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Assembly of carbon nanotubes on a nanoporous gold electrode for acetylcholinesterase biosensor design

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ABSTRACT

An electrochemical sensing platform based on assembly of carbon nanotubes on a nanoporous gold electrode is described for highly sensitive detection of organophosphate pesticides. The nanoporous gold film (NPG) electrode is fabricated by an alloying/dealloying process, which possess high electroactive surface area and is an excellent substrate for sensor design. The NPG functionalized with cysteamine allows the immobilization of carbon nanotubes on the electrode with the self-assembly technique. The carboxylated carbon nanotubes are further linkered with acetylcholinesterase (AChE) for amperometric sensing of pesticides. The immobilized AChE, as a model, shows excellent activity to its substrate and allows a quantitative measurement of organophosphate pesticides. Under the optimal experimental conditions, the inhibition of malathion is proportional to its concentration in the range of 0.001–0.5 $\mu g\,mL^{-1}$ with a detection limit of 0.5 $ng\,mL^{-1}$. The proposed method shows good reproducibility and high stability, which provides a new avenue for electrochemical biosensor design.

1. Introduction

Over the past decades, nanoporous materials have attracted much attention for applications in catalysis, separation, sensors and actuators [1–3]. Among these materials, nanostructured gold films are of special interest because of the excellent stability and biocompatibility of gold for use in sensors and reactors [4]. Nanostructured gold films have a unique porous structure, high specific surface area, good permeability and high conductivity, which offer a large number of adsorption sites for proteins and enzymes and could increase the signal-to-noise ratio in the miniaturization systems [5]. Additionally, the porous structure has the ability to perform electrochemical measurements even in biofouling solutions [6]. Moreover, gold is an excellent substrate for formation of self-assembled monolayers (SAMs) with thiol, sulfide and disulfide groups. Such SAM can be used as an intermediate layer for coupling of a wide range of molecules such as enzymes, DNA and antibiotics [7].

The striking properties of the nanoporous gold have motivated intensive interest in their utilization in biosensor designs. To date, a number of enzymes or large biomolecules (such as DNA and antibody) have been entrapped in or directly immobilized onto the nanoporous structure for sensitive detection [8]. However, the nanoporous gold electrode employed as a substrate for biosensor design via surface grafting of nanomaterials has not yet been reported. Moreover, the sensitivity and stability of the biosensors using nanoporous gold film needs to be improved [7].

Carbon nanotubes (CNTs) represent one kind of carbon-based materials that pocess unique structural and electronic features and are frequently used in electrochemistry [9]. Up to now, CNTs have been widely used in biosensor designs and nanoscale electronic devices owing to their ability to mediate electron-transfer reactions with enzymes and other biomolecules [10–12]. Moreover, CNTs provide an extremely large surface area for biomolecular conjugation and subsequent signal amplification [13]. In addition, shortened CNTs can be aligned to an electrode by self-assembly and be used to enhance the electron-transfer reaction with electroactive species [14]. Herein, multiwall carbon nanotubes (MWCNTs) are grafted onto porous gold via the self-assembled monolayer of cysteamine and used as a template for enzyme loading. CNTs play an important role in both the enzyme immobilization and

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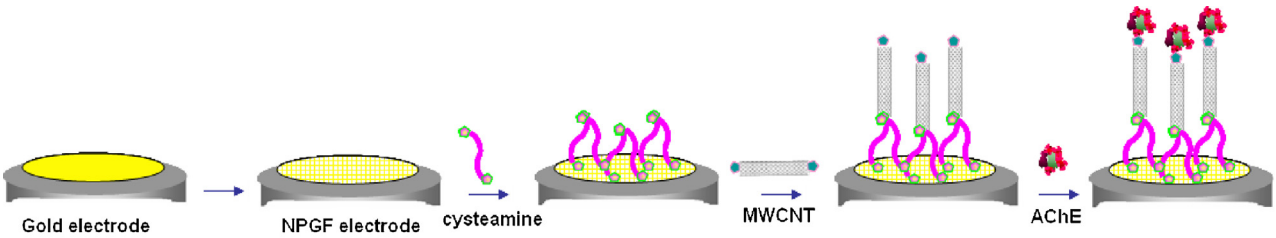

Fig. 1. Schematic illustration of the formation of AChE-MWCNT-CA-NPG.

transduction events. Combining the advantages of nanoporous gold with unique electrochemical properties of CNTs, it is possible to design an amperometric biosensor with good electron transfer properties and a high sensitivity.

Acetylcholinesterase (AChE), which is an essential enzyme responsible for the nervous system functioning and also a major target enzyme of organophosphorus and carbamate pesticides, is selected as a model. With AChE immobilized on the MWCNTs, its interaction with the substrate (acetylthiocholine) produces an electroactive product (thiocholine). The inhibition by organophosphate pesticides in the enzyme system can be monitored by measuring the oxidation current of thiocholine [15–19]. Based on the inhibition of AChE activity, a highly sensitive amperometric biosensor for pesticide was developed.

2. Experimental

2.1. Chemicals

Acetylthiocholine chloride (ATCl) and acetylcholinesterase (Type C3389, 500 U mg^{-1} from electric eel), cysteamine (CA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and N,N-dimethylformamide (DMF) were purchased from Sigma and used as received. Malathion was obtained from AccuStandard (USA). Multiwall carbon nanotubes were obtained from the Institute of Nanometer, Huazhong Normal University. Phosphate buffer solution (PBS, 0.02 M pH 7.0) was prepared with doubly distilled water. All other reagents were of analytical reagent grade.

2.2. Apparatus

Electrochemical measurements were performed on CHI-660C workstation (Chenhua Instruments Co., Shanghai, China) with a conventional three-electrode system comprised of a platinum wire as the auxiliary electrode, a saturated calomel electrode (SCE) as the reference, and a modified electrode as the working electrode. The ac impedance experiment was carried out in 5 mM $Fe(CN)_6^{4-/3-}$ with frequencies ranging from 100 kHz to 0.1 Hz.

2.3. Preparation of the nanoporous gold film electrode

The gold electrode (3 mm in diameter) was polished, cleaned by acetone, alcohol and doubly distilled water and dried before use. Nanoporous gold film electrodes were prepared through multicyclic electrochemical alloying/dealloying processes in a novel electrolyte composed of $ZnCl_2$ and benzyl alcohol according to the method described before [3,5]. The alloying/dealloying processes were carried out in the potential range of 1.8 to −0.8 V (vs Zn) with a scan rate of 10 mV s^{-1}. 25 cycles were applied to the gold working electrode using the electrochemical workstation with a Zn plate as the auxiliary electrode, and a Zn wire as the reference electrode. Before each experiment, the NPG electrode was cleaned in a 0.5 M H_2SO_4 solution by the cyclic scan from 0.4 to 1.5 V (vs SCE) until reproductive curves were obtained.

2.4. Fabrication of the acetylcholinesterase biosensor

MWCNTs were first carboxyl-functionalized and shortened by reflux in HNO_3 for 10 h. The mixture was filtered, washed with water, and then dried and dispersed in DMF. The dispersion was then mixed with 1 mL of 300 mM EDC and 35 mM NHS in a pH 7.0 PBS buffer and vortexed at room temperature for 15 min. The resulting mixture was centrifuged at 15,000 rpm for 5 min, and the supernatant was discarded. The excess EDC was removed by washing with buffer. The functionalized MWCNTs were resuspended in PBS buffer.

The nanoporous gold was immersed in 1 mM cysteamine overnight to prepare the cysteamine modified electrode (CA-NPG). The CA-NPG electrode was placed in the nanotube solution for 4 h so that the amines at the terminus of the SAM formed amide bonds with one end of the tubes (MWCNT-CA-NPG). After that, 5.0 μL AChE solution (100 mU) was dropped onto the modified electrode and dried in air at room temperature for another 2 h to obtain the AChE-MWCNT-CA-NPG. The electrode was washed with PBS twice to remove the AChE that was non-specifically bound (Fig. 1).

2.5. Measurement procedures

The AChE-MWCNT-CA-NPG electrode was first incubated in the PBS solution containing different concentrations of a standard organophosphate pesticide for 12 min, and then transferred to the electrochemical cell of 1.0 mL pH 7.0 PBS containing 0.2 mM ATCl to record the amperometric signals. The inhibition of the organophosphate pesticde was calculated as follows [16]:

$$\text{Inhibition}\,(\%) = 100\% \times \frac{i_{P,control} - i_{P,\exp}}{i_{P,control}}$$

where $i_{P,control}$ and $i_{P,exp}$ are the peak currents of thiocholine with and without organophosphate inhibition, respectively.

3. Results and discussion

3.1. Estimation of the active surface area of the electrode

The electrochemically active surface area is an important factor for potential applications of nanoporous materials. In this work, the surface area of the porous gold was determined first by integrating the reduction peak area of oxidized gold layer formed by cyclic voltammetry (CV) scans from −0.2 to +1.6 V in 0.5 M H_2SO_4 [20,21]. The average value of the electroactive surface area of the electrode can be calculated according to the Randles–Sevcik equation [22]. The surface area of the porous gold was calculated to be 0.24 cm^2, while that of the bare gold electrode was 0.05 cm^2. By integrating the charge consumed in the gold oxide reduction, the roughness factor can be obtained [5]. The roughness factor increased from 1.5