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Acetylcholinesterase Biosensor for Chlorpyrifos Detection Based on Multi-Walled Carbon Nanotubes-SnO_2-chitosan Nanocomposite Modified Screen-Printed Electrode

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An acetylcholinesterase biosensor for organophosphate detection was developed immobilizing the AChE enzyme via MWCNTs-SnO_2-CHIT nanocomposite on screen-printed electrode. MWCNTs provide a flexible conductive film and a much larger pathway due to their high electrical conductivity and large porosity ratio, thus increasing detection sensitivity. SnO_2 decreased the peak voltage, revealed that the SnO_2 nano materials can promote the redox process. Based on the inherent conductive properties of the MWCNTs-SnO_2-CHIT, the immobilized AChE had greater affinity for ATCl and excellent catalytic effect in the hydrolysis of ATCl. Under optimized conditions, the proposed AChE biosensor exhibited sensitive and stable response for the detection of chlorpyrifos, ranging from 0.05 to 1.0×10^3 μg/L with a limit of detection down to 0.05 μg/L. The proposed biosensor was successfully applied in the determination of chlorpyrifos pesticides in cabbage, lettuce, leek and pakchoi samples, obtained acceptable recovery of 89.3~103.3%. With excellent stability, sensitivity, and simplicity, the proposed AChE biosensor showed a feasible quantitative method in detection of chlorpyrifos residues.

Keywords: biosensor; multi-walled carbon nanotubes; tin oxide; acetylcholinesterase.

1. INTRODUCTION

Organophosphates (OPs) have been widely used in modern agriculture due to their remanence and highly toxic [1]. OPs' intensive use in the environment is harmful for human health as they irreversibly inhibit the catalytic of acetylcholinesterase (AChE) by forming a stable complex in the active site of AChE [2]. Various methods have been reported for the detection of pesticide residues, including gas chromatography, high-performance liquid chromatography, and enzyme-linked immunosorbant assay (ELISA) [3-6]. These methods, however, require trained operators, expensive

instruments, and complicated pretreatment process, which is not applicable for on-site chlopyrifos determination. Biosensors have becoming the most excellent biochemical analysis technology due to their good stability and sensitivity, fast response, simple operation, and reproducible results [7, 8].

AChE biosensors have been used widely, the disposable biosensor based on screen-printed electrode has advantages of low cost and miniaturization, which can satisfy the increasing demand for point of care testing and on-spot test in environmental monitoring and industrial applications [9, 10].

For the fabrication of biosensor, the enzyme immobilization is a crucial step [11]. The inorganic oxide nano materials could largely enhance the electron transfer rate [12]. Among these inorganic oxide films, SnO_2 has been applied in the electrochemical fields due to its finer catalytic performance and electrical conductivity characteristics [13, 14].

Multi-walled carbon nanotubes (MWCNTs) are widely used in electrochemical biosensors due to their unique physical and chemical properties [15]. CNTs are consisted of cylindrical graphite sheets with many unique electronic and mechanical properties and they can enhance electron transfer rate on electrochemical reaction [16, 17].

Chitosan (CHIT), a kind of natural macromolecular biopolymer, belongs to the amino polysaccharide [18]. Chitosan (CHIT) has excellent characteristics such as biocompatibility, film-forming ability, high mechanical strength, and good water permeability, which had been used in immobilization of enzymes and the construction of biosensors [19].

In this paper, MWCNTs-SnO_2-CHIT nanocomposites were modified on screen-printed electrode to fabricate the AChE/MWCNTs-SnO_2-CHIT/SPE biosensor, which possessed wonderful stability and sensitivity. The proposed biosensor can be applicable for on-site chlopyrifos determination.

2. MATERIALS AND METHODS

2.1 Reagents

Acetylcholinesterase (Type C2888, 500 UN from electric eel), acetylthiocholine chloride (ATCl) and chlorpyrifos were purchased from Sigma (USA). MWCNTs (purity>95%) was purchased from Shenzhen Nanotech Port Company (China). SnO_2 were obtained from Sinopharm Chemical Reagent Co., Lid. and CHIT was from Shanghai Chemical Reagent Company (China). The 0.1 M pH 7.5 phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Other reagents were of analytical grade. All solutions were prepared using double distilled water.

2.2 Apparatus

Cyclic voltammetry (CV) measurements were performed with CHI660D electrochemical workstation (Shanghai Chenhua Co., China). The commercially available screen-printed electrode (TE100, working diameter was 3 mm) was purchased from Zensor R&D (Taiwan). All experiments were performed with a three-electrode system at room temperature (25±1℃). The morphologies of bare

screen-printed electrode, SnO_2-CHIT, MWCNTs-CHIT and MWCNTs-SnO_2-CHIT nanocomposite were observed by a scanning electron microscope (SEM, SIRION, FEI, Netherlands).

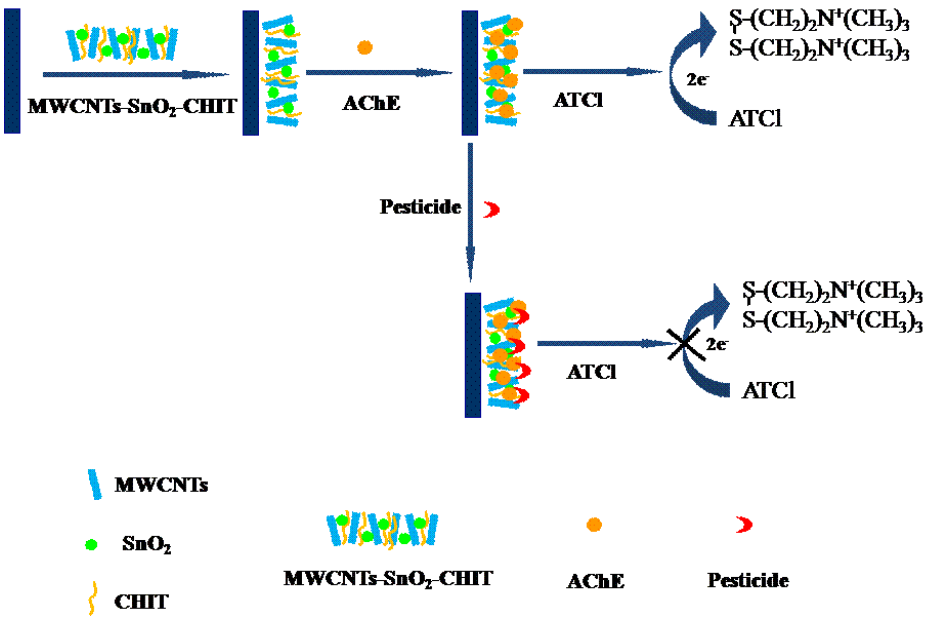
2.3 Determination of acetylcholinesterase activity

Before preparation of the amperometric biosensors, AChE activity was measured using the method described by Crew et al [20]. Using spectrophotometry at a wavelength of 412 nm, the thionitrobenzoate resulting from the reaction of DTNB with thiocholine (the product of the enzymatic hydrolysis of acetylthiocholine substrate) can be measured.

2.4 Preparation of AChE/MWCNTs-SnO_2-CHIT/SPE

Before the experiment, a potential of +1.75 V was applied to the bare SPE, 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 SPE was used for following experiments [21].

The SnO_2 nanoparticles and MWCNTs with a mass ratio of 1:3 were dispersed in 0.2% CHIT solution and stirred at room temperature for 3 h. The obtained highly dispersed black suspension would be named as MWCNTs-SnO_2-CHIT. A 7.5 μL of MWCNTs-SnO_2-CHIT suspension was coated on the screen-printed electrode surface and air-dried naturally to obtain MWCNTs-SnO_2-CHIT/SPE. Similarly, SnO_2-CHIT/SPE and MWCNTs-CHIT/SPE were prepared under the same procedure as illustrated in MWCNTs-SnO_2-CHIT/SPE preparation just without MWCNTs or SnO_2 existing, respectively.



Scheme 1. Schematic illustration of the stepwise AChE biosensor fabrication process and immobilized AChE inhibition in pesticide solution.

The obtained electrode (MWCNTs-SnO_2-CHIT/SPE) was washed thoroughly with ultra-pure water and then dried in air at room temperature. After the water was evaporated, the MWCNTs-SnO_2-CHIT/SPE was coated with 7.5 μL 0.02 U/μL AChE solution to obtain the AChE/MWCNTs-SnO_2-CHIT/SPE. The schematic illustration of the stepwise procedure of the biosensor fabrication was shown in Scheme 1.

2.5 Electrochemical detection of pesticides

The AChE/MWCNTs-SnO_2-CHIT/SPE biosensor was employed for the determination of chlorpyrifos using cyclic voltammetry (CV) method. The performance of the biosensor was tested by its CV response in pH 7.5 PBS solution containing 1.0 mM ATCl. Then the electrode was rinsed with distilled water and incubated in an aqueous solution containing the desired concentration of cholorpyrifos for 14 min. Finally, it was transferred into the 1.0 mM ATCl solution for CV measurements at the same condition. The inhibition rate of pesticides was calculated as follows:

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

For inhibition tests, the original CV signal ($I_{P,\,control}$) was first measured at 1.0 mM ATCl. Then the electrode was rinsed with water and incubated in an aqueous solution containing the cholorpyrifos for 14 min. After incubation, the residual signal ($I_{P,\,exp}$) was recorded at the same condition. Inhibition (%) was plotted against the concentrations of the cholorpyrifos to obtain linear calibration graphs.

2.6 AChE reactivation

After exposure to cholorpyrifos, the AChE/MWCNTs-SnO_2-CHIT/SPE was firstly washed with 0.1 M pH 7.5 PBS, then reactivated by immersing in 5.0 mM pralidoxime iodide for 12 min, and then transferred to 0.1 M pH 7.5 PBS containing 1.0 mM ATCl for CV analysis of the electrochemical response. The reactivation efficiency was calculated as follows:

$$R\ (\%) = (I_r / I_{P,\,control}) \times 100\% \quad (2)$$

Where, I_r was the peak current of 1 mM ATCl on AChE/MWCNTs-SnO_2-CHIT/SPE after 5.0 mM pralidoxime iodide reactivation.

3. RESULTS AND DISCUSSION

3.1 SEM characterizations of MWCNTs-SnO_2-CHIT composite film

The MWCNTs-SnO_2-CHIT was prepared by the sol-gel method [11] and investigated by SEM (Fig. 1). As seen from Fig. 1a, the surface of bare screen-printed electrode is granular and lamellar structure. Fig. 1b and Fig. 1c showed the morphology and structure of MWCNTs and SnO_2. Fig. 1d showed SnO_2 was uniformed distribution in MWCNTs and MWCNTs-SnO_2-CHIT was successfully coated on the surface of screen-printed electrode. They can form a continuous array of MWCNTs,

SnO_2 and CHIT on the electrode, providing necessary conduction pathways of electron-transfer and a beneficial microenvironment for immobilization of AChE.

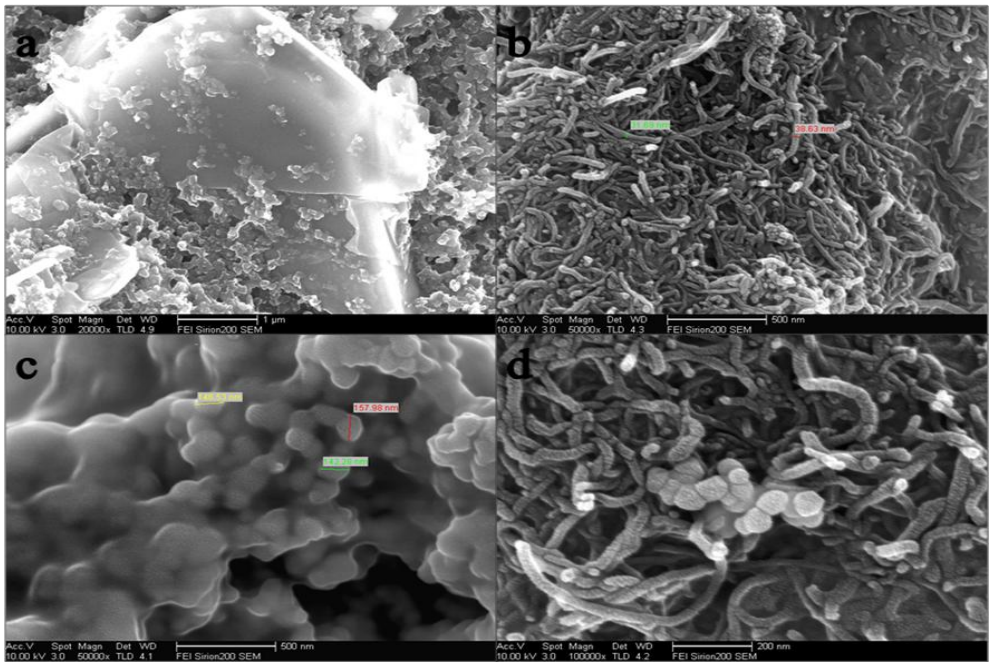


Figure 1. SEM images of (a) bare SPE; (b) MWCNTs-CHIT/SPE; (c) SnO_2-CHIT/SPE; (d) MWCNTs-SnO_2-CHIT/SPE.

3.2 Cyclic voltammetry characterization

Cyclic voltammetry (CV) was used to compare the electrochemical behavior of the nano-composites. CVs of bare SPE, SnO_2-CHIT/SPE, MWCNTs-CHIT/SPE, and MWCNTs-SnO_2-CHIT/SPE in 0.1M PBS (pH 7.4) containing 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol/L KCl were recorded. As shown in Fig. 2, there is a small reversible redox peak at the bare SPE (Fig. 2a). The peak voltage decreased after SnO_2 was modified on the surface of bare SPE (Fig. 2b), revealed that the SnO_2 film can promote the redox process. As seen from Fig. 2c, MWCNTs was modified on the surface of bare SPE, the peak current was increased again. At the MWCNTs-SnO_2-CHIT/SPE biosensor, the peak current of the electrode was extremely large enhanced after deposition of MWCNTs-SnO_2-CHIT on the surface of SPE (curve d). It showed that MWCNTs and SnO_2 could greatly improve catalysis and electron transfer, which increased peak current and decreased the potential and beneficial for avoiding interference from other electroactive species in biological matrix. Obviously, MWCNTs-SnO_2-CHIT/SPE is beneficial to the excellent conductivity and significantly increasing the value of the current response.

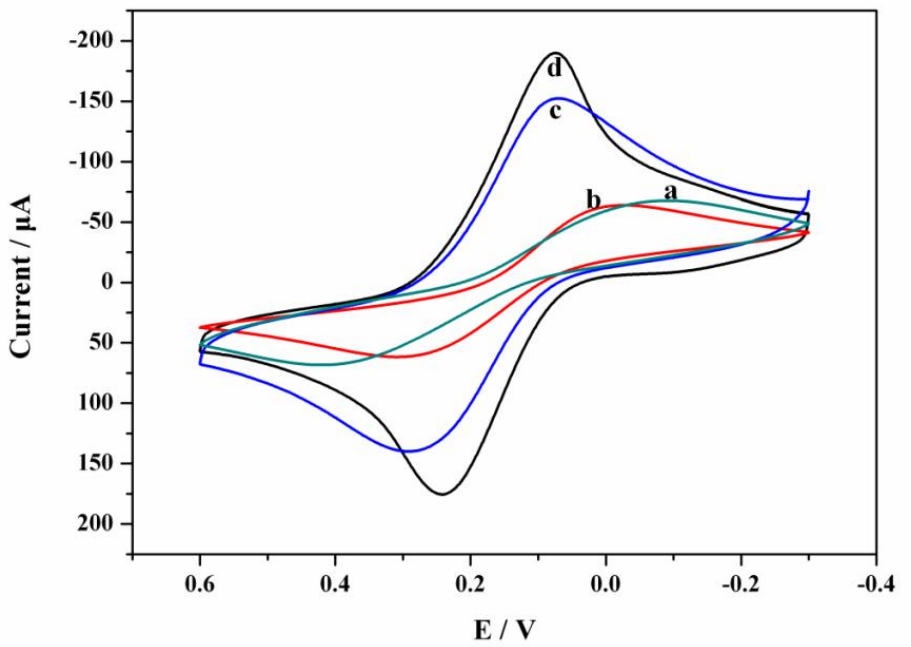


Figure 2. CVs of modified gold electrodes recorded in 0.1M PBS (pH 7.4) containing 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1mol/L KCl: (a) bare SPE; (b) SnO_2-CHIT/SPE; (c) MWCNTs-CHIT/SPE; (d) MWCNTs-SnO_2-CHIT/SPE.

3.3 Optimization parameters of the biosensor performance

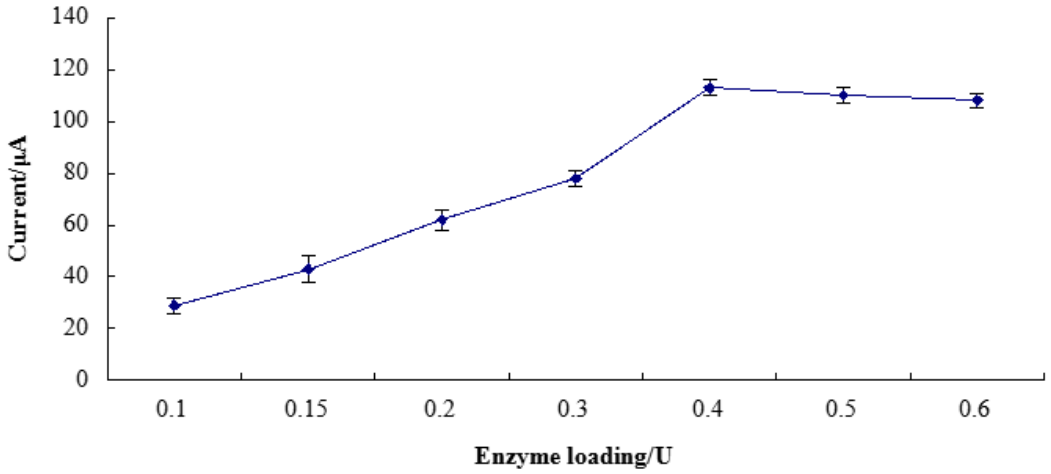


Figure 3. Influence of enzyme loading.

The enzyme amount plays a vital part in influencing the performance of the prepared biosensor The response currents of the enzyme electrode were coated with a series of enzyme amount (enzyme amount from 0.1 U to 0.6 U) in 0.1 M PBS (pH 7.4) containing 1 mM ATCl (see Fig. 3). When the amount of the AChE increasing less than 0.4 U, the current was immediately increased. After that, the oxidation peak current was gradually decreased as the amount of AChE further increased, which was due to the AChE thickening on the electrode surface and hindering electron transfer. Therefore, the optimal enzyme amount for the prepared biosensor was selected as 0.4 U.

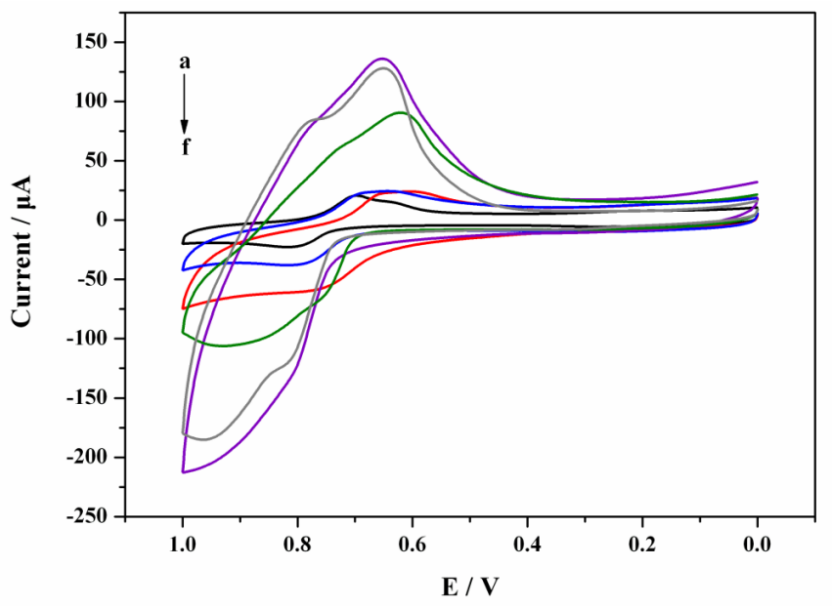


Figure 4. Influence of ATCl concentration: (a) 0.5 mM; (b) 1 mM; (c) 2 mM; (d) 5 mM; (d) 8 mM; (f) 10 mM.

To understand the affinity of immobilized AChE to its substrate, we first study the electrochemical response of AChE/MWCNTs-SnO_2-CHIT/SPE to ATCl using CV measurements. This value showed that the immobilized AChE on MWCNTs-SnO_2-CHIT/SPE matrix maintained its biocatalytic activity and created a more beneficial surface for the attachment of the AChE. Thus, ATCl can be enzymatically hydrolyzed by AChE to thiocholine, which could further be chemisorbed on the surface with an applied potential and giving a measurable peak current (Fig. 4).

With the scan rate increasing, the peak potential shifted slightly and the peak current increased (Fig. 5). The peak currents exhibited a linear dependence on the scan rates ranging from 5 to 200 mV/s (Fig. 5), indicating a typical surface-controlled electrode process.

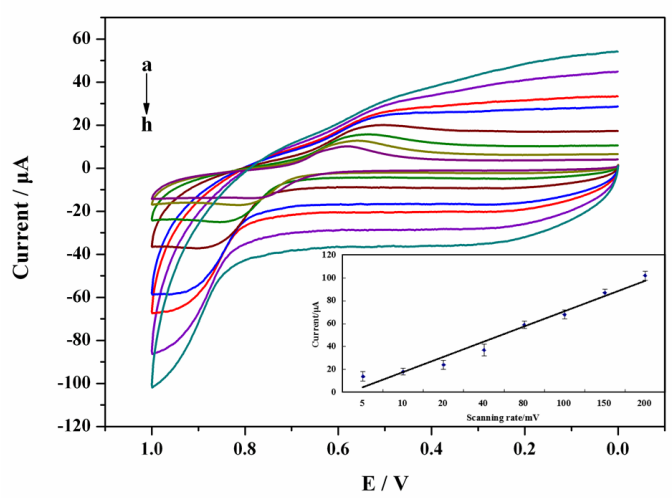


Figure 5. Influence of scanning rate: (a) 5 mV; (b) 10 mV; (c) 20 mV; (d) 40 mV; (e) 80 mV; (f) 100 mV; (g) 150 mV; (h) 200 mV.

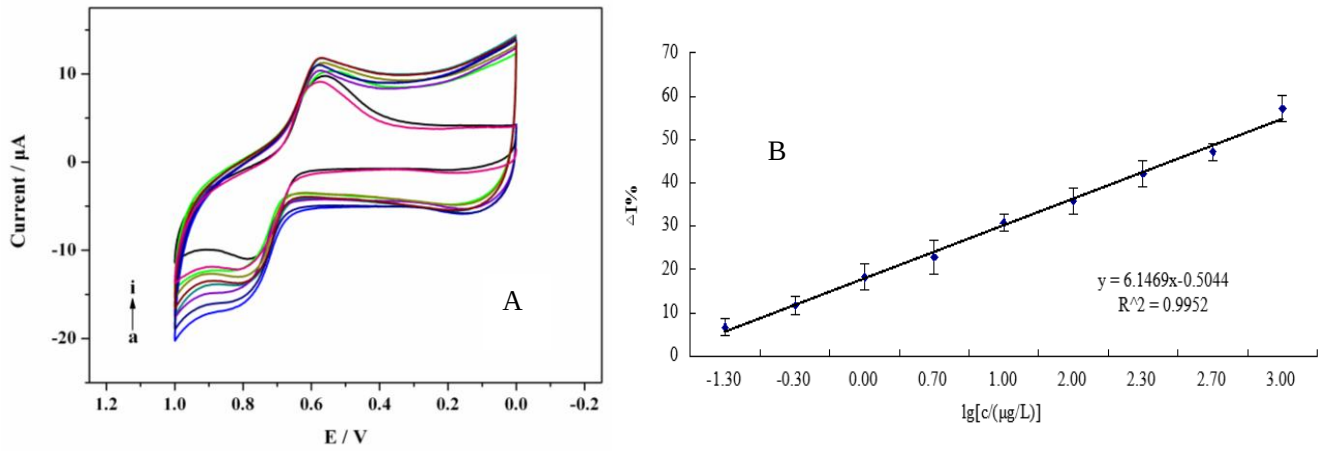


Figure 6. A. Relationship between inhibition rate and chlorpyrifos concentrations:(a) 0.05 μg/L; (b) 0.5 μg/L; (c) 1 μg/L; (d) 5 μg/L; (e) 10 μg/L; (f) 1×10^2 μg/L; (g) 2×10^2 μg/L; (h) 5×10^2 μg/L; (i) 1×10^3 μg/L; B. Relationship between inhibition rate and chlorpyrifos concentrations.

The CV responses were examined after inhibited to different concentration of pesticides (Fig. 6A). Chlorpyrifos was extensively examined as a sample of organophosphate pesticide. Under the optimal experimental conditions, the inhibition of chlorpyrifos on AChE/MWCNTs-SnO_2-CHIT/SPE was proportional to its concentration in the range of 0.05~1×10^3 μg/L (R^2=0.9952), with detection limit of 0.05 μg/L (Fig. 6B). The performance of this AChE/MWCNTs-SnO_2-CHIT/SPE compared with other reported biosensors was summarized in Table 1. The simple method based on enzyme-induced growth of AChE/MWCNTs-SnO_2-CHIT/SPE not only lower the detection limit but also improved the electron transfer in the analysis of enzyme inhibitor quantitatively. Therefore, AChE/MWCNTs-SnO_2-CHIT/SPE biosensor would be an excellent platform for the detection of pesticides.

Table 1. Comparison of the analytical methods for the detection of pesticides

Electrode	Liner range (μg/L)	Detection limit (μg/L)	References
AChE-CdS-G-CHIT/GCE	2-2000	0.7	[22]
AChE/PAMAN-Au/CNTs/GCE	1-20	0.89	[23]
AChE/TCNO/SPE	0.2-166	0.2	[24]
GC/MWCNT/CoPc	66-1322	1.10	[25]
AuNPs/GCE	3.95-23.99	0.99	[26]
AChE/PB-CHIT/GCE	2-80; 200-1000	0.60	[27]
AChE/MWCNTs-SnO_2-CHIT/SPE	0.05-1.0×10^5	0.05	This work

3.4 The determination of the real samples

Fresh vegetables bought from a local supermarket were chopped after removing the rotten leaves and dirt. A 2 g of each sample was added into 10 mL 0.1 M pH 7.5 PBS which was obtained through 3 min of ultrasonic treatment. After the suspensions were centrifuged (10 min, 10,000 rpm), the acquired supernatants were detected by CV directly without extraction or preconcentration. The concentration of pesticides in the samples can be obtained from the calibration curve. As seen from Table 2., the proposed biosensor was successfully applied in the determination of chlorpyrifos pesticides in cabbage, lettuce, leek and pakchoi samples, obtained acceptable recovery of 89.3~103.3%.

Table 2. The recovery of the proposed AChE/MWCNTs-SnO_2-CHIT/SPE biosensor in real samples

samples	Added(μg/L)	Found(μg/L)	Recovery(%)	RSD(%)(n=3)
samples	10	10.15	101.5	3.45
lettuce	10	9.23	92.3	4.36
leek	10	10.33	103.3	5.43
pakchoi	10	8.93	89.3	4.25

3.5 Storage stability

When the enzyme electrode was not in use, it was stored in a refrigerator at 4 ℃ in dry and hermetic surroundings. The response current of the sensor decreased to 97.8% after 7 days. After a 30-day storage period, the sensor retained 83% of its initial current response, which was much better than earlier report [28].

4. CONCLUSIONS

A low-cost screen-printed amperometric biosensor was developed in this study as a simple strategy to quantitatively for pesticides in real samples. Taking advantage of the screen-printed technology, AChE/MWCNTs-SnO_2-CHIT/SPE has been fabricated. Because of the synergistic effects of the SnO_2, MWCNTs and CHIT, the biosensor exhibited excellent performance in the merits of high sensitivity, good stability, good reproducibility, wide linear response range and short response time. Compared with the Nafion/AChE/MWCNTs-SnO_2-CHIT/Au [29], the presence of AChE/MWCNTs-SnO_2-CHIT/SPE biosensor not only exhibited a good conductive ability, but also increased the stability. Moreover, screen-printed electrode is more economical and portable than Aurum disc

electrode. Therefore, AChE/MWCNTs-SnO_2-CHIT/SPE can also be used for direct analysis of practical samples, which would be a new promising tool for pesticides analysis.

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