An electrochemical immunosensor using ferrocenyl-tethered dendrimer

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We report here an enzyme-amplified, sandwich-type immunosensor for detecting the biospecific interaction between an antibody and antigen using redox mediation. We employed biotin/anti-biotin IgG as a model immunosensing pair. Partially ferrocenyl-tethered dendrimer (Fc-D), whose ferrocene moiety acts as a redox mediator, was immobilized to the electrode surface by covalent binding between the dendrimer amines and the carboxylic acids of a self-assembled monolayer. The unreacted amines of the immobilized Fc-D were modified with biotin groups to allow the specific binding of goat anti-biotin IgG. Rabbit anti-goat IgG-conjugated alkaline phosphatase was bound to goat anti-biotin IgG to catalyze conversion of *p*-aminophenyl phosphate monohydrate to *p*-aminophenol. This product is oxidized to quinoimide by the reduction of ferrocenium back to ferrocene, producing an electrocatalytic anodic current. Cyclic voltammograms and surface plasmon resonance experiments showed that the binding of nonspecific proteins is not significant on the biotinylated Fc-D surface. We also examined the change in peak current according to the concentration of anti-biotin IgG and found that the detection range of this immunosensing scheme is between 0.1 and 30 μg mL^{-1}.

Introduction

Immuno- or affinity-sensing techniques to detect biospecific interactions such as antibody–antigen,[1,2] ligand–receptor,[3] and protein–protein[4] interactions have been the focus of many studies. Among these sensing systems, a major focus has been the development of immunosensors for fast and simple measurement of specific biochemical interactions. The detection systems for immunosensors are based on optical, electrochemical, or gravimetric methods.[5,6] In the case of electrochemical immunosensors, the biospecific interaction is electrochemically transformed into an electrical signal.[7,8] Specifically, the interaction signal can be amplified by an enzyme that continuously generates electroactive products.[9] Because of their simple fabrication and good sensitivity, such enzyme-amplified electrochemical immunosensors have been adopted for many miniaturized and microfluidic devices.[10–13]

The electrode surface is very important in electrochemical immunosensors. The modification of electrode surfaces allows highly dense immobilization of biomolecules, long-term stability of attached biomolecules, low nonspecific binding, and proper biomolecular orientation to permit simple and rapid specific interactions. Thiol-linked self-assembled monolayers (SAMs) are a widely used modification method to immobilize biomolecules to gold surfaces[14,15] because they provide a variety of functional groups for easy covalent attachment of biomolecules. However, the additional layers of SAM and biomolecules make electron transfer difficult and lead to blocking of the direct electron transfer between electroactive species and an electrode. In these electrochemical immunosensors, an enzyme is used to generate organic electroactive products and most of these electroactive products are oxidized or reduced on the electrode surface *via* electron transfer.[16] The kinetics of this electron transfer is highly dependent on the surface state of the electrode. If the electron transfer is blocked by the additional layers of SAM, high applied potentials are required for the direct redox reaction of electroactive species. Furthermore, SAMs on gold have a low background current but a limited electrochemical window. Although alkanethiol SAMs have good stabilities between 0.2 and 0.5 V *vs.* a saturated calomel electrode,[17–19] high applied potentials induce a large background current and unwanted change in the SAMs. If the measurement is performed over a long period of time, this change can significantly affect the stability of the sensor. Accordingly, redox mediating functional groups that enable electron transfer between electroactive species and the solid surface should be considered for better signaling.

We have introduced partially-ferrocenyl tethered dendrimer (Fc-D) to solve the problem. Ferrocene in Fc-D acts as a redox mediator. Our previous studies have shown that partially-ferrocenyl tethered dendrimer is a good redox mediator for the oxidation of alkaline phosphatase (ALP)-generated *p*-aminophenol (*p*-AP).[20] The dendrimer part in Fc-D was also used as a good building block. Dendrimers are spherical, and they possess many functional groups (*e.g.*, amines) in their termini so that they can be easily immobilized on a solid surface and modified with other functional ligands.[21–23] Their many and easily modified functional groups are used for the immobilization of biomolecules.

One other way to overcome the problem of electrode surface blocking by organic insulated layers is to separate the surface

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for immobilization of biomolecules from the electrode.[24] In this case, the solid surface and the electrode should be in close proximity to allow rapid diffusion of the enzyme-amplified products. Fritsch's group has accomplished this by using microcavity-containing layered electrodes.[25] However, such a system is structurally more complex than a one-electrode system and therefore difficult to fabricate.

Here we describe an enzyme-amplified electrochemical immunosensor using redox mediation of Fc-D. As shown in Scheme 1, the Fc-D acts not only as a substrate for the attachment of biomolecules but also as a mediator of electron transfer. As a model system, we measured the biospecific interaction between biotin and anti-biotin IgG by a sandwich-type immunosensor using ALP as the enzyme for generating electroactive products. We assessed the performance of the immunosensing layer by measuring the binding of nonspecific antibodies. Finally, we determined the dependence of the sensor signal on the concentration of anti-biotin IgG.

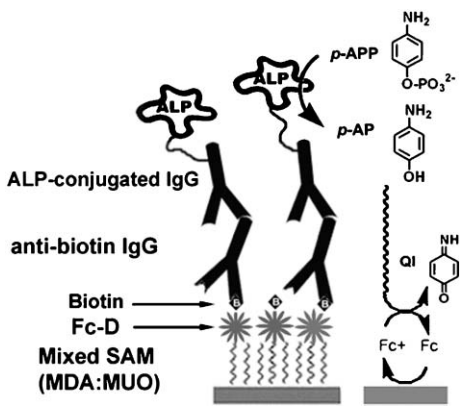
Experimental

Chemicals

Goat anti-biotin IgG, goat anti-mouse IgG, ALP-conjugated goat anti-mouse IgG, and ALP-conjugated rabbit anti-goat IgG were purchased from Pierce Chemicals (Rockford, IL). Poly(amidoamine) dendrimer, (+)-biotin *N*-hydroxy-succinimide ester, ferrocenecarboxaldehyde, and sodium borohydride were purchased from Aldrich. 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride(EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma. *p*-Aminophenyl phosphate monohydrate (*p*-APP) was purchased from Universal Sensors (Cork, Ireland). All buffer salts and other inorganic chemicals were obtained from Sigma or Aldrich unless otherwise stated and were used as received. Ultrapure water (>18 MΩ) from a Modulab water system (US Filter Corp.) was used for these studies.

Preparation of Fc-D

Fc-D was synthesized by an imine formation reaction between partial primary amines of NH_2-terminated G4 poly(amidoamine) (PAMAM) dendrimer and ferrocenecarboxaldehyde as previously described.[21–23] Based on previous studies,[20] the extent of primary amine modification was estimated to be 30%.



Scheme 1 Schematic illustration of an enzyme-amplified immunosensor using redox mediation of Fc-D.

Construction of the biotin-modified Fc-D electrode

Gold electrodes were prepared by electron beam evaporation of 40 nm of titanium and 150 nm of gold onto silicon (100) wafers. The electrode was cleaned in piranha solution (1 : 3 v/v H_2O_2 : H_2SO_4), rinsed with water, and dried under N_2 gas. The clean electrode was immersed in a mixture of 1 mM mercaptododecanoic acid (MDA) and 4 mM mercaptoundecanol (MUO) for 12 h, washed with pure methanol, and dried under N_2 gas. The carboxylic groups were activated by immersion of the electrode into a solution of 10 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride and 5 mM *N*-hydroxysuccinimide for 3 h, and the Fc-D solution (20 μM) was spotted onto the activated electrode and allowed to incubate for 2 h. Nonspecifically absorbed Fc-D was removed by rinsing twice with rinsing buffer (RB; 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris), 0.5 M NaCl, 0.05% (v/v) Tween 20, and 0.05% (w/w) bovine serum albumin (BSA), pH 7.5). Biotin was attached to the Fc-D modified electrode by immersing it for 2 h in a solution containing 1.5 mg mL^{-1} (+)-biotin *N*-hydroxy-succinimide ester, after which it was washed with methanol and water.

Electrochemical and SPR experiments

The electrochemical experiment was performed using a BAS 100B electrochemical analyzer (Bioanalytical Systems, Inc.). The three-electrode electrochemical cell consisted of the modified gold electrode, a Pt wire counter electrode, and a mercury sulfate reference electrode (MSE; Hg/Hg_2SO_4 electrode containing saturated K_2SO_4). The cell was filled with buffer for electrochemical experiments (EB; 50 mM Tris, and 1 g L^{-1} $MgCl_2$, pH 9.0) containing 1 mM *p*-APP and deoxygenated with Ar gas. The *p*-APP solution was prepared daily.

The biotin-modified Fc-D electrode was incubated in binding buffer (BB; 50 mM Tris, 0.3 M NaCl, 0.05% Tween 20, and 1% BSA, pH 7.4) for 30 min to prevent the nonspecific adsorption of antibody and then washed with RB. The electrode was then immersed for 30 min in incubating buffer (IB; 50 mM Tris, 150 mM NaCl, and 1% BSA, pH 7.2) containing 100 μg mL^{-1} goat anti-biotin IgG. After rinsing with RB, the resulting assembly was immersed in IB containing 100 μg mL^{-1} ALP-conjugated rabbit anti-goat IgG, followed by washing in RB.

Surface plasmon resonance (SPR) measurements were performed with a BIAcore X instrument (D. I. Biotech Ltd). Fc-D was immobilized on the mixed SAM–bare gold substrate of the sensor chip using a SIA Kit Au (BIAcore AB). SPR experiments were carried out using RB as the running buffer, a constant flow (5 μL min^{-1}) of solution over the surfaces, and with 100 μg mL^{-1} of antibodies.

Results and discussion

In this study, an enzyme-amplified electrochemical immunosensor for sensing antibody–antigen interaction has been

developed. Scheme 1 illustrates the immunosensing system developed in the current study. For this system, a biotin-modified Fc-D layer was prepared on the gold electrode. Fc-D affords two important advantages. First, Fc-D has an unreacted amine moiety, which provides an efficient site for immobilizing biomolecules to sensor surfaces. Second, Fc-D acts as a redox mediator because it can mediate electron transfer between the electrode and the enzyme-generated electroactive products. Because the compact mixed SAM blocks the approach of the soluble redox reagents to the electrode,[17–19] direct electron transfer reaction on the gold electrode can be hindered. The compact mixed SAM also reduces the background current of the electrode.[26] In this system, the analyte, goat anti-biotin IgG, binds to biotin on the surface and then is bound by the ALP-conjugated anti-goat IgG. ALP enzymatically converts *p*-APP to electroactive *p*-AP.[27,28] The produced *p*-AP then diffuses to the Fc-D where it is electrocatalytically oxidized by the ferrocene of Fc-D. Thus, the electrocatalytic current depends on the concentration of anti-biotin IgG.

To characterize the electrocatalytic performance of the immunosensing layer, the biotinylated electrode was bound to goat anti-biotin IgG followed by ALP-conjugated anti-goat IgG and then incubated for 1 min in EB containing 1.0 mM *p*-APP. Fig. 1A shows a cyclic voltammogram after the 1 min incubation. In the presence of ALP, an irreversible peak appears near −0.1 V *vs.* MSE, indicating redox mediated oxidation of *p*-AP. The oxidation potential of *p*-AP appears near −0.4 V *vs.* MSE at a bare gold electrode, but the potential appears at more positive potential at the dendrimer-modified electrode. As discussed in our previous report,[20] the anodic peak potential positively shifts from the oxidation potential of *p*-AP (−0.4 V *vs.* MSE) to the oxidation potential of the dendrimetric ferrocenyl groups (−0.1 V *vs.* MSE). This is explained by the following mechanism,

$$2\ \text{ferrocene} \rightarrow 2\ \text{ferricenium}^{+} + 2\ e^{-}$$

$$2\ \text{ferricenium}^{+} + p\text{-AP} \rightarrow \text{quinoimide} + 2\ \text{ferrocene} + 2\ H^{+}$$

In the case of a poly(amidoamine) (PAMAM) dendrimer-modified electrode, the broad peak with low current appears at a higher positive potential. The acid SAM (mixed with MUO) efficiently blocks much of the direct electron transfer between *p*-AP and the gold electrode, only a minor portion of *p*-AP directly oxidizes on the dendrimer modified SAM. Although plenty of electroactive labels (*p*-AP) are generated by the hydrolysis reaction of ALP, the electrochemical signal might not increase in proportion to the amount of biocatalytically generated *p*-AP, because the electron transfer between *p*-AP and the electrode is blocked by the organic SAM. However, the introduction of ferrocene into the PAMAM-dendrimer solves this problem, leading to a great increase in the electrochemical signal. More detailed experiments are described in our previous report.[20]

As a negative control, we obtained a cyclic voltammogram in the absence of goat anti-biotin IgG binding to the biotinylated Fc-D layer (Fig. 1B). Without the goat anti-biotin IgG, the peak current was much smaller than in Fig. 1A. This diminished current indicates that the binding of ALP-conjugated anti-goat IgG is much weaker due to the lack of goat anti-biotin IgG on the Fc-D surface. The small peak current seems to be partially due to nonspecific binding of antibodies because the peak current due only to ferrocene oxidation on the Fc-D electrode was about 1 μA.

As additional conditions to test nonspecific binding, we examined the effects of replacing goat anti-biotin IgG with goat anti-mouse IgG (Fig. 1C) or ALP-conjugated anti-mouse IgG in place of ALP-conjugated anti-goat IgG (Fig. 1D). In both cases, the peak currents were very small. The small peak currents indicate that the goat anti-mouse IgG does not bind specifically to the biotin and that ALP-conjugated anti-mouse IgG does not bind the surface-immobilized anti-biotin IgG. The small peak currents appear to be at least partially due to nonspecific binding.

Based on these control experiments, it is evident that the immunosensing layer performs well in terms of specific binding and redox mediation and that there is only weak nonspecific binding of antibodies to the Fc-D. Low nonspecific binding seems due to the hydrophilic nature of the unmodified amine in Fc-D.

To qualitatively monitor the specific and nonspecific binding, we also performed SPR measurements. There was an increase of 2900 RU after between 270 and 690 s when the biotinylated Fc-D surface was treated with goat anti-biotin IgG followed by ALP-conjugated anti-goat IgG. A second increase of 900 RU was observed between 1000 and 1420 s

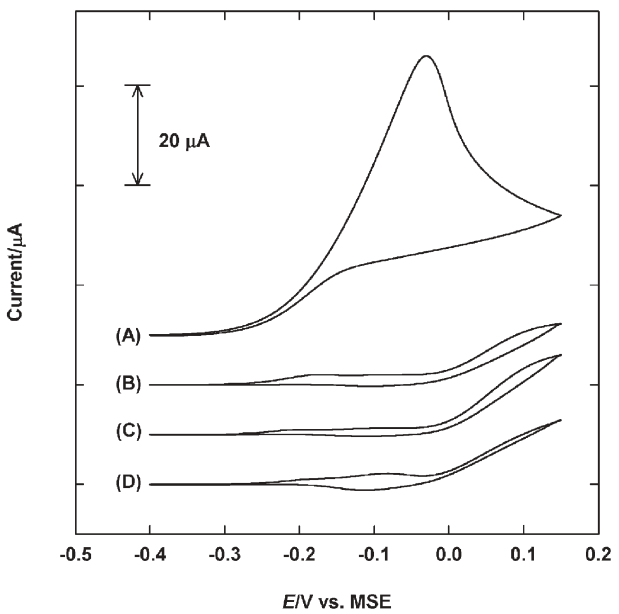


Fig. 1 Cyclic voltammograms for the performance of biotinylated Fc-D layers with (A) normal and (B–D) negative control samples. The electrodes were incubated in (A, D) the presence and (B, C) the absence of goat anti-biotin IgG (100 μg mL^{-1}, 30 min). In (C), goat anti-mouse IgG was used instead of goat anti-biotin IgG. The electrodes were then incubated with (A–C) ALP-conjugated rabbit anti-goat IgG or (D) ALP-conjugated goat anti-mouse IgG (100 μg mL^{-1}, 30 min). All cyclic voltammograms were obtained after incubating for 1 min in EB containing 1 mM *p*-APP and at a scan rate of 50 mV s^{-1}.

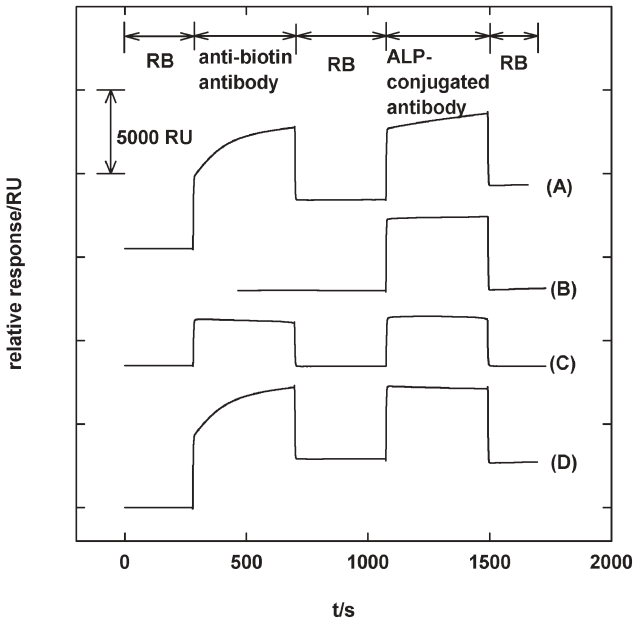


Fig. 2 SPR sensorgrams for the performance of biotinylated Fc-D layers with (A) normal and (B–D) negative control samples. All cases (A–D) have the same antibody incubating sequence as in Fig. 1A–D. The electrodes were incubated in (A, D) the presence and (B, C) the absence of goat anti-biotin (100 μg mL^{-1}, 7 min). In (C), goat anti-mouse IgG was used instead of goat anti-biotin IgG. The electrodes were then incubated with (A–C) ALP-conjugated rabbit anti-goat IgG or (D) ALP-conjugated goat anti-mouse IgG (100 μg mL^{-1}, 7 min). The flow rate was 5 μL min^{-1}.

(Fig. 2A). This stepwise increase indicates a two-step specific binding process: the initial increase is due to the binding of goat anti-biotin IgG to biotin, and the second is due to the binding of the ALP-conjugated anti-goat IgG to goat anti-biotin IgG. A change of 1000 RU in SPR corresponds to 1 ng mm^{-2}.[29] Given that the molecular weight of ALP-conjugated IgG is approximately 270 kDa, the change of 900 RU corresponds to approximately 3.3 fmol mm^{-2} of ALP-conjugated IgG bound to the electrode surface.

On the contrary, when the biotinylated Fc-D surface was treated with only ALP-conjugated anti-goat IgG and without incubation with goat anti-biotin IgG, the change in the SPR signal was only approximately 40 RU (Fig. 2B). The small change seems to be related to nonspecific binding of ALP-conjugated anti-goat IgG. When the biotinylated Fc-D surface was incubated twice with goat anti-mouse IgG (instead of goat anti-biotin) followed by ALP-conjugated anti-goat IgG, there was not a detectable increase in the SPR signal (Fig. 2C). This indicates an absence of specific binding and negligible nonspecific binding. When the biotinylated Fc-D surface was treated with goat anti-biotin IgG followed by ALP-conjugated anti-mouse IgG instead of ALP-conjugated anti-goat IgG, the increase in the SPR signal was also negligible (Fig. 2D). The results of both the cyclic voltammograms and SPR experiments indicate that the binding of anti-biotin IgG to biotin and the binding of ALP-conjugated IgG to anti-biotin IgG are specific and that there is not considerable nonspecific binding of antibody proteins.

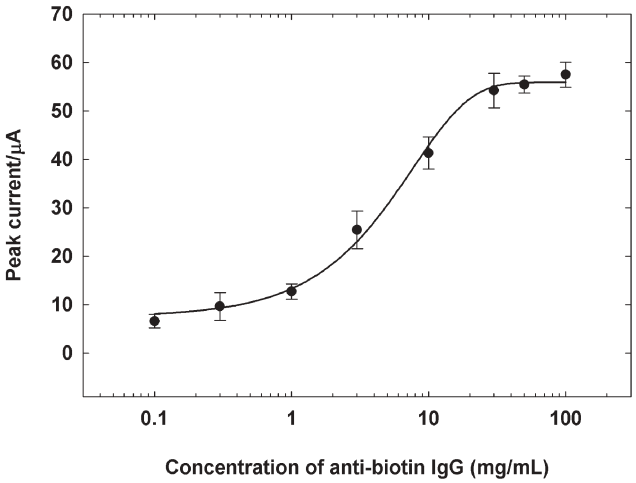


Fig. 3 Calibration plot showing the correspondence between the changes in anodic peak current and the concentration of goat anti-biotin (0.1 μg mL^{-1} to 100 μg mL^{-1}). Cyclic voltammograms were obtained for 1 min after incubation with EB containing 1 mM *p*-APP and at a scan rate of 50 mV s^{-1}.

We next investigated the detection range of the immunosensing system. Cyclic voltammograms were obtained in solutions containing 0.1, 0.3, 1.0, 3.0, 30, 50, and 100 μg mL^{-1} of anti-biotin IgG (Fig. 3). The peak current increased with the concentration of anti-biotin IgG but saturated at concentrations over 30 μg mL^{-1}. The detection limit for qualitative analysis was approximately 0.1 μg mL^{-1}, and the detection range for quantitative analysis was between 0.1 μg mL^{-1} and 30 μg mL^{-1}.

Conclusion

We developed a new electrochemical method to detect the specific interaction between antibodies and antigens. We employed biotin/anti-biotin IgG as a model antigen/antibody pair. In this system, Fc-D acts as a redox mediator and as a biofunctional substrate. Nonspecific binding that is important in terms of immunosensor performance was not significant on the biotinylated Fc-D surface. The redox mediated oxidation of *p*-AP with Fc-D is helpful for the determination of peak current according to concentration. These studies show that Fc-D can be used for enzyme-amplified electrochemical immunosensors.

Acknowledgements

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