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Synergistic effects of nano-ZnO/multi-walled carbon nanotubes/chitosan nanocomposite membrane for the sensitive detection of sequence-specific of PAT gene and PCR amplification of NOS gene

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ABSTRACT

The remarkable synergistic effects of the zinc oxide (ZnO) nanoparticles and multi-walled carbon nanotubes (MWNTs) were developed for the ssDNA probe immobilization and fabrication of the electrochemical DNA biosensor. The ZnO/MWNTs/chitosan nanocomposite membrane-modified glassy carbon electrode (ZnO/MWNTs/CHIT/GCE) was fabricated and the ssDNA probes were immobilized on the modified electrode surface. The preparation method is quite simple and inexpensive. The hybridization events were monitored by differential pulse voltammetry (DPV) using methylene blue (MB) as an indicator. As compared with previous MWNTs-based DNA biosensors, this composite matrix combined the attractive biocompatibility of ZnO nanoparticles with the excellent electron-transfer ability of MWNTs and fine membrane-forming ability of CHIT increased the DNA attachment quantity and complementary DNA detection sensitivity. The approach described here can effectively discriminate complementary DNA sequence, noncomplementary sequence, single-base mismatched sequence and double-base mismatched sequence related to phosphinothricin acetyltransferase (PAT) gene in transgenic corn. Under optimal conditions, the dynamic detection range of the sensor to PAT gene complementary target sequence was from 1.0×10^{-11} to 1.0×10^{-6} mol/L with the detection limit of 2.8×10^{-12} mol/L. The polymerase chain reaction (PCR) amplification of nopaline synthase (NOS) gene from the real sample of one kind of transgenic soybeans was also satisfactorily detected with this electrochemical DNA biosensor, suggesting that the ZnO/MWNTs/CHIT nanocomposite hold great promises for sensitive electrochemical biosensor applications.

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1. Introduction

Carbon nanotubes (CNTs) consist of cylindrical grapheme sheets with nanometer diameter, and show many unique characteristics, such as large ratio of surface area to mass, high-electrical conductivity and remarkable mechanical strength [1]. Since Lijima discovered them in 1991 [2], CNTs have received much attention. It is well known that CNTs have the capability to promote electron-transfer reactions and improve sensitivity in electrochemistry, and thus they are widely used to prepare electrodes. CNTs-modified electrodes have been reported to give super performance in the study of a number of biological species, such as bilirubin oxidase [3], deferiprone [4] and ascorbic acid [5]. CNTs have also been used in the development of electrochemical DNA biosensors [6,7]. For example, Guo et al. [7] detected calf thymus DNA molecules on the surface of multi-walled carbon nanotubes (MWNTs) modified electrode by cyclic voltammetry and electrochemical impedance spectrometry. Most of the CNTs-sensing research has focused on the utilizing of only CNTs to promote electron-transfer reactions with electroactive species [8–12]. Recently, composite materials based on the combination of CNTs with some other materials, which possess properties of the individual components and simultaneously have outstanding synergistic effects, have drawn much attention. Materials for such aims include conducting polymers [13,14], metal nanoparticles [15,16] and oxide particles [17,18]. For instance, coupling CNTs with polyaniline resulted in the remarkable improvement of the electroactivity of the composite materials toward dopamine through synergistic effects [14]. Zhu et al. [15] utilized MWNTs/Pt nanoparticles/Nafion composite system for the fabrication of DNA biosensor. Due to the ability of MWNTs to promote electron-transfer reaction and the high-catalytic activity of Pt nanoparticles for chemical reac-

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tions, the sensitivity of presented electrochemical DNA biosensor was remarkably improved.

Zinc oxide (ZnO) is an inorganic semiconductor oxide with the thermal stability, chemical inertness, lack of toxicity and a high-isoelectric point (~9.5) [19], which is positively charged on the surface under acidic conditions, so it has been widely used for the immobilization of biomolecules [20–22]. Li et al. [20] developed a mediator-free phenol biosensor. The low-isoelectric point tyrosinase was adsorbed on the surface of high-isoelectric point ZnO nanoparticles facilitated by the electrostatic interaction and then immobilized on the glassy carbon electrode (GCE) via the membrane forming by chitosan (CHIT).

Chitosan is a linear hydrophilic polysaccharide composed of linked glucosamine units together with some proportion of *N*-acetylglucosamine units. It is an attractive biocompatible, biodegradable and nontoxic natural biopolymer that exhibits excellent membrane-forming ability [23]. Because of its desirable properties, it has been widely used as an immobilization matrix for biosensors [24,25].

In this paper, the integration of nano-ZnO, MWNTs and CHIT for DNA immobilization and hybridization detection has been explored to enlarge the electrochemical signal of the DNA indicator and increase the sensitivity for DNA detection. The ssDNA probes were immobilized on the surface of GCE modified with the ZnO/MWNTs/CHIT nanocomposite membrane. After the hybridization reaction, the target DNA sequences were detected by differential pulse voltammetry (DPV) using an electroactive indicator, methylene blue (MB), which possesses different affinity for ssDNA and dsDNA [26,27]. This paper reported the experimental results of the preparation of the designed biosensor and its analytical performance for the detection of phosphinothricin acetyltransferase (PAT) gene sequences in transgenic corn and polymerase chain reaction (PCR) amplified real sample of nopaline synthase (NOS) gene of transgenic soybean. Such a novel membrane may be used as a potential sensing platform for the detection of other biomolecules.

2. Experimental

2.1. Apparatus and reagents

A CHI 660C electrochemical analyzer (Shanghai CH Instrument Company, China), which was in connection with a glassy carbon-modified working electrode (diameter 3 mm), a saturated calomel reference electrode (SCE) and a platinum wire counter electrode, was used for the electrochemical measurements. Scanning electron microscopy (SEM) was operated using a JSM-6700F machine (JEOL, Japan). Transmission electron microscopy (TEM) was performed on a JEM-2000EX machine (JEOL, Japan). The pH values of all solutions were measured by a model pHS-25 digital acidimeter (Shanghai Leici Factory, China). The PCR amplification was performed by an Eppendorf Mastercycler Gradient PCR system (Germany).

MWNTs were purchased from Shenzhen Nanotech. Port Co., Ltd. (Shenzhen, China). $Zn(NO_3)_2{\cdot}6H_2O$, CHIT, sodium dodecyl sulfate (SDS) and MB were obtained from Shanghai Reagent Company (Shanghai, China). All the chemicals are of analytical grade and solutions were prepared with ultrapure water.

The 20-base oligonucleotides probe (ssDNA), its complementary DNA sequence (cDNA), single-base mismatched sequence, double-base mismatched sequence and noncomplementary sequence (ncDNA) were synthesized by Beijing SBS Gene Technology Limited Company. Their base sequences were the same as we have used in our previous work [28].

Materials for the PCR amplification of NOS gene sample: one pair of primers (19-base for every primer). Their base sequences are as below:

Primer 1: 5′- ATC GTT CAA ACA TTT GGC A -3′;
Primer 2: 5′- ATT GCG GGA CTC TAT CAT A -3′.

The primer 1 was also used as the probe DNA for the detection of PCR amplification of NOS gene sample. The DNA sample for PCR amplification was extracted from a kind of transgenic soybean according to the method of plant DNA mini prep kit (Shanghai Academy of Agricultural Sciences).

All oligonucleotides stock solutions of 20-base oligomers (1.0×10^{-6} mol/L) were prepared using Tris–HCl buffer solution (5.0 mmol/L Tris–HCl, 50.0 mmol/L NaCl, pH 7.0), which were stored at 4 °C. More diluted solutions were obtained via diluting aliquot of the stock solution with ultrapure water prior to use. The hybridization solution was diluted with 2× SSC (pH 7.5), which was consisted of 0.30 mol/L NaCl and 0.03 mol/L sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$).

2.2. Synthesis of nano-ZnO powder

In a typical procedure, 1.5 g of $Zn(NO_3)_2{\cdot}6H_2O$ and 0.8 g of NaOH were dissolved in 40 mL of ultrapure water. The $Zn(NO_3)_2$ solution was added dropwise to the NaOH solution to form a white slurry. Then, the white slurry was kept at 80 °C for 6 h. The resulted white precipitates were collected, washed with ultrapure water and ethanol several times, and then dried at 60 °C in vacuum for 10 h.

2.3. PCR amplification of DNA template from transgenic soybean

To a 0.2 mL reaction tube 12.0 pmol primer 1, 12.0 pmol primer 2, 2.0 mmol/L $MgCl_2$, 0.2 mmol/L dATP, 0.2 mmol/L dCTP, 0.2 mmol/L dGTP, 0.2 mmol/L dTTP, 1.5 U Taq DNA polymerase (Promega, Wisconsin, USA), DNA template (50 ng DNA), 10× reaction buffer B (Promega) were added. The reaction total volume was 25 μL.

PCR procedure: DNA was denaturated at 94 °C for 3 min. Each of the 40 cycles of amplification consisted of annealing at 56 °C for 20 s, extension at 72 °C for 20 s, denaturation at 94 °C for 30 s. Then another extension at 72 °C for 6 min was carried out.

2.4. Preparation of ZnO/MWNTs/CHIT solution

MWNTs were pretreated as the reference [6]. A 0.2% CHIT solution (pH 5.0) was prepared by dissolving appropriate amount of CHIT flakes into 0.1 mol/L acetic acid and stirring for 3 h at room temperature until complete dissolution. Appropriate amount of ZnO nanoparticles and MWNTs were dispersed in 0.2% CHIT solution. The mass ratio of ZnO:MWNTs:CHIT was 1:3:100. The mixture was sonicated for 20 min after stirring 6 h. Finally, a high dispersed black colloidal solution was formed.

2.5. Fabrication of electrochemical DNA biosensor

For the electrochemical experiments, the GCE surface was freshly polished prior to each experiment with 0.3 and 0.05 μm α-Al_2O_3 paste, respectively, and was rinsed with ultrapure water after each polishing step, finally cleaned ultrasonically in ethanol and water, respectively, for 3 min.

Ten microliters of ZnO/MWNTs/CHIT casting solution was coated on the GCE surface and dried in air. Then, the obtained ZnO/MWNTs/CHIT/GCE was immersed into 2.0 mL Tris–HCl buffer containing 1.0×10^{-6} mol/L probe ssDNA for 2 h at room temperature, followed by washing the electrode with 0.5% SDS solution for

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