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Detection of Carbofuran Pesticide Using an Enzyme Biosensor Based on MWNTs-PDDA Modified Glassy Carbon Electrode

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Abstract

This paper presents the construction of an amperometric biosensor for the highly sensitive detection of the carbamate insecticide carbofuran, based on the inhibition of acetylcholinesterase (AChE). Using an enzyme biosensor based on multi-walled carbon nanotubes (MWNTs) and poly diallyldimethylammonium chloride (PDDA) modified glassy carbon electrode (GCE), then immobilizing the Acetylcholinesterase (AChE) to the modified GCE with layer-by-layer (LBL) technique directly. Detect the inhibition of Aches with the amperometric i-t response. The pesticide concentration is correlated to the percentage inhibition of enzyme activity. Then detect the carbofuran by the enzyme electrode with 0.01U activity value and it shows a good linearity in the range from 10^{-12} g/L to 10^{-6} g/L between the inhibition rates and -lg[carbofuran], and the detection limit is 10^{-12} g/L.

Keywords: Enzyme Biosensor; AChE; Layer-by-layer (LBL); Carbofuran

1 INTRODUCTION

Carbamate pesticides are widely used in agriculture and it is a kind of toxic compounds which is bad for human health [1]. Despite of this fact, several carbamates are highly toxic compounds to humans and other mammals. The mode of action of these pesticides in vertebrates and insects is based on inhibition of the activity of AChE enzyme in the hydrolysis of the neurotransmitter acetylcholine, which is responsible for the transmission of nervous impulses. Thus, there is a growing interest in their fast and accurate identification and quantification [2–4].

The determination of carbamates is usually carried out by gas (GC) or high performance liquid chromatography (HPLC) [5-6], these methods require expensive instrumentation and highly trained personnel. They are, moreover, time consuming and not easily adapted to field analysis.

In order to address these problems, biosensors based on inhibition of the enzyme AChE have been developed [7–10]. It is well known that carbamate pesticides quantitatively inhibit cholinesterase. For this reason, amperometric biosensors based on inhibition of AChE activity have been extensively applied to rapid, simple, and selective analysis of pesticides.

AChE is a serine esterase that is attached to postsynaptic membranes[11]. AChE rapidly converts the neurotransmitter acetylcholine (ACh) to thiocholine (TCh) and acetate after transmission of a nerve impulse. TCh is then captured and recycled by the presynaptic terminal through the high-affinity thiocholine uptake transporter protein. However, carbamate compounds used as pesticides and nerve gas agents (e.g., sarin) are known to inhibit AChE. The decrease in biosensor response is correlated with the amount of pesticide present in the sample and the time of incubation, which has been described in some research reports [12–14].

A controllable modification technique of GCE with MWNTs[15–16] is described in this paper, what's more, immobilizing the Acetylcholinesterase (AChE) to the modified GCE technique is introduced. A new technology of layer-by-layer (LBL) self-assembly modification with immobilized AChE for the determination of Dichlorvos

pesticides is described, then using the amperometric i-t response to detect the inhibition of AChE. This approach produces a simple electrochemical sensor, which can be used for sensitive monitoring of inhibitors of AChE in solution via detection of thiocholine after enzymatic hydrolysis of TCh.

2 EXPERIMENTAL

2.1 Reagents and Instruments

Acetylthiocholine chloride (ATCh), acetylcholinesterase (AChE, 317 U/mg) were obtained from sigma; Poly (diallyldimethylammonium chloride) polymer (PDDA, M_W:100,000-200,000) was obtained from Sigma-Aldrich; MWNTs were obtained from Shenzhen Nanotech Port Co. Ltd., with>95% purity; 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) was obtained from Junchuang bio-technique Co. Ltd. (China). All chemical reagents were of analytical reagent grade and used without further purification. All solutions were prepared with double distilled water.

A three-electrode configuration was used. The glass-carbon electrode (GCE) was the working electrode, the platinum wire was the auxiliary electrode and the saturated calomel electrode was the reference electrode. Using the electrochemical analyzer CHI800 (Chenhua Instruments) to study the Amperometric and cyclic voltammetric measurements, and all the electrochemical experiments were conducted at 30℃.

2.2 Preparation of Thiocholine

The thiocholine (TCh) solution was prepared by the enzymatic reaction of AChE and acetylthiocholine chloride solution for 30 minutes in 0.10 M phosphate buffer (pH 7.4). The dissolved oxygen in stock solution was removed by bubbling with high-purity nitrogen for 15min. Detecting the finial thiocholine concentration with spectrophotometry. The standard solutions of thiocholine for the voltammetric and amperometric studies were prepared by dilution of this stock solution.

2.3 Modification of the Electrode and Immobilization of AChE

1) Surface-cleaning of GCE

Pretreatments should be done before the modification. First, polishing the glassy carbon electrode with 0.3 and 0.05 μm alumina slurries, then rinsing it with double-distilled water, at last immersing the electrode in alcohol, acetone and concentrated HNO_3 (1:1) separately with the aid of ultrasonic agitation to obtain a clean surface. The effect of the preprocessing of glass-carbon electrode was tested by cyclic voltammetric in 1mM $[Fe(CN)_6]^{3+}$ solution.

2) Preparation of MWNTs

Refluxing the Multi-walled carbon nanotubes (MWNTs, 0.10 g) in 150mL concentrated HNO_3 for 24 hours, aimed at shortening and functionalizing the MWNTs, then extensive washing them in double-distilled water until the filtrate was neutral. After that, dry them in vacuum. At last disperse the resulting MWNTs in double-distilled water under the ultrasonic agitation for 30 minutes with a concentration of 0.1mg/mL to obtain a uniform, stable black solution.

3) Electro-deposition and LBL Assembly of MWNTs onto GCE

Under the deposition potential 1.7V, the electrochemical deposition of MWNTs on the GCE was carried with the magnetic stirring for 120 minutes. Then dipping the modified electrode into borate buffer solution (pH 9.18) for 15 minutes to make the functional groups were negatively charged. Afterwards, the GCE was immersed in 1 (wt) % PDDA solution (adjust the ion intensity to 0.8M mol/L with NaCl) and then dispersed the concentration of 1mg/mL MWNTs in pH 9.18 borate buffer solution for 15 minutes either, which is aimed at fabricating the self-assembled bilayer membranes of PDDA/MWNTs. After each dipping step, wash the membranes carefully with double-distilled water. Repeat this sequence until obtain the desired PDDA/MWNTs bilayer number. These modified electrode was stored at 4℃ in PBS before use.

4) Self-Assembly of AChE onto {MWNTs/PDDA}$_5$/GCE

This modified electrode was dipped into 1(wt) % PDDA solution and AChE solution for 15 min alternately after preparing{MWNTs/PDDA}$_5$/GCE. After each dipping step, wash the modified electrode carefully with double-distilled water. Repeat this sequence until obtain the desired AChE/PDDA bilayer number. An AChE membrane was fabricated at the last surface aimed at getting the most activity of the enzyme electrode.

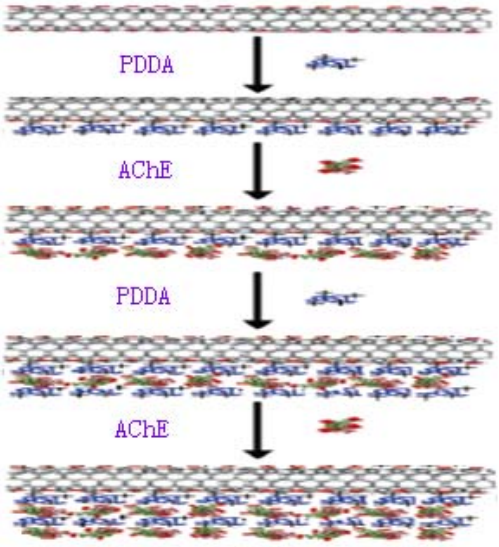

FIG. 1 SCHEMATIC DIAGRAM OF LBL SELF-ASSEMBLED MWNTS MODIFIED GCE AND THE OBTAINED BIOSENSOR OF ACHE/PDDA/{MWNTS/PDDA}$_5$/GCES WERE STORED AT 4℃ IN PBS WHEN NOT IN USE.

2.4 Activation of Modified GCE

Successive scanning with three-electrode system, aiming at the activation process of modified GCE activation process, and this is carried in 6.0mL PBS with scan slope of -0.3-1.0V and scan rate 100mV/s, until stable CV curves were obtained.

3 RESULTS

3.1 Electrochemical Behavior of TCh on Modified GCE

The effect of potential scanning rate on voltammetric behavior of thiocholine on {PDDA/MWNTs}$_5$/GCE electrode was studied by cyclic voltammetry. It was found the oxidation peak potential shifts positively and the peak current increases with the increase of scanning rate in the range from 10 to 100 mV/s. Peak currents vary linearly with the square root of the scan rate (R=0.9999), indicating that the electrode reaction of thiocholine at {PDDA/MWNTs}$_5$/GCE surface is a typical diffusion-controlled process.

3.2 Determination of Additional TCh on Modified GCE

AChE could be immobilized directly by LBL self-assembly after preparing {PDDA/MWNTs}$_5$/GCE, in order to avoid the fall-off of AChE, a PDDA membrane was fabricated at the last surface. According to the formula of A=0.133c and U=0.075A, the activity value of AChE could be obtained. As to the formula, a means the activity value at 412 nm (reaction between DTNB and TCh after 5 minutes), c means the concentration of TCh. What's more, the activity of the immobilized AChE in 4-PDDA/AChE bilayer was stable, reproducible and always 0.01U.

3.3 The Relationship between the Activity of Immobilized Enzyme and the Change in the Current

FIG. 2 shows the amperometric response signals of 1mM TCh on {PDDA/MWNTs}$_5$ multilayer films modified GCE under different activity of immobilized enzyme. It is clear that the steady-state current change rise with the activity of immobilized enzyme and quickly reach the stable value. The change in the current is linear with the activity of immobilized enzyme up to 0.012 from 0.002 with a correlation coefficient of 0.9996.

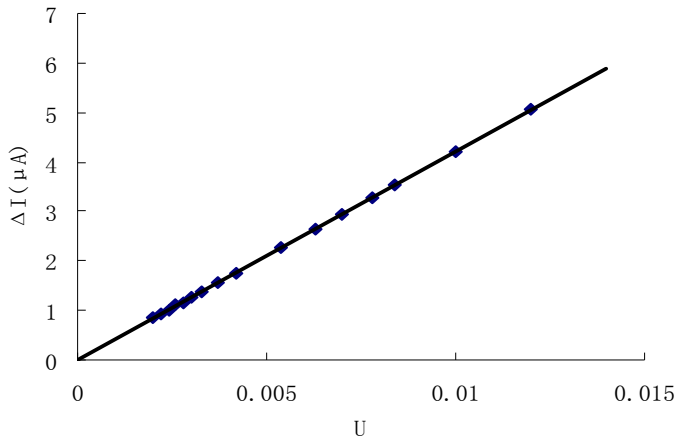

FIG. 2 RELATIONSHIP BETWEEN THE ACTIVITY OF IMMOBILIZED ENZYME U AND THE CHANGE IN THE CURRENT ΔI OBTAINED BY ACHE/PDDA/{MWNTS/PDDA}$_5$ / GCE TO 1MM TCH. SUPPORTING ELECTROLYTE IS PHOSPHATE BUFFER SOLUTION (0.10 M, PH 7.40).

3.4 Linear Range of Amperometric Response of Modified GCE to TCh

In order to more sensitively determine TCh, the chronoamperometric response of AChE/PDDA/{MWNTs/PDDA}$_5$/ GCE on key step changes of the TCh at applied potential -0.30V is described in FIG. 3. It can be seen that the steady-state current increasing when the TCh was added and quickly reached the stable value.

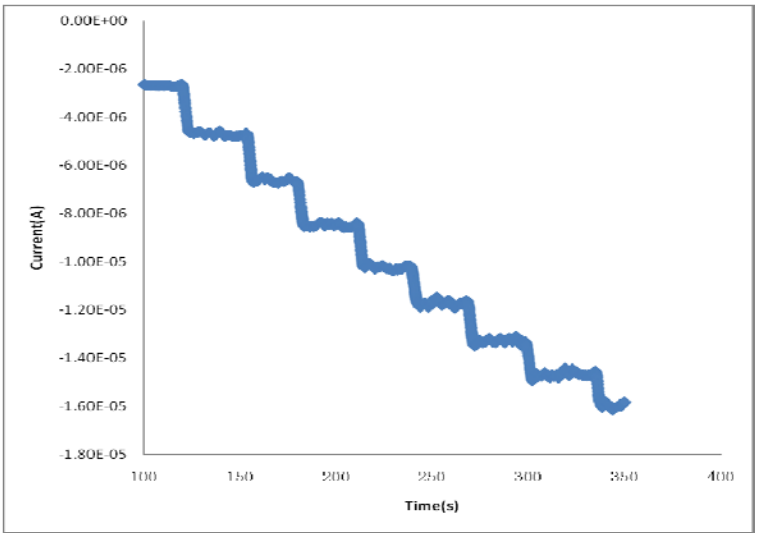

FIG. 3 AMPEROMETRIC RESPONSE OF ACHE/ PDDA /{MWNTS/PDDA}$_5$/GCE TO EVEN REPETITIVE INJECTIONS OF 5ML OF 4.5M TCH. SUPPORTING ELECTROLYTE: PBS (0.10 M, PH 7.40); WORKING POTENTIAL: 0.30V.

3.5 Detection of Carbofuran with AchE/PDDA/{MWNTs/PDDA}$_5$/GCE

Since ATChCl can be hydrolyzed by AChE to produce TCh, the AChE activity can also be indicated with the current value produced by TCh oxidation. I_1 represents the oxidation current value that will get smaller when the AChE activity is inhibited. What's more, it is proved that, with the activity decreasing of immobilized AChE, the current value (I) decreased linearly when every 80 mL ATChCl is added to PBS, and the following formula is the inhibition rate of the AChE: Inhibition%=$[(I_0 - I_1)/I_0]\times100\%$. Hereinto, I_0 is 9.93×10^{-6}A (for 0.01U activity of the immobilized AChE). The inhibition rate can be decided in different concentration solutions of pesticide after 10 minutes immersion of enzymatic electrode.

FIG. 4 shows the relationship between pesticide inhibition rate and concentration. It proved to be a good linearity in the range from 10^{-12} g/L to 10^{-6} g/L between the -lg[carbofuran] and inhibition rate, and the detection limit is 10^{-12} g/L.

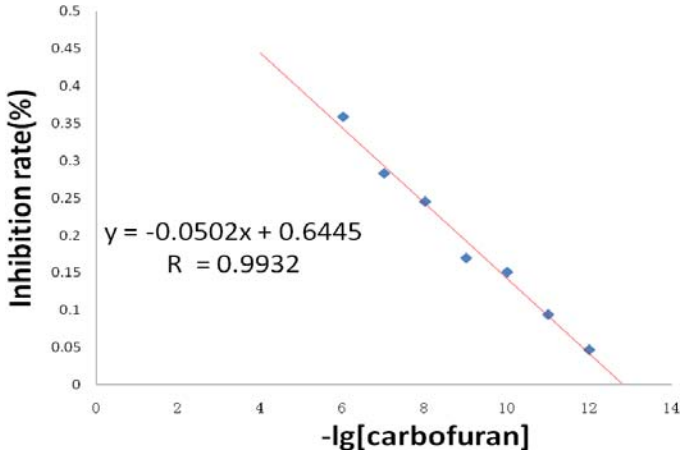

FIG. 4 RELATIONSHIP BETWEEN THE -LG[CARBOFURAN] AND ENZYME INHIBITION RATE . INSERT: LINEARITY OF THE -LG[CARBOFURAN] AND ENZYME INHIBITION RATE BETWEEN THE CONCENTRATION FROM 10^{-12} TO 10^{-6} G L^{-1}.

At the concentration of 10^{-6} g/L, pesticide on the degree of inhibition of the enzyme is too large to bring greater measurement of interference. When the concentration reaches 10^{-3} g/L, because the activity has been completely inhibited, curve tends to parallel.

3.5 The Determination of Recovery

The recovery rate of carbamate was connected with the inhibition of AChE, and it proved that, the recovery ranged from 87.1% to 115% with an average of 98.4% in 0.01 ppm carbamate standard solution. So it is clear that the enzymatic biosensor shows excellent advantages in detection of pesticides.

TABLE 1 RESULTS OF THE DETERMINATION OF RECOVERY OF CARBAMATE

Sample	Carbamate detected ($g \cdot L^{-1}$)	Carbamate obtained ($g \cdot L^{-1}$)	Recovery (%)
Sample 1	10^{-8}	$10^{-7.94}$	115
Sample 2	10^{-8}	$10^{-8.06}$	87.1
Sample 3	10^{-8}	$10^{-8.03}$	93.3

3.6 Stability of the AChE/ PDDA /{MWNTs/PDDA}$_5$ /GCE Electrode

The storage stability and the operational stability of the AChE/PDDA/{MWNTs/PDDA}5/GCE electrode were examined to prove the practicability and the precision of the proposed method. It retained about 80% of its original sensitivity after 3 weeks, which is stored at 4℃ in PBS and daily measured. Because the fabrication process is mild and the enzyme molecules are protected well, so the enzymatic electrode has a good stability.

4 CONCLUSIONS

This work showed that it is possible to quantify the pesticide carbofuran in phosphate buffer. AChE enzymes were used. The introduction of different mutations in the enzyme structure improved the sensitivity of the biorecognition molecules and consequently of the biosensors. Good operational stability, immobilisation reproducibility, and storage stability were obtained for each biosensor. The lifetime of the developed biosensors was substantially improved by immobilizing the enzymes by entrapment in the layer-by-layer self-assembly, because this polymer maintained the activity of the enzyme for a long time, mass production of these kinds of biosensors were enabled.

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