Immunoassay Using a Metal-complex Compound as a Chemiluminescent Catalyst. V. Continuous Immunoassay by the Use of CLCCIA

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Immunoassay, using as a labeling reagent a synthesized metal-complex compound having catalytic activity for a chemiluminescence reaction, was continuously carried out by the sandwich method. The time necessary for all the processes (including: an immune reaction, B/F separation, a sandwich reaction, and a chemiluminescence reaction) was reduced to 50 min. Human serum albumin in the range of 5 ng to 1 μg could be determined (continuously) up to 5 times by the use of the same column (immobilized with an antibody).

In order to determine a trace amount of the component in a biological sample, immunoassay is an important method. So far, enzyme immunoassay and radioimmunoassay have been used for practical purposes. Unfortunately, they have necessitated a tedious batchwise operation. Accordingly, studies regarding automatic and continuous immunoassay have been made for nonlabeled immunoassay such as laser nephelometry and latex-photometric immunoassay. However, few such attempts have been made for a labeled immunoassay, which is theoretically suitable for a very sensitive assay. This is attributed to the instability of labeling reagents such as enzymes and radioactive materials.

Chemiluminescence complex catalyst immunoassay (CLCCIA),[1-4] in which a synthesized metal-complex compound acting as a catalyst for a chemiluminescence reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and hydrogen peroxide (H_2O_2) was used as a labeling reagent, was reported by the authors for the determination of human serum albumin (HSA) as a model compound for the protein. In a previous paper,[3] the immune complex after the immune reaction could be dissociated in a liberating solution(pH 11.4) without losing its catalytic activity for the chemiluminescence reaction since the synthesized metal-complex as a labeling reagent in CLCCIA was stable. Then, the measurement of the catalytic activity of the dissociated labeling reagent resulted in a rapid and accurate immunoassay. However, the method was far from the immunoassay in which all the processes (including: an immune reaction, B/F separation, a chemiluminescence reaction, and the cyclic use of the immobilized antibody) could be continuously carried out.

The present study deals with a method and a support suitable for the immobilization of an antibody and with an immune reaction in order to establish continuous CLCCIA in which all the immunoassay processes (including: an immune reaction, B/F separation, a sandwich reaction, a chemiluminescence reaction, and the cyclic use of the immobilized antibody) are continuously carried out.

Experimental

Preparation of a Labeled Antibody. [1,8,15,22-tetrakis-(chloroformyl)phthalocyaninato]iron(III) ([Fe(tccp)]) as a labeling reagent was synthesized as described in a previous paper.[2] 500-mm³ of a phosphate buffer solution was added to 2 cm³ of an immunoglobulin G(IgG) fraction of anti-human serum albumin(TAGO) so as to maintain the mixed solution at pH 7.2. Five mg of [Fe(tccp)] was dissolved in the above mixed solution, and the resulting solution was then reacted for 1 h at room temperature. Then, the mixed solution was incubated for 18 h at 4 °C. After the reaction, 2.5 cm³ of a potassium dihydrogenphosphate–disodium hydrogenphosphate buffer solution (0.01 mol/dm³, pH 7.2) (Buffer A) was added to the mixture. The mixture was centrifuged at 6000 min^{-1}, and the supernatant liquid was separated from the precipitate. The precipitate was treated with 2.5 cm³ of Buffer A and the supernatant liquid was again obtained by centrifuge. To the combined supernatant liquid, 5 cm³ of saturated ammonium sulfate solution was added and it was left standing at 4 °C overnight. The precipitate was separated by centrifuging and then dissolved in 2 cm³ of Buffer A. After the obtained solution was dialysed against Buffer A, the contents were separated on a column (160 cm³) charged with TOYOPEARL HW-50, and a 20-cm³ fraction corresponding to IgG was collected.

Preparation of Glass Beads Immobilized with Antibody. Five g of porous glass beads (CPG-3000, 120/200 mesh, 31250 nm mean pore diameter, ELECTRO-NUCLEONICS, INC.) were heated at 100 °C for 1 h on a water bath in 75 cm³ of a 1 mol/dm³ hydrochloric acid solution and a nitric acid solution. The beads were filtered, washed with water, and dried at 60 °C in an oven. The purified porous glass beads were suspended in 200 cm³ of 1% [3-(glycidyloxy)propyl]trimethoxysilane solution. The slurry was degassed under vacuum in an ultrasonic bath and then was heated at 90 °C for 2 h with stirring. The pH of the mixture was adjusted to 3 with a 1 mol/dm³ hydrochloric acid solution, and the mixture was heated again at 90 °C for 1 h. The diol-bonded porous glass beads, thus obtained, were washed with water and dried. Two g of the porous glass beads were placed in 20 cm³ of 6 mmol/dm³ of sodium periodate solution. A vacuum was applied for about 2 min to degas the slurry. The slurry was stirred for 1 h at room temperature, and then was washed with 100 cm³ of Buffer A. A 500-mm³ of phosphate buffer solution was added to 2 cm³ of IgG fraction of anti-human serum

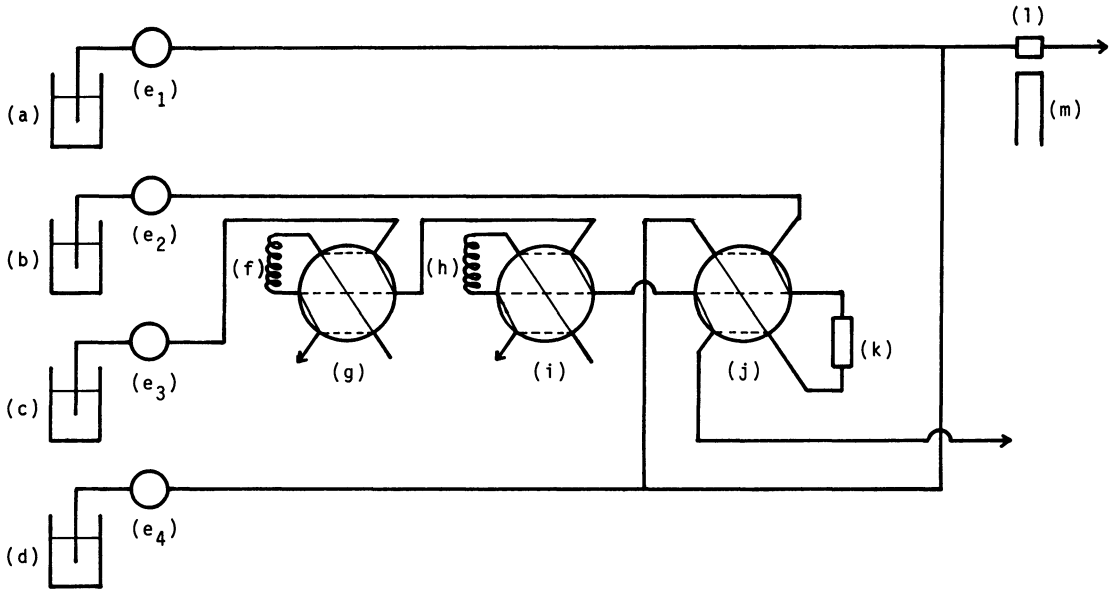
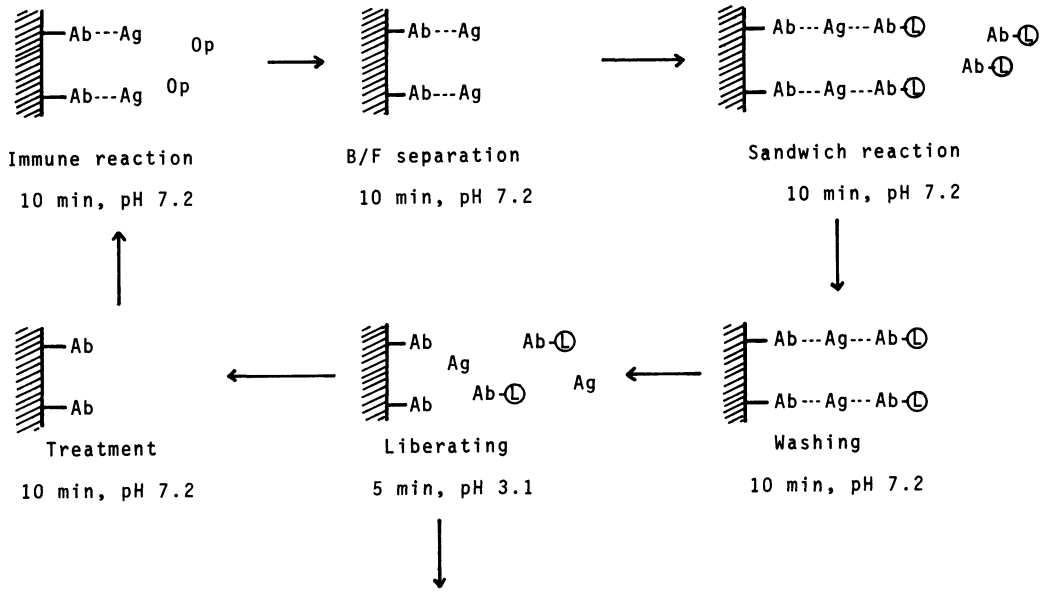


Fig. 1. Schematic flow diagram.
a: Luminol solution, b: liberating solution, c: Buffer A, d: H_2O_2 solution, e_1, e_2, e_3, e_4: pump, f,h: sampling loop, g,i,j: six-way cock, k: immobilized antibody column, 1: flow-cell, and m: photomultiplier.



Scheme 1. Operating procedure for the continuous CLCCIA.
Ab: Antibody, Ag: antigen, Op: other protein, and L: [Fe(tccp)].

albumin(TAGO) to adjust the pH at 8.8. The aldehyde-type porous glass beads recovered from the above slurry were added to the mixed antibody solution and were reacted with the antibody for 50 h at 4 °C. After the reaction, the porous glass beads were washed with Buffer A, and were suspended in 200 cm^3 of Buffer A. Each 100-mm^3 of 40 mg/cm^3 sodium borohydride solution was added four times to the suspended solution every 30 min, and then the mixed solution was reacted for 2 h. The porous glass beads immobilized with the antibody were washed and stored in Buffer A at 4 °C.

Apparatus and Procedure. A schematic flow diagram of the apparatus used for the continuous CLCCIA is shown in Fig. 1. Its operating procedure is shown in Scheme 1.

Each bottle in Fig. 1 was filled with (a) a 1.0×10^{-3} mol/dm^3 luminol solution (pH 10.4, 0.4 mol/dm^3 boric acid-0.4 mol/dm^3 potassium hydroxide buffer solution), (b) a tartrate buffer solution (pH 3.1, 4.45×10^{-3} mol/dm^3 tartaric acid- 1.11×10^{-3} mol/dm^3 sodium tartrate buffer solution) as a liberating solution, (c) Buffer A, and (d) a 7.5×10^{-3} mol/dm^3 H_2O_2 solution. Each solution, except Buffer A(c), was fed at a flow rate of 1.65 cm^3/min by the pump (e_1, e_2, e_4) (Atto,

SJ1211). Buffer A in Fig. 1(c) was fed at a flow rate of 1.65 cm^3/min or 0.24 cm^3/min by the pump(e_3)(Atto, SJ1211). The porous glass beads immobllized with antibody were charged into a 88 mm^3 column(k).

The capacity of the sampling loop(h) was 200 mm^3, and each definite volume of HSA solution as an analyte model was injected into a flow line by the use of a sample injection six-way cock(i). Injected sample(antigen) was introduced into the column(k) at a flow rate of 0.24 cm^3/min and was made to react with the immobilized antibody. After the immune reaction, the six-way cock(i) was operated and B/F separation was accomplished by washing the column with Buffer A for 10 min at a flow rate of 1.65 cm^3/min. Then, 200 mm^3 of labeled antibody fraction(which was 50 times diluted with Buffer A) was taken in a sampling loop(f) and was injected at a flow rate of 0.24 cm^3/min by operating a six-way cock(g) into a flow line. The injected labeled antibody was subjected to a sandwich-type reaction in the column. After the reaction, a labeled antibody which was adsorbed unspecifically was eliminated by washing the column with Buffer A for 5 min at the flow rate of 1.65 cm^3/min by operating again the six-way cock(g). By operating the six-way cock(j), a tartrate buffer solution was fed into the column and the immune complex was liberated from the column. The liberated labeled antibody was mixed with H_2O_2 and luminol, and the chemiluminescence intensity of the mixture was measured by a system consisting of a flow-cell, a photomultiplier, and a photon counter as described in a previous paper.[2]

Results and Discussion

Preparation of Labeled Antibody. In order to label efficiently [Fe(tccp)] to antibody, the conditions of labeling reaction were investigated.

Labeling reactions were produced at various pH of buffer solutions, and the labeled HSAs were obtained by the procedure discussed in the experimental section. By assuming that the molar absorptivity of HSA at 280 nm and of [Fe(tccp)] at 639 nm does not change upon labeling, the approximate mole ratio of [Fe(tccp)] to HSA was obtained by the use of the calibration curves for [Fe(tccp)] and HSA. Here, the mole ratio is referred to as a labeling ratio, and HSA is used as a antibody model only in this part of the present study (to make the experiment easier). The relationship between the pH of a solution in the labeling reaction and a labeling ratio of HSA was studied. The results are shown in Fig. 2.

As can be seen from Fig. 2, the reaction between an acyl chloride group of [Fe(tccp)] and an amine group of HSA proceeded efficiently in a reacting solution at pH 7 to 8. Therefore, the labeling of the antibody was carried out in Buffer A (pH 7.2).

Preparation of Immobilized Antibody. The three methods (glutaraldehyde method, cyanogen bromide method, and periodate method) as an immobilization procedure of antibody were compared with each other.

The glutaraldehyde method by which an immobilization reaction can be easily produced, has been applied to the immobilization of many kinds of enzymes[5] and antibodies.[6] In the previous CLCCIA an

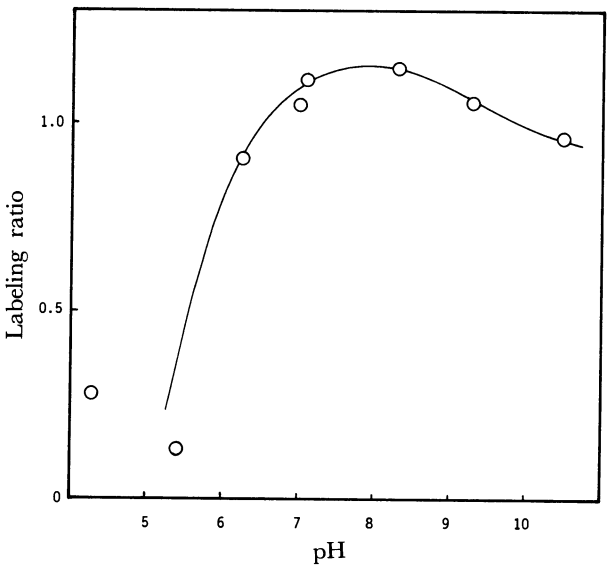


Fig. 2. Relationship between pH of solution in labeling reaction and labeling ratio of HSA.

antibody was immobilized on a glass bead of 4-mm diam. by the glutaraldehyde method. The antibody was combined with the support through an imine-type bond by the use of glutaraldehyde. As this imine-type bond is relatively weak, it dissociates easily in an acidic solution. Therefore, the immobilized antibody glass bead could not be used again after the dissociation of the immune complex on the glass bead with an acidic liberating solution.

There have been many reports in which various kinds of enzymes and proteins were immobilized on activated cellulose with cyanogen bromide. The authors were also able to immobilize albumin and galactose oxidase with good results[7] by the cyanogen bromide method. Immobilization of an antibody onto cellulose beads (Cellulofine, GC-700m, Chisso) by the cyanogen bromide method was further attempted, but the immobilized antibody, thus obtained, did not react with an antigen. This seemed to be due to the fact that the distance between the support and the antibody was too short to allow the antibody to react with the antigen.

Finally, the immobilization of an antibody on glass beads (CPG-3000) was attempted by the periodate method.[8] Glycosil bonded glass beads were converted to their aldehydic form by oxidation with sodium periodate. This formyl group and an amino group of antibodies were made to react. An imine-type bond was formed. The imine-type bond was reduced to amine-type bond with sodium borohydride, and the bond between the antibody and the support was strengthened. The immobilized antibodies, thus obtained by the periodate method, could not only be used repeatedly for an immune reaction, but also they showed little unspecific adsorption of other proteins.[9] In the present study, the periodate method was chosen as the immobilization method of the antibody.

An attempt was made to reduce an antibody im-

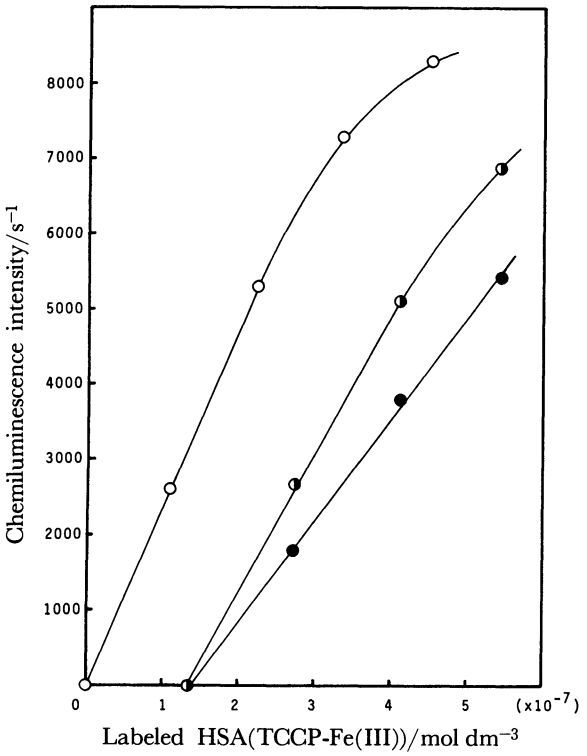


Fig. 3. Effect of volume of immobilized antibody in column.
○: 88 mm^3, ◑: 177 mm^3, and ●: 357 mm^3.

mobilized by the glutaraldehyde method with sodium borohydride, but the immobilized antibody did not show an effective immune reaction.

Immunoassay. There are competitive and sandwich methods as labeled immunoassay. A competitive immunoassay technique was used in a previous CLCCIA because the operation of the immunoassay was simple. However, the conditions of an immune reaction (the mixing ratio of a sample antigen to a labeled antigen, the desired amount of immobilized antibody, *etc.*) must be closely controlled. The control of these conditions of immune reaction is easy in the case of batchwise immunoassay, but it is difficult for continuous immunoassay in which an immobilized antibody is repeatedly used because the activity of an immobilized antibody for an immune reaction may be changed. Therefore, use was made, for immunoassay in this investigation, of the sandwich method which could be expected to be highly sensitive, though it was accompanied with a number of steps.

In order to determine the optimum volume of an immobilized antibody column, the following experiment was carried out. An immune reaction was carried out by passing labeled HSA with [Fe(tccp)] as a sample through the immobilized antibody column (Fig. 1 (k)). Here, three kinds of immobilized antibody columns were used. The catalytic activity of the labeled HSA in a liberating solution was measured for the chemiluminescence reaction between luminol and H_2O_2. The results are shown in Fig. 3. The chemiluminescence intensity increased with a decrease in the volume of the immobilized antibody column, that is, the labeled HSA liberated from the immobilized antibody column increased. Much labeled HSA was found to be unspecifically adsorbed with an increase of the column volume. On the basis of this experimental result, the volume of the immobilized antibody column was determined to be 88 mm^3.

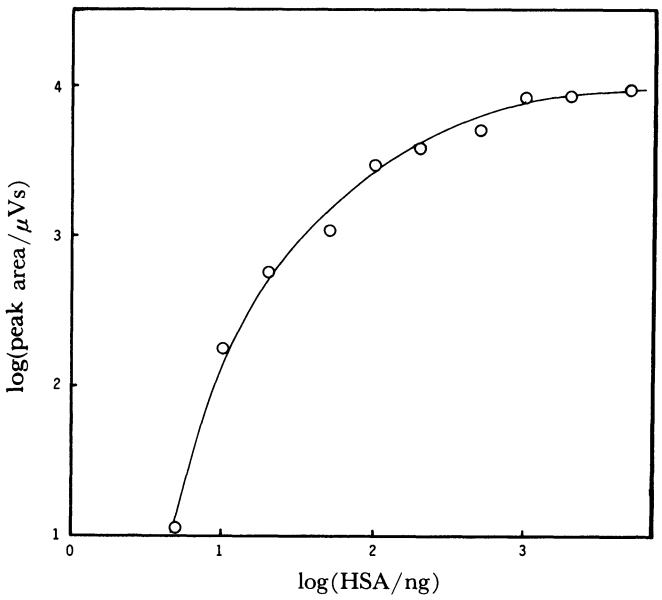


Fig. 4. Standard curve of HSA.

The relationship between the immune reaction time in the immobilized antibody column and a peak area of chemiluminescence was examined. The experiment was made as follows: 1) labeled HSA was used as a sample, 2) the flow rate of Buffer A, which was used to feed labeled HSA into the column was changed so as to give various immune reaction times. The immune reaction time was defined as the residence time of a sample in the column. The peak area of chemiluminescence increased with an increasing immune reaction time. The capacity of pump (e_3) allowed the flow rate of the sample solution to be brought to 0.24 cm^3/min, and the immune reaction time became 11 s.

To determine the washing time necessary for the removal of an excess labeled antibody from the immobilized antibody column, the relationship between washing time and intensity of chemiluminescence blank was examined. The intensity of the chemiluminescence blank was found to be almost constant at a washing time of more than 4 min. Hereafter, the immobilized antibody column was washed with Buffer A for 5 min after the sandwich reaction.

In accordance with the procedure shown in Scheme 1, a standard curve of HSA was obtained (Fig. 4). A peak area of a sample was presented by subtracting the blank peak area of unspecifically adsorbed labeled antibody from the peak area of experimental data. According to the present method, HSA in the range from 5 ng to 1 μg could be determined. The detection limit became a

much smaller value than that in the previous competitive CLCCIA (20 ng). Further, the time necessary for all the immunoassay processes (including: an immune reaction, B/F separation, a sandwich reaction, and a chemiluminescence reaction) could be reduced to 50 min from 10 h or more in the previous CLCCIA. The immunoassay of 20 ng HSA as a sample was by the use of the same immobilized antibody column. The coefficient of variation was 17% for 5 replicate analyses while 40% for 10 replicate analyses. Since the coefficient of variation up to about 20% for the immunoassay of the present level had been generally allowed, it was concluded that a continuous CLCCIA of up to 5 times could be carried out by the use of the same immobilized antibody column. In order to make continuous CLCCIA a practical automatic method of immunoassay, the immobilized antibody has to be further improved so as to increase the number of continuous analyses.

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