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The fabrication of a label-free electrochemical immunosensor using Nafion/carbon nanotubes/charged pyridinecarboxaldehyde composite film

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ABSTRACT

A label-free electrochemical immunosensor based on Nafion/carbon nanotubes (CNTs)/charged pyridinecarboxaldehyde composite film was developed for the detection of hepatitis B surface antigen. Nafion/CNTs/charged pyridinecarboxaldehyde nanocomposites were prepared by dispersing charged pyridinecarboxaldehyde and CNTs in Nafion solution. The nanocomposites were cast on the electrode surface to form aldehyde-terminated composite film that can covalently bind antibody on the film without using other reagent. The immunosensor response was linearly changed with hepatitis B surface antigen concentration in the range from 0.1 to 25 ng ml^{-1} with a detection limit (signal/noise ratio = 3) of 0.04 ng ml^{-1}. Some important advantages such as simple preparation, good stability, reproducibility, and selectivity of the immunosensor were achieved.

Recently, polymers have been proven to be a very suitable matrix for forming composite film [1,2]. Nafion, a sulfonated ion-exchange polymer, has been widely used to fabricate chemical sensors or biosensors due to its various advantages such as mechanical strength, thermal stability, chemical inertness, excellent film forming ability, and antifouling properties [3,4]. In addition, it is a permselective polymer known for its capability of incorporating cations and rejecting anions, making it a good candidate for successful incorporation of various cationic electrocatalysts into the Nafion film at the surface of the modified electrode. However, pure Nafion film was insulated, which obstructed the electron transfer and mass transmission. This limitation is unfavorable for the fabrication of label-free biosensors. A common approach to address this problem is the incorporation of conductive materials into Nafion films [5].

Carbon nanotubes (CNTs)[1] have attracted much attention due to their high chemical stability, high surface area, unique electronic properties, and relatively high mechanical properties. However, CNTs are easy to form aggregates due to strong van der Waals interactions. CNTs and Nafion show a well-known good chemical affinity [6]. In the presence of Nafion, it is relatively simple to uniformly disperse CNTs in the polymeric matrix without the formation of aggregates [7]. Thus, as electrode matrix materials, CNTs can be incorporated into Nafion film for promoting electron transfer and provide a convenient method for fabricating chemical sensors or biosensors [8–10].

In the development of electrochemical immunosensors based on Nafion composite film with good performance, one of the most common issues faced is how to conveniently immobilize antibodies on the Nafion film. It was reported that the immobilization of antibodies on Nafion-containing sensing surfaces usually can be carried out by the following steps [11–13]: (i) introduce the positive-charged materials (e.g., L-cysteine, $Co(bpy)_3^{3+}$) on the electrode surface; (ii) introduce gold nanoparticles; and (iii) immobilize antibodies on the surface of gold nanoparticles. The need for many steps of antibody immobilization is tedious and time-consuming. Thus, it is important to develop a simple immobilization method and new matrices for the fabrication of electrochemical immunosensors. As is well known, aldehyde groups can react with amino

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[1] *Abbreviations used*: CNT, carbon nanotube; HBsAg, hepatitis B surface antigen; DPV, differential pulse voltammetry; IgG, immunoglobulin G; BSA, bovine serum albumin; PBS, phosphate buffer solution; CV, cyclic voltammetry; EIS, electrochemical impedance spectroscopy; SCE, saturated calomel electrode; GCE, glassy carbon electrode; Py-CHO, aldehyde-functionalized pyridinium salt; Ab, antibody; TEM, transmission electron microscopy; AFP, α-fetoprotein; CEA, carcinoembryonic antigen; ELISA, enzyme-linked immunosorbent assay.

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groups directly. Therefore, glutaraldehyde was usually used as a linker reagent for the antibody immobilization [14]. Aldehyde-functionalized pyridinium salt contains aldehyde groups involved in covalent binding of the antibodies, which made it possible to simplify antibody immobilization. In addition, pyridinium salt has a structure of quaternary ammonium salt and possesses positive charges. So, it can be incorporated into Nafion matrix to improve the conductivity of sensing interface and, therefore, to enhance the sensitivity of biosensors. Although immunosensors based on aldehyde-terminated self-assembled monolayers have also been developed [15,16], few articles have reported the use of aldehyde-functionalized pyridinium to fabricate immunosensors.

In this work, a composite film composed of Nafion, CNTs, and aldehyde-functionalized pyridinium was modified on the electrode surface. Nafion was used as a matrix material, CNTs were applied to promote electron transfer, and aldehyde-functionalized pyridinium was used not only to immobilize antibodies, in which no additional reagent was required, but also to improve the conductivity of sensing interface. This electrostatic adsorption method is very simple and does not need any label to biomolecules. Hepatitis B surface antigen (HBsAg) is one of the most common contagious diseases that cause cirrhosis, chronic hepatitis, and primary liver cancer. Thus, HBsAg detection is very helpful to clinical diagnoses. In the current work, HBsAg was used as a model analyte to design a simple label-free immunosensor. The specific binding of antibody to antigen could be directly detected by the decrease of the peak current response of differential pulse voltammetry (DPV) based on ferricyanide ions as a redox probe.

Materials and methods

Reagents and apparatus

Monoclonal anti-HBsAg and HBsAg were purchased from Kehua Bioengineering (China). Human immunoglobulin G (IgG) and bovine serum albumin (BSA) were purchased from Beijing Dingguo Biotechnology (China). Pyridine-4-carboxaldehyde, dichloromethane, methyl iodide, and Nafion (5%, v/v) were obtained from Sigma Chemical (St. Louis, MO, USA). Phosphate buffer solution (PBS, 0.1 M, pH 7.0) was prepared using Na_2HPO_4 and KH_2PO_4.

All electrochemical measurements, including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and DPV, were carried out with a CHI 660 electrochemistry workstation (Shanghai CH Instruments, China). A conventional three-electrode cell, consisting of a Pt electrode as counter electrode, a saturated calomel electrode (SCE) as reference electrode, and a glassy carbon electrode (GCE) modified with aldehyde-functionalized pyridinium salt/Nafion composite film as working electrode, was used. CV measurements were taken at a scanning rate of 100 mV s^{-1} from −0.2–0.6 V relative to SCE. DPV measurements were carried out as follows: the potential range was from −0.4 to 0.1 V, pulse amplitude was 0.05 V, pulse width was 0.05 s, and sample width was 0.02 s. EIS measurements were carried out in the frequency range from 10^{-1}–10^5 Hz under an open potential. The amplitude of the alternative voltage was 5.0 mV.

Preparation of aldehyde-functionalized pyridinium salt

Aldehyde-functionalized pyridinium salt (Py-CHO) can be synthesized according to the reported literature [17]. The synthetic route is shown in Fig. 1A. Typically, pyridine-4-carboxaldehyde (5 ml, 56.9 mmol) in dichloromethane (20 ml) was treated with methyl iodide (7 ml, 112.4 mmol) and stirred for 2 days at room temperature. Yellow crystals were obtained by filtering off.

Preparation of electrochemical immunosensor

The bare GCE (3 mm diameter) was first polished with emery paper and alumina slurry of 0.3 and 0.05 μm, followed by successive sonication in distilled water and ethanol. The electrode was then treated with fresh piranha solution (H_2O_2 and H_2SO_4, 1:3, v/v) for 3 min and washed with distilled water. Film solution was prepared by dispersing 2 mg of aldehyde-functionalized pyridinium salt and 1 mg of CNTs in 1 ml of Nafion ethanol solution (0.25%, v/v, Nafion/CNTs/Py-CHO). The cleaned electrode was coated with 10 μl of Nafion/CNTs/Py-CHO solution and dried in the air. Subsequently, 10 μl of 100 μg ml^{-1} antibody was dropped on the Nafion/CNTs/Py-CHO film modified electrode to be incubated for 30 min at 37 °C. The obtained electrode was thoroughly washed with double-distilled water to remove the physically absorbed antibodies. Then, the electrode modified with antibodies was incubated with 10 μl of BSA (2.0 wt%) for 40 min at 37 °C to eliminate nonspecific binding. The whole process of the immunosensor fabrication is shown in Fig. 1B.

Results and discussion

Electrochemical behaviors of electrode modified with different films

Fig. 2 shows cyclic voltammograms of electrode modified with Nafion (curve a), Nafion/CNTs (curve b), and Nafion/CNTs/Py-CHO (curve c) in 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ with a scanning rate of 100 mV s^{-1} in the potential range from −0.2–0.6 V versus SCE. After bare electrode was modified with Nafion film, the anodic and cathodic peaks were not observed, indicating that Nafion was nearly insulated and there was greatly obstructed electron and mass transfer (curve a). When the bare electrode was modified with Nafion/CNTs, the anodic and cathodic peaks emerged (curve b). The significant increase of redox peak currents can be attributed to high electrical conductivity of CNTs. When the bare electrode was modified with Nafion/CNTs/Py-CHO, redox peak currents further increased, meaning that charged pyridinecarboxaldehyde can improve the conductivity of sensing interface (curve c).

Electrochemical characterization of immunosensor fabrication

To study the stepwise assembly of the immunosensor. CV measurements were performed in 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at a scan rate of 100 mV s^{-1} in the potential range of −0.2–0.6 V. The typical CV spectrum is shown in Fig. 3A. Curve a exhibits CV of the bare electrode. It is a well-defined redox wave resulting from the reversible redox reaction of ferricyanide ions on the GCE (curve a). After bare electrode was modified with Nafion/CNTs/Py-CHO composite film, the peak current increased (curve b). The results illustrate that the conductivity of Nafion/CNTs/Py-CHO composite film was good. After anti-HBsAg antibody (Ab) was adsorbed onto the Nafion/CNTs/Py-CHO composite film, the peak current decreased significantly (curve c). This result demonstrated that the formed protein layer enhanced electron transfer barriers. When BSA solution was dropped onto the Nafion/CNTs/Py-CHO/Ab-modified electrode, the peak current further decreased, indicating that nonspecific active sites were blocked (curve d). Subsequently, the immunosensor was incubated with antigens, and an obvious decrease in peak current was observed (curve e). It was probably due to the formation of antibody–antigen immunocomplex, which blocked the tunnel for mass and electron transfer.

EIS was also used to further investigate the stepwise modified processes. Fig. 3B shows the Nyquist plots of impedance spectra of different electrodes in 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution. Curve a exhibits EIS of the bare electrode. After bare electrode was