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Determination of Carbamate Pesticide Methomyl Using Acetylcholinesterase / MWNTs-Chitosan Modified Glassy Carbon Electrode

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Abstract: An electrochemical biosensor based on immobilization of acetylcholinesterase (AChE) onto multi-walled carbon nanotubes (MWNTs) and chitosan (CS) modified glassy carbon electrode (GCE) has been prepared for the rapid determination of a carbamate pesticide Methomyl. AChE was immobilized via a layer-by-layer self-assembly modification technique with CS. Inhibition effect of methomyl on the AChE activity was proportional to its concentration within the range from 10^{-10} to 10^{-3} g L^{-1} with the detection limit of 10^{-11} g L^{-1}. The {AChE/CS}/{MWNTs/CS}/GCE biosensor represents an effective and alternative tool for the determination of pesticide residues in environment and food samples.

Keywords: Electrochemical biosensor, Methomyl, Acetylcholinesterase, Chitosan, Carbon nanotubes

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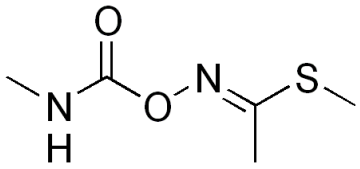
Introduction

With respect to facts that since the 1970s many organ chlorine pesticides were restricted or prohibited, and the number of insect species resistant to the organ chlorine pesticides is increasing, the use of carbamate pesticides in China becomes more and more common. Carbamate compounds like carbaryl, carbofuran, aldicarb, methomyl and others can be used not only as pesticides but can significantly stimulate crop growth. However, a great part of them is very toxic for animals and some of them appear to be much more persistent than previously believed. They have brought a great harm to the environment. Therefore, the carbamate pesticide residues analysis has drawn great attention [1].

Methomyl (other names Lannate, Mesomile, Methomex, Nudrin), methyl *N*-{[(methylamino)carbonyl]oxy}ethanimidothioate (see Scheme 1), is a water-soluble crystalline solid that gives off a sulfurous odor. Highly toxic, methomyl is classified as a carbamate insecticide that is designated as being a Restricted Use Pesticide (RUP) by the U.S. Environmental Protection Agency (EPA). Since the late 1960s, methomyl has been used as a pesticide on commercial fruit and vegetable crops as well as stored products. Its application as an insecticide is highly effective against a wide variety of pests, particularly those that are resistant to organophosphorus pesticides.



Scheme 1: *Structural formula of Methomyl*

Many methods are available for the detection of the pesticides and their residues, where gas chromatography is the most common one. However, this approach requires expensive instrumentation and highly trained personnel, it is time consuming, and is not easily adapted for in field analysis.

Ellman-based colorimetry [2] is rather method for the determination of AChE activity and carbamate pesticide detection, but it is time-consuming and troublesome as well. Enzyme based electrochemical biosensors represent a promising alternative to the conventional methods due to their good selectivity, sensitivity, rapid response, and miniature size.

When acetylcholinesterase (AChE) is immobilized on a working electrode surface, its interaction with acetylthiocholine (ATCh) as the reaction substrate gives thiocholine (TCH), an electroactive product which possesses an irreversible anodic oxidation signal. Based on their inhibitory effect on the enzymatic activity, carbamate pesticides are detected by measuring decrease in the anodic current of thiocholine.

Laschi et al. [3] developed an acetylcholinesterase based biosensor with a cobalt(II) phthalocyanine modified screen-printed carbon electrode as the transducer. The detection limit of 4.9×10^{-10} mol L^{-1} for carbofuran was found at analysis time of 15 min. Valdés-Raminéz et al [4] reported similarly constructed biosensors with three different mutations of AChE which were applied to the determination of the pesticides carbofuran, carbaryl, methylparaoxon, and dichlorvos in phosphate buffer containing 5% acetonitrile. Biosensor stability up to 7 months has been achieved. Upadhyay et al. [5] developed highly sensitive amperometric biosensor based on electrodeposition of gold–platinum bimetallic nanoparticles onto 3-aminopropyltriethoxy silane modified glassy carbon electrode. Paraoxon ethyl, sarin, and aldicarb were detected up to $150–200\times10^{-9}$ mol L^{-1}, $40–50\times10^{-9}$ mol L^{-1}, and $40–60\times10^{-6}$ mol L^{-1} respectively, at the 30–40% inhibition of AChE. The limits of detection obtained are low enough to detect trace amounts of pesticides on the level of their admissible concentrations in environmental as well as food matrices.

Since the discovery of carbon nanotubes (CNTs) in 1991 [6], they have attracted much attention due to their unique mechanical and electrical properties [7,8]. However, to our best knowledge, not many applications of CNTs at the amperometric biosensors with AChE for the carbamate pesticide detection have been reported. Due to its excellent biocompatibility, nontoxicity, cheapness, easy-handling and high mechanic strength; the polymer chitosan (CS) [9] has been widely used as a reagent to immobilize biomolecules like enzymes and to construct amperometric biosensors. For instance, Du et al. [10] developed a simple method for the immobilization of AChE onto a multi walled carbon nanotubes (MWNTs) – CS composite and construction of stable amperometric sensor with fast response to organophosphorous. Under optimum conditions, the inhibition by triazophos was proportional to its concentration within $(0.03 – 7.8)\times10^{-6}$ mol L^{-1} and $(7.8 – 32)\times10^{-6}$ mol L^{-1} with a detection limit of 0.01×10^{-6} mol L^{-1}. Up to 95% reactivation of the inhibited AChE could be achieved using pralidoxime iodide within 8 min. By using similar biosensor, carbaryl was detected [11]. Liu et al. [12] reported an amperometric biosensor prepared by entrapping laccase into a CNTs–CS composite film.

At pH 6.0, the fungi laccase exhibited catalytic activity better than that for the enzyme dissolved in solution. Galandová et al [13] used the MWNTs-CS composite for the construction of nucleic acid based biosensors.

Although the AChE modified electrodes are already widely used for the detection of organophosphates and carbamates pesticides, they are still some problems with them. For instance, lost of the enzyme activity and overall sensitivity of these electrodes should be improved. The aim of this paper is to solve some of these problems via immobilization of AChE and CS by using a self-assembly technique on a MWNTs-CS modified glassy carbon electrode. An analytical procedure based on conventional AChE enzymatic hydrolytic reaction – TCh electrochemical oxidation scheme [14] for the amperometric determination of methomyl residual concentration by using the biosensor is proposed.

Experimental

Apparatus and Reagents

Cyclic voltammetric and amperometric measurements were performed using an electrochemical analyzer CHI800 (Chenhua Instruments, Shanghai, China) connected to a personal computer. A three-electrode configuration was employed consisting of bare glassy carbon electrode (GCE) or chemically modified GCE as a working electrode, a saturated calomel reference electrode (SCE), and a platinum wire auxiliary electrode.

The reagents used were AChE (317 U/mg) and acetylthiocholine chloride (ATChCl) were purchased from Sigma; CS (Mw 15000) was obtained from Zhejiang Aoxing Biotechnology Co., Ltd (China). MWNTs with >95% purity were obtained from Shenzhen Nanotech Port Co., Ltd (China). Methomyl preparation was obtained from Shenzhen Zonewins Special Materials Co., Ltd (China) and its stock solution was prepared in the concentration 10^{-3} g L^{-1} in 0.1 mol L^{-1} phosphate buffer solution (PBS) pH 7.4. All other reagents were of analytical reagent grade and used without further purification. All solutions were prepared with double distilled water.

Preparation of the Biosensor

First, oxidation of MWNTs and pretreatment of GCE were performed as reported previously [15]. The electrode prepared was subsequently immersed into 0.5% CS in glacial CH_3COOH (pH 5.0, adjusted with NaOH) and 1 mg mL^{-1} MWNTs in 0.01 mol L^{-1} borate buffer (pH 9.18) for 15 min alternately 5 times, until a {MWNTs/CS} modifier layer on GCE was obtained.

Afterwards, the {MWNTs/CS}/GCE electrode was dipped into 0.5% CS acetic buffer solution (pH 5.0) and 0.5 U of AChE in 0.1 mol L^{-1} phosphate buffer solution (PBS, pH 7.4) for 15 min alternately. The electrode was carefully washed with double-distilled water after each dipping step. This operation was repeated (typically 4 times) until the desirable modifier layer was obtained. The obtained {AChE/CS}/{MWNTs/CS}/GCE biosensor was stored at 4 ºC in 0.1 mol L^{-1} PBS (pH 7.4) when not in use.

Procedure

For the determination of methomyl, the biosensor was first incubated in the methomyl solution (PBS, pH 7.4 or sea water sample) for 10 min. Then, it was washed with water and transferred into the electrochemical cell containing 1.0×10^{-3} mol L^{-1} ATCh in PBS pH 7.4, and the i-t curve was obtained under stirring at 0.50 V versus SCE. The inhibition of enzyme by the pesticide was calculated as follows:

$$\text{Inhibition [\%]} = (i_0 - i_1) / i_0 \times 100 \qquad (1)$$

where i_0 and i_1 are the TCh anodic currents in the absence and in the presence of methomyl in the incubation medium, respectively.

Results and Discussion

First, the voltammetric behavior of thiocholin (TCh) at bare GCE, MWNTs/GCE and {MWNTs/CS}/GCE electrodes was investigated by cyclic voltammetry (Fig. 1). The peak current of TCh at {MWNTs/CS}/GCE (I_{pa} = 61.0 μA, curve C) was enhanced comparing to that at GCE (I_{pa} = 29.5 μA, curve A) and MWNTs/GCE (I_{pa} = 57.5 μA, curve B). Meanwhile, the peak potential value of TCh at the {MWNTs/CS}/GCE (E_{pa} = 0.35 V) was lower than that at bare GCE (E_{pa} = 0.60 V). This behavior can be attributed to an electrocatalytic activity of MWNTs, which resulted from its edge-plane-like sites at the tube ends. The enhanced anodic peak current of TCh at {MWNTs/CS}/GCE comparing to MWNTs/GCE could be attributed to the electrocatalytic activity of MWNTs positioned at the protonated amino groups of CS. Experimental conditions for the preparation of the biosensor {AChE/CS}/ {MWNTs/CS}/GCE and its use in the amperometric mode have been found. The pH value of PBS (7.4) was kept well above the isoelectric point of AChE (pI = 4.5).

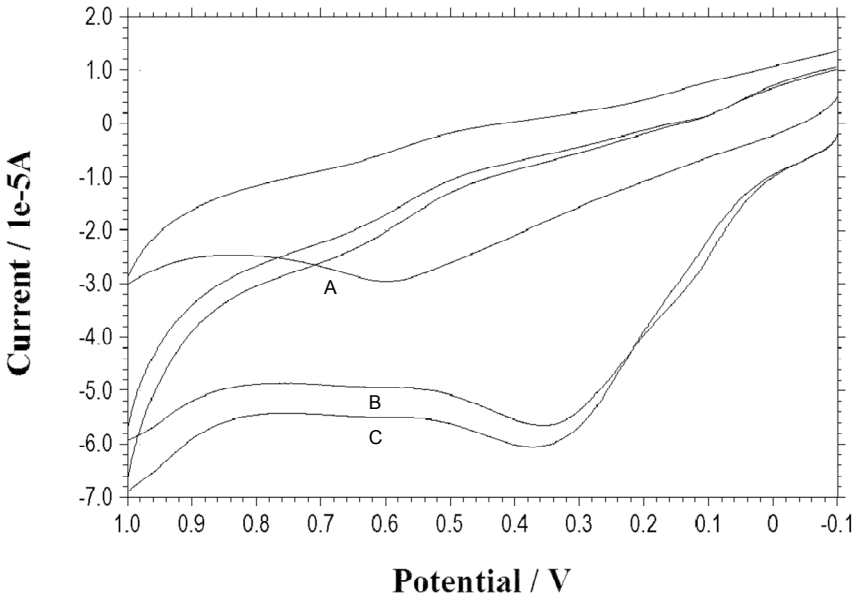


Fig. 1: *Cyclic voltammograms of 5.0×10^{-3} mol L^{-1} TCh recorded at bare GCE* (curve **A**), *MWNTs/GCE* (curve **B**), *and {MWNTs/CS}/GCE* (curve **C**). Exp. conditions: 0.1 mol L^{-1} PBS (pH 7.4), scan rate of 100 mV s^{-1}.

Thus, the enzyme was present as an anionic form, facilitating its adsorption on protonated amino groups of CS. At pH values about 7.0, the catalytic activity of AChE was also reported to be the highest one [11 and references therein]. Upon the addition of 60 μL of 0.1 mol L^{-1} ATCh to 6.0 mL PBS (pH 7.4) under stirring, the anodic current at an amperometric i-t curve obtained with the biosensor at +0.50 V versus SCE has increased rapidly to a stable value reaching 95% of the steady-state-current within 10 s. Fig. 2 shows an effect of number of the AChE and CS deposition steps on the biosensor response. After 4 steps, evidently blocking of electrode surface occurs due to high thickness of the modifier layer.

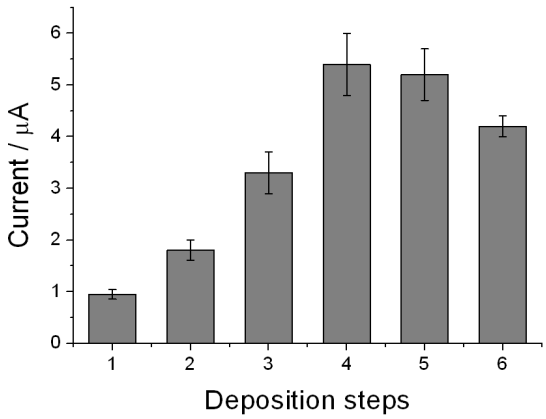


Fig. 2: *Effect of AChE and CS deposition steps on amperometric response of the biosensor {AChE/ /CS}/{MWNTs /CS}/GCE. Exp. conditions: 1.0×10^{-3} mol L^{-1} ATCh in 0.1 mol L^{-1} PBS (pH 7.4), 0.50 V vs. SCE.*

The use of four steps at the formation of the {AChE/CS} recognition layer on the {MWNTs/CS}/GCE transducer has been chosen for the construction of the final biosensor. For the determination of methomyl, the biosensor {AChE/CS}/{MWNTs/CS}/GCE was first incubated in 1.0×10^{-7} g L^{-1} methomyl solution (PBS, pH 7.4) for 10 min which was chosen for further measurements (Fig. 3). Fig. 4 overleaf shows the semilogarithmic dependence between the pesticide concentration and inhibition indicating good linearity within the range from 10^{-10} to 10^{-3} g L^{-1} ($y = -8.64x + 96.89$, $R^2 = 0.9928$) with the detection limit of 10^{-11} g L^{-1}.

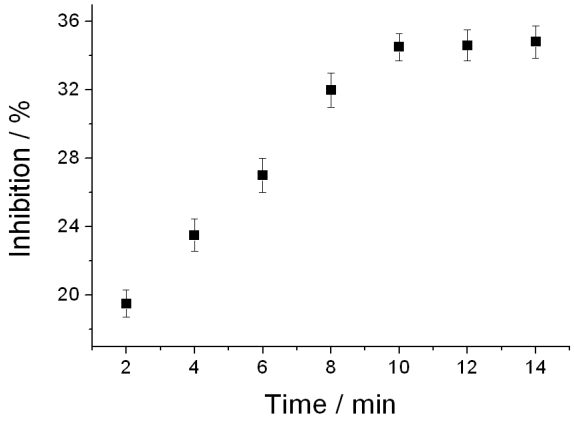


Fig. 3: *Effect of biosensor incubation time in 1.0×10^{-7} g L^{-1} methomyl in PBS (pH 7.4) on AChE inhibition (%).* Exp. conditions: amperometry with 1.0×10^{-3} mol L^{-1} ATCh, 0.1 mol L^{-1} PBS (pH 7.4), 0.50 V vs. SCE.

The recovery of the methomyl was evaluated using seawater (pH 8.2) which was got from the Yellow Sea near Qingdao using standard sampling procedure and used after being filtered for particles only. It was checked experimentally that in the seawater without methomyl added, no AChE inhibition proceeded. The recovery data obtained are summarized in Table I. Generally, for the determination of pesticides when the average recovery is > 80%, the result is considered as acceptable. Taken into account variation in the biosensors prepared and error of the methomyl detection itself, the results obtained can be considered as accurate.

The storage stability of the biosensor was also examined. Three biosensors {AChE/CS/{MWNTs/CS}/GCE have been prepared, stored at 4 ºC in PBS pH 7.4 and used for the repeated amperometric measurement of ATCh concentration every five days. From Fig. 5 it is seen that the AChE activity of the biosensor retained about 80% of its original value even after 3 weeks. The good stability of both the enzyme and the biosensor is attributed to the biosensor fabrication process under mild conditions and the biorecognition layer composition. The biosensor stability is near to that of 10 to 30 days reported for the MWNTs-CS composite based biosensor with covalently bound AChE by using glutaraldehyde as cross linker [11].

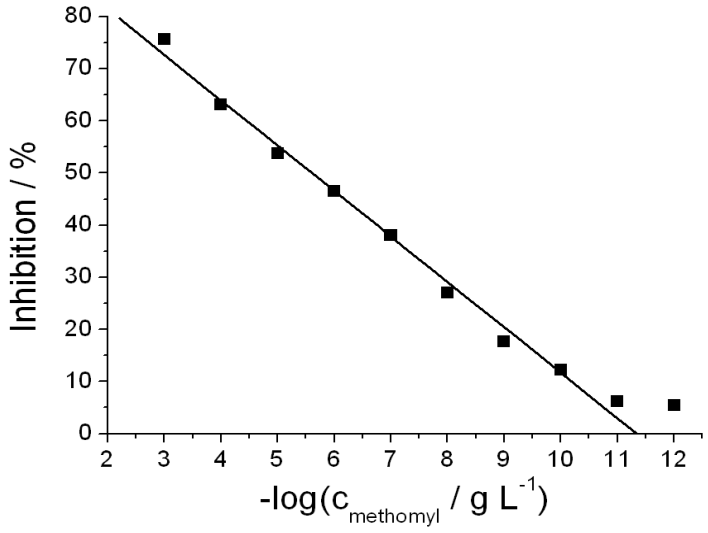


Fig. 4: Dependence of the enzyme inhibition on the methomyl concentration (PBS, pH 7.4) obtained at the biosensor {AChE/CS}/{MWNTs/CS}/GCE. Conditions: incubation time 10 min, amperometric measurement at 0.50 V vs. SCE.

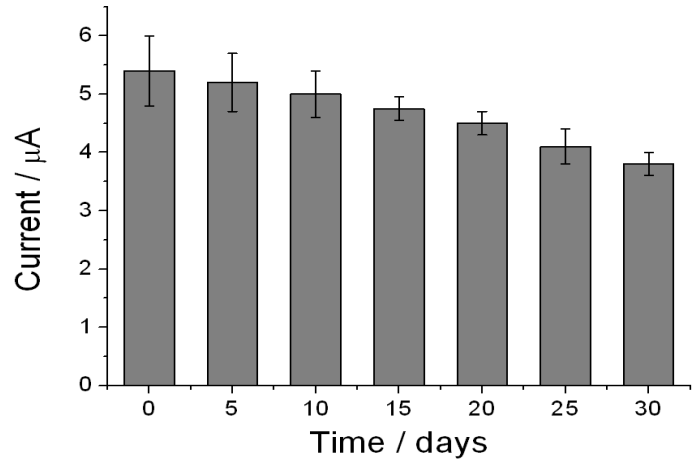


Fig. 5: The storage stability of the {AChE/CS/{MWNTs/CS}/GCE biosensor indicated by the average response value of 3 biosensors obtained every 5 days when stored in 0.1 mol L^{-1} PBS (pH 7.4) at 4 ºC between the days of measurement. Conditions: incubation time 10 min., amperometric measurement in 1.0×10^{-3} mol L^{-1} ATCh at 0.30 V vs. SCE.

Conclusions

In summary, at the construction of AChE biosensor we have integrated good electroconductivity of MWNTs with the biocompatibility of CS by using the layer-by-layer self-assembly modification technique. CS helps to maintain the enzymatic activity of the immobilized AChE.

Table 1. Methomyl recovery obtained in the seawater matrix from the Yellow Sea near Qingdao (pH 8.2) (n=3, P=0.95). Conditions: different biosensors used at individual samples, biosensor incubation time 10 min, amperometric measurement at 0.50 V vs. SCE.

Seawater sample	Spiked methomyl concentration (g L^{-1})	Found methomyl concentration (g L^{-1})	Recovery (%)	Average recovery (%)
1	1.0×10^{-4}	$(1.05\pm0.09)\times10^{-4}$	105	
2	1.0×10^{-4}	$(1.10\pm0.07)\times10^{-4}$	110	104
3	1.0×10^{-4}	$(0.98\pm0.09)\times10^{-4}$	98	

Under optimum conditions, the biosensor exhibits several advantages, such as high sensitivity with low detection limit for the methomyl, good storage stability, and good performance at the methomyl determination in the seawater matrix. Of course, some specific analytes affecting the AChE activity will interfere in the measurements in complex samples and their preliminary separation would therefore be needed. We expect that the method of electrochemical biosensor preparation described here could be useful also for the development of other biosensors.

Acknowledgement

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