Research article

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# A sensitive and regenerable biosensor for organophosphate pesticide based on self-assembled multilayer film with CdTe as fluorescence probe

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**ABSTRACT: A fluorescence biosensor for organophosphorus pesticides was developed. A pH indicator, CdTe quantum dots, were used as an optical transducer of the inhibition of enzyme by analyte. Through the intervening agency of chitosan, the recognition elements (acetylcholinesterase and CdTe) were immobilized onto the surface of quartz by electrostatic attraction to form a self-assembled multilayer film. In the absence of pesticide, acetylcholine was biocatalytically hydrolysed to yield acetic acid and choline. The released acid resulted in pH decrease, which was sensed by the immobilized pH indicator (CdTe). In the presence of pesticide, the action of acetylcholine was reduced; the fluorescence intensity of the film changed and was related to the concentration of pesticide. This multilayer film could be used as the biosensor for monocrotophos, with a detection limit of $3.20 \times 10^{-8}$ mol/L; the sensitivity was 100 times higher than that of CdTe in aqueous solution. The sensor was easily regenerated, and had good stability and selectivity for organophosphorus pesticides. Copyright © 2011 John Wiley & Sons, Ltd.**

**Keywords:** self-assembled multilayer; biosensor; organophosphorus pesticide; CdTe; acetylcholinesterase

## Introduction

Because of the high toxicity of organophosphate pesticides (OPs) and nerve agents, the rapid detection of these toxic agents in the environment, public places or workplaces and the biomonitoring of individual exposures to chemical warfare agents have become increasingly important for homeland security and health protection. There is great interest and concern to develop a biosensor for the detection of organophosphorus pesticides in food and the environment (1–3), especially an enzyme-based sensor (4,5). However, immobilization of the enzyme to a solid surface is a crucial step in the design of the biosensor, since the procedure must maintain the activity of the enzyme while requiring that the interface exhibits reasonable biocompatibility. Unfortunately, at the present time there is no ideal immobilization method generally usable for all enzymes. Therefore, many immobilization methods and different materials have been employed to fabricate enzyme-based biosensors, such as direct physical adsorption, encapsulation into hydrogel and cross-linking. A key requirement of enzyme immobilization is attachment without the bioactivity being sacrificed. Self-assembled monolayers (SAMs) are stable, well-ordered, easy to prepare and low cost, and have been extensively applied for the constructions of chemo- and biosensors (6,7). We previously reported that the SAMs-based fluorescent biosensors showed extremely high sensitivity (8–10) and also developed ratiometric fluorescent sensors containing both dyes to improve detection accuracy (11). Furthermore, layer-by-layer assembly by electrostatic attraction can retain the long-term activity of the enzyme (12,13) and overcome some of the disadvantages of enzyme immobilization, and thus has been used in this study. CdTe quantum dots, with outstanding light features (14,15), were chosen as a fluorescent probe. In this study a novel biosensor was developed to detect organophosphorus pesticides based on the fabrication of enzyme/nanoparticles by layer-by-layer assembly. First, quartz wafers were silanized with γ-aminopropyltriethoxysilane (APES), then CdTe, chitosan (CS) and acetylcholinesterase (AChE) were alternately immobilized onto the quartz substrate to form a quartz/APES/CdTe/CS/AChE/CS/CdTe multilayer film by electrostatic attraction. Under the optimal conditions, the detection limit of the biosensor for monocrotophos was $3.20 \times 10^{-8}$ mol/L, which was 100 times lower than CdTe in aqueous phase. The regeneration, stability and interference studies of the optical device were also studied.

## Materials and methods

### Reagents

Monocrotophos, methylparathion, trichlorfon and acetylcholinesterase were purchased from Sigma-Aldrich. Thioglycolic acid (TGA) was obtained from the Chemical Reagent Co. (Shanghai, China). All chemicals were of analytical grade or higher. Milli-Q water (18.2 MΩ/cm) was used throughout the study.

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### Instruments

Corrected fluorescence spectra were recorded on an American Varian Cary Eclipse fluorescence spectrophotometer with excitation wavelength of 470 nm and excitation and emission monochromator slits of 5 nm. The angle between the quartz plane and the incident excitation light was set at 50° to ensure the maximum efficiency of collecting the emission light while avoiding reflection light interference.

### Preparation of SAMs

**Synthesis of CdTe nanoparticles.** The synthesis of CdTe was performed according to previously published methods (16,17) with some modifications. First, 0.2158 g $KBH_4$, 0.2550 g Te and 10 mL Milli-Q water were added to a suitable flask. The reaction continued for 8 h and the product was stored for following use. Then, 0.9136 g $CdCl_2 \cdot 2.5H_2O$ and 0.9212 g thioglycolic acid were dissolved in 100 mL (DI) water, followed by adjusting the pH to 10 by the addition of 1.0 mol/L NaOH solution. The mixture was degassed by $N_2$ bubbling for 30 min, then the above freshly prepared KHTe solution was quickly injected into the mixture under vigorous stirring, followed by refluxing under open conditions.

**Preparation of CdTe-modified quartz wafers.**

1. *Pre-treatment of quartz wafers*. Quartz plates of 1 × 1 cm size were cleaned by polishing for 5 min in a mixture of $HF/H_2SO_4/H_2O$ (1:1:3, v/v/v) and washed with distilled water, toluene, acetone and ultrapure water. Cleaned wafers were then immersed in piranha solution at 80°C for 1 h to make the quartz surface protonated. The treated wafers were cleaned by sonication in ethanol, dried with $N_2$ and heated in an oven at 80°C for 30 min. **Caution: The piranha solution is dangerous to human health and should be used with extreme caution and handled only in a small quantity**.
2. *Silanization of quartz wafers*. The above-prepared quartz wafers were immersed in 1% v/v aqueous solution of APES for 6 h to allow the quartz surface to be functionalized with amino groups. After cleaning with ultrapure water and drying with $N_2$, the silanized quartz wafers were ready for the next procedure.
3. *Deposition of CdTe nanoparticles on silanized quartz wafers*. The silanized quartz wafer was dipped in freshly prepared CdTe colloidal suspension for 12 h. Then the wafers were taken out and dried in nitrogen for 10 min.

**Immobilization of CdTe and acetylcholinesterase to quartz surface.** The above fabricated quartz/APES/CdTe wafer was immersed in 1 mol/L NaOH solution for 10 min, followed by rinsing thoroughly in ultrapure water and drying with $N_2$, then immersed into 0.1 g/L pH 3–4 chitosan solution for 6 h followed by immersion in 0.5 U/mL acetylcholinesterase in pH 9.0 phosphate buffer for 6 h. Acetylcholinesterase was immobilized by electrostatic attraction with chitosan. The isoelectric point (pI) of acetylcholinesterase is 4.5, and therefore at pH > pI it was negatively charged, whereas at pH < pI it was positively charged (4). Another CdTe layer was adsorbed on the top of the acetylcholinesterase layer, using the same procedure to prevent acetylcholinesterase leaking from the layer surface. CdTe Quantum dots and AChE were alternately immobilized onto the quartz substrate to form the quartz/APES/CdTe/CS/AChE/CS/CdTe nanocomposite structure. Scheme 1 shows the assembly process on the quartz substrate.

**Measuring procedure.** The determination of organophosphate pesticides is based on inhibition of immobilized acetylcholinesterase in the presence of the acetylcholine substrate. With increasing amounts of pesticide, a smaller pH change occurs as the activity of the enzyme is decreased and the change in the fluorescence intensity of the sensor is measured.

After incubation of organophosphate pesticides, the residual enzyme activity was revealed by substrate injection and the percentage inhibition was calculated according to the following equation:

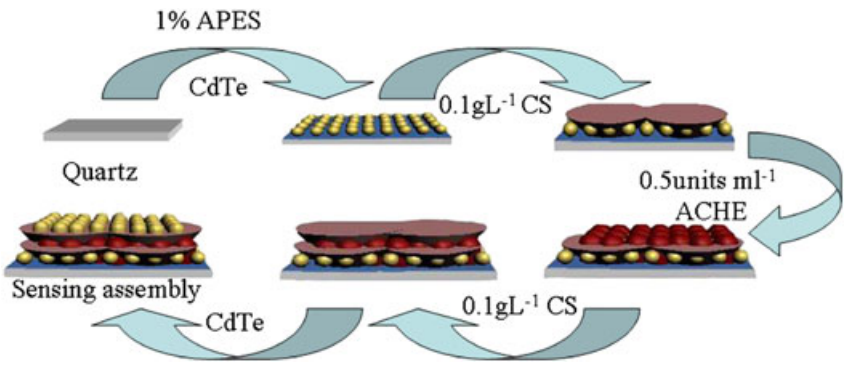
$$I = (\Delta I_i - \Delta I_0)/\Delta I_0 \times 100\%, \ \Delta I_0 = I_1 - I_0 \ , \ \Delta I_i = I_i - I_0$$

where $I_0$ and $I_i$ are the film's fluorescence intensity in the absence and presence of inhibitor, respectively, and $I_1$ is the film's fluorescence intensity in solution containing substrate without inhibitor.

## Results and discussion

### Characteristics of CdTe in aqueous solution

**Influence of pH on CdTe fluorescence intensity.** As shown in Fig. 1, it was found that over the pH range 6.2–9.0, the fluorescence intensity increased with increasing pH. A pH value of about 9.0 was chosen when considering the enzymatic response and the maximum sensitivity of the pH indicator.




**Scheme 1.** Schematic diagram of self-assembly multilayers.



**Detection of monocrotophos.** Using the above experimental method, monocrotophos of different concentration was added into the reaction system (consisting of 8.33 mM CdTe, 0.5 U/mL acetylcholinesterase and 2.85 mmol/L acetylcholine). As shown in Fig. 2, it was confirmed that the fluorescence emission wavelength of CdTe was 561 nm under excitation at 340 nm. It was shown that fluorescence intensity of the reaction system decreased with a corresponding increase of monocrotophos solution concentration. Also, the insert to Fig. 2 shows that there was a good linearity between the inhibitory rate and the concentration of monocrotophos with a detection limit of $1.03 \times 10^{-6}$ mol/L.

Fluorescence interfacial recognition based on SAMs could have higher sensitivity and better regeneration (7–9) and ensure longer-term activity of enzyme in the detection of OPs, compared with that in aqueous solution. Therefore, the experiment was selected to immobilize AChE and CdTe onto the surface of quartz to build quartz/APES/CdTe/CS/AChE/CS/CdTe SAMs.

## Optimization of assembly conditions

**CdTe concentration and assembling time optimization.** It is very important to optimize the concentration of the assembled material. A higher concentration of the material causes disorder; if the concentration is too low, the fluorescence intensity of the modified material is so weak that the detection sensitivity might be affected. The strongest fluorescence signal was obtained when the concentration of CdTe solution was set at 8.33 mmol/L (calculated as the concentration of $Cd^{2+}$); therefore this concentration was selected for assembly. The experimental results also showed that the optimal immersion time was 6 h.

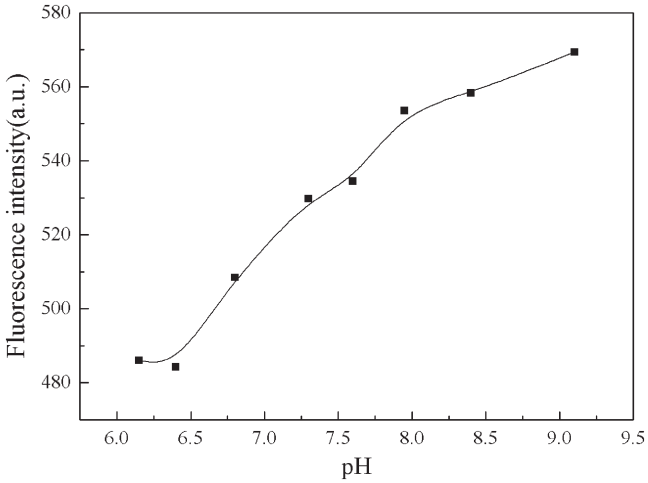



**Figure 1.** Influence of pH on fluorescence intensity of CdTe solution.

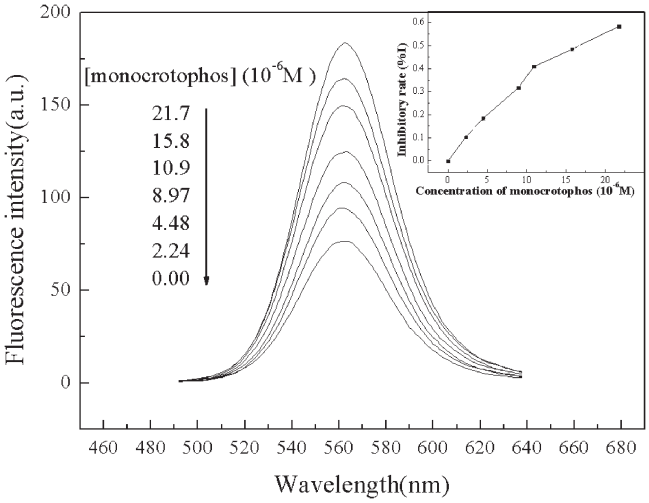



**Figure 2.** Fluorescence spectra of CdTe solutions containing monocrotophos of varied concentration. Insert shows the relationship curve between monocrotophos of varied concentrations and inhibitory rates.

**Chitosan concentration, pH and immersing time optimization.** Chitosan was assembled onto the self-assembled multilayer films of CdTe nanoparticles by electrostatic attraction with CdTe. It is therefore important to choose the right pH for the chitosan solution. The results showed that the quartz/APES/CdTe/CS multilayer was formed by dipping quartz/APES/CdTe in 0.1 g/L chitosan solution, pH 3–4, for 12 h.

**Acetylcholinesterase concentration optimization.** The concentration of acetylcholinesterase is one of the important parameters in sensor performance. A low concentration leads to inactivity of the enzyme, whereas a high concentration leads to a wasteful accumulation of the enzyme on the surface, which reduces the detection sensitivity. The experiment revealed that the optimal concentration was 0.5 U/mL.

## Fluorescence properties of SAMs film

Figure 3 shows the fluorescence spectra of quartz/APES, quartz/APES/CdTe, quartz/APES/CdTe/CS/AChE/CS/CdTe and CdTe solution. CdTe solution had a fluorescence emission at 561 nm; the fluorescence spectra showed a small 4 nm red-shift for CdTe SAMs. There was no fluorescence signal from quartz/APES, which also verified the assembly of CdTe on the quartz surface. It was found that the fluorescence of CdTe could be used as a reference to monitor the assembly of CdTe.

## Organophosphate pesticides detection conditions optimization

**Inhibition time optimization.** Inhibition of organophosphate pesticides needs a certain reaction time. The experiments studied the fluorescence intensity of the system for different inhibition

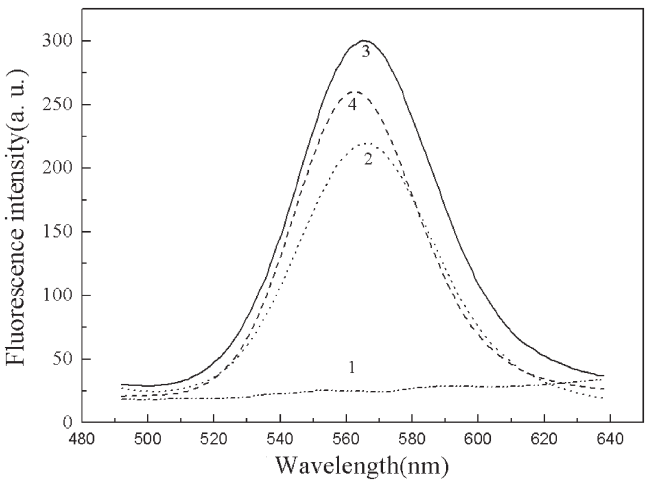



**Figure 3.** Surface fluorescence spectra of: 1, quartz/APES; 2, quartz/APES/CdTe; 3, quartz/APES/CdTe/CS/AChE/CS/CdTe; and 4, CdTe solution.

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times, as shown in Fig. 4; the longer the reaction time, the less was the fluorescence intensity of the system. When the response time increased to 14 min, the reaction system gradually stablilized, therefore 14 min inhibition time was selected.

**Acetylcholine concentration optimization.** It has been reported that pesticide inhibition is a function of both organophosphate pesticides and substrate concentrations, and the optimal acetylcholine concentration value for inhibition measurements is that at which the sensor response is shown to be saturated (18). As shown in Fig. 5, the fluorescence intensity vs. acetylcholine concentration had good linearity between 0.523 and 2.85 mmol/L acetylcholine, and levelled off as acetylcholine concentration became >3.14 mmol/L. The linear response could be fitted to the equation: $I_0/I = 1.06188 - 118.3\,C$, where $I_0$ and $I$ represent the fluorescence intensity of the system in the absence and presence, respectively, of acetylcholine and $C$ represents the concentration of acetylcholine. These SAMs can also be used as a sensor for acetylcholine. An optimal acetylcholine concentration of 2.85 mmol/L was selected for the analysis of organophosphate pesticides.

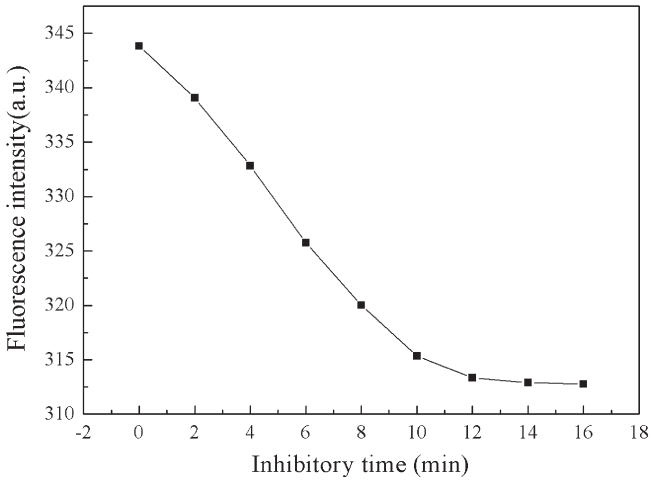


**Figure 4.** Relationship curve between fluorescence intensity of SAMs and different inhibitory times.

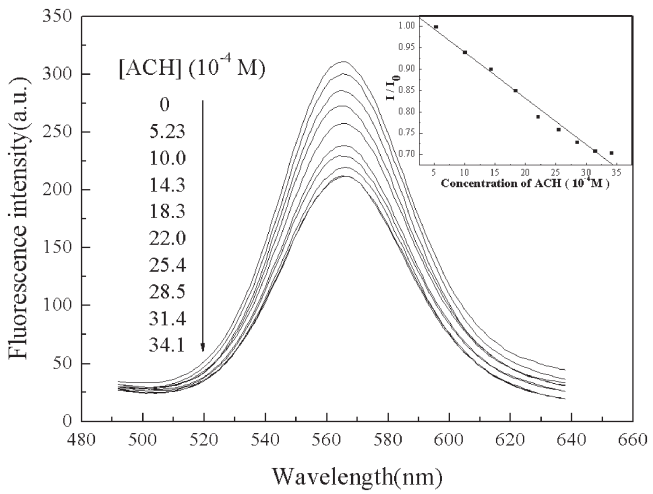


**Figure 5.** Fluorescence spectra of SAMs in aqueous solutions containing different concentrations of ACh; inset shows relative fluorescence intensities of different concentrations of ACh.

### Fluorescence sensing of SAMs for monocrotophos

The acetylcholinesterase, quartz/APES/CdTe and quartz/APES/CdTe/CS/AChE/CS/CdTe the films were studied by fluorescence spectroscopy at the same concentration of acetylcholine. Figure 6a shows that the fluorescence intensity of quartz/APES/CdTe/CS/AChE/CS/CdTe decreased with increasing concentration of monocrotophos, whereas there was no response to a quartz/APES/CdTe film (Fig. 6b), which confirmed that acetylcholinesterase had been assembled to the surface of quartz/APES/CdTe/CS/AChE/CS/CdTe SAMs and also indicated that monocrotophos indeed inhibited acetylcholinesterase, i.e. monocrotophos had a strong inhibitory effect on acetylcholinesterase. Also, a linear calibration curve was available for the determination of monocrotophos, as shown in the inset of Fig. 6a. The percentage inhibition increased linearly with increasing monocrotophos concentration in the range $8.95 \times 10^{-8}$–$6.09 \times 10^{-7}$ mol/L, with a detection limit of $3.20 \times 10^{-8}$ mol/L. Compared with that of CdTe in solution

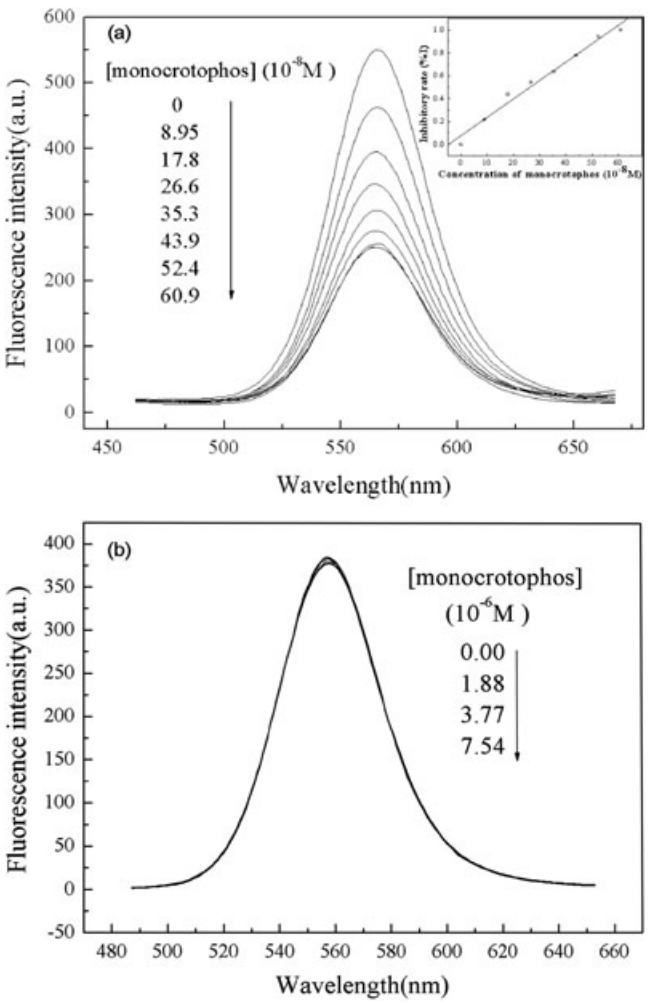


**Figure 6.** Fluorescence spectra of (a) quartz/APES/CdTe/CS/AChE/CS/CdTe and (b) quartz/APES/CdTe films in solutions containing monocrotophos of varied concentrations; insert shows the relationship between monocrotophos of different concentrations and its inhibitory rate to AChE in the SAMs.



  

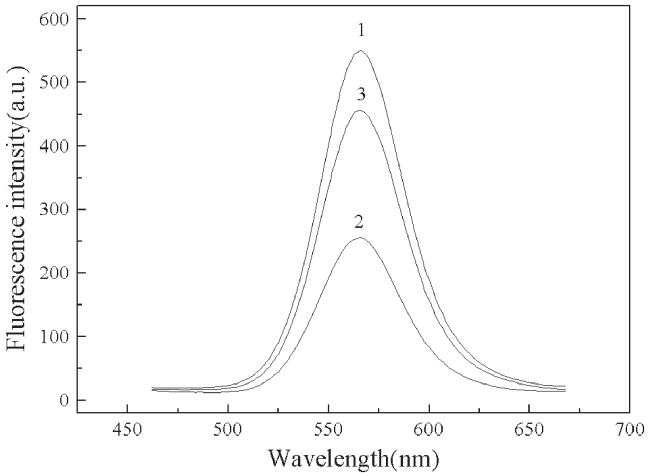


**Figure 7.** Fluorescence spectra of quartz/APES/CdTe/CS/AChE/CS/CdTe: 1, in the absence of monocrotophos; 2, in the presence of $6.09 \times 10^{-7}$ mol/L monocrotophos; 3, after regeneration.

(as low as $1.03 \times 10^{-6}$ mol/L), the sensitivity of SAMs was significantly improved; that is to say, interfacial fluorescence analysis based on SAMs can improve sensitivity in microanalysis.

### Inhibition rate of different organophosphate pesticides to acetylcholinesterase in the SAMs

Comparison of the inhibition of acetylcholinesterase by three organophosphate pesticides was also studied. Different kinds of organophosphate pesticides had different inhibition rates with respect to acetylcholinesterase; the experimental results showed that the inhibition rate of methylparathion and monocrotophos was almost the same, but the trichlorfon inhibition rate was the weakest. This is mainly due to the different molecular characteristics of the inhibitors and this agrees with the quantum chemistry simulations (Gaussian03). It also may be due to changes in the conformation of acetylcholinesterase on the surface of SAMs. This remains to be further explored and studied.

The experimental results also indicated that the presence of 1000 equivalents of $Na^+$, $K^+$, $Mg^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cl^-$ and $SO_4^{2-}$ did not interfere with the fluorometric detection of monocrotophos; the change in the emission intensity was within 10%. But significant interference existed in the presence of 10 equivalents of $Fe^{3+}$and $Cu^{2+}$. The fluorescence quenching of $Cu^{2+}$ may be attributed to effective electron transfer from thioglycolic acid to copper ions. The reduction of $Cu^{2+}$ to $Cu^+$ by thioglycerol forms $CdS^+$–$Cu^+$ species on the surface of the quantum dots (19). The interference of $Fe^{3+}$ may be attributed to an inner filter effect resulting from the strong absorption of the excitation wavelength by $Fe^{3+}$ (20).

SAMs stability is an important parameter for the sensor. The experiment showed that the stability of this SAMs sensor was high and could be used repetitively for 1 month by keeping it at low temperature.

### SAMs regeneration

Oxime compounds (2-PAM) were used as the regeneration agent for acetylcholinesterase (21). SAMs that had been used for monocrotophos determination were placed in 2% 2-PAM solution for 10 min and cleaned with phosphate buffer solution. Figure 7 indicates that the biosensor retained 83% of its original activity and had good regeneration capacity.

## Conclusions

This paper reports the preparation of quartz/APES/CdTe/CS/AChE/CS/CdTe film and fluorescence interfacial recognition for OPs. Using positively charged chitosan, CdTe and acetylcholinesterase were assembled onto the surface of quartz to form an acetylcholinesterase biosensor by a layer-by-layer assembly technique. This process preserved the long-term activity of the enzyme. The self-assembled multilayer film could be used to detect monocrotophos with high sensitivity, good regeneration and stability. This novel method should have high potential for applications in environment microanalysis.

## Supporting information on the internet

The following supporting information may be found on the online version of this article.

### Acknowledgements

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