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## Acetylcholinesterase biosensor based on the mesoporous carbon/ferroferric oxide modified electrode for detecting organophosphorus pesticides †

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In this paper the biosensor modified by ordered mesoporous carbon-chitosan (OMC-CS)/ ferroferric oxide-chitosan ( $\text{Fe}_3\text{O}_4$ -CS) was developed on the surface of the screen-printed carbon electrodes (SPCEs). The acetylcholinesterase (AChE) were modified onto the film to prepare an AChE biosensor. The chitosan was used as dispersant to disperse OMC and  $\text{Fe}_3\text{O}_4$ . The OMC and  $\text{Fe}_3\text{O}_4$  were used to enhance the electrochemical response. Before the detection for organophosphorus (OP) pesticides, the electrochemical behaviour of AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE was studied with cyclic voltammetry, and the results showed that the chitosan can disperse OMC and  $\text{Fe}_3\text{O}_4$  evenly and make them fixed on the electrode surface firmly. OMC and  $\text{Fe}_3\text{O}_4$  have a significant synergistic effect towards electron transfer. The parameters affecting performance, such as pH of the test solution, amount of AChE and the time of inhibition have been optimized. Under optimum conditions, using methamidophos and chlorpyrifos as model compounds, this biosensor showed a wide range, low detection limit, good reproducibility and high stability. Moreover, AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE biosensor can be used for the detection of real samples which was suitable for field test of OP pesticide residue.

### Introduction

Organophosphorus pesticides (OPs) are used in the field of agriculture widely due to their high efficiency.<sup>1</sup> But it also caused the excess of pesticide residues in vegetables. OP compounds are harmful to the health of human beings.<sup>2</sup> So, it is necessary to control pesticide residues in vegetables. Besides controlling the use of pesticides from the source, the timely and accurate detection of pesticide residues in vegetables is an effective way to protect the health of human body. Comparison with the conventional analytical methods, such as gas chromatography,<sup>3</sup> high performance liquid chromatography,<sup>4</sup> gas-mass spectroscopy,<sup>5</sup> the biosensor method with many advantages of miniature size, fast response, low cost, good selectivity is more suitable for the on-site analysis of pesticide residues.<sup>6,7</sup> Acetylcholinesterase (AChE) biosensor is one of the biosensor. The detection mechanism for OPs is as follows: AChE can catalyze the hydrolysis of acetylthiocholine (ATCl). OPs can inhibit the activity of AChE, this made catalytic action of the enzyme to substrate decline, thus the active substances are reduction, as a result, the current decreases which measured by sensor. The oxidation peak current is inversely proportional to the concentration of OPs. By detecting the change of the current before and after inhibition of pesticide, the OPs concentration can be determined.<sup>8,9</sup>

In order to enhance the electrochemical response or the sensitivity of AChE biosensor, various nanomaterials and composites of nanomaterials have been widely used on the surface of electrode. Such as prussian blue (PB), that is a material with

increasing interest for electrochemical sensors,<sup>10,11</sup> multiwall carbon nanotubes (MWCNTs), that can accelerate electron transfer rates on electrochemical reactions,<sup>12,13</sup> gold nanoparticles (AuNPs), that can cause changes in electrical conductivity.<sup>14,15</sup>

In this study, the ferroferric oxide ( $\text{Fe}_3\text{O}_4$ ), the ordered mesoporous carbon (OMC) and chitosan (CS) were used.  $\text{Fe}_3\text{O}_4$  has high electrical conductivity and can enhance the surface area of electrode, thus more AChE can be immobilized with it.<sup>16</sup> OMC is a new type of non-silicon-based mesoporous materials, which has a large specific surface area and a large pore volume. Moreover, compared with other mesoporous materials, OMC has good biocompatibility, conductivity and adsorption properties. In recent years, it has become one of the research hotspots in the field of electrochemical sensors.<sup>17,18</sup> Chitosan as membrane material contains large groups of  $-\text{NH}_2$  and  $-\text{OH}$  which is preferable to maintain the high biological activity of the immobilized biomolecules.<sup>19</sup> The  $\text{Fe}_3\text{O}_4$  and OMC were dispersed in chitosan evenly. Then the  $\text{Fe}_3\text{O}_4$ -CS and OMC-CS were modified on the surface of screen-printed carbon electrodes to immobilize the AChE. To the best of our knowledge, such an enzyme sensor has not been reported. The proposed enzyme sensor has the advantages of high sensitivity and low background current. This electrochemical AChE biosensor is simple, rapid and sensitive, and it was developed for the sensitive detection of methamidophos in real vegetable samples.

### Experimental

#### Apparatus

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed with CHI660D electrochemical workstation (Shanghai Chenhua Co., China). All experiments were performed with a three-electrode system at room temperature. The commercially available screen-printed carbon electrode (TE100,

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working diameter was 3 mm) was purchased from Zensor R&D (Taiwan). The morphologies of OMC-CS were observed by a scanning electron microscope (SEM, SIRION, FEI, Netherlands).

#### Reagents and materials

Acetylcholinesterase (Type C2888, 500 UN from electric eel), acetylthiocholine chloride (ATCl) and chlorpyrifos were purchased from Sigma (USA). Methamidophos were purchased from Lifeholder (USA). Chitosans were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). OMCs were purchased from Nanjing Ji Cang Nano Technology Co., Ltd. (China). Fe<sub>3</sub>O<sub>4</sub> were purchased from Shanghai crystal pure reagent Co., Ltd. (China). The phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of NaH<sub>2</sub>PO<sub>4</sub> and Na<sub>2</sub>HPO<sub>4</sub>. Other reagents were of analytical grade. All solutions were prepared using double distilled water.

#### Preparation of AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE

##### Preparation of Fe<sub>3</sub>O<sub>4</sub>-CS and OMC-CS

2.0 mg Fe<sub>3</sub>O<sub>4</sub> was added into the 4 mL 0.2 % CS solution with ultrasonic treatment for about 3 h to get Fe<sub>3</sub>O<sub>4</sub>-CS solution. The Fe<sub>3</sub>O<sub>4</sub>-CS solution was uniform and steady. Similarly, OMC-CS solution was prepared under the same procedure as illustrated in Fe<sub>3</sub>O<sub>4</sub>-CS solution preparation, the solution of OMC-CS was highly dispersed black suspension.

##### Preparation of screen-printed carbon electrode

Before the experiment, a potential of +1.75 V was applied to the bare 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.<sup>20</sup>

##### Fabrication of the AChE biosensor

8  $\mu$ L Fe<sub>3</sub>O<sub>4</sub>-CS was added onto the surface of bare screen-printed carbon electrode and dried in the air at room temperature (noted as Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE). Then the electrode of Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE was modified with 8  $\mu$ L OMC-CS and dried in the air at room temperature (noted as OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE). The OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE was coated with 5.0  $\mu$ L 0.02 U/ $\mu$ L AChE solution to obtain the AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE and dried in the environment of 4  $^{\circ}$ C, stored at 4  $^{\circ}$ C.

##### Electrochemical detection of pesticides

The electrochemical characteristics of the modified electrode were characterized by the CV in 5 mM [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup> or in pH 7.5 PBS solution containing 1.0 mM ATCl. The AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE biosensor was employed for the determination of methamidophos and chlorpyrifos using DPV method. The performance of the biosensor was tested by its DPV response in pH 8.0 PBS solution containing 1.0 mM ATCl. Then the electrode was rinsed with phosphate buffer solutions (pH 8.0) and incubated in an aqueous solution containing the desired concentration of pesticides for 12 min. Finally, it was transferred into the 1.0 mM ATCl solution for DPV measurements at the same condition as the previous DPV response test. The inhibition rate of pesticides 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 ATCl on AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE,  $I_{p, \text{exp}}$  was the peak current of ATCl on AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE with pesticides inhibition. Inhibition(%) was plotted against the concentrations of the pesticides to obtain linear calibration graphs.

#### AChE reactivation

After exposure to pesticides, the AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE was firstly washed with 0.1 M pH 8.0 PBS, then reactivated by immersing in 5.0 mM pralidoxime iodide for 12 min, and then transferred to 0.1 M pH 8.0 PBS containing 1.0 mM ATCl for DPV analysis of the electrochemical response. The reactivation efficiency was calculated as follows:

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

Where,  $I_r$  was the peak current of 1 mM ATCl on AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE after 5.0 mM pralidoxime iodide reactivation.

#### Pretreatment of real samples

Fresh vegetables (cabbage, rape, lettuce) bought from a local supermarket were chopped after removing the rotten leaves and dirt. 2.0 g of each sample was sprayed with 2 mL different concentrations (0  $\mu$ g/L, 500  $\mu$ g/L) of methamidophos respectively, and storage at 4  $^{\circ}$ C for 24 h. Then the pesticides in vegetables were extracted, the process is as follows: each sample was added into 10 mL 0.1 M pH 8.0 PBS and ultrasonic treatment 3 min. After the suspensions were centrifuged (10 min, 10,000 rpm), the acquired supernatants were detected by DPV directly without extraction or pre-concentration. The concentration of pesticides in the samples can be obtained from the calibration curve. In order to verify the accuracy of the method, the acquired supernatant was also detected by GC.

## Results and discussion

### SEM characterizations of OMC-CS

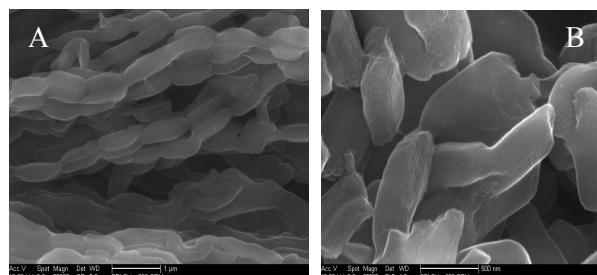


Fig. 1. SEM characterizations of OMC-CS

The morphology of the OMC-CS was studied by SEM, the results were shown in figure 1. It can be seen that mesoporous carbon were dispersed uniformly by chitosan. And it can be seen many small mesoporous structure from the figure 1B. Showing that OMC-CS was successfully modified onto the surface of the electrode.

### Current characterization of the assembly process of the AChE biosensor

Cyclic voltammetry (CV) was used to compare the electrochemical behavior of the nano-composites. CVs of bare SPCE, Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE, OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE, and AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE in 0.1M PBS (pH 7.5) containing 5.0 mmol/L [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup> and 0.1mol/L KCl were recorded. As shown in Fig.2, there was a pair of well-defined redox peaks observed on the bare SPCE (Fig. 2a). The peak currents of the Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE (Fig. 2b) increase since the good electrical conductivity of Fe<sub>3</sub>O<sub>4</sub>, and the peak current was about 94 $\mu$ A. The highest redox peaks were appeared at the OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE (Fig. 2d) due to the large specific surface area and good conductivity of OMC. But, The current was significantly

decreased when the 5  $\mu\text{L}$  AChE (0.02 U) was added to the electrode of OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE (Fig. 2c), because the enzyme is a macromolecular protein, it not only does not conduct electricity, but also hinders the electron transfer in the interface, so the current peak value became smaller, which also proved that the AChE has been successfully immobilized onto the electrode surface.

CVs of bare SPCE,  $\text{Fe}_3\text{O}_4$ -CS/SPCE, OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE, AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE and AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE after inhibition with methamidophos for 10 min in pH 7.5 PBS solution containing 1.0 mM ATCl were recorded. As shown in Fig. 3, there was a pair of well-defined redox peaks on the bare SPCE (Fig. 3a). Chitosan as organic membrane may block the electron transfer,<sup>21</sup> so the current peaks of curve b and curve c showed up shift. The peak current becomes larger because of the good electrical conductivity of  $\text{Fe}_3\text{O}_4$  and OMC. Compared with the Fig. 2, the current has been significantly increased when the 5  $\mu\text{L}$  AChE (0.02 U) was applied to the surface of the electrode of OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE (Fig. 3e), current is formed by the oxidation of the active substance, which was produced by the hydrolysis of ATCl under the catalytic action of acetylcholinesterase. Fig. 3d showed that the surface of the AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE electrode after inhibition with 100  $\mu\text{g/L}$  methamidophos for 10 min, the current reduced significantly, this is because pesticides inhibit the activity of acetylcholinesterase.

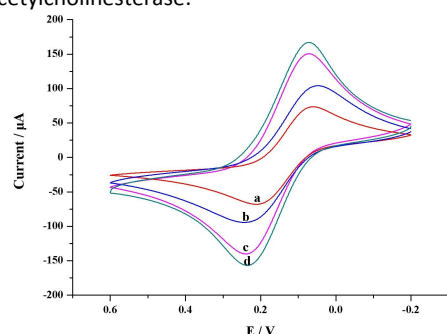


Fig. 2. CVs of modified SPCEs recorded in 0.1M PBS (pH 7.5) containing 5.0 mmol/L  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  and 0.1mol/L KCl: (a) bare SPCE; (b)  $\text{Fe}_3\text{O}_4$ -CS/SPCE; (c) AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE; (d) OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE

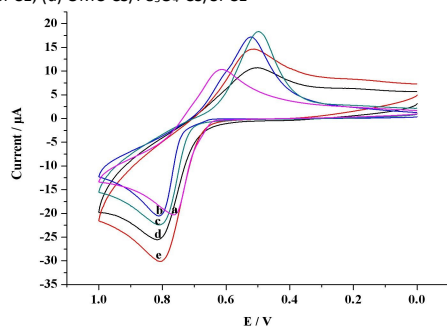


Fig. 3. CVs of modified SPCEs recorded in pH 7.5 PBS solution containing 1.0 mM ATCl: (a) bare SPCE; (b)  $\text{Fe}_3\text{O}_4$ -CS/SPCE; (c) OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE; (d) AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE after inhibition with methamidophos for 10 min; (e) AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE

### Optimization parameters of the biosensor performance

#### optimization of Phosphate buffer pH

The pH of the detection solution plays an important factor in achieving good analytical performance. Therefore, the response currents of the enzyme electrode were investigated in a series of PBS (0.1 M, pH from 6.0 to 9.0) including 1 mM ATCl (See Fig. 4).

The maximum value of the response current was at pH 8.0. Thus, a pH 8.0 of PBS was used in the subsequent experiment.

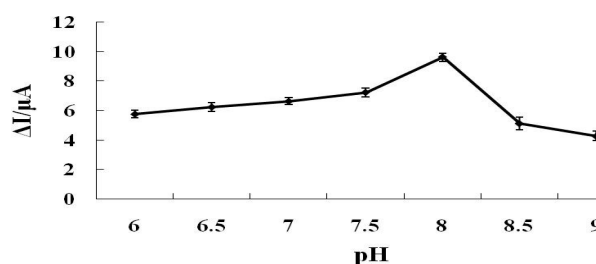


Fig. 4. The influence of pH

#### Optimization of load of AChE

The enzyme amount also plays an important role in achieving good analytical performance. The response currents of the enzyme electrode were coated with a series of enzyme amount (enzyme amount from 0.02 U to 0.14 U) in 0.1M PBS (pH 8.0) containing 1 mM ATCl (see Fig. 5). The results showed that the increasing of the AChE amount, the peak current increased gradually and reached the maximal value at 0.1 U. After that, the amperometric response decreased gradually as the amount of AChE further increased. Therefore, 0.1 U of AChE was chosen as the optimal enzyme amount for preparation of the biosensor.

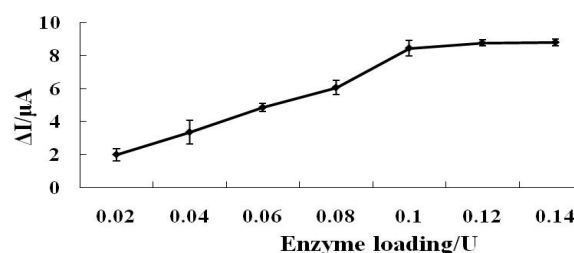


Fig. 5. The influence of enzyme amount on the response of biosensor

#### Optimization of Incubation time on inhibition

Fig. 6 showed that the peak current of AChE/OMC-CS/ $\text{Fe}_3\text{O}_4$ -CS/SPCE decrease greatly with an increase of incubation time in the OP pesticides solution. When the incubation time was longer than 12 min, the curve tended to maintain a stable value, which indicated that the binding interaction with active target groups in enzyme could reach saturation. Thus, the optimum incubation time of 12min was selected.

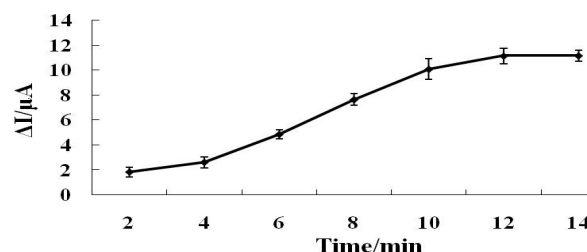


Fig. 6. The influence of incubation time on inhibition

#### Determination of pesticides

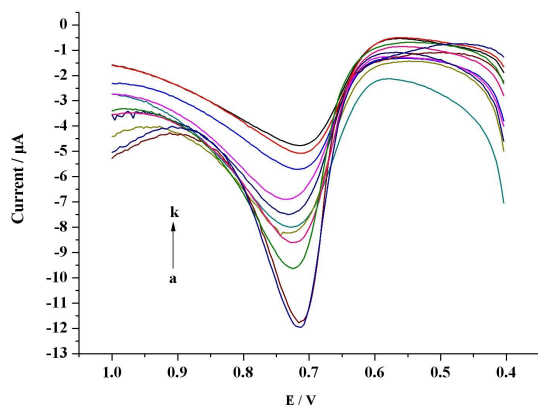
Under optimal experimental conditions (pH 8.0, enzyme load was 0.1 U, incubation time was 12 min), we studied the relationship

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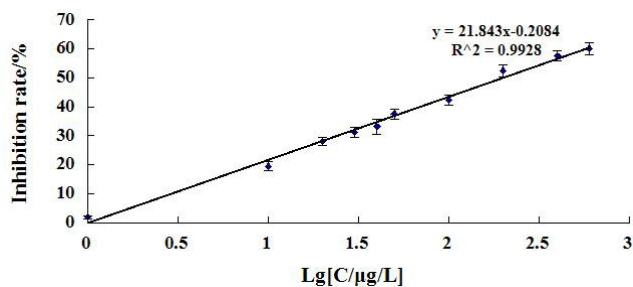
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between the AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE sensor and the different concentrations of pesticides. The DPV responses were examined before and after exposure to different concentration pesticides. The DPV diagram of methamidophos as shown in Figure 7, as the concentration of pesticides increased gradually, the value of the current was decreased gradually (curve a-k). The relationship between inhibition rate and methamidophos concentrations as shown in Figure 8, the linear equation was  $y = 21.843x - 0.2084$ , with a correlation coefficient of 0.9928, the detection limit was 1  $\mu\text{g/L}$  which was lower than colloidal gold immunochromatography strip for detection of methamidophos residue (1.0  $\mu\text{g/ml}$ ).<sup>22</sup> The relationship between inhibition rate and chlorpyrifos concentrations as shown in Figure 9, the linear equation was  $y = 7.8311x + 16.513$ , with a correlation coefficient of 0.9957, the detection limit was 0.05  $\mu\text{g/L}$  which was lower than the biosensor based on enhancement of CdS-decorated graphene nanocomposite (0.7  $\mu\text{g/L}$ ) and the biosensor based on a multi-walled carbon nanotube/cobalt phthalocyanine (1.10  $\mu\text{g/L}$ ).<sup>23,24</sup>

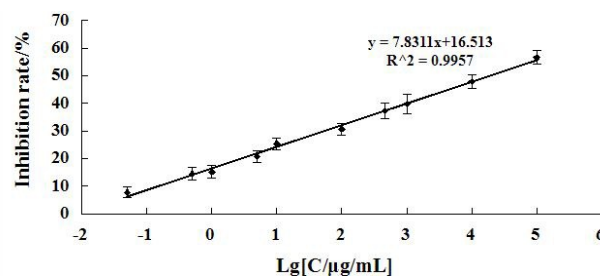
From figure 8 and figure 9, it can be seen that the detection limit of the sensor to different pesticides is different. This shows that the sensitivity of this sensor to different pesticides is different. It is proved that the sensor has a certain specificity.



**Fig. 7** DPV of AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE in pH 8.0 PBS solution containing 1.0 mM ATCl after inhibition with methamidophos for 12 min. methamidophos concentration: a-k: 0  $\mu\text{g/L}$ ; 1  $\mu\text{g/L}$ ; 10  $\mu\text{g/L}$ ; 20  $\mu\text{g/L}$ ; 30  $\mu\text{g/L}$ ; 40  $\mu\text{g/L}$ ; 50  $\mu\text{g/L}$ ; 100  $\mu\text{g/L}$ ; 200  $\mu\text{g/L}$ ; 400  $\mu\text{g/L}$ ; 600  $\mu\text{g/L}$



**Fig. 8.** Relationship between inhibition rate and methamidophos concentrations



**Fig. 9.** Relationship between inhibition rate and chlorpyrifos concentrations

### Repeatability

The repeatability of the sensor was studied by testing the deviation of research. The same method was used to produce 10 electrodes in the screen-printed carbon electrode, methamidophos of 40  $\mu\text{g/L}$  and 400  $\mu\text{g/L}$  were determined respectively. The relative standard deviations were 3.6% and 2.9% respectively, that indicated the AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE sensor has good repeatability.

### Storage stability

When the enzyme electrode was not in use, it was stored in a refrigerator at 4°C in dry and hermetic surroundings. Almost all of the response current of the sensor can reach 98% of the initial current after 7 days. After a 30-day storage period, the sensor retained 80% of its initial current response, which was much better than earlier report.<sup>25</sup>

### Reactivation of the biosensor

we selected pralidoxime iodide as activity recovery agent. After inhibited by a certain concentration methamidophos, the biosensor was immersed in 5.0 mM pralidoxime iodide for 12 min. The results showed that the biosensor could resume higher than 90% of its original activity of AChE.

### The detection of the real samples

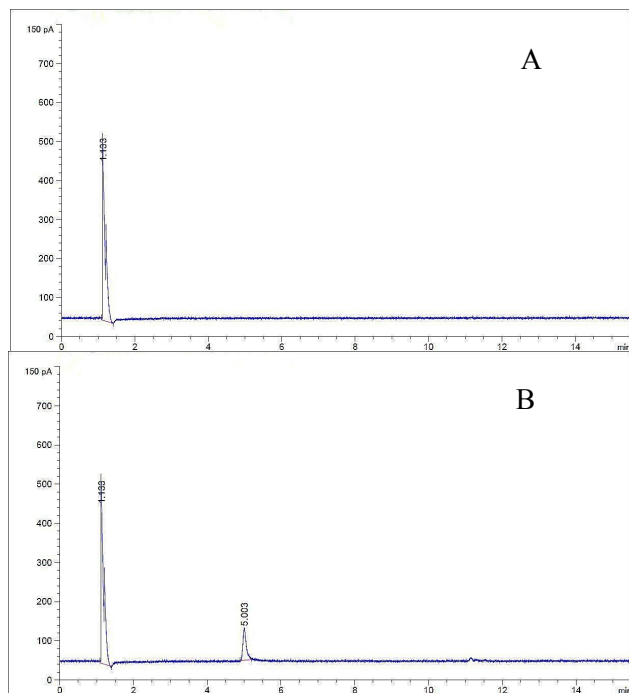
The recovery tests were studied by adding different amounts of pesticides into vegetables samples (cabbage, rape, lettuce), respectively. The detection results of AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE biosensor were shown in Table 1, the relative standard deviations was between 0-4.51, and the recovery rate was between 94%-105%. The detection results of GC were shown in Table 2. And figure 10 (A) shows the background spectra of lettuce, figure 10 (B) shows the spectra of lettuce added methamidophos. From Table 1 and Table 2, it can be seen that the recoveries of AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE biosensor and GC were both bigger than 90%. The results indicated that this method was highly accurate, precise and reproducible. It can be used for direct analysis of practical samples.

**Table 1** The recovery of the proposed AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE biosensor in real samples

samples	Added ( $\mu\text{g/L}$ )	Found ( $\mu\text{g/L}$ )	RSD(%) (n=3)	Recovery (%)
cabbage	0	0	0	—
	100	94	1.78	94
rape	0	0	0	—
	100	105	4.51	105
lettuce	0	0	0	—
	100	97	3.16	97

**Table 2** The detection results of GC

samples	Added ( $\mu\text{g/L}$ )	Found ( $\mu\text{g/L}$ )	RSD%(n=3)	Recovery (%)
cabbage	0	0	0	—
	100	98	2.35	98
rape	0	0	0	—
	100	101	1.55	101
lettuce	0	0	0	—
	100	94	2.17	94

**Fig. 10** The GC chromatograms of lettuce sample: blank (A) and added with methamidophos (B).

## Conclusion

In this work, the sensor of AChE/OMC-CS/Fe<sub>3</sub>O<sub>4</sub>-CS/SPCE had been successfully fabricated for the detection of OP pesticides. Fe<sub>3</sub>O<sub>4</sub>, OMC and CS have good conductive ability, film-forming ability and excellent biocompatibility that can increase the surface area to capture a large amount of acetylcholinesterase, thus increased detection sensitivity. Because of the synergistic effects of the Fe<sub>3</sub>O<sub>4</sub>, OMC and CS, the AChE biosensor exhibited higher sensitivity, better stability, wide linear response range and short response time and the low detection limit. Thus, it is more suitable for trace detection of OP pesticides residue compared with the other biosensor.

## Acknowledge

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