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Acetylcholinesterase biosensor based on prussian blue-modified electrode for detecting organophosphorous pesticides

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ABSTRACT

In this study, a novel acetylcholinesterase (AChE) biosensor was developed based on dual-layer membranes (chitosan membrane and prussian blue membrane) modifying glassy carbon electrode (GCE). A chitosan membrane was used for immobilizing AChE through glutaraldehyde cross-linking attachment to recognize pesticides selectively. A prussian blue (PB) membrane was electrodeposited on the surface of GCE to enhance electron transfer. Before the detection, the chitosan enzyme membrane was quickly fixed on the surface of PB/GCE with O-ring to prepare an amperometric AChE-PB/GCE sensor for organophosphorus (OP) pesticides. The electrochemical behaviour of AChE-PB/GCE was studied, and the results showed that the chitosan membrane as carrier can absorb a large amount of enzyme, and PB has a significant synergistic effect towards enzymatic catalysis. As a result of these two important enhancement factors, the proposed biosensor exhibited extreme sensitivity to OP pesticides compared to the other kinds of AChE biosensor. The influences of phosphate buffer pH, substrate concentration, incubation time of pesticide on the response of the fabricated biosensor were investigated. Under optimum conditions, the inhibition rates of these pesticides were proportional to their concentrations in the range of 0.01–10 $\mu g\,l^{-1}$, 0.05–10 $\mu g\,l^{-1}$, 0.03–5 $\mu g\,l^{-1}$, and 0.05–10 $\mu g\,l^{-1}$, respectively. The detection limits were found to be 2.5 $ng\,l^{-1}$ for dichlorvos, 15 $ng\,l^{-1}$ for omethoate, 5 $ng\,l^{-1}$ for trichlorfon and 10 $ng\,l^{-1}$ for phoxim. Moreover, the biosensor exhibited good reproducibility and stability, and it was suitable for trace detection of OP pesticide residue.

1. Introduction

Organophosphorus (OP) compounds are harmful to the health of human beings, therefore, the development of fast and sensitive detection methods become more and more important. Recently biosensor techniques based on the inhibition of acetylcholinesterase (AChE) activity have gained considerable attention due to the advantages of simplicity, rapidity, reliability and low cost devices (Tanimoto de Albuquerque and Ferreira, 2007; Ramírez et al., 2008). Several types of AChE-based amperometric, potentiometric, or optical biosensors have been described (Mulchandani et al., 1999; Viveros et al., 2006). For amperometric AChE-based biosensor, the inhibition of enzyme activity is monitored by the change of oxidation current of the thiocholine (TCh) at a certain potential. (Liu et al., 2006; Du et al., 2007a,b; Schulze et al., 2003). The reaction equation is shown as follows (Wu et al., 2009):

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid} \quad (1)$$

$$2\text{thiocholine} \rightarrow \text{dithio-bis-choline} + 2H^+ + 2e^- \quad (2)$$

Improvements on amperometric biosensor have mainly focused on two aspects. One is to improve electrocatalytic ability and enhance electron transfer through the modification of working electrode at a lower potential. A variety of modification methods have been employed, such as cobalt phthalocyanine (Parham and Rahbar, 2010), multi-walled carbon nanotubes (MWNTs) (Du et al., 2010; Tsai et al., 2008; Sun et al., 2010), 7,7,8,8-Tetracyanoquinodimethane (TCNQ) (Bucur et al., 2006) and prussian blue (PB) (Shulga and Kirchhoff, 2007; Suprun et al., 2005; Arduini et al., 2006; Wu et al., 2009). Among these methods, prussian blue has attracted enormous interest for the fabrication of electrochemical biosensors that possess conductive sensing interface, catalytic properties and conductivity properties (Suprun et al., 2005; Arduini et al., 2006; Wu et al., 2009)

The other technique is to improve the efficiency of enzyme immobilization (Gao et al., 2005; Sun et al., 2008a,b). Commonly, enzymes were immobilized on electrode surfaces directly (Du et al., 2008; Du et al., 2007a,b; Yin et al., 2009), which has many disadvantages, such as weak protection of the immobilized enzyme and elaborate pretreatment of the electrode surface before the actual immobilization. In contrast, by immobilizing enzyme on a membrane, the enzyme membrane can be easily replaced when enzyme's activity is lost. Moreover, there are multiple options for

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analyte detection based on the choice of enzyme to immobilize on the membrane (one electrode–multiple membranes–multiple enzymes) (Marinov et al., 2009; Ivanov et al., 2010; Shimomura et al., 2009).

Chitosan as membrane material contains large groups of -NH2 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 (Du et al., 2008; Gong et al., 2009).

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 it is a simple operation to replace enzyme membrane, rather than immobilize enzyme on a new electrode when enzyme's activity lost. Using PB membrane to modify the glassy carbon electrode (GCE) can improve electrocatalytic ability and enhance electron transfer, therefore, can obtain high amperometric signals with a corresponding low detection limit. Based on these consideration, we introduced a novel dual-layer membrane (chitosan membrane and PB modified membrane) modified 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. Four kinds of OP pesticides were then analyzed with this biosensor demonstrating the high sensitivity and stability of this method.

2. Experimental

2.1. Apparatus

Cyclic voltammograms and amperometric *i–t* curve were performed with CHI660D electrochemical workstation (Shanghai Chenhua Co., China). The working electrode was glassy carbon electrode ($d = 3$ mm) 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. Cellulose nitrate microporous membrane with an aperture of 0.45 μm was purchased from Hangzhou Rikang Purification Equipment Co. (Hangzhou, China). Pesticides were standard products. Chitosan (95% deacetylation), phosphate buffer (PBS, pH 8.0) and other reagents were of analytical grade.

2.3. Preparation of PB modified 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 (PBS, 0.1 mol l^{-1}, pH 8.0) for 12 h, dried and stored for use (Qiang et al., 2007).

2.3.2. The AChE immobilization

A solution of 100 μl of AChE liquid (100 U ml^{-1}), 30.0 μl of BSA (1.0%), 10 μl of glutaraldehyde (5.0%), and 360 μl of PBS (0.1 mol l^{-1}, pH 8.0) were mixed in a 1 ml of centrifuge tube. A chitosan membrane was immersed in it for 8 h at 4 °C. Finally, enzyme membrane was washed with PBS (0.1 mol l^{-1}, pH 8.0), immersed in PBS (0.1 mol l^{-1}, pH 8.0), and stored at 4 °C before use (Sun et al., 2008a,b).

2.3.3. Electrode modification with PB

The A solution was a mixture of 2 mM $K_3[Fe(CN)_6]$, 2 mM $FeCl_3$, 0.1 M KCl, and 10 mM HCl, and the B solution was a mixture of 0.1 M KCl and 10 mM HCl. First, a potential of +0.4 V was applied to the electrode in solution A for 60 s and then the electrode was transferred to the B solution, and scanned by cyclic voltammetry from −0.05 and 0.35 V at a rate of 50 mV s^{-1} for 12 times. The electrode surface was rinsed with double-distilled water. Finally, the electrode was stored at room temperature (Jin and Hu, 2008).

2.3.4. Preparation of AChE-PB/GCE

Before the detection, the chitosan enzyme membrane was quickly fixed on the surface of PB/GCE with O-ring to prepare an amperometric AChE-PB/GCE sensor for OP pesticides (See supporting information, Fig. S1)

2.4. Amperometric analysis of pesticides

The AChE-PB/GCE biosensor was tested by amperometric *i–t* curve (*i–t*) at a potential of 600 mV (versus SCE). After 100 μl of ATChI (15 mg ml^{-1}) solution was injected into the cell, and the peak current was recorded as I_0. The cell was washed with double-distilled water between measurements.

For OP pesticide detection, the AChE-PB/GCE was incubated in a given concentrations of pesticide for 10 min. Then it was transferred to PBS (0.1 mol l^{-1}, pH 8.0), and 100 μl of ATChI was injected. The peak current was recorded as I_1. The inhibition rate of pesticides was calculated as follows:

$$I\% = \frac{(I_0 - I_1)}{I_0} \times 100\%$$

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

3. Results and discussion

3.1. Electrochemical behaviour of AChE-PB/GCE

Fig. 1 showed the cyclic voltammograms of AChE/GCE and AChE-PB/GCE in the presence and absence of 100 μl of ATChI (15 mg ml^{-1}) in PBS (pH 8.0) at a scan rate of 100 mV s^{-1}. No peak was observed at GCE (curve *a*) and AChE-PB/GCE (curve *b*) in PBS. After 100 μl of ATChI was injected into PBS, the cyclic voltammogram of AChE-PB/GCE identified an oxidation peak at 570 mV (curve *d*), and the non-modified AChE/GCE also appeared an oxidation peak at 900 mV (curve *c*). The oxidation peak (curve *c* and *d*) came from the oxidation of thiocholine, hydrolysis product of ATChI, catalyzed by immobilized AChE. Fig. 1 also showed that this peak current (curve *d*) had a sharp increase and the peak potential shifted negatively compared to those on the electrode without PB (AChE/GCE) (curve *c*). One reason was the presence of PB on the surface of GCE, which possessed a relatively large specific surface area and an inherent, high electricity conducting ability, thus they could enhance the rates of catalyzed reactions as well as the electron transfer rate at a lower potential, which was comparable with that reported electrochemical (Arduini et al., 2006; Wu et al., 2009). The other