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Competitive Immunoassay Using Capillary Electrophoresis with a Chemiluminescence Detector

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We have proposed competitive immunoassay using capillary electrophoresis (CE) with a chemiluminescence (CL) detector, in which a small amount of sample (ca. 20 μL) is required for analysis. Human serum albumin (HSA) and anti-human serum IgG (anti-HSA) were used for immune reaction as a model. A luminol and hydrogen peroxide CL reaction was adopted, and HSA was labeled with isoluminol isothiocyanate (ILITC) for competitive immunoassay. The reactant after the immune reaction was directly subjected to CE with the CL detector, where the labeled HSA was easily and rapidly separated and detected. The amount of labeled HSA indicated a good relationship to that of HSA as an analyte through the immune reaction. The HSA was determined over the range of 0.2–1.2 μM with a detection limit of 0.1 μM (S/N = 3). The present method features high sensitivity, a small sample volume, and easy and rapid operation. The method also shows the possibility to analyze a specific protein in a serum sample.

Analytical techniques using chemiluminescence (CL) reactions have become familiar by degrees to the average analytical researchers.[1,2] There are some advantages in CL-based techniques, such as high sensitivity, wide determinable range, easy operation, and inexpensive reagent and apparatus. Also, because no external light source and spectroscopes are required, the CL techniques lead to only minimal analytical instrumentation. The combination of CL detection and separation methodologies, such as HPLC or capillary electrophoresis (CE), can offer promising analytical possibilities, providing good sensitivity and selectivity, and allowing the resolution and quantification of various analytes in a complex mixture.[3,4]

We have also developed various types of CE with the CL detector, taking advantage of CL reagents, such as luminol, 1,10-phenanthroline, ruthenium(II) complex, and peroxyoxalate.[5–7] We could separate and detect many kinds of samples, including metal ions, metal compounds, amino acids, peptides, proteins, saccharides, nucleic acids, phenolic compounds, fluorescence compounds, and liposomes. Furthermore, the combination of microchip CE with the CL detector was successfully demonstrated by using luminol and peroxyoxalate CL reagents.[8–10]

On the other hand, an immunoassay involving a technique based on a specific reaction between an antigen and an antibody has been extensively studied.[11,12] A competitive immunoassay usually requires bound/free (B/F) separation, leading to tedious and time-consuming procedures. For example, an immunoassay using supports modified with an antibody must carry out B/F separation by removing the supports from a reactant solution after an immune reaction. The supports holding labeled antigen–antibody complexes are washed in order to remove any co-existing compounds. The supports are then subjected to the determination of labeled complexes. Research activities concerning the combination of competitive immune reactions with CE have been reviewed;[13] several groups have reported that competitive immunoassay using CE has been applied to the determination of proteins, using fluorescence-labeled protein and laser-induced fluorescence detection.[14,15]

In this study, we proposed competitive immunoassay using the CE with CL detector, in which a small amount of sample is needed and B/F separation is easily and rapidly brought about through CE. Human serum albumin (HSA) and anti-human serum IgG (anti-HSA) were used for immune reactions as a model. A luminol and hydrogen peroxide CL reaction was adopted, and HSA was labeled with isoluminol isothiocyanate (ILITC) for a competitive immunoassay. The immune reaction was carried out in a micro-vessel. A small amount (ca. 60 μL) of reactant after the immune reaction was sufficient for a sample volume of CE. The reactant was directly subjected to CE with the CL detector. The ILITC-labeled HSA could be detected by on-line CL detection. In the present competitive immunoassay, the CL peak of the labeled HSA increased with increasing the concentration of HSA (analyte). That is, the analyte was determined by detecting the CL peak of the labeled HSA that appeared on the electropherogram.

Experimental

Reagents. All reagents used were of commercially available and special grade. Ion-exchanged water was distilled for use. Human serum albumin (HSA) (MW 66000), rabbit anti-human serum IgG (anti-HSA) (MW 150000), and control human serum were purchased from Sigma Co., Wako Pure Chemical Industries, Ltd., and Sanko Junyaku Co., Ltd., respectively. Luminol and microperoxidase were purchased from Nacalai Tesque. Isoluminol isothiocyanate (ILITC) was purchased from Tokyo Chemical Industry Co.

Labeling Procedure. Labeling using ILITC was carried out as previously described.[16,17] Amounts of 0.025 μmol HSA and 0.5 μmol ILITC were added to a micro-vessel and dissolved in 100 μL of a mixture of water and triethylamine (95:5). The solution was subjected to ultra-sonication for 1 min, and then left in a

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dark place for 20 min with mixing by a vortex mixer. The residue obtained by evaporation from the solution was redissolved in a 10 mM phosphate buffer (pH 7.0) to give a ILITC-labeled HSA solution that comprised excess ILITC.

For further purification to remove excess ILITC, the obtained ILITC-labeled HSA solution was subjected to column separation (PD-10 desalting columns, Amersham pharmacia biotech). ILITC and ILITC-labeled HSA were separated with a 10 mM phosphate buffer (pH 7.0) on the separation column. The fractions including ILITC-labeled HSA were collected to give a pure ILITC-labeled HSA solution not including ILITC.

The ILITC-labeled HSA solution that comprised excess ILITC and the pure ILITC-labeled HSA solution were used for the immune reaction in this study.

Competitive Immunoassay. The competitive immunoassay is illustrated in Fig. 1. HSA and anti-HSA were used for the immune reaction as a model. Solutions of ILITC-labeled HSA, HSA (analyte), and anti-HSA were prepared by being dissolved with a 10 mM phosphate buffer (pH 7.0). A 3.3 μM anti-HSA solution (15 μL) was added to a micro-vessel including a 2.5 μM ILITC-labeled HSA solution (20 μL) and 0.1–1.2 μM HSA solution (20 μL) for a competitive immune reaction. The mixture was stirred for 1 min and left for 1 h at room temperature. The labeled HSA and the free HSA as an analyte were made to react competitively with anti-HSA in a micro-vessel. The reactant was directly subjected to CE with the CL detector.

Apparatus and Procedure of CE with CL Detector. A schematic diagram of the CL detection cell is shown in Fig. 2.[5] The detection cell was constructed of Teflon, and the inner volume was about 8 μL. One of the faces of the cubic cell was equipped with a piece of Pyrex glass (1 mm thick). A fused-silica capillary 50 cm long with 50 μm i.d. (GL Sciences Inc.) and a platinum wire as a grounding electrode were fixed to the detection cell. In other words, the cell also served as an outlet reservoir including an electrolyte solution. The distance between the capillary outlet and the inner face of the glass was maintained at about 0.5 mm.

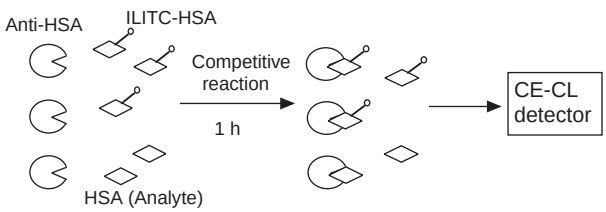

Fig. 1. The illustration of the competitive immunoassay.

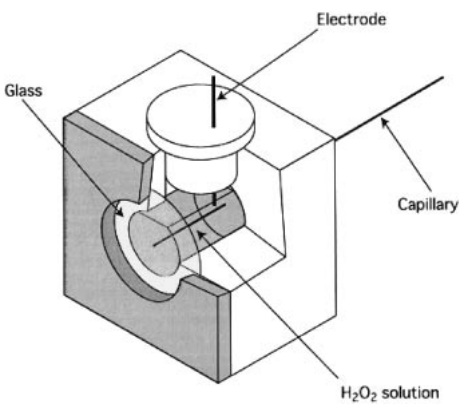

Fig. 2. The schematic diagram of the CL detection cell.

As analytes emerged from the capillary, they reacted with reagents to produce visible light.

A high voltage (12 kV) was applied to electrodes using a DC power supplier (Model HCZE-30PNO. 25, Matsusada Precision Devices Co., Ltd.). The luminol and hydrogen peroxide CL system was used in conjunction with microperoxidase as the catalyst. A 10 mM phosphate buffer (pH 7.0) containing 4 μM microperoxidase was used as a migration buffer. A 10 mM phosphate buffer (pH 10.8) containing 400 mM hydrogen peroxide was added to the outlet reservoir or the detection cell. Sample injections (ca. 5.6 nL) were performed by gravity for 10 s at a height of 20 cm. A sample migrated in the migration buffer toward the CL detection cell and mixed with reagents. The resulting CL at the capillary outlet was directly captured by the photosenser module in the CL detector (Model EN-21, Kimoto Electric, Inc.). For a comparing experiment, a modified spectrophotometric detector of Shimadzu Co. SPD-6A was used as an absorption detector. The output from the detector was fed to an integrator (Chromatopac C-R6A, Shimadzu Co.) to produce electropherograms.

Results and Discussion

Analysis of the Reactant after Labeling Procedure. After the labeling procedure without further purification was carried out, as described in the experimental section, the reactant comprising excess ILITC and ILITC-labeled HSA was directly subjected to the present CE with CL detector. The obtained electropherogram is shown in Fig. 3. The CL peaks of hydrolyzed ILITC and ILITC appeared at around ca. 12–15 min, and the CL peak of ILITC-labeled HSA was detected at ca. 22 min. The peak of ILITC-labeled HSA was reproducibly observed; the relative standard deviations of the retention time and the CL intensity or the peak height were 2.5% and 3.0%, respec-

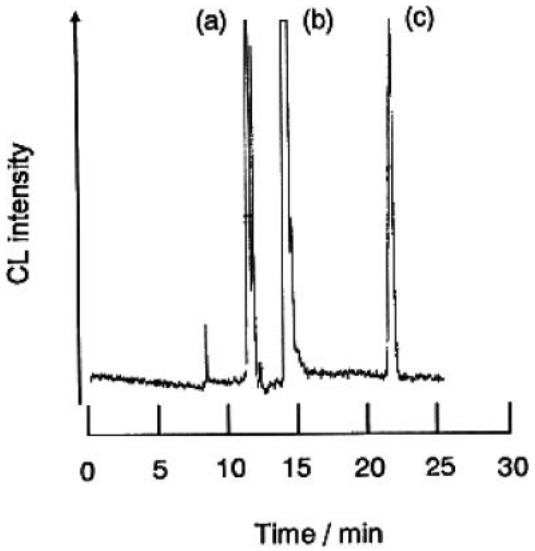

Fig. 3. The electropherogram of the reactant obtained after the labeling procedure. (a) Hydrolyzed ILITC, (b) ILITC, and (c) ILITC-labeled HSA. Conditions: Capillary, 50 cm of 50 μm i.d. fused silica; applied voltage, 12 kV; migration buffer, 10 mM phosphate buffer (pH 7.0) containing 4 μM microperoxidase; reagent in the cell, 10 mM phosphate buffer (pH 10.0) containing 400 mM hydrogen peroxide; sample injection, siphoning (for 10 s at 20 cm height); and ILITC-labeled HSA concentration, 1.0 μM.

tively. The ILITC-labeled HSA was determined over the range of 0.02–5 μM with a detection limit of 0.01 μM (S/N = 3). The CL detection for HSA was about 100-times as sensitive as absorption detection (215 or 280 nm).

Analysis of the Reactant after Immune Reaction. As preliminary experiments before the competitive immunoassay, we carried out an immune reaction between ILITC-labeled HSA and anti-HSA, in which the pure ILITC-labeled HSA was used. Experiments were similarly carried out concerning the competitive immune reaction described in the experimental section, but without using HSA as an analyte. The obtained reactant was subjected to CE with the CL detector. The CL peak of ILITC-labeled HSA was observed on the electropherogram at ca. 22 min, similarly as shown in Fig. 3. However, the CL peak due to the complex of the labeled HSA and anti-HSA was not observed on the electropherogram, at least within 1 h. In order to confirm the electrophoretic behavior of the complex, we examined a reactant by means of the CE equipped with an absorption detector (215 nm); the peak of the labeled HSA was observed, but that of the complex did not appear. There may be an adsorption phenomenon of the complex (relative larger molecular weight, ca. 220000) on the reaction vial or the capillary wall due to a hydrophobic or electrostatic interaction. The reason has not yet been clarified.

The immune reaction was carried out by using a definite concentration of the pure ILITC-labeled HSA solution and various concentrations of anti-HSA solutions: a 0, 8, 20, or 40 μM anti-HSA solution (10 μL) was added to a micro-vessel including a 45 μM ILITC-labeled HSA solution (10 μL) for the immune reaction. The reactants were analyzed by the present CE with the CL detector. The obtained electropherograms are shown in Fig. 4. The CL intensity decreased with increasing the concentration of anti-HSA. It was clearly confirmed that the immune reaction between ILITC-labeled HSA and anti-HSA proceeded under the present condition and the labeled HSA was quantitatively analyzed by the CE with the CL detector. Through further experiments, it was confirmed that the electrophoretic behavior of the ILITC-labeled HSA on the electropherogram obtained by using the ILITC-labeled HSA that comprised excess ILITC was the same as that obtained using the pure ILITC-labeled HSA.

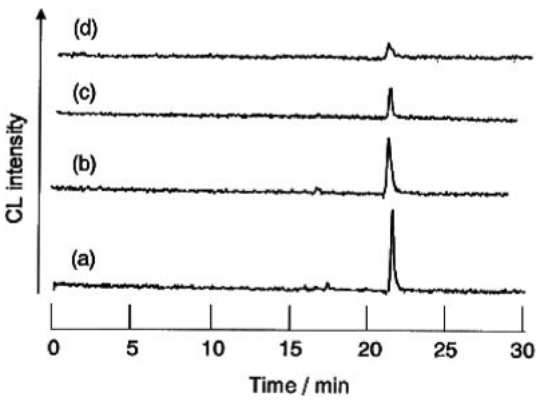

Fig. 4. The electropherograms of the reactants obtained after the immune reaction between the definite concentration of ILITC-labeled HSA and the various concentrations of anti-HSA. (a) 0, (b) 8, (c) 20, and (d) 40 μM anti-HSA. Conditions: Capillary, 50 cm of 50 μm i.d. fused silica; applied voltage, 12 kV; migration buffer, 10 mM phosphate buffer (pH 7.0) containing 4 μM microperoxidase; reagent in the cell, 10 mM phosphate buffer (pH 10.0) containing 400 mM hydrogen peroxide; sample injection, siphoning (for 10 s at 20 cm height); and immune reaction, 45 μM ILITC-labeled HSA solution (10 μL) + 0, 8, 20, or 40 μM anti-HSA solution (10 μL).

Determination of HSA by Means of Competitive Immunoassay. The ILITC-labeled HSA and HSA (analyte) competitively react with anti-HSA in a micro-vessel through the immune reaction, as shown in Fig. 1. The reactant obtained after the competitive immune reaction is directly subjected to CE with the CL detector. A small amount (ca. 60 μL) of reactant after the immune reaction is sufficient for the sample volume of CE, because it is usually of nano-liter order. Because a sample and reagents are often expensive and valuable in immunoassay, it is important that the sample volume required for an analysis is only minimal.

First, we carried out a competitive immune reaction by using the pure ILITC-labeled HSA solution (Fig. 5) under the conditions described in the figure caption. The reactant after the immune reaction would comprise the labeled HSA as well as the complex of the labeled HSA and anti-HSA, which possess for CL property. However, the CL peak of the complex did not appear at least within 30 min, and only the peak of the labeled HSA was observed, similarly to the results as described in the above sections. As shown in Fig. 5, the CL intensity increased with increasing the concentration of HSA through the competitive immune reaction. That is, the competitive immune reaction among ILITC-labeled HSA, anti-HSA, and HSA as an analyte successfully proceeded under the present conditions, and the labeled HSA was analyzed by CE with the CL detector. It was also confirmed from further experiments that there was no difference in the electrophoretic behavior of ILITC-labeled HSA between the electropherograms obtained using the ILITC-labeled HSA, including excess

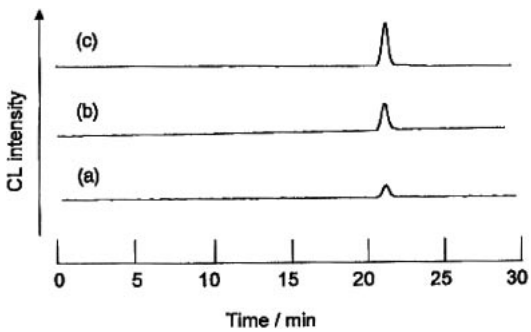

Fig. 5. The electropherograms of the reactants obtained after the competitive immunoassay. (a) 10, (b) 50, and (c) 100 μM HSA. Conditions: Capillary, 50 cm of 50 μm i.d. fused silica; applied voltage, 12 kV; migration buffer, 10 mM phosphate buffer (pH 7.0) containing 4 μM microperoxidase; reagent in the cell, 10 mM phosphate buffer (pH 10.0) containing 400 mM hydrogen peroxide; sample injection, siphoning (for 10 s at 20 cm height); and competitive immune reaction, 25 μM ILITC-labeled HSA solution (20 μL) + 3.3 μM anti-HSA solution (20 μL) + 10, 50, or 100 μM HSA solution (analyte) (20 μL).

ILITC and those obtained using the pure ILITC-labeled HSA. Thus, the following competitive immunoassay for analyzing HSA was carried out by using ILITC-labeled HSA that comprised excess ILITC.

It was briefly noted that the peak shape in CL detection must be influenced by not only the concentration distribution of the sample in a sample zone, but also the sample diffusion and the CL reaction at the capillary outlet in the batch-type cell. That is, the peak shape in the CL detector was based on the CL appearance through the post-column CL reaction at the tip of a capillary which greatly depended on the sample diffusion in the batch-type cell.[18] Tentatively, we calculated an apparent theoretical plate number of ca. 9200 by using the data in Fig. 5.

The calibration curves of HSA by using the competitive immune reaction and CE with the CL detector was obtained under the conditions as described in the experimental section; because ILITC-labeled HSA that comprised excess ILITC was used, electropherograms like those shown in Fig. 3 were obtained. In the calibration curves, the amount of the labeled HSA indicated a good relationship to that of HSA as an analyte through the immune reaction. Under the condition of the competitive immune reaction, the HSA as an analyte was determined over the range of 0.2–1.2 μM ($y = 4.23x + 2.25$; correlation coefficient, 0.998) with a detection limit of 0.1 μM ($S/N = 3$). The CL detection for HSA was about 50-times as sensitive as the absorption detection (215 or 280 nm).

Application to Analysis of HSA in Control Serum. In order to determine the amount of HSA in a serum by the present method, the control serum solution that was diluted to 500 times its volume was added to a micro-vessel including ILITC-labeled HSA and the anti-HSA. After the competitive immune reaction, the reactant was subjected to CE with the CL detector. The amount of HSA obtained by the present method, 6.0×10^{-4} M, almost corresponded to that reported by the maker (5.7×10^{-4} M). This result showed that the present method was applicable to the determination of protein in a serum sample without being interfered with by coexisting constituents.

Although the present immunoassay may need more detailed examinations for optimizing the analytical conditions, it must be one of the most promising ways for the determination of biological constituents with good sensitivity, a small amount of sample volume, and easy and rapid operation.

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