

Electrochemical Immunosensor Using the Modification of an Amine-functionalized Indium Tin Oxide Electrode with Carboxylated Single-walled Carbon Nanotubes

Md. Abdul Aziz and Haesik Yang*

Department of Chemistry, Pusan National University, Busan 609-735, Korea. *E-mail: hyang@pusan.ac.kr
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We have developed an electrochemical immunosensor that combines the electrocatalytic property of carbon nanotube and the low background current of indium tin oxide (ITO) electrode. A partial monolayer of carboxylated single-walled carbon nanotube (CCNT) is covalently formed on an ITO electrode modified with amine-terminated phosphonic acid. Nonspecifically adsorbed avidin on the hydrophobic sidewalls of CCNT is used to immobilize biotinylated antibody and to reduce the nonspecific binding to CCNT. The biotinylated antimouse IgG bound on avidin and the antimouse IgG conjugated with alkaline phosphatase (ALP) sandwiches a target mouse IgG. ALP catalyzes the conversion of *p*-aminophenyl phosphate monohydrate into *p*-aminophenol, which is electrocatalytically oxidized to *p*-quinone imine on CCNT surface. Moderate electrocatalytic electrode obtained with the combination of CCNT and ITO allows low detection limit (0.1 ng/mL).

Key Words : Carbon nanotube, Indium tin oxide, Immunosensor

Introduction

Indium tin oxide (ITO) films have been widely used in optoelectronic devices that require both high optical transparency and high electrical conductivity.¹ The films have also been employed as a working electrode in electrochemical sensors because of their low background current in a wide range of potentials.²⁻⁴ The low background current enables to obtain ultralow detection limits in many biosensors.⁵⁻⁹ Moreover, the micropatterning of ITO electrodes and the formation of stable self-assembled monolayers on ITO electrodes are easily achieved.¹⁰⁻¹² Accordingly, ITO electrodes are highly suitable for electrochemical biosensors. However, their low electrocatalytic properties should be improved.

On the other hand, carbon nanotubes (CNTs) have received a wide attention as an electrode material.^{13,14} The considerable interest is due to unique characteristics of CNTs, including high electrical conductivity, chemical stability, and electrocatalytic property. To apply CNTs to biosensors, the modification of CNTs with biomolecules and their attachment to electrodes are required. The modification usually begins with dispersing CNTs in solutions, and then biomolecules are immobilized on CNT.^{15,16} One example to disperse CNTs is to use highly carboxylated CNTs (CCNTs), which are dispersible even in aqueous solutions without using surfactants or covalent modification of CNTs.^{17,18} The carboxylic groups of CCNT also allow covalent bonding with biomolecules or solid surfaces.

Recently, we have reported an immunosensor that combines the low background current of an ITO electrode, the good electrocatalytic properties of multi-walled CNTs, and the low biofouling properties of poly(ethylene glycol)-silane copolymer.⁷ The stable and uniform modification of an ITO electrode with CNTs is essential for obtaining reliable bio-

sensor performances. Accordingly, more approaches are required to modify ITO electrodes with CNTs.

Here, we have used a covalent bond between CCNT and amine-functionalized ITO electrode to form a partial monolayer of CCNT on an ITO electrode. The amine-functionalized ITO electrode is obtained by forming an amine-terminated phosphonate monolayer on an ITO electrode. First, the preparation of a CCNT-modified ITO electrode is presented. Second, the background current and electrocatalytic properties of modified ITO electrodes are evaluated. Finally, the dependence of cyclic voltammograms on the concentration of mouse IgG is compared, and the detection limit is determined.

Experimental Section

ITO electrode was supplied by Geomatec (www.geomatec.co.jp). Carboxylated single-walled CNT (catalog No. 652490) was obtained from Aldrich, and *p*-aminophenyl phosphate (APP) was obtained from Universal Sensors (Cork, Ireland). All other reagents were purchased by Sigma, Aldrich, or Fluka. Doubly distilled water was used for preparing aqueous solutions, and all chemicals were used without further purification.

Phosphate buffered saline (PBS) solution consisted of 0.01 M phosphate, 0.138 M NaCl, and 0.0027 M KCl (pH 7.4). PBSB solution contained all of the ingredients of PBS and additionally 1% (w/v) albumin-bovine serum (pH 7.4). Rinsing buffer (RB) was comprised of 50 mM tromethamine, 40 mM HCl, 0.5 M NaCl, and 0.05% (w/v) albumin-bovine serum (pH 7.6). Tris buffer for electrochemical experiments consisted of 50 mM tromethamine, 10 mM KCl, 1 g/L MgCl₂, and 7 mM HCl (pH 9.0).

ITO electrodes were cleaned with acetone, 2-propanol and water successively, and dried at 60 °C, followed by pretreat-

ment with a mixed solution of water, NH_4OH (30%), and H_2O_2 (30%) in a ratio of 5:1:1. The pretreated ITO electrodes were immersed in an aqueous solution containing 0.1 mM 3-aminopropylphosphonic acid (APPA) for 24 h at room temperature to form an amine-functionalized monolayer. After being washed with water and dried at room temperature, the amine-functionalized ITO electrodes were dipped in a dimethylformamide solution containing 0.2 mg/mL CCNT and 0.8 mg/mL dicyclohexylcarbodiimide at 50 °C for 12 h. The CCNT-modified ITO electrodes were washed with ethanol and dried with nitrogen gas.

The CCNT-modified ITO electrodes were immersed for 6 h in a PBS solution containing 100 $\mu\text{g/mL}$ avidin and washed twice with RB. The avidin-coated electrodes were incubated in a PBSB solution containing 0.05% (v/v) Tween-20 for 30 min to minimize the nonspecific adsorption of proteins. After being washed with RB, the electrodes were incubated for 30 min in a PBSB solution containing 100 $\mu\text{g/mL}$ biotinylated goat antimouse IgG and then washed with RB. Afterward, the electrodes were immersed for 30 min in a PBSB solution containing various concentrations of mouse IgG. Subsequently the electrodes were rinsed with RB and immersed in a PBSB solution containing 100 $\mu\text{g/mL}$ alkaline phosphatase (ALP)-conjugated goat antimouse IgG, followed by washing the electrodes with RB.

The electrochemical experiments were performed using a CHI 617 (CH instruments). The electrochemical cell consisted of a modified ITO working electrode, a Pt counter electrode, and an Ag/AgCl reference electrode.

Results and Discussion

The modification of ITO surface begins with the formation of an amine-functionalized monolayer on ITO electrodes (Figure 1). APPA was used to form the amine-functionalized phosphonate monolayer. Derivatives of phosphonic acid that undergo easy formation of organic monolayers on metal oxide surfaces are useful for introducing polar functional groups on the surfaces.^{19,20} Contact angle measurement (Figure 2) reveals that water contact angle decreases with the formation of an amine-functionalized monolayer. The contact angle plateaus, and 24-h immersion is enough to form a

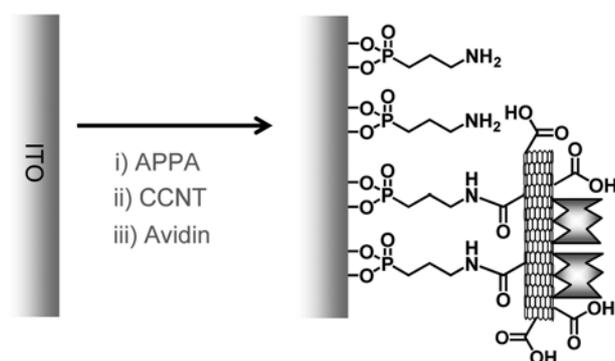


Figure 1. Schematic representation for the preparation of a CCNT/APPA/ITO electrode.

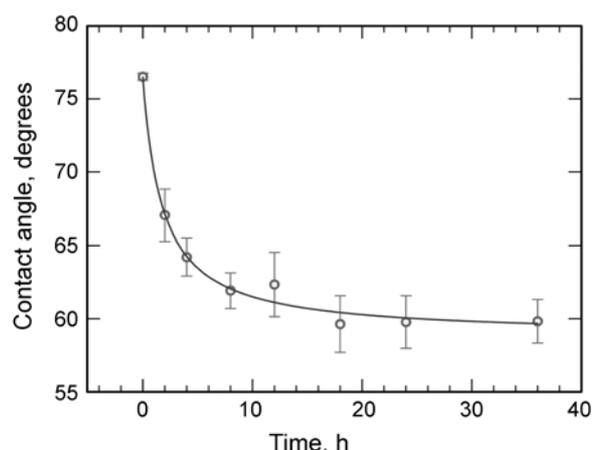


Figure 2. Water contact angles of APPA/ITO electrodes as a function of immersion time.

monolayer on ITO electrodes. The attachment of CCNTs to the amine-functionalized ITO (APPA/ITO) electrode was achieved by using a coupling reagent of dicyclohexylcarbodiimide in a dimethylformamide solution.

After the modification of an ITO electrode with CCNTs, avidin was bound nonspecifically on the hydrophobic sidewalls of CCNTs. Because of its carbohydrate content, avidin has relatively high nonspecific binding properties. In addition, the basic isoelectric point of avidin (pI 10) allows attractive electrostatic interaction with CCNT in a PBS solution (pH 7.4).

To investigate background current and electrocatalytic property of CCNT-modified electrodes, cyclic voltammograms were recorded in a Tris buffer solution (pH 9.0) (Figure 3a). In the cyclic voltammograms, the background current arises from both double-layer charging and redox reaction of electrolytes. The background current of an APPA/ITO electrode is low at potentials between -0.2 V and 0.8 V (Figure 3a), indicating low redox current of electrolytes in a wide range of potentials. After the modification with CCNT, the background current increases at potentials more than 0.4 V, but there is no significant change in the background current at potentials between 0.4 V and -0.1 V. Considering that CNT films significantly increase background currents, this current behavior means that the surface coverage of the CCNT film on ITO electrode is not high. When the background current is compared to that of our previous report using multi-walled CNTs, the current in this study is smaller.⁷ Considering that the surface coverage of multi-walled CNTs on ITO electrode was 0.03, the surface coverage of CCNTs in this study seems to be very low. It verifies that a partial monolayer of CCNT is formed on an APPA/ITO electrode. In case of an avidin/CCNT/APPA/ITO electrode, the background current is low and similar to that of the APPA/ITO electrode. The low background current indicates that the electrocatalytic surface of CCNT is well covered with avidin. It is also interesting to note that the background current is very flat at a range of potentials between -0.2 and 0.6 V. This low background current and

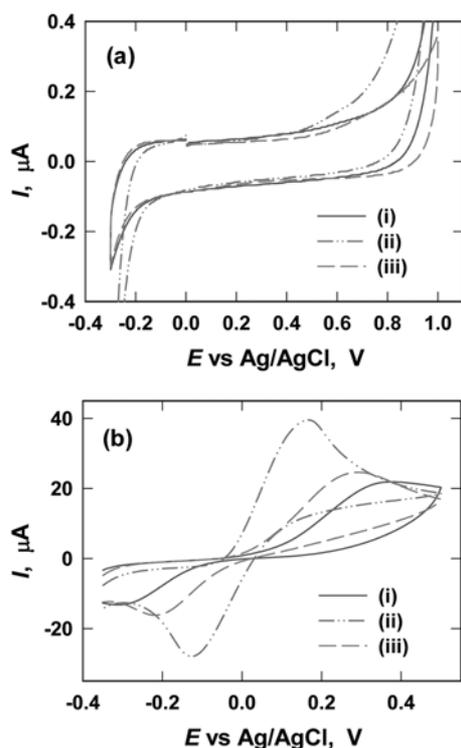


Figure 3. Background currents and electrocatalytic properties of modified ITO electrodes. Cyclic voltammograms of modified ITO electrodes were obtained at a scan rate of 10 mV/s in (a) a Tris buffer solution (pH 9.0) and (b) a Tris buffer solution containing 1.0 mM AP (i) APPA/ITO electrode, (ii) CCNT/APPA/ITO electrode, and (iii) Avidin/CCNT/APPA/ITO electrode).

the flat current behavior of the avidin/CCNT/APPA/ITO electrode are beneficial for achieving a low detection limit.

p-Aminophenol (AP) that is generated by enzymatic reaction of ALP is commonly employed as an electrochemically signaling species in biosensors. The electrocatalytic oxidation of AP at low potentials is very important in obtaining low background current. A comparison of the electrocatalytic properties for AP on modified ITO electrodes are shown in Figure 3b. Although the formal potential of AP is around 0 V, the rate of electron transfer is slow on ITO electrodes, leading to oxidation at high potentials. In a cyclic voltammogram of APPA/ITO electrode, the difference between peak potentials is larger than 0.6 V. The large difference is due to slow electron-transfer rate. In the case of CCNT/APPA/ITO electrode, the difference is reduced considerably, *ca.* 0.3 V. On the other hand, the modification of the CCNT/APPA/ITO electrode with avidin shows an unfavorable effect on electron-transfer rate. The difference between peak potentials becomes larger, because CCNTs are covered with avidin. However, the difference between peak potentials is still smaller than at the APPA/ITO electrode, and the anodic peak appears at potentials less than 0.4 V. Accordingly, the avidin/CCNT/APPA/ITO electrode provides moderate electrocatalytic properties, which is good enough for electrochemical biosensors using AP electrooxidation.

The CCNT and avidin-modified ITO electrode was used for an electrochemical immunosensor (Figure 4). A sand-

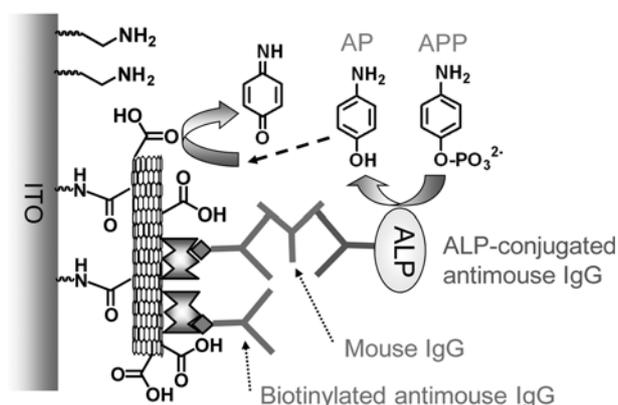


Figure 4. Schematic illustration of an electrochemical immunosensor using an avidin/CCNT/APPA/ITO electrode for detecting mouse IgG.

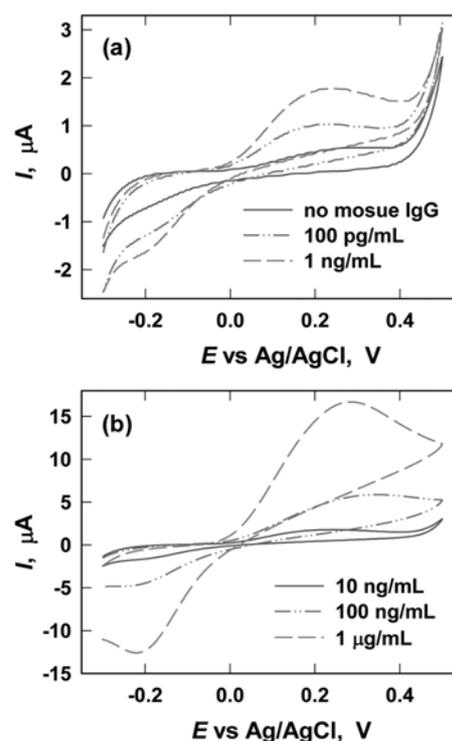


Figure 5. Dependence of cyclic voltammograms on the concentration of mouse IgG. Cyclic voltammograms were obtained at a scan rate of 10 mV/s after incubation for 10 min in a Tris buffer containing 1.0 mM APP at (a) 0, 100 pg/mL, and 1 ng/mL mouse IgG; (b) 10, 100 ng/mL, and 1 μg/mL.

wich-type immunosensor measuring mouse IgG was devised. Biotinylated antimouse IgG was immobilized on avidin to allow the specific binding of mouse IgG. ALP-conjugated antimouse IgG was bound to mouse IgG to catalyze conversion of APP into AP. This product was electrocatalytically oxidized to *p*-quinone imine on the CCNT-modified ITO electrode.

Figure 5 shows cyclic voltammograms obtained in various concentrations of mouse IgG. The cyclic voltammograms were obtained after the fully-assembled electrodes were immersed in a Tris buffer solution (pH 9.1) containing 1.0

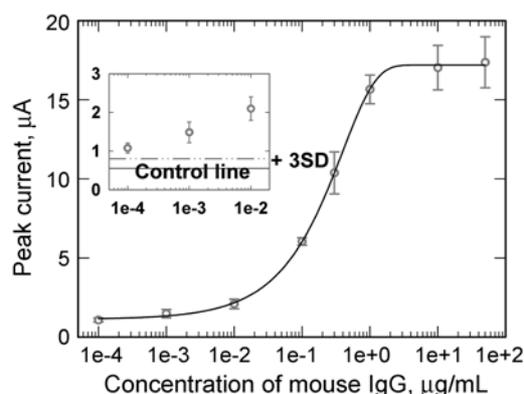


Figure 6. Dependence of anodic peak current on mouse-IgG concentration. The inset represents a magnified graph at low concentrations. The control line corresponds to the mean current in the absence of mouse IgG, and the dashed line corresponds to the mean current plus three times the standard deviation (SD).

mM APP for 10 min. At low concentration, the sharp increase of oxidation current at potentials higher than 0.4 V corresponds to the oxidation of APP. The anodic peak potential of AP is much less than 0.4 V in all concentrations. Accordingly, the anodic peak current of AP can be obtained without interference of APP electrooxidation. As the concentration of mouse IgG increases, the anodic peak current of AP also increases.

The anodic peak currents of cyclic voltammograms in Figure 5 were plotted with respect to mouse-IgG concentration (Figure 6). The detection limit of the immunosensor is 0.1 ng/mL, which is comparable to that of commonly employed enzyme-linked immunosorbent assay (pM range; 1 pM correspond to 150 pg/mL).

Conclusion

A sandwich-type electrochemical biosensor was developed using the covalent attachment of CCNTs to an amine-functionalized ITO electrode. A partial monolayer of CCNT on an ITO electrode enables us to obtain good electrocatalytic properties as well as low background currents. As a result, a low detection limit (0.1 ng/mL) is achieved.

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