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A mediator-free screen-printed amperometric biosensor for screening of organophosphorus pesticides with flow-injection analysis (FIA) system

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Abstract

A mediator-free amperometric biosensor for screening organophosphorus pesticides (OPs) in flow-injection analysis (FIA) system based on anticholinesterase activity of OPs to immobilized acetylcholinesterase enzyme (AChE) has been developed. The enzyme biosensor is prepared by entrapping AChE in Al_2O_3 sol–gel matrix screen-printed on an integrated 3-electrode plastic chip. This strategy is found not only increase the stability of the embedded AChE, but also effectively catalyze the oxidative reaction of thiocholine, making the Al_2O_3–AChE biosensor detects the substrate at 0.25 V (versus Ag/AgCl), hundreds mini-volt lower than other reported mediator-free ones. The Al_2O_3–AChE biosensor is thus coupled to FIA system to build up a simple and low-cost FIA–EC system for screening OPs in real samples. A wide linear inhibition response for dichlorvos, typical OP, is observed in the range of 0.1–80 μM, corresponding to 7.91–84.94% inhibition for AChE. The detection limit for dichlorvos is achieved at 10 nM in the simulated seawater for 15 min inhibiting time, which allows the biosensor quantitatively detects the ecotoxicological effect of the real samples from the seaports in eastern China, where the OPs pollution is confirmed by GC–MS.

Keywords: Mediator-free; Organophosphorus; Enzymatic inhibition; Screen-printed; Amperometric; Flow-injection analysis

1. Introduction

Organophosphorus pesticides (OPs), known as a typical enzyme inhibitor, have been widely used for decades in agriculture, medicine, industry and even as chemical warfare agents in military practice. Meanwhile, global attention has been paid on the problems caused by OPs in environmental surveillance and protection. The methodology optimization study for the detection of OPs is thus quite crucial for analytical researchers, especially when the maneuverability and the cost of the analytical system are taken into account [1–12].

Electrochemistry affords high sensitivity, simple sample treatment, inexpensive instrument, and easily operation procedure, and is an ideal analytical technique for in situ analysis. Amperometric AChE biosensors based on the inhibition of acetylcholinesterase enzyme (AChE) using screen-printed electrodes (SPE) have shown satisfied results for real samples analysis [13–25], in which the anticholinesterase activity is employed as an indicator of quantitative measurement of OPs. The relevant reaction is described as in Eqs. (1) and (2).

$$\text{Acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid} \quad (1)$$

$$2\text{Thiocholine} \xrightarrow{\text{anodic oxidation}} \text{dithio} - \text{bischoline} + 2H^+ + 2e^- \quad (2)$$

The amperometric response of the AChE biosensors, i.e. the anodic oxidation current resulting from the thiocholine (TCh) formed in the enzymatic hydromantic hydrolysis of acetylthiocholine (ATCh), is inversely proportional to the concentration of OPs in samples, and the exposed time as well. To optimize the performance of the amperometric AChE biosensors, various mediators, like cobalt phthalocyanine (CoPC) [15], 7,7,8,8-tetracyanoquinodimethane [16–18,25]

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and Prussian Blue [19] or $[Fe(CN)_6]^{3-}$ [20], have been used to decrease the applied potentials for the electrocatalytic oxidation of thiocholine for the detection of OPs. Undoubtedly, the use of the mediators increases the selectivity and sensitivity for sensing OPs in real samples; however, it also involves the interference caused by the added chemicals. So, mediator-free biosensors [21–24] have been developed to increase the S/N ratio and decrease the interference. The matrix for the immobilization of enzymes in such mediator-free biosensors is considered as a key factor to promote the direct electron transfer (ET) between the enzyme and the electrode, and to maintain the bioactivity of the enzyme in films as well. Al_2O_3 sol–gel has been proved as an ideal one to couple with enzymes not only due to its hydrophilicity necessarily for the retention of the tertiary structure of the enzyme and subsequent enzyme stability, but also due to its efficiency as a promoter of direct ET [26]. In our previous work, Al_2O_3 sol–gel has been successively used to encapsulate tyrosine [26] or antibody to sensitively detect phenols or liver fibrosis markers [27]. So, in this work, Al_2O_3 sol–gel is adopted to immobilize AChE, and thus a novel mediator-free screen-printed amperometric biosensor coupled with flow-injection analysis (FIA) system for detection of OPs is developed. The simple low cost self-made screen-printed electrodes covered with an enzymatic Al_2O_3 gel membrane are built in a homemade flow cell, and the facility of the system has been evaluated by monitoring the inhibition response to OPs in simulated sea water (prepared by Mocledon prescription) and other real samples, comparing with the results analyzed by GC–MS. The Al_2O_3 sol–gel based AChE biosensor is found to effectively catalyze the oxidative reaction of thiocholine with no loss of the sensitivity and minimization of the interference.

2. Experimental

2.1. Apparatus and reagents

Electrochemical measurements were performed with CHI-1030 electrochemical workstation (Chenghua Instrumental Co., Shanghai, China). The FIA system was coupled with a periststic pump (Baoding Longer, Hebei, China), 7725 injection valve (Rheodyne, USA) and a self-made flow cell. A semiautomatic screen-printer (Guixing Co., Shanghai, China) was used for fabricating the screen-printed electrodes.

Purified acetylcholinesterase (AChE, VI-S, 244 IU/mg), acetylthiocholine (ATCh) chloride, dichlorvos (2,2-dichlorovinyl dimethyl phosphate), trichlorfon([2,2,2-trichloro-1-hydroxyethyl]-phosphonic acid dimethyl ester), rogor (dimethoate, *O*,*O*-dimethyl-*S*-methylcarbamoylmethyl phosphorodithioate), malathion (carbofos, *S*-1,2-bis(ethoxycarbonyl)ethyl-*O*,*O*-dimethyl phosphoro-dithoate) were purchased from Sigma–Aldrich. Carbon, silver and insulating inks were obtained from Baoying Company (Shanghai, China). All of other chemicals were analytical grade and were used without further purification. Buffer solutions used in experiments were prepared with doubly distilled water.

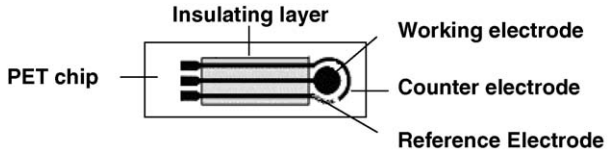

Fig. 1. The diagram of the integrated three screen-printed electrodes. Diameter of the working electrode and the counter electrode are 4 mm and 10 mm, respectively.

2.2. Fabrication of the screen-printed AChE biosensors

Before screen-printing, the AgCl particle was prepared: excess HCl (0.1 mol L^{-1}) was titrated slowly into the stirred 0.1 mol L^{-1} $AgNO_3$ solution. Then the AgCl deposit was filtrated and incubated to dry. Grinded AgCl particle (can pass through 0.2 mm mesh) was mixed thoroughly with the silver printing ink (1/1 to g/g).

The screen-printed electrodes with integrated three electrodes (Fig. 1) were fabricated as follows: firstly, the carbon ink was printed through the mesh to the PET support to form the conducting layer and also to work as the working electrodes and the counter electrodes, and then it was dried at 120 °C for 15 min. Secondly, the Ag/AgCl ink was printed on the reference electrodes position, and then dried at 120 °C for 45 min. Lastly, the insulating ink was printed to cover the non-working and non-conducting region of the SPE, and then it was dried under the UV lamp for 5 h. By using this procedure, batch production of SPE with low cost and stable quality were prepared.

Al_2O_3 sol–gel was prepared as literature [26]. The AChE was immobilized by casting a 10 μL droplet of AChE (0.5 U/μL in Al_2O_3 sol) onto the screen-printed carbon working electrode surface. The enzymatic membrane was subsequently gelatinized completely in the refrigerator (4 °C) for 24 h. All the prepared AChE–Al_2O_3–SPE biosensors were stored in buffer solution (pH 8.0, 0.1 mol L^{-1} phosphate containing 0.1 mol L^{-1} KCl) in the refrigerator (4 °C) before use.

2.3. The flow-injection analysis (FIA) system

The flow injection cell was constructed with two Perspex and a silicone rubber gasket to ensure the cell was watertight. The screen-printed electrode was placed tightly between the silicone rubber gasket and the back Perspex. There was an empty circle at the silicone rubber gasket just on the SPE region to act as the flow cell. The inlet and outlet for carrying solutions were just at the two opposite end of the cell to minimize the dead volume. The total volume of the flow cell was approximately 100 μL. The injection loop of the valve in FIA was 20 μL.

2.4. Measurement procedures

OPs determination was carried out as follows: the initial amperometric response (A_0: peak area of the oxidation current) to 1 mmol L^{-1} acetylthiocholine (ATCh) chloride was measured in the working buffer (pH 8.0, 0.1 mol L^{-1}phosphate in 0.1 mol L^{-1} KCl) stream; then 20 μL OPs sample was injected into the flow cell and stopped to let the biosensor incubated with the OPs for 5 min. Then the amperometric response to 1 mmol L^{-1} ATCh (A_i) was measured again. Thus the inhibition percent (I%) was calculated as the equation: I (%) = $(A_0 - A_i)/A_0 \times 100$. Total four kinds of OPs, i.e. dichlorvos, trichlorfon, rogor and malathion, were tested in this work. Among them, dichlorvos showed the highest inhibition effect to AChE, and thus was chosen as a model for the others experiments. All measurements were performed at room temperature (about 25 ± 2 °C). The real samples were obtained from the Beilun Seaport and the branch rivers of Yangtzejiang River at Ninbo, Zhejiang province located in Eastern China. All the samples were filtered with 0.22 μm filter as soon as possible and stored at 4 °C before use to avoid degradation.

3. Results and discussions

3.1. Catalytic effect and promoted electron transfer for Al_2O_3–SPE

The electrochemical behavior of Al_2O_3–SPE was tested by cyclic voltammetry (CV) in pH 8.0 buffer solution containing 5 IU AChE. Fig. 2 shows the cyclic voltammograms of the bare SPE and the Al_2O_3–SPE to thiocholine. It can be observed that the Al_2O_3–SPE shows a lower background

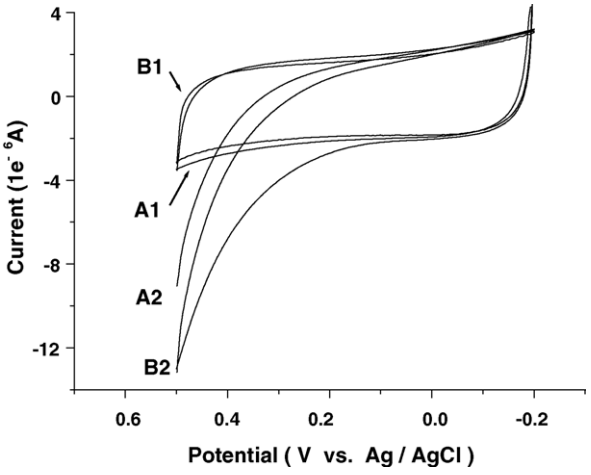

Fig. 2. Cyclic voltammograms of the thiocholine for bare SPE in buffer solution containing 5 IU AChE (A1), adding 0.667 mmol L^{-1} ATCh (A2), and for sol–gel immobilized SPE in buffer solution containing 5 IU AChE (B1), adding 0.667 mmol L^{-1}ATCh (B2). Scan rate: 0.1 V/s.

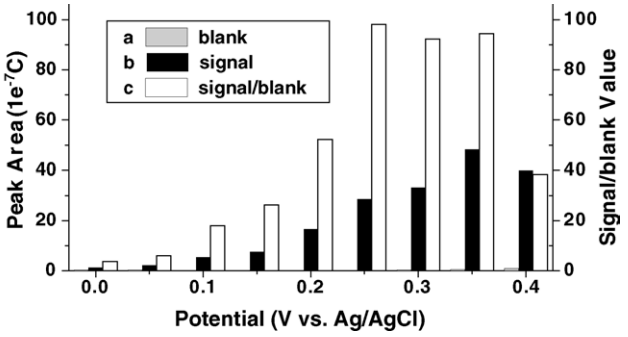

Fig. 3. Peak area via potential and signal/blank value-potential curves of the biosensors obtained under batch condition with (a) blank SPE immobilized with Al_2O_3 sol–gel; (b) AChE biosensor. Flow rate: 0.347 mL/min, ATCh concentration: 1 mM.

current compared to that of the bare SPE. When the ATCh substrate was added, the oxidative current of the Al_2O_3–SPE changed dramatically, being obviously larger than those of the bare SPE. This result indicates that the Al_2O_3 sol–gel can effectively catalyze the oxidative reaction of thiocholine. A similar phenomenon was also observed in our previous work using Al_2O_3 sol–gel tyrosinase biosensors to detect phenols [26].

Amperometric responses of the AChE–Al_2O_3–SPE biosensors at different potentials were tested in FIA–EC system as shown in Fig. 3. It can be observed, in the range from 0.0 V to 0.4 V (versus Ag/AgCl), that the responses for AChE–Al_2O_3–SPE were much larger than those of the control, indicating that the Al_2O_3 sol–gel is beneficial to AChE immobilization and retains the activity of the AChE. Meanwhile, a higher amperometric response with distinctly increased background current and noise was obtained when the working potential was more positive than 0.3 V. The highest signal/blank ratio was obtained at 0.25 V and it was further selected as working potential. This working potential showed to be several hundreds mini voltages lower than those obtained by other reported mediator-free amperometric biosensors for OPs analysis and would be helpful to minimize interferences from other coexisting electroactive impurities [21–24].

The specific surface area, pore size distribution and polarity of the Al_2O_3 sol–gel had a great influence on the performance of the AChE-biosensor and on the hydrophilic and positively charged porous Al_2O_3 sol–gel matrix. In fact, the Al/H_2O ratio is the most important factor. The effect of Al/H_2O ratio (mol/mol) on the biosensor response was tested and the optimum ratio was found to be 1:100, higher or lower ratio than this value leaded to poorer sensitivity of the biosensor.

3.2. Kinetic characteristics of AChE–Al_2O_3–SPE in FIA

The kinetic response of AChE–Al_2O_3–SPE in FIA system was also tested to investigate the enzymatic activ-

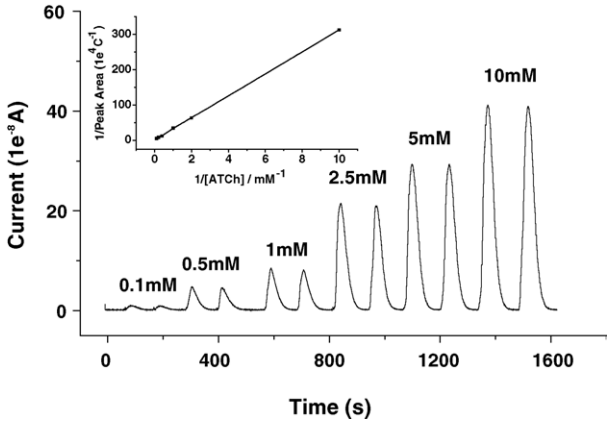

Fig. 4. Responses of the biosensor for successive injection ATCh. Inlet is the Line-weaver-Burk electrochemical representation of 1/peak area versus 1/[ATCh]. Working potential: 0.25 V vs. Ag/AgCl.

ity retaining in the embedded sol–gel. Fig. 4 shows the dependence of the current on the concentration of ATCh. The K_M value of the immobilized AChE was calculated by fitting experiment data to the Michaelis–Menten equation using the Lineweaver-Burk electrochemical representation of 1/[peak area] versus 1/[ATCh]. At pH 8.0 and at room temperature (about $25 \pm 2\,^{\circ}C$), the K_M of the immobilized AChE was of 15.59 mM and the linear correlation coefficient R was of 0.99994. It demonstrates that the immobilized AChE is suitable for the Michaelis–Menten kinetic equation. Besides, 1 mmol L^{-1} ATCh was already used in numerous experiments by using AChE-based biosensors to detect anticholinesterase pesticides considering that it was within the linear response range with high sensitivity.

3.3. Probing OPs using AChE–Al_2O_3–SPE

The inhibition responses of OPs spiked into different substrates to the AChE–Al_2O_3–SPE were tested in FIA–EC system. Fig. 5 gives the inhibition results in simulated seawater, the detailed calibration characteristics of inhibition results in simulated seawater and the others are showed in Table 1. It can be seen that when dichlorvos is present in simulated seawater and in buffer solution, a wider linear range and a larger inhibition response can be obtained as much as in double distilled water or ethanol. This phenomena of inhibition decreasing in water or ethanol may be caused by changes in the ionic strength and dielectric constant of the enzyme active site microenvironment [19,28]. When it was exposed to the mixture of the water solution with a certain amount of ethanol or lower ion strength solution, the tertiary structure of the enzyme might had a little change compared to that in the buffer solution.

In addition, the influence of the blank on the activity of the AChE was also tested as a control in the high salinity solution (simulated sea water and 0.3 mol L^{-1} phosphate in 0.3 mol L^{-1} KCl), lower ionic strength buffer (0.01 mol L^{-1} phosphate in 0.01 mol L^{-1} KCl) and 20% ethanol working buffer. The control measurement was processed after the above-mentioned solution passing through the cell for 5 min (flow rate: 0.347 mL/min) followed by exposing the AChE–Al_2O_3–SPE in OPs-free working buffer solution for 1 min at the same flow rate. After this, it was observed that the activity of the AChE had no measurable change with such operation (see Fig. 6). It was also observed that the higher salinity of the simulated seawater had no significant influence on the amperometric signal, compared to the OPs dissolved in the working buffer solution. So, it is promising for the

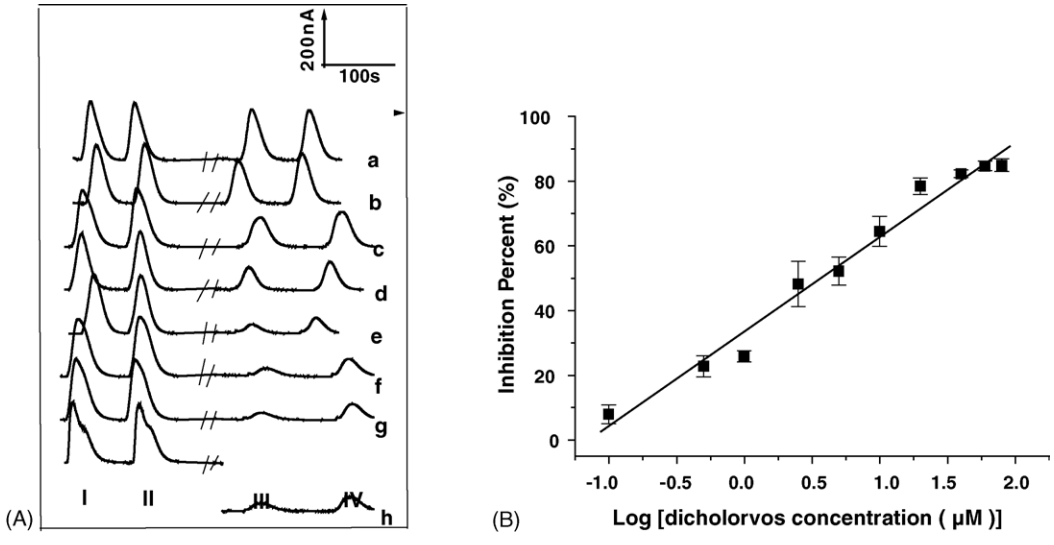

Fig. 5. Dichlorvos in simulated seawater inhibition response to the biosensor. Operating potential: 0.25 V; flow rate: 0.347 mL/min immobilized 5 IU AChE on each electrode. Incubation time: 5 min (a) 0.1 μM; (b) 1 μM; (c) 5 μM; (d) 10 μM; (e) 20 μM; (f) 40 μM; (g) 60 μM; (g) 80 μM dichlorvos. (A) The inhibition responses. (B) The working curve of inhibition responses.

Table 1
Coefficient of calibration curves: I (%) = $A + B$ log(Ci/μM), range of pesticide concentration and detection limit to be determined with the Al_2O_3–AChE biosensors

Dichlorvos in	B	A	R	C-range (μmoL L^{-1})	D-limit (μmol L^{-1})
S-seawater	29.31 ± 1.55 (N = 10)	33.59 ± 1.81	0.98905	0.1–80	0.08
Work buffer	30.68 ± 2.43 (N = 8)	31.83 ± 3.39	0.97867	0.1–80	0.08
D-water	28.24 ± 3.96 (N = 6)	3.84 ± 4.82	0.96283	1–60	0.8
Ethanol	17.78 ± 1.97 (N = 5)	7.14 ± 2.11	0.98206	1–40	0.8

S-seawater: simulated seawater; D-water: distilled water; B: slope; A: intercept; R: linear correlation coefficient; C-range: dichlorvos linear concentration range; D-limit: detection limit.

biosensor to monitor the OPs pollution state of seawater. The detection limit was obtained by measuring the response after injection of 20 μL 10 nM dichlorvos (dissolved in the simulated seawater) into the flow cell and enzyme incubation during 15 min. An apparent inhibition signal three times higher than the noise with inhibition degree about 6.81% was also observed. This detection limit value is much lower compared to others reported mediator-free electrochemical sensors for dichlorvos analysis [19–20,30].

3.4. Reactivation of the enzyme at the biosensors

In the continuous experiments, it was found that the inhibited AChE could be partially reactivated when the AChE–Al_2O_3–SPE was exposed to the ATCh substrate after the detection of OPs. As shown in curves b–h of Fig. 5, the latter amperometric response (peak IV) was higher than the first response (peak III) by measuring the amperometric response after inhibition. This similar effect was also observed by Neufeld [20]. In order to investigate this phenomenon and to see whether the inhibited biosensors can be fully reusable at certain conditions, we exploited this substrate reactivation effect to reuse the biosensor when the inhibition ratio was not higher than 80%. In Fig. 7, it can be seen that the biosensor with 79% inhibition degree can get fully reactivation after incubated with the substrate for 4 min.

The substrate reactivation effect can be explained based on the irreversible inhibition kinetic reaction of the enzyme. The inhibition procedure can be described as Eq. (3):

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdots I \xrightarrow{k_2} E - I \tag{3}$$

The inhibitor and the correspondence part of the activity site of the enzyme will form a reversible, non-covalent binding at the first, and then form a stable covalent complex. The irreversible inhibition equation [29] is:

$$\Delta \ln \alpha = k_i[I]t \tag{4}$$

where k_i is the biomolecular inhibition rate constant and $\Delta \ln \alpha$ is the logarithmic difference of AChE activities measured before and after inhibition with the OPs. So, the inhibition degree is proportional to the rate of formation the covalently phosphorylaled enzyme complex. The inhibition time in the experiment was only of 5 min, not enough to the formation of the stable irreversible covalent complex in lower dichlorvos contents. When the substrate ATCh was injected, a competition between the substrate and the organophosphorus pesticides to the activity site of the AChE was verified. The reactivation degree was based mainly either on the inhibition degree or on the reactivation time and ATCh loading. Thus, we employed this strategy to reactive the biosensors those inhibition degrees were lower than 80%. We believe this approach could also be used in other enzyme inhibition systems.

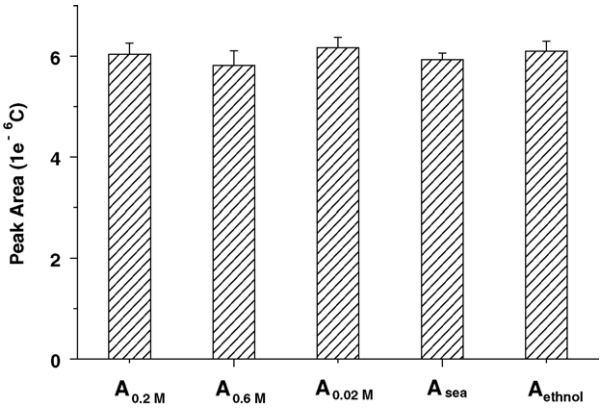

Fig. 6. Amperometric responses of the biosensors before and after the flowing pass of different solutions. The detailed experimental conditions see text. The R.S.D. of different amperometric responses is 2.31%.

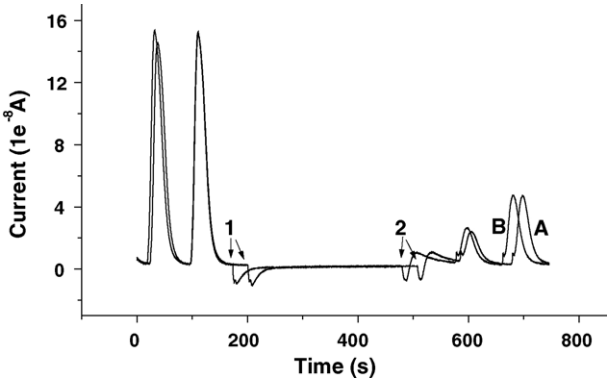

Fig. 7. The reactivation effect of the biosensors. (A) The initial inhibition results when the biosensor was exposed to 40 μM dichlorvos. (B) The inhibition results of the reactivated same biosensor after it was incubated with 1 mM ATCh for 4 min. (1) stop for incubate dichlorvos with the biosensor, (2) flow again. Other conditions were same as Fig. 5.

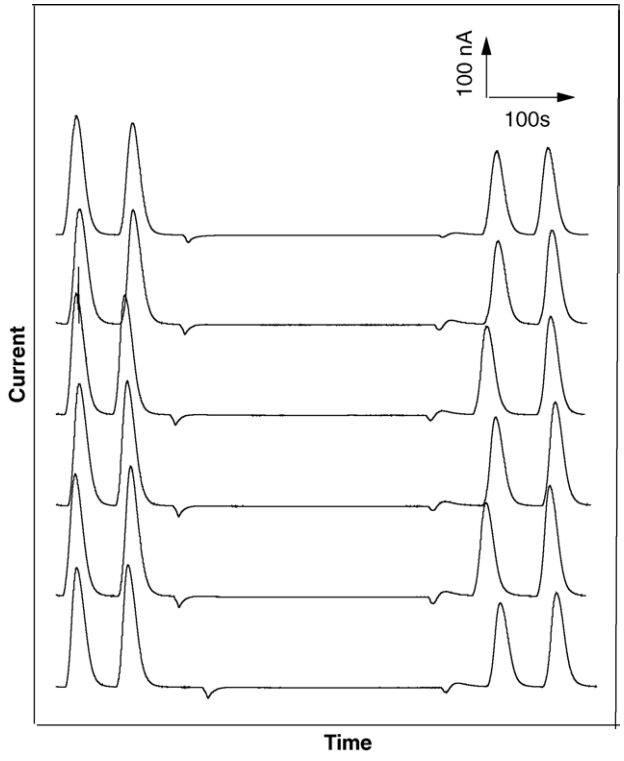

Fig. 8. Reproducibility of the inhibition responses. The biosensor was incubated with 20 ng dichlorvos for 5 min. Other conditions were the same as Fig. 5.

3.5. Stability and reproducibility of the screen-printed biosensors

Stability and reproducibility tests for the biosensors were performed by measuring consecutive amperometric responses nine times, and the relative standard deviation was 4.92%. Furthermore, ideal Michaelis–Menten enzymic kinetic response was found for the immobilized AChE. About 93% activity of the biosensor was maintained after 25 times testing and about 90% activity was kept after stored for 5 months.

The reproducibility of the inhibition responses (Fig. 8) was tested by injecting 20 μL of 1 μM dichlorvos and incubating the biosensor into it for 5 min. The relative standard deviation of the inhibition percent was 2.87% ($N = 6$), indicating that inhibition reproducibility of the proposed biosensors was relatively ideal, compared with other reported mediator-free OPs biosensors [21–24].

Table 3
Recovery test of dichlorvos spiked in seawater obtained from Beilun Seaport

Concentration (μM)	Recovery percent (%)	R.S.D. (%)
1	89.28	7.65 ($n = 4$)
5	77.59	5.76 ($n = 4$)
10	88.88	3.24 ($n = 4$)
20	83.86	1.27 ($n = 4$)
50	73.22	3.82 ($n = 4$)
60	98.33	3.05 ($n = 4$)

R.S.D. (%): relative standard deviation.

3.6. Analysis of real samples

Real samples obtained from Beilun Seaport and the branch rivers of YangtzeJiang River (Ninbo, Zhejiang province of China) were analyzed using the proposed AChE-based biosensor. The obtained data was compared with the results obtained by GC–MS (HP 6890 series GC systems, HP 5973 Mass selective Detector) combined with solid phase microextraction (SPME) as shown in Table 2. It can be seen that the biosensor system has shown enough sensitivity to detect OPs or other AChE inhibitors in real water samples. Either the FIA–EC or GC–MS provided negative results rejecting the presence of OPs in the samples obtained from Beilun Seaport, but positive results for the samples from the other four rivers. Here, it should be mentioned that dichlorvos was only selected as the model to this experiment system, and in the real samples, other OPs, carbamates pesticides and some organic compounds, etc. also have the inhibition effect to the AChE biosensors. So when the real samples were determined by Al_2O_3–AChE biosensors, the sum inhibition effect was measured, which is reflected the toxicology degree of the samples and further confirmed by the results obtained from GC–MS as shown in Table 2.

The response of the samples with dichlorvos spiked into Beilun Seaport seawater to evaluate the effect of salinity on the biosensors was also tested as shown in Table 3. The recover ratio was smaller than 100%. It may be due to the rapid hydrolysis of dichlorvos in this seawater environment, and attempts were made to measure the samples as quickly as possible after they had been prepared.

Table 2
The inhibition results of different real samples with Al_2O_3–AChE biosensors and the potential AChE inhibitors got from GC–MS

Water position	I (%)	R.S.D. (%)	GC–MS result
Beilun seaport A	≤0 ($n = 3$)	n.a.	No OPs present
Beilun seaport B	≤0 ($n = 3$)	n.a.	No OPs present
Yongjinag river A	6.50	4.02 ($n = 3$)	Phosphonoacetic acid-tritms ester
Yongjinag river B	4.37	5.68 ($n = 3$)	1-Methyl-4-[4,5-dihydroxyphenyl]-hexahydropyridine; 3-methyl-1,1′-biphenyl
Fenhuajiang river	8.75	2.95 ($n = 3$)	Phosphorodithioic acid,*S*-[(4,6-diamino-1, 3,5-triazin-, 2-yl)methyl]*O*,*O*-dimethyl ester
Infall site	2.64	9.44 ($n = 3$)	5-(4-Fluorophenyl)-1-(4-chlorophenyl)-3-trifluoromethyl-1H-1,2,4-triazole

Experimental conditions of Al_2O_3–AChE biosensors were same as Fig. 5. *Distance between sample A and B was about several kilometers away; n.a.: not analyzed; I (%): inhibition percent; R.S.D. (%): relative standard deviation.

4. Conclusions

In summary, a simple strategy to quantitatively screen the OPs in the environment by using low-cost screen-printed amperometric biosensors at relatively low potential were presented. The hydrophilic and porous Al_2O_3 sol–gel matrix used to embed the enzyme have provided not only a friendly microenvironment for the immobilization of AChE to retain its activity for a long time, but also an effective promotion of the electron transfer between the thiocholine and the electrode. This promoting effect greatly decreased the overpotential to the detection of the thiocholine and minimized the interference from other co-existing impurities. The feasibility of the FIA–EC system was demonstrated in screening runs with real samples, especially for the high salinity sea water, demonstrating the toxicological effect of these pesticides.

Acknowledgements

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