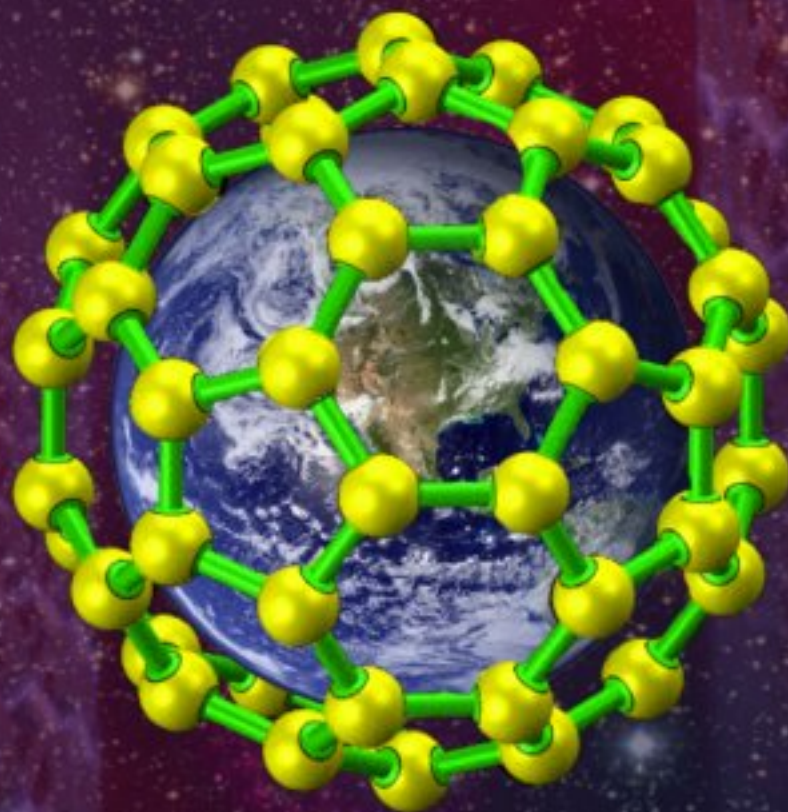


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Amperometric Acetylcholinesterase Biosensor Based on Multilayer Multiwall Carbon Nanotubes-chitosan Composite

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Abstract: A simple method for immobilization of acetylcholinesterase (AChE) onto the glassy carbon electrode (GCE) modified with five layers of multiwall carbon nanotubes (MWNTs)-chitosan (CHIT) composite was proposed, and thus a fast, sensitive and stable amperometric sensor for quantitative determination of pesticides was developed. Five layers of MWNTs-CHIT promoted electron transfer reactions at a lower potential and catalyzed the electro-oxidation of thiocholine, thus, it improved the detection sensitivity of biosensor. Based on the inhibition of pesticides to the enzymatic activity of AChE, using carbofuran as a model compound, under optimal conditions, the inhibition of carbofuran was proportional to its concentration in two ranges, from 5×10^{-4} to 7.5 $\mu\text{g/mL}$ and 7.5 to 20 $\mu\text{g/mL}$ with a detection limit of 1×10^{-4} $\mu\text{g/mL}$. The constructed biosensor showed prominent characteristics and performances such as good precision, acceptable stability, fast response and low detection limit, which provided a new promising tool for pesticide analysis. Copyright © 2011 IFSA.

Keywords: Biosensor, Acetylcholinesterase, Multiwall carbon nanotubes, Chitosan.

1. Introduction

Pesticides are the most abundant environmental pollutants found in soil, water, the atmosphere and agricultural products. They are therefore important target molecules in the search of fast and sensitive analytical methods for determination of pesticides [1]. Various inhibition and non-inhibition biosensor systems, based on the immobilization of AChE or organophosphorus hydrolase onto various electrochemical or optical transducers, have been used to determine pesticides [2]. Among these, amperometric enzymatic biosensors have been considered to be the most suitable for biochemical

analysis due to their good selectivity, sensitivity, rapid response, miniature size, and reproducible results [3]. The toxic action of pesticides is due to their ability to irreversibly modify the catalytic serine residue in AChE, and subsequent inhibition of the AChE effectively prevents nerve transmission by blocking breakdown of the transmitter choline [4]. When AChE was immobilized on the working electrode surface, its interaction with the substrate obtained an electro-active product of thiocholine, which produced an irreversible oxidation peak. A decrease in enzyme activity below normal values can serve as a biomarker which is an indicator of possible pesticide poisoning [5]. Amperometric AChE biosensors have shown satisfactory results for pesticide analysis based on their inhibition on AChE [6], in which the enzymatic activity is employed as an indicator of quantitative measurement of pesticides.

A significant challenge to develop sensitive and stable biosensors comes from the effective immobilization of the enzyme onto the solid electrode surface [7, 8]. Generally, direct adsorption of enzyme onto bare glassy carbon electrode (GCE) surface can frequently result in their denaturation and the loss of bioactivity. CHIT containing large groups of $-NH_2$ and $-OH$ has been widely used as a modifying reagent to prepare modified electrodes due to its excellent biocompatibility, nontoxicity, cheapness, easy-handling and high mechanical strength [9]. Therefore, it is preferable to maintain the high biological activity of the immobilized biomolecules and enhance the sensitivity of the sensor. MWNTs have demonstrated to be an excellent material for the development of electrochemical sensors [10]. MWNTs are attractive because of their unique properties, such as high electrical conductivity, great chemical stability and extremely high mechanical strength [11]. Recently, Sun et al. used MWNTs-based conductive polymer for designing AChE biosensor for detecting dichlorvos, the detection limit was 3 $\mu g/L$ [12]. Du et al. designed the immobilization of acetylcholinesterase (AChE) on the GCE modified with one layer multiwall carbon nanotubes-chitosan composite, using triazophos as a model compound, the detection limit was 0.01 μM at a 10 % inhibition [13]. Sun et al. reported that with the increase of layers of MWNTs-CHIT, the sensitivity and reversibility of electrodes were improved [14]. However, no research about the effect of the number of MWNTs layer on the detection performance of AChE biosensor in the detection of pesticide residues has been reported.

Motivated by these observations, in this study, after a series of experiments and optimization of the optimal test parameters, a stable AChE biosensor was designed based on the immobilization of AChE onto the electrode surface modified with five layers of MWNTs-CHIT composite. Compared with other kinds of electrochemical AChE biosensor design, this method was simple, rapid and more sensitive for pesticide determination with a much lower detection limit [15, 16]. The proposed sensor showed acceptable stability and sensitivity, which had potential application in AChE-inhibitors (organophosphates or carbamates pesticides) analysis and environmental monitoring.

2. Materials and Methods

2.1. Apparatus

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

2.2. Reagents

Acetylcholinesterase (Type C3389, 500 U/mg from electric eel), and acetylthiocholine iodide (ATChI) were purchased from Sigma (St. Louis, USA). Carbofuran was obtained from solarbio Co. (Beijing,

China). Functional MWNTs was obtained from Nanotech Port Co. (Shenzhen, China). 0.1 M pH 7.0 phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 . CHIT (95 % deacetylation), ethanol and other reagents were of analytical grade. All solutions were prepared using double distilled water (DDW).

2.3. Preparation of Sensor

1.0 % (w/v) chitosan stock solution was prepared by dissolving 0.5 g chitosan flakes in aqueous solution of 50 mL 1.0 % acetic acid. 1 mg functional MWNTs was weighed and put into 0.3 mL absolute ethyl alcohol, after sonicating the solution, 1 mL 1.0 % chitosan solution was added in, and the miscible liquid should be sonicated for another 8 h. Eventually it will be stable black solution (MWNTs-CHIT composite), and the concentration of MWNTs was 0.769 g/mL [14]. Preparation was stored in a refrigerator when not in use.

A GCE was carefully polished to a mirrorlike with 0.3 μm and 0.05 μm Al_2O_3 paste and washed sonication with ethanol, nitric acid and DDW. Before modification, the electrode was applied a potential scanned from -0.6 to 1.0 V in 0.5 mol/L H_2SO_4 for 300 s until a steady-state curve was obtained. Activation of the GCE involved formation of a new phase containing a substantial amount of microcrystallinity and graphite oxide, thus increasing the hydrophilicity of the surface [17]. Then coated 3 μL of MWNTs-CHIT composite on a pretreated GCE dried it in air under natural condition for 4h, keeping the surface dry and clean, repeated the same work until there are five layers of MWNTs-CHIT composite. After being washed thoroughly with DDW, the electrode was coated with 7.0 μL AChE solution (100 mU), which was incubated at 20°C for 30 min to obtain the AChE/MWNTs-CHIT/GCE. Shown in Fig. 1. The obtained biosensor was stored at 4 °C when not in use.

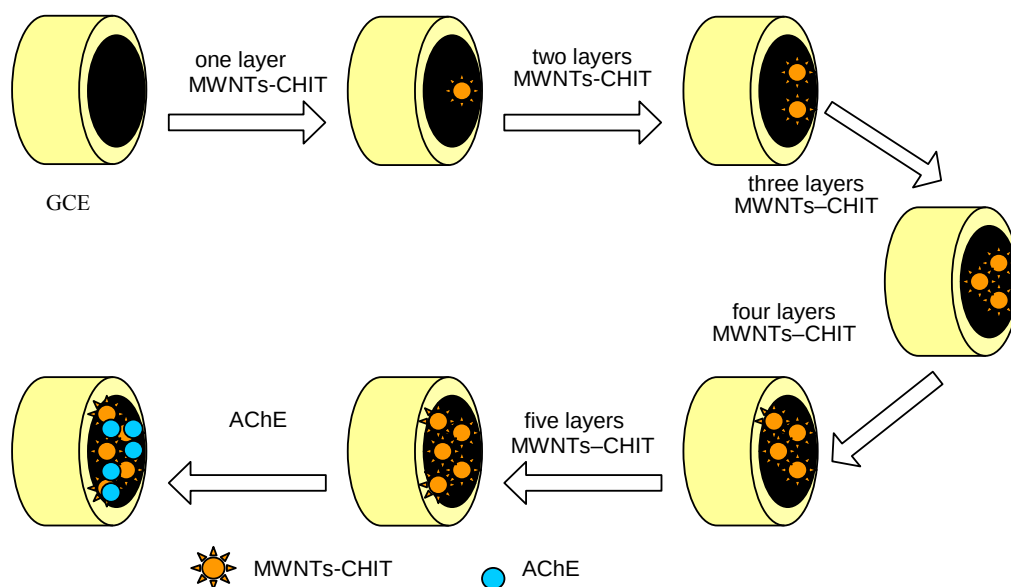


Fig. 1. Preparation of AChE/MWNTs-CHIT/GCE.

2.4. Electrochemical Detection of Pesticide

For the measurement of carbofuran, the obtained AChE/MWNTs-CHIT/GCE was first immersed in pH 7.0 PBS containing different concentrations of standard carbofuran solution for 10 min, and then transferred to the electrochemical cell of pH 7.0 PBS containing 2 mM ATChI to study the

electrochemical response by cyclic voltammetry (CV) between 1.0 and 0 V. The inhibition of pesticide was calculated as follows:

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

where, $i_{p, \text{control}}$ was the peak current of ATChI on AChE/MWNTs-CHIT/GCE without carbofuran inhibition, $i_{p, \text{exp}}$ was the peak current of ATChI on AChE/MWNTs-CHIT/GCE with carbofuran inhibition. Inhibition (%) was plotted against the concentrations of the carbofuran to obtain a linear calibration graph.

2.5. Preparation and Determination of Real Samples

The cabbage and lettuce bought from a supermarket were rinsed three times with double-distilled water. Then, different concentrations of carbofuran solution were sprayed on their surfaces. Seal with plastic wrap at 4 °C, after 24 h, 10 g of each sample were weight, chopped and meshed. After that, each sample was mixed with the mixed solution of 10 ml 0.1 M phosphate buffer (pH 7.0), which was obtained through 15 min of ultrasonic treatment. After the suspensions were centrifuged (10 min, 10000 rpm), the acquired supernatants were detected by CV directly without extraction or preconcentration. The content of carbofuran in the samples can be achieved from the calibration curve [15].

3. Results

3.1. Electrochemical Impedance Measurements

Electrochemical impedance spectroscopy (EIS) is a high effective method for probing the features of surface-modified electrodes [18]. Fig. 2 illustrated the EIS obtained from bare GCE (curve a), MWNTs-CHIT/GCE (curve b), AChE/MWNTs-CHIT/GCE (curve c) using $\text{Fe}(\text{CN})_6^{3-/4-}$ as the redox probe. A very big interfacial resistance on bare GCE was exhibited (curve a), after coated with MWNTs-CHIT on the electrode surface, we observed that resistance decreased obviously compared with the bare GCE (curve a). The phenomena likely because that the presence of MWNTs-CHIT could promote electron transfer due to their unique excellent and electronic properties. However, after AChE was immobilized on the electrode, the interfacial resistance increased remarkably (curve c) due to the increase of the thickness of the interface. This was the direct evidence of successful binding of enzyme on the electrode surface [19].

3.2. Electrochemical behaviour of AChE/MWNTs-CHIT/GCE

Fig. 3 shows the cyclic voltammograms of various electrodes in absence and presence of 2 mM ATChI in pH 7.0 PBS. No peak was observed at bare GCE electrode (curve a), MWNTs-CHIT/GCE (curve b) in pH 7.0 PBS. When 2 mM ATChI was added into PBS, the cyclic voltammograms of AChE/MWNTs-CHIT/GCE showed an irreversible oxidation peak at 800 mV (curve d). However, no detectable signal was observed at MWNTs-CHIT/GCE (curve c). Obviously this peak came from the oxidation of thiocholine, hydrolysis product of ATChI, which was catalyzed by immobilized AChE. The produced current by thiocholine was used as a quantitative measurement of the enzyme activity, which reflected the biological effect of carbofuran pesticide involved in the inhibition action [20]. Thus, the AChE/MWNTs-CHIT/GCE was used in following experiments of carbofuran determination.

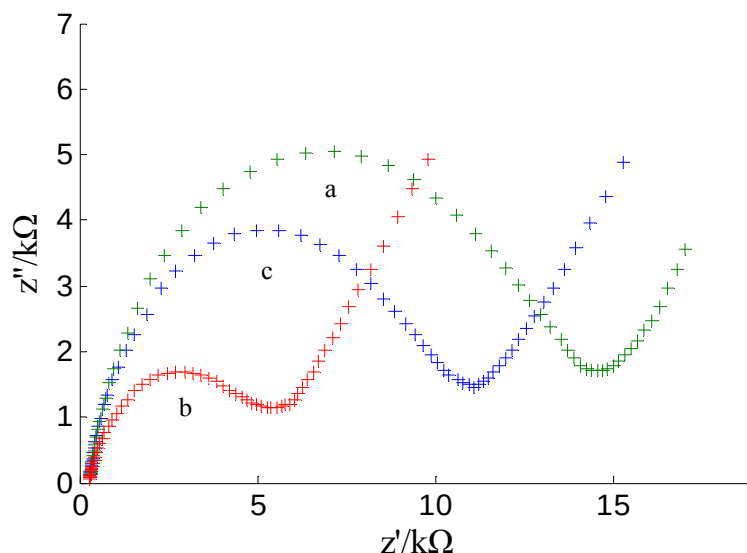


Fig. 2. Electrochemical impedance spectra of (a) bare GCE; (b) MWNTs-CHIT/GCE, (c) AChE/MWNTs-CHIT/GCE recorded in pH 7.0 PBS containing 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ and 0.1 M KCl.

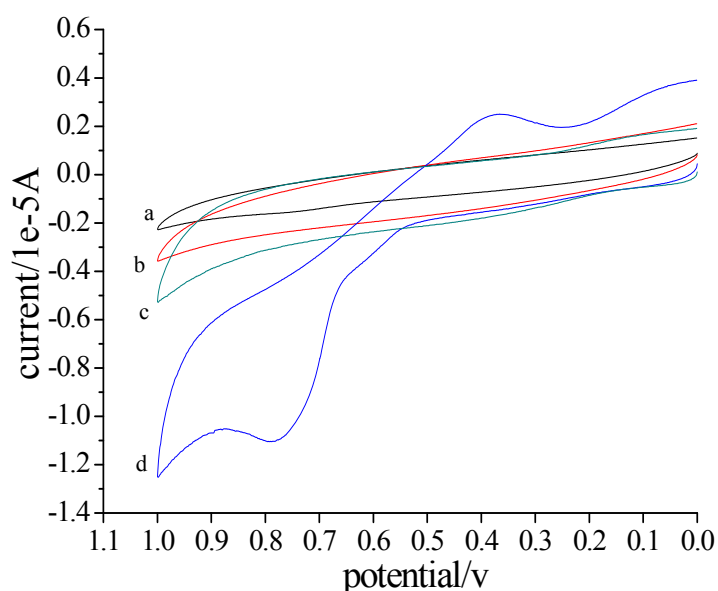


Fig. 3. Cyclic voltammograms: (a) bare GCE and (b) MWNTs-CHIT/GCE in pH 7.0 PBS; (c) MWNTs-CHIT/GCE and (d) AChE/MWNTs-CHIT/GCE in pH 7.0 PBS containing 2 mM ATChI. Scan rate: 50 mV/S.

3.3. Optimization Parameters of the Biosensor Performance

3.3.1. Influence of MWNTs-CHIT Composite Layers

The amount of MWNTs-CHIT composite layer was an important parameter influencing peak current. As shown in Fig. 4(A) the current of bare electrode is minimum (curve a), and it increased along with the increase of MWNTs-CHIT layer (curve b-e; g), but after the sixth layer was modified, it decreased (curve f). The results showed that, the increase of modified layer's number enhanced the electrochemical response of the modified GCE, when the number was five, the response was most sensitive. Therefore, five layers were selected for subsequent experiments.

3.3.2. Influence of Incubation Time on Inhibition

With an increase of immersing time (2-14 min at an interval of 2 min) in the 10 $\mu\text{g/mL}$ carbofuran solution, the inhibition of the enzyme was increased (up to 50 %). As shown in Fig. 4(B), carbofuran displayed increasing inhibition to AChE with immersing time. When the immersing time was longer than 10 min, the curve trended to a stable value, indicating the binding interaction between pesticides and active target groups in the enzyme reaches saturation. However, the maximum value of inhibition of pesticides was not 100 %, which is attributable to the binding equilibrium between pesticides and binding sites in the enzyme [21]. Thus, 10 min incubation time was used in subsequent experiments.

3.3.3. Influence of Detection Solution pH

For the electrochemical biosensors, the pH value was a crucial factor influencing the sensitivity and stability [22]. Fig. 4(C) showed the relation between the catalytic peak current of AChE to ATChI and solution pH. Obviously, the maximum peak current was obtained at pH 7.0 in the pH range from 5.5 to 8.5. The enzyme lost activity irreversibly at the lower or higher pH values. Therefore, PBS of pH 7.0 was selected for subsequent experiments.

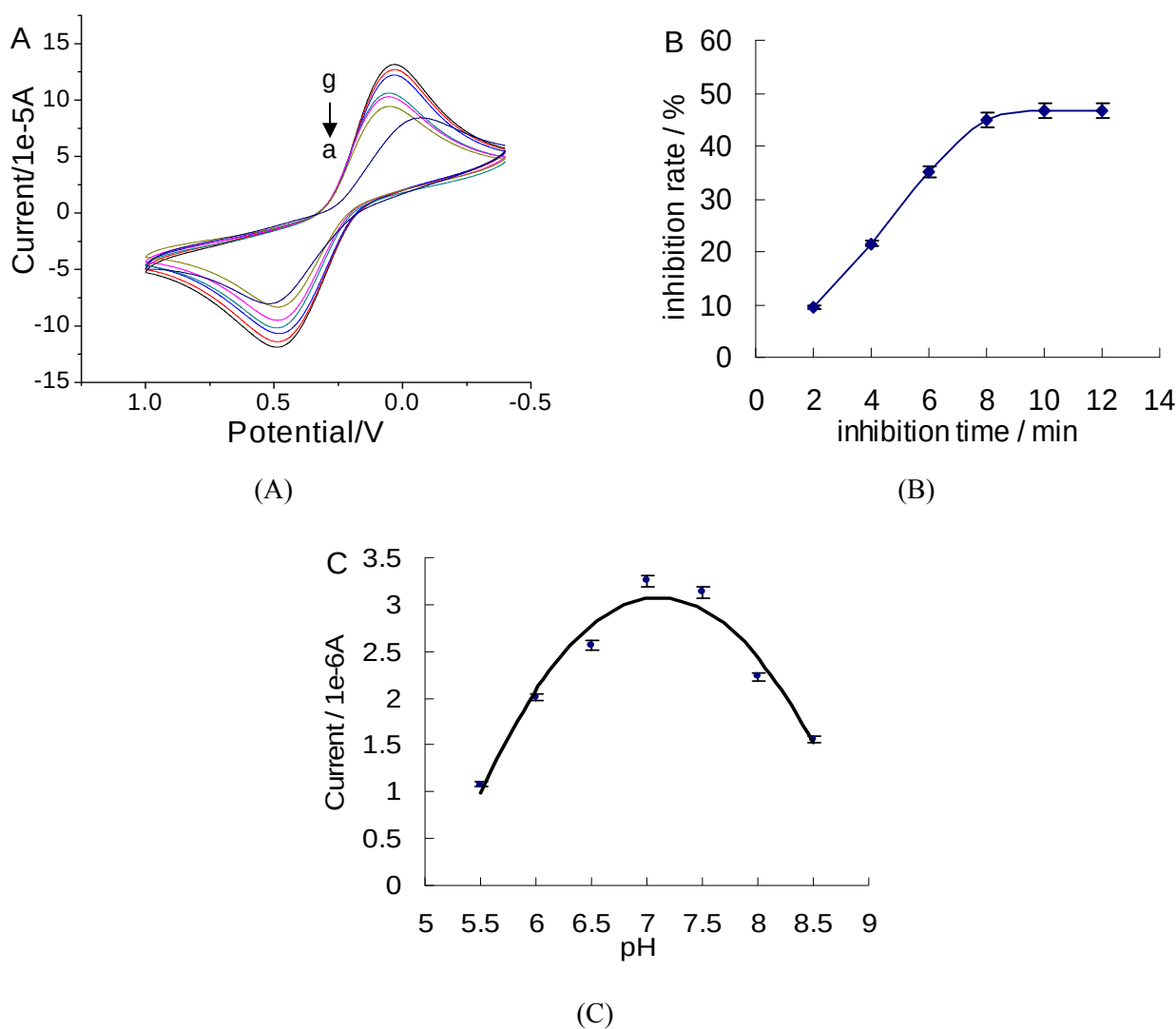


Fig. 4. (A) Cyclic voltammograms of GCE with different layers of MWNTs-CHIT in pH 7.0 PBS containing 5 mM $\text{Fe(CN)}_6^{3-/4-}$ and 0.1 M KCl. (a) GCE; (b) one layer; (c) two layers; (d) three layers; (e) four layers; (g) five layers; (f) six layers. (B) Relationship between inhibition rate and inhibition time. (C) Relationship between peak current and pH 5.5 to 8.5.

3.4. Detection of Carbofuran

As seen from Fig. 5, the peak current of 2 mM ATChI on AChE/MWNTs-CHIT/GCE after incubation with 0.05 $\mu\text{g/mL}$ carbofuran for 10 min decreased drastically (curve b) comparing with the control (curve a). The peak current observed in the simple electronic voltammetric sensing system reflected the activity of immobilized AChE and could be used for pesticide analysis.

Under the optimum conditions, determination of carbofuran has been achieved. The inhibition of carbofuran was proportional to its concentration in two ranges, from 5×10^{-4} to 7.5 $\mu\text{g/mL}$ and 7.5 to 20 $\mu\text{g/mL}$ (Fig. 6), the linear equation is $I\% = 3.759c + 17.528\%$ and $I\% = 1.085c + 39.863\%$, respectively, with the correlation coefficients of 0.9906 and 0.9943, respectively. The calibration sensitivity was 3.759 and 1.085 % $\mu\text{g/mL}$, respectively. The detection limit was 1×10^{-4} $\mu\text{g/mL}$ taken as the inhibition rate is 10 % in signal, which was significantly lower than those of 0.01 μM at GCE by using one layer MWNTs-CHIT composite modified electrochemical biosensor [13], and was also lower than the High Performance Liquid Chromatography (HPLC) method (1.7 $\mu\text{g/kg}$) [23], indicating that the proposed AChE/MWNTs-CHIT/GCE biosensor is reliable for the determination of pesticides.

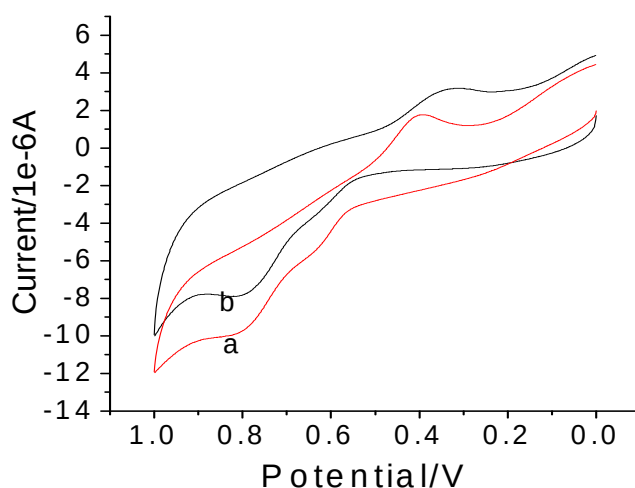


Fig. 5. Cyclic voltammograms of AChE/MWNTs-CHIT/GCE in pH 7.0 PBS containing 2 mM ATChI before (a) and after (b) inhibition with 0.05 $\mu\text{g/mL}$ carbofuran for 10 min. Scan rate: 50 mV/S.

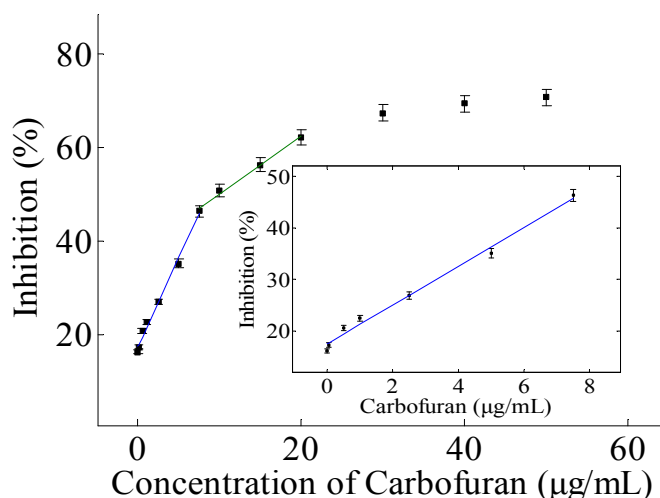


Fig. 6. Relationship between inhibition rate and carbofuran concentrations.

3.5. Precision of Measurements and Stability of Biosensor

The inter-assay precision was estimated through determining the response of 2 mM ATChI at six different electrodes, which were treated in 10 µg/mL carbofuran solutions for 10 min, respectively. The R.S.D. of inter-assay was found to be 3.5 %, indicating acceptable precision and reproducibility. When the enzyme electrode was not in use, it was stored in a refrigerator at 4°C in dry condition for 7 days, no obvious decrease in the response of ATChI was observed. After a 30-day storage period, the sensor retained 83 % of its initial current response, indicating acceptable stability of biosensor.

3.6. The Detection of the Real Samples

To further demonstrate the practicality of the proposed method, the recovery test was studied by adding different amounts of carbofuran into cabbage and lettuce samples. Results are summarized in Table 1. The recoveries were from 91.4 % to 105.0 %. The performance of this proposed biosensor was more stable than the performance of the enzyme-linked immunoassay [24], and better than that detection of carbofuran by the Ab/SiSG/GCE immunosensor [25]. The results indicated that the proposed method was highly accurate, precise and reproducible. It can be used for direct analysis of practical samples.

Table 1. Recovery studies of carbofuran in cabbage and lettuce samples.

Sample	Concentration		Recovery (%)
	Taken (µg/mL)	Found (µg/mL)	
Cabbage	0.1	0.0923	92.3
	0.5	0.477	95.4
Lettuce	0.1	0.105	105.0
	0.5	0.457	91.4

4. Conclusions

In summary, this paper presents an efficient and simple method for immobilization of AChE on five layers of MWNTs-CHIT composite modified electrode. The multilayer MWNTs-CHIT composite on the electrode provides a favorable environment to the AChE, electrocatalytic characteristics and fast electron transfer, increasing the sensitivity and facilitating the amperometric response of the sensor. The proposed method was sensitive, fast, showed good linear relationship, low detection limit and acceptable stability in determination of trace enzyme inhibitor. We are currently facing the disadvantage of the low selectivity of AChE because the degree of inhibition on AChE by pesticide is almost the same. However, this does not necessarily compromise its value when extended toward the preparation of other electrochemical sensors based on this mode. The ease of performance and the cost effectiveness of the system for carbofuran detection show a high potentiality in pesticide analysis.

Acknowledgements

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