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A novel biosensor based on photoelectro-synergistic catalysis for flow-injection analysis system/amperometric detection of organophosphorous pesticides

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ABSTRACT

In this study, a highly sensitive amperometric biosensor based on photoelectro-synergistic catalysis for detecting organophosphorus pesticides (OPs) in flow-injection analysis (FIA) system has been developed. The acetylcholinesterase enzyme (AChE) was immobilized by adsorption into the nanostructured $PbO_2/TiO_2/Ti$, which also acted as the working electrode. This strategy was found to catalyze the oxidative reaction of thiocholine effectively, make the AChE/$PbO_2/TiO_2/Ti$ biosensor detect the substrate at 0.30 V (vs. SCE), hundreds milli-volts lower than others reported. $PbO_2/TiO_2/Ti$ and TiO_2/Ti electrodes were prepared and investigated with atomic force microscope (AFM). Factors influencing the performance were optimized. The resulting flow system offered a fast, sensitive, and stable response. A value of 1.34 mM for the apparent Michaelis–Menten constant (K_M^{app}) was obtained. A wide linear inhibition response for trichlorfon was observed in the range of 0.01–20 μM with the detection limit of 0.1 nM. The results using this biosensor agreed very well with chromatographic method and we also examined the real samples successfully in this work.

1. Introduction

Organophosphorous pesticides are well known as highly toxic compounds which are widely used in agriculture and for chemical warfare agents [1]. Because of the inherent toxicity of these neurotoxic compounds, there is a considerable interest in the development of highly sensitive, selective, rapid and reliable analytical methods in their detection. Current analytical techniques like gas chromatography (GC), liquid chromatography (LC), mass spectrometry (MS) or combinations thereof (GC–MS or LC–MS/MS), are very sensitive and reliable, but they are very time-consuming and expensive and can be only performed by highly trained technicians and currently not suitable for field use [2].

Enzyme-based biosensors have emerged in the past few years as the most promising alternative for direct monitoring of pesticides [3,4]. Various inhibition and noninhibition biosensor systems, based on the immobilization of acetylcholinesterase (AChE) or OP hydrolase (OPH) onto various electrochemical or optical transducers, have been proposed for field screening of OP neurotoxins [5–9]. Although OPH-based noninhibition biosensors provide a direct biosensing route (OPH catalyzes the hydrolysis of OP compounds, resulting in electroactive species, such as *p*-nitrophenol), OPH is not commercially available, which limits widespread applications. A preferred indirect electrochemical biosensing route based on the inhibition of target enzymes has been widely developed by different groups [10–18]. Because of their high sensitivity, amperometric transducers have been the transduction principle of choice in many of these biosensors [10].

In the AChE enzyme system (Eq. (1) and (2)), thiocholine (TCh) ester, acetylthiocholine (ATCh), are preferred as substrate.

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

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

The amperometric response of the AChE biosensors, i.e. the anodic oxidation current resulting from the thiocholine formed in the enzymatic hydromantic hydrolysis of acetylthiocholine, is inversely proportional to the concentration of organophosphorus pesticides (OPs) in samples, and the exposed time as well.

There are many efforts to develop these inhibition biosensors, certain analytical characteristics of these systems require further improvement to meet the required specifications, such as higher sensitivity, selectivity, and stability.

One approach to improve the performance characteristics of the AChE-based inhibitor biosensors would be the design and production of the appropriate enzymes with characteristics more suitable for biosensor applications. It was only recently that biotechnology allowed the highly efficient production of pure AChE from different sources thus enabling their thorough study [11] and use [12]. Initial biochemical studies revealed that *Drosophila melanogaster* acetyl-

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cholinesterase (Dm. AChE) is the most sensitive enzyme toward OPs [13]. The Dm. AChE-based inhibitor biosensors show great promise to improve the sensitivity of the biosensor system.

Another possible approach for improving the performance characteristics of the AChE-based inhibitor biosensors is to improve the design of the biosensor and the electrochemical detection of the enzymatic product. Most of the approaches for AChE immobilization on electrode surfaces in the reported literature are covalent binding [14], direct adsorption [15,16], or entrapment in different substrate materials [17,18]

Here, we describe for the first time the application of a biosensor based on photoelectro-synergistic catalysis, a combination approach of photocatalysis and electrocatalysis, together with flow-injection analysis (FIA) for highly sensitive insecticide detection. An AChE/PbO_2/TiO_2/Ti biosensor was prepared and its electrocatalysis, photocatalysis and photoelectro-synergistic catalysis were investigated. Electrochemical technique was adopted to collect the electrical signals produced during photoelectro-synergistic catalytic oxidation, which also succeeded in determining OPs directly. As an example, we used the organophosphate insecticide, dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (trichlorfon), which has been widely used as an insecticide for more than 40 years. The PbO_2/TiO_2/Ti based on AChE biosensor was found to effectively catalyze the oxidative reaction of thiocholine with no loss of the sensitivity and with the detection limit 0.1 nM of trichlorfon.

2. Materials and methods

2.1. Materials

Purified acetylcholinesterase (VI-S, 1000 IU mg^{-1}) from electric eel, pyridine 2-aldoxime methiodide (PAM), acetylthiocholine chloride and chitosan were purchased from Sigma–Aldrich. Trichlorfon ([2,2,2- trichloro-1-hydroxyethyl]-phosphonic acid dimethyl ester) was purchased from Dr. Ehrenstorfer Co. Titanium rod (99.7%, diameter 2.5 mm) was purchased from the Far East Ti Equipment Co. Titanium tetraisopropoxide, ethanol, polyglycol, lead nitrate, perchloric acid were purchased from Shanghai Chemical Reagent Co., China. All of other chemicals were analytical grade. All solutions used in experiments were prepared with doubly distilled water.

2.2. The experimental setup

The setup of the FIA system for OPs detection is shown in Fig. 1. The flow carrier which was held in a reservoir was propelled by a D100B/C peristaltic pump (Huxi Instrumental Co., Shanghai, China) through an injector (7520, Rheodyne) and a photochemical cell with a three-electrode unit installed, before being transported to a waste tank. The three-electrode unit was composed of an AChE/PbO_2/TiO_2/Ti photoelectro-synergistic catalysis biosensor, a SCE electrode and a Pt electrode, serving as the working, reference and counter electrode, respectively. A 12-W quartz UV lamp (λ_{max} = 253.7 nm) was used as an excitation source. The surface of the photoelectro-synergistic catalysis cell facing the UV lamp was made of quartz, and the inlet, outlet and the salt bridge were all connected to the interlayer (0.5 mm in thickness). The photoelectrocurrent produced by the working electrode during photoelectro-synergistic catalysis process was recorded with a CHI-1030 electrochemical station (CHI, America).

2.3. AChE/PbO_2/TiO_2/Ti biosensor preparation

Titanium tetraisopropoxide (12 mL) and ethanol (48 mL) were mixed under an inert atmosphere. Subsequently, a solution of diethanolamine (4 mL) and water (0.5 mL) were added to the mixture under stirring for 60 min at ambient temperature, which led to the formation of a sol–gel of TiO_2 [19]. Then, polyglycol was added to the sol–gel solution before dip-coating the bare Ti rod. The TiO_2-coated Ti rod was then dried at 100 °C for 5 min. The operation of coating and drying was repeated three times. Then it was heated at 400 °C for 3 h. After that, the PbO_2 film was formed in 1 M $HClO_4$ solution containing 0.01 M $Pb(NO_3)_2$ by cycling the potential between 1.4 and 1.8 V at low scan rate for 10 min. After modification, the electrode was removed from the modifying solution and rinsed with doubly distilled water.

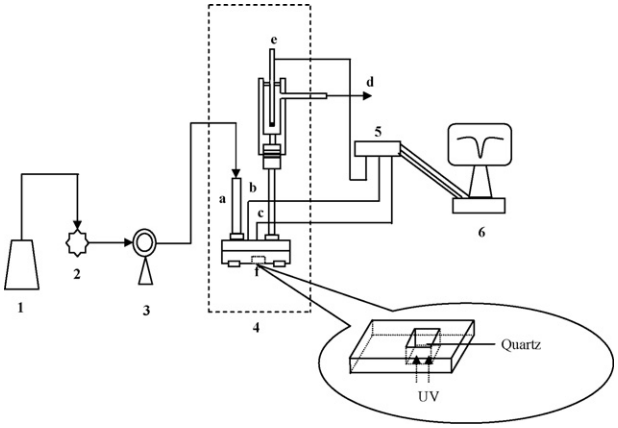

Fig. 1. Schematic diagram of the FIA apparatus (1) carrier (0.1 M KH_2PO_4/Na_2HPO_4 (PBS), pH 8.0)); (2) pump; (3) injector; (4) a thin-layer amperometric detector (a: flow inlet, b: counter electrode, c: working electrode, d: flow outlet, e: reference electrode, f: quartz window); (5) CHI-1030 electrochemical system; (6) personal computer.

The 0.1 M KH_2PO_4/Na_2HPO_4 (PBS, pH 8.0) was used to dissolve the enzyme (2.2 mg in 0.5 mL PBS). The resulting solution was stored at 4 °C in refrigerator. The AChE was immobilized by casting a 5 μL droplet of AChE mixed with 1.0 μL 1.0 mg mL^{-1} chitosan onto the PbO_2/TiO_2/Ti working electrode surface and air dried. The enzymatic membrane was subsequently gelatinized completely in the refrigerator (4 °C) for 24 h. All the prepared AChE/PbO_2/TiO_2/Ti biosensors were stored in PBS (pH 8.0) in the refrigerator (4 °C) when not in use.

2.4. Flow-injection amperometric detection of trichlorfon

Flow-injection amperometric detection of trichlorfon was performed with the above-mentioned flow-injection system. First, 20 μL 1.0 mM ATCh substrate was injected to obtain the initial response under UV irradiation (A_0: peak area of the oxidation current) of the biosensor after a steady-state value was obtained. Then, 20 μL of a specific concentration of trichlorfon was injected. After the pesticide reached the cell, the flow was stopped to let the biosensor incubated with the OPs for 10 min. Following the inhibition step, the response of the AChE/PbO_2/TiO_2/Ti biosensor to the same concentration substrate (A_i) was recorded again. It was noted during the inhibition that the electrochemical record of current via time and UV irradiation were paused. The inhibition percentage was calculated from the remaining activity after each inhibition step and was plotted against the log [trichlorfon].

3. Results and discussions

3.1. Atomic force micrographs of the electrodes

The surface of the electrodes which was characterized by atomic force microscope (AFM, Shanghai AJ Nanoscience Development Co., Ltd.) as shown in Fig. 2(A) illustrates the diameter of the prepared TiO_2 particle was about 50 nm. Meanwhile, PbO_2 particles whose

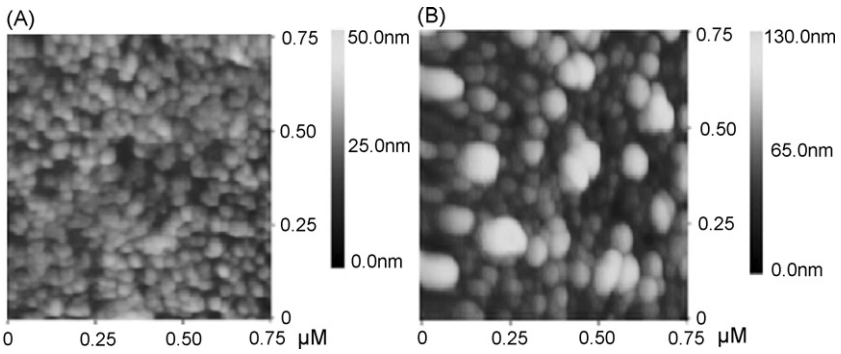

Fig. 2. AFM images of TiO_2/Ti (A) and PbO_2/TiO_2/Ti (B) electrodes.

diameter was around 100 nm were formed on the surface of the electrode, as shown in Fig. 2(B). Fig. 2 provides strong evidence for the formation of PbO_2/TiO_2/Ti nanocomposite which can be used to catalyze the oxidative reaction of thiocholine effectively.

3.2. Photoelectro-synergistic catalysis on AChE/PbO_2/TiO_2/Ti biosensor

When TiO_2 particles were irradiated by UV (<387.5 nm), they generated h^+/e^- pairs and these species migrated to the solid surface. It was accepted commonly that the strong oxidant, photogenerated hole could oxidize water adsorbed on TiO_2 surface to create another oxidizing agent hydroxyl radical ($^{\bullet}OH$) [20]. The thiocholine could be oxidized by the photogenerated hole and hydroxyl radicals which were generated from the anodic discharge of water adsorbed on PbO_2 surface at a certain potential.

Fig. 3 shows the effect of UV illumination to the current responses of AChE/TiO_2/Ti biosensor and AChE/PbO_2/TiO_2/Ti biosensor. In Fig. 3(A), at a working potential of 0.30 V (vs. SCE) AChE/TiO_2/Ti biosensor had a larger amperometric response with the injection of ATCh (arrows in figure) with UV irradiation than without it. This was because the production of photo-generated electrons could catalyze the oxidative reaction of thiocholine. In Fig. 3(B), AChE/PbO_2/TiO_2/Ti biosensor had a great larger amperometric response with UV irradiation than without it. It demonstrates the photoelectro-synergistic catalysis has largely improved the oxidation current than that just of electrocatalysis. It could be summarized that the photoelectro-synergistic catalysis of AChE/PbO_2/TiO_2/Ti biosensor can enlarge the oxidation current of thiocholine effectively, thus it can detect largely lower concentration of OPs.

3.3. Kinetic characteristics of AChE/PbO_2/TiO_2/Ti biosensor in FIA

The kinetic response of AChE/PbO_2/TiO_2/Ti biosensor in FIA system was tested to investigate the enzymatic activity retained on the surface of PbO_2/TiO_2/Ti electrode. Fig. 4 shows the dependence of the current on the concentration of ATCh. The K_M^{app} value of the immobilized AChE was calculated by fitting experiment data to the Michaelis–Menten equation [21] using the Lineweaver-Burk electrochemical representation of – [peak area] vs. [ATCh]. At pH 8.0 and at room temperature (about 25 ± 2 °C), the K_M^{app} of the immobilized AChE was of 1.34 mM and the linear correlation coefficient r^2 was of 0.9969. It demonstrates that the immobilized AChE is suitable for the Michaelis–Menten kinetic equation. Besides, 1.0 mM ATCh was already used in the following experiments by using AChE-based biosensors to detect anticholinesterase pesticides considering that it was within the linear response range with high sensitivity.

3.4. Optimal operating conditions

The proposed AChE/PbO_2/TiO_2/Ti biosensor was used as an amperometric detector in an FIA system. Accordingly, an optimization study involved the best experimental parameters was performed. Fig. 5 shows the influences of both the working potential and the pH value of PBS on the response to substrate ATCh. It can

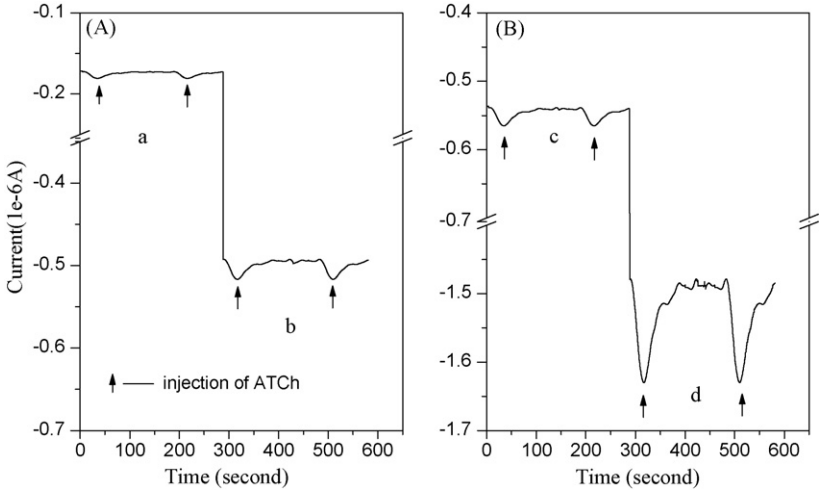

Fig. 3. The influence of biosensor on the current response in the FIA system (ATCh concentration: 1.0 mM; flow rate: 0.50 mL min^{-1}; carrier: PBS, pH 8.0; working potential: 0.30 V vs. SCE) (A) AChE/TiO_2/Ti sensor (a) without UV illumination, (b) with UV illumination; (B) AChE/PbO_2/TiO_2/Ti sensor (c) without UV illumination, (d) with UV illumination.

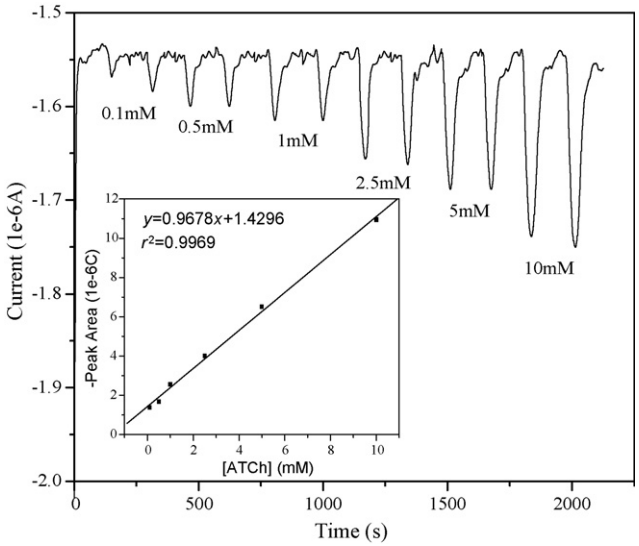

Fig. 4. Amperometric responses of the AChE/PbO_2/TiO_2/Ti biosensor for successive injection ATCh in FIA system (the inlet shows the calibration curve) (ATCh concentration: 1.0 mM; flow rate: 0.50 mL min^{-1}; carrier: PBS, pH 8.0; working potential: 0.30 V vs. SCE).

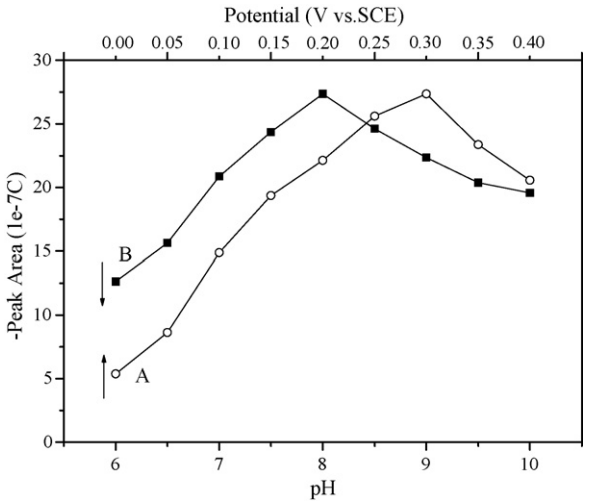

Fig. 5. The influences of potential (○) and pH value (■) on the response of the AChE/PbO_2/TiO_2/Ti biosensor (ATCh concentration: 1.0 mM; flow rate: 0.50 mL min^{-1}; carrier: PBS, pH 8.0; working potential: 0.30 V vs. SCE).

be observed in Fig. 5(A), in the range from 0.00 to 0.30 V (vs. SCE), that the responses of AChE/PbO_2/TiO_2/Ti biosensor were increased to reach the maximum at 0.30 V. This working potential showed to be several hundreds milli-volts lower than those obtained by other reported amperometric biosensors for OPs analysis and would be helpful to minimize interferences from other coexisting electroactive impurities [18–24]. Thus, a potential of 0.30 V was selected for subsequent studies. The effect of pH value of PBS on the performance of the AChE/PbO_2/TiO_2/Ti biosensor was studied as shown in Fig. 5(B). The experimental results indicated the increased amperometric responses were obtained for 1 mM ATCh in PBS, pH 6.0–8.0; the signal decreased with the increase of the buffer pH value at the range of 8.0–10.0. The reason of the decreasing signal may be attributed to the decreased activity of AChE on the electrode surface. The pH 8.0 at which the signal was maximum was used for subsequent studies.

3.5. Trichlorfon detection with AChE/PbO_2/TiO_2/Ti biosensor

The optimized biosensor was subsequently used for the detection of the pesticide trichlorfon. Incubation time of 5, 10, 15 and 20 min were examined and based on these initial results an incubation time of 10 min was chosen as the best compromise between signal and incubation time. The inhibition curve of the biosensor to different concentrations of trichlorfon is presented in Fig. 6(A). The most remarkable characteristic of the system is the limit of detection (LOD) 0.1 nM trichlorfon. This value is significantly lower than the values reported so far [25] and it is achieved within 10 min of incubation without any sample pretreatment. In our opinion, this decrease in the lower LOD can be attributed to the photoelectrosynergistic catalysis of PbO_2/TiO_2/Ti.

AChE/PbO_2/TiO_2/Ti biosensor was also used to detect other OPs and the effect of interferences of oxygen-containing inorganic ions during the detection of trichlorfon. The control experiments were performed with injection of 0.1 μM dichlorvos, 0.1 μM methamidophos, 0.1 μM parathion in PBS (pH 8.0) with the absence of trichlorfon, and 0.1 M NO_3^- or 0.1 M SO_4^{2-} in 0.1 μM trichlorfon in PBS (pH 8.0), respectively. 95–106% current responses at 0.30 V were remained in the above solutions.

The validation of AChE/PbO_2/TiO_2/Ti biosensor was performed by using gas chromatography as the reference method. Samples spiked with trichlorfon at five different concentrations: 0.05, 0.1, 0.2, 0.5 and 1.0 μM were analyzed by both methods. The variability of the method was studied by injecting three replicate of each trichlorfon concentration. The linear regression analysis provided the calibration equation ($y = 1.1604x - 0.0280$) as shown in Fig. 6(B). The correlation data indicated a good agreement between methods ($r^2 = 0.9964$) and no significant discrepancy was found when com-

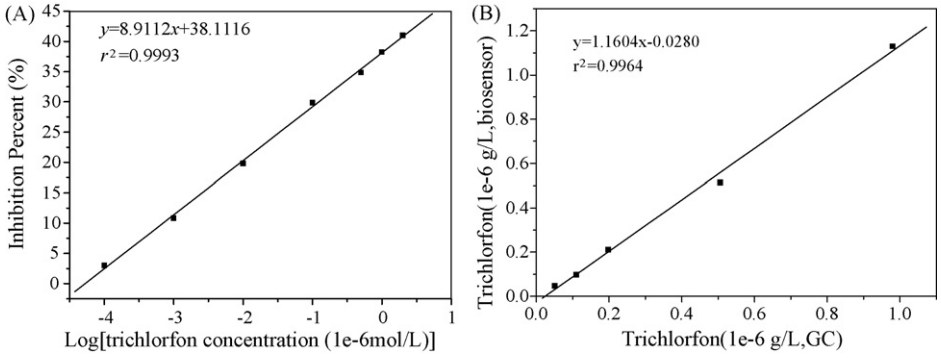

Fig. 6. Trichlorfon detection with AChE/PbO_2/TiO_2/Ti biosensor (A) (ATCh concentration: 1.0 mM; flow rate: 0.50 mL min^{-1}; carrier: PBS, pH 8.0; working potential: 0.30 V vs. SCE) and a correlation of the concentrations of trichlorfon determined by the biosensor directly with that determined by GC (B) (carrier gas: N_2, 99.999%; flow rate: 1.1 mL min^{-1}; injection volume: 1.0 μL).

Table 1
The detection results of real samples.

Sample	The amount of trichlorfon sprinkled on the vegetables (y) (nM)	The amount of trichlorfon detected by the biosensor (x) (nM)
Celery	0	Nd[a]
	200	178
	400	372
	600	540
	800	730
	1000	920
Cauliflower	0	Nd
	200	180
	400	376
	600	538
	800	725
	1000	890
Leek	0	Nd
	200	180
	400	375
	600	542
	800	720
	1000	900

[a] Not detected.

paring the biosensors and GC methods, as shown by the slope of the linear regression analysis (1.16). However, the deviation from ideal value (1.0) might be due to the difference on the recovery factor as a result of the random errors that could be occurred in both methods.

3.6. Application to real samples

To evaluate the performance of the developed modified sensor, the contents of trichlorfon in real samples of celery, cauliflower and leek were detected by AChE/PbO_2/TiO_2/Ti biosensor. Additionally, a blank sample was prepared from the noncontaminated vegetables and analyzed with the above method. From the data in Table 1, it can be seen that the trichlorfon amount in the vegetable samples were about 10% lower when analyzed by a modified sensor compared to the amount of trichlorfon sprinkled on the vegetables. The lower results can be attributed to the loss of trichlorfon in the process of preparation of extracts and the removing of solids of the vegetables. The amount of trichlorfon sprinkled on the vegetables is linear related with the amount of detected by the modified biosensor. The calibration equation is $y = 1.1075x - 2.2397$ ($R^2 = 0.9998$).

3.7. Reactivation of the enzyme at the biosensors

Reactivation of the enzyme is one of the key issues in the development of inhibition enzyme biosensors. Different approaches, such as simple rinsing with buffer, incubating with PAM regeneration reagents, and injection of high concentration of ATCh, had been applied [17]. In the continuous experiments, it was found the reactivation characteristics of inhibited enzyme depend on the inhibitor concentration and exposure time as was reported by Okazaki et al. [26]. The enzyme weakly inhibited (30% inhibition) by trichlorfon was reactivated by rinsing with phosphate buffer without PAM. It means that the weakly inhibited enzyme shows good reversibility. We tried the above approaches to regenerate the AChE/PbO_2/TiO_2/Ti biosensor after a complete measurement (50% inhibition). We found that only 10%, 22%, 30% responses were recovered after a short regeneration time (2 min) by rinsing with buffer and incubating ATCh, respectively. But the whole response of the biosensor was regenerated after successive injections of 0.1 mM PAM and 10 mM ATCh. So the successive injections of 200 μL of 0.1 mM PAM and 10 mM ATCh were used to reactivate the enzyme. The enzymatic activity was also recovered without PAM even in the case of the inhibition by trichlorfon of high concentration if exposing time was short enough. On the other hand, strongly inhibited enzyme could not be regenerated without the help of PAM treatment. Repeated inhibition and reactivation test with this treatment revealed that almost stable recovery rate and sensitivity were guaranteed. The reactivation methods proposed by the present research would provide more reliable pesticide detection with a reusable enzyme electrode.

3.8. Stability and reproducibility of the biosensor

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 enzymatic 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.

4. Conclusion

In summary, we described an effective AChE-based biosensor amperometric flow detector for rapid and sensitive measurements of OPs. We investigated the photoelectro-synergistic catalysis oxidation of substrate by comparing with electrocatalysis and photocatalysis oxidation. The catalytic activity of AChE/PbO_2/TiO_2/Ti was improved greatly because photocatalysis, electrocatalysis and biocatalysis were carried out at the same time and they were synergistic. The flow-injection analysis system was utilized with the proposed biosensor to detect the amount of OPs. This method presented a low detention limit of 0.1 nM and the results were in excellent correspondence with those determined by GC ($r^2 = 0.9964$, $n = 5$).

Acknowledgements

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