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A screen-printed, amperometric biosensor for the determination of organophosphorus pesticides in water samples

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Abstract

An amperometric biosensor based on screen-printed electrodes (SPEs) was developed for the determination of organophosphorus pesticides in water samples. The extent of acetylcholinesterase (AChE) deactivation was determined and quantified for pesticide concentrations in water samples. An enzyme immobilization adsorption procedure and polyacrylamide gel matrix polymerization were used for fabrication of the biosensor, with minimal losses in enzyme activity. The optimal conditions for enzyme catalytic reaction on the SPEs surfaces were acetylthiocholine chloride (ATChCl) concentration of 5 mmol/L, pH 7 and reaction time of 4 min. The detection limits for three organophosphorus pesticides (dichlorvos, monocrotophs and parathion) were in the range of 4 to 7 μg/L when an AChE amount of 0.1 U was used for immobilization.

Key words: organophosphorus pesticides; acetylcholinesterase; enzyme immobilization; screen-printed electrodes; biosensor
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Introduction

Organophosphorus (OP) pesticides have been widely used for decades in agriculture, medicine and industries due to their high efficiency as insecticides or for enzyme inhibition (Gogol et al., 2000; Mulchandani et al., 2001). These pesticides comprise a diverse group of chemicals and the majority have been shown to result in high levels of acute neurotoxicity and carcinogenicity (Kamanyire and Karalliedde, 2004; Alavanja et al., 2004). The OP pesticides are one of the more abundant environmental and food chain pollutants, and have attracted increased global attention with respect to human, animal and insect health (Kamanyire and Karalliedde, 2004). Determining the sources of pollutants and magnitude of the threat is important as a protective measure. Therefore, the detection methods for OP pesticides have become a top concern (Diehl-Faxon et al., 1996).

Currently, most pesticide residues are measured by gas or liquid chromatographic methods (Molina et al., 1994; Pérez-Ruiz et al., 2005; Yao et al., 1991), usually coupled with mass spectrometric detection (Jeannot and Sauvard, 1999). The determination of individual analyte species by these standard reference methods are accurate and of high sensitivity (Jeannot and Sauvard, 1999; Hooijschuur et al., 2002). However, the analyses must be performed by highly trained technicians and cannot be carried out in the field. Moreover, the above methods require a long time and extensive sample preparation, and no information about the toxicity of pesticides can be obtained (Duttaa et al., 2008).

Biological compounds, typically enzymes or other biological ligands, can be used as specific recognition agents in analytical techniques. Immunoassays have been reported as alternative biological methods for determination of OP pesticides (Sherma, 1993). Immunoassay detection methods such as the enzyme-linked immunosorbent assay (ELISA) have been tested for the detection of pesticides in soil and water matrices (Walker et al., 2000). However, the long analysis time (1–2 hr) and extensive sample handling procedures required for this method are not particularly suitable for field-based analysis.

On the other hand, use of enzymes like acetylcholinesterase (AChE) or organophosphate hydrolase (OPH) has been previously reported for the measurement of OP pesticides (Velasco-Garcia et al., 2003, Deo et al., 2005). With high sensitivity, simple sample treatment, and easy operational procedure, electrochemical detection is an ideal analytical technique for *in situ* analysis. The screen-printed, amperometric AChE biosensor based on the inhibition of AChE shows a great application prospect among the enzyme-based biosensors developed.

AChE, employed as an indicator for quantitative measurement of OP pesticides, is essential for terminating the action of the neurotransmitter acetylcholine (Khayyami et al., 1998; Laschi et al., 2007). The hydrolysis of acetylth-

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iocholine chloride (ATChCl) can be catalyzed by AChE, while cholines generated by the reaction can be oxidized in the presence of hexacyanoferrate. The catalytic reaction of AChE and the oxidation of its products are described in Eqs. (1) and (2) (Ciucu et al., 2003; Neufeld et al., 2000; Shi et al., 2006).

$$CH_3COS(CH_2)_2N^+(CH_3)_3Cl^- + H_2O \xrightarrow{AChE} CH_3COOH + HS(CH_2)N^+(CH_3)_3Cl^- \quad (1)$$

$$2\,[Fe(CN)_6]^{3-} + 2HS(CH_2)N^+(CH_3)_3Cl^- \longrightarrow 2\,[Fe(CN)_6]^{4-} + 2S(CH_2)N^+(CH_3)_3Cl^- \quad (2)$$

The inhibition of AchE activity by OP pesticides can be characterized by the anodic oxidation current on the surface of SPEs. With various mediators optimizing the performance, similar amperometric AChE biosensors using SPEs have shown satisfactory results for the analysis of environmental samples (Andreescu et al., 2002; Schulze et al., 2004; Li et al., 1999; Petrov et al., 2002; Bucur et al., 2005).

The use of the mediators can undoubtedly increase the selectivity and sensitivity for OP pesticides in environmental samples; however, interference can be caused by the added chemicals. To maintain the bioactivity of the enzyme in the films, methods involving less chemicals or a harmless matrix for enzyme activity are preferred choices for enzyme immobilization on the electrode.

In recent years, sol-gel encapsulation of enzyme species has provided encouraging results and has shown potential advantages for enzyme immobilization on electrochemical biosensors (Albareda-Sirvent and Hart, 2002; Andreescu et al., 2002; Anitha et al., 2004). Sol-gel polymerization can be performed at low temperatures without any kinds of modifications and the resulting thin films exhibit a tunable pore size, allowing small molecules and ions to diffuse into the matrix (Anitha et al., 2004; Waibel et al., 2006). This immobilization method provides better enzyme activity and stability in contrast to covalent binding or crosslinking.

In this study, we report an amperometric biosensor based on simple, low cost and self-made SPEs for OP pesticides detection. Two immobilization methods, adsorption and sol-gel polymerization, were used for fabrication of the screen-printed AChE biosensor. A relatively simple preparation for the biosensor, optimized conditions of enzyme reaction and inhibition studies are presented and discussed based on three OP pesticides (dichlorvos, monocrotophs and parathion). The cost of the total detection process, suitability for on-site application and complexity of operational procedures were prior considerations in developing these sensors for the determination of neurotoxic pesticide residues in water samples.

1 Experimental

1.1 Apparatus and reagents

Electrochemical measurements were performed with a model CHI440A electrochemical workstation (Chenhua Instrumental Co., Shanghai, China). A semiautomatic screen-printer (Tingxuan Co., Shanghai, China) was used for fabricating the SPEs. Ultraviolet (UV) light was generated with an UVP gel imaging system (UVP Co., USA).

Purified AChE (500 U) and ATChCl were purchased from Sigma-Aldrich Corporation and stored at –20°C. Parathion, dichlorvos (2,2-dichlorovinyl dimethyl phosphate) and monocrotophs standards (100 mg/L) were obtained from Environmental Research and Monitoring of the Ministry of Agriculture, Tianjin, China. Acrylamide and N′-N′-methylene-bis-acrylamide was supplied by Dingguochangsheng Biotechnology Co., Beijing, China. Carbon inks (SC-1010), silver inks (EA-1854), insulating inks (UG-360) and polyethylene terephthalate (PET) were purchased from Advanced Electronic Materials Inc., Tainan, China. All reagents used in this study were of analytical or higher grade. The pH was adjusted with buffer solution (0.1 mol/L phosphate buffer and 0.2 mol/L KCl), and double distilled (dd) H_2O was used for all the experiments.

1.2 Fabrication of the screen-printed AChE biosensors

A series of PET chips (width 1.5 cm, length 4 cm and height 0.125 mm) were prepared before screen printing. The SPEs with three integrated electrodes were fabricated as follows: first, silver ink was printed through mesh to form three conducting channels, followed by drying at 130°C for 35 min. Second, carbon ink was printed to form circular working electrodes and annular auxiliary electrodes. The conducting layer for wire connection was printed as shown in Fig. 1, and then the chips were dried at 120°C for 30 min. Lastly, insulating ink was printed to cover the non-working and non-conducting region of the SPEs. Then, the chip was exposed under the UV lamp for 4 hr followed by a heat treatment for 30 min at 120°C for stabilization. The schematic design of the fabrication procedure for the integrated three-electrode screen-printed sensor is shown in Fig. 1. The diameter of the working electrode and the counter electrode were 4 and 10 mm, respectively (Fig. 1).

The AChE was immobilized directly by bulk copolymerization of acrylamide and N′-N′-methylene-bis-acrylamide on the screen-printed carbon working electrode surface. A small amount of AChCl substrate was also

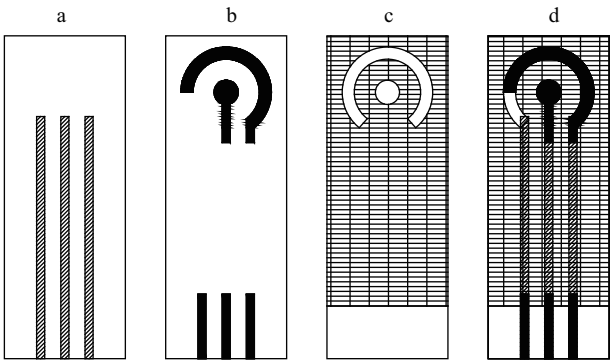

Fig. 1 Fabrication procedure of the integrated three-electrode screen-printed sensor. (a) silver ink layer; (b) carbon ink layer; (c) insulating ink layer; (d) schematic of three-electrode screen-printed sensor.

added to protect enzyme activity. The polymerization was initiated by UV light at a wavelength of 365 nm for 30 min, and the enzymatic membrane was gelatinized completely in the refrigerator at 4°C for 3 hr.

A solution mixture of 100 μL (10 mg/L) acrylamide monomer, 40 μL (10 mg/L) of N′-N′-methylene-bis-acrylamide and 20 μL (5 mmol/L) of AChCl was prepared. Subsequently, 10 μL of solution mixture and 10 μL of enzyme solution were added onto the screen-printed AChE sensor and mixed for polymerization. Then, 20 mL of enzyme solution was directly added to the screen-printed AChE sensor and dried at 4°C for 3 hr. The screen-printed AChE sensors were stored in the freezer at –20°C.

1.3 Optimization of AChE reaction conditions

A series of experiments was performed to optimize the enzyme catalytic reaction conditions. Standard solution 40 μL of potassium ferricyanide and 40 μL of ATChCl substrate solution were added to the surface of the SPEs, and mixed with 30 μL AChE solutionscontaining various concentrations (0.08, 0.1 and 0.15 U). Consecutive monitoring was carried out for 15 min. The optimum amount of ATChCl was determined by keeping a fixed amount of AChE and an unchanged pH with varying amounts of ATChCl (0.5, 1, 2, 5, 8 and 10 mmol/L solutions) and the response current was recorded every minute. AChE inhibition by pH and effects on the reaction were examined for various pH values ranging from 5.5 to 8.5. Based on this, the best reaction time was defined, which showed significant current response under optimal enzyme concentration, pH and substrate concentration. Based on AChE inhibitory activity, quantitative analysis of dichlorvos, monocrotophos and parathion was performed.

1.4 Amperometric measurements

All amperometric measurements were carried out by the electrochemical workstation at room temperature. Phosphate buffer (0.1 mol/L) and KCl (0.1 mol/L) supporting electrolyte were used during the whole detection process. Cyclic voltammetry tests were performed to assess the consistency of the SPEs, while stable current responses of the biosensors were measured by chronoamperometry at 0.08 V. Each sample was stirred to ensure uniform distribution during the measurement process.

To measure the inhibition of AChE under the same conditions, 30 μL pesticide solution (0.6 to 3000 μg/L) was added to the surface of the biosensors with immobilized AChE. After an incubation time of 8 min, 40 μL potassium ferricyanide standard solution and 40 μL ATChCl substrate solution were added and mixed. As the stationary state current is related to activity of the enzyme, the current intensity was recorded at every 1 min. Phosphate buffer solution 30 μL was used as a control to replace the pesticide solution. The percentage inhibition (I, %), which corresponds to a fixed concentration of water sample, was calculated as follows:

$$I = \frac{I_{ss} - I_p}{I_{ss}} \times 100\% \quad (3)$$

where, I_{ss} and I_p represent the response current intensity of the control sample and the water sample, respectively.

2 Results and discussion

2.1 Electrochemical behavior of the enzyme catalytic reaction

The electrochemical behavior of the enzyme reaction was characterized through chronoamperometry in pH 7.5 buffer solution on the surface of the SPEs. Figure 2 shows the relevant current intensity of the enzymes. It is clear that the electrochemical signals decreased with time, and stabilized after 10 min. The currents changed dramatically with evident first-order reaction kinetics in the first few minutes, which indicate that the enzyme could effectively catalyze the hydrolysis reaction of ATChCl. A similar phenomenon was also observed in our previous work for different amounts of AChE, but more rapid catalytic reaction was seen for 0.15 U enzyme. With the magnitude of the error bars shown in Fig. 2, the standard solution of potassium ferricyanide and SPEs used here were sufficient for generation of suitable and reliable data.

2.2 Optimization of detection conditions

Figure 3a shows the response of enzyme reaction currents on SPEs for different pH ranging from 5.5 to 8.5. Clearly, AChE has a broad optimal pH in the range of 7.0 to 8.5 with maximum response at pH 8.0. As reported by Liu et al. (1985), the reagents and measuring systerm used in this study were suitable for enzyme activity and evaluation of OP pesticides concentrations. Varying detection time (4, 5 and 6 min) showed a similar trend, but more time meant higher current intensity. Concerning that OP pesticides could be decomposed under alkaline conditions, pH 7.0 and 7.5 was chosen for further reaction analysis in the presence of OP pesticides. Corresponding changes were

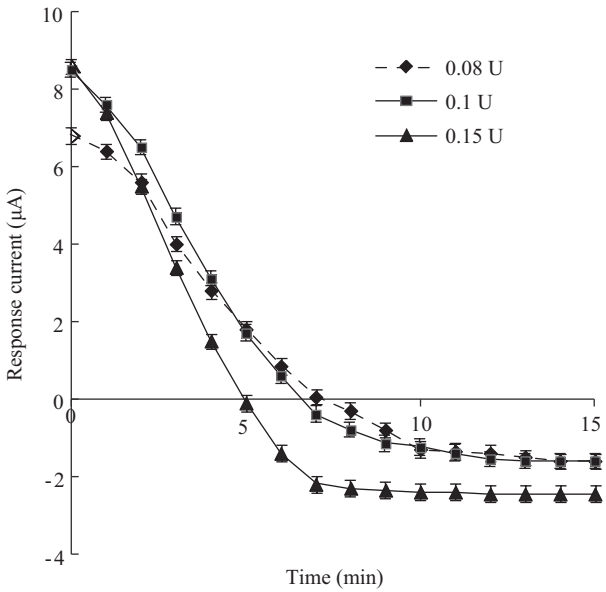

Fig. 2 Response currents of the biosensor using different amounts of AChE in 3 mmol/L ATChCl substrate concentration at pH 7.5 (working potential: 0.08 V vs. Ag/AgCl).

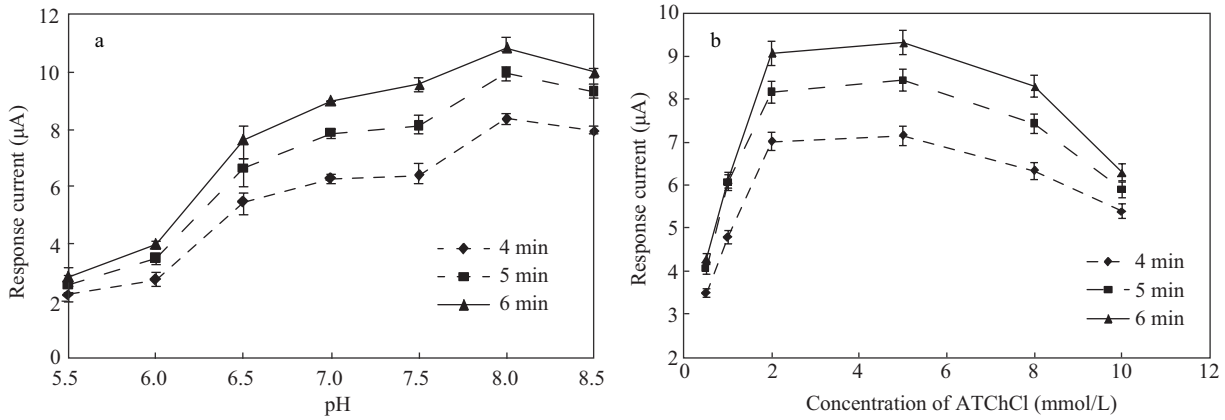

Fig. 3 Response currents of the biosensor at different pH for 3 mmol/L ATChCl substrate concentration (a) and different ATChCl substrate concentrations at pH 7.0 (b) (working potential: 0.08 V vs. Ag/AgCl).

obtained for different concentrations of pesticides at pH 7 and this value was used in the following experiments.

The response of SPEs in the absence of pesticide analytes was determined with ATChCl concentrations ranging from 0 to 10 mmol/L. As shown in Fig. 3b, it was clear that the best sensitivity and accuracy of measurements were achieved when the ATChCl concentration was 5.0 mmol/L. With a similar trend as a function of detection time, response currents increased sharply when the substrate concentrations were less than 2.0 mmol/L, whilst a small decline in sensor output was observed at concentrations in excess of 5.0 mmol/L. This may be due to enzyme inhibition at higher concentrations of the enzyme substrate. Taking into account economic factors, the substrate concentration of 5.0 mmol/L was chosen in the subsequent experimental study. Meanwhile, to achieve significant reaction results, a shorter time of 4 min was chosen as the optimal reaction time for further analysis.

Given the acceptable performance of SPEs towards ATChCl within the non-inhibited system, the performance of the sensor in the presence of dichlorvos was examined. With various concentrations of AChE (0.08, 0.1 and 0.15 U) and dichlorvos added, the results obtained from the reaction was evaluated. From Fig. 4, it could be found that dichlorvos inhibited the activity of enzyme in a distinct way. It was difficult to detect low concentrations of dichlorvos with 0.15 U AChE, due to weak inhibition of the enzyme. An amount of 0.08–0.1 U was therefore used, because less enzyme could be inhibited more easily, which would result in achieving a lower detection limit. Enzyme activity was reduced during the process of immobilization and a relatively higher amount of enzyme resulted in shorter response times, thus a AChE concentration of 0.1U was found to besuitable for immobilization.

2.3 Detection of OP pesticides

In order to measure the inhibition rate of AChE, different concentrations within the range of 0.6 to 3000 μg/L of pesticides were tested under the optimum conditions mentioned above. Dichlorvos, monocrotophs and parathion are typical pesticides that were evaluated in this study. The entire calibration curves (Fig. 5) revealed that all the OP pesticides used in this study resulted in measurable

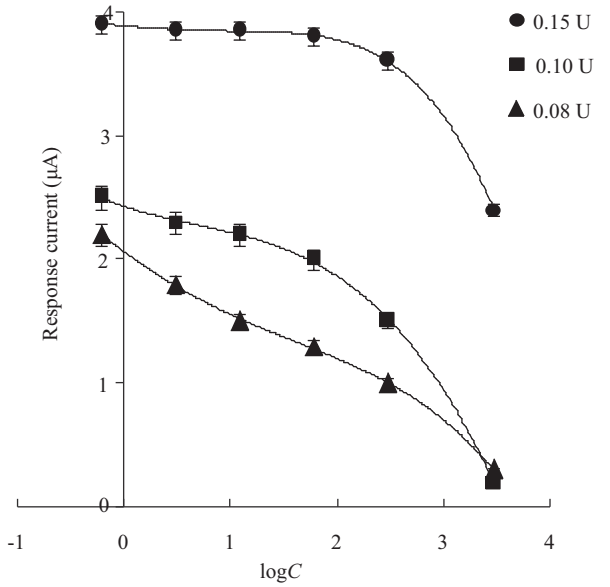

Fig. 4 Response currents of different AChE concentrations for dichlorvos at pH 7.0 (working potential: 0.08 V vs. Ag/AgCl).

inhibition within the range of 0.6 to 3000 μg/L. Response currents and the logarithm of the inhibitor concentrations showed an approximate first-order correlation for all the OP pesticides tested. The current intensity for enzyme immobilized by adsorption was higher than that immobilized by sol-gel polymerization, while the same approximate current span was achieved. This was possibly attributed to the negative impact on hexacyanoferrate in the presence of sol-gel substances. These calibrations were used to calculate *I* and construct the inhibition plots. The corresponding inhibitor concentration giving a 10% inhibition rate was defined as the theoretical detection limit (Fig. 5).

Detection limits of dichlorvos, monocrotophs and parathion with enzyme immobilized by adsorption were 4.0, 5.9 and 4.2 μg/L, respectively, while the values for sol-gel polymerization were 4.7, 5.8 and 6.5 μg/L, respectively. The enzyme AChE, essential for the functioning of the central nervous system, plays an important role in the human body (Musilek et al., 2008). The presence of any pesticide species in water samples has a potential toxic effect on the environment and human life. Thus, the inhibition series observed in this study could serve as a method

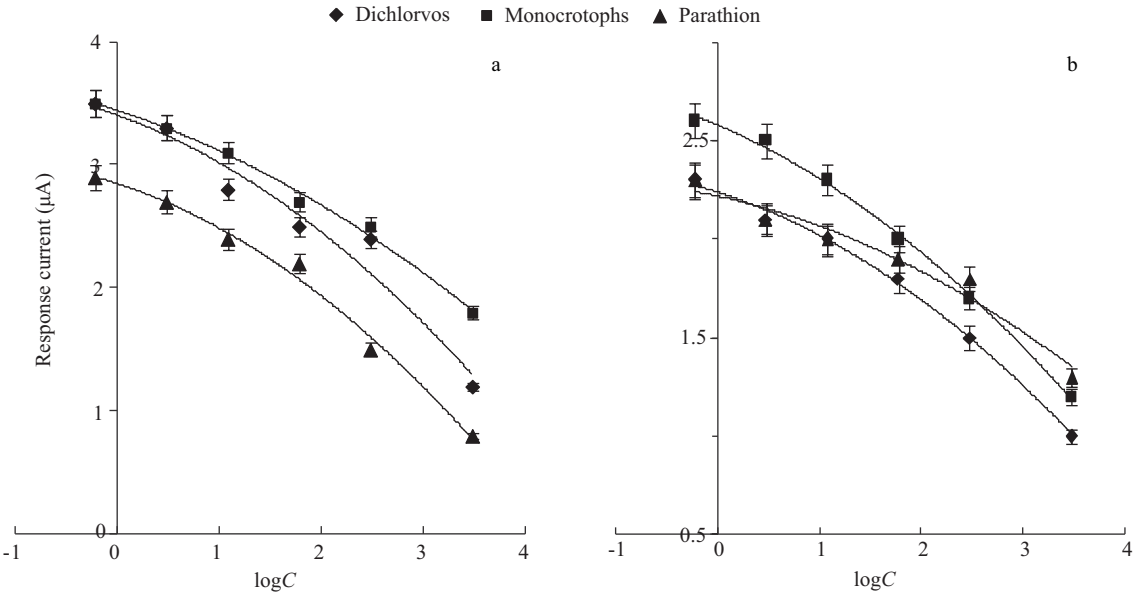

Fig. 5 Calibration curves for different pesticides obtained with optimal experimental conditions for adsorption (a) and sol-gel polymerization (b).

to measure the relative OP toxicity and potential health threat. As a simple, convenient, low-cost and repeatable screening tool, the biosensors developed in this study will be suitable for analysis of pesticides in the field. Duttaa et al. (2008) fabricated a biosensor based on SPEs of a two-electrode system with a similar enzyme immobilization procedure to detect four kinds of organophosphate and carbamate pesticides. A range of 0 to 10 μg/L pesticides was obtained for various inhibition rates, and the highly sensitive biosensor was targeted for low-cost, field-based determination of pesticides. Compared to a conventional three-electrode system, the potentiometric detector based on a two-electrode system provided reliable data.

A mediator-free amperometric biosensor for screening OP pesticides in a flow-injection analysis (FIA) system has been reported by Shi et al. (2006). The enzyme biosensor was prepared by entrapping AChE in a Al_2O_3 sol-gel matrix screen-printed on an integrated 3-electrode polyethylene terephthalate (PET) chip. A linear inhibition response at the range of 0.1–80 μmol/L (22 to 1768 μg/L), corresponding to 7.91% to 84.94% inhibition for AchE, was observed for dichlorvos. With 15 min of inhibition time, a 10 nmol/L (2.2 μg/L) detection limit was achieved for dichlorvos in simulated seawater. The Al_2O_3 sol-gel matrix with embedded enzyme provided a friendly microenvironment for immobilization and promoted electron transfer between the thiocholine and the electrode. This feature is similar to our polyacrylamide gel matrix polymerization, with potassium ferricyanide as mediator for electron transfer. Good stability and reproducibility were achieved for high salinity sea water and it has also been proven that a long inhibiting time led to a lower detection limit. We believe that the simple and broad-range detection SPEs biosensor developed in this study would be more suitable for field testing .

Waibel et al. (2006) developed a highly sensitive screen-printed bienzymatic sensor for the detection of phosphorothionates in food. These compounds evolve their inhibitory activity towards AChE only after oxidation (by P450 monooxygenases), thus a triple mutant of cytochrome P450 BM-3 (CYP 102-A1) and *Nippostrongylus brasiliensis* AChE (NbAChE) were immobilized on the sensor using a fluoride-catalyzed sol-gel process. Different sol-gel types were fabricated and characterized regarding enzyme loading capacity and enzyme activity containment. Specifically, 7,7,8,8-tetracyanoquinodimethane (TCNQ) as a modified particle was employed for the sol-gel technique. The detection limits achieved were 1 μg/L for paraoxon and 10 μg/L for parathion while the incubation time was varied from 30 to 90 min. Transducers with cobalt(II) phthalocyanine (CoPC)-modified screen-printed carbon electrodes (SPCEs) were used by Laschi et al. (2007). The AChE was immobilized by crosslinking with glutaraldehyde, bovine serum albumin (BSA) and Nafion onto the surface of the modified SPCE. A dynamic range for carbofuran detection was 10^{-10} to 10^{-7} mol/L (0.02 to 22.1 μg/L) under optimized conditions, with a detection limit of 4.9×10^{-10} mol/L (0.1 μg/L) and an analysis time of 15 min (potential 0.1 V versus pseudo-Ag/AgCl reference electrode). The limits of detection obtained were small enough to detect trace amounts of pesticides but at the cost of a large amount of enzyme (1.4 U). Recently, Istamboulie et al. (2010) obtained a new electrochemical mediator for acetylcholinesterase-based biosensors directly by screen-printing a poly(3,4-ethylenedioxythiophene) suspension on the surface of thick film carbon electrodes. The high conductivity polymer was shown to be suitable for thiocholine oxidation, allowing the measurement of AChE activity at 0.1 V vs. Ag/AgCl on SPCEs. An accurate detection of the OP insecticide chlorpyrifos-oxon at a concentration of 1.3 μg/L was achieved at inhibition rates as low as 5%. However, with a low construction cost and a simple operation procedure, our biosensor provides a detection threshold suitable for routine testing of OP pesticides.

3 Conclusions

A low-cost screen-printed amperometric biosensor was developed in this study as a simple strategy to quantitatively screen for OP pesticides in water samples. Changes in the degree of enzyme inhibition were related to the amount of pesticides. Two enzyme immobilization procedures, adsorption and polyacrylamide gel matrix polymerization, coupled with electrochemical detection methods, proved to be feasible for OP pesticide determination. It was observed from the experiments that an ATChCl concentration of 5 mmol/L at pH 7 and 4 min reaction time were optimal conditions for the enzyme catalytic reaction on SPEs surfaces. An AChE amount of 0.1 U was used for immobilization, making the detection limits for three OP pesticides in the range of 4 to 7 μg/L. With a relatively wide detection range and short detection time, this technique allowed the immobilization of different amounts of enzyme and incubation time depending on specific requirements. Future studies will focus on the development of a biosensor for rapid and direct determination of pesticides in soil extracts, potable water and other environmental samples.

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