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Highly Sensitive Electrochemiluminescence Immunoassay Using the Ruthenium Chelate-Labeled Antibody Bound on the Magnetic Micro Beads

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The newer electrochemiluminescence (ECL) immunoassay system was established by using both an antibody coated on paramagnetic micro-beads (Capt-MB) as a carrier of the immunoassay and an antibody labeled with ruthenium(II) tris-bipyridine-NHS (Ru-Ab). The ECL excitations were designed to be generated upon the surface of the working electrode which collected the reacted Capt-MB by magnetic force. As a model of the immunoassay, the reaction between alpha-fetoprotein (AFP) and anti-AFP antibodies was used in accordance with the so-called sandwich method, where the sandwich conformation of AFP-(Ru-Ab) was made on the surface of the Capt-MB. The ECL immunoassay system revealed the following results: a) the ECL signal intensity was obtained in proportion to the AFP concentration of each specimen; b) the dynamic range of this ECL immunoassay system was extended to 10000 times of magnitude in the 2-step assay; and c) the detection sensitivity reached to the level of 5 pg/ml in the AFP concentration after 15 min in the 1-step immunoassay.

Keywords Electrochemiluminescence, immunoassay, ruthenium(II) tris-bipyridine, magnetic-beads, alpha-fetoprotein

Since the ECL phenomenon with ruthenium(II) tris-bipyridine ($Ru(bpy)_3^{2+}$: Ru-chelate) in an aqueous solution was identified by Rubinstein in 1981[1], Bard and his coworkers showed many applications of ECL for the measurement of chemical and/or biological substances such as oxalate and total DNA measurement.[2-5]

A preliminary ECL immunoassay was reported by Bard in 1984, using a newly synthesized Ru-chelate having *N*-hydroxysuccinimide residue (Ru-chelate-NHS), which enabled the protein moiety to be labeled[6] (Fig. 1). Further studies on ECL immunoassays were carried out by Igen International. In 1990, Leland improved the sensitivity of ECL detection by adding tripropylamine (TPA) to the electrolyte solution; this showed a strong reductant activity after electrochemical oxidation.[7] This catalytic reaction of oxidized TPA extended the detection limit of the Ru-chelate to 0.2 pmol, which was approximately 10 thousand times more sensitive than that initially reported by Ege in 1984.[8]

Meanwhile, the first actual ECL immunoassay was reported by Ikariyama *et al.* in 1985, using the pyrene labeled human serum albumin (HSA) as the competitive and homogeneous immunoassay method.[9] Their report demonstrated that the intensities of the photon emission were correlated with the amounts of the analyte antigen that did not emit the photon.

Generally, an electrochemical reaction occurs within a limited reaction layer, the so-called electrical double layer and/or the electrical diffuse layer at several nm distances from the surface of the working electrode (WE). Consequently, the ECL emission might be interfered with by the increase in molecule size of the labeled HSA, if they bonded with the corresponding antibody molecule having a size of ten and several nm. Thus, the major ECL emission reported by Ikariyama *et al.* might be caused by non-reacted labeled HAS, because its molecular size is approximately 30% of the labeled HSA binding with the antibody.

Based on the above considerations, only an extremely small quantity of the analyte that contacts with the sur-

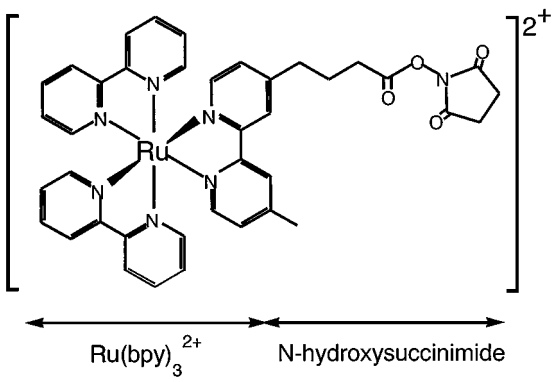

Fig. 1 Ruthenium(II) tris-bipyridine *N*-hydroxysuccinimide ester.

face of the WE may be utilized for the ECL detection. Accordingly, if all the ECL labeled materials could be collected over the surface of the WE, much more ECL emission might be generated and a highly sensitive ECL immunoassay could be realized.

Thus, one of the authors as well as Leland *et al.* developed new, highly sensitive ECL immunoassay methods using the paramagnetic beads (MB) as a solid phase carrier for the sandwich immunoassay using antibody labeled with Ru-chelate-NHS (Ru-Ab).[10] And the ECL excitations were made by collecting the immune-reacted MB over the surface of the WE by magnetic force (Particle Based ECL).

In this report, further detailed studies on the above-mentioned newer ECL immunoassay methods to detect the alpha feto-protein (AFP) using the MB will be introduced.

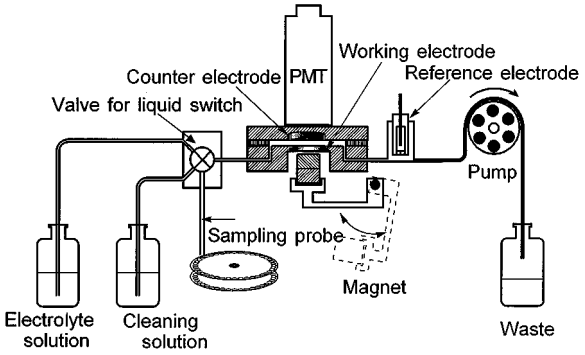

Fig. 2 Schematic composition of ECL detection system specified for the Particle Based ECL measurement. The flow-cell was 0.5 mm in thickness and 60 μl in capacity. In a typical sequence, while the peristaltic pump is drawing in the measurement sample, the magnet arm moves upward and contacts with the backside of the electrode to collect the paramagnetic beads over the electrode surface.

Experimental

MB coated with antibody (Capt-MB)

The monoclonal anti-AFP antibody was used as the first antibody (1st-Ab) for the sandwich immunoassay. As a solid phase carrier for the 1st-Ab, paramagnetic micro-beads (MB) with a diameter of 4.5 μm and specific gravity of 1.5 (Dynabead® D-450, Dynal, Oslo, Norway) were used. The 1st-Ab was coated over the surface of the MB by a physical adsorptive method. A 1 ml solution of the 1st-Ab (2 mg/ml) in the 0.15 M phosphate buffer saline pH 7.5 (PBS) containing 0.2% NaN_3 was added to 2 ml of the MB with a concentration of 30 mg/ml in the PBS, followed by over-night stirring by turning at room temperature. Then the treated MB was collected on the inner wall of the reaction tube by a magnet, repeatedly washed by adding the PBS, and all the liquids were removed from the tube by suction (the Magnetic-washing Procedures). Thereafter, the treated MB was suspended in a 2 ml PBS containing 3% bovine serum albumin (BSA) in order to block the surface of the MB, followed by stirring procedures by turning for 3 h. Finally, after several magnetic washing procedures, the MB coated 1st-Ab was suspended in a 60 ml solution (1 mg/ml of beads concentration containing 12 million of particles per 1 ml) of same PBS, containing 10% normal rabbit serum (NRS) to be used as the Capt-MB.

Antibody labeled with Ru-chelate-NHS (Ru-Ab)

As the 2nd antibody (2nd-Ab), another anti-AFP antibody whose epitope was different from the 1st-Ab was used. The labeling of the Ru-chelate-NHS (Igen International, Gaithersburg, MD, USA) to the 2nd-Ab was carried out according to the following procedures. To prepare the labeled antibody whose molecular ratio of Ru-chelate and antibody (TAG-ratio) was several, 35 to 70 μg of Ru-chelate-NHS was dissolved into 35 μl of anhydrous dimethyl sulfoxide (Aldrich Chemical Co., Milwaukee, USA) just before being added into a 0.5 ml of 0.15 M PBS (pH 7.8) containing 1 to 0.5 mg of the 2nd-Ab, and the mixture was shaken at room temperature for 30 min in the dark. The reaction was terminated by the addition of 25 μl of 1 mol/l glycin solution followed by further incubation for 10 min. The reacted protein mixture was subjected to gel filtration in a Shepadex G-25 column eluted with the same PBS. The first eluted colored fraction was collected and each concentration of protein and Ru-chelate-NHS was determined by means of Lowry's method and absorption at 455 nm respectively. To confirm the TAG-ratio (molecular ratio between the Ru-chelate and IgG), the absorptive value at 455 nm was divided by 13700, and further divided by the molecular value of IgG obtained by Lowry's method. Thereafter, the collected fraction was diluted from 500 to 2000 fold with PBS (pH 7.5) containing 10% of NRS and 0.2% of NaN_3 before use as the antibody labeled with Ru-chelate-NHS (Ru-Ab).

MB coated with Ru-Ab (Hot-MB)

As the material to investigate the Particle Based ECL detection without any immunoassay procedures, the MB coated with Ru-Ab (Hot-MB) was prepared. The gel filtrated Ru-Ab that was diluted from 50 to 200-fold with PBS containing 1% of NRS was coated on the MB by the same physical adsorptive methods as described in the method for preparing the Capt-MB. These prepared MBs were suspended into the electrolyte solution with a concentration of 80 μg/ml (containing 1 million particles per milliliter), after the Magnetic-washing Procedures. The Hot-MB suspensions with different ECL activities were made by serial dilution with the non-coated MB (Cold-MB) having the same particle concentration as that of the Hot-MB.

Electrolyte solution and flow-cell cleaner

The electrolyte solution was composed of a 0.2 M

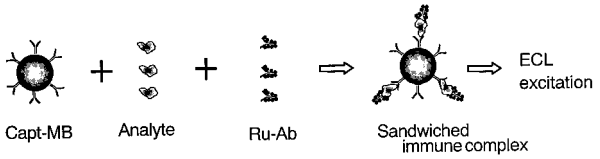

Fig. 3 Schematic reaction format of the sandwich immunoassay using the MB. The Capt-MB, analyte and Ru-Ab were reacted at the same time in the 1-step assay. But in the 2-step assay, the Capt-MB and analyte were reacted first, then the washed Capt-MB was reacted with the Ru-Ab in the 2nd incubation. After the formation of the sandwich complex, the non-reacted Ru-Ab was removed by the Magnetic-washing Procedures and the ECL activities were measured.

potassium phosphate buffer (pH 7.5), which contained 3 mM of NaCl, 0.1% of NaN_3, 0.05% of Triton X-100, 0.05% of Tween 20, and 100 mM of TPA. A 0.5 M KOH aqueous solution containing 1.25% Triton X-100 was used as a flow-cell cleaning solution.

ECL detection apparatus and ECL measurement methods

Figure 2 shows an outline of the composition of the ECL detection apparatus. A peristaltic pump draws various fluids through the flow-cell chamber. A photon multiplier tube (PMT: R 1104, Hamamatsu Co., Hamamatsu, Japan) was operated at 650 to 850 V of potentials supplied to detect the light emitted by the electrochemical reaction. The photo-signals detected by the PMT were processed by a personal computer after being digitized by the analog to digital converter. Each 10 ms of photo-signal was used as the ECL intensity.

The flow-cell electrode was composed of a thin layer cell chamber with a thickness of 0.5 mm and a capacity of 60 µl. The WE, a platinum (Pt) disc with a diameter of 6.0 mm, was placed in the center of the bottom side of the flow-cell chamber. The counter-electrode, a Pt ring with an inner diameter of 7.0 mm and an outer diameter of 9.0 mm, was placed on the counter side of the WE. A reference-electrode, Ag/AgCl (CIMW-2021, ABS, Weatlayette, USA), was placed on the outlet of the flow-cell chamber. A movable magnet operation arm was placed under the backside of the WE. While the peristaltic pump was drawing the respective specimen to be measured for ECL intensity, the magnet arm moved upward and contacted with the backside of the WE to collect the MB over the surface of the WE.

For ECL detection, a 250 µl solution of the respective 300 µl Hot-MB or the immune-reacted Capt-MB resuspended into a 300 µl electrolyte solution was transferred into the flow-cell chamber by suction. Then, a ramp voltage was applied to the electrode and the ECL excitations were generated and measured. After the ECL excitations, the flow-cell cleaning solution with a high pH was drawn into the flow-cell chamber and an electrochemical cleaning procedure was performed. For conditioning, the electrolyte solution was then aspirated into the flow-cell chamber and a voltage waveform was applied to the electrode. This process kept the surface of the electrodes in a highly reproducible state, ready for the next ECL measurement cycle.

Immunoassay

AFP standard solutions in a concentration of 20 µg/ml to 5 pg/ml were prepared by dilution with NRS from 200 µg of purified AFP in 1 ml of 1% BSA-PBS (pH 7.5) whose concentration was calibrated by WHO standard. To study the correlation and hook phenomena, AFP positive clinical serum samples were used. All ECL measurements were carried out in duplicate assay. In the 2-step immunoassay, a 50 µl AFP positive specimen and a 25 µl Capt-MB solution containing about 0.3 million pieces of beads were added into the reaction tube, containing a 150 µl reaction medium, and the mixture was incubated for 15 min. Thereafter, the Capt-MB was washed repeatedly by the Magnetic-washing Procedure, and then all of the media including the specimens were removed from the tube. At the 2nd incubation, 200 µl of Ru-Ab solution was added into the tube and the mixture was reacted for 15 min. Then, the same Magnetic-washing Procedures were performed in order to remove the non-reacted Ru-Ab. The washed Capt-MB was led into the magnet-mounted flow-cell electrode, together with a 300 µl electrolyte solution, to measure the quantity of the ECL emission.

For the 1-step immunoassay, all of a 25 µl Capt-MB solution, a 50 µl specimen and a 200 µl Ru-Ab in relatively high concentration were reacted at the same time. After the 15 min incubation, all liquid containing the specimens and the non-reacted Ru-Ab was removed from the reaction tube by the Magnetic-washing Procedures before the ECL excitation. Figure 3 shows the schematic ECL reaction format for the sandwich ECL immunoassay, in which the sandwiched immune complex is formed on the surface of the Capt-MB.

Results and Discussion

ECL emitting time course for Hot-MB collected over the surface of the WE

The ECL exciting mechanism for Ru-chelate using the oxidative reduction by TPA is shown in Fig. 4 schematically. Although TPA is consumed through the electrical oxidation, Ru-chelate at the exiting stage returns to a ground stage after emitting the photon. Therefore, the excitation processes for Ru-chelate occurred repeatedly. Figure 5 shows the typical ECL emitting time course for the Hot-MB settled over the surface of the WE by natural gravity or by magnetic force. The ECL photon emission was observed when the applied electric potential reached 1.0 V, and the peak ECL emission was observed at the electric potential of approximately 2.0 V, where the increasing electric current waveform due to the oxidation of TPA dis-

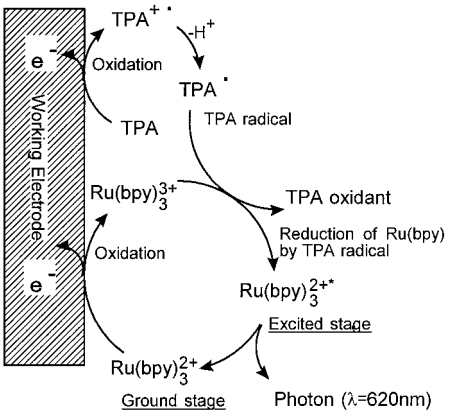

Fig. 4 Mechanism of ECL excitation: $Ru(bpy)_3^{2+}$ and TPA are oxidized at the surface of working electrode, forming $Ru(bpy)_3^{3+}$ and $TPA^{+}\cdot$, respectively. The $TPA^{+}\cdot$ spontaneously loses a proton, forming $TPA^{+}\cdot$. The TPA*, a strong reductant, reacts with $Ru(bpy)_3^{2+}$, a strong oxidant, forming the excited state of the label, $Ru(bpy)_3^{2+}$*. The excited state decays to the ground state through a normal fluorescence mechanism, emitting a photon having a wavelength of 620 nm.

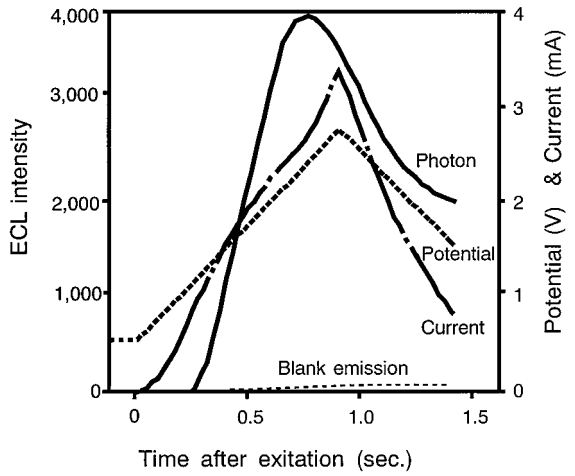

Fig. 5 The typical waveform observed for the Particle Based ECL. A large solid line shows the photon emission, the dotted line is the applied voltage, the dotted and broken line is electric current, and the small solid line is a blank emission.

appeared. For the blank specimen, a weak blank photon emission due to water hydrolysis was observed when the electric potential exceeded 1 V, and the peak emission of ten plus level appeared at the time after the highest electric potential of 2.5 V was applied. Except for the weak ECL emission, it was observed that the ECL peak appearance time (Peak Time) after the excitation was constantly the same, regardless of the analyte concentrations. On the basis of these observations, the integrated ECL intensity was calculated as the accumulated value of all 40 sampling signals of each 10 ms ECL intensity during the period from 200 ms before the Peak Time to 200 ms after the Peak Time.

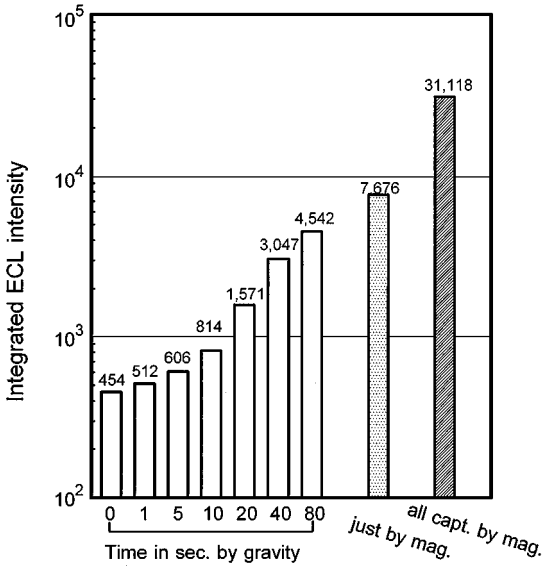

Fig. 6 Enhanced effects of ECL intensity by settling the Hot-MB over the electrode surface with gravity and/or magnetic force. The numbers below the horizontal axis show the time periods in seconds for precipitation of the Hot-MB in the flow-cell chamber by natural gravity. The dotted bar shows magnetic precipitation. The slanted line bar shows the magnet capturing the Hot-MB while the specimen passes through the flow-cell. Each integrated ECL intensity value shows here minus the background value.

Enhanced effect of ECL emission by capturing the Hot-MB over the WE

To evaluate the enhanced effect on the ECL intensity of the Hot-MB by collecting over the surface of the WE, three different collection methods were applied before the ECL excitation. In the first collection method, the sedimentation of Hot-MB by natural gravity was applied after the 60 µl Hot-MB specimen filled the flow-cell chamber. In the second method, the sedimentation of Hot-MB by magnetic force was employed, here the magnet was attached to the backside of the WE for a moment after the 60 µl Hot-MB specimen filled the flow-cell chamber. In the third method, the magnet was attached to the backside of the WE until the entire 250 µl of the specimen passed through the flow-cell chamber.

Figure 6 shows the enhanced effects of ECL intensity by settling or capturing the Hot-MB over the WE. The integrated ECL intensity of the specimen containing the Hot-MB still in a state of suspension in the flow-cell chamber showed a value at only the 450 level. In accordance with the time period for the settlement, the ECL intensity increased by duration. For the specimen having an 80 s period for settling, the value was increased to 4500, which was about ten times that of a 0 s period. In the method that the magnet was attached for a moment to the back side of the WE, which was similar to the one with a long time period settling by gravity, the value was observed at the 7600 level, which was 17 times that of the zero time for sedimentation.

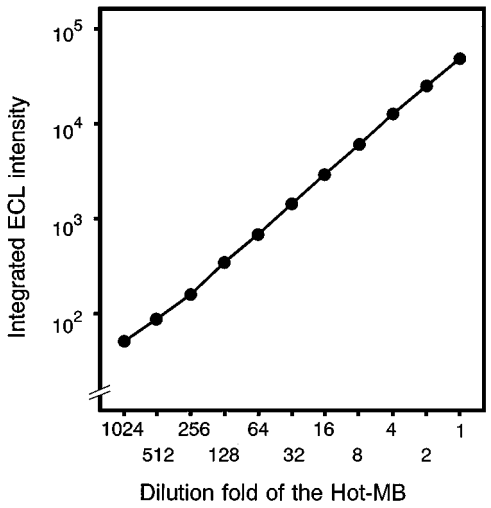

Fig. 7 ECL linearity responses of the Hot-MB: 250 µl of the Hot-MB solution diluted serially by the Cold-MB was aspirated into the magnet-mounted flow-cell. Each integrated ECL intensity value shows here minus the background value.

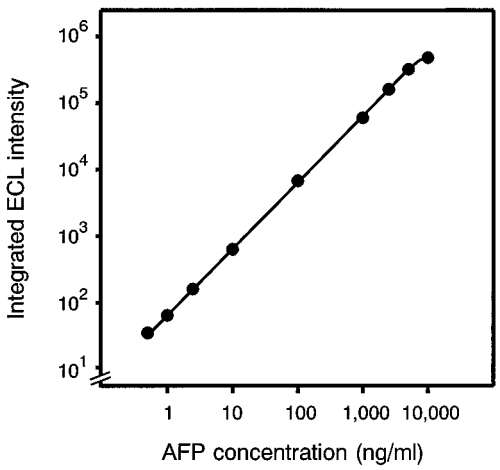

Fig. 8 Standard curve of the 2-step AFP ECL sandwich assay. A 50 µl AFP positive specimen and 25 µl Capt-MB were reacted with a 150 µl reaction medium for 15 min. After the magnetic washing procedure, reacted beads were incubated with a 200 µl Ru-Ab for 15 min. The detection limit of 0.2 – 0.4 ng/ml was always obtained. Each integrated ECL intensity value shows here minus the background value.

Further enhanced ECL emission at the 31000 level was obtained when all the Hot-MB contained in the total 250 µl specimen were collected over the surface of the WE by attaching a magnet to the backside of the WE until the specimen passed through the flow-cell chamber. The value of 31000 was about 4 times that of the ECL intensity level obtained for the 60 µl of Hot-MB filled into the flow-cell chamber. This increased ratio of 4 times in intensity corresponds to that of the sample volume.

Figure 7 shows the dose responses of the integrated ECL linearity for the Hot-MB which were made into a serial dilution of up to 1024 fold by Cold-MB, under the normal operation of the magnetic electrode. The integrated ECL intensity was dramatically correlated proportionally with the concentrations of the Hot-MB in the respective specimens. In particular, it was remarkable that a constant value of 40000 and 2.3% in coefficient value (CV) were obtained when each integrated ECL intensity was multiplied by the corresponding dilution fold number, from the 1/1 specimen to 1/256 dilution fold. This result indicates that both the capturing of the particles by a magnet over the surface of the WE and the succeeding ECL excitation worked quite precisely.

Results of ECL immunoassay

In the preliminary studies, Particle-Based ECL immunoassay showed such extremely wide detecting ranges that optimization studies were carried out to satisfy both the clinical sensitivity and clinical distribution range of AFP. Although the ECL signal intensities against AFP were enhanced with the increase of Ru-Ab concentration and/or TAG-ratio, higher concentration of Ru-Ab with high TAG-ratio elevated the reagent blank in both 1-step and 2-step assay. On the contrary, Ru-Ab with high concentration and low TAG-ratio improved the sensitivity rather than the measuring ranges in both 1-step and 2-step assay, but the reagent blank values in 2-step assay were usually 3 to 5 time higher than that of 1-step assay. Figure 8 shows the calibration curve obtained by the 2-step ECL immunoassay that was optimized by using Ru-Ab with 100 ng/ml and 14:1 of TAG-ratio. It was clearly demonstrated that the integrated ECL intensities were dramatically increased, and the results corresponded to the respective AFP concentrations. Namely, the range of the integrated ECL intensity from the 48 level to the 450000 level was linear to the respective AFP concentrations within a range of 0.5 ng/ml to 8000 ng/ml. In other words, the linearity of these ECL signals was extended to over 10000 times of magnitude. The blank emission caused by the remains and/or adsorption of the Ru-Ab was weak at the level of 480, reflecting the effectiveness of the incorporation of the Magnetic-washing Procedures before the ECL excitation. The analytical sensitivity (DL>/=(the mean value of the blank+2SD)) was calculated to be 0.2 ng/ml. In contrast, the 1-step ECL immunoassay using Ru-Ab with 400 ng/ml and 6:1 of TAG-ratio with the simultaneous sandwich conformation of (Capt-MB)-AFP-(Ru-Ab) demonstrated that the integrated ECL intensity ranges were restricted to 1000 times of magnitude, while the highest sensitivity of 5 pg/ml was obtained, as shown in Fig. 9.

Clinical serum samples within the concentration of 20000 ng/ml of AFP which were determined by RIA (Daiichi Isotope Co, Ltd., Tokyo, Japan) were measured by the 2-step ECL immunoassay; the result is shown in Fig. 10. There is 14% difference in the AFP concentration, but the extremely good correlation of two methods

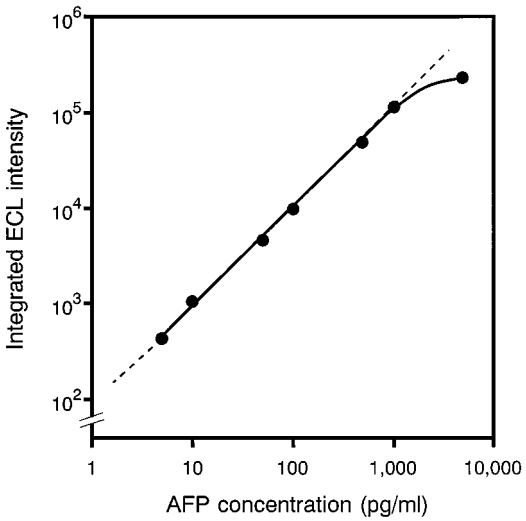

Fig. 9 Standard curve of the 1-step AFP ECL immunoassay. In the 1-step immunoassay, all of a 25 µl Capt-MB solution, a 50 µl specimen and a 200 µl Ru-Ab were reacted at same time for 15 min. All liquids containing the specimens and the non-reacted Ru-Ab were removed by the magnetic washing procedures before ECL excitation. Each integrated ECL intensity value shows here minus the background value.

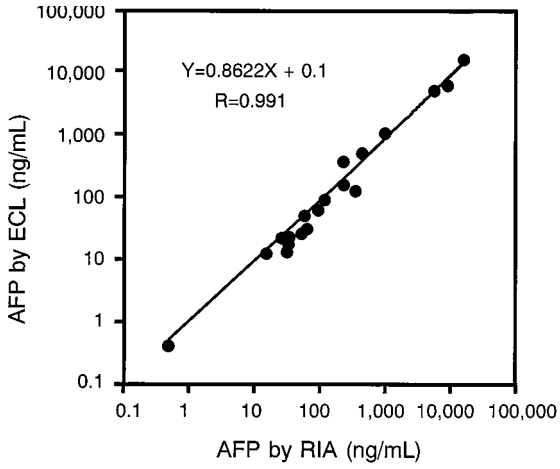

Fig. 10 Correlation of AFP concentration in 19 clinical serum specimens measured by RIA and ECL.

is expressed as ((ECL)=0.8622×(RIA)+0.1) with the correlation coefficient of 0.991. For a specimen with the AFP concentrations of 200 µg/ml, which was so high that it exceeded the measurement ranges in this 2-step assay, the ECL signals did not increase linearly but there was no appearance of any hook effect due to the excessive antigen. On the other hand, in the 1-step ECL immunoassay, the ECL signal for the specimen with the AFP concentration of 20 ng/ml was decreased to a lower level than that of the ECL value obtained for 10 ng/ml of AFP.

ECL emitting efficiency by molecular size of ECL-producing materials

In the past 15 years, many analytical applications of ECL using the Ru chelate have been studied for measuring chemical materials such as carbonate compounds, amine compounds and others[2,3], and for detecting the total DNA.[4,5]

In the former measurement system, the ECL signal linearity was 1000 to 10000 times of magnitude in accordance with the analyte concentration. It is reasonable that such a wide range of linearity could be obtainable with ECL detection, because the ECL linearity responses of free Ru-chelate cover the range of 7 orders of magnitude.[11] On the other hand, however, in the latter system of the ECL measurement for total DNA, which utilizes the binding affinity between the DNA double strands and the Ru chelate, the linearity of ECL intensities is somehow limited to such a small range as only 2 times. Additionally, the ECL signal intensities are decreased against the DNA concentration. Likewise, in the case of the ECL competitive immunoassay using the pyren labeled antigen, it was also observed that the measurable range was very narrow (*i.e.*, *S*/*N*<2.5) and ECL signals were decreased by the reaction of the labeled HAS to the ant-HAS antibody.[9]

Although the discrepancy observed in the above two different types of analysis has rarely been discussed in the previous studies, the reason why the ECL signal intensities are proportional or inversely proportional to the analyte concentrations might be explained as follows: In the former system, there was no change in the molecular size of the ECL-producing material at ECL detection, and the ECL-producing material only acted as the indicator for ECL emission. On the other hand, in the latter system, the ECL-producing materials were incorporated into the analyte, such as DNA double strands or labeled HSA bound with the corresponding antibody, and consequently the decrease in ECL signal has been observed. The ECL emission must be reduced with the increase in the molecular size of ECL-producing materials, since the range of the electrical double diffusion layer is approximately a 10 nm distance from the surface of the WE.[12]

The present studies did not demonstrate the phenomenon that the ECL emission efficiency was reduced with the increase in the molecular size of ECL producing materials. As shown in Figs. 6 and 7, however, the ECL signals of the Hot-MB were increased according to the collecting efficiency and/or concentration of the Hot-MB. In the ECL sandwich immunoassay using the MB as the solid phase carrier, it was quite surprising that the linearity was expanded to 10000 times of magnitude for the AFP concentration range of 0.5 ng/ml to 8000 ng/ml, by the 2 step AFP assay (Fig. 8). In the case of the 1-step immunoassay that was optimized for higher sensitivity rather than a wider measuring range, the highest sensitivity of 5 pg/ml was obtained in a 15 min reaction. On the other hand, in this 1-step assay, the linearity range was restricted to about 1000 times of magnitude. Although this 1000 times of magnitude lin-

earity range was slightly narrow compared with the results obtained by the 2-step assay, this is markedly wider compared with those in conventional immunoassays, such as enzyme immunoassay and chemiluminescent immunoassay.

The reasons why these wider linearity ranges could be obtained must be ascribed to the following two improvements. One improvement is that the major ECL producing material was excited close to the WE, like the measurement of carbonate compounds or amine compounds by ECL. The other is that blank emissions were prevented by washing out the non-reacted Ru-Ab before ECL excitation in this newer particle based ECL immunoassay.

The newer ECL immunoassay is superior in reproducibility to chemiluminescent (CL) methods, because the photo-exciting procedures in the ECL can be controlled by the electrode potential alone; on the other hand, that of the CL may be influenced by several factors such as temperature and the timing of adding and/or mixing of reagents.

In the present study, the detection sensitivity at the picogram level was achieved by capturing 4.5 μm of immune reacted MB on the electrode, while the size of MB was so huge compared with the size of the electric double diffusion area that Ru-Ab bound to MB surface might be partly excited by electrochemical reactions. It is also suggested that the sensitivity of Particle Based ECL may be more improved by reducing the size of the particles and/or by extending an electrical double diffusion area electrochemically.

The outline of this study was presented at the Mt. Fuji Conference on Molecular and Polymer Based Electrodes, the Post-Meeting of the 49th ISE (International Society of Electrochemistry), September 19 – 20, 1998.[13]

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