Synthesis of Electroactive Protein Hybrid, Fec-BSA-Dig, and Its Application to a Novel Homogeneous Electrochemical Immunoassay

Toshiyuki Suzawa, Yoshihito Ikariyama,# and Masuo Aizawa*

Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 227

(Received February 8, 1994)

A novel homogeneous electrochemical immunoassay using ferrocene- (Fec-), digoxin- (Dig-) doubleconjugated protein, has been developed. The system is based on the principle that electrochemical activity of the conjugate is suppressed in the presence of an antibody, and restored in the presence of free digoxin. Hence, a new homogeneous immunoassay can be carried out when the electroactivity of the conjugate is determined. Here, we synthesize the conjugate by controlling the numbers of ferrocene and digoxin on individual carrier proteins simultaneously. Bovine serum albumin (BSA) is selected as a carrier protein to which the electrochemical labels and the several hapten molecule are bound by Schiff's base formation. Since the conjugate synthesized here behaves as a multivalent antigen, and is capable of binding to antidigoxin antibody, therefore the antigen having electrochemical activity loses its activity depending on antibody concentration. When the conjugate, in which 39 molecules of ferrocene and 8 molecules of digoxin are bound, is used for the inhibition experiment of electrochemical reaction before and after the immunoreaction, peak height currents, responsible for the redox reactions of the bound ferrocene, decreased depending on the added antibody in a dose-dependent fashion. This system is feasible as a homogeneous electrochemical immunoassay, since the method may provide a simple and a rapid approach toward a homogeneous assay of bioactive substances.

Immunoassays have become an indispensable technique for the sensitive determination of many substances of physiological importance.[1—8] In recent years, a variety of immunoassays have been developed with enzymes' entry into immunoassay.[9] However, most of the enzyme immunoassays were performed with heterogeneous formats which essentially required the separation of a solid phase-bound antibody (or antigen) from free antibody (or antigen).[10,11]

Although highly sensitive assays can be carried out, heterogeneous immunoassays, which require a troublesome separation step, should be simplified further. Homogeneous immunoassays, on the other hand, have a few advantages, mainly due to the lack of the separation procedure. In the last two decades, a number of homogeneous immunoassays have been developed.[12—18]

We have already reported an electrochemical luminescence-based homogeneous immunoassay by taking advantage of steric hindrance of antibody molecule after antigen–antibody reaction, where pyrene-labeled antigen and free antigen were competitively reacted with a constant amount of antibody.[19] As the detection limit should still be improved, we have been screening sensitively detectable labels. Luminol labelling has made the assay more sensitive because of the high quantum efficiency of luminol.[20,21] We propose that a sensitive homogeneous immunoassay would be developed, if only a more effective probe with high quantum efficiency could be exploited.

In this paper, a novel homogeneous immunoassay using ferrocene as an electrochemically amplifiable probe is described to assess the relation of the extent of sensitization to the number of labelling probes from the viewpoint of amperometric amplification. In addition, the strategy is novel because the method takes advantage of steric hindrance against antibody binding with hapten–protein conjugate. This hapten-carrying albumin behaves as a multivalent antigen which easily forms a lattice complex with an antibody. The immunocomplex between multilabelled albumin and antibody, which has less efficient electrochemical communication due to the lattice formation, seems to dissociate when the immunocomplex coexists with free hapten (antigen). Therefore one can easily expect that electrochemical communication will be restored depending on the hapten concentration. Furthermore electrochemical detection techniques have been gaining practical use in recent immunoassays,[22,23] since these techniques can be operated in an inexpensive and simplified manner. Electrochemical immunoassays of heterogeneous format have been investigated by detecting enzyme products

#Present address: Research Institute, National Rehabilitation Center, 4-1, Namiki, Tokorozawa 359.

electrochemically.[24,25]

For example, adsorptive stripping voltammetry was also evaluated in the determination of analytes such as immunoglobulin E[26] and hapten.[27]

Electroactive labels such as metal chelate[28] and ferrocene, which can be detected by electrochemical method, were also evaluated.[29,30] Here we make the hybrid protein conjugated with ferrocene and hapten, which are electroactive and antigenic respectively. It is then probable that the prepared hapten-conjugate with ferrocene multilabelling is amplifiable depending on the number of incorporated ferrocenes. The concept of the method is evaluated by choosing digoxin as a model hapten and ferrocene as an electrochemically amplifiable label. It seems likely that optimum numbers of digoxin and ferroecene play an important role in performing sensitive assays. Therefore an optimum ratio of digoxin to ferrocene labelled to the same carrier protein should be investigated in detail from the viewpoint of sensitivity of the homogeneous assay. Digoxin, which is a cardiac glycoside widely used for treating congestive heart failure and other acute cardiac diseases, is a potent drug having a therapeutic effect. The drug is known to be effective at a trace concentration; therefore, it requires a rapid and sensitive detection method.[1,2,4]

The carrier protein employed here is bovine serum albumin (BSA), which has a variety of functional groups such as α- and ε-amino residues, can be exploited for the conjugation of ferrocene and digoxin.

We investigate optimal conditions for the preparation of Fec-BSA-Dig conjugate to elucidate the possibility of a novel homogeneous electrochemical immunoassay where electrochemical ferrocene regeneration is inhibited in case Dig-conjugated BSA and anti-Dig antibody undergo complex formation.

Experimental

Materials. Bovine serum albumin (BSA) and sodium borohydride were purchased from Wako Chemicals (Osaka). Ferrocenecarbaldehyde and digoxin were from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO), respectively. Sodium periodate and potassium hexacyanoferrate(II) trihydrate were from Nacalai Tesque (Kyoto, Japan). Antidigoxin polyclonal antibody (rabbit) (protein concentration: 1.91 mg ml^{-1}, titer : 1 : 3300) and antidigoxin monoclonal antibody (mouse No: 20-9f6, 1.8 mg ml^{-1}) were the gifts from CIBA Corning Diagnostic (Medfield, MA). Peroxidase-labelled anti-mouse IgG (H+L) (rabbit, ZYMED, San Francisco), *o*-phenylenediamine (Wako Chemical, Osaka), skimmed milk (Yukijirushi Nyugyo, Tokyo), and Tween 20 (Kanto Chemicals, Tokyo) were used for enzyme immunoassay (ELISA) for the conjugate. Human IgG (I-4506, Sigma Chemical, St. Louis, MO) was employed for the adjustment of protein concentration in electrochemical measurement. Na_2HPO_4 (0.085 M, pH=7.0) (1 M=1 mol dm^{-3}) was used as a buffer solution throughout the experimentation. Other chemicals were analytical reagent grade.

Synthesis of Fec-BSA-Dig Conjugate. Fec-BSA-Dig conjugate was prepared by the method described by Butler et al.[1] Digoxin (1-50 mg) was added in 2 ml of acetonitrile under magnetic stirring, which was followed by the dropwise addition of sodium periodate solution (1 ml, 10 mg ml^{-1}) under magnetic stirring. After stirring for 30 min, ethylene glycol (30 μl, 1 M) was added to the mixture to terminate the reaction. The termination was performed under magnetic stirring for 5 min. Then ferrocenecarbaldehyde from 5 to 120 mg was mixed with the above solution under continuous stirring.

BSA solution was separately prepared by dissolving 50 mg of the protein in 4 ml of a buffer solution whose pH was adjusted to 9.3 by 5% K_2CO_3. Into the protein solution, a mixture of digoxin and ferrocene was added in a dropwise way, and the pH was controlled at 9.3 by the addition of 5% K_2CO_3. The coupling reaction was continued for 1 h at 30 °C. The reaction was terminated by the addition of sodium borohydride (10 mg) to the mixture. The termination was performed under magnetic stirring for 10 min, and the mixture was kept at room temperature for 6 h. Finally, the pH of the resulting mixture was adjusted to 6.5 to decompose the remaining borohydride. The pH of the mixture was readjusted to 8.5 by the dropwise addition of 1 M NaOH.

The mixture was then centrifuged at 3000 rpm for 20 min at 4 °C, and the resulting supernatant was filtrated by a membrane filter having 0.45 μm pore size. The filtrate was dialyzed against PB for a few days at 4 °C to separate unreacted ferrocene, digoxin and other low molecular weight substances.

Determination of Binding Ratio of Digoxin to BSA. The average number of digoxin moieties bound to BSA was determined by the method described by Butler et al.[1] The method was based on the color change of digoxin sample in a solution of concentrated sulfuric acid. The sample turned reddish brown in concentrated H_2SO_4. After standing for a few hours at room temperature, the H_2SO_4 solution containing digoxin was provided for spectrophotometric measurement at 338 and 470 nm by a UVIDEC-610C spectrophotometer (Japan Spectroscopic Co, Tokyo).

Determination of Binding Ratio of Ferrocene to BSA. In order to determine the average number of ferrocene molecules bound to BSA, the iron content of the conjugate solution was determined by an SPS-7000 atomic absorption spectroscopy (Seiko Instrument, Tokyo). An aqueous solution of potassium ferrocyanide was exploited as a standard solution of iron atom.

Determination of Protein Content. The protein concentration contained in the conjugate solution was determined by using protein assay kit (Bio Rad, Richmond, CA). A BSA solution prepared in PB was used as a standard solution.

ELISA Procedure for Fec-BSA-Dig Conjugate. Immnoactivity of double conjugated BSA was ascertained in a similar way to that of Fec-, Dig-double conjugated GOD.[33] A polystyrene cuvette (Costar Co, Cambridge) was coated with antidigoxin polyclonal antibody. The IgG coating was performed by adding 500 μl of a diluted antibody solution (1/100 dilution). The IgG coating was performed overnight at 4 °C. The cuvette was then rinsed three times with the phosphate buffer containing 0.05% (v/v) Tween 20 and 0.05% (w/v) skimmed milk. A solution of skimmed milk (0.5 ml, 1% (w/w)) was then added to the cuvette to block

the active sites for nonspecific adsorption at room temperature for 2 h. After thorough washing of each cuvette, 500 μl of diluted conjugate was placed in the cuvette and incubated for 2 h.

After thorough washing, 500 μl of antidigoxin monoclonal antibody (1/1500 dilution) was added and incubated for 2 h; this was followed by the addition 500 μl of peroxidase-labelled antimouse IgG (1/2000 dilution). The binding of the enzyme-labelled second antibody was also performed for 2 h. After each binding reaction, the cuvette was washed thoroughly. Four hundred μl of 10 mM *o*-phenylenediamine dissolved in 50 mM sodium acetate buffer (pH=5.0) was added to the cuvette. The enzyme amplification was initiated by the addition of 100 μl of a solution containing 0.1% (w/v) hydrogen peroxide.

At five min after the addition of hydrogen peroxide, the enzyme reaction was terminated by adding 500 μl of 1 M H_2SO_4 containing 2 g dm^{-3} Na_2SO_3, and the resulting change in absorbance at 491 nm was measured by the spectrophotometer.

Electrochemical Characterization of Ferrocene-, Digoxin Double Conjugated Protein. Electrochemical characterization was performed in 1 ml of each sample solution by a P-100 polarographic analyzer (Yanagimoto, Kyoto) and an HA301 potentiostat (Hokuto Denko, Tokyo). All the electrode potentials were referred to an Ag/AgCl/KCl. A glassy carbon having a diameter of 3 mm (BAS, West Lafayette, IN) was used as a working electrode, and a platinum wire was employed as an auxiliary electrode. The glassy carbon electrode was polished with pasted alumina of 0.05 μm diameter, which was followed by sonication in distilled water for 5 min. The electrode was then scanned several times from 0 to 800 mV at a rate of 100 mV s^{-1} in PB prior to each electrochemical measurement.

The electroactivity of the ferrocene moiety in the conjugate was determined by voltammetric techniques. In the case of cyclic voltammetry, the second cyclic voltammogram was adopted, because a stable and reproducible voltammogram was observed from the second sweeping. In the case of differential pulse voltammetry, the first voltammogram during the scanning in positive and negative directions was adopted. However, the differential technique was preceded by one cycle of cyclic voltammetry.

Electrochemical Assessment of the Inhibition of Electron Transfer between the Conjugate and Electrode after Immunoreaction. Each one ml sample prepared, contained 0.015 mg ml^{-1} of Fec(39)-BSA-Dig(8) conjugate, 0-150 μl of anti-digoxin monoclonal antibody and human IgG. The total IgG concentration was adjusted to 0.225 mg ml^{-1} by using human IgG. After standing at room temperature for a few hours, each sample was employed to electrochemical assessment.

Results and Discussion

Synthesis of Fec-BSA-Dig Conjugate. Digoxin can be chemically conjugated to a carrier protein having free amino groups, as described by Butler et al.[1] Digoxin has three pharmacologically less important digitoxose residues at the 3-position of its steroid structure. The digitoxose residues were oxidized by periodate to generate two formyl groups. The periodate derivative was conjugated in the coexistence of ferrocenecarbaldehyde to the carrier protein. The coupling was carried out at slightly alkaline pH (9.3) for 1 h. Covalent binding of ferrocene and digoxin to the carrier protein was ascertained by dialyzing the conjugate against 3 M urea solution, because the noncovalently bound ferrocene and digoxin were completely desorped from the carrier protein in the denaturing solution. The resulting yellowish-brown solution was provided for determining the contents of ferrocene and digoxin in the conjugated BSA molecule. In Fig. 1(a), ferrocenecarbaldehyde was increased from 5 to 120 mg against 50 mg BSA, while digoxin was kept constant at 15 mg. Similarly, the amount of digoxin was increased from 1 to 50 mg against 50 mg BSA, whereas the amount of the ferrocene derivative was held constant at 80 mg in Fig. 1 (b). These results indicate that labelling of ferrocene and digoxin to one BSA molecule was easily controlled by changing the amount of the labelling agents.

When 120 mg of ferrocenecarbaldehyde, 15 mg of digoxin and 50 mg of BSA were employed for the synthesis of the conjugate (Fig. 1(a)), the carrier protein having 39 molecules of ferrocene and 8 molecules of digoxin, Fec(3)-BSA-Dig(8), was obtained. At this binding state, approximately 80% of lysine residues of the BSA molecule was exploited for the conjugation. Here we can emphasize the advantage of the coupling by Schiff's base formation, because crosslinking of the protein molecules can be avoided.

Binding of Fec-, Dig-Multilabelled Protein with Antibody The immunoreactivity of the Fec-, Dig-multilabelled protein was investigated by utilizing antidigoxin antibody by the conventional sandwich ELISA, as described in Experimental. The assessment is done in a similar manner to that in Fec-GOD-Dig preparation, where glucose oxidase (GOD) was chosen as a carrier. The assay was performed by using the following four conjugates: Fec(28)-BSA-Dig(2), Fec(30)-BSA-Dig(3), Fec(27)-BSA-Dig(5), and Fec(25)-BSA-Dig(6). Although the number of bound digoxins is different, these conjugates were capable of binding the antidigoxin specific antibody (Data not shown). However, among them, Fec(28)-BSA-Dig(2) conjugate demonstrated the lowest immunoreactivity, presumably due to low reactivity of two digoxin residues for the binding with antibody in ELISA of sandwich format.

Electrochemical Characterization of the Conjugate. The cyclic voltammogram shown in Fig. 2 was obtained for Fec(39)-BSA-Dig(8) conjugate sample whose concentration was 3 mg ml^{-1} in 0.085 M PB. The conjugate demonstrated a pseudoreversible electrochemical behavior: It demonstrated electron shuttling, i.e., $E_{1/2}$=350 mV, E_{pa}=450 mV, and E_{pc}=250 mV vs. Ag/AgCl. The cyclic voltammogram shown in the dotted line in Fig. 2 was obtained for 10^{-4} M ferrocenecarbaldehyde. The electrochemical behavior of ferrocenecarbaldehyde was remarkably different from

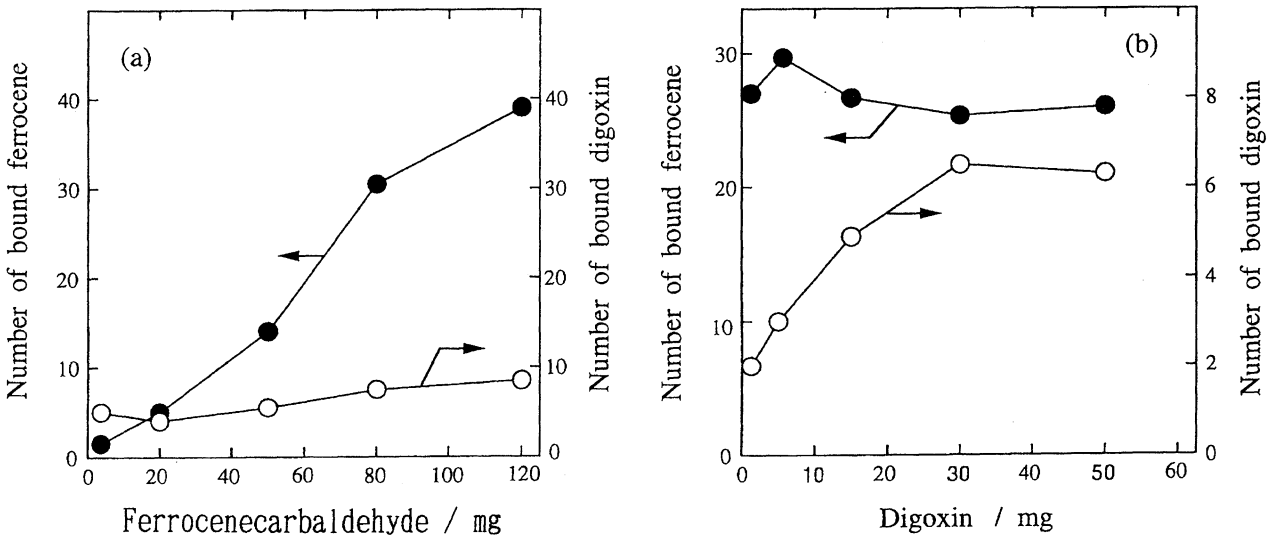

Fig. 1. Dependence of the number of labelled ferrocene (-●-) and digoxin (-○-) on reactive ferrocene and activated digoxin. (a) 50 mg of BSA and 15 mg of digoxin were reacted with an aliquot amount of ferrocenecarbaldehyde. (b) 50 mg of BSA and 80 mg of ferrocenecarbaldehyde were reacted with an aliquot of activated digoxin.

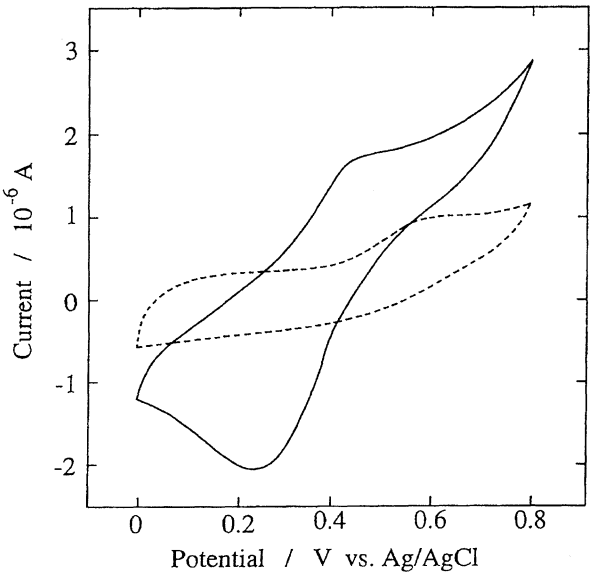

Fig. 2. Cyclic voltammogram of Fec(39)-BSA-Dig(8) conjugate. Three mg ml^{-1} of the multilabelled conjugate in phosphate buffer of pH 7 (85 mM) was scanned at a rate of 100 mV s^{-1}. The voltammogram (----) of 10 mM ferrocenecarbaldehyde was obtained to compare the redox behaviors of the conjugate with free ferrocenecarbaldehyde at same conditions.

that of the conjugate. In addition, the electrochemical behavior of the aldehyde derivative of ferrocene was irreversible: Oxidation peak was observed at $E_{pa}=600$ mV (vs. Ag/AgCl). There is a discrepancy on the reversibility in the voltammetric behavior of the ferrocene covalently attached to the carrier protein. One of the reasons for the discrepancy may be the following. The carbaldehyde is attached to the carrier protein via Schiff's base, and then reduced by sodium borohydride. So the redox behavior can not be directly compared with that of free carbaldehyde. The comparison with ferrocenyl methanol would be more reasonable. Figure 3 shows the peak oxidation and reduction currents of the conjugate (3 mg ml^{-1} solution) having various numbers of ferrocene labels. Each peak was obtained by differential pulse voltammetry. The oxidation peak appeared at a potential of ca. 350 mV and the peak height increased with the increase in the number of bound ferrocenes. The reduction peak current at around 350 mV increased in the same way, but the peak current at each point was approximately 40% smaller than that of oxidation peak current.

Inhibitory Effect of Digoxin-Specific Antibody on the Electrochemical Reaction of Dig-, Fec-Multilabeled BSA. The homogeneous immunoassay is based on the concept that the electrochemical activity of the conjugate decreases due to the steric hindrance of antibody after immunoreaction. In addition, attenuation of the diffusion of hapten to electrode surface is also anticipated. Both of them are expected to be caused by the binding of antibody to the electroactive conjugate. For performing a sensitive assay, the concentration of the conjugate should be decreased as low as possible. Therefore, we have studied the relationship between the peak oxidation current and the conjugate concentration in differential pulse voltammetry. Figure 4 shows standard curves of the three conjugates; Fec(39)-BSA-Dig(8), Fec(31)-BSA-Dig(7), and Fec(15)-BSA-Dig(5). These data indicate that low detection limit of each conjugate was improved as the amount of ferrocene label increased. In the case of Fec(39)-BSA-Dig(8) conjugate, 3×10^{-3} mg ml^{-1} (3.8×10^{-8} M) of the conjugate was determined. Ferrocenecarboxylic acid whose redox potential is 300 mV (vs. Ag/AgCl) could not be detected beyond 1×10^{-6} M, when it was determined in the same manner. Therefore chemical

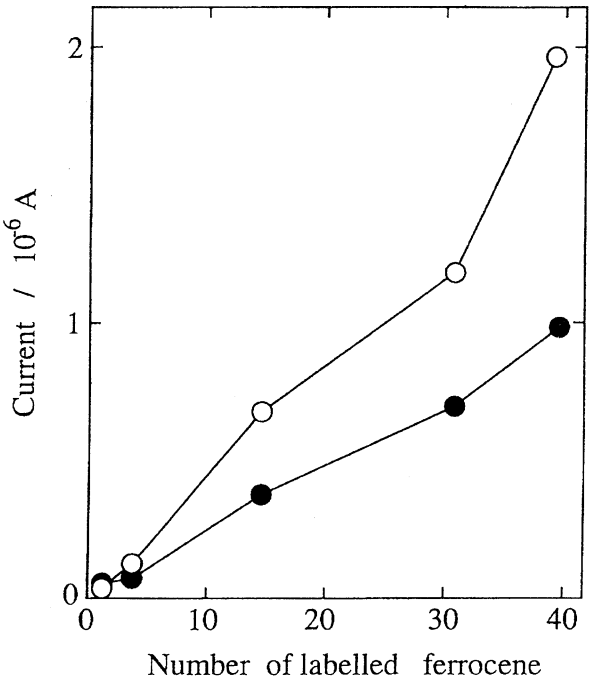

Fig. 3. Relationship between the number of labelled ferrocene and the peak current at 350 mV (vs. Ag/AgCl). Three mg ml^{-1} Fec(39)-BSA-Dig(8) was prepared in 85 mM phosphate buffer of pH 7 to get a reduction peak current (-○-) and an oxidation peak current (-●-) by differential pulse voltammetry. The excitation wave form for the voltammetry consists of a small amplitude pulse (50 mV) summed with a slowly moving linear potential ramp (2 mV s^{-1}) from 0 to 0.8 V and from 0.8 to 0 V to obtain negative-going and positive-going voltammograms, respectively. The excitation signal, with a frequency of 1 Hz, lasted for 50 ms, following which the sampling current was monitored at 40 ms.

amplification was attained by taking the multilabeling technique. The nonliner relationship between the peak height current and the conjugate concentration suggests an isothermic adsorption. At this stage of investigation, our understanding of this phenomenon is a kind of speculation rather than a reasonable interpretation based on experimental results. In the case of reduction peak (Fig. 5), the relation seems to provide a two-step curve. This may be explained by the formation of monolayer and the following bilayer protein adsorption in the course of potential sweeping from extreme potential (+0.8 V) to less extreme potential (0 V).[31] As the Fec(39)-BSA-Dig(8) conjugate gave the largest current, we employed the multilabelled conjugate at the concentration of 0.015 mg ml^{-1} for the investigation of steric hindrance and diffusion attenuation caused by the binding of the antibody against digoxin.

Figure 6 showed the differential pulse voltammograms in the presence and absence of the digoxin specific antibody. Also shown is the voltammogram of the Fec(39)-BSA-Dig(8) in the presence of human IgG as

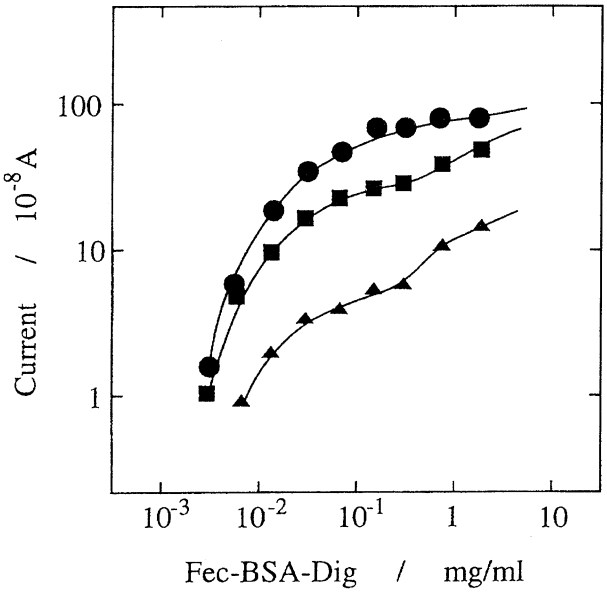

Fig. 4. Relationship between the conjugate concentration and the oxidation peak current. Each peak was obtained by differential pulse voltammetry in the same conditions as described in Fig. 3. Fec(39)-BSA-Dig(8) (-●-), Fec(31)-BSA-Dig(7) (-■-), and Fec(15)-BSA-Dig(5) (-▲-) were used in the experiment.

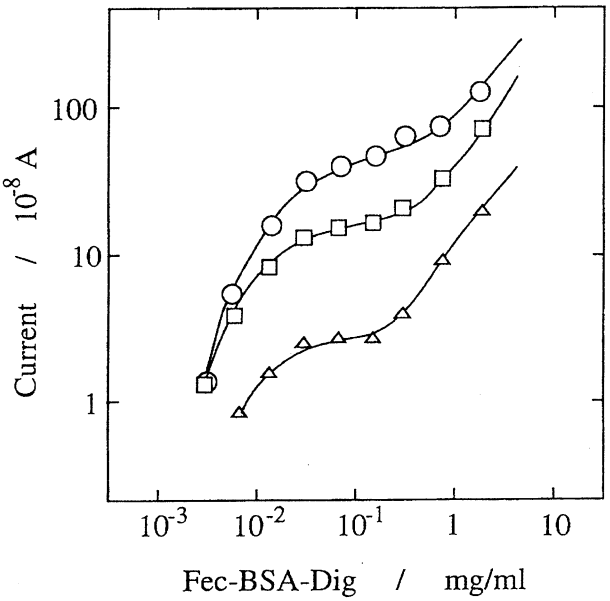

Fig. 5. Relationship between the conjugate concentration and the reduction peak current. Each peak was obtained by differential pulse voltammetry in the same conditions as described in Fig. 3. Fec(39)-BSA-Dig(8) (-○-), Fec(31)-BSA-Dig(7) (-□-), and Fec(15)-BSA-Dig(5) (-△-) were used in the experiment.

a control experiment. When the control protein, human IgG, was present in the conjugate solution, a considerable decrease of peak current was observed. This result suggests that physical adsorption of nonspecific IgG interferes with the electronic reaction between elec-

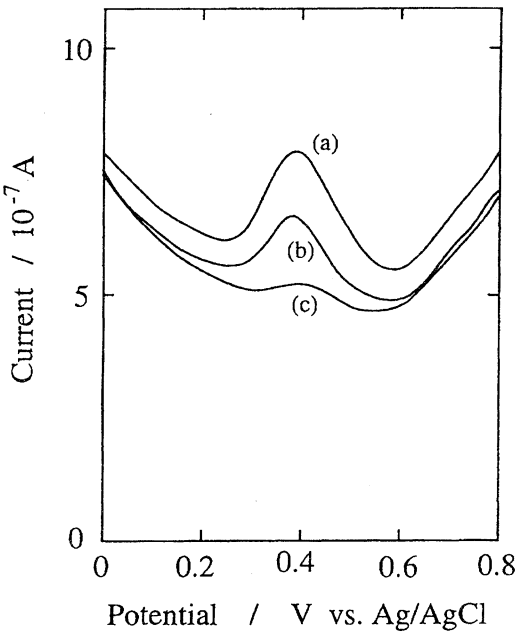

Fig. 6. Oxidation peak of the conjugate in the presence and absence of digoxin specific antibody. The voltammetry was performed in the same conditions as described in Fig. 3. (a) 0.015 mg ml^{-1} Fec(39)-BSA-Dig(8) conjugate, (b) in the presence of 0.225 mg ml^{-1} human IgG (control), (c) in the presence of 1/20 dilution of anti-digoxin monoclonal antibody (total IgG concentration is 0.225 mg ml^{-1}).

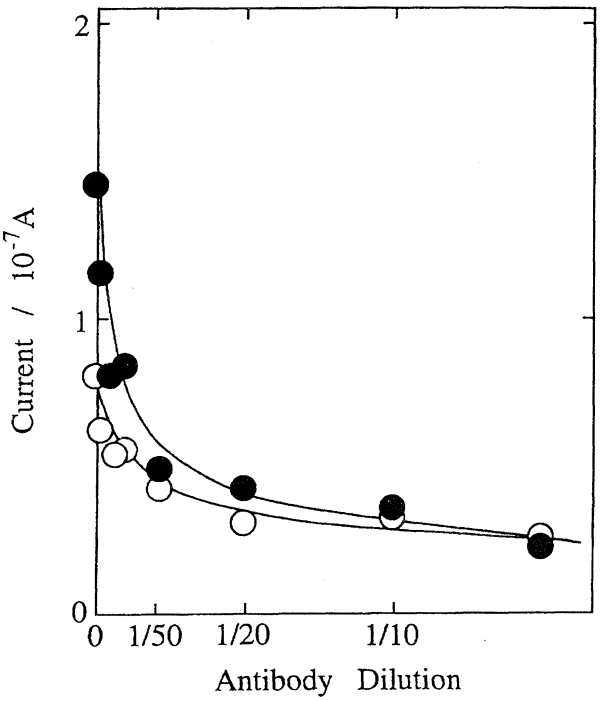

Fig. 7. Relationship between antibody concentration and electrochemical activity of the conjugate. Oxidation peak current (-●-) and reduction peak current (-○-) in differential pulse voltammetry were plotted against the dilution ratio of the conjugate. The voltammetry was done in the same way as in Fig. 3.

trode surface and the ferrocene moieties of the conjugate. However, when the digoxin specific antibody was added, the oxidation peak at ca. 0.4 V almost disappeared. We have then examined the oxidation peak of the Fec-, Dig-doublelabelled albumin in the presence and absence of antidigoxin antibody. In this experiment, nonspecific human IgG was also added to cancel the effect of physical adsorption of the nonspecific globulin over the surface of the electrode. The voltammetric curves in Fig. 6 were obtained by adjusting the concentration of the corresponding globulin to be the same, since nonspecific response due to the physical adsorption was canceled to evaluate the net specific response. Figure 7 shows that the current response of the doublelabelled conjugate was dependent on the dilution ratio of the specific antibody: The response decreased with the increase of antidigoxin specific antibody. It is highly probable that the decrease of the response current in an exponential fashion was the reflection of the steric hindrance and/or the decrease of diffusion after immunocomplex formation. As the Fec-labelled conjugate having 8 digoxin moieties seems to behave as a multivalent antibody, steric hindrance due to lattice formation and the decrease in diffusion transport due to antigen–antibody complexation are enough to render the electric signal smaller. By taking these effects into consideration, we conclude that free digoxin and the digoxin conjugate were competitively reacted with the antibody. We can evaluate the feasibility of the electrochemical homogeneous assay in the determination of the hapten. And the resulting low detection limit was 10^{-5} M digoxin. In the clinical laboratory from 6×10^{-10} M to 2.6×10^{-9} M digoxin (0.5—2.0 ng in one mL) should be determined, so that further sensitization is strongly anticipated. Since the signal is not necessarily large, the method presented here may be employed to determine antibody concentration rather than to quantify the hapten concentration.

Conclusions

A novel electrochemical strategy toward immunoassay if homogeneous format was demonstrated by exploiting a multilabelled conjugate where several ferrocene molecules and digoxins were labelled. By multilabelling, the electrochemical signal was enhanced. In addition, colabelling of hapten to electrochemically activated carrier protein was effective, as steric hindrance against electronic reaction was remarkably provided. In general, monovalent hapten becomes multivalent antigen if several hapten molecules are attached to one carrier protein. One can easily expect lattice formation between multivalent antigen and divalent antigen. And the resulting antigen–antibody complex is a huge substance where less efficient electronic communication occurs between the electroactive label and the electrode.

By the presented strategy, the binding molecules and ratio of the electroactive label and hapten label were easily controlled by changing the amount of the activated labelling agent and the reaction time. By increasing the labelled hapten, the diffusion of the resulting antigen–antibody conglomerate seemed to be attenuated. This may be one of the reasons of the decrease in electrochemical signal.

Further sensitization may be accomplished by taking advantage of mediator function of electrochemical label in the combination of enzyme molecule where the enzyme is to act as a carrier protein and signal amplifier. The combination of ferrocene and glucose oxidase system may be one of the most reasonable combinations for sensitizing the strategy presented here.[28,32] This project is now in progress in our laboratory.

We thank Brenda Manning of CIBA Corning Diagnostics for supplying the anti digoxin antibody.

References

1) V. P. Butler, *Methods Enzymol.*, **84**, 558 (1982).
2) P. H. Hinderling, J. O. Magnusson, and L. Molin, *J. Pharm. Sci.*, **75**, 517 (1986).
3) D. R. Clark and H. H. Sussman, *Clin. Chem. (N. Y.)*, **32**, 381 (1986).
4) M. Gault, L. L. Henly, M. D. Madonna, and V. C. Sudesh, *Clin. Chem. (N. Y.)*, **31**, 1272 (1985).
5) P. Helsingius, H. Likka, and L. Yimo, *Clin. Chem. (N. Y.)*, **32**, 1767(1986).
6) M. Plevani and A. Burlina, *Clin. Chem. (N. Y.)*, **31**, 1879 (1985).
7) E. P. Diamandis, R. C. Morton, E. Reichstein, and M. J. Khosrau, *Anal. Chem.*, **61**, 48 (1989).
8) R. W. Nadeau and D. J. Liberato, *Anal. Chem.*, **61**, 1732 (1989).
9) R. Lejeune, L. Thunus, F. Gomez, F. Frankenne, J. L. Cloux, and G. Hennen, *Anal. Biochem.*, **189**, 217 (1990).
10) T. G. Kingan, *Anal. Biochem.*, **183**, 283 (1989).
11) F. Coutlee, R. H. Yolken, and R. P. Viscidi, *Anal. Biochem.*, **181**, 153 (1989).
12) K. E. Rubenstein, R. S. Schneider, and E. F. Ullman, *Biochem. Biophys. Res. Commun.*, **47**, 846 (1972).
13) W. R. Heineman, *Anal. Chem.*, **58**, 2995 (1986).
14) W. E. Hornby and D. Rawson, *Anal. Chem.*, **53**, 658 (1981).
15) G. Gorog, A. Gandlfi, G. Paradisi, E. Rolleri, E. Klasen, V. Dessi, R. Strom, and F. Celada, *J. Immunol. Methods*, **123**, 131 (1989).
16) J. M. Dicks and A. P. F. Turner, *Anal. Chim. Acta*, **182**, 103 (1986).
17) A. U. Kabanov, M. M. Khrutskaya, S. A. Eremin, N. L. Klyachko, and A. V. Leuashou, *Anal. Biochem.*, **181**, 145 (1989).
18) T. T. Ngo and H. M. Lenhoff, *Appl. Biochem. Biotechnol.*, **6**, 53 (1981).
19) Y. Ikariyama, H. Kunoh, and M. Aizawa, *Biochem. Biophys. Res. Commun.*, **128**, 987 (1985).
20) T. Kuwana, B. Epstein, and E. T. Seo, *J. Phys. Chem.*, **67**, 2243 (1963).
21) S. H. Jenkins, W. R. Heineman, and H. B. Halsall, *Anal. Biochem.*, **168**, 292 (1988).
22) C. A. Broyres and G. A. Rechnitz, *Anal. Chem.*, **58**, 1241 (1986).
23) A. Borrachero, J. V. Rodriguez, and A. F. Sanchez, *Analyst*, **113**, 1795 (1988).
24) S. H. Jenkins, H. B. Halsall, and W. R. Heineman, "Advances in Biosensors," ed by A.P.F. Jurner, JAI Press, London (1991), Vol. 1, p. 171.
25) Rodriguez and A. Sanchez, *Analyst*, **144**, 393 (1989).
26) M. J. Doyle, H. B. Halsall, and W. R. Heineman, *Anal. Chem.*, **54**, 2318 (1982).
27) A. Heller, *Acc. Chem. Res.*, **23**, 128 (1990).
28) K. D. Gleria, H. A. O. Hill, and C. J. McNeil, *Anal. Chem.*, **58**, 1203 (1986).
29) J. A. Hinds and P. Duffy, *Clin. Chem. (N. Y.)*, **32**, 16 (1986).
30) Y. Degani and A. Heller, *J. Phys. Chem.*, **91**, 1285 (1987).
31) T. Suzawa, Y. Ikariyama, and M. Aizawa, *Anal. Sci.*, **9**, 641 (1993).
32) Yao and G. A. Rechnitz, *Biosensor*, **3**, 307 (1986).
33) T. Suzawa, Y. Ikariyama, and M. Aizawa, *Anal. Chem.*, in press.