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ORIGINAL PAPER

Rapid determination of triazophos using acetylcholinesterase biosensor based on sol–gel interface assembling multiwall carbon nanotubes

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Abstract A sensitive and stable amperometric sensor has been devised for rapid determination of triazophos based on efficient immobilization of acetylcholinesterase (AChE) on silica sol–gel (SiSG) film assembling multiwall carbon nanotubes (MWNTs). The sol–gel matrix provided a biocompatible microenvironment around the enzyme and efficiently prevented leakage of the enzyme from the film. In the presence of acetylthiocholine chloride (ATCl) as a substrate, MWNTs promoted electron transfer reactions at a lower potential and catalyzed electrochemical oxidation of enzymatically formed thiocholine, thus increasing detection sensitivity. Based on the inhibition of organophosphorous compound on the enzymatic activity of AChE, using triazophos as a model compound, the effects of pH, temperature, and MWNTs contents were explored. Under optimum conditions, the inhibition of triazophos was proportional to its concentration from 0.02 μM to 1 μM and from 5 μM to 30 μM, with a detection limit of 0.005 μM. The determination of triazophos in garlic samples showed acceptable accuracy. Fabrication reproducibility of the sensor was good and stability was acceptable. The sensor is a promising new tool for pesticide analysis.

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Keywords Acetylcholinesterase · Amperometric determination · Biosensor · Multiwall carbon nanotube · Sol–gel · Triazophos

1 Introduction

The great success in agricultural applications of organophosphorous (OP) compounds has led to an increase in production and spread of these insecticides [1]. OP compounds exhibit acute toxicity and can irreversibly inhibit acetylcholinesterase (AChE), which is essential for the functioning of the central nervous system, often causing respiratory paralysis and death [2]. For these reasons, rapid determination and reliable quantification of trace level of OP compounds are significant to health and the environment [3]. Common analytical techniques for determination of OP compounds such as gas and liquid chromatography [4–7] are sensitive and reliable; however, these methods are time-consuming and require expensive equipment, highly trained personnel, complicated sample pretreatments and are not suitable for field conditions.

Enzyme based electrochemical biosensors represent a promising alternative to the classical methods due to their good selectivity, sensitivity, rapid response, and miniature size [8]. A variety of enzymes such as organophosphorous hydrolase, alkaline phsosphatase, ascorbate oxidase, tyrosinase and acid phosphatase have been employed [9]. During pesticide analysis, amperometric AChE biosensors have shown satisfactory results for OP and carbamate determination [10]. When AChE was immobilized on a working electrode surface, its interaction with the substrate of acetylthiocholine gave an electro-active product of thiocholine, which produced an irreversible oxidation peak. Based on inhibition of OP on the enzymatic activity,

triazophos was monitored by measuring the oxidation current of thiocholine. Aldridge [11] has described the inhibition mechanism.

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

$$2\ \text{thiocholine (red)} \rightarrow \text{thiocholine (ox)} + 2e^- + 2H^+$$

$$E + PX \rightarrow EP + X$$

(E = enzyme, PX = organophosphate or carbamate, X = leaving group).

In order to obtain a sensitive and stable enzyme sensor, a key requirement of enzyme immobilization is attachment without the bioactivity being sacrificed [12]. Sol–gel technology provides an attractive method due to attractive features including low temperature process, chemical inertness, physical rigidity, negligible swelling and tenable porosity [13]. Many biological entities including full cells, enzymes, proteins and antibodies or antigens have been successfully immobilized to solid electrode surfaces using sol–gel films [14–16]. Since discovered in 1991 [17], carbon nanotubes (CNTs) have attracted much attention due to their unique mechanical and electrical properties [18–20]. Because of the advantages in the sensing field: small size with larger surface, easy immobilization of protein with retention of activity [21] and particularly the ability to facilitate electron transfer when being used as electrode [22, 23], these kinds of materials have been widely used for preparation of amperometric biosensors and the study of the electrochemical properties of biomolecules [24, 25]. However, to our best knowledge, no application of CNTs with sol–gel composites in organophosphorous analysis has been reported.

Because of the excellent biocompatibility, nontoxicity, low cost, easy-handling, and high mechanical strength of chitosan, it is a preferred matrix for maintaining the high biological activity of biomolecules and enhancing the sensitivity of the sensor. In this work multiwall carbon nanotubes (MWNTs) was assembled on chitosan modified sol–gel-derived silicate network for immobilization of AChE, leading to a stable AChE sensor for rapid determination of triazophos, a model compound of OP insecticides. This matrix provided a biocompatible microenvironment around the enzyme molecule to stabilize its biological activity to a large extent. The immobilized AChE film was fragile and easily became detached if chitosan was not used in the membrane. The presence of nanoparticles reduced the working potential by catalyzing the electrochemical oxidation of enzymatically formed thiocholine. The sensor showed acceptable stability and sensitivity to the determination of triazophos in garlic samples, which has potential application in pesticide analysis and environmental monitoring.

2 Materials and methods

2.1 Reagents

Acetylthiocholine chloride (ATCl), acetylcholinesterase (Type C3389, 500 U mg^{-1} from electric eels) and Tetrathoxysilane (TEOS) were purchased from Sigma-Aldrich (St. Louis, USA) and used as received. Triazophos was acquired from AccuStandard (USA). Multiwall carbon nanotubes were a gift from the Institute of Nanometer, Central China Normal University. Chitosan (95% deacetylation), phosphate buffer solution (PBS, pH 7.0) and other reagents used were of analytical reagent grade. All the chemicals were of analytical grade and aqueous solutions were prepared with double distilled water.

2.2 Preparation of the biosensor

A glassy carbon electrode (GCE) was polished to mirror finish using a BAS-polishing kit with 0.3 and 0.05 μm Al_2O_3 paste. After sonication with ethanol and water, a potential of +1.75 V was applied under string 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 [26].

The MWNTs used in the experiments were heated under reflux in HNO_3 for 10 h. It has been shown that this treatment causes segmentation and carboxylation of the MWNTs at their terminus. After separated from the mixture, the sediment was washed with water till pH 7.0 and redispersed in water to obtain suspension of MWNTs with aid of ultrasonic agitation. A homogenous silica sol–gel assembling MWNTs composite was prepared by mixing 20 μL of TEOS, 50 μL of ethanol, 200 μL of MWNTs, 600 μL of 0.5 mg mL^{-1} chitosan solutions (final concentration 0.3%). This mixture was stirred for 1 h until a clear sol–gel composite was formed and its pH was adjusted to 4.0–6.0 using 130 μL 0.1 M NaOH solution.

The sol–gel composite (3.0 μL) was applied to a pretreated GCE and left to react at 20 °C for 4 h. The electrode was then coated with 4.0 μL AChE solution, and incubated at 25 °C for 30 min. After evaporation of water, the sensor was washed with PBS to remove unbound AChE. The AChE-MWNTs-SiSG/GCE obtained was stored at 4 °C when not in use.

2.3 Electrochemical measurement

Electrochemical measurements were performed with a Bioanalytical System (BAS, USA) cv-50w with a conventional

three-electrode system comprising platinum wire as auxiliary electrode, saturated calomel electrode (SCE) as reference and AChE immobilized on a chitosan-silica sol–gel (SiSG) assembling MWNTs composite modified glass carbon electrode (AChE-MWNTs-SiSG/GCE) as working electrode.

For the measurements of triazophos, the obtained AChE-MWNTs-SiSG/GCE was first immersed in the PBS solution containing different concentrations of standard triazophos or samples for 12 min, and then transferred to the electrochemical cell of 1.0 mL pH 7.0 PBS containing 0.5 mM ATCl to study the electrochemical response by cyclic voltammetry (CV). The inhibition of triazophos was calculated as follows:

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

where $i_{\text{P, control}}$ is the peak current of ATCl on AChE-MWNTs-SiSG/GCE, $i_{\text{P, exp}}$ is the peak current of ATCl on AChE-MWNTs-SiSG/GCE with triazophos inhibition.

2.4 High-pressure liquid chromatography measurement

HPLC detections at 235 nm were carried out on an Alliance 2695 Separation Module comprising an XDB-C8 column (150×4.6 mm^2) equipped with a UV–vis photodiode-array detection system using a mobile phase consisting of 20% CH_3OH and 80% H_2O at a flow rate of 0.8 mL min^{-1}.

3 Results and discussion

3.1 Cyclic voltammetric behavior of AChE-MWNTs-SiSG/GCE

Figure 1 shows the cyclic voltammograms of different electrodes in the absence and presence of 0.5 mM ATCl in pH 7.0 PBS. No peak was observed when the GCE (curve a) and AChE-MWNTs-SiSG/GCE (curve b) were placed in pH 7.0 PBS. When 0.5 mM ATCl was added to the PBS, the cyclic voltammograms of AChE-MWNTs-SiSG/GCE showed an irreversible oxidation peak at 566 mV (curve e), whereas no detectable signal was observed at MWNTs-SiSG/GCE without immobilization of AChE (curve c). Obviously this peak arose from the oxidation of thiocholine a hydrolysis product of ATCl, which was catalyzed by immobilized AChE.

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

$$2 \text{ thiocholine (red)} \rightarrow \text{thiocholine (ox)} + 2e^- + 2H^+ .$$

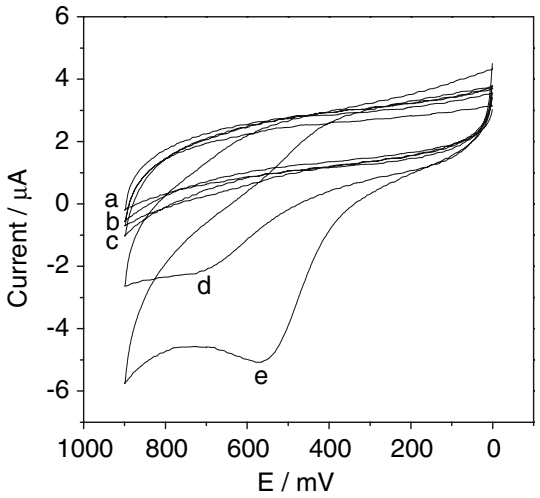

Fig. 1 Cyclic voltammograms. (**a**) GCE, (**b**) AChE-MWNTs-SiSG/GCE in pH 7.0 PBS, (**c**) MWNTs-SiSG/GCE, (**d**) AChE-SiSG/GCE, (**e**) AChE-MWNTs-SiSG/GCE in pH 7.0 PBS containing 0.5 mM ATCl

In comparison, when AChE was immobilized on a silica sol–gel modified electrode without MWNTs (AChE-SiSG/GCE), there was also a small peak (curve d). However, this peak current was much lower and the peak potential shifted positively by about 150 mV compared with those on the AChE-MWNTs-SiSG/GCE (curve e). This was because of the presence of MWNTs in the sol–gel film, which provided a conductive pathway for electron transfer and promoted electron-transfer reactions at a lower potential. Thus AChE-MWNTs-SiSG/GCE was used in the following experiments.

After the AChE-MWNTs-SiSG/GCE was immersed in a solution of triazophos for 12 min, the current decreased drastically compared with the control (Fig. 2). The decrease in peak current increased with increasing triazophos concentration. This was because triazophos, as a kind of OP pesticide, exhibits acute toxicity and is involved in the irreversible inhibition action on AChE, thus reducing the activity of the enzyme to its substrate. Due to the notable change in voltammetric signal of the AChE-MWNTs-SiSG/GCE, a simple method for determination of triazophos was established.

With increasing scan rate, the peak current increased and the peak potential shifted slightly Fig. 3). The peak currents exhibited a linear dependence on the scan rate from 5 mV s^{-1} to 200 mV s^{-1}, indicating a typical surface-controlled electrode process instead of a diffusion-controlled process. If the enzyme dissolved from the film surface into the reaction buffer and the soluble enzyme catalyzed ATCl hydrolysis in solution, the peak current should exhibit a linear dependence on the square root of

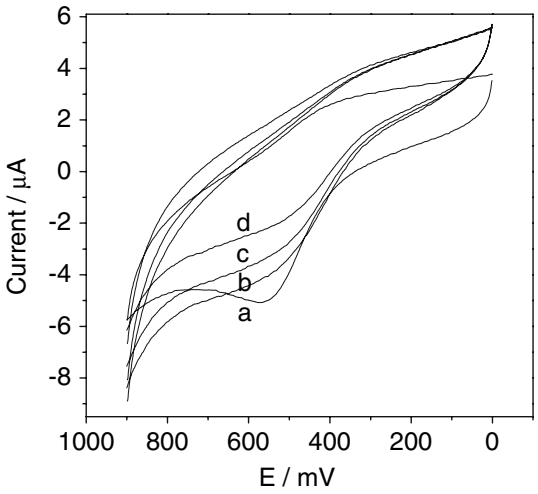

Fig. 2 Cyclic voltammograms of AChE-MWNTs-SiSG/GCE in pH 7.0 PBS containing 0.5 mM ATCl after incubation in triazophos solution for 12 min. (**a**) 0 μM, (**b**) 0.5 μM, (**c**) 2.0 μM, and (**d**) 25 μM

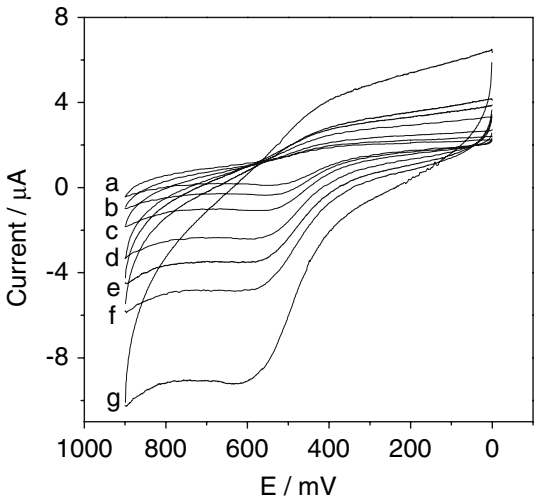

Fig. 3 Cyclic voltammograms of AChE-MWNTs-SiSG/GCE in pH 7.0 PBS containing 0.5 mM ATCl at different scan rate. (**a**) 5, (**b**) 10, (**c**) 20, (**d**) 50, (**e**) 80, (**f**) 100, (**g**) 200 mV/s

scan rate instead of on the scan rate. This result showed efficient immobilization of enzyme on the matrix.

3.2 Effect of MWNTs content on preparation of biosensor

With increasing amount of MWNTs, the anodic peak current increased and reached a maximum at a volume ratio of 20% (V_{MWNTs}/V) (Fig. 4). Further increasing the volume of MWNTs (more than 200 μL) led to a decrease in the response, possibly because of increased resistance and double layer capacitance of the modified electrode. As a result of these experiments, 20% (V_{MWNTs}/V) MWNTs was used for preparation of the biosensor.

3.3 Effect of pH on response of the AChE-MWNTs-SiSG/GCE

The effect of pH on the response of the biosensor contains two factors: the activity of the immobilized enzyme and the peak potentials of the electrode reaction. It was clearly observed that the maximum current occurred at pH 7.0 (Fig. 5). This same result was close to that reported for soluble AChE, indicating that the MWNTs-SiSG matrix did not alter the optimum pH for electron-transfer of the immobilized enzyme. The microenvironment surrounding the enzyme in the sol–gel pores was easily accessed by the substrate. Thus pH 7.0 was used in the detection solution.

3.4 Effect of temperature on response of the AChE-MWNTs-SiSG/GCE

The peak current of ATCl at AChE-MWNTs-SiSG/GCE increased in the temperature range 0–20 °C, indicating an increasing activity of the immobilized enzyme. The optimum region occurred from 20 °C to 50 °C (Fig. 6). No obvious decrease in response was observed during this temperature interval, indicating an excellent activity of the immobilized enzyme and no denaturation. The thermal stability and activity could be attributed to the biocompatible microenvironment around the enzyme molecules provided by the sol–gel matrix, which stabilizes their biological activities to a large extent. For practical convenience 25 °C was therefore selected for immobilization of AChE.

3.5 Effect of inhibition time of triazophos on response of the AChE-MWNTs-SiSG/GCE

One of the most influential parameters in pesticide analysis is inhibition time. With increase in immersion time in triazophos solution, the peak current of ATCl on the AChE-MWNTs-SiSG/GCE decreased greatly. Triazophos displayed an increasing inhibition with increasing time. As shown in Fig. 7, when the immersion time was longer than 12 min the curve trended to a stable value, indicating that binding interactions with active target groups in the enzyme were reaching saturation. This tendency of the peak current to decrease reflects a change in enzymatic activity, which decreases interactions with the substrate. Therefore, the change observed in the simple electronic voltammetric sensing system can be used as an indicator for quantitative measurement of triazophos pesticide. However, the maximum value of inhibition of triazophos was not 100%, which is attributable to the binding equilibrium between pesticide and binding sites in the enzyme.

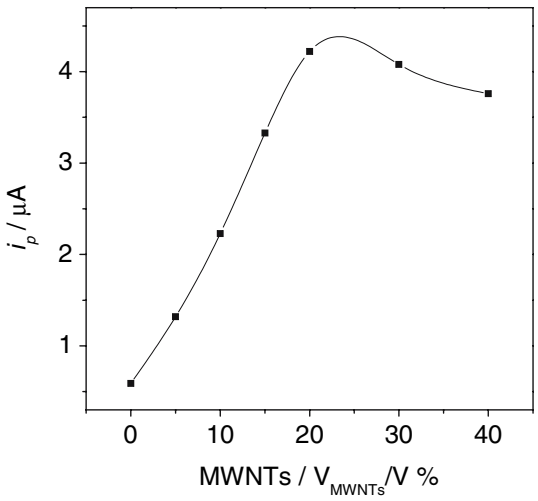

Fig. 4 Effect of MWNTs content on peak current

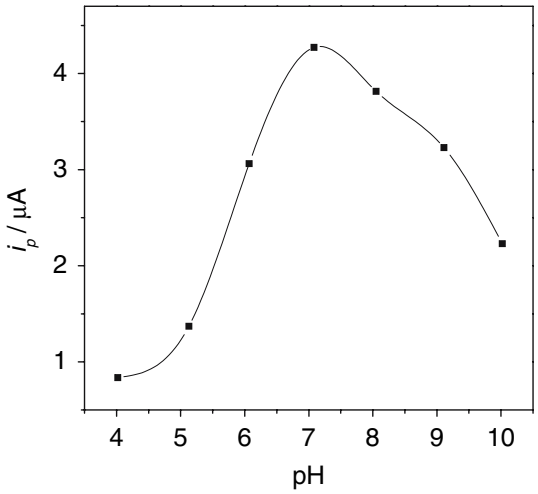

Fig. 5 Effect of pH on peak current

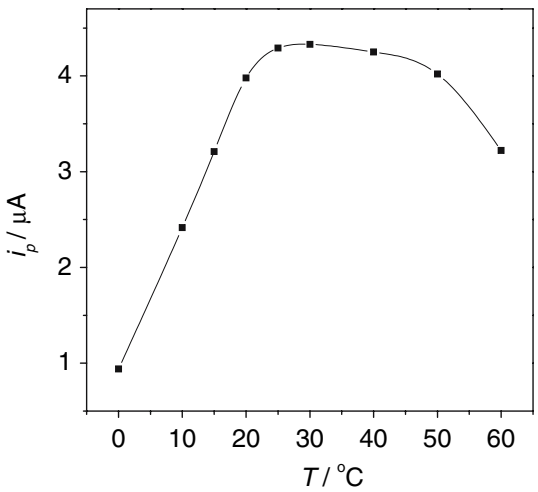

Fig. 6 Effect of temperature on peak current

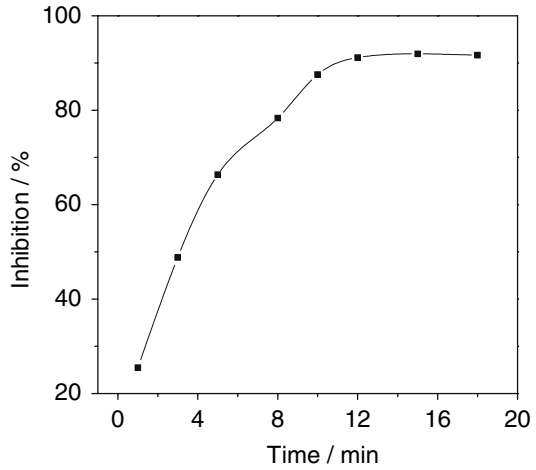

Fig. 7 Effect of immersing time on inhibition of triazophos

3.6 Measurement of triazophos

With increasing triazophos concentration in the immersing solution, the current decreased. As shown in Fig. 2, the responses of ATCl at AChE-MWNTs-SiSG/GCE decreased by 33% and 58% after inhibition with 0.5 μM triazophos and 2.0 μM triazophos, respectively. At higher triazophos concentration (25 μM), the response decreased by up to 89%. The inhibition of triazophos increased with concentration. Under optimum experimental conditions, the inhibition of triazophos was proportional to its concentration from 0.02 μM to 1 μM and from 5 μM to 30 μM, with correlation coefficients of 0.9957 and 0.9986, respectively (Fig. 8). The detection limit was 0.005 μM at a 10% inhibition [2, 26].

3.7 Application of the biosensor

Triazophos concentrations in garlic samples were detected from their inhibition to AChE. In parallel, the concentrations

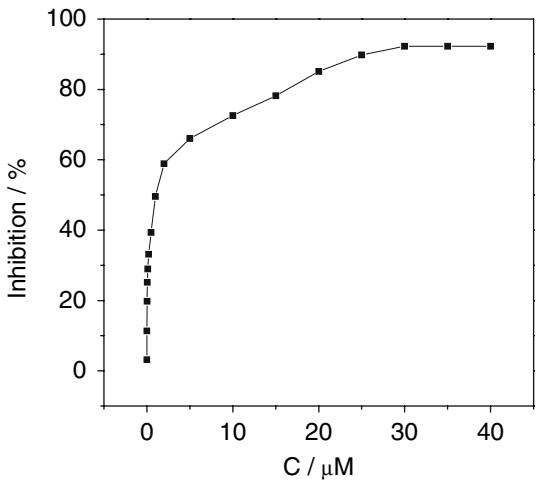

Fig. 8 Calibration curve for triazophos determination

Table 1 Comparison of triazophos levels in samples determined using two methods

Samples	1	2	3	4	5	6	7
Biosensor/μM	0.027	0.032	0.056	0.066	0.075	0.079	0.099
HPLC/μM	0.025	0.035	0.061	0.069	0.077	0.080	0.095
Relative deviation/%	8.0	–8.6	–8.2	–4.3	–2.6	–1.2	4.2

were also detected with HPLC. The results are shown in Table 1. The relative deviations between the two methods are in the region of 8.0% to –8.6%, indicating acceptable agreement. Thus, the biosensor may be satisfactorily applied to the determination of triazophos levels in practical samples.

3.8 Reproducibility, precision and stability of biosensor

The reproducibility of the biosensor was estimated by determining the response of 0.5 mM ATCl at eight different electrodes, which were immersed in 0.5 and 5.0 μM triazophos for 12 min, respectively. The coefficient of variation was found to be 2.9% and 1.5%, respectively, indicating acceptable fabrication reproducibility. The intra-assay precision of the sensors was calculated by assaying one enzyme electrode for eight replicate determinations, and the RSD was 0.5% at 0.5 mM ATCl.

After the AChE-MWNTs-SiSG/GCE was stored in a refrigerator at 4 °C for 3 weeks, no obvious decrease of ATCl was observed. After storage for 40 days, the sensor retained 80% of its initial current response. This indicated that silica sol–gel provided a biocompatible microenvironment around the enzyme to stabilize its biological activity to a large extent [16]. The large quantities of hydroxyl groups in the sol–gel hybrid material were able to form strong hydrogen bonds. These hydrogen bonds and the intermolecular interactions between enzyme molecule and specific sites of the silica sol–gel prevented the immobilized enzyme from leaking from the film.

4 Conclusions

A AChE biosensor for quantitative determination of triazophos, a model compound of OP insecticides was developed. The porous structure of the silica sol–gel matrix provides a biocompatible microenvironment around the enzyme. This is very efficient for retaining the enzyme activity and preventing its leaking from the film. The assembled multiwall carbon nanotubes on a sol–gel network resulted in a high affinity to the substrate, a fast response rate of the immobilized enzyme and a high catalytic effect to the hydrolysis of ATCl to form thiocholine, which was then oxidized to produce a detectable response. The biosensor possessing good precision and fabrication reproducibility, acceptable stability, fast response and low detection limit has potential application in the characterization of enzyme inhibitors and the detection of toxic compounds.

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