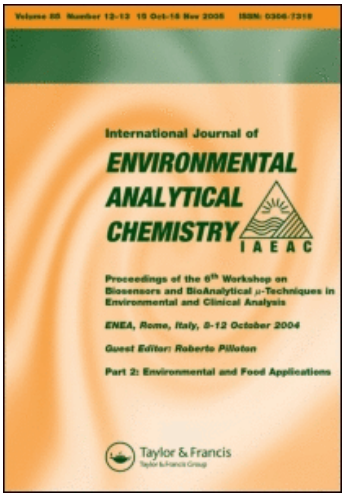

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Determination of pesticides in vegetable samples using an acetylcholinesterase biosensor based on nanoparticles ZrO_2/chitosan composite film

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An amperometric pesticides inhibition biosensor has been developed and used for determination of pesticides in vegetable samples. To eliminate the interference of ascorbic acid, multilayer films of polyelectrolyte (chitosan/polystyrensulfonate) were coated on the glass carbon electrode. Then, acetylcholinesterase was immobilized on the electrode based on surface-treated nanoporous ZrO_2/chitosan composite film as immobilization matrix. As a modified substrate, acetylthiocholine was hydrolysed by acetylcholinesterase and produced thiocholine which can be oxidized at +700 mV vs. SCE. Pesticides inhibit the activity of enzyme with an effect of decreasing of oxidation current. The experimental conditions were optimized. The electrode has a linear response to acetylthiocholine within 9.90×10^{-6} to 2.03×10^{-3} M. The electrode provided a linear response over a concentration range of 6.6×10^{-6} to 4.4×10^{-4} M for phoxim with a detection limit of 1.3×10^{-6} M, over a range of 1.0×10^{-8} to 5.9×10^{-7} M for malathion, and over a range of 8.6×10^{-6} to 5.2×10^{-4} M for dimethoate. This biosensor has been used to determine pesticides in a real vegetable sample.

Keywords: Pesticides; Amperometry; Inhibition biosensor; Acetylcholinesterase; Nanoparticles ZrO_2; Chitosan

1. Introduction

The extensive use of pesticides to protect agricultural crops necessitates reliable tools for the detection of residues in products and water, thus ensuring environmental protection and consumer safety. Organophosphorous pesticides (OPs), such as phoxim, are widely used in agriculture due to their high efficiency, low bioaccumulation and limited persistence in the environment [1, 2]. Since they not only inhibit insect

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cholinesterase (actelcholinesterase, AChE, EC 3.1.1.7, and butyrylcholinesterase, BChE, EC 3.1.1.8) but also interfere with neural transmission in other organisms, including humans, OPs can also be used for chemical warfare and are a potential hazard for human health and environmental food chains because they contaminate food and drinking water. In China, the maximum concentration of OP type pesticides permissible in vegetable is 0.05 mg/kg. Consequently, there is a growing interest in fast and sensitive detection systems for this type of compunds. Methods traditionally used for the detection of pesticides are based on gas chromatography (GC) [3] or high-performance liquid chromatography (HPLC) [4], but these methods are relatively expensive, time-consuming and hardly suitable for field use [5]. AChE inhibition tests, and the AChE biosensors, in particular, have been shown to be suitable alternatives. Numerous AChE inhibition biosensors have been developed that are based on potentiometric [6, 7], amperometric [8, 9] and piezoelectric [10] transducers.

Two approaches are possible when using amperometry to detect pesticides. A two-enzyme system can be employed using choline oxidase and either AChE or BChE. The internal transducer can be an oxygen [11] or hydrogen peroxide [12] sensor. When a single enzyme is used, the substrate has to be modified so that a reaction product is electroactive. Acetylthiocholine (ATCh) is an ideal substitute for the original substrate, since it is very specific for the acetylcholinesterase and is hydrolysed in the same proportion [13]. However, ATCh can only be oxidized at a high potential, which will cause the oxidation of the interferents such as ascorbic acid (AA) in real samples. This is the reason that an inhibition biosensor is difficult to use in real-sample detection [14]. In this respect, Albareda *et al.* [15] have reported a disposable amperometric AChE or BchE biosensor based on thick-film technology.

In the present paper, an amperometric AChE biosensor with improved selectivity to eliminate the interference of AA based on multilayer of polyelectrolyte (chitosan/polystyrensulfonate) is reported. A surface-treated nanoporous ZrO_2/chitosan composite matrix, which was developed in the author's previous work [16], is used to immobilize the enzyme AChE. The nanoporous ZrO_2 was treated with an anionic surfactant (sodium dodecylbenzene sulfonate) to improve the dispersion of ZrO_2 in chitosan solution. This material combined the advantages of inorganic nanoparticles, ZrO_2, and the organic polymer, chitosan. Another advantage of this new composite film is that the immobilization of enzyme is based on the absorption on nanoporous ZrO_2, so, the usage of glutaraldehyde can be avoided. The resulting biosensor is based on the electrochemical oxidation of thiocholine, produced by the enzymatic hydrolysis of acetylthiocholine. This biosensor has been used in pesticide assays in real vegetable samples with satisfactory results.

2. Experimental

2.1 *Reagents*

Acetylcholinesterase(EC 3.1.1.7 from electric eel, 317 U/mg), acetylthiocholine(ATCh), pyridine-2-aldoxime methoiodide(2-PAM) and chitosan (CHIT,MW $\sim 1 \times 10^6$, 75–85% deacetylation) were supplied by Sigma (St. Louis, MO). Polystyrensulfonate (PSS, MW 10 000) was purchased from Aldrich (Milwaukee, WI). Nanoporous ZrO_2 was produced by the Nano Material Application Engineering Technology Center

(Zhejiang, China). Phoxim, malathion and dimethoate were produced by the Liming Chemical actory (Jiansu, China). All other chemicals used, such as sodium dodecylbenzene sulfonate, were of analytical-reagent grade and used as received without further purification. All solutions were prepared with doubly distilled water. The supporting electrolyte was 0.067 M phosphate buffer (PB), which was prepared with KH_2PO_4 and Na_2HPO_4.

2.2 *Apparatus*

Cyclic voltammetric experiments were performed using a VMP2 Mutltichannel potentiostat (Advanced Measurement Technology Inc., USA). The three-electrode system consisted of a GC/ZrO_2/CHIT/AChE electrode as working electrode, an SCE as reference electrode and a Pt foil as counter-electrode. All electrochemical experiments were carried out in 10 mL of phosphate buffer (pH 7.4) at ambient temperature under air-saturated conditions, unless otherwise stated. All potentials were measured and reported vs. the SCE. A magnetic stirrer and bar provided convective transport.

Transmission electron micrographs of ZrO_2/CHIT matrix were taken using a Hitachi H-800 transmission electron microscope (Hitachi, Tokyo), using an accelerating voltage of 200 kV.

2.3 *Surface treatment of nanoporous zirconia*

Nanoporous zirconia can be treated by adding 0.5 g of nanoporous zirconia to 100 mL of 0.7% sodium dodecylbenzene sulfonate and adjusting to pH 4.0. After stirring for 6 h, the resulting mixture was filtered. The surface-treated nanoporous zirconia was obtained by washing with water and drying.

2.4 *Preparation of CHIT/PSS solution*

Equal volumes of 4 mg/mL PSS solution and 0.1% chitosan in 0.05 M acetic acid solution were mixed and sonicated for 5 min to form a CHIT/PSS casting solution.

2.5 *Preparation of ZrO_2/CHIT solution*

An appropriate amount of surface-treated nanoporous zirconia was dispersed in 0.1% of chitosan (0.05 mM acetic acid); the mass ratio of ZrO_2 : chitosan was 1 : 100. The mixture was sonicated for 15 min after stirring for 1 h. Finally, a highly dispersed colloidal solution was formed.

2.6 *Preparation of inhibition biosensor*

A glass carbon (0.2 mm diameter) electrode was polished before each experiment with 0.05 μm α-alumina powder, successively rinsed thoroughly with absolute alcohol and distilled water in ultrasonic bath, and dried in air. To form a CHIT/PSS film, 8 μL of CHIT/PSS casting solution was coated on the glass carbon electrode surface and dried in air. The same procedure was repeated four times to form an anti-interference electrode. Then, a solution of 7 mg/mL AChE was prepared in 0.067 M phosphate buffer at pH 7.4. An enzyme casting solution was obtained by mixing 10 μL of

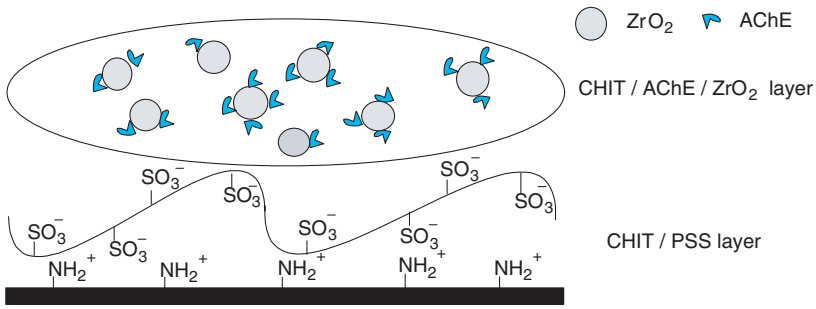

Figure 1. Schematic diagram of the preparation of the AChE electrode.

ZrO_2/CHIT composite solution and 10 μL of enzyme solution. A volume of 10 μL of resulting casting solution was pipetted onto the surface of the above modified electrode. The casting solution was allowed to dry at 4°C overnight. Finally, the enzyme electrode was immersed in 0.067 M phosphate buffer (pH 7.4) to wash out the unimmobilized AChE from the electrode surface. When not in use, the electrode was stored dry at 4°C in a refrigerator. The schematic diagram of the preparation procedure of electrode was illustrated in figure 1. The immobilization of enzyme is based on the absorption of nanoporous ZrO_2. Therefore, the usage of glutaraldehyde, which can denature enzyme, could be avoided. In addition, the nanoporous structure of ZrO_2 greatly enhances the active surface available for enzyme binding over the geometrical area [17].

2.7 *Procedure for enzyme-inhibition studies*

The AChE-modified GCE was dipped into a stirred phosphate buffer solution (pH 7.4), and an initial baseline current was recorded. An appropriate amount of substrate (0.1 M ATCh standard solution) was added to give a final concentration which was 1.0 mM, and a steady-state current was recorded (I_0). Finally, a solution of pesticide was added in succession to inhibit the enzyme activity. After 15 min, the current decrease (I_1), which was proportional to the final concentration of inhibitor in solution, was recorded.

The percentage inhibition ($I\%$) as defined by Palleschi *et al.* [18] due to selected levels of pesticide was calculated using the relationship.

$$I(\%) = \frac{I_0 - I_1}{I_0} \times 100$$

2.8 *Pretreatment of the samples*

For pesticide-recovery tests in vegetables, the real samples were pretreated according to the following procedure: A weighed amount of crushed sample was extracted with 10 mL PB solution for 10 min. The mixture was then filtered. The residue was washed with 5 mL of PB and combined with the filtrate. The resulting filtrate solution was used in the recovery test and real-sample analysis with the enzyme electrode.

For pesticide determination, the real sample was sprayed with pesticide and extracted according to the following procedure [19]. A weighed amount of sample, crushed and ground into powder with absolute sodium sulfate, was extracted with 60 mL acetone for 30 min. The solution was then filtered. The dregs were washed with 20 mL acetone and incorporated into the filtrate. The resulting filtrate solution was concentrated to 14 mL with a water bath at 40°C and extracted with 30 mL dichloroethane and 30 mL NaCl (60 g/L). The organic phase was concentrated to almost dry with water bath at 40°C. Acetone (0.35 mL) were added to dissolve the sample and used in gas-chromatographic analysis.

3. Results and discussion

3.1 *Morphologies of the surface-treated ZrO_2/CHIT composite film*

To investigate the microstructure of the synthesized surface-treated ZrO_2/chitosan matrix, morphologies of surface-treated ZrO_2/CHIT films was characterized by TEM. Figure 2 is a typical TEM photograph of a surface-treated zirconia/chitosan film, which shows a uniform porous structure, consisting of particles about 20 nm. When ZrO_2 was treated with surface-active agent molecules, sodium dodecylbenzene sulfonate absorbed on the ZrO_2 surface and resulted in a molecule layer coated on the surface, thus helping ZrO_2 to disperse in the solution of chitosan [20]. This provided a significantly enhanced effective electrode surface for high enzyme loading.

3.2 *Elimination of the interference of ascorbic acid*

The organic polyelectrolyte is a selectively permeable membrane which can effectively suppress the interference of many interferents [21]. To eliminate the interference caused by the oxidation of ascorbic acid in the real sample at the applied potential (+700 mV), a few layers of the polyelectrolyte (chitosan/polystyrensulfonate) were coated on the glass carbon electrode. Figure 3a–d illustrates the effect of the numbers of layers of CHIT/PSS films in terms of the response of the electrode to

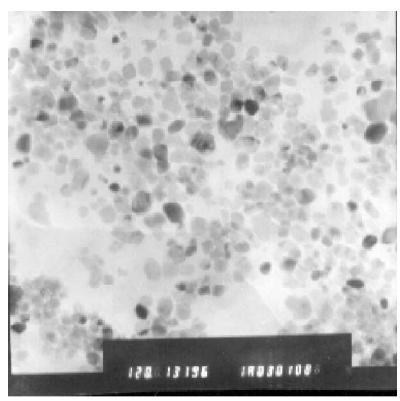

Figure 2. TEM of the surface-treated ZrO_2/CHIT film.

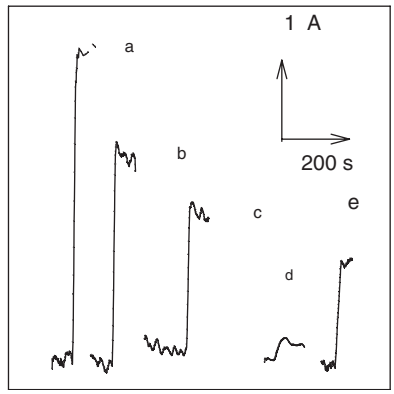

Figure 3. Effect of different numbers of CHIT/PSS layers on the response of the enzyme electrode in 0.067 M phosphate buffer (pH 7.4) containing 0.1 mM ascorbic acid. (a) Without CHIT/PSS layer; (b) one layer of CHIT/PSS; (c) two layers of CHIT/PSS; (d) four layers of CHIT/PSS; (e) response of enzyme electrode with four layers of CHIT/PSS in 0.067 M phosphate buffer (pH 7.4) containing 0.1mM ATCh.

ascorbic acid. This clearly shows that the response of the electrode (coated with four layers of CHIT/PSS) to ascorbic acid was suppressed. Figure 3e shows the response of the enzyme electrode with four layers of CHIT/PSS to ATCh. At the same concentration, the response to ATCh is 6.65 times greater than the response to ascorbic acid. The high selectivity of the electrode was realized, based on the selective permeation of thiocholine over the interferents due to a size-exclusion mechanism.

3.3 *Cyclic voltammetric characterization*

Figure 4 shows cyclic voltammograms of the sensor in different solutions. The response of the enzyme electrode in 0.067 M PB (pH 7.4) displays a background current (figure 4a). When PB contains 2.44 mM ATCh, the anodic current peak attributed to the redox process of ATCh at the enzyme electrode (figure 4b) was increased. Figure 4c displays the response of the enzyme electrode in 0.067 M PB containing 2.44 mM ATCh with 0.2 mM phoxim added. The decreased current response indicated the inhibition of the activity of enzyme.

The typical enzyme-dependent catalytic process can be expressed as follows:

$$\underset{\text{Acetylthiocholine}}{(CH_3)_3\overset{+}{N}CH_2CH_2SCOCH_3} \xrightarrow{\text{AChE}} \underset{\text{thiocholine}}{(CH_3)_3\overset{+}{N}CH_2CH_2SH} + CH_3COOH$$

$$\underset{\text{Thiocholine (oxidation state)}}{2(CH_3)_3\overset{+}{N}CH_2CH_2SH} \xrightarrow{\text{electrode}} \underset{\text{(reduction state)}}{SCH_2CH_2\overset{+}{N}(CH_3)_3} + 2H^+ + 2e^-$$

Thiocholine can be oxidized on the electrode surface, and an oxidation current, which is proportional to the concentration of thiocholine, can be observed. This current

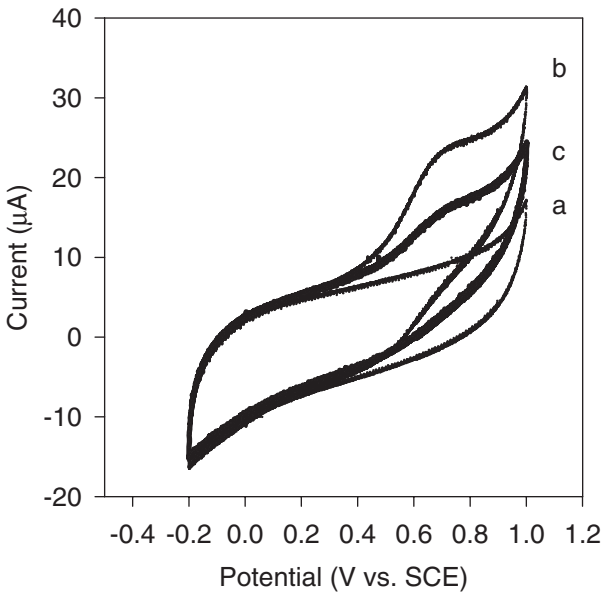

Figure 4. Cyclic voltammograms of the electrode at different stages: (a) CHIT/ZrO_2/AChE in 0.067 M phosphate buffer (pH 7.4) without ATCh; (b) same as (a) with 2.44 mM ATCh added; (c) same as (b) with 0.20 mM phoxim added, using a scan rate 100 mV/s.

is related to the activity of AChE enzyme. Organophosphorous pesticides are effective inhibitors to AChE because the hydrolysis rate of the produced phosphorylated AChE is so slow that releasing active AChE again is hardly possible. Thus, OPs are the irreversible inhibitors of AChE [22]. When a pesticide is added, the oxidation current decreases due to its inhibition to AChE.

Typical cyclic voltammograms of the sensors in 0.067 M PB (pH 6.5) containing 2.44 mM ATCh at different scan rates from 10 to 100 mV/s are shown in figure 5. The peak currents are proportional to the square root of the scan rate, showing typical diffusion-controlled electrochemical behaviour.

3.4 *Optimization of the conditions of enzyme electrode preparation*

Since the biosensor performance may be related to the enzyme loading, the effect of the amount of immobilized AChE on the analytical characteristics of the biosensor was investigated. When the concentration of substrate (ATCh) is fixed, the current increases with the degree of enzyme loading. Thus, the enzyme-loading amount can influence the linear range. Both the sensitivity and the linear range reached an optimum when the AChE concentration was 7 mg/mL. Thus, this degree of AChE loading was used for the subsequent experiment.

3.5 *Optimization of measurement variables*

The experimental variables, which can affect the amperometric determination of ATCh and phoxim, include the pH of the supporting electrolyte, applied potential and concentration of substrate.

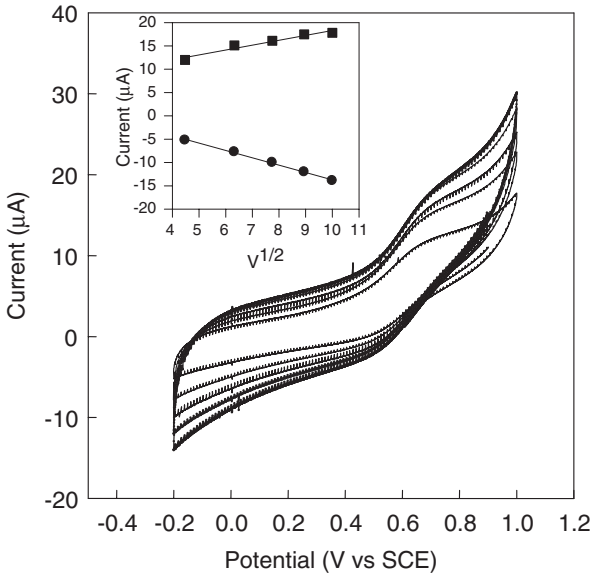

Figure 5. Cyclic voltammograms of the electrode in 0.067 M PB (pH 7.4) in the presence of 2.34 mM ATCh at various scan rates (from inner to outer curves: 20, 40, 60, 80, 100 mV/s). Inset: plots of peak current vs. $v^{1/2}$.

3.5.1 Effect of pH. The influence of the pH of the assay solution over the range 5.5–8.0 on the amperometric response of the biosensor to ATCh at a fixed concentration of 1.0 mM in PB was investigated. The result shows that the pH optimum was 7.4 (data not shown). Therefore, pH 7.4 was fixed for the rest of the experiments.

3.5.2 Effect of applied potential. The effect of applied potential on the biosensor response was also studied. With the increasing potential from 500 to 800 mV, the sensitivity of the biosensor increased significantly. To avoid interference at high applied potentials, a potential of 700 mV (vs. SCE) was selected as the applied potential for amperometric measurements.

3.5.3 Concentration of ATCh used for pesticide determination. The influence of the concentration of substrate on pesticide determination was investigated. The results are shown in table 1. With the concentration of ATCh increasing, the sensitivity for pesticides determination decreased, while the correlation coefficient increased. An overly high substrate concentration would cause all active centres to be occupied by the substrate and insensitive to the inhibitor. Thus, 1.0 mM of ATCh was selected for pesticide amperometric measurement.

3.6 *Electrode-response characteristics*

3.6.1 Calibration of the AChE-modified electrode. Figure 6 displays a typical chromoamperometry of the AChE biosensor for successive additions of a fixed amount of ATCh under optimized experimental conditions. The biosensor exhibited a rapid

Table 1. Effect of concentration of ATCh on phoxim determination.

Concentration of ATCh (mM)	Regression equation	Correlation coefficient
0.5	$I\% = 48.60 \times \log C + 257.967$	0.979
1.0	$I\% = 44.96 \times \log C + 248.196$	0.986
2.0	$I\% = 42.66 \times \log C + 223.736$	0.996

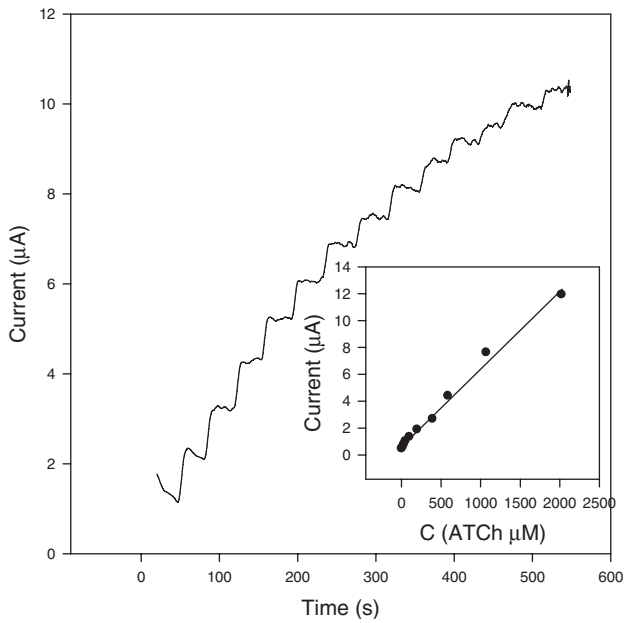

Figure 6. Dynamic response of the enzyme electrode to successive addition of ATCh with a 0.1 mM step in 0.067 M phosphate buffer (pH 7.4) at an applied potential of 0.70 V vs. SCE. The inset shows the calibration curve.

and sensitive response to the change in ATCh concentration. This indicated the good electrocatalytic behaviour of the AChE enzyme electrode. The response time is less than 10 s. The calibration curve is shown in figure 6 (inset). The response current increases as the concentration is elevated, and the current reaches a saturation value at high ATCh concentrations, which suggests that the active sites of enzyme units are saturated at those ATCh levels. Over a concentration range of 9.90×10^{-6} to 2.03×10^{-3} M, the electrode provided a linear response to ATCh with a sensitivity of 5.785 μA/(mM).

3.6.2 Calibration of the AChE-modified electrode for pesticides. Pesticide inhibition of acetylcholinesterase has long been established [9, 12]. Figure 7 demonstrates the time-dependent response of the AChE-modified electrode. The rising part of the response curve shows the anodic peak current increase as the ATCh concentration is increased. The falling part shows the current decrease due to the addition of a different concentration of pesticide to an ATCh solution, as pesticide inhibits the activity of AChE immobilized on the electrode. It is known that the degree of inhibition

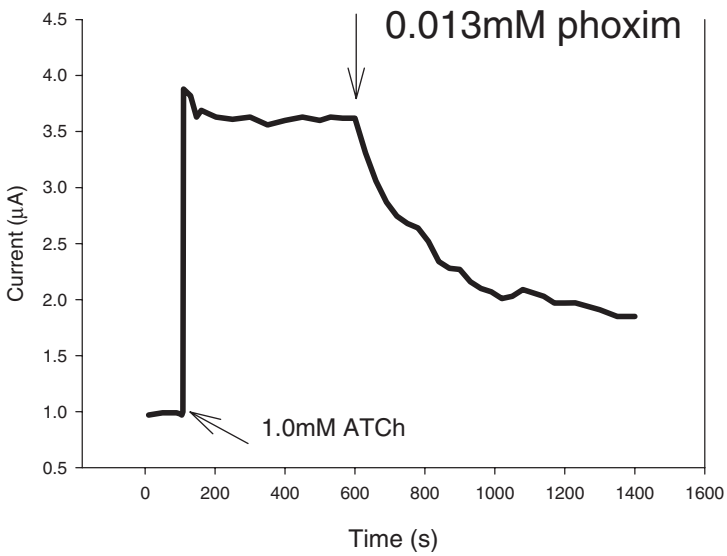

Figure 7. Dynamic response of the enzyme to additions of 1.0 mM ATCh and 0.013 mM phoxim in 0.067 M phosphate buffer (pH 7.4) at an applied potential of 0.70 V vs. SCE.

Table 2. Response characteristics of enzyme electrode.

Parameters	Phoxim	Malathion	Dimethoate
Regression equation	$I\% = 44.96 \times \log C + 248.20$	$I\% = 28.89 \times \log C + 222.27$	$I\% = 10.86 \times \log C + 61.58$
Linear range	6.6×10^{-6} to 4.0×10^{-4} M	1.0×10^{-8} to 5.9×10^{-7} M	8.6×10^{-6} to 5.2×10^{-4} M
Detection limit	1.3×10^{-6} M	5.0×10^{-9} M	1.7×10^{-6} M
Correlation coefficient	0.986	0.984	0.991

increases as the concentration of pesticides is elevated [12]. Thus, the determination of pesticides can be achieved according to the degree of inhibition of AChE. Table 2 lists the response characteristics of the AChE biosensor to the pesticides. Figure 8 shows the calibration curves of the inhibition biosensor for phoxim, malathion, and dimethoate. All three pesticides studied showed a linear dependence between the logarithm of their concentration and the inhibition (%) due to the irreversible inhibition [23]. Considering the error of the measurement, 5% inhibition degree was regarded as a standard to determine the detection limit [9]. In a previous work [15], detection limits in the order of 10^{-10}–10^{-11} M pesticide (paraoxon and carbofuran) have been achieved. However, 0.28 mg BChE was needed to construct each disposable biosensor, which is costly. Although the incubation method used by Alegret *et al.* [15] does not represent any problem regarding ascorbic acid, removing the enzyme electrode from the measuring system and inserting a fresh buffer after coming into contact with the sample may produce errors. The direct method defined by Palleschi *et al.* [18] and used in this paper can prevent such errors. Thus, it is possibe to convert to a flow-injection analysis.

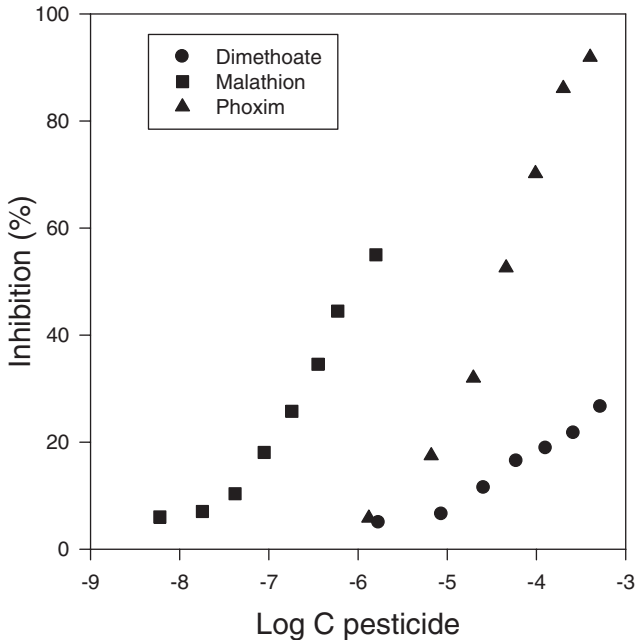

Figure 8. Calibration curves of three different pesticides.

3.6.3 Interference. Besides ascorbic acid, other possible interferents were also investigated. At the applied potential, glycine, salicin, citric acid and oxalic acid have no significant interference owing to the specificity of the enzyme.

3.7 *Reactivation of the enzyme electrode with pralidoxime*

Experiments show that after coming into contact with a pesticide solution, it is difficult for the enzyme activity to recover due to the irreversible inhibition of organophosphate to AChE. However, the enzyme membrane can be reactivated by immersion in a solution of cholinesterase reactivator (pyridine-2-pralidoxim methoiodide, 2-PAM) (0.1 mM) to recover the activity of AChE after the determination of pesticide [21]. The activity of AChE could be recovered to 100% of its original value. This takes only about 10–20 min.

3.8 *Repeatability and lifetime*

The repeatability of the response current of the AChE enzyme electrode was investigated at a phoxim concentration of 0.13 mM. The variation coefficient (RSD) was 2.46% for five successive assays. The stability and lifetime of the sensor are investigated by measuring the biosensor response with 0.13 mM of phoxim over a 1-month period. The response current of the sensor decreased to 70% after 10 days. After 20 days of testing, the biosensor retained about 60% of its original response. The electrode can be used to determine pesticides at least 106 times. When not in use, the electrode was stored dry at 4°C in a refrigerator.

Table 3. Recovery tests for pesticide in spinach sample.

Pesticides	Recovery (%)	RSD (%)
Phoxim	102.7	5.98
Malathion	105.4	4.67
Dimethoate	98.9	5.02

Table 4. Determination of pesticides in a vegetable sample.

Phoxim found by AChE electrode (mg/kg)			Average value (mg/kg)	RSD%	Phoxim average value found by gas chromatogram (mg/kg)
1.59	1.77	1.76	1.71	5.92	1.70

3.9 *Recovery tests*

To use this enzyme inhibition sensor for detecting pesticides in the vegetable samples, a certain amount of phoxim was added to the sample and processed accordingly. The results are shown in table 3. The average recovery was 102.6%, and the RSD was 5.98%.

3.10 *Real-sample analysis*

The feasibility of practical use of the AChE-modified biosensor was assessed by determining the pesticide concentration in spinach samples. The RSD was 5.92%. The results were compared with those obtained by gas chromatography [19]. Table 4 shows that the results determined by AChE electrode were in satisfactory agreement, indicating that the AChE-modified electrode developed can be used to determine pesticides in spinach samples.

4. Conclusion

An inhibition AChE biosensor for determination of pesticides, based on a surface treated nanoporous ZrO_2/Chitosan composite film as immobilization matrix, has been developed. With four layers of polyelectrolyte (chitosan/polystyrensulfonate) coated on the glass carbon electrode, the interference of ascorbic acid could be suppressed. The other possible interferents such as glycine, salicin, citric acid and oxalic acid have no significant interference owing to the specificity of the enzyme. Therefore, this biosensor could be used to determine pesticides in real samples directly. This inhibition biosensor is reusable because the enzyme activity could be recovered through being treated with cholinesterase reactivator (pyridine-2-pralidoxim methoiodide, 2-PAM) just 10 min after coming into contact with the inhibitor. The determination of pesticides in a vegetable sample was satisfactory. The low-cost, simple and fast fabrication of the sensor makes it superior to other techniques.

Acknowledgements

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