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A Nanocatalyst-Based Assay for Proteins: DNA-Free Ultrasensitive Electrochemical Detection Using Catalytic Reduction of *p*-Nitrophenol by Gold-Nanoparticle Labels

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An ultrasensitive and simple method for detecting and quantifying biomarkers is essential for early diagnosis of diseases.[1] Much attention has been focused on signal amplification without using enzymes; for example, the ultrasensitive detection of proteins has been achieved by employing DNA as an amplified signal reporter.[1,2] The signal in the immuno-PCR assay[2b] is amplified by polymerase chain reaction (PCR) after target recognition of capture antibody, whereas in the bio-barcode assay[2c,d] and the DNA-amplified electrochemical assay,[2e] it is preamplified by using nanoparticles with a high ratio of DNA to capture antibody. Furthermore, the sensitivity of the bio-barcode assay is improved by using post- as well as preamplification. A recently developed liposome-PCR assay[2f] also employs both post- and preamplification. While all DNA-based amplification methods have very low detection limits (even much less than 1 fM), their practical application is restricted due to the complex detection procedures or conjugation chemistries.[2a]

Nanoparticles have received wide attention as electrocatalysts for electrochemical reaction and catalysts for organic synthesis. The electrocatalytic property of nanoparticle labels can be used for signal amplification.[3] A short distance between electrodes and nanoparticles is required for facile electron transfer, which limits the usefulness of electrocatalysis-based assays. Semi-heterogeneous nanocatalysts lead to very fast and highly selective reactions,[4] which can be applied to signal amplification in biosensors. For example, the catalytic reduction of silver ions by gold-nanoparticle labels and the subsequent silver precipitation enables ultrasensitive detection.[5] To date, however, there is no other scheme that uses the catalytic reaction of nanocatalysts to detect proteins or nucleic acids.

Here we report an ultrasensitive and simple electrochemical method (without using DNA) for signal amplification that is achieved by catalytic reduction of *p*-nitrophenol (NP) to *p*-aminophenol (AP) using gold-nanocatalyst labels. In addition, the electrochemical signal is amplified by chemical reduction of *p*-quinone imine (QI) to AP by $NaBH_4$.

For the preparation of a sandwich-type heterogeneous electrochemical immunosensor, an IgG layer was formed on an indium tin oxide (ITO) electrode (Figure 1a). First, partially ferrocenyl-tethered dendrimer (Fc-D)[6] was immobilized to the ITO electrode by covalent bonding between dendrimer amines and carboxylic acids of a phosphonate self-assembled monolayer. Some of the unreacted amines of Fc-D were modified with biotin groups to allow the specific binding of streptavidin. Afterward, biotinylated antibodies were immobilized to the streptavidin-modified ITO electrode. An IgG−nanocatalyst conjugate was prepared via direct adsorption of IgG on 10 nm gold nanoparticles.[7] This simple conjugation method is effective in retaining IgG activity after conjugation.[7a]

Mouse IgG or prostate specific antigen (PSA) was chosen as a target protein (Figure 1b). The IgG−nanocatalyst conjugate and the immunosensing layer sandwich the target protein. The gold-

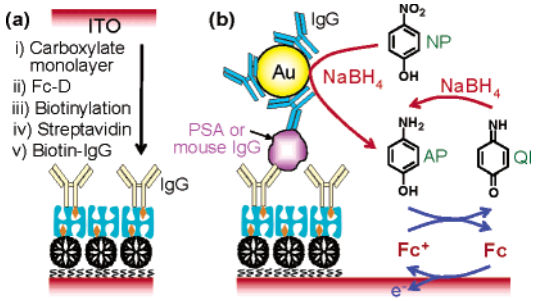

Figure 1. (a) Schematic representation of the preparation of an immunosensing layer. (b) Schematic view of electrochemical detection of mouse IgG or PSA.

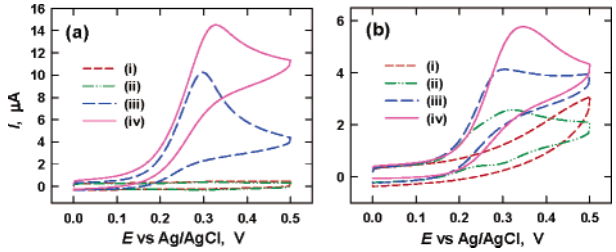

Figure 2. (a) Cyclic voltammograms of a Fc-D-modified ITO electrode in (i) a Tris buffer solution (pH 9.0) and a Tris buffer solution containing (ii) 5 mM NP, (iii) 0.1 mM AP, or (iv) 0.1 mM AP and 5 mM $NaBH_4$ at a scan rate of 50 mV/s. (b) Cyclic voltammograms of immunosensing electrodes obtained after incubating for 10 min in a Tris buffer solution containing 5 mM NP and 5 mM $NaBH_4$ at a scan rate of 50 mV/s. Before this measurement, the electrodes were (i, ii) not incubated or (iii, iv) incubated with (iii) 0 and (iv) 1 fg/mL mouse IgG (30 min) and then with IgG−nanocatalyst conjugate (30 min). In i, dendrimer was used instead of Fc-D.

nanocatalyst label generates AP by catalytic reduction.[8] This reaction is very fast, and its kinetic values (k_{cat} and k_{cat}/K_M) are large and not decreased significantly even after IgG conjugation to gold nanocatalyst (Figures S1 and S2 in Supporting Information). We believe that this is due to the easy access of small NP molecules to the gold-nanocatalyst surface through pinholes of IgG−nanocatalyst conjugates. The high reaction rate arises from the large number of catalytic sites per nanocatalyst label as well as the fast catalytic reaction. Thus generated AP molecules are electrochemically oxidized to QI via an electron mediation of ferrocene. The oxidized QI is then reduced back to AP by $NaBH_4$. Such a chemical reduction can be monitored by cyclic voltammetry (iii and iv of Figure 2a). The oxidation current of AP increases significantly upon adding $NaBH_4$ to the electrolyte solution. The greater current in the $NaBH_4$-containing solution is related to the electrochemical reoxidation of the AP that is reduced from QI by $NaBH_4$, which is the so-called electrochemical catalytic (EC′) reaction.[9] It is worth noting that the current amplification has been obtained without using

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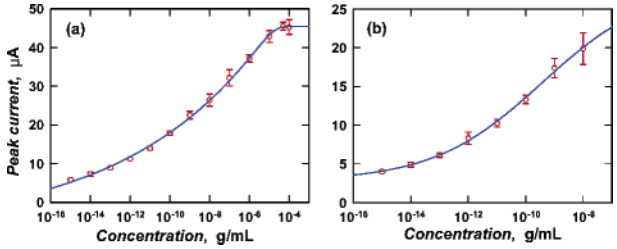

Figure 3. Dependence of the anodic peak current on the concentration of (a) mouse IgG and (b) PSA.

a complex interdigitated electrode array[10] or a two-enzyme system.[11] The oxidation potential of NP is much higher than that of AP, which enables us to measure the oxidation current of AP without the interference of NP oxidation (i and ii of Figure 2a).

To accomplish an ultrahigh sensitivity, a high signal-to-noise ratio is required. In this study, the signal is amplified by the catalytic reduction of NP along with the chemical reduction of QI (Figure 1b). The noise should also be lowered for the ultrasensitive detection. $NaBH_4$, however, is electrochemically oxidized on commonly employed platinum, gold, and carbon electrodes at potentials where AP is oxidized, which causes high background current (i.e., high noise). On the contrary, the electron transfer kinetics of $NaBH_4$ on ITO electrodes is so slow that the oxidation current of $NaBH_4$ can be lowered significantly (Figure S3 in Supporting Information). Whereas ITO electrodes are favorable for low background current of $NaBH_4$, they are unfavorable for electrochemical oxidation of AP. The slow electron transfer kinetics of AP on ITO electrodes causes AP oxidation at a high potential where the background current of electrolytes is considerable. The ferrocene moiety of Fc-D acts as an electron mediator and was used to shift the oxidation potential of AP less positive. In addition, the ratio of ferrocene groups to dendrimer amines was minimized because the oxidation current of ferrocene may be another noise source. The ferrocene of Fc-D does not significantly increase the rate of $NaBH_4$ oxidation (i and ii of Figure 2b). As a result, Fc-D-modified ITO electrodes facilitate the slow electrooxidation of $NaBH_4$ as well as the fast electrooxidation of AP.

For heterogeneous phase biosensors, nonspecific binding of nontarget proteins to the immunosensing layer could also be a source of noise. The peak current without binding of both mouse IgG and the IgG−nanocatalyst conjugate is less than that in the absence of mouse IgG (ii and iii of Figure 2b). The difference in peak current resulting from nonspecific binding of the IgG−nanocatalyst conjugate to the immunosensing layer is not substantial. In the case of 1 fg/mL mouse IgG, the peak current is $6.0 \pm 0.4\ \mu$A, which is clearly higher than the current at zero concentration of mouse IgG ($4.5 \pm 0.5\ \mu$A) (iii and iv of Figure 2b). As a control, we examined the effect of replacing mouse IgG with goat IgG, in which case the peak current is similar to that at zero concentration of mouse IgG (Figure S4 in Supporting Information). The similar current indicates that the nonspecific binding of goat IgG is not significant. The low nonspecific binding to the immunosensing layer is due to the hydrophilic nature of IgG layer, streptavidin, and unreacted amine groups of Fc-D.[6c]

Figure 3 shows the dependence of the anodic peak current on the concentration of the two target proteins. The detection limit for mouse IgG is 1 fg/mL (Figure 3a), which corresponds to ca. 7 aM. Importantly, the concentration of mouse IgG can be detected ranging from 1 fg/mL to 10 μg/mL with a single assay format, which covers a 10-order concentration range. We also achieved a 1 fg/mL detection limit for PSA (Figure 3b), which is comparable to that of the bio-barcode assay.[2c]

In summary, we have described a nanocatalyst-based electrochemical assay with an ultrahigh sensitivity without using DNA or enzymatic amplification. The ultrasensitive detection is achieved by signal amplification combined with noise reduction: the signal is amplified both by the catalytic reduction of NP by gold-nanocatalyst labels and by the chemical reduction of QI by $NaBH_4$; the noise is reduced by employing an Fc-D-modified ITO electrode and a hydrophilic immunosensing layer. The Fc-D-modified electrode offers the slow electrooxidation of $NaBH_4$ as well as the fast electrooxidation of AP, while the hydrophilic layer reduces the nonspecific binding of proteins. The wide range of concentrations can be detected in a single assay format. The simple preparation of the IgG−nanocatalyst conjugate and the easy detection procedure make our approach practically appealing.

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Note Added after ASAP Publication. After this paper published ASAP on November 23, 2006, the nomenclature for QI (third paragraph) and the presentation of its structure (Figure 1b and table of contents graphic) were modified. The corrected version was published ASAP on December 1, 2006.

Supporting Information Available: Detailed experimental procedures, kinetic data, and cyclic voltammograms in various concentrations of mouse IgG and PSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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