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Gold Nanoparticles/Chitosan Dual-Layer Membranes Modified Acetylcholinesterase Biosensor

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Abstract: In this paper, a novel acetylcholinesterase (AChE) biosensor for organophosphorus (OP) pesticides detection was constructed by modifying glassy carbon electrode (GCE) with gold nanoparticles/chitosan dual-layer membranes. The dual-layer membranes were included in chitosan membrane coated with physically bound AChE to recognize pesticides selectively and gold nanoparticles (GNPs) membrane directly deposited on the surface of GCE to enhance electron transfer rate. During the detection, the chitosan membrane was quickly fixed on the surface of GNPs/GCE with O-ring. Since GNPs process high electron-transfer ability, moreover, chitosan membrane can provides a microenvironment around the enzyme molecule to stabilize its biological activity and prevents the enzyme leaking out effectively. The combination of GNPs and chitosan dual-layer membranes promoted electron transfer and catalyzed the electro-oxidation of thiocholine, thus amplified the detection sensitivity. Using dichlorvos omethoate, trichlorfon and phoxim as model compounds, the inhibition rates of these pesticides were proportional to their concentrations in the range of 0.05~10 μg/l, 0.1~50 μg/l, 0.1~50 μg/l, and 0.2~50 μg/l with a detection limit of 30 ng/l for dichlorvos, 80 ng/l for omethoate, 60 ng/l for trichlorfon and 100 ng/l for phoxim respectively. The biosensor exhibited high sensitivity, good reproducibility and stability, and it was suitable for trace detection of OP pesticide residue. *Copyright © 2012 IFSA.*

Keywords: Biosensor, Gold nanoparticles, Acetylcholinesterase, Pesticides residues.

1. Introduction

Organophosphorus (OP) pesticides have been used extensively in agriculture for pest control. OP pesticides are highly toxic and their accumulation in living organisms can be a cause of serious diseases

[1-3]. Therefore, rapid determination and reliable quantification of trace level of OP pesticides have become increasingly important for human health protection. Traditional analytical methods, such as gas and liquid chromatography, are sensitive, reliable and precise. These methods, however, require expensive instrumentation, complicated pretreatment procedure and professional operators, and are not applicable for on-site pesticides determination [4-5]. Acetylcholinesterase (AChE) based biosensors have emerged the past few years as the most promising alternative for direct detecting of pesticides [6-7], which is commonly based on quantification of the inhibition of enzyme activity in the presence of pesticides [8]. When AChE is immobilized on the working electrode surface, its interaction with the substrate of acetylthiocholine (ATCh) can produce the electroactive product of thiocholine. Thus, the concentrations of the pesticides can be monitored by measuring the change of the oxidation current of thiocholine, and this change is the result of the inhibition of enzyme activity after exposure to the pesticides. However, the oxidation generally requires high potential values in a suitable electrode [9], which easily lead to the electrochemical interference of other oxidable compounds. Therefore, many reports have focused on electrode modified method to realize oxidable reaction at a lower potential.

Gold nanoparticles (GNPs) acting as electron transfer "wires" have widespread used for constructing electrochemical biosensors through their high electron-transfer ability between enzyme active centers and electrode surface, therefore, GNPs modified electrode for detecting OP pesticides have been reported in many literatures[1, 4-5, 8, 10-17]. These reports have shown satisfactory results for GNPs employing as electron transfer "wires" to amplify the detection sensitivity. However, most of these methods rely on enzyme immobilization directly onto the electrode surface. It cannot overcome the biofouling of the electrode surface, which would eventually lead to the deactivation of the biosensor or at least to worsening of the electrochemical response [18-20]. Our previous investigation results have shown that a replaceable membrane as supports to immobilize enzyme have many advantages [21-22]. For example, enzyme membrane can be easily replaced when enzyme's activity is lost. Moreover, there are multiple options for analyte detection based on enzyme immobilization on the membrane [23].

Chitosan contains large groups of $-NH_2$ and -OH which is preferable to maintain the high biological activity of the immobilized biomolecules. In addition, chitosan also has the properties of nontoxicity and low cost. Therefore, it is widely used as carrier material for enzyme immobilization [10-11, 13, 24-25].

As mentioned above, using a replaceable chitosan membrane as carrier material can provide a favorable microenvironment, load a large amount of enzyme and prolong the enzyme storage life, and simply operation. Using GNPs membrane to modify the GCE can improve electrocatalytic ability and enhance electron transfer. Based on these considerations, we introduced a novel gold nanoparticles/chitosan dual-layer membranes AChE biosensor. To the best of our knowledge, this kind of biosensor has not yet been reported. The aim of this work was to develop a fast, simple, inexpensive, stable and highly sensitive AChE biosensor for OP pesticides. The experimental conditions related to the performance of the fabricated biosensor (pH, substrate concentration, inhibition time) were investigated in detail.

2. Materials and Methods

2.1. Apparatus

Electrochemical measurements were performed with CHI660D electrochemical workstation (Shanghai Chenhua Co., China). The working electrode was glassy carbon electrode (d=3mm) or modified glassy carbon electrode. A saturated calomel electrode (SCE) and platinum electrode were used as reference and auxiliary electrodes, respectively.

2.2. Reagents

Acetylcholinesterase was purchased from Nuoyawei Biology Tech. Co. (Shanghai, China). Acetylthiocholine iodide (ATChI), glutaraldehyde (25%) and bovine serum albumin (BSA) were provided by Sigma. Pesticides were the product of Dacheng Pesticide (Shandong, China). Cellulose nitrate microporous membrane was purchased from Hangzhou Rikang purification equipment Co. (Hangzhou, China). Chitosan (95 % deacetylation), phosphate buffer (PBS, pH 8.0) and other reagents were of analytical grade.

2.3. Preparation of AChE Biosensor

2.3.1. Preparation of Chitosan Membrane

A solution was prepared with 0.1 g chitosan added to 10 ml of acetate solution (1 %, mass ratio), and the mixture was centrifuged for 5 min in high-speed centrifuge at 3000 rpm to remove insoluble particles. Finally, the pretreated cellulose nitrate microporous membrane was immersed in this sol for 12 h, and then immersed in phosphate buffer solution (PBS, 0.1 mol/l, pH 8.0) for 12 h, dried and stored for use [26].

2.3.2. The AChE Immobilization

A solution of 100 μl of AChE liquid (100 U/ml), 30.0 μl of BSA (1.0 %), 10 μl of glutaraldehyde (5.0 %), and 360 μl of phosphate buffer (PBS, 0.1 mol/l, Ph 8.0) were mixed in a 1 ml of centrifuge tube. A chitosan membrane was immersed in it for 8 h at 4 ^{o}C. Finally, enzyme membranes was washed with PBS (0.1 mol/l, pH 8.0), immersed in PBS (0.1 mol/l, pH 8.0), and stored at 4 ^{o}C before use [27].

2.3.3. Electrode Modification with GNPs

0.01 % $HAuCl_4$ solution was heated to boiling, and quickly added 1 ml 1 % sodium citrate. After about 1min, the color of solution changed from yellowish to light rose red. The working electrode was immersed in 10 ml of GNPs solutions for 24 h at 4 °C, after that the surface of working electrode was rinsed with double-distilled water for use [1].

2.3.4. Preparation of AChE-GNPs/GCE

The AChE was immobilized on the chitosan membrane to prepare enzyme membrane, and GNPs was deposited on the surface of GCE to prepare GNPs/GCE. The enzyme membrane was fixed on the surface of GNPs/GCE with O-ring during electrochemical detection, thus the AChE-GNPs/GCE biosensor was obtained.

2.4. Electrochemical Detection of Pesticide

The AChE-GNPs/GCE was tested with amperometric i-t curve (i-t) at a potential of +600 mV (versus SCE). After 100 μl of ATChI (15 mg/ml) solution was injected into the cell, and the peak current was recorded as I_0. The cell was washed with distilled water between measurements.

For the measurement of pesticides, the pretreated AChE-GNPs/GCE was first incubated in a given concentrations of pesticide for 10 min. Then it was transferred to PBS (0.1 mol/L, pH 8.0), and 100 μl of ATChI was injected after the current stabilized. The peak current was recorded as I_1. The inhibition of pesticides was calculated as follows:

$$I\% = \left(I_0 - I_1\right) / I_0 \times 100\%, \tag{1}$$

where, I_0 was the initial peak current of ATChI at the AChE-GNPs/GCE biosensor. I_1 was the peak current of ATChI at the AChE-GNPs/GCE biosensor with pesticide inhibition.

3. Results

3.1. Electrochemical behavior of AChE-GNPs /GCE

Fig. 1 showed the cyclic voltammograms of AChE/GCE and AChE-GNPs/GCE in the presence and absence of 100 μl of ATChI (15 mg/ml) in PBS (pH 8.0) at a scan rate of 100 mV/s. No peak was observed at GCE (curve a) and AChE-GNPs/GCE (curve b) in PBS. After 100 μl of ATChI (15 mg/ml) was injected into PBS, AChE-GNPs/GCE was found an oxidation peak at 510 mV (curve d), and the non-modified AChE/GCE also appeared an oxidation peak at 900 mV (curve c). Compared with the oxidation peak current of ATChI at AChE/GCE, one can see that the electrochemical response at AChE-GNPs/GCE was dramatically enhanced, with the oxidation overpotential reduced by 390 mV. The oxidation overpotential was lower than the literature reports, such as those of 796 mV at AChE-Au-PPy/GCE [5], those of 700 mV at AChE-AuNPs-SF/Pt [4] and those of 600 mV at AChE-CdTe–GNPs-Chitosan-GCE [13]. The oxidation peak current was comparable with that reported GNPs modified AChE biosensor [4, 11, 19]. This could be attributed to the combined effect of gold nanoparticles/ chitosan dual-layer membranes. One reason was the presence of GNPs in the surface of GCE, which possessed the huge specific surface area and inherent high electricity conducting ability, thus can provide a conductive pathway for electron transfer and promote electron transfer reactions at a lower potential. The other reason was use the chitosan membrane, which provided a microenvironment around the enzyme molecule to stabilize its biological activity and prevented the enzyme leaking out from chitosan membrane effectively. Dual-layer membranes had synergistic effects towards enzymatic catalysis, thus, the AChE-GNPs/GCE biosensor obtain higher oxidation peak current compared with previously reported at a lower potential.

3.2. Effect of Phosphate Buffer pH on AChE-GNPs/GCE

Fig. 2a showed that the current response increased with an increase of pH value up to 8.0, and then it decreased at higher pH value. Obviously, the maximum response of peak current appeared at pH 8.0. The phenomena was because that the pH value of electrolyte had great influence on the activity of enzyme, which led to the change of the anodic peak current at AChE-GNPs/GCE. Thus, the optimum pH value of 8.0 was used in the subsequent experiment.

3.3. Effect of ATChI Concentration on AChE-GNPs /GCE

Fig. 2.b showed the effect of different ATChI concentration on anodic peak current of AChE-GNPs/GCE. The peak current increase when the ATChI concentration was less than 15 mg/l, Whereas the peak current have no change with further the increasing of the concentration of ATChI. It was likely because that the velocity of enzyme catalyzing substrate reaches to the equilibrium when the

substrate added to some concentration, so subsequent increased the substrate concentration, the velocity of enzyme catalyzing substrate did not increase. In this work, the ATChI concentration of 15 mg/l was selected.

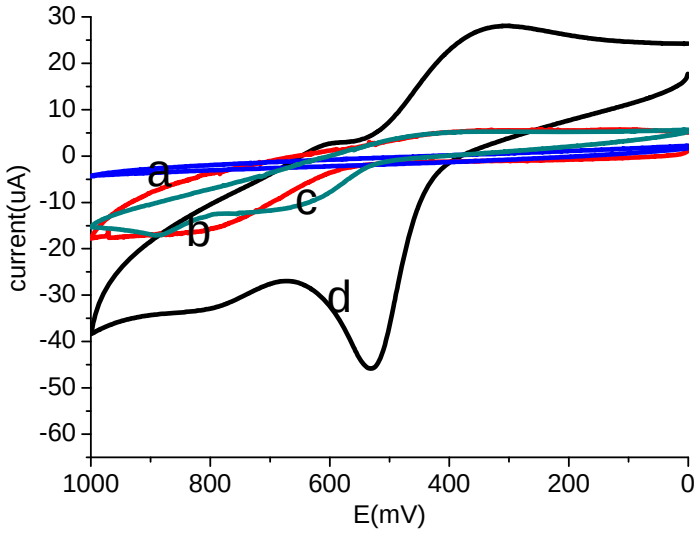

Fig. 1. Cyclic voltammograms.GCE(a) and GNPs/GCE in pH 8.0 PBS (b); AChE /GCE (c) and AChE-GNPs/GCE (d) in pH 8.0 PBS containing 100 μL of ATChI (15 mg/mL). Scan rate: 100 mV/s.

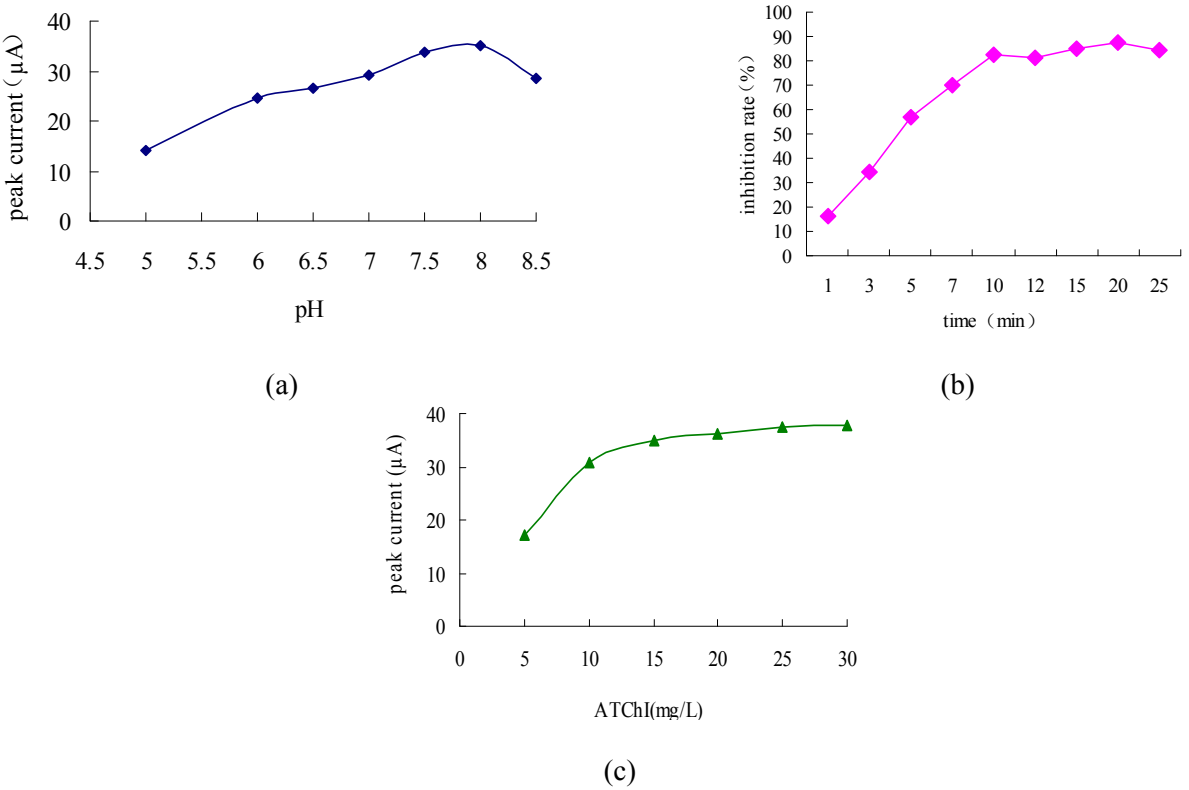

Fig. 2. Effect of phosphate buffer pH on AChE-GNPs/GCE (a), effect of ATChI concentration on AChE-GNPs/GCE (b) and effect of incubation time on the response of ATChI after the AChE-GNPs/GCE (c).

3.4. Effect of Incubation Time on Inhibition

Fig. 2c showed that the peak current of ATChI on the AChE-GNPs/GCE decreased greatly with an increase of incubated time in the OP pesticides solution, OP pesticides displayed increasing inhibition to AChE with incubated time. When the incubated time was longer than 10 min, the curve trended to maintain a stable value, which indicated that the binding interaction with active target groups in enzyme could reach saturation. This change tendency of the peak current value showed the alteration of enzymatic activity, which resulted in the change of the interactions with its substrate. In this work, the optimum incubation time of 10 min was selected.

3.5. Determination of Pesticides

As shown in Fig. 3, when substrate was injected to PBS, an anodic current at potentials of 600 mV was observed (a-g). AChE-GNPs/GCE was incubated in the standard solution of omethoate at a certain concentration for 10 min respectively, the current decreased drastically (curve b-f) compared with no inhibitor (curve a). The response current of decreased to 38.5 % after inhibition with 0.5 μg/l of omethoate. At higher omethoate concentration (50 μg/l), the current decreased up to 89.7 %. However, the maximum value of inhibition rate was not 100 %, which was likely to attribute to the binding equilibrium between pesticides and binding sites in enzyme [28].

The inhibition rate of AChE activity and the logarithm of omethoate concentration had a certain linear relationship in the range from 100 ng/l to 50 μg/L [29], with the correlation coefficient of 0.9963 (Fig. 3 insert). The detection limit was 80 ng/l.

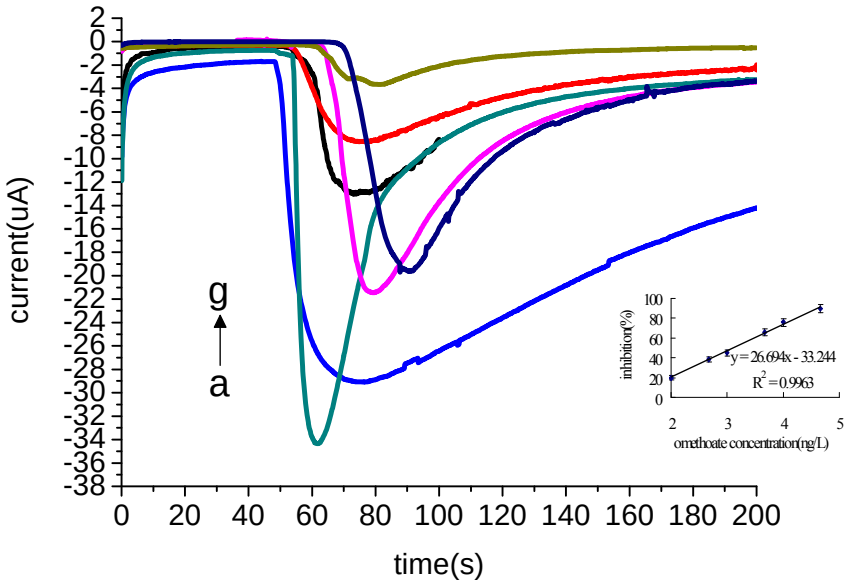

Fig. 3. Amperometric i-t curve of AChE-GNPs/GCE in PBS(0.1 mol/L, pH 8.0) injected 100 μL of ATChI (15 mg/mL), after incubation in 0 ng/L (a), 100 ng/L(b), 500 ng/L(c), 1 μg/L(d), 5 μg/L(e),10 μg/L(f) and 50 μg/L(g) omethoate solution with 10 min.

The detection of dichlorvos, trichlorfon and phoxim with the AChE-GNPs/GCE were shown in Table 1. The results showed that the inhibition rate of dichlorvos, trichlorfon, and phoxim were proportional to their concentrations in the range of 0.05~10 μg/l, 0.1~50 μg/l, and 0.2~50 μg/l respectively. The detection limits were found to be 30 ng/l for dichlorvos, 60 ng/l for trichlorfon, and 100 ng/l for phoxim, respectively. The detection limit obtained is comparable with that reported so far with an enzyme-based

inhibitor electrochemical sensor [10, 14], and it is significantly lower than those of 2 ng/mL at GCE by using Au-polypyrrole nanocomposite modified electrochemical biosensor [5], and also lower than 0.6 ng/mL at the gold nanoparticles embedded in sol-gel film AChE biosensor [12], indicating that the proposed gold nanoparticles/ chitosan dual-layer membranes biosensor is reliable for the determination of OP pesticides.

Table 1. Detection of four kinds of pesticides with the AChE-AuNPs/GCE.

Pesticides	Linear Range	Equation of Linear Regression	Correlation Coefficient	Detection Limit
dichlorvos	50 ng/l~10 μg/l	I=22.804 lgc-6.3489	0.9928	30 ng/l
omethoate	100 ng/l-50 μg/l	I=26.694 lgc-33.244	0.9963	80 ng/l
trichlorfon	100 ng/l~50 μg/l	I=20.566 gc-4.5166	0.9918	60 ng/l
phoxim	200 ng/l~50 μg/l	I=19.684 lgc-3.0872	0.9899	100 ng/l

3.6. Reactivation of the Biosensor

The AChE inhibited by OP pesticides can be reactivated by immersion in PBS (0.1 mol/L, pH 8.0). The experiment results showed that AChE modified electrode inhibited by pesticides sample solution can resume 97.1 % original activity after immersion in PBS (0.1 mol/L, pH 8.0) for 20 min (See supporting information, Fig. S2).

The experimental results also indicated that PBS itself play a role as a reagent of reactivation of AChE, which had a good reactivation effect with immersion in PBS (0.1 mol/L, pH 8.0) for sufficient time. Compared with using TMB-4 or 2-PAM as reagent of reactivation previously reported [30-31], this method was simple and reliable.

3.7. Precision of Measurements and Stability of Biosensor

The precision intra-assay of the biosensors was evaluated by assaying three enzyme membranes on the same electrode for ten replicate determinations after exposure to a certain concentration pesticides respectively. Similarly, the inter-assay precision was estimated by assaying three enzyme membranes on six different electrodes. The average relative standard deviation (R.S.D.) of intra-assay and inter-assay were found to be 5.58 and 3.1 %, respectively, indicating acceptable reproducibility.

The stability of the enzyme electrode could be maintained by being stored at 4 ^{o}C in PBS (0.1 mol/L, pH 8.0). No obvious decrease in the response of ATChI was observed in the first 10-day storage. After a 15-day storage period, the sensor retained 83% of its initial current response. The responses of the sensors had decreased with the time of storage. Specifically, after a 25 days storage period, the sensor lost 35% of its initial current response. It indicated the sensors have good stability.

4. Conclusions

We have successfully used GNPs/chitosan dual-layer membranes to fabricate an amperometric AChE biosensor. AChE is immobilized on the chitosan membrane, which provides a microenvironment around the enzyme molecule to stabilize its biological activity and prevents the enzyme leaking out effectively. Moreover, GNPs are deposited on the surface of GCE, which can provide a conductive pathway for electron transfer and promote electron transfer reactions at a lower potential. The dual-layer modified

membranes exhibit a significant synergistic effect towards enzymatic catalysis. Therefore, this biosensor have obtained significantly higher sensitivity and a lower detection limit, it is a promising tool for the trace detection of OP compounds.

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