Full Paper

Platform for Highly Sensitive Alkaline Phosphatase-Based Immunosensors Using 1-Naphthyl Phosphate and an Avidin-Modified Indium Tin Oxide Electrode

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Abstract
We report a versatile platform for highly sensitive alkaline phosphatase (ALP)-based electrochemical biosensors that uses an avidin-modified indium tin oxide (ITO) electrode as a sensing electrode and 1-naphthyl phosphate (NPP) as an ALP substrate. Almost no electrocatalytic activity of NPP and good electrocatalytic activity of 1-naphthol (ALP product) on the ITO electrodes allow a high signal-to-background ratio. The effective surface covering of avidin on the ITO electrodes allows very low levels of nonspecific binding of proteins to the sensing electrodes. The platform technology is used to detect mouse IgG with a detection limit of 1.0 pg/mL.

Keywords: Immunosensor, Alkaline phosphatase, 1-Naphthyl phosphate, Avidin, Nonspecific binding, Biosensors, Enzymes

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1. Introduction

The modification of electrodes with (bio)molecular layers is essential for electrochemical biosensors [1–4]. The modified electrode surfaces should provide i) good immobilization of biomolecules, ii) low levels of nonspecific binding, and iii) high electrocatalytic activities for signaling species. However, it is not easy to achieve all these requirements via simple surface modification. In many cases, surfaces for good immobilization and low levels of nonspecific binding require complex preparation procedures [5]. Moreover, such surfaces can potentially decrease electrocatalytic activities of underlying electrodes. Therefore, a thin and simply preparable surface layer is essential for meeting the 3 requirements.

Generally, phosphonate and silane monolayers are used to obtain thin functional layers on indium tin oxide (ITO) electrodes [5–11]. However, the monolayers are less dense and less uniform than thiol monolayers on gold electrodes. This low surface density and nonuniformity could yield many defect sites and cause high levels of nonspecific binding of proteins. One of the strategies to overcome this problem is to thoroughly cover the electrodes with nonbiofouling layers. In our previous studies, amine-terminated dendrimer layers were used to fully cover the ITO electrodes [5, 12]. The dense and uniform dendrimer layers on the ITO electrodes allowed very low levels of nonspecific protein binding. Nevertheless, the complex preparation procedures inherent to dendrimer layers limit their practical application.

The conjugation between biotin and biotin-binding protein (avidin, streptavidin, or neutravidin) is a common strategy to immobilize or modify biomolecules [13–16]. Biotin-binding proteins are rigid and hard [17]. Accordingly, minor conformational changes occur upon their adsorption, and chemical modification has little effect on their activities [15, 18]. Although avidin is cheaper and has higher water-solubility, it is less popular than streptavidin as it has a high isoelectric point (pH 10 to 10.5) and its carbohydrate content causes high levels of nonspecific binding [15]. Nevertheless, there is a possibility that the high nonspecific binding properties of avidin could allow a thorough covering of the ITO electrodes with avidin and that the rigid and hard properties of avidin could allow long-term stability. In this case, avidin layers on ITO could allow both effective immobilization of biotinylated biomolecules and low levels of nonspecific binding.

Indium tin oxide (ITO) electrodes are becoming more and more popular as working electrodes in electrochemical biosensors, as they allow low and reproducible background-current levels [5, 19]. However, in many cases, low electrocatalytic activities of ITO electrodes require modification of the ITO electrodes with electrocatalytic materials such as ferrocene [5, 12], carbon nanotubes [19], and gold nanoparticles [6, 20, 21]. The modification makes the preparation of sensing surfaces more complex and the sensors impractical.

In enzyme-based biosensors, alkaline phosphatase (ALP) has been widely used as an enzyme label for signal

amplification. ALP substrate and product should be electrochemically inactive and active respectively within electrochemical potential windows to obtain high signal-to-background ratio, which is required for achieving low detection limits. In this case, ITO electrodes could be used without modifying them with electrocatalytic materials. *p*-aminophenyl phosphate has been the most popular ALP substrate in ALP-based electrochemical biosensors [19, 22, 23]. However, in aqueous solutions, *p*-aminophenyl phosphate slowly decomposes to *p*-aminophenol, a signaling product, without enzymatic reaction [24–26]. The decomposed *p*-aminophenol causes high and less reproducible background-current levels. Compared to *p*-aminophenyl phosphate, 1-naphthyl phosphate (NPP) is stable, inexpensive, and electrochemically inactive over a wide range of potentials [24, 25, 27].

Here, we have presented a versatile platform for highly sensitive ALP-based electrochemical biosensors that use NPP as an ALP substrate and an avidin-modified ITO electrode as a sensing surface. First, the electrocatalytic properties of NPP and 1-naphthol (NP) on bare and modified ITO electrodes were compared. Second, the level of nonspecific binding of proteins on avidin-modified electrodes was compared with that on streptavidin-modified electrodes. Finally, the dependence of the cyclic voltammograms on the concentration of a model target protein, mouse IgG, was obtained and the detection limit was determined.

2. Experimental

2.1. Chemicals

Avidin, streptavidin, biotinylated goat antimouse IgG, mouse IgG from serum, rabbit IgG from serum, and ALP-conjugated goat antimouse IgG were obtained from Sigma. All other reagents were supplied by Sigma, Aldrich, or Fluka and used as received. All aqueous solutions were prepared in twice distilled water. ITO electrodes were obtained from Geomatec (Yokohama, Japan).

The phosphate buffered saline (PBS) solution consisted of 0.01 M phosphate, 0.138 M NaCl, and 0.0027 M KCl (pH 7.4). The PBSB buffer contained all the ingredients of PBS along with 1% (w/v) albumin-bovine serum (pH 7.4). The rinsing buffer (RB) consisted of 50 mM tris(hydroxymethyl)aminomethane, 40 mM HCl, 0.5 M NaCl, and 0.05% (w/v) albumin-bovine serum (pH 7.6). For the preparation of Tris buffer (pH 9.8), 50 mM tris(hydroxymethyl)aminomethane, 10 mM KCl, 10.5 mM $MgCl_2 \cdot 6H_2O$ were dissolved in water and pH was adjusted by adding 1 M HCl solution.

2.2. Preparation of Immunosensing Electrodes

ITO electrodes were successively cleaned with trichloroethylene, ethanol, and water with 15-min sonication, followed by drying at 50 °C. The cleaned electrodes were pretreated in a mixture of 5:1:1 H_2O/H_2O_2 (30%)/NH_4OH (30%) (v/v/v) at 70 °C for 1.5 h. The electrodes were then washed with copious amounts of water and dried at 50 °C for 20 min. The formation of 3-aminopropylphosphonic acid (APPA) monolayer on ITO electrode has been carried out according to our previous report [9]. Briefly, the pretreated ITO electrodes were immersed in an aqueous solution containing 0.1 mM APPA for 24 h to form APPA-modified ITO electrodes. It was then washed thoroughly with water and dried at room temperature. Afterward, the APPA-modified electrodes were dipped in dimethylformamide containing 1.5 mg/mL (+)-biotin *N*-hydroxysuccinimide ester for 2 h to immobilize biotin [5]. The biotin- and APPA-modified ITO electrodes were then washed with methanol and water, successively.

To immobilize avidin or streptavidin, the biotin- and APPA-modified ITO electrodes were immersed for 30 min in PBS (pH 7.4) containing 100 μg/mL avidin (or streptavidin) and then washed twice with PBS [5, 6, and 19]. Subsequently, the avidin-, biotin-, and APPA-modified ITO electrodes were immersed in a PBSB solution containing 0.05% (v/v) Tween-20 (pH 7.4) for 30 min to minimize nonspecific binding of proteins. After being washed with RB, the avidin-, biotin-, and APPA-modified ITO electrodes were incubated for 30 min in PBSB containing 10 μg/mL biotinylated goat antimouse IgG and washed with RB. The immunosensing electrodes (antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes) were then treated in a PBSB solution containing different concentrations of mouse IgG for 30 min. After being rinsed with RB, the resulting assemblies were dipped in PBSB containing 10 μg/mL ALP-conjugated goat antimouse IgG for 30 min, followed by washing with RB. Finally, the electrode was incubated in Tris buffer (pH 9.8) containing 4.0 mM NPP at 30 °C for 10 min.

2.3. Electrochemistry

The electrochemical experiment was performed using a CHI 617B (CH instruments). The electrochemical cell consisted of a modified ITO working electrode, a Pt counter electrode, and an Ag/AgCl reference electrode. All cyclic voltammograms were recorded at 30 °C.

3. Results and Discussion

To obtain high signal-to-background ratios, an ALP substrate should be electrochemically inactive, but an ALP product should be electrochemically active within potential windows. The appropriate selection of an enzyme-reaction couple (ALP substrate and product) is essential for highly sensitive electrochemical detection. Thus, the NPP/NP couple was selected as the enzyme-reaction couple. Although NPP has been used in electrochemical biosensors [25, 27, 28], there is no report on electrochemical character-

ization of NPP on ITO electrodes. Thus, the electrochemical activities of NP and NPP were examined on bare and modified ITO electrodes.

NPP was electrochemically inactive on bare ITO electrodes over a wide range of potentials (curve i of Fig. 1a), whereas NP was highly electrochemically active although ITO electrodes generally show low electrocatalytic activities (curve i of Fig. 1b). The peak potential in curve i of Figure 1b appeared at a potential more negative than 0.4 V. The current decreased significantly during the reverse cathodic scan because the oxidation of NP causes the formation of insulating polymer films that ultimately leads to electrode fouling [29–31].

On avidin-, biotin-, and APPA-modified ITO electrodes, there was no significant change in cyclic voltammograms compared to the bare ITO electrodes (Fig. 1a). Curve ii of Fig. 1a shows a small anodic peak also observed on the APPA-modified ITO electrodes. Although its origin is unclear, the current increase was not high. The peak potential in curve ii of Fig. 1b was shifted to a more positive potential by ca. 0.1 V compared to that in curve i of Figure 1b. However, there was no significant change in the peak current. These results show that there is almost no background current due to NPP and that the avidin/biotin/APPA layer on the ITO electrodes does not significantly change electrocatalytic activities of the electrodes. More importantly, these results show that ALP product (NP) can be measured without electrochemical interference of the ALP substrate (NPP), even without modifying ITO electrodes with any electrocatalytic materials.

Figure 2 shows a schematic diagram of an ALP-based electrochemical biosensor using NPP and an avidin-modified ITO electrode. To test the feasibility of the sensor, a sandwich-type immunosensor for detecting mouse IgG was designed. Biotinylated antimouse IgG was immobilized on avidin-, biotin-, and APPA-modified ITO electrodes to capture target mouse IgG [5, 6, 19]. ALP-conjugated antimouse IgG was attached after the mouse IgG was captured [5, 6, 19]. The ALPs bound on the electrode converted electrochemically inactive NPP into electrochemically active NP. After the fully assembled electrode was incubated for 10 min in Tris buffer (pH 9.8) containing 4 mM NPP, the generated NP was electrooxidized on the ITO electrode. The resulting anodic current depends on i) the concentration of mouse IgG, ii) the background-current level, and iii) the level of nonspecific binding of ALP-conjugated antimouse IgG. The use of NPP and an avidin-modified ITO electrode allowed the minimization of both the background-current level and the level of nonspecific binding. As a result, the current became mainly dependent upon the concentration of the mouse IgG.

To investigate the level of nonspecific binding to the immunosensing electrodes (antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes), cyclic voltammograms (curves i and ii of Fig. 3) were obtained both without and with the treatment of ALP-conjugated antimouse IgG. The current levels of the 2 curves were similar, indicating that the level of nonspecific binding of the ALP-conjugated

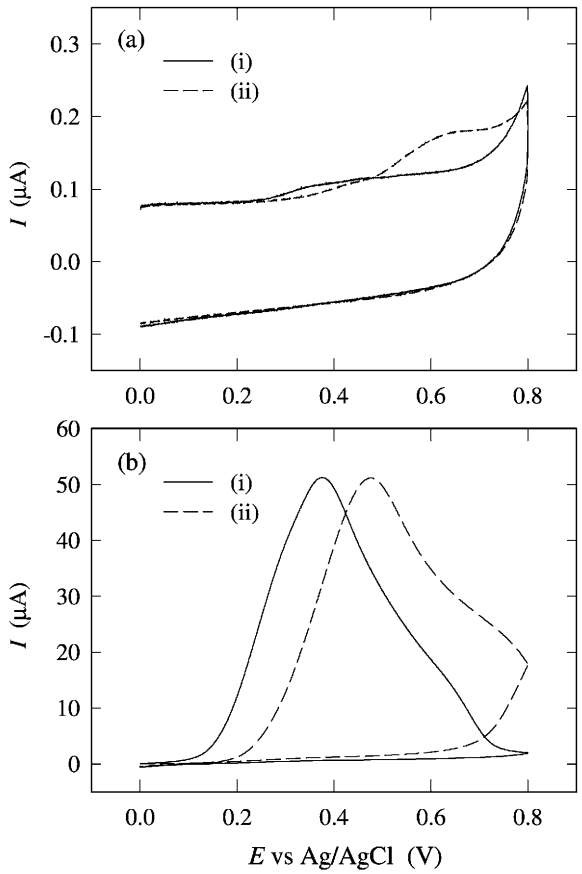

Fig. 1. Cyclic voltammograms of a) NPP or b) NP obtained on i) bare ITO electrodes or ii) avidin-, biotin-, and APPA-modified ITO electrodes at a scan rate of 20 mV/s in a Tris buffer (pH 9.8) containing 1.0 mM NPP or NP.

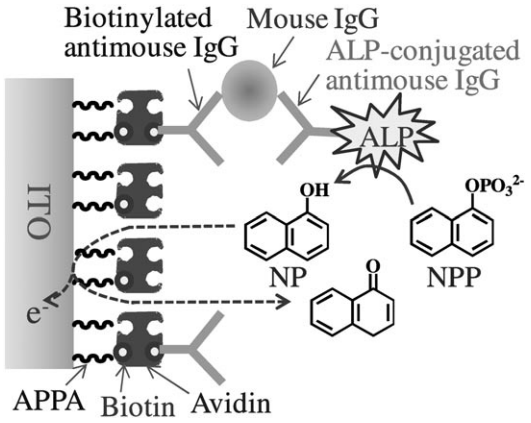

Fig. 2. Schematic diagram of an ALP-based electrochemical biosensor using NPP and an avidin-modified ITO electrode.

antimouse IgG to the immunosensing electrodes was very low.

Moreover, the level of nonspecific binding was tested by replacing avidin with streptavidin. The peak current in curve iii of Figure 3 was 2.9 ± 0.2 μA (mean ± standard deviation), much larger than the peak current in curve ii of Figure 3 (0.32 ± 0.09 μA), indicating that the level of nonspecific

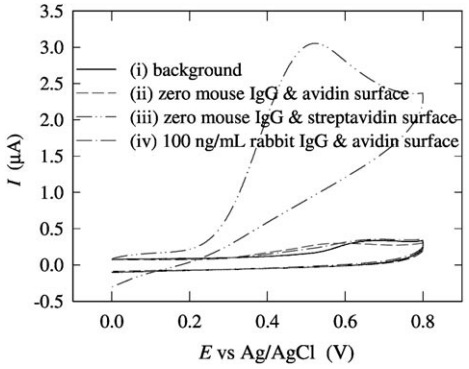

Fig. 3. Cyclic voltammograms obtained on antimouse IgG-, avidin-, biotin-, APPA-modified ITO electrodes and antimouse IgG-, streptavidin-, biotin-, APPA-modified ITO electrodes. Cyclic voltammogram for background was obtained at a scan rate of 20 mV/s in a Tris buffer (pH 9.8) containing 4.0 mM NPP, without target and ALP treatment. All other cyclic voltammograms were obtained at a scan rate of 20 mV/s after incubating target- and ALP-treated immunosensing electrodes for 10 min in a Tris buffer (pH 9.8) containing 4.0 mM NPP.

binding to the streptavidin-modified electrodes is much higher than that to the avidin-modified electrodes. In our previous studies, amine-terminated dendrimer layers were used to fully cover ITO electrodes [5, 12]. For the immobilization of biotinylated antibodies, dendrimer layers were modified with streptavidin. The dense, uniform, and hydrophilic dendrimer layers allowed very low levels of nonspecific binding of proteins, although streptavidin was used. In the case of antimouse IgG-, streptavidin-, biotin-, and APPA-modified ITO electrodes, because the level of nonspecific binding of streptavidin to biotin- and APPA-modified ITO electrodes was low, many defect sites of biotin- and APPA-modified ITO electrodes remained uncovered, causing high levels of nonspecific binding of ALP-conjugated antimouse IgG to the streptavidin-modified electrodes. On the other hand, in the case of antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes, avidin well-covered the biotin- and APPA-modified ITO electrodes via nonspecific binding along with specific binding to biotinylated surfaces, because of the high nonspecific binding properties of avidin [15]. This effective surface covering allowed very low levels of nonspecific binding to the avidin-modified electrodes.

To test the effect of nontarget analyte on the sensor, a cyclic voltammogram (curve iv of Fig. 3) was obtained when 100 ng/mL rabbit IgG was used instead of mouse IgG. The current behavior is similar to that in the zero mouse IgG (curve ii of Fig. 3). This results shows that the sensor is selective to mouse IgG.

Figure 4a shows the dependence of cyclic voltammograms on the concentration of mouse IgG. The peak current increases with the concentration of mouse IgG. Because of the high nonspecific binding properties of avidin, it could be immobilized on APPA-modified ITO electrodes without biotin modification. The preparation of antimouse IgG-, avidin-, and APPA-modified ITO electrodes is simpler than

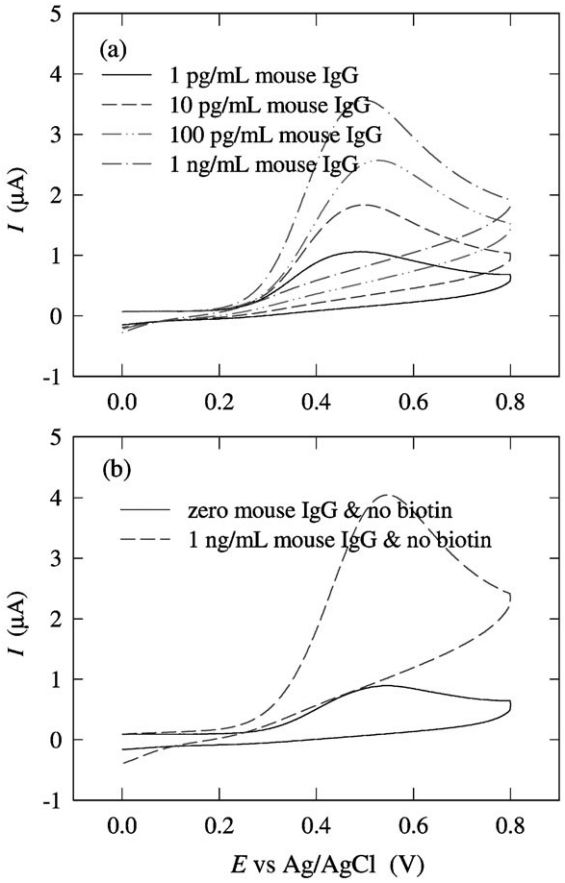

Fig. 4. Dependence of cyclic voltammograms on the concentration of mouse IgG obtained on a) antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes and b) antimouse IgG-, avidin-, and APPA-modified ITO electrodes. Cyclic voltammograms were obtained at a scan rate of 20 mV/s after incubating target- and ALP-treated immunosensing electrodes for 10 min in a Tris buffer (pH 9.8) containing 4.0 mM NPP.

that of antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes. However, the level of nonspecific binding on antimouse IgG-, avidin-, and APPA-modified ITO electrodes was higher than that on the antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes. The peak current in the zero mouse IgG on the biotin-unmodified electrodes (Fig. 4b) (0.75 ± 0.17 μA) was higher and less reproducible than that on the biotin-modified electrodes (curve ii in Fig. 3) (0.32 ± 0.09 μA). Furthermore, the peak current for 1.0 ng/mL mouse IgG on the biotin-unmodified electrodes (Fig. 4b) (5.0 ± 1.1 μA) was also higher and less reproducible than that on the biotin-modified electrodes (Fig. 4a) (3.6 ± 0.4 μA). The higher currents at both zero and 1.0 ng/mL concentrations are due to the nonspecific binding of the ALP-conjugated antimouse IgG to the biotin-unmodified electrodes. As a result, the antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes are better for low levels of nonspecific binding.

Figure 5 represents the dependence of peak currents on the concentration of the mouse IgG. All concentration data

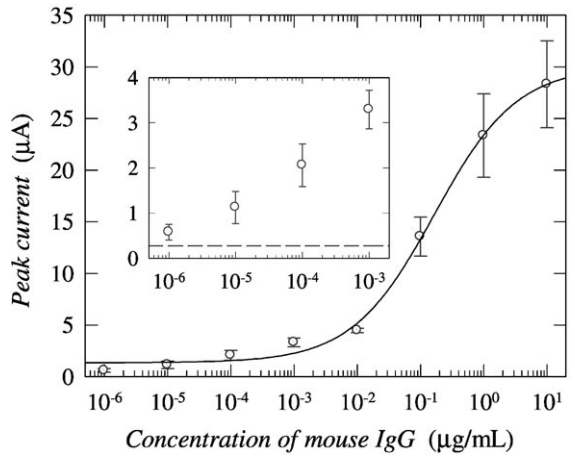

Fig. 5. Dependence of peak currents on the concentration of mouse IgG obtained on antimouse IgG-, avidin-, biotin-, and APPA-modified ITO electrodes. All concentration data were subtracted by the mean current at a zero concentration of mouse IgG. The inset represents a magnified graph at low concentrations. The dashed line corresponds to 3 times the standard deviation of the current at a zero concentration of mouse IgG.

were subtracted by the mean current at zero mouse IgG (0.32 μA). The current at 1.0 pg/mL mouse IgG was 0.90 ± 0.17 μA, higher than the current at zero mouse IgG (0.32 ± 0.09 μA). As a result, the detection limit of the sensor was 1.0 pg/mL, better than that of general ELISA (sub ng/mL) and better than that obtained without the redox cycling in our previous studies (10 pg/mL) [5, 6, 19]. This very low detection limit indicates that it can be obtained with a good selection of an enzyme substrate and a simply prepared immunosensing electrode.

4. Conclusions

We have shown a simple method for highly sensitive ALP-based electrochemical immunosensors that uses NPP and an avidin-modified ITO electrode, which offer the minimization of both the background-current level and the level of nonspecific binding of proteins. Almost no electrocatalytic activity of NPP and good electrocatalytic activity of NP on ITO electrodes allowed high signal-to-background ratios. Avidin-modified electrodes allowed very low levels of nonspecific binding of proteins to the sensing electrodes. Finally, the combined effect of a high signal-to-background ratio and a low level of nonspecific binding enabled a very low detection limit for detecting mouse IgG (1.0 pg/mL) to be achieved.

5. Acknowledgements

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