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Optical detection of organophosphorus compounds based on Mn-doped ZnSe d-dot enzymatic catalytic sensor

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ABSTRACT

In this paper, we report a sensitive and selective method for detection of organophosphorus compounds (OPs) based on Mn:ZnSe d-dots-enzyme-hydrogen peroxide (H_2O_2) fluorescence quenching system. Acetylcholine esterase (AChE) can hydrolyze acetylcholine (ACh) to choline. Subsequently, choline oxidase (ChOx) oxidizes choline to generate H_2O_2. The enzyme-generated H_2O_2 can quench the fluorescence of Mn:ZnSe d-dots. When paraoxon are introduced in solution, it can interact with the active centers of AChE and decrease the enzyme activity. This leads to the decrease of the H_2O_2 production and then the fluorescence quenching rate of Mn:ZnSe d-dots. Experimental results showed that the enzyme inhibition percentage of Mn:ZnSe d-dots-ChOx-AChE-ACh system was proportional to the logarithm of paraoxon in the range 4.84×10^{-11} to 4.84×10^{-6} mol/L with the detection limit (S/N=3) of 1.31×10^{-11} mol/L. The proposed biosensor has been employed for quick determination of paraoxon in tap water and milk samples with satisfactory reproducibility and accuracy. This nano-biosensor was proved to be sensitive, rapid, simple and tolerance of most interfering substances.

1. Introduction

Quantum dots (QDs) have emerged as the attractive fluorescent probes for biological detection and biotechnology, due to the tunable size-dependent emission, broad excitation spectra, and narrow emission bandwidths. Moreover, the high quantum yield and photostability make QDs desirable fluorescent tags in vitro and in vivo (Michalet et al., 2005; Howarth et al., 2008; Yan et al., 2008). The controlled quenching of fluorescent QDs has led to the development of probes in biology, medicine, and analytical chemistry, e.g. protease-activation (Chang et al., 2005), DNA (Dyadyusha et al., 2005), spironolactone (Liang et al., 2006), glucose (Cordes et al., 2006), copper ion (Jin et al., 2007), and catecholamine (Ma et al., 2005) sensors. Although the assay with cadmium based QDs has been performed in vitro, in the long term, any leakage of cadmium from cadmium-based QDs would be fatal to biological systems, and eventually cadmium containing products would cause environmental problems. Therefore, in recent years, the emphasis has shifted toward the fabrication of non-cadmium-based QDs for applications in biology. The transition-metal-ion-doped quantum dots (d-dots) have attracted increased attention due to their low toxicity (Pradhan et al., 2005), moreover, d-dot emitters can also overcome a couple of intrinsic disadvantages of undoped QD emitters, that is, strong self-quenching caused by their small ensemble Stokes shift (Kagan et al., 1996; Achermann et al., 2003) and sensitivity to thermal, chemical, and photochemical disturbances (Pradhan et al., 2005; Empedocles et al., 1996; Li et al., 2003). Consequently, the d-dots, such as Mn:ZnSe d-dots, have been widely used in both optoelectronic and biological applications.

Organophosphorus compounds (OPs) are widely used in the agriculture all over the world as pesticides and insecticides (Yang et al., 1995). Due to the widespread use of OPs, their residues have been frequently found in soil, atmosphere, groundwater, as well as agricultural products. Therefore, public concern about the development of detection devices for effectively monitoring OPs and evaluating the human health risk of OPs exposure has grown steadily in recent years. The maximum residue limits (MRLs) for paraoxon in food stipulated by Food Safety Authority of Ireland (FSAI) are 0.01 mg/kg. As a matter of fact, OPs exert their toxicity through nonreversible phosphorylation and inactivation of esterase in the central nerve system. OPs readily inhibit important cholinergic enzymes, such as acetylcholinesterase (AChE), through an attack on the serine residue of AChE, forming a phosphorylated adduct. When the function of AChE is inhibited, accumulation of acetylcholine (ACh) will result in overstimulation of corresponding muscarinic and nicotinic cholinergic receptors, which may lead to serious health problems or even death (Quinn, 1987; Jarv, 1984). Paraoxon is typical OPs, which can inhibit the AChE activity (Zhang and Malhotra, 2005; Aubert et al., 2004). In

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most AChE-based sensors, OPs detection is mainly based on the irreversible inhibition of AChE activity by OPs (Anitha et al., 2004). The degree of inhibition has been calculated by comparing the residual activity of the enzyme with the initial activity (Neufeld et al., 2000). In order to avoid possible harm to humans and animals, there is an increasing need to develop fast and sensitive methods for detecting low concentrations of OPs in the daily-consumed foods and drinking water. Many analytical methods have been devised to detect OPs, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and electrical techniques. But most analytical methods are time-consuming, expensive, and must be manipulated by trained personnel. Therefore, many enzyme-based sensors are developed, such as enzyme organophosphorus hydrolase (OPH) electrode (Rainina et al., 1996), fluorescence spectroscopy (Viveros et al., 2006) and electrochemistry (Deo et al., 2005). Leblanc's group used simpler biosensor architecture to detect paraoxon and achieved lower detection limits (Constantine et al., 2003a,b; Celeste et al., 2003; Ji et al., 2005; Orbulescu et al., 2006). It is believed that enzymatic biosensors can overcome those disadvantages with their high efficiency and sensitivity.

Bi-enzyme systems have been generally considered as simple, inexpensive, selective and sensitive technique, and have been utilized for the construction of biosensors (Delvaux et al., 2005; Castillo et al., 2003; Shi et al., 2003). For example, electrochemical biosensor based on horseradish peroxidase (HRP) and glucose oxidase (GOD) was utilized to detect glucose (Bucur et al., 2004; Li et al., 2009). The electrocatalytic reactions with coupling of bi-enzymes or multi-enzymes can mimic the sequential electron transfer reactions found in many enzyme pathways of living cells (Conrado et al., 2008). Bi-enzyme layer-by-layer (LbL) films have been used in biosensors, bioreactors, and other industrial applications (Sheldon, 2007; Brady and Jordaan, 2009). Several works have been reported concerning on the QDs-enzyme based determination (Huang et al., 2007, 2008; Ji et al., 2005; Gill et al., 2006). However, QDs were rarely utilized in the bi-enzyme biosensors. Compared with other bi-enzyme systems, the QDs based bi-enzyme system has the advantages of simple detection procedure without the need of enzyme immobilization or QDs modification and can detect analytes optically.

In this study, we reported a highly sensitive optical biosensor for the detection of OPs. The proposed biosensing system is designed on the basis of fluorescence quenching of QDs and enzyme inhibition. The ACh can be catalyzed by AChE to choline and then choline was catalyzed by ChOx to H_2O_2 (Sharareh et al., 2009). Accordingly, H_2O_2 that produced from enzymatic reaction quenches the fluorescence of Mn:ZnSe d-dots. Paraoxon can inhibit AChE activity, thus the fluorescence quenching of Mn:ZnSe d-dots by H_2O_2 should be inhibited, and the enzyme inhibition percentage is proportional to the concentrations of paraoxon.

2. Experimental section

2.1. Materials and apparatus

All chemicals were of analytical reagent grade and used without further purification. Mercaptopropionic acid (MPA) (99%), selenium powder (~200 mesh, 99.9%), $Zn(NO_3)_2 \cdot 6H_2O$ (99.9%), $MnCl_2 \cdot 4H_2O$ (99.9%), $NaBH_4$ (99%), the acetylcholinesterase (AChE), choline oxidase (ChOx) and acetylthiocholine chloride (ACh) were purchased from Sigma-Aldrich. Paraoxon standard reagent (1000 μg/mL, acetone solution) was purchased from Shenzhen Shidejia Biotechnology Company. The water used in all experiments had a resistivity higher than 18 MΩ/cm. Fluorescence measurements were performed on a Shimadzu RF-5301 PC spectra fluorophotometer (Shimadzu Co., Kyoto, Japan), and 1 cm path-length quartz cuvette was used in experiments. All pH measurements were made with a PHS-3C pH meter (Tuopu Co., Hangzhou, China).

2.2. Preparation of Mn:ZnSe d-dots

Mn:ZnSe d-dots were prepared in aqueous solution by nucleation doping method based on our previous report (Wang et al., 2009). The synthesis procedure of Mn:ZnSe d-dots was performed as follows: 0.2 mL $MnCl_2$ solution (1.25×10^{-2} M) and 90 mL H_2O were loaded into a 250 mL three neck flask and degassed for 30 min by bubbling with nitrogen. Fresh NaHSe solution was added to N_2-saturated $MnCl_2$ solution at pH 11.2 in the presence of MPA as stabilizing agent. The reaction was then switched from nitrogen bubbling to nitrogen flow and subjected to a reflux at 100 °C for 40 min. 10 mL of $Zn(NO_3)_2$ stock solution (1.25×10^{-2} M) was injected and the reaction was refluxed for 5 h. The Mn-to-Se-to-Zn and Zn-to-MPA precursor ratios were 1:25:50 and 1:24, respectively. Finally, the reaction mixture was allowed to cool down to room temperature and the as-prepared Mn:ZnSe d-dots were used directly without any post-treatments.

2.3. Paraoxon detection

Mn:ZnSe d-dots, and then ChOx, AChE and different concentrations of ACh solution were successively added into 2.0 mL calibrated test tube, then diluted to the mark with phosphate buffer solution (pH=8.0) and mixed thoroughly within 10 min at 30 °C. Mn:ZnSe d-dots, ChOx, AChE, ACh, and paraoxon were successively added into 2.0 mL calibrated test tube, then diluted to the mark with phosphate buffer solution (pH=8.0). The relationship between the percentage enzyme inhibition and the paraoxon concentration was plotted as a calibration curve, which was used in detection of paraoxon in the real samples.

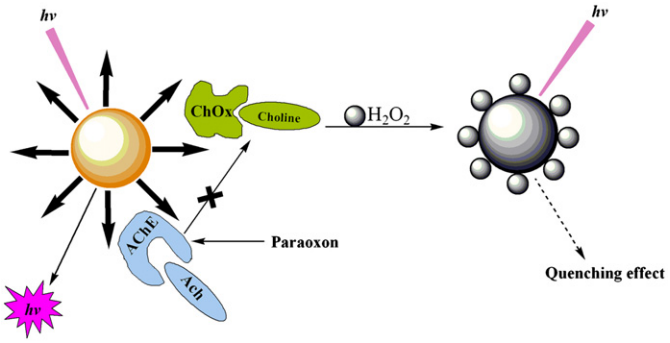
Two samples, one tap water sample from natural lakes, and the other milk sample from the market were filtered through a 0.22 μm membrane. The sample (10 mL) was mixed with 10 mL of 2 mM phosphate buffer (pH=8.0) to ensure the pH value. After simple pretreatment, different concentrations of paraoxon were added to study the percentage enzyme inhibition under the optimal conditions.

3. Results and discussion

3.1. The quenching effect of the H_2O_2 on Mn:ZnSe d-dots

In the semiconductor nanocrystals lattices of Mn:ZnSe d-dots, the doping ions Mn^{2+} substitute for the cations Zn^{2+} and act as a emission center due to $^4T_1(^4G) \rightarrow {}^6A_1(^6S)$ transition of the Mn^{2+} (Xue et al., 1998). Its PL is much higher than that of their bulk counterpart. Moreover, the emission has excellent thermal, chemical and photochemical stability, which are attributed to the nature of the atomic-like emission states (Narayan and Peng, 2007; Pradhan et al., 2007). Fig. S1 (see Supplementary Materials) showed a TEM micrograph of the Mn:ZnSe d-dots used in this study, It can be seen that the average particle diameter of the Mn:ZnSe d-dots is 3.8 nm.

The basic principle of our paraoxon biosensor was shown in Scheme 1. Paraoxon is structurally similar to other OPs like sarin and soman. Hence, in the present work, it has been chosen as standard OPs for the AChE inhibition. As shown in Scheme 1, AChE can hydrolyze ACh to choline, and then ChOx oxidizes choline to betaine with the concomitant generation of H_2O_2. Therefore, we firstly investigated the quenching effect of H_2O_2



Scheme 1. Schematic illustration of measuring principle of paraoxon biosensor.

on the fluorescence of Mn:ZnSe d-dots. Fluorescence spectra changes of Mn:ZnSe d-dots upon the interaction with different amounts of H_2O_2 were shown in Fig. S2 (see Supplementary Materials). As shown in Fig. S2, the fluorescence intensity of Mn:ZnSe d-dots decreased gradually with the increasing concentrations of H_2O_2. When H_2O_2 got to the surface of the Mn:ZnSe d-dots, the electron-transfer reaction happened immediately between H_2O_2 and Mn:ZnSe d-dots, thus the fluorescence signal of Mn:ZnSe d-dots was quenched.

3.2. Effect of pH, reaction time and temperature

Fig. S3 (see Supplementary Materials) showed the effect of pH and temperature on the fluorescence quenching of Mn:ZnSe d-dots-ChOx-AChE system in the presence of ACh. The fluorescence intensity ratio I/I_0 (I_0 and I refer to the fluorescence intensity of Mn:ZnSe d-dots-ChOx-AChE system in the absence and presence of ACh) at pH 8.0 was the highest (Fig. S3A), which indicated that the quenching effect of ACh on the fluorescence of Mn:ZnSe d-dots-ChOx-AChE system was much greater in alkaline medium than that in acidic or neutral medium. This result was caused by the different optimum pH values of these enzymes. The optimum pH values for ChOx was pH 7.0–8.0 (Nachmansohn and Wilson, 1991) and 8.0–9.0 for AChE (Eisenthal and Danson, 1993). High acidity will affect the fluorescence intensity of Mn:ZnSe d-dots and the protein denaturation of AChE in the higher pH (>9.0) may contribute to its lower activity. Therefore, 2 mmol/L pH 8.0 PBS was used in the further experiment.

Initial experiments demonstrated that the quenching effect of ACh on the fluorescence of Mn:ZnSe d-dots-ChOx-AChE system was finished within 10 min and the fluorescence intensities were stable for more than 20 min. We recorded the fluorescence intensity of the system after reaction time of 10 min. As shown in Fig. S3B, the quenching effect of ACh on the fluorescence intensity ratio I/I_0 of Mn:ZnSe d-dots-ChOx-AChE system at 30 °C was the highest, which was consistent with the optimum temperature of the catalytic behavior of cholinesterase (Tumturk et al., 2007). We chose 30 °C as the optimal reaction temperature in this work.

3.3. Effect of the concentrations of ChOx and AChE

ACh hydrolysis reaction catalyzed by AChE was carried out under a kinetically controlled condition, while choline oxidation reaction catalyzed by ChOx was performed based on diffusion-controlled conditions. So the amount of choline produced depends on AChE activity, not on the ACh concentration. And the amount of the H_2O_2 production is controlled by AChE activity. In order to optimize the concentrations of ChOx and AChE to get better reaction activity of AChE to produce choline and sufficient activity of ChOx to convert all the choline. The effect of the concentrations of ChOx and AChE on the ACh determination was studied. Fig. 1 showed the fluorescence spectra of Mn:ZnSe d-dots upon interaction with different concentrations of ACh and ChOx/AChE for a fixed time interval of 10 min. It can be seen that the fluorescence intensity of Mn:ZnSe d-dots-ChOx-AChE system decreased with the increasing concentration of ACh. The inset in Fig. 1 showed the relationship between fluorescence intensity ratio I/I_0 (I_0 and I refer to the fluorescence intensity of Mn:ZnSe d-dots-ChOx-AChE system in the absence and presence of ACh) and the ACh concentration. The linear relationship of ACh was entirely attributable to the amount of H_2O_2 production, and the generation of H_2O_2 was a step-by-step catalytic reaction via two enzymes. When the ACh concentration was lower, the high concentration of enzyme was not conducive to the quenching effect of H_2O_2, because the excess enzyme covered on the surface of Mn:ZnSe d-dots would inhibit the quenching effect. The plots exhibited a better linear relationship (0.017–2.33 mM) when the concentration of ChOx and AChE were 0.3 U/mL and 2 U/mL, respectively. Therefore, we used 0.3 U/mL ChOx and 2 U/mL AChE in the further experiment.

3.4. Detection of paraoxon by Mn:ZnSe d-dots-ChOx–AChE–ACh system

The control experiments of paraoxon detection system (Fig. S4 in the Supplementary Materials) showed the fluorescence of Mn:ZnSe d-dots changed slightly in the presence of ChOx, AChE, ChOx and AChE, ACh or AChE and paraoxon, respectively. The results proved that the formations of H_2O_2 were essential to quench the fluorescence of the Mn:ZnSe d-dots.

It can be seen from Fig. 2 that the fluorescence intensity of Mn:ZnSe d-dots was not influenced by the addition of paraoxon in the concentration range from 0.024 to 2.42 μmol/L. Therefore, paraoxon did not have quenching effect on the fluorescence signal of Mn:ZnSe d-dots.

To optimize the concentration of ACh for paraoxon detection, we use the absolute quenching rate (K_{10}) of the biosensor for the first 10 min as a parameter for paraoxon detection, $K_{10}=(F_0-F_{10})/10$, where F_0 and F_{10} are the fluorescence intensity recorded at 570 nm before and after addition of ACh (first 10 min), respectively. The enzyme inhibition percentage can be calculated from $I\ (\%)=(K_{10\ \text{without}}-K_{10\ \text{with}})/K_{10\ \text{without}}\times 100$, where $K_{10\ \text{with}}$ and $K_{10\ \text{without}}$ are the absolute quenching rates with and without ACh in a certain concentration of paraoxon, respectively. Fig. S5 (see the Supplementary Materials) showed the inhibition percentage increased with increasing ACh concentration from 0.017 to 0.67 mM and then decreased with further increasing ACh concentration. This was probably due to that the interaction between AChE and ACh was greater than the AChE inhibition, when the ACh concentration was excess. So AChE must keep a significant portion of its activity in the solution. Therefore, in the present study, an ACh concentration of 0.67 mM was chosen for paraoxon determination.

Fig. 3 showed the fluorescence intensity of Mn:ZnSe d-dots-ChOx–AChE–ACh system after the incubation with variable concentrations of paraoxon. The inset in Fig. 3 showed the relationship of inhibition percentage and the paraoxon concentration. It can be seen that the inhibition percentage was proportional to the logarithm of paraoxon in the concentration range 4.84×10^{-11} to 4.84×10^{-6} mol/L (the inset of Fig. 3), with the detection limit (S/N=3) down to 1.31×10^{-11} mol/L. The regression equation was $I\ (\%)=162.92+14.91\ \log C_{\text{paraoxon}}$ ($R^2=0.998$). Fig. S6 (see the Supplementary Materials) further compared the inhibition efficiencies of four kinds of OPs at the concentration of 4.84×10^{-8} mol/L. It can be seen that the percentages of inhibition were only 30.79% and 21.90% for methyl parathion and omethoate, respectively, which were considerably smaller than those of parathion (43.89%) and paraoxon (55.69%). These results

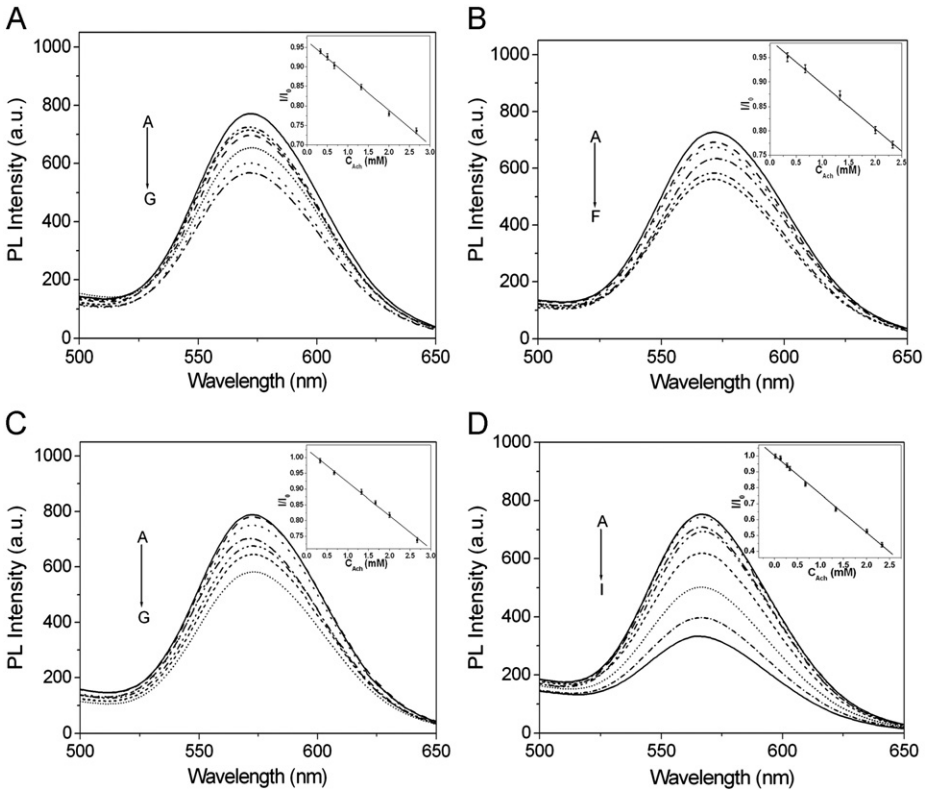


Fig. 1. Fluorescence spectra of Mn:ZnSe d-dots in the presence of varying concentrations of ChOx, AChE and ACh. (A) 0.3 U/mL ChOx and 4 U/mL AChE, A–G: 0, 0.33, 0.5, 0.67, 1.33, 2, 2.67 mM ACh. (B) 0.6 U/mL ChOx and 2 U/mL AChE, A–F: 0, 0.33, 0.67, 1.33, 2, 2.33 mM ACh. (C) 1.5 U/mL ChOx and 2 U/mL AChE, A–G: 0, 0.33, 0.67, 1.33, 1.67, 2, 2.67 mM ACh. (D) 0.3 U/mL ChOx and 2 U/mL AChE, A–I: 0, 0.017, 0.13, 0.27, 0.33, 0.67, 1.33, 2, 2.33 mM ACh. The inset shows the relationship between fluorescence intensity ratio I/I_0 and the ACh concentration.

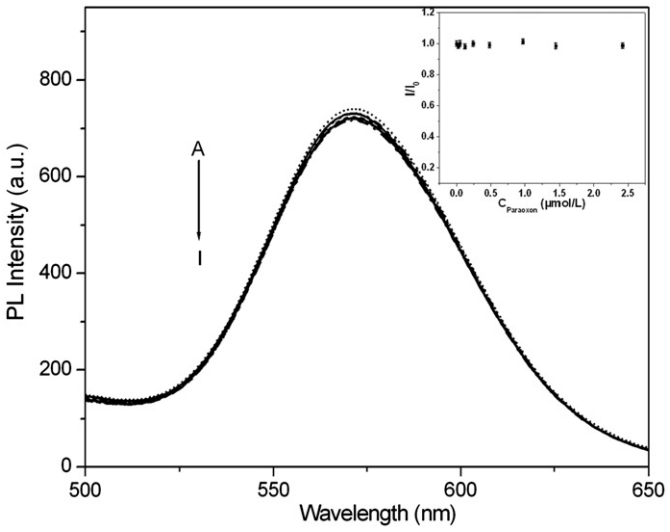


Fig. 2. Effect of paraoxon concentrations on the fluorescence intensity of Mn:ZnSe d-dots. The inset shows the relationship between I/I_0 with the paraoxon concentration. I_0 and I are the fluorescence intensity of Mn:ZnSe d-dots in the absence and presence of paraoxon.

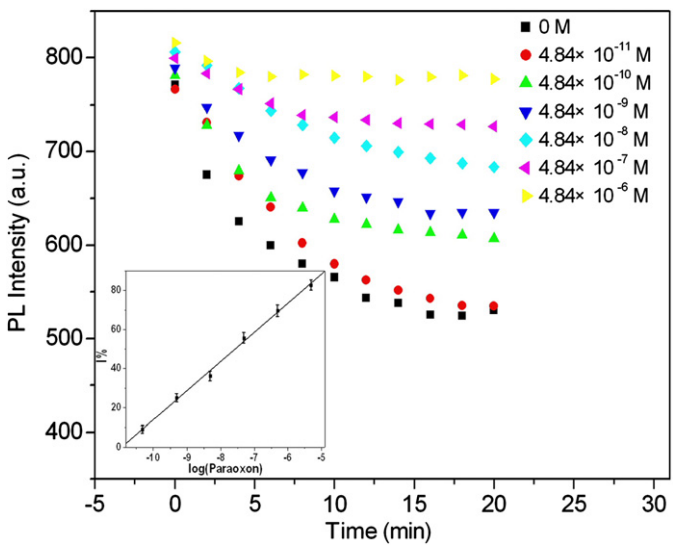


Fig. 3. Fluorescence intensity of Mn:ZnSe d-dots-ChOx-AChE system in the present of 0.67 mM ACh after incubation with variable concentrations of paraoxon: (a) 0, (b) 4.84×10^{-11}, (c) 4.84×10^{-10}, (d) 4.84×10^{-9}, (e) 4.84×10^{-8}, (f) 4.84×10^{-7}, and (g) 4.84×10^{-6} mol/L. The inset shows the relationship between the enzyme inhibition percentage and the paraoxon concentration. All measurements were performed in a 2 mM PBS solution, pH=8.0. The ChOx concentration is 0.3 U/mL, the AChE concentration is 2 U/mL.

suggested that the established biosensors could be used for determination of different kinds of OPs.

3.5. Interference study

The selectivity of the proposed method for paraoxon detection was further evaluated with various coexistence substances added. Table S1 (see Supplementary Materials) showed the interference effect of inorganic ions, sugar, nitrophenol and organic acids on the determination of paraoxon, a relative error of $\pm5.0\%$ was considered to be tolerable. As shown in Table S1, the results showed that the tolerable concentration ratios of coexisting substances to 4.84×10^{-7} mol/L paraoxon was over 10^6-fold for

Table 1
Determination of paraoxon in tap water and milk samples.

Sample	Added (10^{-7} mol/L)	Found[a] (10^{-7} mol/L)	Recovery (%)	RSD (%, $n=3$)
Tap water	0.484	0.497	102.7	0.9
	4.84	4.64	95.9	1.3
Milk	0.484	0.498	102.9	1.0
	4.84	4.70	97.1	0.6

[a] Mean of three replicates.

Table 2
Comparison of different methods for determination of paraoxon.

Methods	System	Linear range (mol/L)	LOD (mol/L)	References
Electrochemistry	Multiwalled carbon nanotubess/Screen printed electrode (SPE)	$1.0\times10^{-9}-6.9\times10^{-9}$	0.5×10^{-9}	Joshi et al. (2005)
Electrochemistry	ChOx/Prussian blue/SPE	$0.1\times10^{-6}-1\times10^{-6}$	0.1×10^{-6}	Sharareh et al. (2009)
Localized surface plasmon resonance	AChE/AuNPs	$3.63\times10^{-9}-0.36\times10^{-6}$	0.85×10^{-9}	Lin et al. (2006)
Fluorometry	CdSe QDs/Organophosphorus hydrolase (OPH)	Not mentioned	10^{-9}	Celeste et al. (2003)
Spectrophotometry	Fluorescent dye/OPH	$10^{-9}-10^{-5}$	5×10^{-9}	Jhony et al. (2006)
Electrochemistry	QDs/OP/AChE	$1.1\times10^{-9}-1.1\times10^{-6}$	5.5×10^{-10}	Wang et al. (2008)
Electrochemistry	Tyrosinase enzyme	$0.01\times10^{-6}-0.1\times10^{-6}$	5.0×10^{-9}	Campanella et al. (2007)
Electrochemistry	Butyrylcholinesterase and ChOx	$0.0\times10^{-6}-0.5\times10^{-6}$	15.0×10^{-9}	Campanella et al. (2007)
Fluorometry	Mn:ZnSe d-dots/ChOx/AChE/ACh	$4.84\times10^{-11}-4.84\times10^{-6}$	4.19×10^{-12}	This work

NaCl and KCl, 500-fold for $Zn(NO_3)_2$, $CaCl_2$ and $CdCl_2$, 10^4-fold for glucose, sodium acetate and sodium citrate, 250-fold for glycerol and 2-nitrophenol. It was found that there was little interference from commonly existed substances. Thus, the new sensitive method displays high selectivity for the determination of paraoxon.

3.6. Determination of paraoxon in the tap water and milk samples

In order to evaluate the accuracy, repeatability and selectivity of the proposed method, different concentrations of paraoxon in the spiked tap water and milk samples were determined and the results were listed in Table 1. From Table 1, it can be seen that the recoveries of the two kinds of samples were found to be in the range 95–103%, and the RSDs were less than 2.0%. The results revealed that it was an applicable method for organophosphate compound-contaminated environmental samples. A comparison between this method and other reported methods for paraoxon determination in detection limit and linear range was summed up in Table 2. It could be seen from Table 2 that the sensitivity of this method was better than most of the well-known methods including electrochemistry method. In contrast to other biosensors, the present method did not require sample enrichment of OPs from the real samples. Both fast sample preparation and low detection limit were very important for development of new sensing techniques with low cost and high-throughput screening.

4. Conclusion

In this study, a sensitive and selective OPs detection method based on fluorescence quenching and enzyme inhibition was developed. The detection limits of the proposed biosensor are much lower than that of reported detection methods. We successfully detect paraoxon in the tap water and milk samples with satisfactory results. This nano-biosensor based on enzyme inhibition and fluorescence quenching is also useful to detect other OPs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.03.042.

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