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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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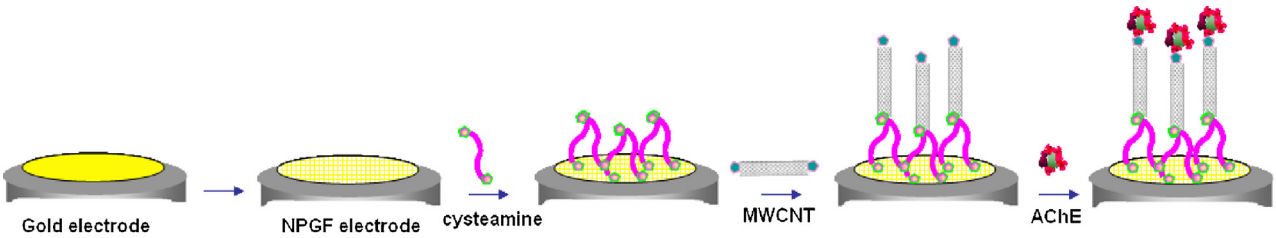

Fig. 1. Schematic illustration of the formation of AChE-MWCNT-CA-NPG.

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

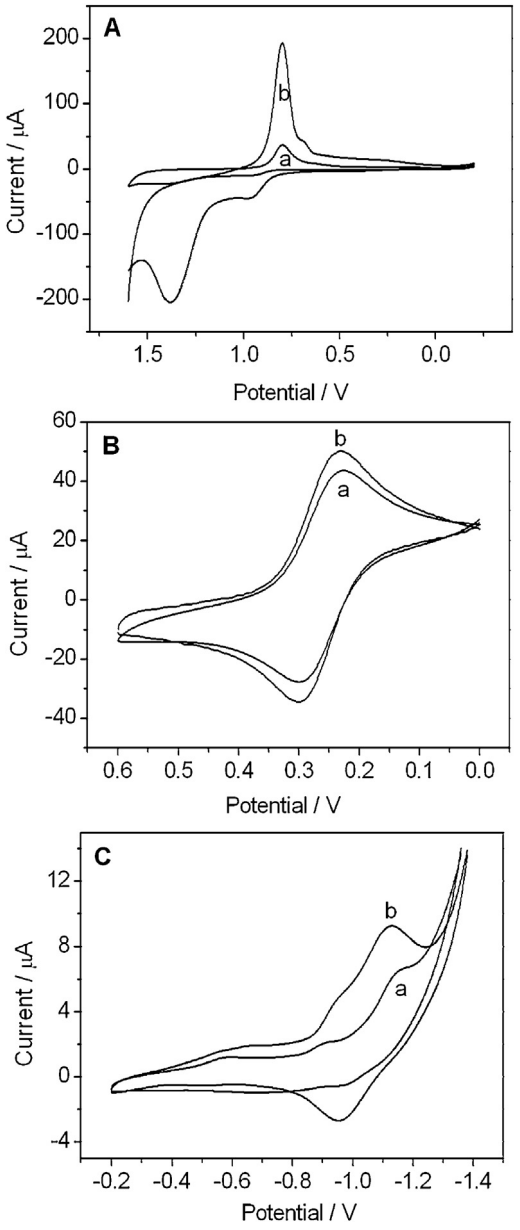

Fig. 2. CV curves recorded in (A) 0.5 M H_2SO_4, (B) 5.0 mM $Fe(CN)_6^{4-/3-}$ for (a) the polished gold electrode and (b) the nanoporous gold electrode, and (C) 0.5 M KOH for (a) the polished gold electrode and (b) the nanoporous gold electrode after being immersed in cystamine for 12 h. The scan rate was 50 mV s^{-1}.

of the bare gold electrode to 7.5 of the porous gold (Fig. 2A). The active surface area was 5-fold higher than that of the bare gold.

$Fe(CN)_6^{4-/3-}$ as one of the most extensively studied redox couples in electrochemistry was further used to confirm the properties and changes of the porous surface. When this couple is used on a flat electrode, a linear increase of the signal as a function of the active surface area, usually proportional to the geometric area, can be observed. Unlike the flat electrode, the porous one does not show such a behavior for a given geometric area (Fig. 2B). The active surface area of the porous gold is only 1.2-fold higher than that of the bare gold. Due to the high reaction rate of the $Fe(CN)_6^{4-/3-}$ redox couple, the molecules react completely at the outermost pore before they are able to diffuse into the inner pores, thus only a small part of the whole active area is used for the reaction [23,24]. Although the advantage of the NPG electrode could not fully stand out on analyzing redox reactions with fast electron-transfer

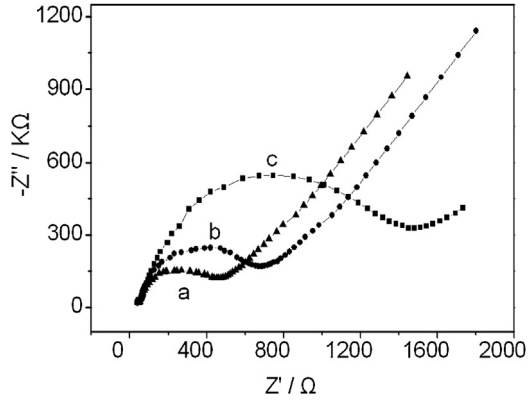

Fig. 3. EIS for (a) MWCNT-CA-NPG, (b) CA-NPG and (c) AChE-MWCNT-CA-NPG in 10 mM pH 7.4 PBS containing 5.0 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl.

kinetics, we expect that a remarkable increase in electrochemical signal at the NPG electrode could be observed for redox reactions with low electron-transfer kinetics.

The electrochemical behavior of monolayers of cysteamine chemisorbed on the surface of porous gold was investigated. The NPG electrode was immersed in 1 mM cysteamine overnight to prepare the SAMs. The CA-NPG electrode was characterized by its reductive desorption peak of thiols. The cyclic voltammogram of a cysteamine modified gold electrode displays a reduction peak at −1.11 V in 0.5 M KOH at 100 mV s^{-1} (Fig. 2C). This peak is attributed to the reductive desorption of the thiolated compounds bound to gold [25,26], indicating the presence of the cysteamine molecules on the gold surface. From the integration of the reductive desorption peak, the surface coverage, Γ ($\Gamma = Q/nFA$, where Q is the charge (C), n is the number of transfer electrons, F is the Faraday constant, and A is the geometric area of the electrode), was calculated to be 2.3×10^{-10} mol/cm^2. The coverage of bare gold was 1.3×10^{-10} mol/cm^2. Therefore, the active surface area is 1.8-fold higher than that of the bare gold, which indicates that more cysteamine SAM is accessible in the porous gold electrode. Since the pore cannot be fully occupied by cysteamine. The results obtained with the cysteamine reduction are smaller than those obtained with the gold oxide reduction.

3.2. AC impedance measurements

To check if the MWCNTs and AChE were formed on the surface, electrochemical impedance spectra (EIS) were used to characterize the resulting surface. As shown in Fig. 3, the electron transfer resistances of $Fe(CN)_6^{4-/3-}$ at CA-NPG (curve a), MWCNT-CA-NPG (curve b), and AChE-MWCNT-CA-NPG are 681, 454, and 1456 Ω, respectively. These results indicate that: the amines at terminus of the SAM form amide bonds with one end of the MWCNTs other activated sites of MWCNTs can be used to bond AChE. The decrease in the interfacial resistance of MWCNT-CA-NPG indicates that MWCNTs facilitate the electron transfer across self-assembled monolayers and act as molecular wires [9]. Moreover, the combination of nanoporous gold and MWCNTs yields better electron transfer properties. The significant change was observed for AChE-MWCNT-CA-NPG, indicating that the immobilized AChE layer induces a barrier to electrochemical process.

3.3. Cyclic voltammetric behaviors of the biosensor

Although AChE can be immobilized on the self-assembled monolayer of cysteamine, the previous report has shown that a number of active proteins can be immobilized on per CNT [27]. Therefore, CNTs could play an important role in both the

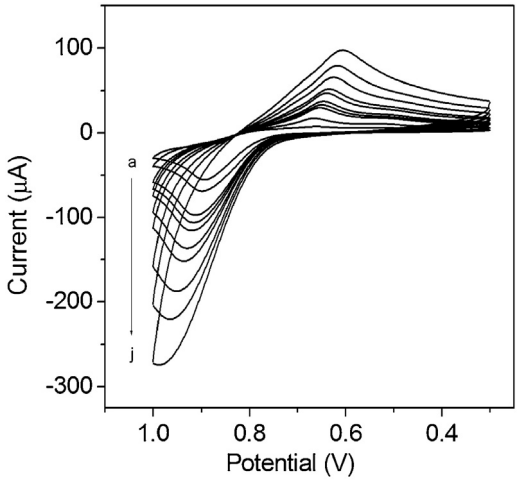

Fig. 4. Cyclic voltammograms of AChE-MWCNT-CA-NPG in pH 7.0 PBS containing 0.2 mM ATCl at different scan rates. (a) 10, (b) 20, (c) 40, (d) 60, (e) 80, (f) 100, (g) 150, (h) 200, (i) 300 and (j) 400 mV s^{-1}.

enzyme immobilization and signal amplification. Moreover, integrating carbon nanotubes (CNTs) with biological systems to form hybrid functional assemblies is promising in different fields such as nanotechnology, medicine, materials science, and biology [28]. In this article, AChE was selected as a model and immobilized on MWCNTs. The reaction between AChE-MWCNT-CA-NPG and the ATCl substrate is a CE (chemical and electrochemical) coupled reaction, including a chemical reaction: EH (enzyme) + RS-COR_2 (substrate) → RSH + E-COR_2 and an electrode reaction: RSH – $2e^-$ → RSSR + $2H^+$ [16]. The effect of scan rate on peak current was examined. For the AChE-MWCNT-CA-NPG electrode, the peak current increases and the peak potential shifts slightly with increasing scan rate (Fig. 4). The peak currents exhibit a linear dependence on the scan rate ranging from 10 to 300 mV s^{-1}, indicating a typical surface-controlled electrode process [16].

3.4. Cyclic voltammetric behaviors of the AChE-MWCNT-CA-NPG electrode

Experiments showed that no redox peaks were observed at MWCNT-CA-NPG (curve a) and at AChE-MWCNT-CA-NPG (curve b) in pH 7.0 PBS. In the presence of 0.2 mM ATCl, an oxidation peak at 913 mV was observed for the AChE-MWCNT-CA-NPG electrode (Fig. 5d), while no detectable signal was found for MWCNT-CA-NPG electrode (curve c). Obviously, this peak is caused by the oxidation

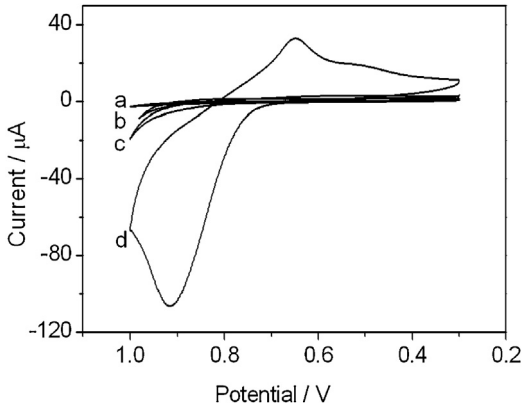

Fig. 5. Cyclic voltammograms of (a) MWCNT-CA-NPG, (b) AChE-MWCNT-CA-NPG in pH 7.0 PBS; (c) MWCNT-CA-NPG, (d) AChE-MWCNT-CA-NPG in pH 7.0 PBS containing 0.2 mM ATCl. Scan rate: 50 mV s^{-1}.

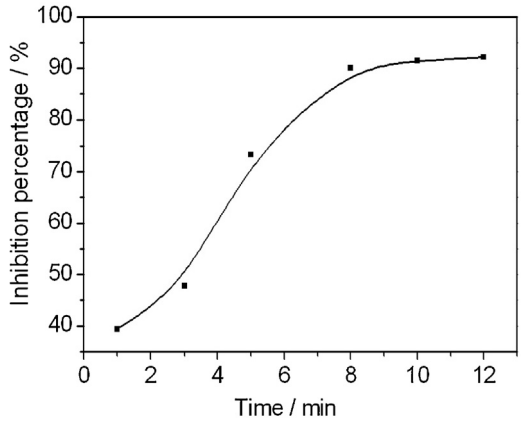

Fig. 6. Effect of the immersing time on inhibition of 0.01 μg mL^{-1} malathion.

of thiocholine (the hydrolysis product of ATCl), which is catalyzed by the immobilized AChE. AChE immobilized at the surface of the biosensor exhibits a fast response and high affinity to its substrate. The produced current by thiocholine can be used for quantification of the enzyme activity, which reflects the biological effect of an organophosphate pesticide involved in the inhibition action. The amount of ATCl directly influences the amount of reduced thiocholine, which changes the oxidative peak current. In this work, saturate amount of ATCl was used. It should be noted that the cyclic voltammogram of AChE-MWCNT-CA-NPG displayed a pair of peaks in the presence of ATCl (Fig. 5d). This phenomenon was also observed by others [29–31]. The cathodic peak might be attributed to the reduction of the thiocholine and the mechanism is currently being investigated.

3.5. Effect of incubation time on the response of the AChE-MWCNT-CA-NPG electrode

Incubation time is a very important parameter for enzyme-inhibition based biosensing systems. For irreversible inhibition, a low detection limit can be obtained with a longer incubation time. When in contact with malathion, the produced current of ATCl on the AChE-MWCNT-CA-NPG electrode decreases drastically. The decrease in peak current is related to the increase in the incubation time (Fig. 6). When the incubation time is longer than 12 min, the curve trends to a stable value, indicating the binding interaction with active target groups in enzyme reaches saturation. This change tendency of peak current reflects the alteration of enzymatic activity, which results in the change of the interaction with its substrate. However, the maximum value of inhibition of malathion is not 100%, which is likely to attribute to the binding equilibrium between the pesticide and the binding sites in the enzyme.

3.6. Calibration curve for malathion determination

Due to the notable change in voltammetric signal of the AChE-MWCNT-CA-NPG electrode, a simple method for determination of malathion can be established. It should be noted that the dissociation constant for the initial reversible enzyme inhibitor-complex is ca 10^{-5} M^{-1} [32]. Therefore, malathion may show a weak reversible inhibition at lower concentrations. However, there are malathion transformation products which are generally formed during storage or through natural or photochemical degradation. These impurities include OP esters that bear one thiolester ligand (P-S-R), the oxidation product malaoxon, and the isomerization product isomalathion. These impurities are far more potent anti-cholinesterase agents, which inhibit free and immobilized AChE in a concentration-dependent manner [32,33]. This

Table 1
Comparison of analytical characteristics of amperometric AChE biosensors for malathion with the present biosensor.

Electrode	Incubation time (min)	Linearity (nM)	Detection limit (nM)	Reference
AchE/chitosan/Au	10	0.3–15	0.15	[17]
AchE/AuNPs–$CaCO_3$/silica sol–gel/Au	10	0.1–100	0.1	[29]
AchE/ZnS/Pin5COOH/Au	10	0.1–50	0.1	[36]
AchE/Fe_3O_4 NPs/c-MWCNT/ITO	10	0.1–40	0.1	[37]
AchE/ZrO_2/chitosan/GC	15	10–59	5	[41]
AchE/sol–gel/SPE	10	10–500	3.6	[42]
AchE/MWCNT/CA/NPG	12	3–150	1.5	Present

phenomenon was also observed by others [34,35]. With increasing the malathion concentration, the produced current of ATCl on the AChE-MWCNT-CA-NPG electrode decreases (Fig. 7A). Under the optimal experimental conditions, the inhibition of malathion on the AChE-MWCNT-CA-NPG electrode is proportional to its concentration in the range of 0.001–0.5 μg mL^{-1} with a correlation coefficient of 0.998 (Fig. 7B). The detection limit is 0.5 ng mL^{-1} taken as the concentration equivalent to a 10% decrease in signal. Recently, a number of AChE biosensors have been developed for malathion. However, the detection limits were calculated in different ways [29,36,37]. Our reported value is much lower or comparable to those in the references [30,38]. The inhibition rate of malathion decreased when increasing the concentration of malathion, indicating that the binding interactions between the pesticide and the enzyme could reach an equilibrium. In the presence of pesticide at high concentrations, the inhibition percentage change is not so pronounced [39]. So, two linear ranges on the calibration curve were observed. This phenomenon was also observed by others [40].

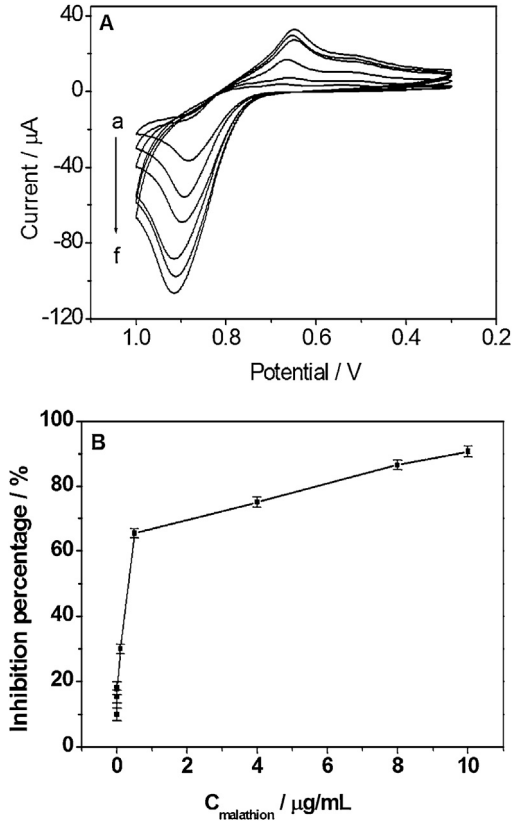

Fig. 7. (A) Cyclic voltammograms of AChE-MWCNT-CA-NPG in pH 7.0 PBS containing 0.2 mM ATCl after immersed in (A) malathion solution with different concentrations of (a) 0, (b) 0.001, (c) 0.01, (d) 0.1, (e) 0.3 and (f) 0.5 μg mL^{-1}. (B) Linear relationships between the inhibition percentage and malathion concentration . Error bars represent 1 standard deviation from three measurements.

So far, carbon nanotube-based nanocomposites have been widely used for acetylcholinesterase sensors. Unlike previous reports, shortened CNTs can be aligned to an electrode by self-assembly and be used to enhance the electron-transfer reaction with electroactive species [14]. The performance of this AChE-MWCNT-CA-NPG compared with other reported AChE biosensors for malathion is summarized in Table 1. In our previous research, we reported an electrochemical sensor based on enzyme-induced growth of AuNPs [17]. The $[Fe(CN)_6]^{3-/4-}$ redox system needs to be added and used as an additional reporter. Moreover, the method can only be used for a system including a reductant. Compared with that sensor, the present sensing platform is more general and can avoid the addition of other signal reporters. Since the oxidized form of malathion shows a stronger inhibitory effect toward AChE than unoxidized, an oxidative treatment of malathion can improve the detection limit further [43,44].

3.7. Reproducibility and stability of the AChE-MWCNT-CA-NPG electrode

The inter-assay precision was estimated by determining the responses of 0.2 mM ATCl at five different electrodes. The enzyme electrodes were immersed in 0.1 μg mL^{-1} and 2.0 μg mL^{-1} malathion for 12 min, respectively. The coefficients of variation were calculated to be 4.6% and 2.7%, respectively. The intra-assay precision of the sensors was evaluated by assaying one enzyme electrode for five replicate measurements, and the RSD was 3.9% at the ATCl concentration of 0.2 mM.

When the enzyme electrode was not in use, it was stored at 4 °C in dry conditions. No obvious decrease in the response to ATCl was observed for the first 5-day storage. After two weeks, the sensor retains 85% of initial current response to ATCl. For practical applications, the interferences such as heavy metal ions should be taken into consideration. Since the AChE-based biosensor can be commonly used for the detection of most organophosphorous pesticides, the selectivity can be further improved by using organophosphorus hydrolase. Moreover, various mediators can be employed to lower the oxidation potential.

4. Conclusions

In summary, we have shown that multiwall carbon nanotubes (MWCNTs) can be linked to a nanoporous gold electrode by using the self-assembly technique. The MWCNTs can enhance the electron-transfer reaction with electroactive species generated from enzymatic reactions. In addition, many enzymes could be assembled on MWCNTs and used to amplify the detection signals. The immobilized AChE, as an enzyme model, shows excellent activity to its substrate and allows a quantitative measurement of organophosphate pesticide. By combining the advantages of nanoporous gold with unique electrochemical properties of CNTs, we believe that such a constructed interface has potential applications for fabrication of amperometric biosensors.

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