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A Simple Acetylcholinesterase Biosensor Based on Ionic Liquid/Multiwalled Carbon Nanotubes-Modified Screen-Printed Electrode for Rapid Detecting Chlorpyrifos

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A simple, low cost and sensitive electrochemical biosensor was developed for detection of chlorpyrifos, based on an acetylcholinesterase/multi-walled carbon nanotubes/ionic liquid composite modified screen-printed electrode (AChE/MWCNTs/IL/SPE). In this study, incorporating MWCNTs and IL formed a relative uniform microarchitecture on the electrode surface to promote electron transfer and enhance electrochemical response, hence, the fabricated biosensor exhibited obvious electrochemical signal in the redox probe $[Fe(CN)_6]^{3-/4-}$ during the detection. AChE as a biorecognition element was immobilized onto the successfully modified SPE, which enabled sensitive determination of chlorpyrifos owing to high affinity of organophosphate pesticides toward the AChE. The morphologies and electrochemistry behaviors of AChE/MWCNTs/IL/SPE were investigated by using cyclic voltammetry (CV) and scanning electron microscopy (SEM), respectively. Under optimal conditions, chlorpyrifos could be detected in the wide linear range of 0.05 -1.0×10^5 μg/L with a detection limit of 0.05 μg/L. The biosensor also showed other advantages such as convenient preparation, good reproducibility, excellent stability and high sensitive. Moreover, the biosensor could also be used for directly analysis of real vegetable samples, which would provide a new promising tool for pesticide residues tests.

Keywords: AChE; ionic liquid; multi-walled carbon nanotubes; chlorpyrifos

1. INTRODUCTION

Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate, CPF), an organophosphate insecticide (OPs), is widely used in modern agriculture, which is known as toxic

agricultural waste [1] and classified as restricted pesticide with strict maximum residue limited standard (MRLs) for use in farming in many countries [2]. It is generally known that the repeated application of CPF leads to its accumulation in food, water and soil, which would considerably impact safety of agricultural products and consumers' health, especially in developing countries [3-5]. As with other OPs, the CPF and its derivatives inhibit the acetylcholinesterase (AChE) by phosphorylation of its active site [6], which might result in a potentially poisoning [1]. Therefore, a sensitive, accurate, fast and cost-effective detection is of great importance.

According to previous literatures, the analytical methods reported for the detection of CPF included colorimetry [7], high performance liquid chromatography (HPLC) [8], gas chromatography (GC) [9] and high-performance liquid chromatography/mass spectrometry (HPLC/MS) [10], etc. Though these methods are sensitive and reliable, they have some kinds of limitations such as time consuming, need for expensive equipment and trained personnel, not adapted for in situ detection, and so forth. [11]. In order to make the process more convenient and easy during the pesticide determination, electrochemical biosensors have been proposed as alternative analytical tools for on-site testing [12]. Among them, amperometric biosensors based on the inhibition of AChE are particularly attractive due to their good selectivity, sensitivity, rapid response and miniature size [13]. The AChE immobilized on an electrode surface can catalyze the hydrolysis of acetylthiocholine chloride (ATCl) to produce the electro-active species thiocholine, and the thiocholine can produce oxidation current upon a certain applied potential to quantitative measure the enzyme activity [14,15]. The reaction equation are shown as follows [16]:

$$\text{Acetylthiocholine chloride} + H_2O \xrightarrow{\text{AChE}} \text{Thiocholine(red)} + \text{acetic acid} + Cl^-$$

$$2\text{Thiocholine(red)} \xrightarrow{\text{anodic oxidation}} \text{nthiocholine(ox)} + 2e^- + 2H^+$$

With the rapid development of nano manufacturing technology, multi-walled carbon nanotubes (MWCNTs) have been synthesized, which open new horizons to amplify the signal of biosensors [17,18]. In the past few decades, they have been effectively used as sensing materials [19] in electrochemical biosensors due to their attractive properties of high electrical conductivity, outstanding electrocatalytic activity [20-22] and good biocompatibility [19]. At the same time, on account of the excellent ionic conductivity, high chemical and thermal stability, low toxicity, low volatility and wide electrochemical windows [20,23-27], research on ionic liquid (IL) has also become one of the most rapidly growing fields. The ability of IL combining with carbon materials to form conductive composites has gain massive attention in the preparation of various sensors [27]. In the study of pesticide residue detection, multifarious biosensors have been fabricated by the nanocomposite (IL-carbon). For example, a biosensor modified with MWCNTs/IL-Au electrodes to detect paraoxon has been described; a direct CPF determination also was performed with IL-carbon nanotubes gels to coat carbon paste electrode (CPE) [6].

By the use of screen-printing technology, the screen-printed electrode (SPE) manufacture process shows apparent superiority of cheap, rapid, simple and factory production. Therefore, it has gained mass attention in electrochemical sensing [27]. Compared with conventional electrodes, SPE may be the most suitable for in situ analysis since they have design flexibility, reproducibility and

excellent conductivity [28,29]. However, to the best of my knowledge, the MWCNTs/IL-modified SPE for detecting CPF has never been reported until now.

In this paper, AChE and MWCNTs/IL were modified on SPE to construct a novel biosensor for CPF detection. The amperometric biosensor included three main components: SPE was chosen as the electrode material, which permit the biosensor to be disposed after a single use [30,31] and make the biosensor more portable; MWCNTs/IL nanomaterials, which grafted the advantages of individual, possessed big electrode surface active area and super conductivity [19,21,32] to increase the electron transfer rate at the SPE surface; and the AChE was immobilized onto modified electrode to build AChE biosensor for specific detection of CPF. The easy fabrication, less expensive, good repeatability and high sensitivity biosensor has a good practical value in the determination of CPF in vegetable samples.

2. EXPERIMENTAL

2.1. Apparatus

Cyclic voltammetry (CV) measurements were performed on a 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). The morphologies of all bare or modified SPEs were observed by a scanning electron microscope (SEM, SIRION, FEI, Netherlands). All experiments were carried out with a three-electrode system at room temperature (25±1°C).

2.2. Reagents and materials

Acetylcholinesterase (AChE, Type C2888, 500 UN from electric eel), acetylthiocholine chloride (AChCl) and chlorpyrifos (CPF) were supplied by Sigma (USA). Ionic liquid noctylpyridinum hexafluorophosphate (OPPF), multi-walled carbon nanotubes (MWCNTs, purity⩾ 95%) and Chitosan (CHIT) were purchased from Shanghai Chengjie Co., Ltd (China), Shenzhen Nanotech Port Company (China) and Shanghai Chemical Reagent Company (China), respectively. The 0.1 M pH 7.5 phosphate buffered saline (PBS) was prepared by mixing the solutions of NaH_2PO_4 and Na_2HPO_4. All other reagents were of analytical grade. All solutions were prepared using double distilled water.

2.3. Preparation of AChE/MWCNTs/IL/SPE

Before modification, SPE was pretreated according to a previous reported procedure [13,14]. Briefly, the SPE was rinsed carefully with ethanol and distilled water in an ultrasonic bath, and dried in N_2 atmosphere. A potential of +1.75 V was applied to the SPE, with stirring in pH 5.0 PBS for 300 s

and 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 the follow-up experiments.

To gain a homogeneous ionic liquid, 4 mg ionic liquid OPPF was dispersed in 4 mL absolute ethyl alcohol by ultrasonic agitation for about 6 h. CHIT solution (0.2%) was obtained by dissolving CHIT in an aqueous solution of 1.0% acetic acid (50 mL) and magnetic stirring for further 6 h. 2 mg MWCNTs were then added in 4 mL CHIT solution and sonicated at room temperature for 6 h to obtain a black suspension. These solutions were stored under refrigeration (4℃) when not in use.

6 μL of 1.0 mg/mL IL suspension solution was dropped gently on the surface of the SPE and dried with air. Next, 6 μL of MWCNTs suspension (0.5 mg/mL) was casted onto the IL/SPE electrode surface and air-dried naturally to obtain MWCNTs/IL/SPE. Then, the modified SPE was coated with 6 μL 0.02 U/μL AChE solution to obtain the AChE/MWCNTs/IL/SPE with the help of CHIT as the cross linking agents. After the water was evaporated, the electrode surface was washed thoroughly with PBS (pH 7.5) to remove unbound enzyme. The enzyme electrode was stored at 4℃ for further use. Under the catalysis of AChE, the substrate ATCl is converted into thiocholine and aceticacid. Thiocholine is oxidized by the applied voltage, and current will flow. When the pesticide is present, conversion of ATCl is decreased or even null. The schematic diagram of the proposed biosensor is shown in Figure 1.

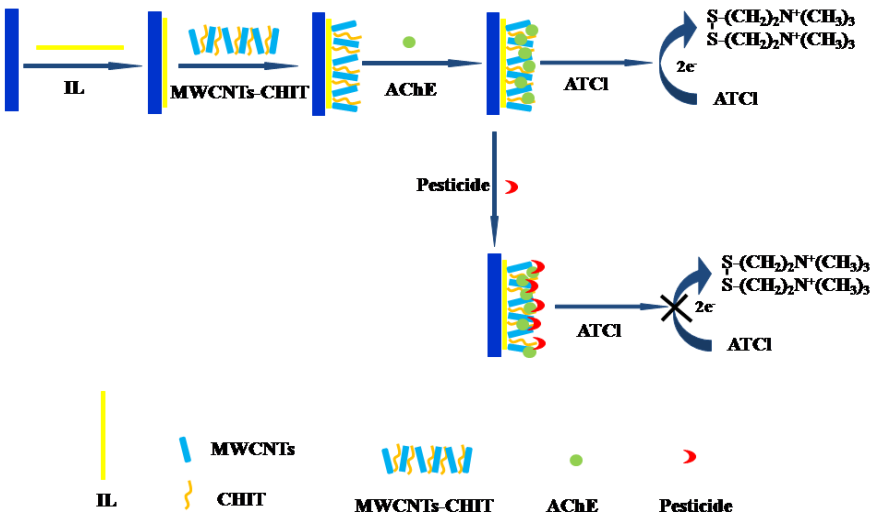

Figure 1. Schematic representation of the stepwise AChE/MWCNTs/IL/SPE biosensor fabrication and principle for pesticide determination

2.4. Electrochemical detection of pesticides

The AChE/MWCNTs/IL/SPE biosensor was employed for the determination of CPF. After the biosensor was immersed in a electrochemical cell of pH 7.5 PBS containing 1.0 mM ATCl, the cyclic voltammograms were recorded from 1.2V to 0V at a scan rate of 50 mV/s, which can investigate the properties of the biosensor. Then, the biosensor was incubated for 14 min in different concentrations of CPF solution. After that, the biosensor was rinsed with PBS and distilled water. Finally, it was

transferred to the cell for cyclic voltammetry measurement at the same condition. The inhibition rate of pesticide was calculated as follows [1,11,33]:

$$\text{Inhibition}(\%) = [(I_0 - I_i)/I_0] \times 100\%$$

Where, I_0 denotes initial peak current of ATCl on AChE/MWCNTs/IL/SPE, and I_i refers to the peak current of ATCl on AChE/MWCNTs/IL/SPE with CPF inhibition.

2.5. Sample preparation

Vegetable samples were bought from a local supermarket. 2 g of rapes were spiked with 10 μg/L of CPF. After they were left to dry for 4 h at room temperature, adsorbed CPF was extracted in mixed solution (1 mL acetone and 9 mL PBS), and then the measured degree of inhibition was compared with the calibration curve to quantify the CPF concentration in real sample.

3. RESULTS AND DISCUSSION

3.1. Morphological characterizations of the modified electrode

Scanning electron microscope (SEM) was used to characterize the morphologies of the films modified onto the SPE surface (Figure 2). From Figure 2a, we can see that the surface of bare SPE is isolated and irregularly shaped flakes, which would block the charges transfer along the vertical direction of planes. Figure 2b indicated that uniform and smooth surface has been achieved due to the IL being infiltrated into the void spaces of the bulk graphite of the SPE. It was clear that MWCNTs were in bundles with a disordered arrangement (Figure 2c), providing necessary conduction pathways of electron-transfer and a favorable microenvironment for immobilization of AChE.

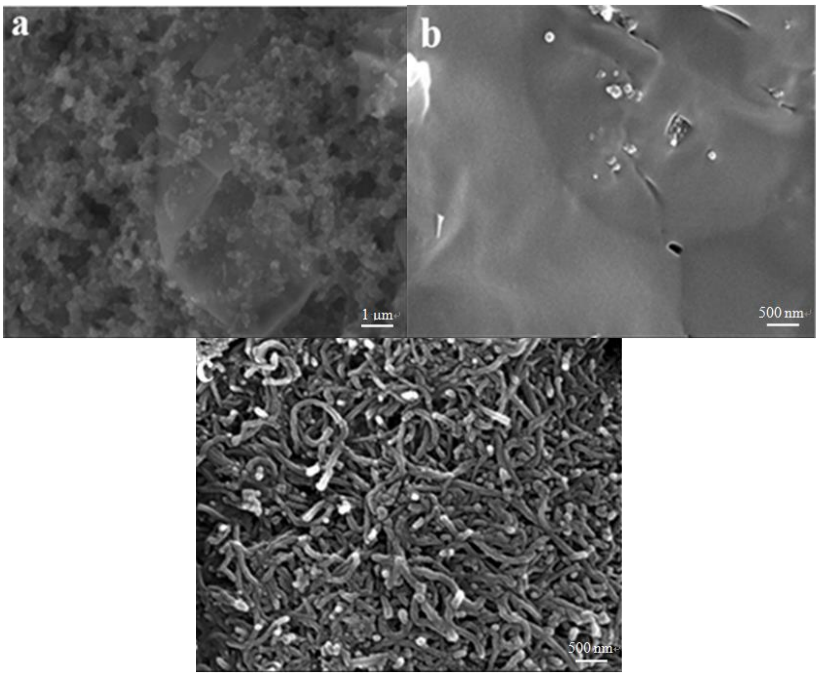

Figure 2. SEM images of (a) bare SPE; (b) IL/SPE; (c) MWCNTs/IL/SPE

3.2. Electrochemical characterizations of the sensing electrode

The cyclic voltammetric (CV) behavior of different sensing electrode in pH 7.5 PBS containing 1.0 mM ATCl was studied at a potential scan rate of 50 mV/s (Figure 3). There was a pair of well-defined redox peaks on the bare SPE with the anodic (Epa) and cathodic (Epc) peak potential of 0.47 V and -0.24 V, respectively (Figure 3a). The peak current increased obviously after IL was modified on the surface of bare SPE (Figure 3c), which could be ascribed to the high ionic conductivity. This result is consistent with previous literature [28]. MWCNTs were modified on the surface of bare SPE, the peak current was also increased (Figure 3d,). As seen from Figure 3e, amperometric signals of IL-MWCNTs on the SPE surface was higher compared to the electrodes modified with IL or MWCNTs. It indicated that the hybrid film of IL and MWCNTs can combine the advantages of all of them and greatly improve electron transfer. As reported previously, MWCNTs/IL can support extremely hydrophilic surface as sensitive layer and support for AChE immobilization [20]. Figure 3b showed that the redox peaks decreased drastically when AChE was cast on MWCNTs/IL/SPE, which may be due to the fact that most biological molecules (including enzymes) have poor conductivity and cause interference to the electron transfer [34]. This was also the direct evidence of successful binding of AChE on the electrode surface.

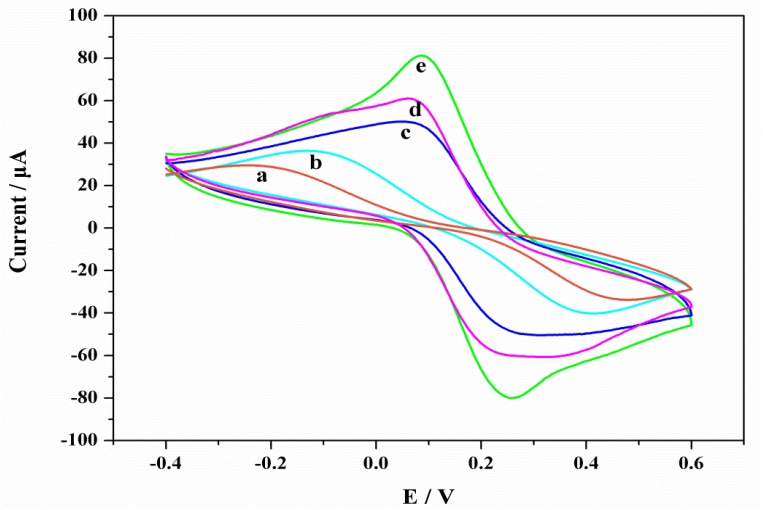

Figure 3. CVs for 0.1M PBS (pH 7.5) containing 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1mol/L KCl obtained with a (a) bare SPE; (b) AChE/MWCNTs/IL/SPE; (c) IL/SPE; (d) MWCNTs/SPE; (e) MWCNTs/IL/SPE. Scan rate: 0.05mV/s.

3.3. Optimization parameters of the biosensor performance

3.3.1. Optimization of phosphate buffer pH

The pH of the PBS plays an important role in affecting the performance of AChE/MWCNTs/IL/SPE. The fabricated biosensor was immersed in various pH (6, 6.5, 7, 7.5, 8) PBS including 1.0 mM ATCl to measure the current density response. As shown in Figure 4, the peak current kept rising from 6 to 7.5 and decreased as the pH value further increased.

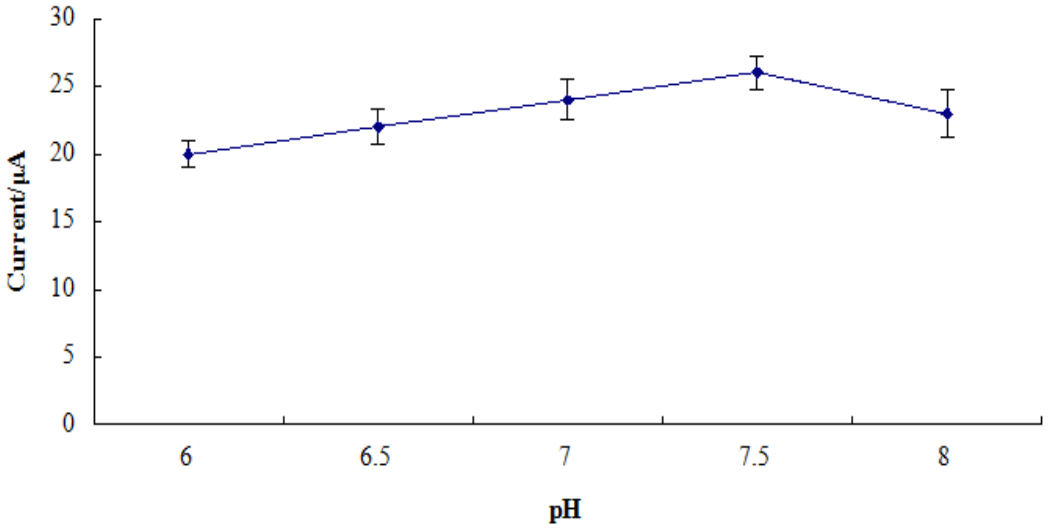

Figure 4. The influence of pH

The maximum value of the response current appeared at pH 7.5 and it is similar with other literature data [33]. Thus, a pH 7.5 of PBS was used in the subsequent experiments.

3.3.2. Optimization of load of AChE

Next the load of AChE was optimized to obtain the best performance of the biosensor. The current response was increasing with the increased of AChE amount from 0.05 to 0.25 U. When the AChE amount further increased, the obtained current signal showed slightly drop. Figure 5 showed that the peak current reached the maximal value at 0.25 U. The reason might be that the immobilized AChE was not enough stable when it more than 0.25 U, indicating a saturation of enzyme loading. Therefore, 0.25 U of AChE was chosen as the optimal amount for preparation of the biosensor.

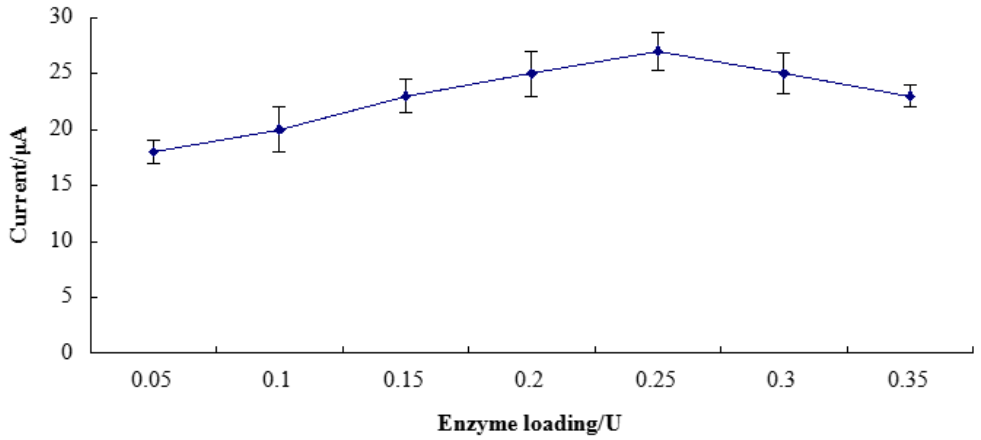

Figure 5. The influence of enzyme amount

3.3.3. Optimization of incubation time

The incubation time showed great influence on the changes of the peak current. The time is defined as the inhibition time of the CPF with AChE. Different inhibition times were tested in the range of 6-18 min. These results suggest that the peak current of ATCl on the AChE/MWCNTs/IL/SPE was descended greatly with the increase of incubation time. When the incubation time was longer than 14 min, the inhibition curve trended to a stable value (Figure 6), which showed that the interaction between CPF and AChE reached equilibrium [35]. Thus, the time of 14 min was the optimum incubation time. This value is in accordance with data reported by other reports [6,13].

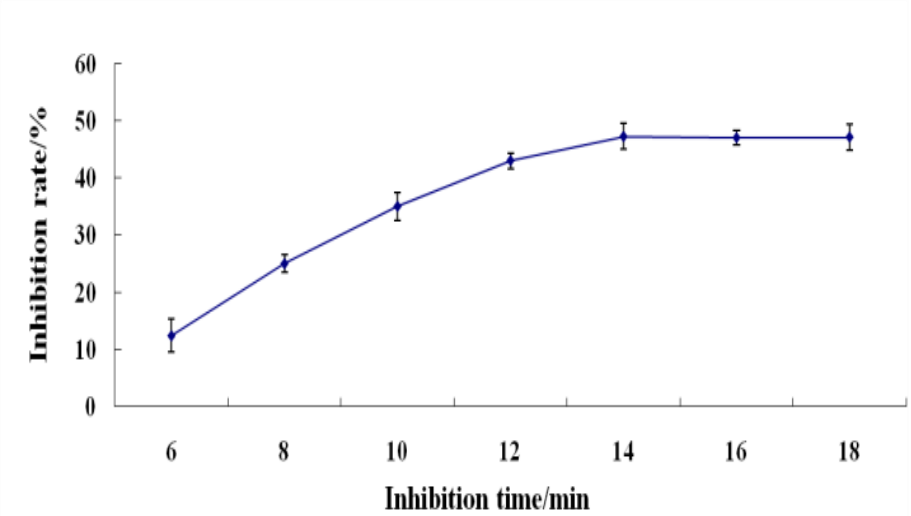

Figure 6. The influence of incubation time

3.3.4. Optimization of scanning rate

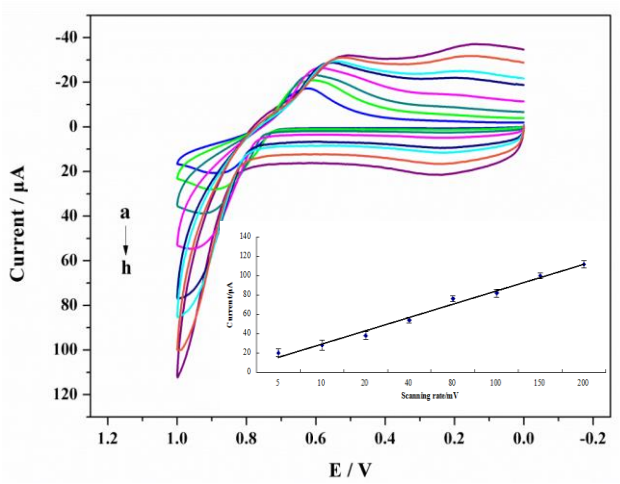

Figure 7. Cvs of AChE/MWCNTs/IL/SPE in pH 7.5 PBS containing 1.0 mM ATCl with different scan rates: (a) 5 mV; (b) 10 mV; (c) 20 mV; (d) 40 mV; (e) 80 mV; (f) 100 mV;(g) 150 mV; (h) 200 mV. Inset: the relationship between scan rates and peak currents.

CVs of AChE/MWCNTs/IL/SPE at different scan rates were recorded and shown in Figure 7. The results showed that with the increase of scan rates, the peak current increased and the peak potential shifted slightly, indicating that the electrode process was an irreversible process. Additionally, the peak currents vary linearly with the scan rate in the range from 5 to 200 mV/s, indicating that the electrode process was a surface control process [35]**.**

3.4. Chlorpyrifos determination

Under the optimal experimental conditions, different concentrations of CPF were determined by AChE/MWCNTs/IL/SPE with cyclic voltammetry. The biosensor was exposed to CPF standard solution of different concentration for 14 min, the produced CV response decrease with the increasing of CPF concentration (Figure 8, curve a-j). This was because that CPF can combine with active site of AChE and inhibit irreversibly the activity of AChE. Thus, the thiocholine from the hydrolysis of ATCl catalyzed by AChE decreased, which led to lower electrochemical response [15]. As illustrated in Figure 8B, the reduction current as a result of AChE inhibition was proportional to the log of CPF concentration in the range of 0.05-1.0×10^5 μg/L, and the linear regression equation was y=3.7794x+8.3039 (R^2=0.9926), in which y was % inhibition and x was the log of CPF concentration. The detection limit was 0.05 μ g /L, which is comparable to our previous studies [36].

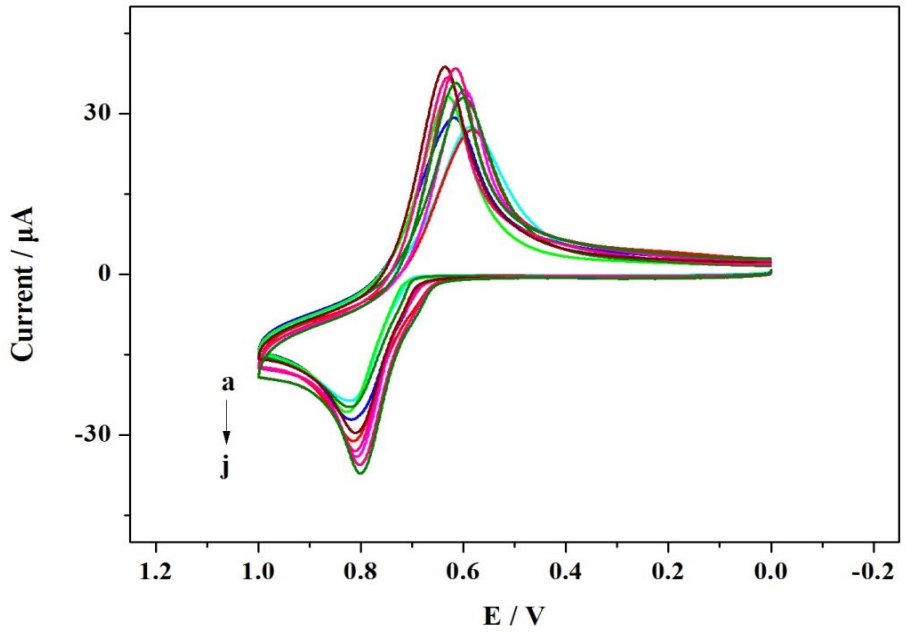

Figure 8(A). CVs of the AChE/MWCNTs/IL/SPE in pH 7.5 PBS containing 1.0 mM ATCl after incubation in different chlorpyrifos concentrations solution for 14 min: (a) 1×10^5 μg/L; (b) 1×10^3 μg/L; (c) 5×10^2 μg/L; (d) 2×10^2 μg/L; (e) 1×10^2 μg/L; (f) 10 μg/L; (g) 5 μg/L; (h) 1 μg/L; (i) 0.5 μg/L; (j) 0.05 μg/L. Scan rate: 0.05 mV/s.

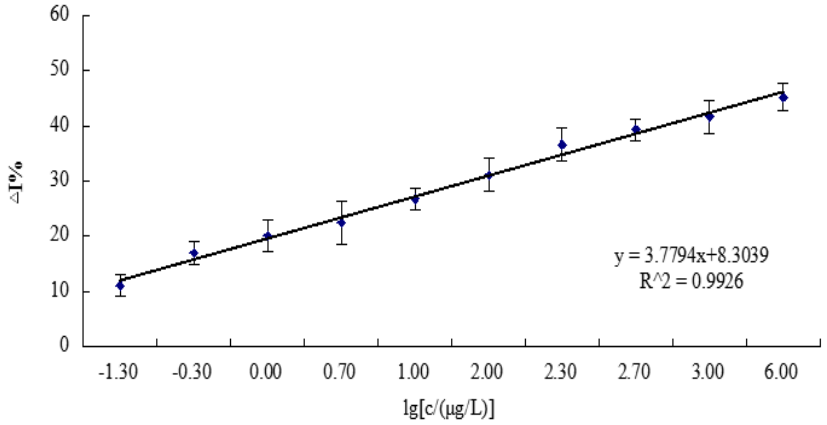

Figure 8(B). Relationship between inhibitions rate and chlorpyrifos concentrations

The performance of AChE/MWCNTs/IL/SPE compared with previously reported AChE biosensors for CPF test was summarized in Table 1. The results demonstrated that the proposed biosensor exhibited good analytical performance with a wider range and lower detection limit. The MWCNTs/IL composite not only improve the electron transfer rate, but also amplify electrochemical signal in the quantitative analysis of CPF, so the present method is higher sensitive. As a consequence, AChE/MWCNTs/IL/SPE biosensor would be an excellent platform for the detection of CPF.

Table 1. Comparison of the response of the fabricated biosensor with other biosensors for detection of chlorpyrifos

Electrode material analytical methods	Liner range (μg/L)	Detection limit (μg/L)	References
AChE-Pin5COOH/ZnS/AuE	0.53-14.02	0.53	[34]
CLDH–AChE/grapheme-AuNPs/GCE	0.05–150	0.05	[36]
AChE/Fc-F/GCE	1.75-262.9	1.05	[37]
AChE/L-Cys/HGNs/Chits/GCE	0.1-150	0.06	[38]
AChE/CMCS-AuNPs/GCE	0.1-100	0.07	[39]
AChE-[BMIM][BF_4]-MWCNT/CPE	3.5-350	1.4	[6]
AChE/MWCNTs/IL/SPE	0.05-1.0×10^5	0.05	This work

3.5. The detection of the real samples

For purpose of evaluating the validity of the present AChE biosensor, CPF concentrations in vegetable samples (rape) were detected by using the introduced biosensor and the conventional gas chromatography (GC). The results were presented in Figure 9, Table 2 and Table 3. Table 2 suggested that an excellent CPF recovery values ranged from 87% to 101%, demonstrating acceptable accuracy of the developed biosensor. Good agreement between present biosensor and GC method was obtained, indicating the practical applicability of the AChE/MWCNTs/IL/SPE biosensor for CPF detection and quantification in real samples.

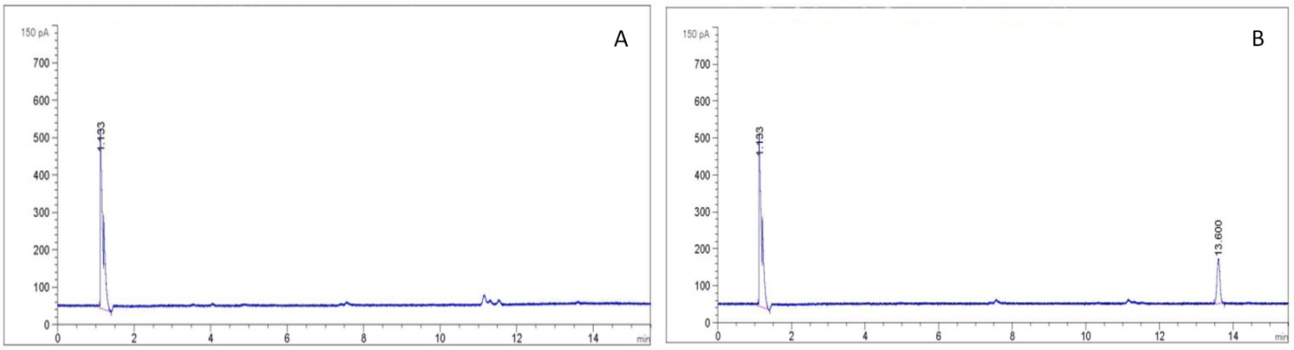

Figure 9. The GC chromatograms of rape sample: blank (A) and added with chlorpyrifos (B)

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

samples	Added (μg/L)	Found (μg/L)	RSD(%)(n=3)	Recovery (%)
cabbage	0	0	0	—
	100	101	4.35	101
rape	0	0	0	—
	100	87	2.57	87
lettuce	0	0	0	—
	100	98	3.17	98

Table 3. The detection results of GC

samples	Added (μg/L)	Found (μg/L)	RSD(%)(n=3)	Recovery (%)
cabbage	0	0	0	—
	100	98	1.73	98
rape	0	0	0	—
	100	81	2.51	81
lettuce	0	0	0	—
	100	94	1.98	94

3.6. Reproducibility and stability study

The reproducibility of the fabricated biosensor was evaluated using the PBS including 1 mM ATCl. To estimate the reproducibility of the intra-assay, the relative standard deviation (RSD) was calculated by assaying one enzyme electrode for six consecutive studies in PBS after being immersed in 100 μg/L CPF solution for 14 min, and the RSD was found to be 5.3%. Similarly, six different enzyme electrodes which were prepared under the same conditions were employed to study the inter-assay reproducibility, and the RSD was calculated as 7.6%. Results indicated the proposed biosensor exhibited good reproducibility with acceptable precision for the determination of CPF.

To study long-term stability, the biosensor responses were recorded during a span of 30 days in the presence of 1.0 mM ATCl. It was stored in a refrigerator at 4 ℃ in dry condition. The electrochemical response reached 97.8% from the initial value after one week, besides, after a month storage period, the response current of the biosensor still retained 83% of its stability. These results showed a good stability of the biosensor, which will help to increase the application for a longer period.

4. CONCLUSION

In this work, the AChE/MWCNTs/IL/SPE biosensor had been successfully fabricated for the detection of CPF. Compared with IL/SPE or MWCNTs/SPE, the experimental results showed that the MWCNTs/IL/SPE could effectively increase the electron transfer, improve the microarchitecture and exhibit a high electrocatalytic activity, which capable served as a classic and powerful tool for pesticide residues analysis. This disposable electrode made the AChE biosensor more suitable for field detection. In addition, because of the synergistic effects of the IL and MWCNTs, the biosensor exhibited excellent performances of high sensitivity, good stability and short response time. The linear range for the determination of CPF was from 0.05 μg/L to 1.0×10^5 μg/L and the detection limit was 0.05 μg/L. According to the results of studies in vegetables, the presented biosensor was a rapid, inexpensive and on-site method for CPF determination in practical samples.

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