  Bull. Chem. Soc. Jpn., 62, 2844—2848 (1989) [Vol. 62, No. 9

A Highly Sensitive Fiber-Optic Immunosensor Using a Metal-Complex Compound as a Chemiluminescent Catalyst

Tadashi HARA,* Kazuhiko TSUKAGOSHI, Akihiro ARAI, and Yuji IMASHIRO
Department of Chemical Engineering, Faculty of Engineering, Doshisha University, Karasuma Imadegawa, Kamigyo-ku, Kyoto 602
(Received March 28, 1989)

A highly sensitive fiber-optic immunosensor using an antibody-immobilized optical fiber was developed and introduced into a chemiluminescence complex catalyst immunoassay (CLCCIA) which had been reported by the authors in a previous paper. After optimization of the experimental conditions, human serum albumin as model protein was determined by both competitive immunoassay and sandwich immunoassay, using the fiber-optic immunosensor. Human serum albumin could be determined in the concentration range 1×10^{-4}—1×10^{-1} g dm^{-3} with a lower limit of 400 pg by competitive immunoassay while in the concentration range 1×10^{-5}—1×10^{-1} g dm^{-3} with a lower limit of 40 pg by sandwich immunoassay. The new fiber-optic immunosensor developed by the authors can be characterized as follows: 1) Highly sensitive for the determination of a small amount of protein, 2) applicable to a wide concentration range of protein sample, 3) feasible with a microvolume of sample, 4) suitable for automated operation, and 5) excellent regarding safety, cost, and treatment.

A new immunoassay, chemiluminescence complex catalyst immunoassay (CLCCIA), in which iron(III) 2,9,16,23-tetrakis(chlorocarbonyl)phtalocyanine (TCCP-Fe(III)) was used as a labeling agent, was previously developed by the authors[1-3] using the fact that TCCP-Fe(III) markedly accelerates the chemiluminescene (CL) reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and hydrogen peroxide (H_2O_2). Their immunoassays were carried out by a batchwise method, a flow-through method, and a chromatographic method using antibody-immobilized glass beads. The CLCCIA was highly sensitive and superior regarding safety, cost, and easy treatment to radioimmunoassay and enzyme immunoassay.

An optical fiber has recently been applied to a chemical sensor and a biosensor since the optical fiber makes in situ and in vivo measurements possible and allows very weak light to transfer without any accompanying light loss. However, a few reports have been published with regard to a fiber-optic immunosensor in which an antibody is directly immobilized on the plain end of an optical fiber,[4,5] or a CL reaction is used for the determination of an antigen.[6-8]

In the present study, an attempt was made to develop a new fiber-optic immunosensor using an antibody-immobilized optical fiber and to introdue it into CLCCIA. The fiber-optic immunosensor gave satisfactory results regarding sensitivity for detection, concentration range for determination, amount of sample, and cost.

Experimental

Reagents. All reagents were of commercially available special grade. Ion-exchanged water was distilled before use.

A 1.0×10^{-3} mol dm^{-3} luminol solution contained 1.8×10^{-1} mol dm^{-3} sodium carbonate, 2.0×10^{-1} mol dm^{-3} sodium hydrogencarbonate, and 1.0×10^{-3} mol dm^{-3} ethylenediaminetetraacetic acid (EDTA). A 7.5×10^{-3} mol dm^{-3} H_2O_2 solution was prepared by diluting 30 wt% H_2O_2 with water.

Since the purpose of the present study was to describe the establishment of a new immunoassay methodology, all experiments were carried out using easily available and inexpensive human serum albumin (HSA) as model protein. Rabbit anti-HSA (DAKO) was used without further purification. HSA (MilesLabolatories, Inc.), bovine serum albumin (BSA)(Sigma Chemical Co.), human serum γ-globulin-(HγG)(Sigma Chemical Co.), bovine serum γ-globulin (BγG) (Sigma Chemical Co.), bovine serum α-globulin (BαG) (INC Pharmaceuticals, Inc.), and ovalbumin (Ova)(Sigma Chemical Co.) were dissolved and diluted with a phoshate buffer solution consisting of 2.78×10^{-3} mol dm^{-3} potassium dihydrogenphosphate, 5.56×10^{-3} mol dm^{-3} disodiumhydrogenphosphate, and 0.05 wt% sodium azide (pH 7.3) (Buff-A) or a Buff-A containing 1 wt% BSA (Buff-B).

Iron(III) 2,9,16,23-tetracarboxyphtalocyanine (TCP-Fe(III)) and TCCP-Fe(III) were synthesized as described in the previous paper.[1,9] TCCP-Fe(III) was used as a labeling reagent. TCP-Fe(III) was used to optimize the conditions for the measurement of the CL intensity because TCCP-Fe(III) was hydrolyzed in water and changed to TCP-Fe(III).

Preparation of labeled HSA and labeled anti-HSA were carried out as described in previous papers.[1,3] The concentrations of these albumins were determined on the basis of the calibration curves of their absorbance.

Preparation of the Optical Fiber Immobilized with Anti-HSA. The plain end of an optical fiber (GC. 800/1000L, Fujikura Co.; 1 mm i.d.×5 cm) was polished with a sand paper (1200 CC-CW, SANKYO RIKAGAKU Co.) and was followed by the immobilization of anti-HSA, as described in the literature.[4,10] After the end part of an optical fiber was activated by strong mineral acid, the surface was treated by 3-(2,3-epoxypropoxy)propyltrimethoxysilane, and anti-HSA was immobilized by a periodic acid method on the surface, followed by the reduction of the imine-type bond to the amine-type bond by sodium borohydride. The antibody-immobilized optical fiber, thus obtained, was washed by Buff-A and stored at 4 °C in Buff-B. According to the present procedure, 160 pieces of antibody-immobilized optical fibers were obtained using 10 mg of antibody.

Apparatus and Procedure. Both the competitive immunoassay and sandwich immunoassay are schematically shown in Fig. 1.

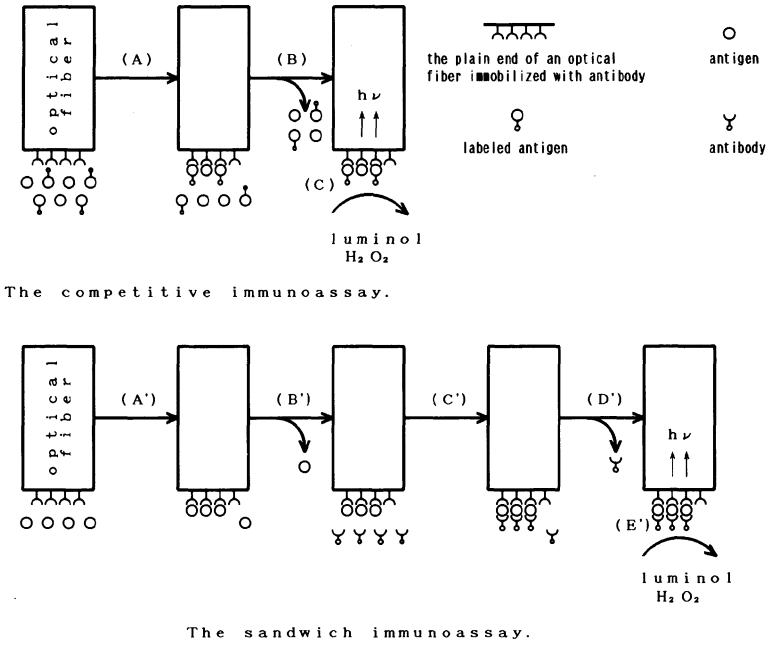

Fig. 1. Schematic diagram of the immunoassay.
(A),(A'),(C'): Antigen-antibody reaction, (B),(B'),-(D'): separation of bound and free fractions, and (C),(E'): measurement of CL intensity.

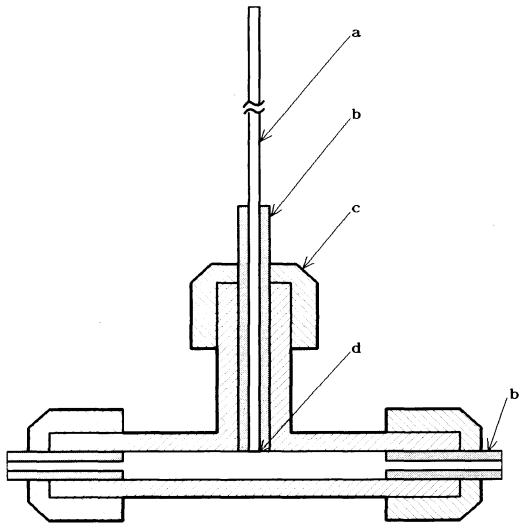

Fig. 2. The cell set with an optical fiber.
a: optical fiber, b: silicone tube (3 mm o.d.×1 mm i.d.), c: Teflon-made three-way joint (3 mm i.d.), and d: immobilized antibody.

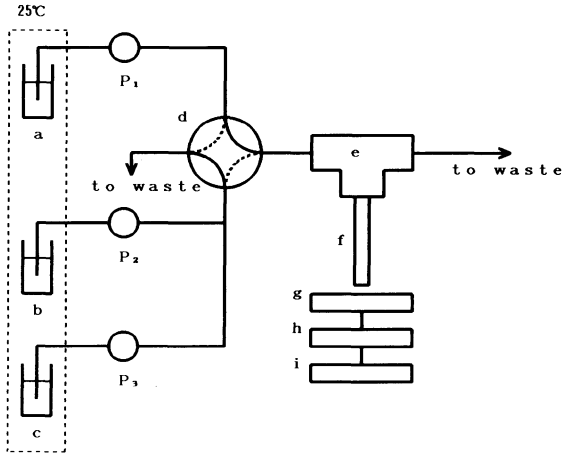

Fig. 3. Schematic flow diagram of a fiber-optic immunosensor.
a: Buffer solution, b: H_2O_2 solution, c: luminol solution, d: four-way cock, e: cell, f: optical fiber, g: photomultiplier, h: photoncounter, i: integrator, and P_1, P_2, P_3: pump.

Competitive immunoassay was carried out as follows: The end part of an antibody-immobilized optical fiber was immersed in a mixed solution containing 2.4 ng of labeled HSA in 4 mm^3 of Buff-B and a definite amount of HSA in 4 mm^3 of Buff-B, and was made to react with antigen (HSA) in the mixed solution at 4 °C for 2 hours (A). The optical fiber was twice washed with 50 mm^3 of Buff-C (2.78×10^{-3} mol dm^{-3} potassium dihydrogenphosphate, 5.56×10^{-3} mol dm^{-3} disodium hydrogenphosphate) (B) and positioned in the cell as shown in Fig. 2. Sandwich immunoassay was carried out as follows: The end part of an antibody-immobilized optical fiber was immersed in a solution containing a definite amount of HSA in 4 mm^3 Buff-B, and was made to react with antigen (HSA) in the solution at 25 °C for 2 hours (A'). The optical fiber was twice washed with 50 mm^3 of Buff-B (B') and was made to react with 12 ng of labeled HSA in 4 mm^3 of Buff-A at 4 °C for 4 hours (C'). The optical fiber was positioned in the cell as shown in Fig. 2 after washing twice with 50 mm^2 Buff-C (D').

The CL emitted from the surface of the optical fiber set to the cell was measured ((C) and (E')) in accordance with the following procedure: The measurement of the CL intensity was made using the immunosensor system shown in Fig. 3 in which all the tubes and connectors were made of Teflon. Each solution of Buff-C (a), H_2O_2 (b), and luminol (c) was fed at a flow rate of 1.5, 0.25, and 0.25 $cm^3\ min^{-1}$ by means of pumps (P_1), (P_2), and (P_3) (Atto, SJ1211). The optical fiber set to the cell was washed with Buff-C for 1 min and fed with a mixed solution of H_2O_2 and luminol by operating a four-way cock (d). The CL emitted from the surface of the optical fiber was immediately detected with the photon-counter (h) and was integrated for 1 min with the integrator (i) to give the CL intensity as a peak area (V s).

Results and Disccusion

Determination of Analysis Conditions. An adequate investigation was not made in previous studies,[1-3,9] regarding the type and pH of the buffer solution used for a luminol solution in the lunimol-H_2O_2-TCP-Fe(III) system. To improve the sensitivity of immunoassay, a CL system by which TCP-Fe(III) could be determined more sensitively was necessary. Then, the type and pH used for a luminol solution were optimized. The apparatus used for this experiment is shown schematically in Fig. 4. Each solution of luminol, H_2O_2 and carrier(Buff-C) was fed at a flow rate of 2.0, 2.0, and 0.5 $cm^{-3}min^{-1}$. A 50 mm^3 portion of a 1.0×10^{-8} mol dm^3 TCP-Fe(III) catalyst solution was injected into the line of carrier solution through the sampling loop and six-way cock. The CL intensity of the solution passing through the flow-cell (0.8 mm i.d.×45 cm; poly (vinyl chloride) tube) was detected with a photoncounter and recorded on a recoder. The peak area obtained by injecting the catalyst sample solution was integrated with the integrator to give the CL intensity as a peak area (V s). As can be seen from the experimental results (Fig. 5) obtained using the above-mentioned apparatus, the CL intensity showed a maximum value when a 0.2 mol dm^{-3} carbonate buffer solution (pH 10.8) was used. Since the value of the base line which corresponded to the CL intensity due to both a luminol solution and a H_2O_2 solution in the absence of a catalyst solution was large, a 1.0×10^{-2}—1.0×10^{-4} mol dm^{-3} EDTA solution was added to a luminol solution so as to minimize the value of the base line. The relationship between the EDTA concentration and the value of the base line or the CL intensity of a TCP-Fe(III) catalyst solution was obtained. The addition of the EDTA solution lowered the value of the base line by one-hundred times, but had almost no affect on the catalytic activity of the

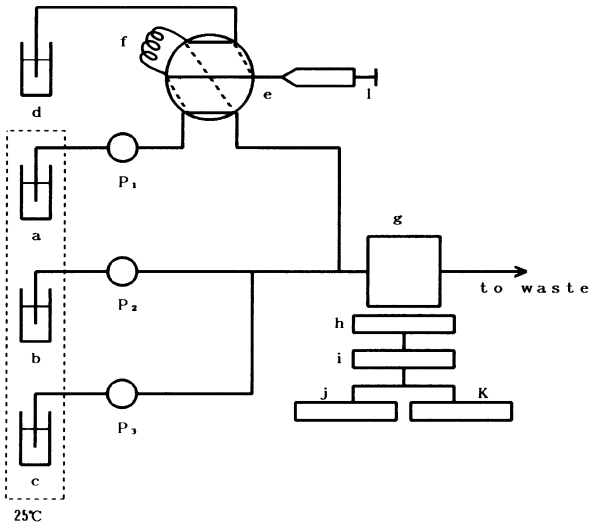

Fig. 4. Flow-injection system.
a: Buffer solution, b: H_2O_2 solution, c: luminol solution, d: sample solution, e: six-way cock, f: sampling loop, g: flow-cell, h: photomultiplier, i: photon-counter, j: recorder, k: integrator, l: syringe, and P1, P2, P3: pump.

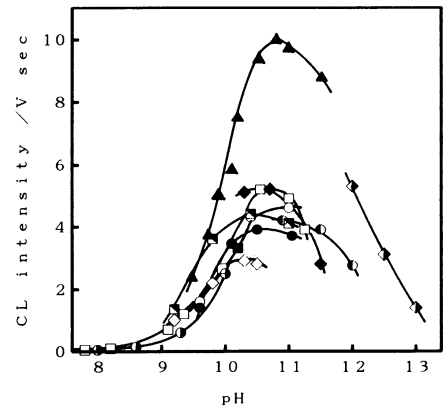

Fig. 5. Effect of various buffer solutions on the CL intensity of the luminol-H_2O_2 system.
■: H_3BO_3-KOH buffer, ◪: $Na_2B_4O_7$-Na_2CO_3 buffer, □: H_3BO_3-KCl-Na_2CO_3 buffer, ◇: $Na_2B_4O_7$-NaOH buffer, ◆: NaOH-KCl buffer, ◆: HCl-Na_2CO_3 buffer, ○: $NaHCO_3$-NaOH buffer, ◐: Na_2HPO_4-NaOH buffer, ◑: H_3BO_3-KCl-NaOH buffer, ▲: 0.2 mol dm^{-3} (Na_2CO_3-$NaHCO_3$) buffer, and ●: 0.02 mol dm^{-3} (Na_2CO_3-$NaHCO_3$) buffer.

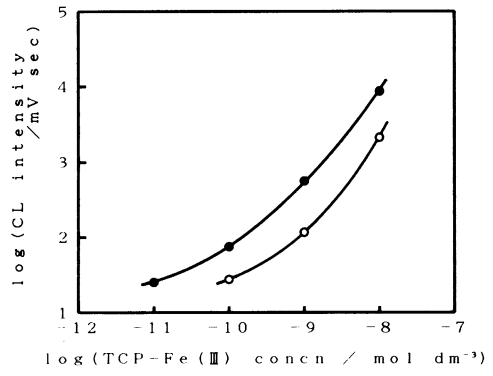

Fig. 6. The relationship between concentration of TCP-Fe(III) and CL intensity.
●: The present luminol solution and ○: the conventional luminol solution.

TCP-Fe(III) solution. Therefore, the detection limit of TCP-Fe(III) using the present luminol solution consisting of 1.0×10^{-3} mol dm^{-3} EDTA in a 0.2 mol dm^{-3} carbonate buffer solution (pH 10.8), was about one-tenth that of TCP-Fe(III) using the luminol solution mentioned in a previous paper.[3] Two calibration curves for TCP-Fe(III) using the present luminol solution and same luminol solution as in the previous paper[3] were shown in Fig. 6.

The relationship between the CL intensity and the flow rate of a 1:1 mixed solution of a luminol solution and a H_2O_2 solution was examined by the measuring system shown in Fig. 3. In this experiment the CL emitted from an antibody-immobilized optical fiber, which had been made to react with 4 mm^3 of 1.0×10^{-2} g dm^{-3} labeled HSA at 4 °C for 20 min, was measured for 1 min at various flow rates of the 1:1 mixed solution of a luminol solution and a H_2O_2 solution. The maxium CL intensity was observed when the flow rate of the 1:1 mixed solution was 0.5 cm^3 min^{-1}; the change of the CL intensity in the flow rate range of 0.1—2.0 cm^3 min^{-1} was within 10%. A flow rate of 0.5 cm^3 min^{-1} was used for the experiment.

The relationship between the repetitive use and the activity change of an antibody-immobilized optical fiber was examined as follows: The antibody-immobilized optical fiber was made to react with 4 mm^3 of 1.0×10^{-2} g dm^{-3} labeled HSA at 4 °C for 20 min and the CL emitted from the optical fiber was measured for 1 min. Then, the labeled HSA was eluted with a 8.0×10^{-3} mol dm^{-3} hydrochloric acid —3.0×10^{-2} mol dm^{-3} potassium chloride buffer solution (pH 2.3), and the antibody-immobilized optical fiber was subjected to the next immune reaction, followed by measuring (using a similar method as before) the CL. This operation was repeated severall times. According to the obtained results, about a 20% reduction of the CL intensity was observed for one-time use. A newly prepared antibody-immobilized optical fiber was used for every measurement in the present study.

The conditions necessary for the immune reaction (A) in competitive and the immune reaction (C′) in sandwich immunoassay were chosen by taking the reaction time and nonspecific adsorption into consideration,[11,12] as follows: Immune reaction (A) 3.0×10^{-3} g dm^{-3} labeled HSA, 4 °C, and 2 hours; immune reaction (C′) 3.0×10^{-2} g dm^{-3} labeled anti-HSA, 4 °C, and 4 hours.

The relationship between the reaction time and the CL intensity was examined for the immune reaction (A′) in sandwich immunoassay. The immune reaction (A′) was found to almost attain equilibrium in 2 hours. Therefore, the immnue reaction (A′) in the subsequent experiment was carried out for 2 hours. The relationship between the sample amount used for sandwich immunoassay and the CL intensity was examined. The CL intensity was nearly definite for a sample solution of more than 4 mm^3. Therefore, a 4 mm^3 sample solution was used in the subsequent experiment.

Calibration Curve of HSA. The calibration curve for HSA was obtained by competitive immunoassay under the optimized conditions mentioned above (Fig. 7). HSA could be determined in the concentration range 1×10^{-4}—1×10^{-1} g dm^{-3}, with a lower limit of 400 pg. The coefficient of variation for five analyses of a 1.0×10^{-3} g dm^{-3} HSA solution was 6.5%.

The calibration curve for HSA was also obtained by sandwich immunoassay under the optimized conditions mentioned above (Fig. 8). HSA could be determined in the concentration range 1×10^{-5}—1×10^{-1} g dm^{-3}, with the lower limit of 40 pg. The

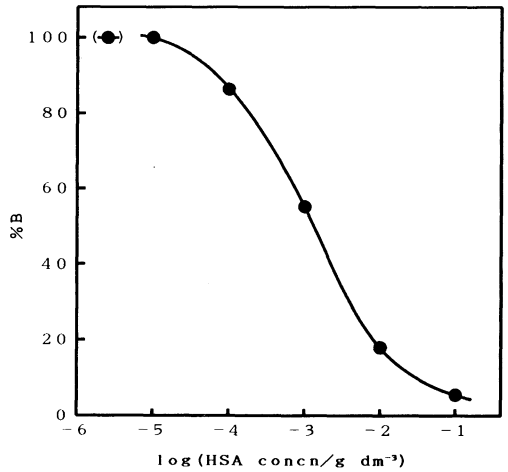

Fig. 7. Calibration curve for HSA obtained by the competitive immunoassay.
$\%B=\{(I_s-I_{ba})/(I_{b1}-I_{ba})\}\times100$. I_s, I_{b1}: CL intensity of the sample in the presence or absence of HSA, I_{ba}: CL intensity of background, and (-●-): Blank value.

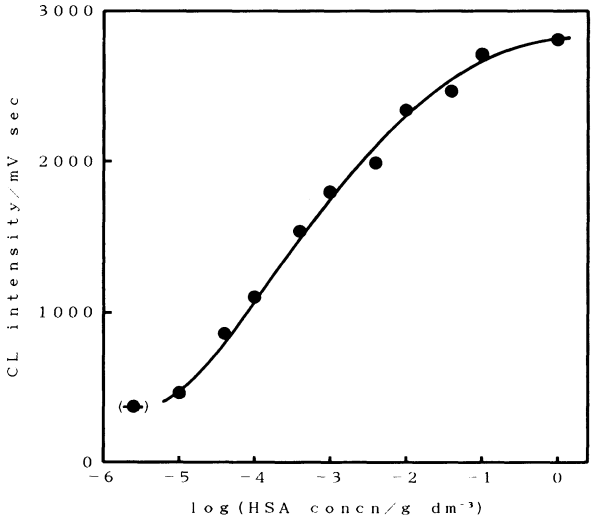

Fig. 8. Calibration curve for HSA obtained by the sandwich immunoassay. (-●-): Blank value.

Table 1. Selectivity of a Fiber-Optic Immunosensor

Protein	Relative CL intensity
HSA	1.00
HγG	0.14
BγG	0.07
BγG	0.00
Ova	0.00

Protein concn=1.0×10^{-2} g dm^{-3}.

coefficient of variation for five analyses of a 1.0×10^{-3} g dm^{-3} HSA solution was 5.0%. Though sandwich immunoassay was a tedious method, it was about 10 times more sensitive than competitive immunoasay.

Selectivity. The selectivity of the fiber-optic immunosensor in sandwich immunoassay was examined by measuring the adsorption amount of various proteins onto an anti-HSA immobilized optical fiber (Table 1). Each 1.0×10^{-2} g dm^{-3} of protein was used throughout this experiment and the relative CL intensity was shown based on the CL intensity of HSA (=1.00). As can be seen from Table 1, BαG and Ova showed no nonspecific adsorption, whereas HγG and BγG showed somewhat nonspecific adsorption with values of 0.14 and 0.07. Such nonspecific adsorption would be decreased by the ues of a monoclonal antibody, since a polyclonal antibody was used in the present experiment.

Comparison with Other Methods. The method for the determination of protein, which is based on the combination of CLCCIA and an immune column, has already been reported by the authors.[3] The concentration range for the determination of protein, the detection limit, and the sample amount necessary for the determination mentioned in the paper[3] were 2.5×10^{-5}—5×10^{-3} g dm^{-3}, 5 ng, and 200 mm^3, while those in the sandwich immunoassay in the present study were 1×10^{-5}—1×10^{-1} g dm^{-3}, 40 pg, and 4 mm^3.

Sepaniak et al. reported the determination of IgG in the concentration range 3.7×10^{-4}—1.8×10^{-1} g dm^{-3} by use of a fiber-optic immunosensor (D-I)[4] with fluorescence detection in which an antibody was directly immobilized on the plain end of an optical fiber. Aizawa et al. also reported the determination of protein in the concentration range 6.9×10^{-2}—2.1 g dm^{-3} HSA using a fiber-optic immunosensor (D-II)[6,7] with electro-chemical luminescence detection and in the concentration range 1×10^{-6}—1×10^{-2} g dm^{-3} IgG they used a fiber-optic immunosensor (D-III)[8] with chemiluminescence detection, in which an aromatic hydrocarbon or enzyme was used as a labeling agent, though the antibody was not directly immobilized on an optical fiber. In the present study, a highly sensitive immunosensor using an optical fiber immobilized with antibody directly on its plain end was newly developed; it was introduced into CLCCIA using a synthetic metal-complex catalyst for CL reaction as a labeling agent. The sensitivity of the present method was inferior to that of D-III but superior to those of D-I and D-II by more than 10 times.

The new fiber-optic immunosensor was characterized by the followings: 1) Highly sensitive for the determination of a small amount of protein, 2) applicable to a wide concentration range of protein sample, 3) feasible with a microvolume of sample, 4) suitable for the automation of operation, and 5) excellent regarding safety, cost, and treatment.

The authors wish to thank Mr. Masakatsu Imaki (Horiba, Ltd.) for providing the apparatus necessary for the present study.

The present study was supported by a Grant-in-Aid for General Scientific Research No. 63550567 from the Ministry of Education, Science and Culture.

References

1) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **56**, 2965 (1983).
2) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **57**, 587 (1984).
3) T. Hara, M. Toriyama, and M. Imaki, *Bull. Chem. Soc. Jpn.*, **58**, 1299 (1985).
4) B. J. Tromberg, M. J. Sepaniak, T. Vo-Dinh, and G. D. Griggin, *Anal. Chem.*, **59**, 1226 (1987).
5) R. D. Petrea, M. J. Sepaniak, and T. Vo-Dinh, *Talanta*, **35**, 139 (1988).
6) Y. Ikariyama, H. Kunoh, and M. Aizawa, *Biochem. Biophys. Res. Commun.*, **128** (1985).
7) M. Aizawa, Y. Ikariyama, M. Tanaka, and H. Shinohara, *Pro-Electrochem. Soc.*, **1987**, 362.
8) M. Aizawa, *Protein, Nucleic Acid and Enzyme, Bessatsu*, No. 31, 272 (1987).
9) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **56**, 2267 (1983).
10) J. R. Sportsman and G. S. Wilson, *Anal. Chem.*, **52**, 2013 (1980).
11) N. Hibi and H. Hirai (1978) in Enzyme immunoassay (E. Ishikawa, T. Kawai, and K. Miyai) pp. 136—146, Igaku-shoin, Tokyo.
12) E. Ishikawa, "Koso-meneki-sokutei-ho no saishin-gijutsu to monokuronaru-kotai heno oyo no jissai", Kogaku-joho-senta.