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A Sensitive Acetylcholinesterase Biosensor Based on Screen Printed Electrode Modified with Fe_3O_4 Nanoparticle and Graphene for Chlorpyrifos Determination

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Biosensor based on enzyme inhibition for the determination of organophosphate pesticides residue was developed and fabricated, which explored to modify screen-printed electrode (SPE) with acetylcholinesterase (AChE) and a composite membrane of magnetic nanoparticle (Fe_3O_4) and graphene (GR). Due to the large specific surface area and high electron transfer of graphene and the strong absorbability and affinity of Fe_3O_4 for the phosphoric group, the nanocomposite film provided much active site and suitable microenvironment to improve the combination with AChE and maintain the enzymatic activity. The AChE/Fe_3O_4/GR/SPE was characterized by different methods including cyclic voltammetry and differential pulse voltammetry, indicating the biosensor had some excellent properties, such as high sensitivity, reproducibility and repeatability. Under optimum conditions, the inhibition rates of AChE were proportional to the concentrations of chlorpyrifos in the range from 0.05 μg/L to 100 μg/L with the coefficients of 0.981 and the detection limits were found to be 0.02 μg/L (S/N=3). This proposed biosensor exhibited high accuracy, reproducibility and regeneration, which was suitable for trace detection of chlorpyrifos residue in vegetable.

Keywords: Organophosphorus pesticide, Graphene, Fe_3O_4 nanoparticle, Acetylcholinesterase, Bisensor, SPE

1. INTRODUCTION

Food demand in developing countries is rising sharply in combination with the increasing population. To overcome the food shortages, a large amount application of fertilizers and pesticides

has been used to eliminate the pests and improve the production. The unreasonable and unscientific use of chemical pesticides can result in serious environmental problems and food pollutions, affecting the ecosystem balance and human being health [1]. According to statistics, the average consumption of pesticides in the world reaches 2 million tons per year, but pesticide use per unit area is much higher in less developed countries. Effective utilization of pesticides reported in China is about 30% and the rest of pesticide residues exist in the environment. As a class of pesticides, organophosphorus pesticides are a high neurotoxic compound [2, 3], which have been used throughout the world over the past few decades. Meanwhile, substantial amounts of residues accumulated in ambient environment, polluting soil, water, crops, livestock, fishery, fruit products [4] and further enriched into the human body through the food chain, indirectly threaten people's health [5]. Therefore, it is necessary to develop a discriminative and high sensitive technique for these toxicant substance determination in the past few decades.

Some spectrometric methods are relatively mature techniques and have been used to analyze organophosphorus pesticides by testing agencies, which contain UV-visible spectroscopy(UV) [6], gas chromatography (GC) [7], gas chromatography-mass spectrometry(GC-MS) [8], high performance liquid chromatography(HPLC) [9], liquid chromatography - tandem mass spectrometry(LC-MS/MS) [10, 11]. However, the devices of these methods mentioned above are high price, large volume and complex operation, which limits the widespread use on site. A simple, rapid and low cost method is desired to overcome the shortages of spectrometric methods. Biosensor [12], especially enzyme sensor [13], was one of the most economic and sensitive method for organophosphorus pesticide determination due to the activity of enzyme inhibited by organophosphorus pesticide. In the past few years, many enzyme sensors using metallic and carbonic materials [14, 15] had been investigated and reported, the price of which is little higher than SPE [16, 17].

Graphene[18, 19], one of carbon nano-materials in a wide research and application, owns bulk distinctive physical and chemical properties include extraordinary carrier mobility and capacitance, high electron transfer rate, excellent transparency, large surface-to-volume ratio, high thermal and chemical stability, robustness and flexibility, which is ascribe to its unique structure that is a two-dimensional nanosheet comprised of sp^2 bonded carbon atoms [20, 21]. All of these excellent properties made it an attractive candidate for the application of various novel sensors.

Magnetic nanoparticles (Fe_3O_4) [22, 23] is widely available material and easily synthesized that has been used in magnetic absorbing, magnetic recording, ferrofluid, catalyst and biomedical applications. Because it has many specific characteristics including large surface area, quantum size effect, high adsorption ability, good biocompatibility, strong super paramagnetic property, low toxicity and easy preparation, resulting in a broader range of applications in catalysis, biotechnology as well as analytical chemistry [24]. Various electrochemical biosensors decorated Fe_3O_4 nanoparticles have been reported, Balwinder Kaur [25] explored a novel biosensor using hybrid composite material of ionic liquid and Fe_3O_4 nanoparticle to determinate DNA bases.

In this work, we proposed a simple and low cost biosensor, which applied the use of a nano composite film of GR and Fe_3O_4 nanoparticles to modify SPE and then immobilized AChE on the surface of Fe_3O_4/GR/SPE. To improve electrochemical characteristics of this biosensor, some affecting factors were investigated and optimized by cyclic voltammetry and differential pulse voltammetry.

Finally, an electroanalytical method using AChE/Fe_3O_4/GR/SPE was established for chlorpyrifos determination in vegetables.

2. EXPERIMENT

2.1 Apparatus

Electrochemical measurements were collected by CHI660D electrochemical workstation (CHI Instrument Company, Shanghai, China). Three electrode system was selected and used for chlorpyrifos detection (the modified and unmodified screen-printed electrode as the working electrode, a bright platinum column as auxiliary electrode and a saturated calomel electrode as reference electrode). All experiments were performed at room temperature (25±1 °C).

2.2 Reagents and materials

Acetylcholinesterase (Type C3389, 500 U/mg from electriceel), acetylthiocholine chloride (ATCl), and chlorpyrifos were bought from Sigma-Aldrich Corporation (USA). Graphene was offered by XFNANO co., LTD (China). Nano-Fe_3O_4 was offered by Shanghai Aladdin biological technology co., LTD (China). NaH_2PO_4 and Na_2HPO_4 were obtained from were provided by National Standard Reference Materials Center of China. Chitosan was supplied by Shanghai Chemical Reagent Company (China). Phosphate buffer solution (0.1 mol/L) was selected as the supporting electrolyte for experiments, which was obtained by mixing the solutions of NaH_2PO_4 and Na_2HPO_4 together in definite proportions. Chitosan solution was prepared by adding and dissolving 0.2 g chitosan flakes into aqueous solution of 100 mL 1.0 % acetic acid. Other reagents were of analytical grade. Millipore-Q (18.2 MΩ) water was used for all experiments.

2.3 Preparation of ACHE/Fe_3O_4/GR/SPE

The fabrication and modification process of ACHE/Fe_3O_4/GR/SPE was shown in Fig.1. Firstly, SPE was pretreated using Millipore-Q water by ultrasonic processing and dried in the air. Then, 0.1 mg Fe_3O_4 was added into 10 mL 0.2 wt% chitosan solution and dispersed for a certain time to obtain a stable dispersed liquid by ultrasonic treatment. 0.5 mg graphene was ultrasonically dispersed in 5 mL dimethylformamide for 30 min to form a homogeneous ink. 4 μL GR solution was coated on the SPE surface and dried at room temperature to get GR/SPE. After the DMF was evaporated, the GR/SPE was spread with 2 μL Fe_3O_4-CS mixing solution, then put Fe_3O_4/GR/SPE in an oven of 80 °C for 30 min. After that, 10 μL AChE solution was dropped on Fe_3O_4/GR/SPE and incubated at 4 °C for 6 hours. Finally, 2.5 μL 0.5 % nafion solution was dipped on ACHE/Fe_3O_4/GR/SPE to maintain the stability of modified electrode.

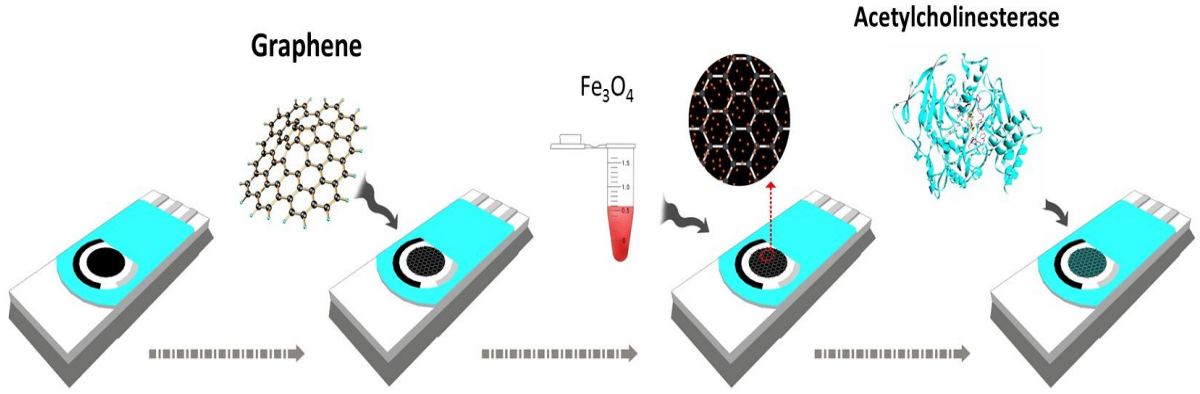


Figure 1. Fabrication and modification process.

2.4 Electrochemical detection of pesticides

Differential pulse voltammetry (DPV) was applied to determinate chlorpyrifos,(Increase E: 0.01 V; Amplitude: 0.05 V; Pulse Width: 0.2 s; Sample Width: 0.0167s; Pulse Period: 0.5 s; Quiet Time: 2 s). Firstly, ACHE/Fe_3O_4/GR/SPE was immersed into pH 7.5 PBS containing 0.5 mM ATCl and tested by the DPV. After that, the biosensor was rinsed with pH 7.5 PBS and incubated in an aqueous solution containing the desired concentration of cholorpyrifos for 20 min. Finally, this biosensor was dipped into pH 7.5 PBS containing 0.5 mmol ATCl and measured again at the same condition. The inhibition rate of ACHE/Fe_3O_4/GR/SPE was calculated as follows [26]:

$$\Delta I\ (\%) = (I_0 - I_1)\ /I_0 \times 100\%$$

Where ΔI was the inhabited rate, I_0 was the response current value of 0.5 mmol ATCl on ACHE/Fe_3O_4/GR/SPE without cholorpyrifos inhibited and I_1 was the response current value of 0.5 mmol ATCl on ACHE/Fe_3O_4/GR/SPE inhibited in the leaching solution of vegetable or different concentration of cholorpyrifos for 15 min.

2.5 AChE reactivation

With the aim of multiple usage and cost-saving, the activity of ACHE/Fe_3O_4/GR/SPE was recovered by pralidoxime iodide. The steps was described as follows: the surface of biosensor was rinsed mildly with pH 7.5 PBS after cholorpyrifos inhabited. Then, the electrode was immersed into an aqueous solution containing 5.0 mmol/L pralidoxime iodide for 20 min. After this recovery process, the biosensor can be reuse again. The reactivation efficiency is calculated as the following equation[26]:

$$\text{Recovery}\ (\%) = (I_2/I_0) \times 100\%$$

Where I_2 was the response current value on ACHE/Fe_3O_4/GR/SPE after 5.0 mmol/L pralidoxime iodide reactivation.

3. RESULTS AND DISCUSSION

3.1 Characters of ACHE/Fe_3O_4/GR/SPE

To investigate the properties of ACHE/Fe_3O_4/GR/SPE, the unmodified and modified SPE were characterized by some electrochemical methods.

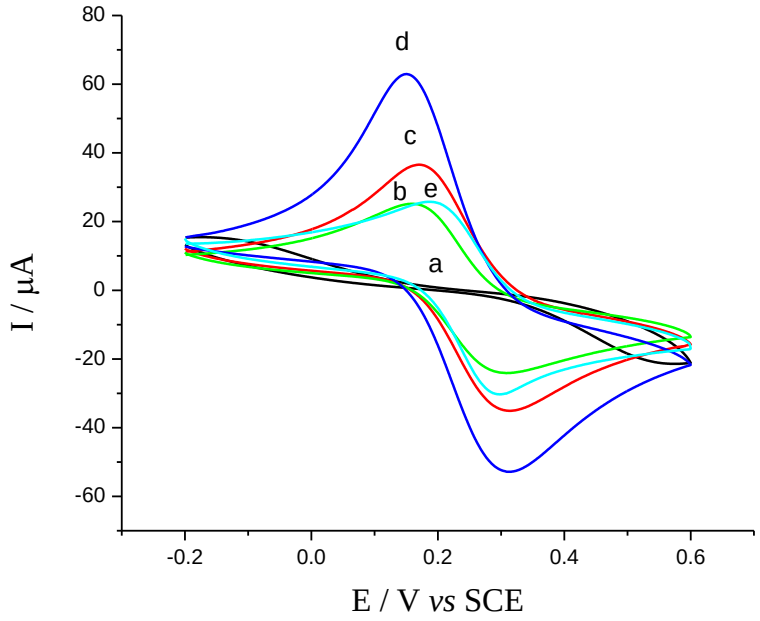


Figure 2. Cyclic voltammograms of different electrodes in 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol/L KCl solution with scan rate 50 mV/s. (a) SPE, (b)GR/SPE, (c)Fe_3O_4/SPE, (d)Fe_3O_4/GR/SPE, (e)ACHE/Fe_3O_4/GR/SPE.

Cyclic voltammograms of SPE before and after modification in 5.0 mmol/L $K_3[Fe(CN)_6]$ and 0.1 mol/L KCl solution show in Fig. 2. It is obvious that SPE (curve a) had a pair of weak redox peak currents with the peak-to-peak separation (∆E) of 600 mV, representing the sluggish electron transfer rate on the SPE's interface. After modifying GR on SPE (curve b), the redox peak currents increased and ∆E decreased in the view of the large specific surface area and good conductivity of graphene that bound to SPE' surface using π-π bond [27]. While on Fe_3O_4/SPE (curve c), the redox peak currents also increased, demonstrating that nano-material membrance covered on SPE can improve the surface area and conductivity because the presence of Fe_3O_4 have specific surface area and excellent conductivity for accelerating the charge transfer[28]. The highest redox peaks at the Fe_3O_4/GR/SPE (curve d), which was ascribed to the effective integration of individual advantages of GR and Fe_3O_4 that revealed synergistic effect for an electrochemical response. For the non-conductive property of protein, the redox peak currents became much weaker after modified AChE on the surface of Fe_3O_4/GR/SPE (curve e), which illustrated that AChE film has formed on the surface of Fe_3O_4/GR/SPE [29].

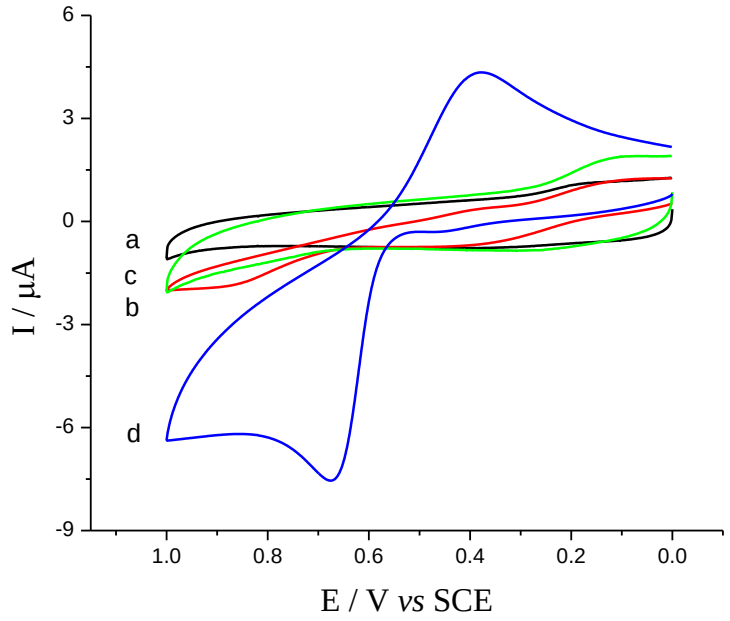
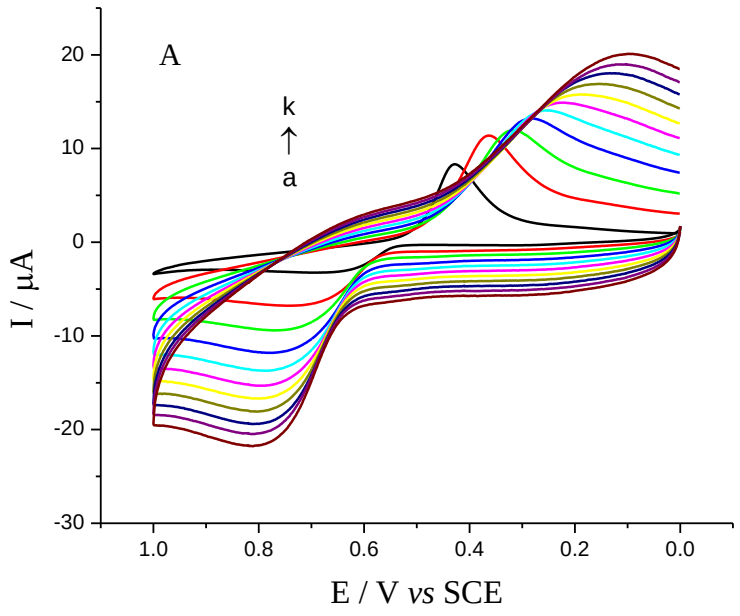


Figure 3. Cyclic voltammograms of different electrodes in pH 7.5 PBS (a)Fe_3O_4/GR/SPE, (c)ACHE/Fe_3O_4/GR/SPE and presence of 0.5 mM ATCl in pH 7.5 PBS: (b)Fe_3O_4/GR/SPE, (d)ACHE/Fe_3O_4/GR/SPE. Scan rate: 50 mV/s;

As shown in Fig. 3, no current response was found at 0.7 V when Fe_3O_4/GR/SPE was employed to measure phosphate buffer solution with 0.5 mmol/L ATCl (curve c) or without (curve a). The main reason is that Fe_3O_4/GR/SPE without AChE cannot catalyze the hydrolysis of acetylcholine chloride into acetylcholine that can determinate by the electrochemical electrode.



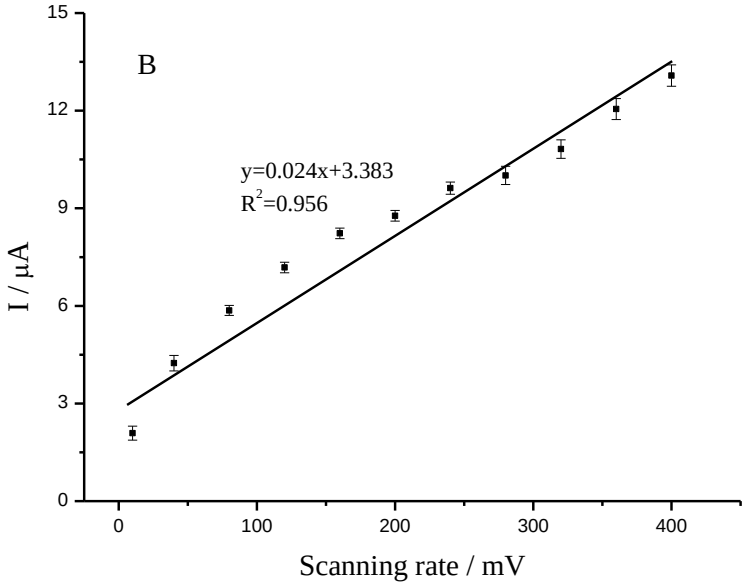


Figure 4. (A) Cyclic voltammograms of 0.5 mmol/L ATCl with different scan rate on AChE/Fe_3O_4/GR/SPE from a to k are 10, 40, 80, 120, 160, 200, 240, 280, 320, 360 and 400 mV/s respectively; (B) Linear relationship of peak current (I) versus scanning rate (*v*)

A small peak current was found on AChE/SPE (curve d), indicating that acetylcholine chloride was catalyzed and hydrolyzed but the low sensitivity of SPE was difficult to detect the low concentration of acetylcholine. While on ACHE/Fe_3O_4/GR/SPE, the current response increased sharply on the electrode, which was estimated that the synergistic effect between Fe_3O_4 and GR promote the electron transfer rate and sensitivity of the prepared electrode.

The relationship between scan rate and peak current was investigated in pH 7.5 PBS containing 0.5 mM ATCl on ACHE/Fe_3O_4/GR/SPE and the results shown in Fig. 4. As depicted in Fig. 4(A), it can easily get that peak current was in good positive relationship with scanning rate in the range from 10 mV/s to 400 mV/s, presenting a good linear relationship. The linear regression equations between peak current and scanning rate were I (μA) = 0.024 *v* (*v*: V/s) +3.383(r=0.977) in Fig. 4(B). It also demonstrated that the electron transfer reaction was surface process control in the solution [30].

The relationship between enzyme amount and the peak current of ACHE/Fe_3O_4/GR/SPE was investigated shown in Fig. 5. The results showed the peak current with the increasing of the AChE amount increased gradually from 0.2 U to 1.0 U and obtain the maximum current at 1.0 U. But the amperometric response decreased when the amount of AChE further increased. The reason that the membrane of AChE thickens can increase the electron transfer distance and affect the interface diffusion of acetylcholine [26]. Therefore, 1.0 U of AChE was the optimal enzyme amount.

The performance of biosensor is closely related to the pH value of electrolyte solution. As shown in Fig.6, the peak currents of AChE/Fe_3O_4/GR/SPE were studied in different pH values of PBS containing 0.5 mmol/L ATCl. The peak current increased gradually with pH value from 6.0 to 7.5 and had an opposite tendency when pH value exceeded 7.5. This is mainly because the activity and

immobilization of AChE can be easily restrained or destroyed in overly acidic or alkaline solution [26]. The result is also very similar to the reported pH values of other biosensor [31, 32]. In order to keep the bioactivity of AChE and obtain higher response, the optimum value of 7.5 was selected.

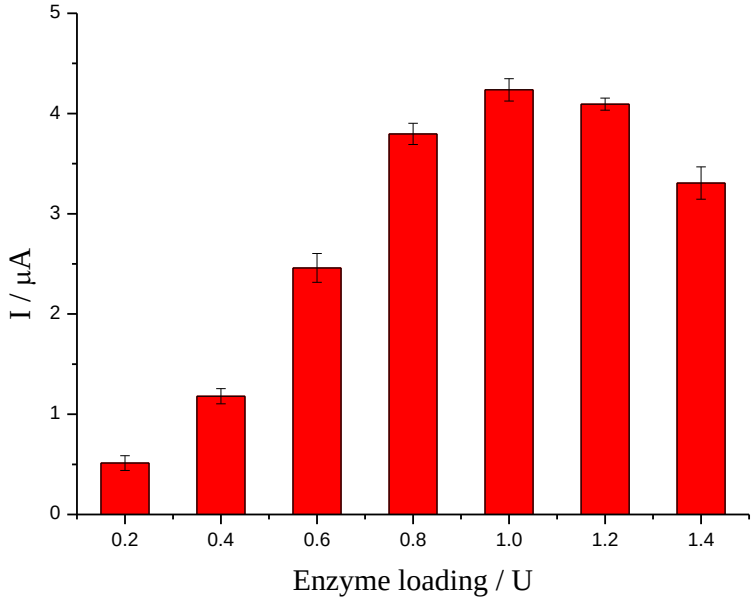


Figure 5. Influence of enzyme loading in pH 7.5 PBS containing 0.5 mmol/L ATCl.

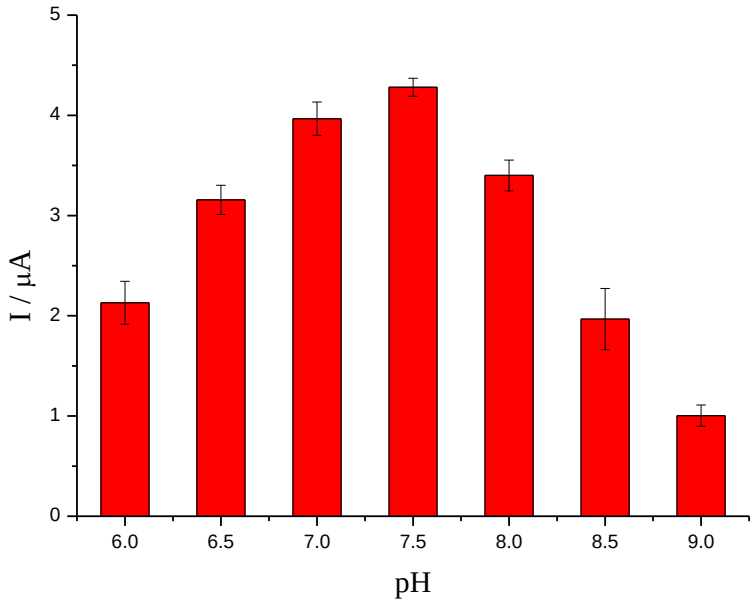


Figure 6. The relationship between pH and peak current of AChE/Fe_3O_4/GR/SPE in 0.1 mol/L PBS containing 0.5 mmol/L ATCl.

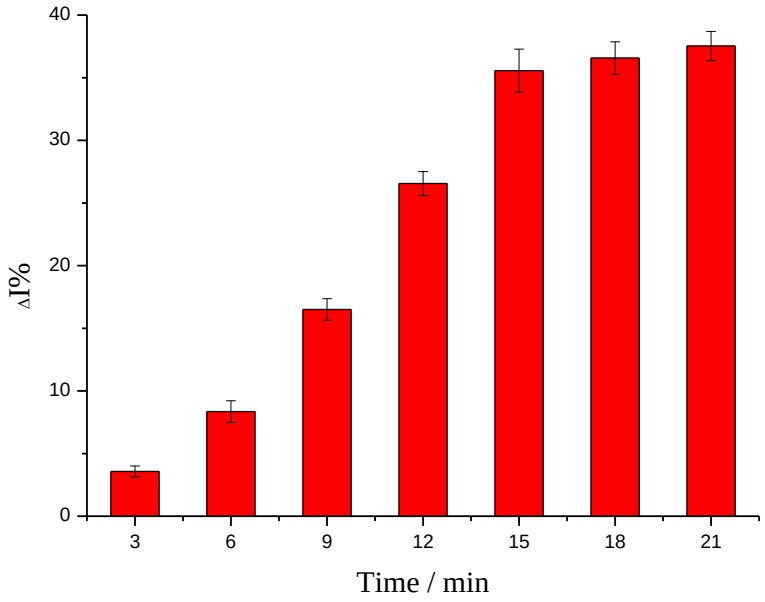
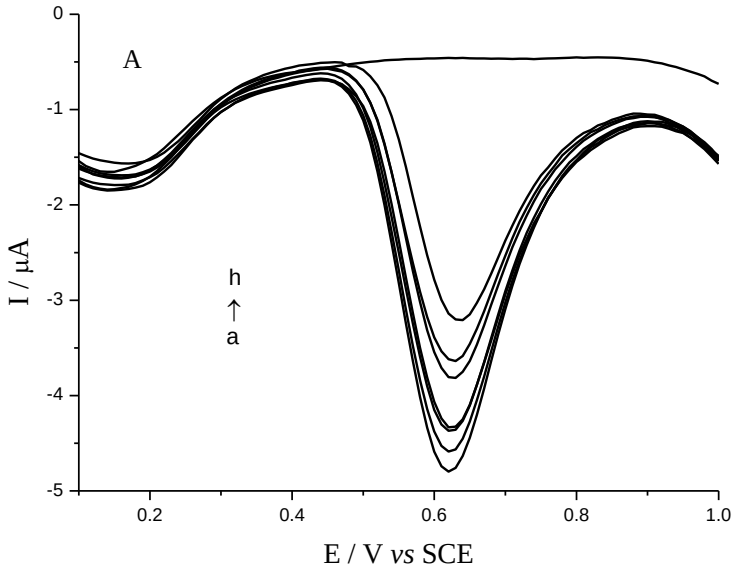


Figure 7. Effect of incubation time on AChE/Fe_3O_4/GR/SPE incubated with 100 ug/mL chlorpyrifos response by the relative change in peak current of DPV (ΔI%) in pH 7.5 PBS containing 0.5 mmol/L ATCl.

The inhibition time is one of the most critical influences on response current. The inhibition ratio of AChE/Fe_3O_4/GR/SPE is studied in range from 3 min to 21 min. From Fig. 7, the inhibition rate was obviously proportional to the inhibition time in certain range from 3 min to 15 min. However, the inhibition rate increased slightly even the deposition time was longer than 15 min due to the binding interaction between chlorpyrifos and active target groups on AChE reached saturation [33]. In consideration of the measure efficient and time, 15 min was selected as optimal inhibition time.



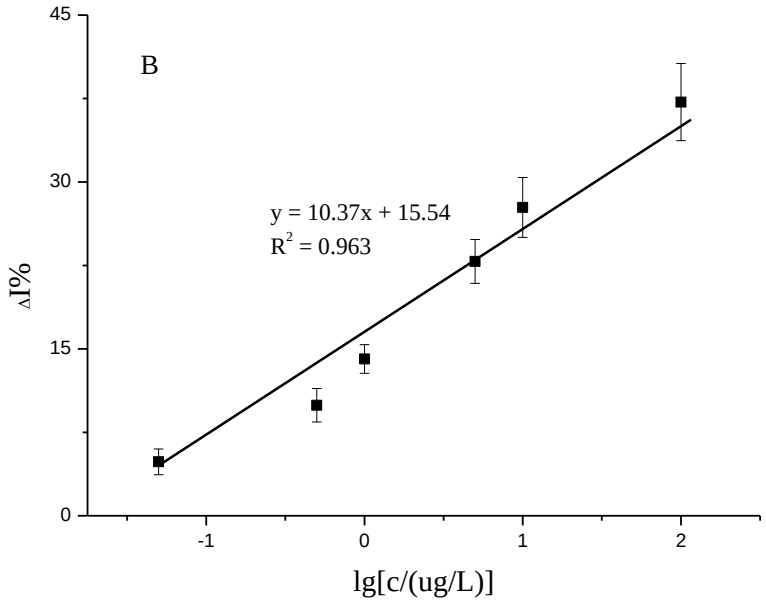


Figure 8. (A) Relationship between inhibition rate and chlorpyrifos concentrations from a to g: 0, 0.05, 0.5, 1.0, 5.0, 10.0, 100.0 μg/L; (B)The calibration curve of the relative change in peak current of DPV (ΔI%) of AChE/Fe_3O_4/GR/SPE versus the logarithm of chlorpyrifos concentration

The DPV was used for quantitative determination, which was examined before and after inhibited the biosensor in different concentration of chlorpyrifos under the optimal conditions. From Fig. 8(A), the peak currents of DPV were found at 0.65 V. The values of peak current had a certain functional relation between the inhibition rate of AChE and the content of chlorpyrifos in the view that the serine hydroxyl groups of AChE can be blocked by covalently binding of pesticides [34]. As shown in Fig. 8(B), a linear relationship was found in the range from 0.05 μg/L to 100 μg/L with the linear regression equations as ΔI %=10.37*lgC* (μg/L)+15.54 (r=0.981) and the detection limit was estimated to be 0.02 μg/L(S/N=3).

Table 1. Comparison of performances of different biosensors for pesticide detection

Electrode	Analyte	Liner rang (μg/L)	LOD (μg/L)	Inhabition time (min)	References
AChE/CB-CS/SPE	paraoxon	0.1～0.5	0.05	20	[35]
AChE/OMC-CS/Fe_3O_4-CS/SPCE	chlorpyrifos	1～600	0.05	12	[36]
Nafion/AChE/ MWCNTs-SnO_2CHIT/Au	chlorpyrifos	0.05～10^5	0.05	25	[37]
AChE/Fc-F/GCE	chlorpyrifos	1.75～350	0.14	15	[38]
HRP-Ab /BSA-Ag/Pt/SiO_2/SPCE	chlorpyrifos-methyl	0.4～20	0.02	40	[39]
AChE.Pin5COOH/Fe_3O_4NP/GCE	chlorpyrifos	0.52～24.5	0.52	10	[40]
AChE/Fe_3O_4/GR/SPE	chlorpyrifos	0.05～100	0.02	15	This work

The characteristic of AChE/Fe_3O_4/GR/SPE was compared with some reported biosensor for chlorpyrifos determination in the last few years. As displayed in Table 1, it is clear the ACHE/Fe_3O_4/GR/SPE has a relative wide linear response range and lower detection limit, but the inhibition time was higher than parts of biosensor.

3.2 Reproducibility, repeatability and regeneration of the biosensor

The reproducibility of AChE/Fe_3O_4/GR/SPE was evaluated by measuring in a pH 7.5 PBS containing 0.5 mmol/L ATCl. The relative standard deviation (RSD) was 6.23 % for five different biosensor by the same way. To investigate the repeatability, the same AChE/Fe_3O_4/GR/SPE inhabited by 100 μg/L chlorpyrifos was used to detect PBS containing 0.5 mmol/L ATCl for five times and the RSD was 4.35 %, which presented a good repeatability. After each measurement, the biosensor was immersed into pralidoxime chloride solution to regenerate the enzyme activity, resuming 90 % of its original activity for one regeneration measurement.

3.3 Real Sample Analysis

In order to evaluate the practicability of AChE/Fe_3O_4/GR/SPE in real sample, the proposed biosensor was applied to determinate the concentration of chlorpyrifos under the optimized conditions using standard addition method. Vegetable samples (cabbage, spinach) purchased from a local supermarket were washed using deionized water and dried in the air. The washed vegetables were sprayed with different concentration solution of chlorpyrifos homogeneously. After that, 10 g of vegetable was added into 20 mL mixed solution of acetone pH 7.5 PBS (1/9, v/v). Chlorpyrifos was extracted using ultrasonic treatment for 30 min.

Table 2. Results of chlorpyrifos determination and recovery in vegetable samples

Sample	Add (μg/L)	Measure (μg/L)	RSD (%)	GC (μg/L)	RSD (%)	Recovery (%)
Cabbage	0	-	-	-	-	-
	10.0	9.40	3.86	9.79	0.57	94.0
	30.0	32.52	0.05	28.56	0.71	108.3
Spinach	0	-	-	-		-
	10.0	9.83	2.46	9.31	0.65	98.3
	30.0	27.42	3.03	28.31	0.43	91.14

Each extract solution was detected by using the standard addition method and the analytical results were listed in Table 2, which undergoes three parallel determinations. The results between the proposed biosensor and gas chromatographic method show that there are no significant changes. And the recovery varied from 91.14% to 108.3%, demonstrating that the proposed method established above has high accuracy for chlorpyrifos detection in different real samples with high accuracy.

4. CONCLUSION

A high-performance biosensor based on AChE and Fe_3O_4/GR film modified SPE has been developed to determine chlorpyrifos residual. Compared with bare SPE, the experimental results indicated that synergistic effect of Fe_3O_4/GR not only improved conductivity and accessible surface area also effectively promoted immobilization of AChE. Moreover, it has been demonstrated that this biosensor exhibited excellent performance in the merits of wide linear response range, lower detection limit, short detection time, good reproducibility and repeatability. At last, this proposed biosensor was applied to detect chlorpyrifos on vegetable with satisfactory results, which offered a new promising tool for pesticides analysis.

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