Amperometric immunosensing using an indium tin oxide electrode modified with multi-walled carbon nanotube and poly(ethylene glycol)–silane copolymer†

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Received (in Cambridge, UK) 25th January 2007, Accepted 12th March 2007
First published as an Advance Article on the web 3rd April 2007
DOI: 10.1039/b701190c

We describe a sensitive electrochemical immunosensor that takes advantage of the low background current of an indium tin oxide electrode, the good electrocatalytic properties of multi-walled carbon nanotubes, and the low biofouling properties of poly(ethylene glycol)–silane copolymer.

Indium tin oxide (ITO) electrodes have been used as working electrodes in electrochemical sensors because of their wide potential window and their low capacitive current (*i.e.*, a low background current over a wide range of potentials).[1] Importantly, the self-assembled monolayers formed on ITO electrodes are more chemically and electrochemically stable than thiol-based monolayers on gold electrodes.[2] Furthermore, the easy micropatterning of ITO electrodes allows their application to microchip-based electrochemical sensors.[3] Nevertheless, ITO electrodes are not commonly employed in electrochemical sensors because electron-transfer kinetics of most electroactive species is much slower on ITO electrodes than on noble metal and carbon electrodes.[1c] This problem intensifies when the electrodes are covered with biosensing layers.

Carbon electrodes, for example, glassy carbon electrodes, are widely used in electrochemical sensors because of their good electrocatalytic properties;[4] however, the high background current of carbon electrodes, which is caused by surface faradaic reactions, together with double-layer charging results in low sensitivity.[4a] Recently, carbon nanotubes (CNTs) have received a great deal of attention as an electrode material because they have good electrocatalytic properties.[5] CNT electrodes are generally constructed as non-oriented thick films or vertically aligned films.[6] Thick CNT films, however, have large capacitive background currents, and the fabrication of vertically aligned films is difficult. Consequently, these CNT films are not suitable for producing sensitive and inexpensive electrochemical biosensors, and a simple method for forming thin CNT films is needed.

Protein-resistant biosensing layers are essential for obtaining low detection limits in biosensors because they minimize unwanted background signals. A common strategy for minimizing the nonspecific binding of proteins is to use poly(ethylene glycol) (PEG)-containing biosensing layers.[7] For example, reliable non-biofouling surfaces can be easily produced by modifying SiO_2-based substrates with self-assembled polymeric monolayers of PEG–silane random copolymer.[8]

Here, we describe an electrochemical immunosensor that employs an ITO electrode modified with multi-walled CNTs (MWCNTs) and PEG–silane random copolymer. The preparation scheme for the electrochemical immunosensor is depicted in Fig. 1. In the immunosensor, the partial monolayer of MWCNT on the ITO electrode provides both a low background current and good electrocatalytic properties, and the monolayer of PEG–silane copolymer formed on vacant regions of the MWCNT-coated ITO electrode minimizes nonspecific protein adsorption. In addition, the avidin bound on the sidewalls of the MWCNTs provides docking sites for various biotinylated proteins and minimizes nonspecific binding of other proteins onto the MWCNTs.

For the formation of MWCNT layers, we used carboxylated MWCNTs obtained by acid treatment.[9] The carboxylated MWCNTs were physically immobilized on an ITO electrode by immersing the electrode for 4 h in an aqueous solution containing 1 mg mL^{-1} of carboxylated MWCNTs. The resulting physisorbed MWCNT layers were sufficiently stable to resist detachment even after sonication for 10 min (see ESI†). Although it is known that carboxyl-terminated compounds spontaneously form monolayers on ITO surfaces,[10] the exact mechanism for the formation of MWCNT layers is still under investigation. The presence of MWCNTs on the ITO electrode was confirmed by SEM (Fig. 2). The surface coverage of the MWCNT layer (*ca.* 0.03) was much less than the maximum coverage for a full monolayer, and the

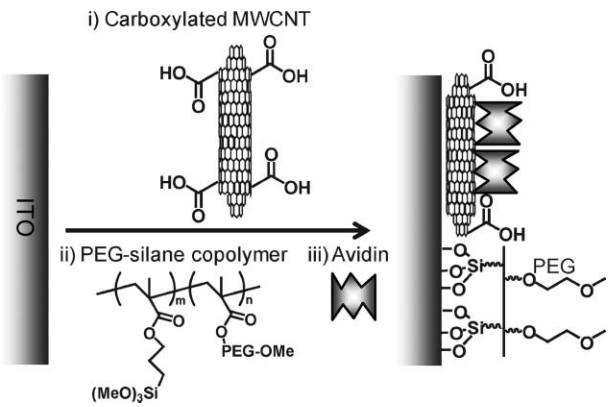

Fig. 1 Schematic representation of the preparation of an ITO electrode modified with MWCNT, PEG–silane copolymer, and avidin.

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† Electronic supplementary information (ESI) available: Experimental details, SEM images, a cyclic voltammogram of APP. See DOI: 10.1039/b701190c

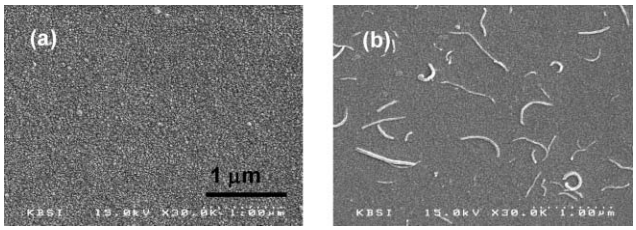

Fig. 2 SEM images of (a) a bare ITO electrode and (b) a MWCNT-modified ITO electrode.

MWCNTs were randomly distributed. Increasing the incubation time did not affect the coverage. After the deposition of MWCNTs on the ITO electrode, the unmodified, vacant regions of the electrode were covered by a monolayer of PEG–silane copolymer (Fig. 1). Because proteins are known to adsorb well to the surfaces of CNTs,[11] avidin was attached to the hydrophobic sidewalls of the MWCNTs simply by dipping the resulting electrode in a solution of avidin.

We next examined the nonspecific binding of proteins by the PEG–silane copolymer-modified ITO electrodes using fluorescein isothiocyanate-labeled avidin and antimouse IgG (Fig. 3). A monolayer of PEG–silane copolymer showed much less nonspecific binding of avidin and antimouse IgG than a bare ITO electrode, indicating that the copolymer substantially reduces nonspecific binding.

To evaluate the background current and electrocatalytic properties of modified ITO electrodes, we carried out cyclic voltammetry measurements in a Tris buffer (pH 9.0) (Fig. 4). The background current of a bare ITO electrode was low at potentials between −0.3 and 0.8 V (Fig. 4(a)) and was due to low capacitive and redox currents of the ITO electrode and the electrolyte over a wide range of potentials. The background current is almost flat in this potential range, which should help achieve low detection limits.[12] The MWCNT-modified electrode shows a higher background current due to the presence of the MWCNTs, but further modification with PEG–silane copolymer and avidin resulted in a slight decrease in the background current, which is sufficiently low to give the sensor an adequate detection limit.

Generation of *p*-aminophenol (AP) from *p*-aminophenyl phosphate (APP) by alkaline phosphatase (ALP) is commonly used for electrochemical signaling in electrochemical biosensors. To achieve low detection limits, AP must be electrooxidized at low potentials, which prevents the electrooxidation of APP from affecting the anodic current. As shown in Fig. 4(b), the

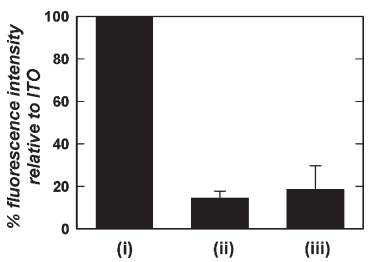

Fig. 3 Adsorption of (i) avidin or antimouse IgG to a bare ITO electrode (100% fluorescence intensity) or of (ii) avidin or (iii) antimouse IgG to a monolayer of PEG–silane copolymer. Avidin and antimouse IgG were labeled with fluorescein isothiocyanate, and adsorption was measured by fluorescence intensity.

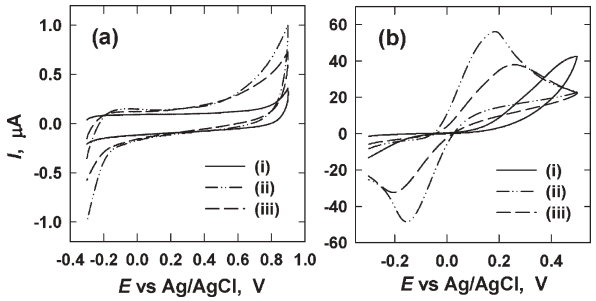

Fig. 4 Background current and electrocatalytic property of bare and modified ITO electrodes. Cyclic voltammograms were obtained at a scan rate of 20 mV s^{-1} in Tris buffer (pH 9.0) without (a) and with (b) 1.0 mM AP and using (i) a bare ITO electrode, (ii) a MWCNT-modified electrode, or (iii) an electrode modified with MWCNT, PEG–silane copolymer, and avidin.

electrooxidation of AP on a bare ITO electrode started at potentials above 0.1 V, and an anodic peak was not observed at potentials up to 0.4 V. Considering that the formal potential of AP is around 0 V, this indicates that the electron-transfer kinetics of AP electrooxidation is very slow on the ITO electrode. Considerable electrooxidation of APP occurs at potentials above 0.4 V (see ESI†). Accordingly, the bare ITO electrode is not suitable as a working electrode for the detection of AP. The cyclic voltammogram of a MWCNT-modified ITO electrode showed quasireversible current behavior (ii in Fig. 4(b)). The good electrocatalytic properties are due to the presence of MWCNTs on the ITO electrode, although the surface coverage by MWCNTs is not high. When the MWCNT-modified electrode is covered with PEG–silane copolymer and avidin, however, the electrocatalytic properties become inferior because the avidin adsorbs to the electrocatalytic sites of the MWCNTs (iii in Fig. 4(b)). Regardless, the electrocatalytic properties are still sufficient to electrooxidize AP at low potentials (*i.e.*, the anodic peak potential is slightly higher than 0.2 V). Consequently, the partial monolayer of MWCNTs on the ITO electrode provides good electrocatalytic properties as well as a low background current.

To test the feasibility of applying the modified ITO electrode to biosensors, we designed a sandwich-type immunosensor for detecting mouse IgG (Fig. 5). Biotinylated antimouse IgG was immobilized on avidin to allow the specific binding of a mouse IgG. Next, ALP-conjugated antimouse IgG was bound to the mouse IgG. The bound ALP catalyzed the conversion of APP to AP, which is electrooxidized on the MWCNTs.

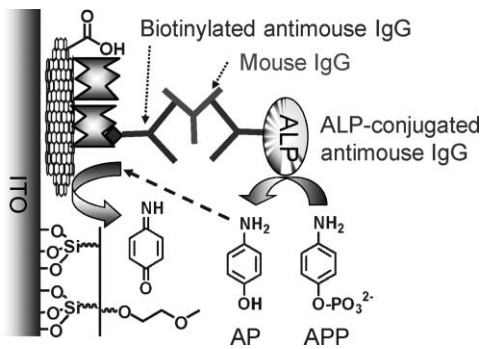

Fig. 5 Schematic illustration of an electrochemical immunosensor for detecting mouse IgG.

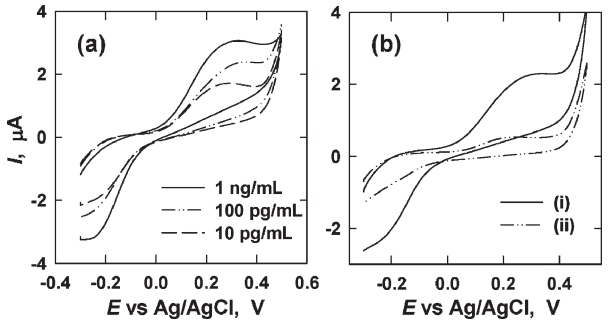

Fig. 6 (a) Dependence of cyclic voltammograms on the concentration of mouse IgG. Cyclic voltammograms were obtained at a scan rate of 20 mV s^{-1} after incubation for 10 min in a Tris buffer containing 1.0 mM APP. (b) Evaluation of nonspecific binding to the immunosensor. Cyclic voltammograms were obtained (i) in the absence of PEG–silane copolymer on the ITO electrode and (ii) in the presence of PEG–silane copolymer and in the absence of mouse IgG.

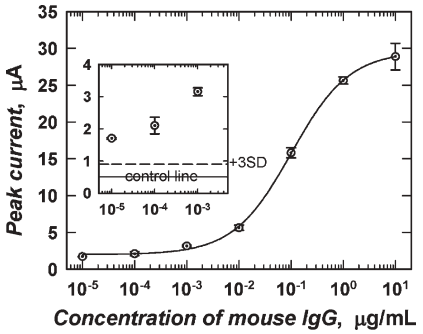

Fig. 7 Dependence of anodic peak current on mouse-IgG concentration. The inset represents a magnified graph at low concentrations. The control line corresponds to the mean current in the absence of mouse IgG, and the dashed line corresponds to the mean current plus three times standard deviation (SD).

Fig. 6(a) shows cyclic voltammograms obtained at three concentrations of mouse IgG. The cyclic voltammograms were recorded after the electrodes had been incubated for 10 min in a Tris buffer containing 1.0 mM APP. As the concentration of mouse IgG increased, the anodic peak current increased. The sharp increase in the anodic current at potentials above 0.4 V is due to the electrooxidation of APP (see ESI†). The peak anodic potential at low concentrations is much lower than 0.4 V. Accordingly, the peak anodic current is independent of the electrooxidation of APP, but is related to the anodic current of AP and the capacitive current.

The nonspecific binding of non-target proteins to the immunosensor could be a problem. To test the nonbiofouling effect of the PEG–silane copolymer, we performed cyclic voltammetry measurements in the absence and presence of copolymer on the ITO electrode and in the absence of mouse IgG (Fig. 6(b)). When the copolymer was absent, the anodic peak current was large because of nonspecific binding of ALP-conjugated antimouse IgG. In the presence of the copolymer, the anodic peak current is much lower. These results clearly show that the PEG–silane copolymer plays a crucial role in reducing the nonspecific binding of proteins and that nonspecific binding of antimouse IgG to the avidin-adsorbed sidewalls of MWCNT is insignificant.

At 10 pg mL^{-1} of mouse IgG, the peak current was 1.71 $\pm$ 0.25 μA (mean $\pm$ SD), which is higher than the current in the absence of mouse IgG (0.51 $\pm$ 0.13 μA) (Fig. 6). Fig. 7 shows the dependence of the anodic peak current on the concentration of mouse IgG. The detection limit for mouse IgG was 10 pg mL^{-1}. General immunoassays such as ELISA (enzyme-linked immunosorbent assay) have detection limits in the pM range; 1 pM corresponds to 150 pg mL^{-1} for IgG. Our electrochemical immunosensor is highly sensitive compared to ELISA, and it is comparable to the immunosensor using funtionalized liposomes.[13]

In summary, we have developed an ITO electrode modified with MWCNTs, PEG–silane copolymer, and avidin, and used it in an electrochemical immunosensor for detecting mouse IgG. The partial monolayer of MWCNTs is formed on the ITO electrode by simply dipping the electrode in a solution containing carboxylated MWCNTs. The modified electrode offers both a low background current and good electrocatalytic properties, and the PEG–silane copolymer minimizes the nonspecific binding of proteins. The nonspecifically bound avidin molecules on the sidewalls of the MWCNTs provide efficient sites for immobilizing biotinylated proteins, and minimize the nonspecific binding of proteins.

This work was supported by the Nano/Bio science & Technology Program (M10536090000-05N3609-00000) of the Ministry of Science and Technology (MOST), the Healthcare & Biotechnology Development Program (A020605) of the Korea Health Industry Development Institute, and by Pusan National University Research Grant, 2004.

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