercially available and analytical grade. Ion-exchanged water was distilled for use. Luminol, microperoxidase, glycine, glycylglycine, and glycylglycylglycine were purchased from Nacalai Tesque. Hydrogen peroxide solution (30 wt%) was purchased from Wako Pure Chemical Industries, Ltd.

2.2. Labeling procedure

Labeling using isoluminol isothiocyanate was performed as previously described [10,11]. A known amount of amino acid or oligopeptide (micromole order) was added in conjunction with ILITC to a microvessel and dissolved in 100 μl of a mixture of water and triethylamine (95:5). The solution was subjected to ultrasonication for 1 min and then left in a dark place for 20 min with mixing by a vortex mixer. The residue obtained by evaporation from the solution was redissolved in 10 mM phosphate buffer (pH 10.8) to give ILITC-labeled sample solution.

2.3. CL detection cell

A batch-type CL detection cell was used in the CE system incorporating the UV/CL dual detector. The detection cell was made of quartz and the inner volume was ca. 0.8 ml. The CL detection cell was enclosed in a small light-tight box together with a photomultiplier tube to produce a compact CL detector. A schematic diagram of the CL detector is shown in Fig. 2. A fused-silica capillary and a platinum wire as a grounding electrode were fixed to the detection

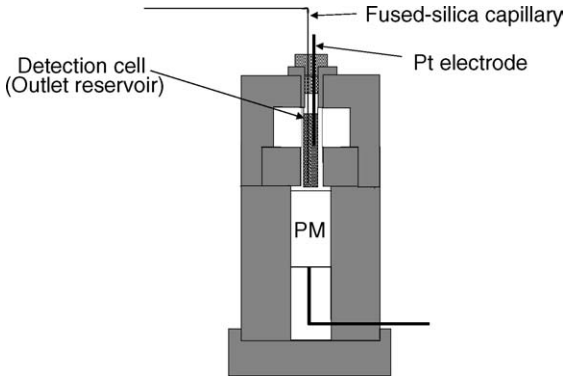

Fig. 2. Schematic diagram of a compact CL detector including the CL detection cell and the photomultiplier.

cell. In other words, the cell also worked as an outlet reservoir including an electrolyte solution. The distance between the capillary end and the cell bottom was kept about 1 mm. As analytes emerged from the capillary, they reacted with reagents to produce visible light. The CL light was detected by the photomultiplier tube located at the bottom of the cell.

2.4. Analytical procedure

The running buffer and the solution in the cell were prepared to detect luminol, ILITC, and ILITC-labeled compounds as follows: A 10 mM phosphate buffer (pH 10.8) containing 4×10^{-6} M microperoxidase was prepared as a running buffer and a 10 mM phosphate buffer (pH 10.8) containing 4.0×10^{-1} M hydrogen peroxide was added to the CL detection cell (cell buffer). The running and cell buffers were exchanged for every measurements.

A high voltage (10 kV) was applied to electrodes using a dc power supplier (Model HCZE-30PNO. 25, Matsusada Precision Devices Co., Ltd.). A fused-silica capillary of 75 μm i.d. and 75 cm length was used; 50 cm length for UV detection (282 nm) and 75 cm length for CL detection. Sample injections were performed by gravity for 15 s at a height of 25 cm. The sample migrated into the running buffer toward the CL detection cell and mixed with the reagents. The absorption detection was carried out on-capillary by a modified SPD-6A spectrophotometric detector (Shimadzu Co.), followed by end-capillary CL detection by the CL detector. The outputs from the detectors were fed to an integrator (Chromatopac C-R8A, Shimadzu Co.) to produce electropherograms.

3. Results and discussion

3.1. The effect of the sample injection time on UV/CL dual detection

CL detection was carried out via the chemical reaction between luminol (ILITC or ILITC-labeled compound)

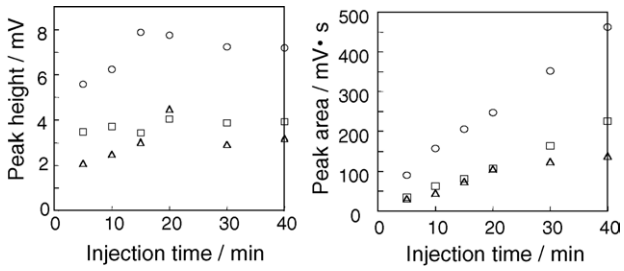

Fig. 3. The effects of sample injection time on the UV and CL responses. (◯) CL for 1.0×10^{-4} M; (△) CL for 1.0×10^{-5} M; and (□) UV for 1.0×10^{-4} M ILITC-labeled glycine. Conditions: fused-silica capillary of 75 μm i.d.; effective length of 50 cm for UV detection and effective length of 75 cm for CL detection; applied voltage, 10 kV; reagent, 10 mM phosphate buffer (pH 10.8) containing 4.0×10^{-6} M microperoxidase as a migration buffer and 10 mM phosphate buffer (pH 10.8) containing 4×10^{-1} M hydrogen peroxide in the outlet reservoir; and sample, 1.0×10^{-5} and 1.0×10^{-4} M ILITC-labeled glycine. The plots are averages for 4–7 measurements.

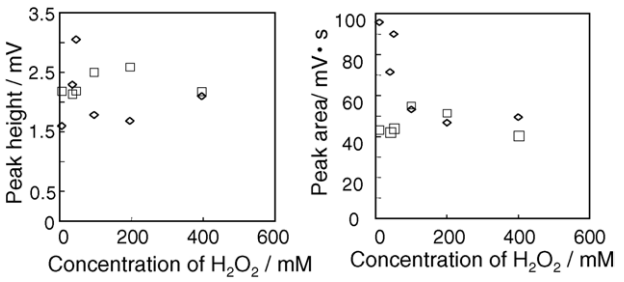

Fig. 4. The effects of hydrogen peroxide concentration on the UV and CL responses. (◊) CL for 1.0×10^{-5} M and (□) UV for 1.0×10^{-5} M ILITC-labeled glycine. Conditions: fused-silica capillary of 75 μm i.d.; effective length of 50 cm for UV detection and effective length of 75 cm for CL detection; applied voltage, 10 kV; reagent, 10 mM phosphate buffer (pH 10.8) containing 4.0×10^{-6} M microperoxidase as a migration buffer and 10 mM phosphate buffer (pH 10.8) containing 4×10^{-1} M hydrogen peroxide in the outlet reservoir and sample, 1.0×10^{-5} M ILITC-labeled glycine. The plots are averages for 4–7 measurements.

and hydrogen peroxide, where microperoxidase served as a catalyst. Naturally, CL detection due to a chemical reaction depends on the reagent volumes and concentrations. Previously, we reported CE–CL detector system using luminol-microperoxidase-hydrogen peroxide reagent [4]. We examined the effect of the reagents concentrations on CL intensities in detail. In this study we selected the concentration of microperoxidase recommended in our previous work and we examined the effects of sample injection time and hydrogen peroxide concentration on the CL response, and compared the results with those of the UV response. The effects of the sample injection time on UV and CL responses are shown in Fig. 3. The peak areas of UV and CL detection increased with increasing injection time. The peak height in CL detection increased with the increased injection time of ca. 15 s, and did not change or slightly decreased above that time, while the peak height in UV detection produced almost identical responses. All related experiments were thus carried out with a 15 s injection time.

3.2. The effect of hydrogen peroxide concentration on UV/CL dual detection

The effects of hydrogen peroxide concentration on the UV and CL responses are shown in Fig. 4. Both UV responses of peak area and height changed little. On the other hand, the maximum peak area and height of the CL responses were observed at around about 4.0×10^{-2} M hydrogen peroxide. However, even a slight concentration change from 4.0×10^{-2} M led to markedly lower CL intensity, which was easily understood by the sudden CL response changes around 4.0×10^{-2} M in Fig. 4. The usage of 4.0×10^{-2} M in the present study brought about poor reproducibility for CL analysis. To ensure reproducibility, 4.0×10^{-1} M hydrogen peroxide was used for related experiments.

3.3. Analysis of luminol

Analogs of luminol, such as ILITC and *N*-(4-aminobutyl)-*N*-ethylisoluminol, are well known to be useful labeling reagents in luminol CL detection analysis. First, we examined luminol and ILITC with the present CE system incorporating a UV/CL dual detector. Luminol was detected at ca. 12 and 18 min with UV and CL, respectively, and was determined over the range of 1×10^{-6} to 5×10^{-4} M and 1×10^{-9} to 1×10^{-7} M, respectively. CL detection was about 10^3 times as sensitive as UV detection for luminol. Although the CL signal of luminol was also observed with sufficient CL intensity at higher concentrations than 1×10^{-7} M, the CL intensity did not increase in proportion to the luminol concentration. As described in the experimental section, the distance between the capillary end and the cell bottom was kept about 1 mm and the running and cell buffers were changed for every experiment in this study. The CL intensities might be influenced with the position of capillary end or continuous measurements. We will examine them in detail in the future in order to confirm the performance of the system.

3.4. Analysis of ILITC

An ILITC sample was also analyzed with the present system. The resultant electropherograms are shown in Fig. 5. The two main peaks were observed at ca. 12 and 14 min with UV detection, and one main peak was observed at ca. 18 min with CL detection. Judging from their migration times, the first peak in the UV detection data corresponded to the main peak in the CL detection data. Subsequent experiments determined that the component producing the second peak in the UV detection data did not function as a labeling reagent. That is, the ILITC sample included natural ILITC and an impure component that showed absorption behavior but did not have CL properties and labeling performance. The ILITC sample was commercially obtained, however the possibility

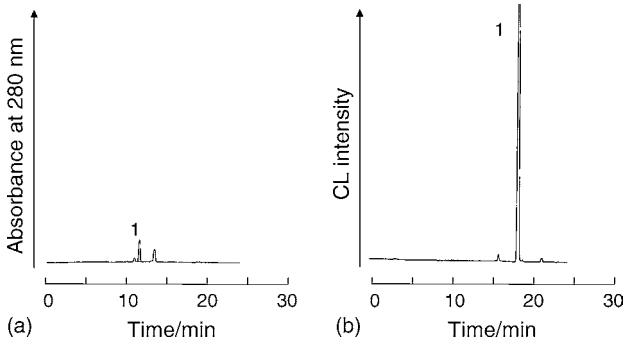

Fig. 5. Electropherograms of ILITC obtained using the CE system incorporating the UV/CL dual detector. (a) UV detection and (b) CL detection. Peak identification: (1) ILITC. Conditions: fused-silica capillary of 75 μm i.d.; effective length of 50 cm for UV detection and effective length of 75 cm for CL detection; applied voltage, 10 kV; reagent, 10 mM phosphate buffer (pH 10.8) containing 4.0×10^{-6} M microperoxidase as the migration buffer and 10 mM phosphate buffer (pH 10.8) containing 4×10^{-1} M hydrogen peroxide in the outlet reservoir; and sample, 1.0×10^{-5} M ILITC.

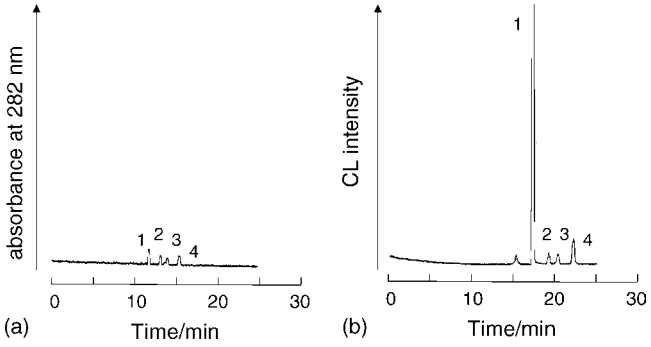

Fig. 6. Electropherograms of ILITC-labeled compounds obtained using the CE system incorporating a UV/CL dual detector. (a) UV detection and (b) CL detection. Peak identifications: (1) ILITC; (2) glycylglycylglycine; (3) glycylglycine; and (4) glycine. Conditions: fused-silica capillary of 75 μm i.d.; effective length of 50 cm for UV detection and effective length of 75 cm for CL detection; applied voltage, 10 kV; reagent, 10 mM phosphate buffer (pH 10.8) containing 4.0×10^{-6} M microperoxidase as a migration buffer and 10 mM phosphate buffer (pH 10.8) containing 4×10^{-1} M hydrogen peroxide in the outlet reservoir; and sample, 1.0×10^{-5} M ILITC-labeled compounds.

exists that the impurity was generated during storage in our laboratory (manufacturer not disclosed here). In any event, the present system quickly and precisely provided the information concerning the impurity or decomposition product via the simultaneous UV/CL detection. The system will be applied to detect a trace amount of compound possessing the CL property selectively among many compounds having the absorption property.

3.5. Analysis of ILITC-labeled compounds

ILITC is one of the reagents used to label amino groups. Like other isothiocyanates, this reagent has potential application in protein sequencing. Here, a mixed sample of glycine, glycylglycine, and glycylglycylglycine, all of which were labeled with ILITC, was analyzed by the present system. The resultant electropherograms are shown in Fig. 6. ILITC, glycylglycylglycine, glycylglycine, and glycine were separated and detected in this order. At a running buffer pH of 10.8, ILITC has a neutral charge and all of the labeled compounds would have negative charges due to the one carboxyl group in their molecules. The magnitude of electrophoretic mobility toward the inlet was glycine > glycylglycine > glycylglycylglycine > ILITC, and electroosmotic flow to the outlet was larger than electrophoretic mobility. Thus, the migration order on the electropherogram, ILITC > glycylglycylglycine > glycylglycine > glycine, was reasonably expected.

3.6. Consideration of CL quantum yield of ILITC

As described above, CL detection can detect luminol with much higher sensitivity than UV detection. However, in Fig. 6, ILITC-labeled compounds were observed with almost the same response intensities on the electropherograms for both detection methods, although an excess of ILITC provided a much larger CL response than the absorption response. The data immediately indicated a unique CL performance in which the CL quantum yield of ILITC was extremely lower under the present conditions, due to the labeling procedure. In order to improve the CL quantum of ILICL-labeled HSA, one may have to examine the analytical CL conditions for ILITC-labeled HSA, including pH, solvent, reagent concentrations, etc.

3.7. Calculation of labeling percentage

When glycine was labeled with ILITC (mole ratio of glycine:ILITC = 1:1), the labeling percentages were examined using the UV and CL detection data. For UV detection, the peak areas of free ILITC and ILITC-labeled glycine after the labeling procedure were used for the calculation. For CL detection, the peak areas of free ILITC before and after labeling were used. Almost the same values of 80–85% as a labeling percentage were obtained with both detection methods under the labeling conditions described.

4. Conclusion

We developed a capillary electrophoretic system that incorporates a UV/CL dual detector, taking advantage of the CL reaction of luminol-hydrogen peroxide and the batch-type CL detection cell. The UV detection was carried out on-capillary and the UV detection was performed at end-capillary. Our original compact CL detector, which enclosed the CL detection cell and a photomultiplier in a light-tight box, enabled the present dual detection system. Conditioning of the capillary inner wall is one of the most important steps in the CE system. The difficulty in controlling the state of the inner wall often leads to poor reproducibility in CE analysis, and thus comparing data obtained using different

CE systems is problematic. However, in the present system, the same sample is subjected to one capillary electrophoretic procedure, and then detected by both UV and CL detectors. The UV and CL data are simultaneously obtained for the same sample, and may be easily and accurately compared. We have successfully demonstrated that the present CE system, incorporating a UV/CL dual detector, provided useful and interesting information regarding isoluminol isothiocyanate (ILITC) and ILITC-labeled compounds. This system will be applied to detect a trace amount of compound possessing the CL property selectively among many compounds with the absorption property. Furthermore, a comparison of peak shapes obtained using UV detection and CL detection will be interesting.

Acknowledgements

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