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Immunoassay Using a Metal-complex Compound as a Chemiluminescent Catalyst. II. An Improvement of the Analytical Method for Practical Use

Tadashi HARA,* Motohiro TORIYAMA, and Kazuhiko TSUKAGOSHI
Dapartment of Chemical Engineering, Faculty of Engineering, Doshisha University, Karasuma Imadegawa, Kamigyo-ku, Kyoto 602
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Immunoassay using a synthesized metal-complex compound as a labeling reagent has been investigated. Iron(III) 2,9,16,23-tetrakis(chlorocarbonyl)phthalocyanine, which showed a marked catalytic action in the chemiluminescence reaction between luminol and H_2O_2, was synthesized, and albumin was labeled with it. Albumin in the range from 50 ng/tube to 50 μg/tube could be determined by measuring the chemiluminescence intensity in a competitive immunoassay using glass beads immobilized with an antibody. The present method became about 40 time as sensitive as the previous method by optimizing the labeling procedure, the chemiluminescence reaction, and the immune reaction.

A chemiluminescence complex catalyst immunoassay (CLCCIA), in which a synthesized metal-complex compound acting as a catalyst for chemiluminescence reaction was used as a labeling reagent, was previously reported by the authors for the determination of human serum albumin(HSA) as a model compound for the protein.[1] Since the CLCCIA was carried out by measuring the catalyst effect of conjugated iron(III) 2,9,16,23-tetracarboxyphthalocyanine(TCP–Fe(III)) on the chemiluminescence reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione(luminol) and hydrogen peroxide(H_2O_2), CLCCIA was expected to have the following advantages: 1) A sensitive and rapid analysis can be made by the use of a simple and inexpensive apparatus; 2) the TCP–Fe(III) used as a labeling reagent is more stable and cheaper than a radioactive isotope and enzyme; 3) no steric hindrance of the immune reaction is caused by TCP–Fe(III) because its molecular weight is very small as compared with that of enzyme, and 4) no counterplan is necessary for hazards of radioactivity. These advantages were almost all verified in the previous work, but its sensitivity was still unsatisfactory for practical use in immunoassay.

In order to make the CLCCIA a practical method of immunoassay, the following were examined: 1) The carboxyl group in TCP–Fe(III) used as a labeling reagent in the previous work was converted into acyl chloride, and the product, iron(III) 2,9,16,23-tetrakis-(chlorocarbonyl)phthalocyanine(TCCP–Fe(III)), was used as a labeling reagent in the present work. Labeled HSA was easily obtained by only adding TCCP–Fe(III) to a HSA solution; it was more homogeneous than that obtained by the tedious and complicated carbodiimide method. 2) Optimization for chemiluminescence reaction was made with regard to the experimental conditions, such as the luminol concentration, the H_2O_2 concentration, and the total volume of the mixed solution. 3) Optimization was also made for the amounts of labeled HSA and the sample which were used for the competitive immunoassay. As a result, an improved CLCCIA was found to be about 40 times as sensitive as the previous method. This sensitivity enabled an improved CLCCIA to determine HSA on a picomole level which corresponded to the practical sensitivity in a common immunoassay.

Experimental

Reagents. TCCP–Fe(III) was synthesized by the acyl chlorination of TCP–Fe(III).[2] The labeling of HSA with TCCP–Fe(III) was made by the following method: Thirty milligrams of HSA (Sigma, Fraction V) was dissolved in 10 cm^3 of a potassium dihydrogenphosphate-disodium hydrogenphosphate buffer solution (pH 7.2), a 5-mg portion of TCCP–Fe(III) was added to it, and the mixed solution was incubated for 48 h at 4 °C. After the reaction, the mixture was centrifuged at 10000 *g*, and the precipitate was removed. To the supernatant we then added 10 cm^3 of a saturated ammonium sulfate solution ,and the mixed solution was held at 4 °C overnight. The supernatant was removed by centrifugation at 10000 *g*, and the precipitate thus obtained was dissolved in 1 cm^3 of a phosphate buffer solution (pH 7.0), after which the solution was dialyzed to remove the ammonium sulfate. After dialysis, the precipitate was removed by centrifugation, and the supernatant was submitted to gel chromatography on a TOYOPEARL HW-50 (TOYO SODA) column (150 cm^3), using a phosphate buffer solution (pH 7.0) as an eluent. Twenty cubic centimeters of an albumin fraction containing HSA labeled with TCCP–Fe(III) were thus obtained.

The glass beads immobilized with the antibody (Ab-bead) were prepared by the previously reported method.[1] The other reagents used were of a commercially available analytical grade.

Apparatus. The same apparatus as in the previous paper was used for the measurement of the chemiluminescence intensity (CL intensity).[1]

Procedure. Seventy cubic millimeters of a phosphate buffer solution (pH 7.2), containing a definite amount of HSA as an analyte and 30 mm^3 of a labeled HSA solution (containing about 200 ng of conjugated TCCP–Fe(III)), were placed in a 50×6 mm diam. test tube, and one particle of the Ab-beads was soaked in its solution. Thus, the labeled HSA and the free HSA used as an analyte were made to react competitively overnight at 4 °C with the antibody immobilized on the Ab-beads. After the immune reaction, the Ab-beads were twice washed with a phosphate buffer solution (pH 7.2). On the other hand, 0.5 cm^3 of a 1.0×10^{-3} mol/dm^3 luminol solution (pH 10.2; a boric acid–potassium hydroxide buffer solution) and 0.5 cm^3 of a 7.5×10^{-3} mol/dm^3 H_2O_2 solution were mixed in a 90×10 mm diam. test tube; the mixture was then allowed to stand for 30 min before measurement to stabilize

the chemiluminescence background. The above Ab-beads were put into the mixed solution, and the CL intensity was immediately measured by means of the photon counter.

Results and Discussion

Labeling with TCCP-Fe(III). The labeling of HSA with TCP-Fe(III) in the previous paper was carried out by allowing the carboxyl group of TCP-Fe(III) to combine covalently with the amino group of HSA by the use of the water-soluble carbodiimide reagent. However, this labeling method resulted in lowering the homogeneity of labeled HSA because in HSA containing both carboxyl and amino groups the two groups easily combined with each other.

The labeling of HSA with TCCP-Fe(III) in the present work was easily carried out by adding TCCP-Fe(III) as an acyl chloride to a HSA solution, and no linkage was produced between one HSA molecule and others. The absorption spectra of HSA, TCP-Fe(III), TCCP-Fe(III), HSA labeled with TCP-Fe(III) by the carbodiimide method, and HSA labeled with TCCP-Fe(III) by the present method were measured by the use of their respective phosphate buffer solutions (pH 7.0); the results are shown in Fig. 1. Each albumin fraction prepared by gel chromatography was used for the respective HSA labeled with TCP-Fe(III) and TCCP-Fe(III) (*cf.* Fig. 1). The spectrum of TCCP-Fe(III) was obtained after TCCP-Fe(III) had been dissolved in a phosphate buffer solution (pH 7.0) and the solution kept at 4 °C for 48 h. As can be seen from Fig. 1, the spectrum of TCCP-Fe(III) agreed well with that of TCP-Fe(III). Each shoulder at 675 nm, which was not quite observed for TCP-Fe(III) and TCCP-Fe(III) alone, was newly found for HSA labeled with TCP-Fe(III) and TCCP-Fe(III) respectively. In addition, slight peak shifts at 325 and 639 nm were also observed for both labeled HSA's. It still remains to be determined if the above-mentioned phenomena are based on either the formation of a covalent bond between HSA and a labeling reagent or the formation of a coordination linkage between the functional group of HSA and the residual coordination sites of the iron(III) complex as a labeling reagent. The absorption spectrum of HSA labeled with TCCP-Fe(III) was rather more similar to that of TCP-Fe(III) than to that of HSA labeled with TCP-Fe(III). By assuming that the molar absorptivity of HSA at 280 nm and of TCCP-Fe(III) and TCP-Fe(III) at 639 nm do not change upon labeling, the approximate mole ratio of a labeling reagent to HSA was obtained by the use of the calibration curves for TCCP-Fe(III), TCP-Fe(III), and HSA, the results being TCCP-Fe(III) : HSA=1 : 1 and TCP-Fe(III) : HSA=1 : 5. It is concluded from the amount of the conjugated iron(III) complex that the present method using TCCP-Fe(III) as a labeling reagent is superior to the carbodiimide method using TCP-Fe(III) as a labeling reagent.

Optimum Conditions for the Measurement of the CL Intensity. To find out the optimum conditions for the measurement of the CL intensity, the effects of the concentrations of H_2O_2 and luminol and of the total volume of a H_2O_2–luminol mixed solution on the CL intensity were examined. The CL intensity was measured by the use of Ab-beads which had been submitted to an immune reaction overnight at 4 °C in 100 mm^3 of a phosphate buffer solution (pH 7.2) containing 30

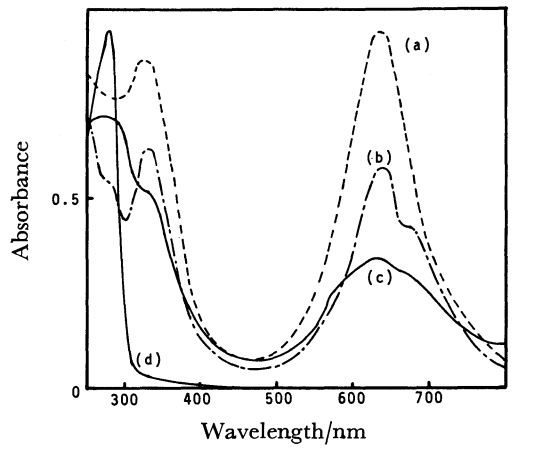


Fig. 1. Absorption spectra.
a: TCP-Fe(III) and TCCP-Fe(III), b: HSA labeled with TCCP-Fe(III), c: HSA labeled with TCP-Fe(III), and d: HSA.

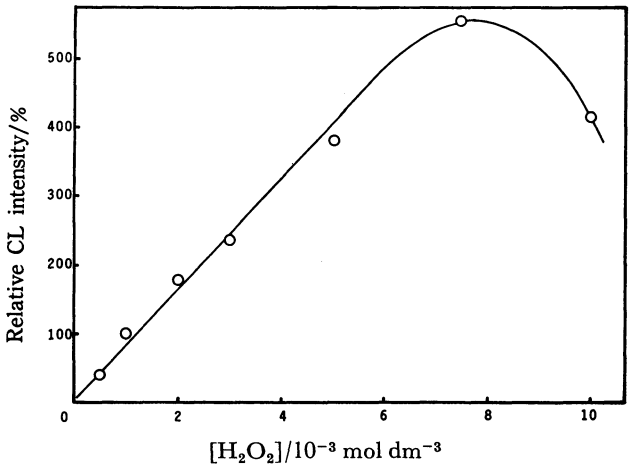


Fig. 2. Effect of H_2O_2 concentration on relative CL intensity at 1.0×10^{-3} mol/dm^3 luminol.

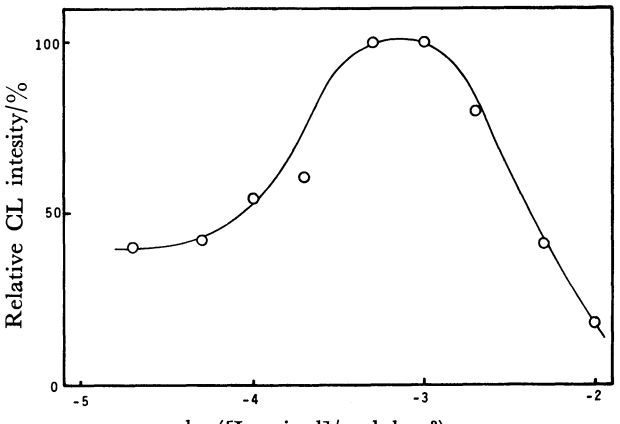
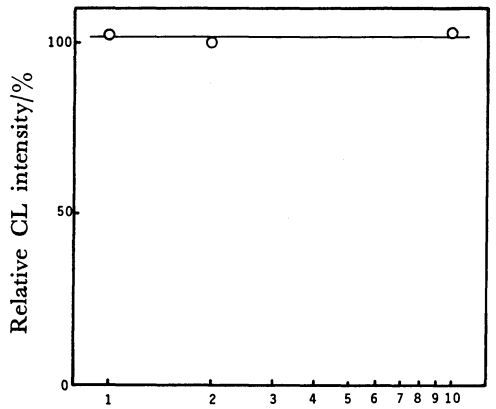
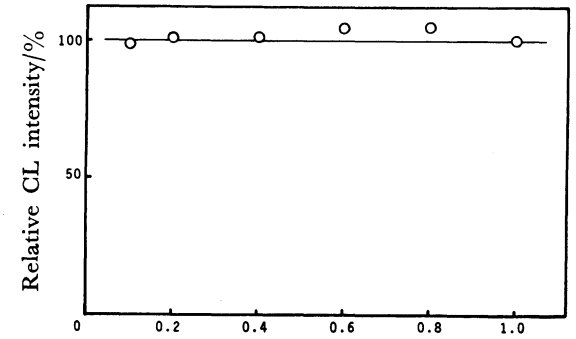


Fig. 3. Effect of luminol concentration on relative CL intensity at 7.5×10^{-3} mol/dm^3 H_2O_2.



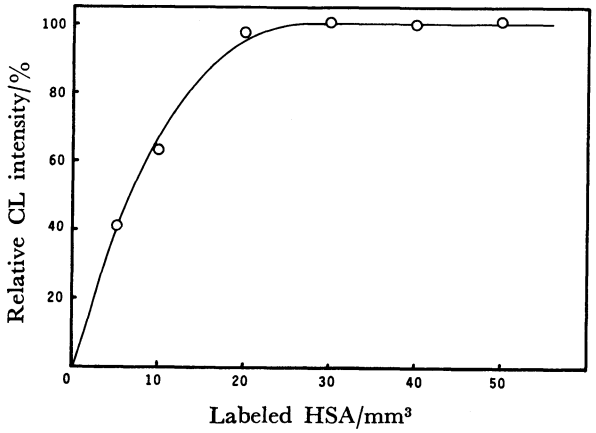
log(Volume of a mixed solution/cm³)

Fig. 4. Relationship between volume of luminol–H_2O_2 mixed solution and relative CL intensity at 1.0×10^{-3} mol/dm³ luminol and 7.5×10^{-3} mol/dm³ H_2O_2.



Volume of immune reaction solution/cm³

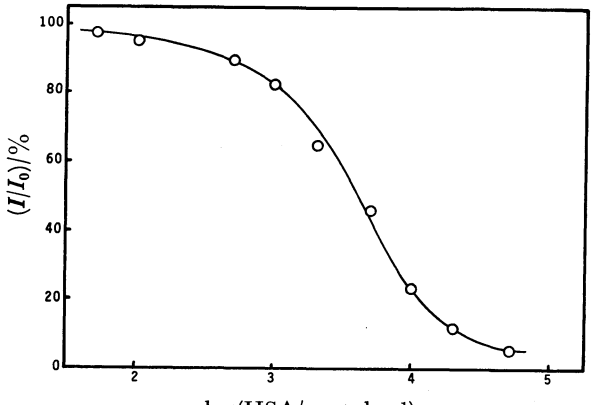
Fig. 5. Relationship between total aqueous volume in immune reaction and relative CL intensity in a mixed solution of luminol (0.5 cm³, 1.0×10^{-3} mol/dm³) and H_2O_2 (0.5 cm³, 7.5×10^{-3} mol/dm³).



Labeled HSA/mm³

Fig. 6. Relationship between volume of labeled HSA and relative CL intensity.

mm³ of a labeled HSA solution. The results are shown in Figs. 2, 3, and 4. The CL intensity increased proportionally with an increase in the concentration of H_2O_2 and gave a maximum value at 7.5×10^{-3} mol/dm³ H_2O_2, followed by its decrease. The maximum CL intensity was also obtained at 1.0×10^{-3} mol/dm³ luminol. The total volume of a luminol–H_2O_2 mixed solution did not affect the CL intensity.



log(HSA/ng tube^{-1})

Fig. 7. Standard curve for HSA.
I, I_0: CL intensity of the sample in the presence or absence of HSA.

On the basis of these results, 0.5 cm³ of a 7.5×10^{-3} mol/dm³ H_2O_2 solution and 0.5 cm³ of a 1.0×10^{-3} mol/dm³ luminol solution were mixed to obtain an optimum result.

Optimum Conditions for a Competitive Immune Reaction. The relationship between the CL intensity and the total aqueous volume for an immune reaction is shown in Fig. 5. A definite concentration of labeled HSA was allowed to react with the antibody on Ab-beads at a total aqueous volume of from 0.1 to 1 cm³. After the immune reaction, each Ab-bead showed approximately the same CL intensity. Since a minimum aqueous volume of 0.1 cm³ was necessary to keep an Ab-bead in a solution, a total aqueous volume of 0.1 cm³ was recommended for practical use.

The amount of labeled HSA required for a competitive immune reaction with HSA was examined by changing the amount of labeled HSA in a 0.1 cm³ aqueous solution and by measuring the CL intensities, the results are shown in Fig. 6. As can be seen from Fig. 6, the active site of an antibody on an Ab-bead was completely saturated with more than 30 mm³ of labeled HSA. To satisfy the requirements that 1) the total amount of antigen and labeled antigen has to surpass the corresponding amount of the immobilized antibody in a competitive immunoassay and 2) the sensitivity for a competitive immunoassay has to be enhanced as much as possible, 30 mm³ of labeled HSA was chosen for the immunoassay.

Immunoassay. The immunoassay of HSA was carried out by the use of HSA labeled with TCCP–Fe(III), and a standard curve was obtained for HSA of from 50 ng/tube to 50 μg/tube (Fig. 7). For the preparation of the standard curve for HSA, the CL intensities of five equal amounts of HSA were separately measured, and their mean value(I) was obtained. The relative CL intensity(I/I_0) was represented as the ratio of I to the CL intensity(I_0) of a labeled HSA alone. The CL intensity of a HSA of more than 50 ng/tube was always smaller than the I_0 value, while the CL intensity of a HSA of less than 50 ng/tube was often larger than the I_0 value. Therefore, 50 ng/tube of HSA was defined

TABLE 1. IMMUNOASSAY USING A METAL COMPLEX COMPOUND AS A LABELING REAGENT

Immunoassay	Method		Chemical amplification[a)]	Detection limit	Sample	Analyte
	Component	Measurement				
CLCCIA	Catalytic activity	Chemiluminescence	+	50 ng/tube (7.6×10^{-9} mol/dm^3)	70 mm^3	HSA
VIA	In	Differential pulse anodic stripping voltammetry	−	5.0 μg/cm^3 (7.6×10^{-8} mol/dm^3)	5000 mm^3	HSA
MIA	Fe, Mn	Atomic-absorption spectrometry	−	0.1 μg/cm^3 (3.5×10^{-7} mol/dm^3)	30 mm^3	Oestrogen steroids (hapten)

a) +, −: Shows the presence or absence of chemical amplification.

as the detection limit. Judging from the detection limit for the previous and the present methods, the present method was about 40 times as sensitive as the previous method. This enhancement is attributed to 1) the optimization of the chemiluminescence reaction and the immune reaction, and 2) the use of HSA labeled sufficiently with TCCP–Fe(III). Voltammetric immunoassay (VIA)[3] and metalloimmunoassay (MIA)[4,5] have previously been reported as immunoassays using a metal-complex compound as a labeling reagent. In Table 1 they are compared with the present method. According to the present method, the picomole level of albumin can be determined; this sensitivity satisfies the sensitivity required for a practical immunoassay. Since the present method makes use of the catalytic action of a metal-complex compound as a labeling reagent, its sensitivity would be much more improved by using a sandwich method or some other such method. Further study is now in progress.

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