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ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 2007

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Anal. Chem., **2007**, 79 (7), 2790-2796 • DOI: 10.1021/ac062291l

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Electrochemical Immunosensor Using *p*-Aminophenol Redox Cycling by Hydrazine Combined with a Low Background Current

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Signal amplification and noise reduction are crucial for obtaining low detection limits in biosensors. Here, we present an electrochemical immunosensor in which the signal amplification is achieved using *p*-aminophenol (AP) redox cycling by hydrazine, and the noise level is reduced by implementing a low background current. The redox cycling is obtained in a simple one-electrode, one-enzyme format. In a sandwich-type heterogeneous immunosensor for mouse IgG, an alkaline phosphatase label converts *p*-aminophenyl phosphate into AP for 10 min. This generated AP is electrooxidized at an indium tin oxide (ITO) electrode modified with a partially ferrocenyl-tethered dendrimer (Fc-D). The oxidized product, *p*-quinone imine (QI), is reduced back to AP by hydrazine, and then AP is electrooxidized again to QI, resulting in redox cycling. Moreover, hydrazine protects AP from oxidation by air, enabling long incubation times. The small amount of ferrocene in a 0.5% Fc-D-modified ITO electrode, where 0.5% represents the ratio of ferrocene groups to dendrimer amines, results in a low background current, and this electrode exhibits high electron-mediating activity for AP oxidation. Moreover, there is insignificant hydrazine electrooxidation on this electrode, which also results in a low background current. The detection limit of the immunosensor using a 0.5% Fc-D-modified electrode is 2 orders of magnitude lower than that of a 20% Fc-D-modified electrode (10 pg/mL vs 1 ng/mL). Furthermore, the presence of hydrazine reduces the detection limit by an additional 2 orders of magnitude (100 fg/mL vs 10 pg/mL). These results indicate that the occurrence of redox cycling combined with a low background current yields an electrochemical immunosensor with a very low detection limit (100 fg/mL). Mouse IgG could be detected at concentrations ranging from 100 fg/mL to 100 μg/mL (i.e., 9 orders of magnitude) in a single assay.

The sensitivity of biosensors depends on their signal-to-noise ratio.[1] Various signal amplification methods have been developed to maximize the signal,[2] with enzyme labeling being most commonly employed to amplify the number of signal-reporting molecules per biospecific binding between a target biomolecule and an enzyme-labeled biomolecule.[3]

Combining electrochemical and enzymatic amplification in electrochemical biosensors provides much higher signal amplification.[4] The electrochemical amplification can be obtained by redox cycling, which is related to the regeneration of enzyme-amplified electroactive species after their electrochemical oxidation or reduction.[4b] The redox reaction of the regenerated species provides an enhanced electrochemical signal. Redox cycling can be achieved using two working electrodes,[5] such as in an interdigitated array electrode in which an electroactive species generated at one microband electrode diffuses across a band gap to the second microband electrode, with the reverse reaction occurring at the second electrode. However, an interdigitated array electrode requires a microfabricated microband electrode with a short band gap and numerous band pairs to obtain a high redox-cycling efficiency, and the potentials of the two microband electrodes need to be controlled separately.[5a] These requirements limit the wide use of interdigitated array electrodes in electrochemical biosensors. Although a scanning electrochemical microscope using two electrodes increases that redox-cycling efficiency,[6] it is not suitable for biosensor applications because of its operational complexity.

Multiple enzymes can also produce redox cycling via enzymatic reduction or oxidation of electrooxidized or electroreduced species.[7] The redox-cycling efficiency is highly dependent on the enzyme kinetics and the amount of a dissolved or immobilized

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10.1021/ac062291l CCC: $37.00
Published on Web 02/21/2007

enzyme. Rational design of a layer containing two enzymes could result in a high redox-cycling efficiency.[8]

It is well-known that electrochemical catalytic (EC′) reactions enhance the redox current of electroactive species.[9] Redox cycling by enzymes is an example of an EC′ reaction. In general EC′ reactions, an excess of nonelectroactive species that act as reducing or oxidizing agents is required to regenerate a starting electroactive species.[9a] We recently employed an EC′ reaction to enhance signal amplification.[10] The chemical reduction of *p*-quinone imine (QI) to *p*-aminophenol (AP) by $NaBH_4$ results in ultrasensitivity, but a reducing agent that is more stable in aqueous solutions is required because $NaBH_4$ is unstable at neutral and acidic pH values.

Alkaline phosphatase (ALP) is commonly employed as an enzyme label in biosensors,[11] and *p*-aminophenyl phosphate (APP) has been used as an enzyme substrate in such sensors.[4b] The reaction rate of ALP remains linear for long incubation times, and the resulting linear accumulation of enzyme product yields a high sensitivity.[11] However, AP is easily oxidized by air and is light-sensitive, which limits the time of linear accumulation.[7c] Thus, deaerated buffer solutions or AP-stabilizing buffer solutions are necessary for long incubation times.

Noise reduction is also required to increase the sensitivity. The noise in electrochemical biosensors is dominated by the background current of the electrode. Highly electrocatalytic electrodes usually exhibit large background currents because the redox reactions of water, electrolyte, and other nonanalytes are considerable at low potentials and near the formal potentials of their redox reactions. Interestingly, indium tin oxide (ITO) electrodes exhibit low background current because of their low electrocatalytic activity and low capacitive current.[12] Although the electrooxidation of $NaBH_4$ can be diminished by employing an ITO electrode modified with a partially ferrocenyl-tethered dendrimer (Fc-D), the background current due to $NaBH_4$ is still considerable.[10] Accordingly, a reducing agent that is electrochemically inactive is also required. Herein, we present an electrochemical immunosensor using hydrazine as a reducing agent for redox cycling and an Fc-D-modified ITO electrode for achieving a low background current. First, the background current of an Fc-D-modified ITO electrode is investigated in terms of its (i) capacitive current and (ii) redox current and (iii) the redox current of hydrazine. Then, AP redox cycling by hydrazine is demonstrated, and its kinetics is assessed. Finally, the effects of hydrazine and the ratio of ferrocene groups to dendrimer amines on the detection limits of an immunosensor are assessed.

EXPERIMENTAL SECTION

Materials. Amine-terminated G4 poly(amidoamine) dendrimer, ferrocene carboxaldehyde, 3-phosphonopropionic acid, AP, hydrazine monohydrate, *N*,*N*-dimethylformamide, H_2O_2, and NH_4OH were purchased from Aldrich. Mouse IgG from serum, biotinylated goat antimouse IgG, ALP-conjugated goat antimouse IgG, (+)-biotin *N*-hydroxysuccinimide ester, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, and streptavidin from *Streptomyces avidinii* were purchased from Sigma. APP monohydrate was purchased from Universal Sensors (Cork, Ireland), and *N*-hydroxysuccinimide was obtained from Fluka. All buffer reagents and other inorganic chemicals were supplied by Sigma, Aldrich, or Fluka except where stated otherwise. All chemicals were used as received. All aqueous solutions were prepared in doubly distilled water.

The PBS solution consisted of 0.01 M phosphate, 0.138 M NaCl, and 0.0027 M KCl (pH 7.4). The PBSB solution contained all of the ingredients of PBS plus 1% (w/v) bovine serum albumin (pH 7.4). The rinsing buffer (RB) was composed of 50 mM tromethamine (Tris), 40 mM HCl, 0.5 M NaCl, 0.05% (w/v) bovine serum albumin, and 0.05% Tween 20 (pH 7.4). The Tris buffer for the electrochemical experiment comprised 50 mM Tris, 10 mM KCl, 1 g/L $MgCl_2$, and 7 mM HCl (pH 9.0).

Three Fc-Ds with differing ratios of ferrocene groups to dendrimer amines were synthesized as described previously.[13] On the basis of NMR and UV/vis spectra, the ratios were estimated at 0.5%, 6%, and 20% in the three synthesized Fc-Ds (these are referred to henceforth as 0.5% Fc-D, 6% Fc-D, and 20% Fc-D, respectively).

Preparation of an Immunosensing Layer. We constructed three sandwich-type heterogeneous immunosensors using 0.5% and 20% Fc-Ds. A schematic of the preparation of an immunosensing layer is shown in Figure 1a. ITO electrodes obtained from Geomatec (Japan) were successively cleaned with ethanol, acetone, 2-propanol, and water and then dried at 60 °C. The cleaned ITO electrodes were pretreated in a mixture containing H_2O, H_2O_2 (30%), and NH_4OH (30%) in a ratio of 5:1:1 at 70 °C for 1.5 h to ensure the presence of active hydroxyl groups on the surface. The electrodes were then washed with copious amounts of water and dried. The pretreated ITO electrodes were immersed in an aqueous solution containing 0.1 mM 3-phosphonopropionic acid for 36 h at room temperature to allow the formation of a carboxylate monolayer. The substrates were then washed with distilled water. The carboxylic groups were activated by immersing the electrodes in a mixture of 50 mM 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride and 25 mM *N*-hydroxysuccinimide for 2 h. The Fc-D solution (100 μM) was dropped onto the activated electrodes and incubated for 2 h. Nonspecifically ad-

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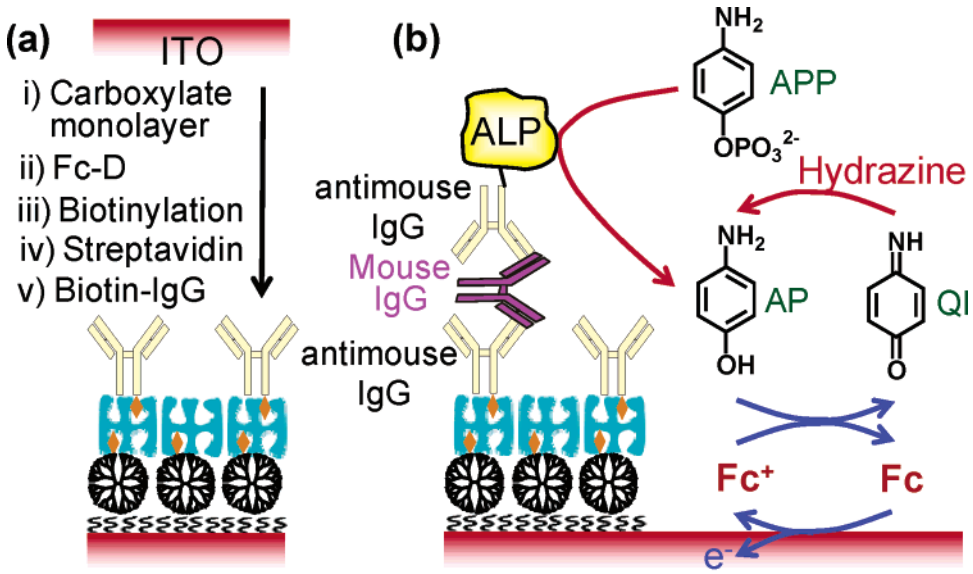


Figure 1. (a) Schematic representation of the preparation of an immunosensing layer. First, a carboxylate monolayer on a cleaned ITO electrode was formed using 3-phosphonopropionic acid, and then Fc-D was immobilized by covalent bonding between dendrimer amines and carboxylic groups of the carboxylate monolayer. Some of the unreacted amines of Fc-D were modified with biotin groups. Afterward, streptavidin and biotinylated antimouse IgG were immobilized. (b) Schematic view of electrochemical detection for mouse IgG. The biotinylated antimouse IgG and the ALP-conjugated antimouse IgG sandwich the target mouse IgG. ALP generates AP by enzymatic reaction. Thus-generated AP molecules are electrochemically oxidized to QI via the electron mediation of ferrocene. The oxidized QI is then reduced back to AP by hydrazine. Fc and Fc^+ represents the ferrocene of Fc-D and its oxidized form, respectively. The immunosensing and electrochemical detection were performed on the same electrode.

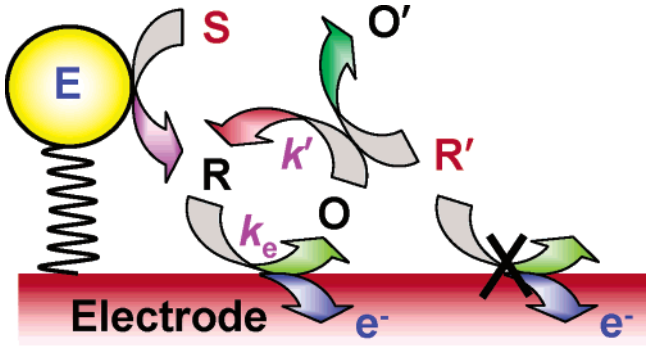
sorbed Fc-D was removed by rinsing twice with RB. Biotin was attached to the Fc-D-modified electrode by immersing it for 2 h in an *N*,*N*-dimethylformamide solution containing 1.5 mg/mL (+)-biotin *N*-hydroxysuccinimide ester, after which it was washed with methanol and water. The biotin-modified electrodes were immersed in PBS containing 100 μg/mL streptavidin for 30 min and washed twice with distilled water. The streptavidin-immobilized electrodes were incubated in a PBSB solution containing 0.05% (v/v) Tween 20 (pH 7.4) for 30 min to minimize the nonspecific binding. After being washed with RB, the electrodes were immersed for 30 min in PBSB containing 100 μg/mL biotinylated goat antimouse IgG and then washed with RB. The substrates were then incubated for 30 min in PBSB containing mouse IgG at various concentrations. After being rinsed with RB, the resulting assembly was immersed in PBSB containing 100 μg/mL ALP-conjugated goat antimouse IgG for 30 min and subsequently washed with RB.

Electrochemical Measurements. The electrochemical experiments were performed using a CHI617B device (CH Instruments). The electrochemical cell consisted of the immunosensing working electrode, a platinum wire counter electrode, and an Ag/AgCl (3 M KCl) reference electrode. The cell was filled with a Tris buffer solution containing 1 mM APP or a mixture of 1 mM APP and 10 mM hydrazine. The hydrazine solution was freshly prepared for each experiment.

RESULTS AND DISCUSSION

Redox Cycling. Scheme 1 illustrates the redox cycling by a reducing agent (R′), in which enzyme E converts substrate S into electroactive product R. After R accumulates for the given incubation time, an electrochemical current is measured by oxidizing R at the electrode. The electrochemically oxidized

Scheme 1



species O is reduced back to R when R′ is present in the solution, and the regenerated R is reoxidized at the electrode. This redox cycling amplifies the redox current. To obtain a low background current, R′ should not be electrooxidized at the electrode. Thermodynamically, the formal potential of the O/R redox couple is more positive than that of the O′/R′ redox couple, which enables R′ to reduce O. Thus, R′ could be electrooxidized at potentials where R is electrooxidized. However, the anodic current of R′ is negligible if the kinetics of R′ electrooxidation is very slow (Scheme 1). In this case, R′ acts as an electrochemically inactive species.

The actual immunosensing scheme is shown in Figure 1b. In the sandwich-type heterogeneous immunosensor for mouse IgG, the biotinylated antimouse IgG and the ALP-conjugated antimouse IgG sandwich the target mouse IgG. The enzyme ALP converts APP into AP. An Fc-D-modified ITO electrode enables AP electrooxidation without large overpotentials,[10,14] and it exhibits very slow electrooxidation of hydrazine. Hydrazine reduces QI

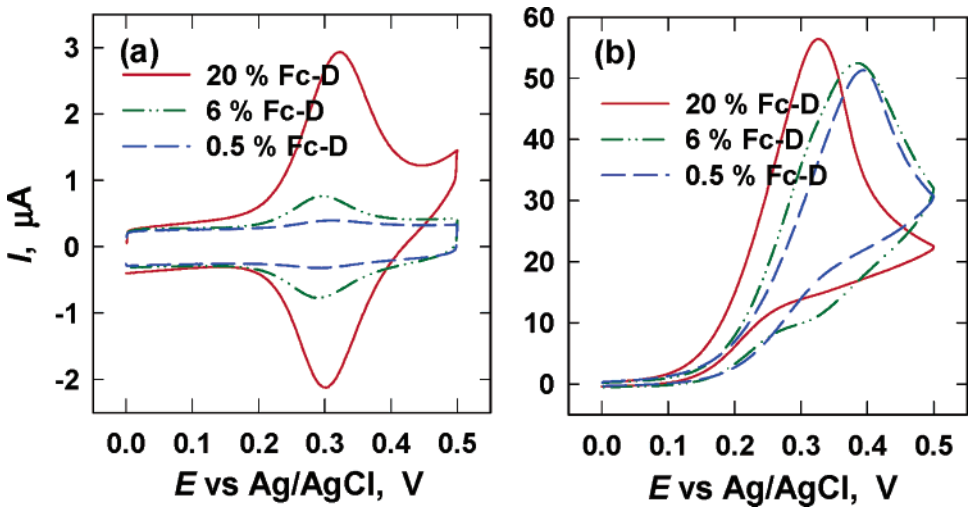


Figure 2. Cyclic voltammograms obtained with 20%, 6%, and 0.5% Fc-D-modified ITO electrodes in (a) Tris buffer solution (pH 9.0) and (b) Tris buffer solution containing 0.5 mM AP at a scan rate of 50 mV/s.

to AP, resulting in redox cycling. The redox cycling increases the current signal, leading to a high sensitivity.

Reduced Background Current. Combining low noise with a large signal results in a high sensitivity. The noise consists of (i) the capacitive current of the electrode, (ii) the redox current of the electrode, and (iii) the redox current of the electrolyte solution. The capacitive current of an electrode will inevitably result in a large amount of noise. Generally, the capacitance current varies with the electrode potential, resulting in highly potential-dependent capacitive current.[15] The capacitive current of ITO electrodes is smaller than those of other metal and carbon electrodes[12b] and is nearly flat over a wide range of potentials, which results in a low background current. Moreover, the capacitive current is highly reproducible compared to those of other electrodes. The flat current behavior is also important in obtaining a high sensitivity.[15]

The redox current of the electrode is a major noise source in many cases, because it results in a large current and unflat current behavior. The redox current during oxide formation at metal electrodes[16] and the surface faradaic current of carbon electrodes[17] are examples of this. In the case of electrodes modified with electroactive species, the redox reaction of the surface-bound electroactive species induces peak-shaped current behavior in cyclic voltammetry, which reduces the sensitivity when the peak potential is close to potentials at which the redox reaction of a signal-generating species occurs. Fc-D-modified electrodes also exhibit peak-shaped current behavior (Figure 2a). The tethering of ferrocene to the dendrimer is employed to lower the overpotential of AP electrooxidation, because ITO electrodes show poor electrocatalytic activity for AP. However, the peak current of ferrocene needs to be reduced to lower the background current. We therefore assessed the three Fc-Ds (0.5%, 6%, and 20% Fc-D) in terms of their peak currents and electron-mediating activities. Figure 2a shows that the peak current of ferrocene electrooxidation is much smaller for the 0.5% Fc-D-modified ITO electrode than for the other two electrodes. Moreover, the 0.5% Fc-D-modified electrode exhibits a redox current of ferrocene that is much smaller than the capacitive current, which results in nearly flat current behavior. It is interesting that the anodic peak currents of AP electrooxidation do not differ significantly between the three electrodes (Figure 2b), even though the peak potential at the 0.5% Fc-D-modified ITO electrode (around 0.4 V) is more positive than that at the 20% electrode. This indicates that the electron-mediating activity of the 0.5% Fc-D-modified ITO electrode is not poor.

A gold, platinum, or glassy carbon electrode cannot be used as a working electrode because of the large redox current of hydrazine (Figure S2 the Supporting Information). In contrast, the background current for a bare ITO electrode in the presence of hydrazine is much smaller (Figure 3),[18] and the current behavior is similar to that in the absence of hydrazine. This background current is clearly smaller than that in the presence of $NaBH_4$, which was used as a reducing agent in our previous work.[10] The ferrocene moiety of Fc-D acts as an electron mediator and makes the oxidation potential of AP less positive. The current behavior of the 0.5% Fc-D-modified ITO electrode in the presence of hydrazine is similar to that of the bare ITO electrode (Figure 3). This demonstrates that the ferrocene of Fc-D does not significantly increase the rate of hydrazine electrooxidation.

Considering that the redox current of hydrazine (Figure 3) and the capacitive current of the Fc-D-modified ITO electrode (Figure 2a) are not large around 0.4 V, the 0.5% Fc-D-modified ITO electrode is good for obtaining a low background current. Moreover, this electrode provides good electron-mediating activity for AP electrooxidation.

Signal Amplification by Redox Cycling. Signal amplification by redox cycling is generally achieved using two working electrodes[5] or multiple enzymes.[7] In this study, we attempted to implement redox cycling by adding only hydrazine to a solution, without using an additional working electrode or enzyme, as

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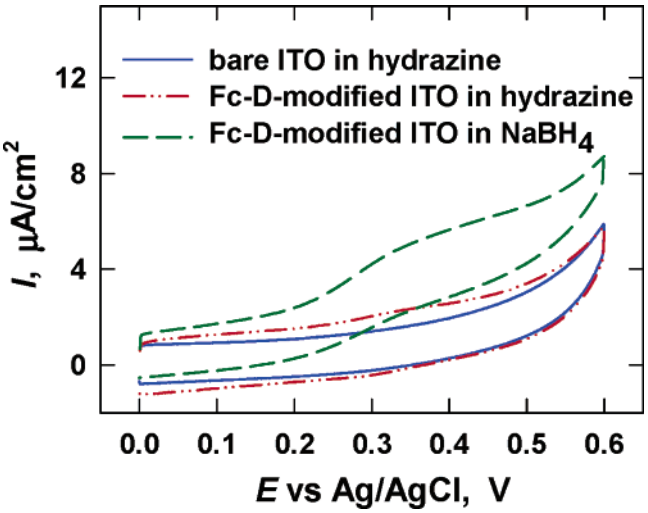

Figure 3. Cyclic voltammograms obtained with bare ITO and 0.5% Fc-D-modified ITO electrodes in Tris buffer solution containing 10 mM hydrazine or 10 mM $NaBH_4$ at a scan rate of 50 mV/s.

shown in Figure 1b. First, the redox cycling by hydrazine was investigated with the 0.5% Fc-D-modified ITO electrode in the presence and absence of hydrazine. Figure 4a shows that the anodic current of AP increases significantly when hydrazine is present in the electrolyte solution, which is related to the electrochemical reoxidation of the AP that is reduced from QI by hydrazine. The difference between the two cyclic voltammograms in the presence and absence of hydrazine is more evident at a low scan rate (Figure 4b). The cyclic voltammogram in the presence of hydrazine at a scan rate of 5 mV/s shows limiting-current behavior rather than peak-current behavior, indicating the presence of the redox cycling (i.e., the EC′ reaction). QI is easily hydrolyzed to *p*-benzoquinone in acidic solutions, but it is stable in the pH 9 Tris buffer solution.[19] Consequently, the redox cycling is not affected by the hydrolysis of QI.

The current in the presence of hydrazine depends on the kinetics of AP oxidation by Fc-D (k_e in Scheme 1), as well as the kinetics of QI reduction by hydrazine (k' in Scheme 1). Because the amount of ferrocene in 0.5% Fc-D is very small, the kinetics of AP oxidation could be slower than that of QI reduction. To check this possibility, a cyclic voltammogram obtained using 6% Fc-D in the presence of hydrazine was compared to a cyclic voltammogram obtained using 0.5% Fc-D (Figure 4b). Although the half-wave potential in the 6% Fc-D case is more negative than that in the 0.5% Fc-D case, the two limiting currents are similar to each other. The limiting current is independent of the amount of ferrocene (i.e., the kinetics of AP oxidation). This indicates that the kinetics of AP oxidation is much faster than that of QI reduction even in the 0.5% Fc-D case. The limiting current depends mainly on the kinetics of QI reduction.

The rate constant of QI reduction by hydrazine (k') can be calculated from the limiting current ($i_{lim} = 11.6\ \mu A$) by[9a]

$$i_{lim} = nFAC^*_{AP}(Dk'C^*_{hydrazine})^{1/2} \quad (1)$$

where C^*_{AP} and $C^*_{hydrazine}$ are the bulk concentrations (mol/cm^3) of AP and hydrazine, respectively, and D is the diffusion coefficient of AP (6.47×10^{-6} cm^2/s).[20] The calculated value of k' is 7.1×10^4 cm^3/mol·s. The rate is represented by

$$\text{rate} = k'[\text{QI}][\text{hydrazine}] \approx k[\text{QI}] \quad (2)$$

The second-order reaction can be simplified to a pseudo-first-order reaction, because the concentration of hydrazine (10 mM) is much larger than that of AP (0.1 mM). In this case, the first-order rate constant, k, is equal to k'[hydrazine], and the calculated k value is 0.71 s^{-1}. In the pseudo-first-order reaction, the half-life of reaction, $t_{1/2}$, is given by[21]

$$t_{1/2} = (\ln 2)/k \quad (3)$$

The calculated half-life is approximately 1 s, which means that the concentration of QI generated by AP electrooxidation halves every second. Although this half-life is not short, the amount of redox cycling is still considerable, and if the half-life were shorter, even higher redox cycling could be obtained.

The AP solution turns black shortly after being prepared because AP is easily oxidized by air, which limits the time for linear accumulation.[7c] Thus, deaerated buffer solutions or AP-stabilizing buffer solutions are usually used for long incubation times. Hydrazine is widely used as an oxygen scavenger for air oxidation and corrosion.[22] In this study, the AP solution did not turn black when hydrazine was present in the solution (Figure S3 in the Supporting Information), which indicates that the hydrazine used for redox cycling prevents the oxidation of AP by air, enabling longer incubation times.

Although $NaBH_4$ can be used to reduce QI to AP, its instability at neutral and acidic pH values makes hydrazine more suitable (because it is stable over a wider range of pH values). Furthermore, we have shown that the redox current of hydrazine for the 0.5% Fc-D-modified ITO electrode is much smaller than that of $NaBH_4$. Thus, hydrazine is better than $NaBH_4$ in terms of both stability and background current.

Immunoassay Using an Fc-D-Modified ITO Electrode and Redox Cycling. We constructed three sandwich-type heterogeneous immunosensors using 0.5% and 20% Fc-D-modified ITO electrodes to investigate the effect of the amount of ferrocene in Fc-D on the detection limit. The effect of hydrazine on detection limits was also examined with the 0.5% Fc-D-modified electrode by comparing cyclic voltammograms in the presence and absence of hydrazine. Cyclic voltammograms for mouse IgG at various concentrations were obtained with each electrode after incubation for 10 min in a Tris buffer solution containing 1 mM APP or 1.0 mM APP and 10 mM hydrazine (Figure 5 and Figures S4 and S5 in the Supporting Information). In Figure 5, the anodic peaks at the 0.5% Fc-D-modified electrode in the presence of hydrazine result mainly from the electrooxidation of AP that is generated by both ALP and redox cycling. The peak current increases with the concentration of mouse IgG. The magnitude of these peak

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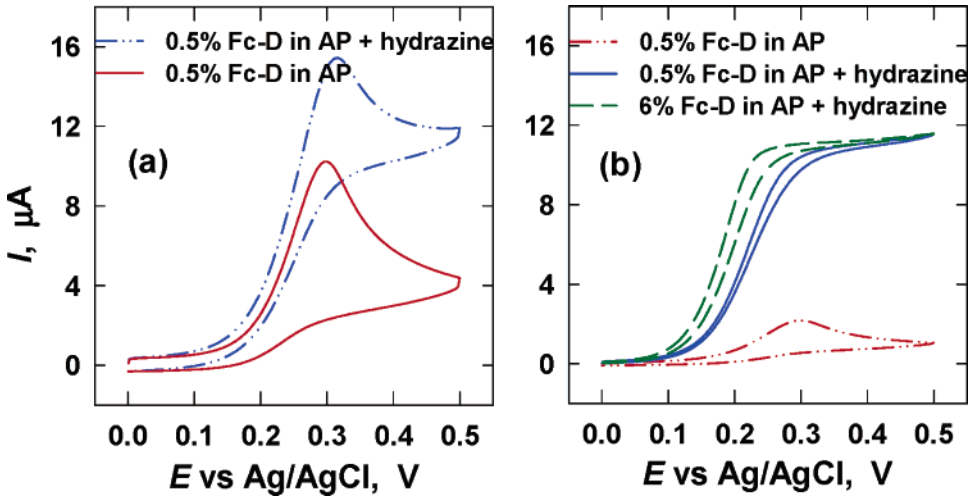

Figure 4. Cyclic voltammograms of a 0.5% and 6% Fc-D-modified ITO electrodes in Tris buffer solution (pH 9.0) containing 0.1 mM AP in the presence and absence of 10 mM hydrazine at scan rates of (a) 50 and (b) 5 mV/s.

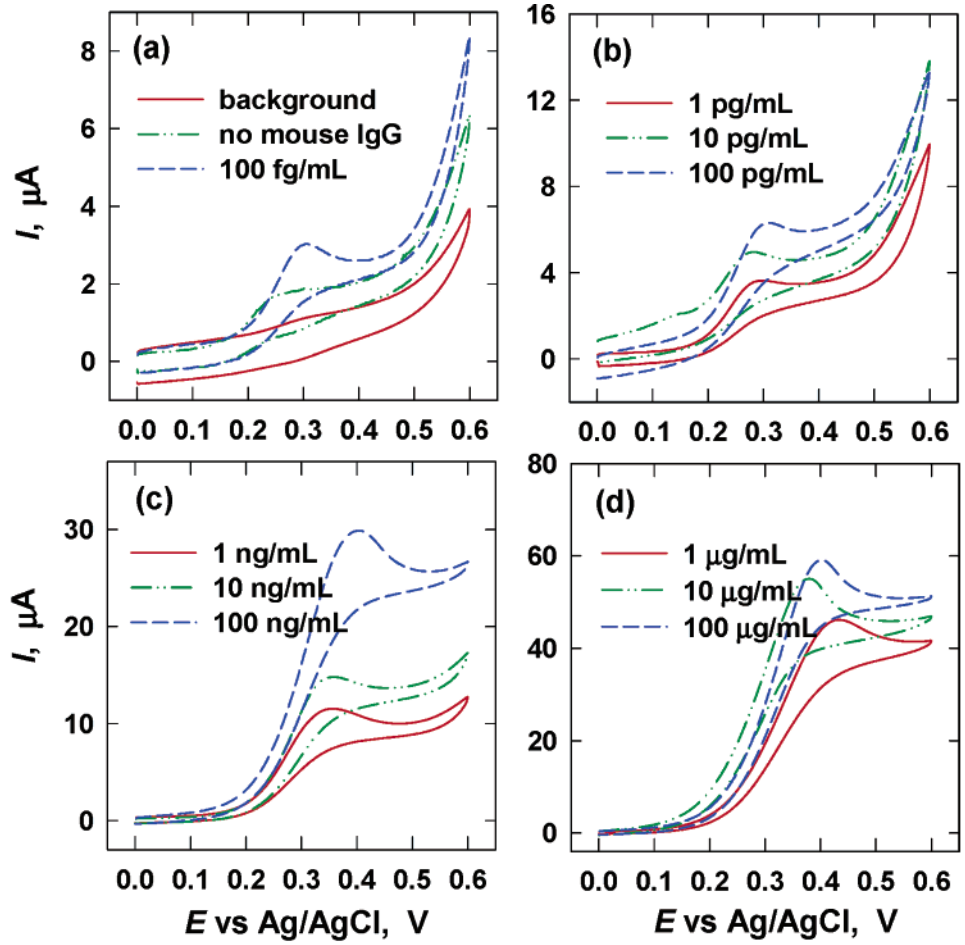

Figure 5. Dependence of cyclic voltammograms on mouse IgG concentration. Cyclic voltammograms of immunosensing electrodes obtained with a 0.5% Fc-D-modified ITO electrode at a scan rate of 50 mV/s after incubation for 10 min in Tris buffer solution containing 1 mM APP and 10 mM hydrazine at (a) 0 and 100 fg/mL mouse IgG; (b) 1, 10, and 100 pg/mL mouse IgG; (c) 1, 10, and 100 ng/mL mouse IgG; and (d) 1, 10, and 100 μg/mL mouse IgG. The background data in part a were obtained without treating the immunosensing electrode with target mouse IgG and ALP-conjugated antimouse IgG.

currents indirectly reflects the amount of the ALP-conjugated IgG that is bound biospecifically to mouse IgG (i.e., the concentration of mouse IgG). The sharp increase in current above 0.5 V is due to APP and hydrazine oxidation.

The cyclic voltammograms of the 20% Fc-D-modified electrode in the absence of hydrazine (Figure S4 in the Supporting Information) are more complex, and the background current of the electrode is very high because of the large redox current of

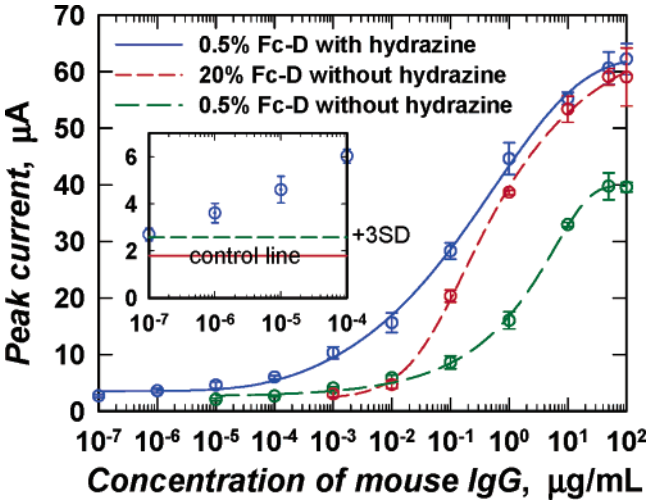

Figure 6. Dependence of anodic peak current on mouse IgG concentration obtained with a 0.5% Fc-D-modified ITO electrode in the presence and absence of hydrazine and a 20% Fc-D-modified electrode in the absence of hydrazine. The inset represents a magnified graph at low concentrations for 0.5% Fc-D with hydrazine. The control line corresponds to the mean current at zero concentration of mouse IgG, and the dashed line corresponds to the mean current plus 3 times the standard deviation (SD).

ferrocene. For the 0.5% Fc-D-modified electrode in the absence of hydrazine (Figure S5 in the Supporting Information), the peak current at a given concentration of mouse IgG is smaller than that in Figure 5, which is due to the absence of redox cycling.

Nonspecific binding of nontarget proteins to the immunosensing layer could be an interfering signal. We have shown that nonspecific binding on an Fc-D-containing immunosensing layer is not significant.[10,14b] In Figure 5a, the background current is less than that in the absence of mouse IgG, indicating the inevitable small amount of nonspecific binding of ALP-conjugated IgG. For mouse IgG at a concentration of 100 fg/mL, the peak current is $2.73 \pm 0.22\ \mu$A (mean ± SD), which is clearly higher than the current when mouse IgG is absent ($1.80 \pm 0.26\ \mu$A). Considering that mouse IgG can be detected at 100 fg/mL, the amount of nonspecific binding is small.

Figure 6 shows the dependence of the anodic peak current on the concentration of mouse IgG. The detection limits for mouse IgG in a 20% Fc-D-modified immunosensor in the absence of hydrazine, a 0.5% Fc-D-modified immunosensor in the absence of hydrazine, and a 0.5% Fc-D-modified immunosensor in the presence of hydrazine are 1 ng/mL, 10 pg/mL, and 100 fg/mL, respectively. The detection limit of the 0.5% Fc-D-modified immunosensor in the presence of hydrazine is very low: 100 fg/mL corresponds to about 0.7 fM. Importantly, the detection limit is reduced by 2 orders of magnitude by using 0.5% Fc-D instead of 20% Fc-D (from 1 ng/mL to 10 pg/mL), which is due to the low redox current of ferrocene. In addition, the detection limit is reduced by a further 2 orders of magnitude by using hydrazine (from 10 pg/mL to 100 fg/mL), which is due to redox cycling. Consequently, a low background current and the presence of redox cycling facilitate a very low detection limit. In the 0.5% Fc-D-modified immunosensor in the presence of hydrazine, mouse IgG could be detected at concentrations ranging from 100 fg/mL to 100 μg/mL (i.e., 9 orders of magnitude) in a single assay.

General immunoassays such as ELISA (enzyme-linked immunosorbent assay) have detection limits in the picomolar range;[2] 1 pM corresponds to 150 pg/mL for IgG. Immuno-PCR (polymerase chain reaction)[23] allows much lower detection limits (femtomolar). The recently developed bio-barcode assay[24] and liposome-PCR[25] provide ultrasensitive detection limits (greater than attomolar). On the other hand, the detection limit of our method is 0.7 fM, indicating that our electrochemical immunosensor is very sensitive.

CONCLUSIONS

We have developed an electrochemical immunosensor that combines redox cycling with a low background current to lower the detection limit. The redox cycling is achieved simply by adding hydrazine to a solution, without using two working electrodes or multiple enzymes. The small amount of ferrocene in Fc-D crucially lowers the background current, and the slow kinetics of hydrazine electrooxidation on Fc-D-modified ITO electrodes facilitates a low background current. Because hydrazine protects AP from oxidation by the air, longer incubation times are possible that would further reduce the detection limit. This approach could be easily applied to all electrochemical biosensors that employ ALP labeling by simply adding hydrazine to the solution.

ACKNOWLEDGMENT

This work was supported by the Korea Health Industry Development Institute (A020605 and A050426) and the Nano/Bioscience & Technology Program (M10536090000-05N3609-00000) of the Ministry of Science and Technology (MOST).

SUPPORTING INFORMATION AVAILABLE

Contact-angle changes during monolayer formation on an ITO electrode, cyclic voltammograms of noble-metal and carbon electrodes in the presence of hydrazine, photographs of AP solutions in the presence and absence of hydrazine, cyclic voltammograms at various concentrations of mouse IgG. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review December 2, 2006. Accepted January 22, 2007.

AC062291L

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