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ORIGINAL PAPER

A disposable sensor based on immobilization of acetylcholinesterase to multiwall carbon nanotube modified screen-printed electrode for determination of carbaryl

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Abstract A simple method has been devised for immobilization of acetylcholinesterase (AChE) covalent bonding to a multiwall carbon nanotube (MWNT)-cross-linked cellulose acetate composite on a screen-printed carbon electrode (SPCE) and a sensitive and disposable amperometric sensor for rapid determination of carbaryl pesticide is proposed. The immobilized enzyme was preserved on this film because of the excellent biocompatibility and nontoxicity of cellulose acetate. Based on the inherent conductive properties of the MWNT, the immobilized AChE had greater affinity for ATCl and excellent catalytic effect in the hydrolysis of ATCl. MWNT improved the interface enzymatic hydrolysis reaction and increased the amperometric response of the sensor. Under optimum conditions, the inhibition of carbaryl on AChE increased linearly with the increasing concentration of carbaryl in two ranges, from 0.01 to 0.5 μg mL^{-1} and from 2 to 20 μg mL^{-1}, with the correlation coefficients of 0.9985 and 0.9977, respectively. The detection limit was 0.004 μg mL^{-1} taken as the concentration equivalent to 10% decrease in signal. The sensor showed acceptable stability, accuracy and could be fabricated in batches, thus it is economic and portable. This type of disposable enzyme-based amperometric sensor has extensive application potential in environmental monitoring of pesticides.

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1 Introduction

Carbamate and organophosphate pesticides have come into widespread use in agriculture because of their high insecticidal activity and relatively low environmental persistence [1]. Among these, carbaryl insecticide, one of the carbamate pesticides, has been extensively used for pest control due to its efficacy, low bioaccumulation, and moderately rapid degradation in the environment [2]. The great success in agricultural applications has led to an increase in the production and spread of these pesticides. Traditional methods available for determination of carbamate include high-performance liquid chromatography (HPLC) and gas chromatography (GC) [3, 4]. However, they present several disadvantages for environmental monitoring including high cost, long analysis time, and the necessity of highly trained personnel. Because electrochemistry is a powerful tool for real-time detection, amperometric sensors have been regarded as most suitable for biochemical analysis, because of their good selectivity, rapid response, miniature size, and reproducible results [5]. Combination of enzymatic reactions with electrochemical methods has enabled the development of a variety of enzyme-based electrochemical biosensors for sensitive and rapid determination of environmental pollutants [6]. The main applications have been specifically described for acetylcholinesterase (AChE)-based biosensors, which were based on enzymatic inhibition by the pesticides [7–9]. When AChE was immobilized on the working electrode surface its interaction with the substrate of acetylthiocholine produced the electro-active

species thiocholine. The current produced by thiocholine could be used as a quantitative measure of the enzyme activity, which was a biomarker for the biological effect of some pesticides (organophosphate and carbamate) involved in the inhibition action [8]. The reactions that occur on the surface of a biosensor are:

$$\text{Acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{HA (acetic acid)}$$

$$2\,\text{Thiocholine (red)} \rightarrow \text{Thiocholine (ox) (dimeric)} + 2H^+ + 2e^-$$

During fabrication of biosensors, immobilization of the enzyme onto the solid electrode surface is a crucial step [10]. Cellulose acetate is a promising natural polymer for use as an immobilization matrix because of its excellent biocompatibility, non-toxicity, chemical inertness, easy handling and low cost [11, 12]. It is preferable to maintain the high biological activity of the enzyme and enhance the sensitivity of the sensor. Of the different types of nanotubes, carbon nanotubes (CNTs) are highly conductive (rapid electron transfer) nano materials with promising applications in the biochemical sensing domain [13]. This work proposes a novel method for immobilization of acetylcholinesterase (AChE) by covalent bonding to a multiwall carbon nanotube (MWNT)-cross-linked cellulose acetate composite (CAMC), leading to a stable AChE sensor for determination of carbaryl pesticide. The presence of MWNT reduces the working potential by catalyzing the electrochemical oxidation of enzymatically formed thiocholine.

Screen-printing is a standard technology in electronics and has been used to produce disposable electrochemical sensors. Compared with conventional electrodes, screen-printed electrodes have several advantages, such as simplicity, convenience, low cost and the avoidance of contamination between samples [14–16]. Biosensors based on screen-printed carbon electrode (SPCE) have been extensively used for detection of pesticides [8], antigens [17, 18], anions [19] and DNA [20].

The objective of the current work is to develop a convenient and sensitive screen-printed three-electrode system as the basis of an amperometric portable sensor for fast determination of carbaryl pesticide.

2 Materials and methods

2.1 Reagents

Acetylthiocholine chloride (ATCl), acetylcholinesterase (Type C3389, 500 U/mg from the electric eel) and cellulose acetate were purchased from Sigma-Aldrich (St. Louis, USA) and used as received. Carbaryl was obtained from AccuStandard (USA). Multiwall carbon nanotube (MWNT) was a gift from the Institute of Nano-Science and Technology, Central China Normal University, and then refluxed for 10 h in concentrated HNO_3 before use. It has been known that this treatment causes segmentation and carboxylation of MWNT at their terminus [21]. Phosphate-buffered saline (PBS, pH 7.0) and other reagents used were of analytical reagent grade. Aqueous solutions were prepared with double distilled water.

2.2 Instruments

Electrochemical measurements were performed with a Bioanalytical System (BAS, cv-50w, USA) with a three-electrode system comprising a graphite auxiliary electrode, an Ag/AgCl reference electrode and an AChE immobilized on CAMC modified screen-printed carbon electrode (AChE-CAMC/SPCE) as working electrode. High pressure liquid chromatography (HPLC) measurement were carried out on an Alliance 2695 Separation Module comprising an XDB-C8 column (150 × 4.6 mm) equipped with a UV–Vis photodiode-array detection system using a mobile phase consisting of 20% CH_3OH and 80% H_2O at a flow rate of 0.8 mL min^{-1}.

2.3 Fabrication of SPCE system

The combined three-electrode system with graphite working electrode (diameter 4.0 mm), graphite auxiliary electrode, and Ag/AgCl reference electrode was fabricated according to Ye and Ju [20]. First, a layer of silver ink was printed on a polyvinyl chloride (PVC) membrane (0.025 mm thickness), then a carbon layer about 0.5–1 mm wider than the silver layer was printed to cover the silver layer except for the area that served as the reference electrode which was oxidized electrochemically in KCl solution to obtain the Ag/AgCl reference. Finally, the conductive bands were insulated by overlaying a silicone rubber layer to expose the conjunction tips and the three-electrode areas.

Before the experiment, a potential of +1.75 V was applied to the SPCE, with stirring, in pH 5.0 PBS for 300 s and the electrode was then scanned from +0.3 V to +1.25 V and from +0.3 V to −1.3 V until a steady state current-voltage curve was obtained. The pretreated SPCE was used for the following experiments.

2.4 Preparation of AChE-CAMC/SPCE

Chitosan stock solution (0.50 (w/v) %) was prepared by dissolving chitosan in an aqueous solution of 2.0 M acetic

aced and the pH was adjusted to 4.0–6.0 by addition of concentrated NaOH solution. The cellulose acetate solutions were stored under refrigeration (4 °C) when not in use. The pretreated MWNT (1.0 mg) was then added to 1.0 mL cellulose acetate and sonicated thoroughly until a homogeneous suspension was obtained. 2.0 μL of the mixture was coated on working electrode of pretreated SPCE and allowed for reaction at 20 °C for 4 h. The CAMC modified electrode (CAMC/SPCE) was washed thoroughly with double distilled water and coated with 5.0 μL AChE solution (100 mU). After evaporation of water, it was washed with PBS to remove unbound AChE. The sensor was stored at 4 °C when not in use.

2.5 Measurement procedure

The treatment of a garlic sample included salting out and separation processes. Briefly, 5 g NaCl was added to 10 powdered garlic samples, followed by the salting out process for 30 min. The sample was then diluted with 5 mL double distilled water and filtered. The resulting liquid was adjusted to pH 7.0 using 0.5 M NaOH.

The obtained AChE-CAMC/SPCE was first activated in pH 7.0 PBS by cyclic voltammetric sweeps from 0.1 to 1.0 V until a stable curve was obtained. For the measurements of carbaryl, the pretreated AChE-CAMC/SPCE was first immersed in the PBS solution containing different concentrations of standard carbaryl for 15 min, and then transferred to the electrochemical cell of 1.0 mL pH 7.0 PBS containing 0.5 mM ATCl to study the electrochemical response by cyclic voltammetry (CV). The inhibition of carbaryl was calculated as follows:

$$\text{Inhibition}\,(\%) = 100\% \times \left(i_{\text{P, control}} - i_{\text{P, exp}}\right)/i_{\text{P, control}} \quad (1)$$

where $i_{\text{P, control}}$ is the peak current of ATCl on the AChE-CAMC/SPCE, $i_{\text{P, exp}}$ is the peak current of ATCl on the AChE-CAMC/SPCE with carbaryl inhibition.

3 Results and discussion

3.1 Cyclic voltammetric behavior of ATCl on AChE-CAMC/SPCE

The cyclic voltammograms of ATCl on AChE-CAMC/SPCE was examined, as shown in Fig. 1. No peak was observed when the SPCE (curve a) and AChE-CAMC/SPCE (curve b) were placed in pH 7.0 PBS. When 0.5 mM ATCl was added to the PBS, the cyclic voltammograms obtained from the AChE-CAMC/SPCE contained an irreversible oxidation peak at 535 mV (curve d), whereas no detectable signal was observed for the CAMC/SPCE without immobilized AChE (curve c). Obviously this peak

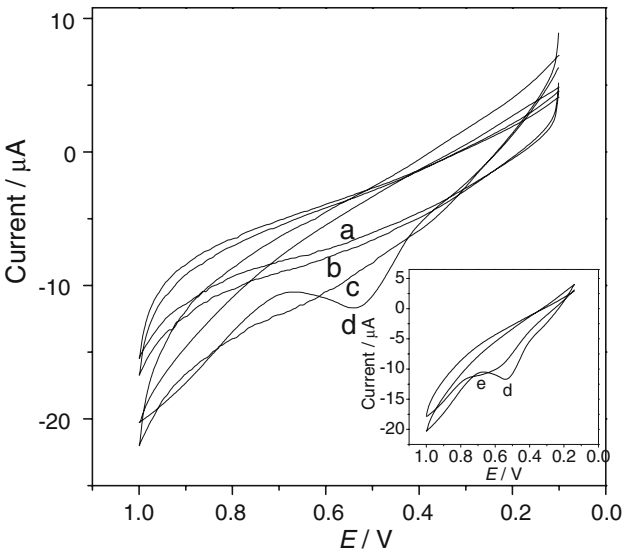

Fig. 1 Cyclic voltammograms. SPCE (**a**); AChE-CAMC/SPCE (**b**) in pH 7.0 PBS. CAMC/SPCE without immobilized AChE (**c**); AChE-CAMC/SPCE (**d**); AChE-cellulose acetate/SPCE without MWNT (**e**) in pH 7.0 PBS containing 0.5 mM ATCl. Scan rate: 50 mV s^{-1}

arose from the oxidation of thiocholine, hydrolysis product of ATCl, catalyzed by the immobilized AChE. The current produced by thiocholine was used as a quantitative measurement of the enzyme activity, which reflected the biological effect of carbaryl pesticide involved in the inhibition action [9]. This peak current was also much higher and the peak potential shifted negatively compared with those on the cellulose acetate modified electrode without MWNT (AChE-cellulose acetate/SPCE) (curves d and e in inset of Fig. 1). This was due to the presence of MWNT with their inherent conductive properties and catalytic behavior, in the CAMC composite film; the MWNT thus provide a conductive pathway for electron transfer and promote electron-transfer reactions at a lower potential. When MWNT was directly coated onto the electrode surface instead of entrapped in the cellulose acetate membrane, a much higher background current was observed. The AChE-CAMC/SPCE was therefore used in subsequent experiments. With increasing scan rate, the peak current increased and the peak potential shifted slightly (Fig. 2). The peak currents were a linear function of scan rate from 5 to 100 mV s^{-1} (inset in Fig. 2), indicative of a typical surface-controlled electrode process [22].

3.2 Optimization of the preparation of the AChE-CAMC/SPCE

The effect of MWNT content on response current was studied. The anodic current gradually increased with increasing MWNT content and reached a maximum at 1.0 mg mL^{-1}, as shown in Fig. 3a. Further increasing the

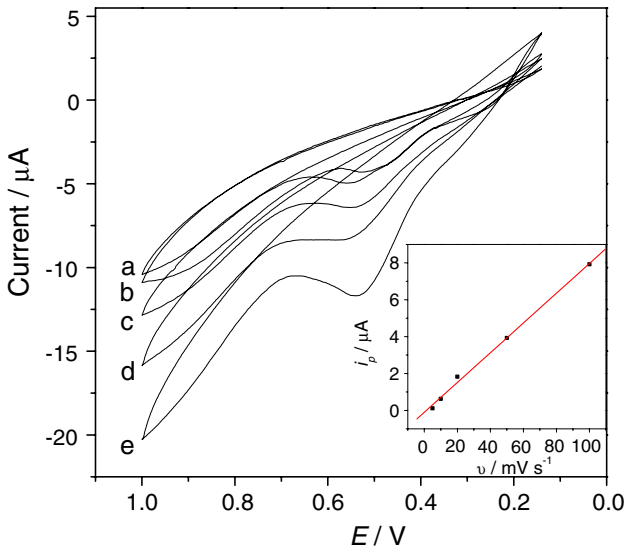

Fig. 2 Cyclic voltammograms of AChE-CAMC/SPCE in pH 7.0 PBS containing 0.5 mM ATCl at different scan rate. 5 (**a**); 20 (**b**); 40 (**c**); 60 (**d**); 100 (**e**) mV s^{-1}. Inset: the relationship between scan rates and peak currents

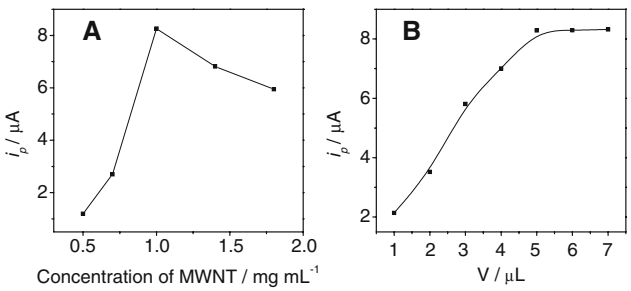

Fig. 3 Effect of different conditions on peak currents. Concentration of MWNT (**a**); volume of AChE (**b**)

amount of MWNT led to a decrease of the response, possibly because of increased resistance and double layer capacitance of the modified electrode. As a result of this experiment 1.0 mg mL^{-1} MWNT was used for preparation of the sensor.

The amount of enzyme immobilized on the electrode surface was another important aspect of the preparation. Figure 3b displays the effect of enzyme loading on the amperometric response. With increasing volume of AChE, the current increases and then tends to a constant value. More than 5.0 μL adsorbed enzyme was not sufficiently stable, owing to imitated electrode area, indicating saturation of enzyme loading.

3.3 Effect of pH on response of ATCl on AChE-CAMC/SPCE

The bioactivity of the immobilized AChE depended on solution pH. Figure 4 shows the relationship between

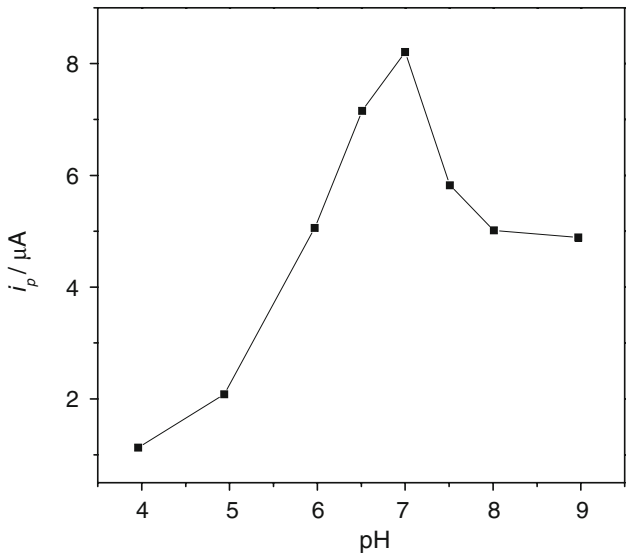

Fig. 4 Effect of solution pH on peak current

catalytic peak current of ATCl on the AChE-CAMC/SPCE and solution pH. Obviously, the maximum peak current was obtained at pH 7.0 in the pH range 4.0–9.0. This result is close to that reported for the soluble enzyme, indicating that CAMC composite did not alter the optimum pH for catalytic behavior of the enzyme. pH 7.0 was therefore used in the detection solution.

3.4 Effect of carbaryl on electrochemical response of AChE-CAMC/SPCE

The peak current observed in the simple electronic voltammetric sensing system could be used to characterize the viability of immobilized enzyme, which can be used as a quantitative measure of carbaryl pesticide. As shown in Fig. 5, after incubation of the AChE-CAMC/SPCE with the standard solution of carbaryl at a known concentration for 15 min, the current of the same ATCl on the AChE-CAMC/SPCE decreased drastically (curve b, c, d and e), compared with the control (curve a). The decrease in peak current increased with increasing carbaryl concentration. This is because carbaryl is a carbamate pesticide involved in the inhibition action on AChE, thus reducing the enzymatic activity to its substrate. Because of the inhibition of AChE by carbaryl the decrease in the oxidation peak current reflects a change in the interface enzymatic activity in the hydrolysis reaction. The voltammetric response can therefore be used to monitor compounds toxic to AChE and provide a simple way of studying the effect of pesticides on the enzyme.

With increase of immersing time in the carbaryl solution, the peak current of ATCl on the AChE-CAMC/SPCE also decreased greatly. As shown in Fig. 6, carbaryl

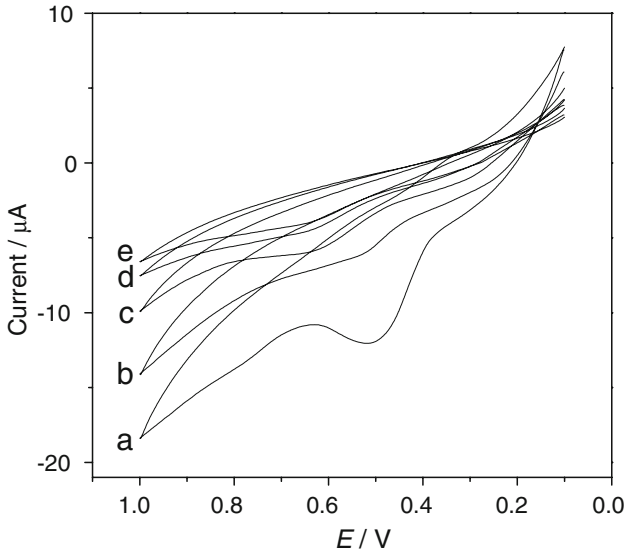

Fig. 5 Cyclic voltammograms of AChE-CAMC/SPCE in pH 7.0 PBS containing 0.5 mM ATCl after immersed in different carbaryl solution for 15 min. 0 (**a**); 1 (**b**); 5 (**c**); 10 (**d**); 15 (**e**) μg mL^{-1}

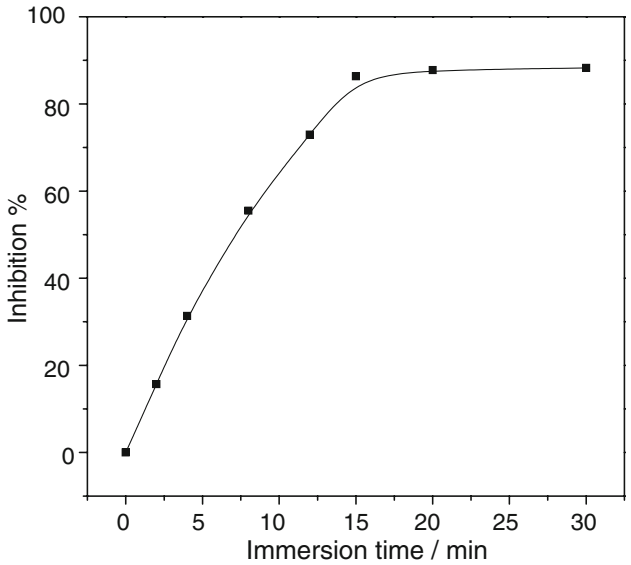

Fig. 6 Effect of immersion time on inhibition of carbaryl

displayed increasing inhibition on AChE with immersing time. When the immersion time was longer than 15 min the curve trended to a stable value, indicating that the binding interaction with active target groups in the enzyme reached saturation. This change in tendency of the peak current reflected the alteration of enzymatic activity, which results in a change in the interactions with the substrate. However, the maximum value of inhibition of carbaryl was not 100%, which is likely to attributable to the binding equilibrium between pesticide and binding sites in the enzyme.

The modified electrodes exposed to the pesticides could not regain their enzyme activity once removed from the pesticide solution. However, the inhibited AChE can be completely reactivated when using nucleophilic compounds such as pralidoxime iodide. This phenomenon may be due to the irreversible inhibition of the pesticides.

3.5 Calibration curve

With increasing carbaryl concentration in the solution, the current of the same ATCl on the AChE-CAMC/SPCE decreased. Under the optimized experimental conditions, the inhibition of carbaryl on AChE-CAMC/SPCE was proportional to its concentration in two ranges, from 0.01 to 0.5 μg mL^{-1} and from 2 to 20 μg mL^{-1}, with the correlation coefficients of 0.9985 and 0.9977, respectively. The linear regression equations were $I\% = 6.81\% + 54.3$ c (μg mL^{-1}) and $I\% = 52.1\% + 1.86$ c (μg mL^{-1}). The detection limit was calculated to be 0.004 μg mL^{-1} taken as the concentration equivalent to a 10% decrease in signal.

3.6 Application of the biosensor

Carbaryl concentrations in garlic samples were detected by their inhibitions on AChE. In parallel, the concentrations were also detected with HPLC. The results are shown in Table 1. The relative deviations between the two methods are in the range 7.1% to −8.3%, indicating acceptable agreement. Thus, the biosensor can be satisfactorily applied to the determination of carbaryl levels in practical samples.

3.7 Precision, reproducibility and stability of AChE-CAMC/SPCE

The inter-assay precision was estimated by determining the response of 0.5 mM ATCl at six different electrodes which were immersed in 0.2 and 5 μg mL^{-1} carbaryl for 15 min, respectively. The coefficient of variation was found to be 3.6 and 2.2%, respectively, indicating acceptable detection precision and fabrication reproducibility. The sensor could

Table 1 Comparison of carbaryl levels in samples determined using two methods

Samples	1	2	3	4	5	6
Biosensor (μg mL^{-1})	0.015	0.017	0.022	0.024	0.029	0.037
HPLC (μg mL^{-1})	0.014	0.018	0.024	0.025	0.028	0.039
Relative deviation (%)	7.1	−5.6	−8.3	−4.0	3.6	−5.1

be fabricated in batches, thus it was economic, disposable and portable.

When the enzyme electrode was not in use it was stored at 4 °C under dry condition. No obvious decrease in the response to ATCl was observed in the first 10-days of storage. After storage for 30 days, the sensor retained 85% of its initial current response. Thus retention of the activity of AChE by the CAMC composite film was acceptable, and the AChE was prevented from leaking from the film. This indicates that this homogeneous composite film provides a biocompatible microenvironment around the enzyme, effectively stabilizing its biological activity.

4 Conclusions

This paper presents a simple and efficient method for immobilization of AChE on a CAMC modified screen-printed electrode and for developing a sensitive, rapid and inexpensive sensor for detection of carbaryl pesticide. Because of its excellent biocompatibility and lack of toxicity, this composite film is very efficient for retaining the enzymatic activity and preventing its leakage from the film. MWNT has good conducting properties which favors interface enzymatic hydrolysis reactions forming electroactive substances; this increases the sensitivity and facilitates the amperometric response of the sensor. The proposed sensor possesses good precision and fabrication reproducibility in batches and acceptable stability and accuracy. The sensor has potential application in the characterization of enzyme inhibitors and detection of compounds toxic to the enzyme.

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