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Short communication

Highly-sensitive organophosphorous pesticide biosensors based on nanostructured films of acetylcholinesterase and CdTe quantum dots

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ABSTRACT

The optical transducer of CdTe semiconductor quantum dots (QDs) has been integrated with acetylcholinesterase enzyme (AChE) by the layer-by-layer (LbL) assembly technique, resulting in a highly sensitive biosensor for detection of organophosphorus pesticides (OPs) in vegetables and fruits based on enzyme inhibition mechanism. The detection limits of the proposed biosensors are as low as 1.05×10^{-11} M for paraoxon and 4.47×10^{-12} M for parathion, which are significantly better than those of the conventional GC/MS methods or amperometric biosensors (0.5 nM). These biosensors are used for quick determination of low concentrations of OPs in real vegetable and fruit samples and exhibit satisfactory reproducibility and accuracy. Moreover, the stock stability of the biosensors are very good due to the stabilizing environment for the enzyme in the nanostructures made by LbL technique. Many advantages provided by these biosensors, like fluorescent change recognized by naked eyes and mass production with low cost, will facilitate future development of rapid and high-throughput screening of OPs.

1. Introduction

Organophosphorus pesticides (OPs) are the most popular insecticides in use today due to their high efficiency for insect elimination, easy synthesis, and low cost. Due to the widespread use of OPs, their residues have been frequently found in soil, atmosphere, groundwater, as well as agricultural products. The statistical data of the Pesticide Data Program published in 2005 demonstrated that overall 73% of fresh fruits and vegetables contained the detectable residues (USDA, 2005). The residues are highly toxic substances that have been found to cause serious problems to human health even at very low concentrations (Heath, 1997). 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.

Currently, detection of OPs is performed under laboratory conditions with the assistance of expensive instruments and trained personnel. To circumvent these issues, simple methods were proposed to realize quick and sensitive detection of pesticides by taking advantage of the inhibition mechanism of acetylcholinesterase enzyme (AChE) using different configurations and transduction technologies, such as electrochemistry (Kok and Hasirci, 2004; Schulze et al., 2003; Joshi et al., 2005), field-effect transistors (Ristori et al., 1996; Singh et al., 1999), fluorescence (Diaz and Peinado, 1997; Tsai and Doong, 2005; Vamvakaki and Chaniotakis, 2007; Constantine et al., 2003; Ji et al., 2005; Li and Qu, 2007) or colorimetric probes (Andreou and Clonis, 2002; Andres and Narayanaswamy, 1997; Wong et al., 2006; Xavier et al., 2000). All these methods proved to be sensitive, portable, easy to use, and capable of providing reliable analytical information, especially when used in measurement of water samples. However, to the best of our knowledge, until now there has been no report on detection of OP in real food sample like fruits and vegetables using biosensors. It is easily understood that foods themselves contain many interferents, e.g., redox and chromatic substances, for both electrical and optical transduction. Hence, to apply the biosensors to OPs' determination in real fruits and vegetables remains a big challenge.

In this study, we report a highly sensitive optical biosensor for the detection of OPs in foods and water. The biosensor is composed of nanostructured multilayers of the enzyme AChE and photoluminescent (PL) CdTe QDs, and fabricated using the layer-by-layer (LbL) assembly technique (Scheme S1). The LbL technique is an environment-friendly method and ideal for cost efficient mass production (Decher, 1997; Podsiadlo et al., 2007), being a very attractive and powerful technology for development of biosensors (Li et al., 2009; Yu et al., 2005). Our results demonstrate that this proposed biosensor can be applied to determine low concentrations of OPs in real vegetable and fruit samples, and the

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demonstrated detection limits of the developed biosensors are significantly better than those of reported biosensor so far.

2. Materials and methods

2.1. Materials and methods

$Cd(ClO_4)_2{\cdot}6H_2O$, Al_2Te_3 powders, and mercaptopropionic acid (MPA) were purchased from Alfa Aesar. AChE (EC 3.1.1.7 from E. electricus, lyophilized, specific activity 435 U/mg), acetylthiocholine, sodium polystyrenesulfonate (PSS, MW 70,000), and poly(allylamine hydrochloride) (PAH, MW 8000–11,000) were purchased from Sigma-Aldrich. The standard samples of paraoxon, parathion, dichlorvos, and omethoate (100 μg/mL in acetone) were purchased from Weiyekechuang Keji CO. Negatively-charged CdTe QDs with PL peaks at 585 nm and 592 nm were synthesized according to our previous report (Li et al., 2009).

The UV–vis absorption spectra were measured using a Varian Cary 4000 UV–vis spectrometer, and the PL spectra were recorded with a Cary Eclipse fluorescence spectrophotometer. If not specifically stated, the samples were excited at 380 nm, and the exciting slit and the emission slit were both 5 nm. Static water contact angles were obtained with a contact angle and surface tension meter (CAM101, KSV). The gas chromatography in combination with mass spectrometry (GC/MS) analysis was performed on an Agilent 7890A equipped with a MSD 5975 mass spectrometer and a split/splitless injector.

2.2. Fabrication of the proposed biosensors

Quartz or glass was used as the substrates and cleaned with Piraha solution [*Caution! Piraha solution is highly corrosive and toxic*]. Scheme S1 showed a scheme of the fabrication process of biosensors: (1) The PAH/QD multilayers were alternatively deposited from 1 mg/mL PAH aqueous solutions (pH 7.5, 0.5 M NaCl) and as-prepared QD solution; (2) three bilayers of PAH/PSS were deposited from 1 mg/mL PAH and PSS aqueous solution (pH 7.5, 0.5 M NaCl), each for 5 min. These three bilayers of PAH/PSS behaved like selective channels (Clark et al., 1997; Liu et al., 2002), because only small molecules rather than large molecules such as enzymes could transport in or out the underlying PAH/QD and avoid possible influences of the AChE-catalyzed hydrolysis reaction of acetylthiocholine on the structures of PAH/QD multilayers; (3) at the end, capping PAH/AChE multilayers were deposited on top of the $(PAH/CdTe)_x(PAH/PSS)_3$, which would contact and react with OPs and acetylthiocholine in solution. In order to enhance the permeability to OPs and the substrate, the PAH solution without NaCl and 0.5 mg/mL AChE in 20 mM of PBS were used for fabrication of the capping multilayers. The pH of PBS solution was 8.0 and the isoelectric point of AChE was 4.5, so the enzyme was negatively charged in PBS solution. The electrostatic interaction between PAH and AChE provided a biocompatible nano-environment in the film to encapsulate the water-soluble enzymes, which greatly improved their stability against unfolding, denaturation, and dilution effects.

2.3. OP detection

The procedure for OP detection was as follows: (1) the biosensor was first incubated with 3.0 mL of the PBS solution (20 mM, pH 8.0, 2 mM Mg^{2+}) containing different concentrations of pesticide for 15 min; (2) 4 mM acetylthiocholine was added to above stirred solution and the PL signal was monitored over time; (3) the measurements were repeated 6 times for each concentration, and the relationship between the percentage enzyme inhibition and the OP concentration was plotted as a calibration curve, which was used in detection of OP in the real vegetable samples.

2.4. OP detection in real vegetable/fruit samples

The procedure for OP determination in vegetable samples was as follows: (1) the vegetable/fruit sample was first chopped and extracted with 20 mL PBS; (2) the biosensor was incubated with the extracted solution at 38 °C for 15 min; (3) after being rinsed with PBS and dried under N_2 flow, the PL spectrum of the biosensor was measured in PBS solution; (4) finally, 4 mM acetylthiocholine was injected in the mixed solution and the kinetic measurements of PL at 592 nm were recorded. The decrease rates of PL after incubation with fruit or vegetable samples were compared with the calibration curve, and the OP concentration in the real sample was obtained.

The accuracy of the proposed biosensor in this study was compared with GC/MS. GC/MS measurement followed the procedure for the modified AOAC Official Method 985.22 (see Part S1 in Supporting Information).

3. Results and discussion

3.1. Fabrication of PAH/CdTe QDs/AChE nanostructured film with good response to acetylthiocholine

The PAH/CdTe QDs/AChE nanostructured film was fabricated by LbL technique. The LbL assembly process of PAH and QDs was monitored by the signal increase in both UV–vis absorption spectroscopy (not shown) and PL spectroscopy (Fig. S1 in Supporting Information). Both the absorbance shoulder at 530 nm and PL intensity at 585 nm of $(PAH/CdTe\ QDs)_x$ multilayers increase linearly with the number of bilayers, *x*, which confirm irreversible adsorption and regular layer growth of PAH and QDs on the substrate. The deviation from linear behavior for the initial bilayers can be explained by incomplete coverage of QDs and partial filling of the polyelectrolyte into the empty space between QDs during the early stage of LbL deposition (Jiang et al., 2004).

Fig. 1A depicts the PL spectra of $(PAH/CdTe)_8(PAH/PSS)_3(PAH/AChE)_3$ multilayers before (curve a) and after exposure to 4 mM acetylthiocholine (curves b–k). Before addition of acetylthiocholine, the PAH/CdTe QDs multilayer has a strong PL peak at 592 nm. It is noticed that the PL signal of the PAH/CdTe QDs multilayer in PBS solution increases to almost 3 times that in air (Fig. S2). The increase of the PL signal of the PAH/CdTe QDs multilayer in PBS solution is possibly due to the adsorbed water molecules on the surface of QDs, which act to passivate surface traps and increase the luminescence efficiency (Cordero et al., 2000). After addition of acetylthiocholine, the PL intensity at 592 nm initially decreases linearly with time (inset in Fig. 1A). When a certain period of time (ca. 10 min) has elapsed, the rate of decrease gradually becomes smaller and finally reaches zero. Control experiments reveal that addition of acetylthiocholine does not yield any obvious PL change in the $(PAH/CdTe)_8(PAH/PSS)_3$ multilayer (Fig. S3). As comparison, thiocholine can quench PL of $(PAH/CdTe)_8(PAH/PSS)_3$ multilayer (Fig. 1B). The PL intensity decrease is accompanying with blue-shift of the peak maxima with time, which is believed to originate from preferential PL quenching of CdTe QDs with large sizes (see detailed discussion in Part S4). Moreover, the quenching rate of thiocholine is enhanced with incubation temperature increased to 38 °C and with increasing thiocholine concentration (Fig. S4). Thus, the quenching of PL of the PAH/CdTe QDs/AChE nanostructured film is attributed to the AChE-catalyzed hydrolysis of acetylthiocholine to acetate and thiocholine. The latter product acts as donors for the holes generated in the valence band of CdTe QDs upon photoexcitation (Pardo-Yissar et al., 2003; Willner et al., 2007), and thus quenches PL of QDs (Fig. S5).

We use the absolute quenching rate (K_{10}) of the biosensor for the first 10 min as a signal for detection of OP, $K_{10} = (F_0 - F_{10})/10$,

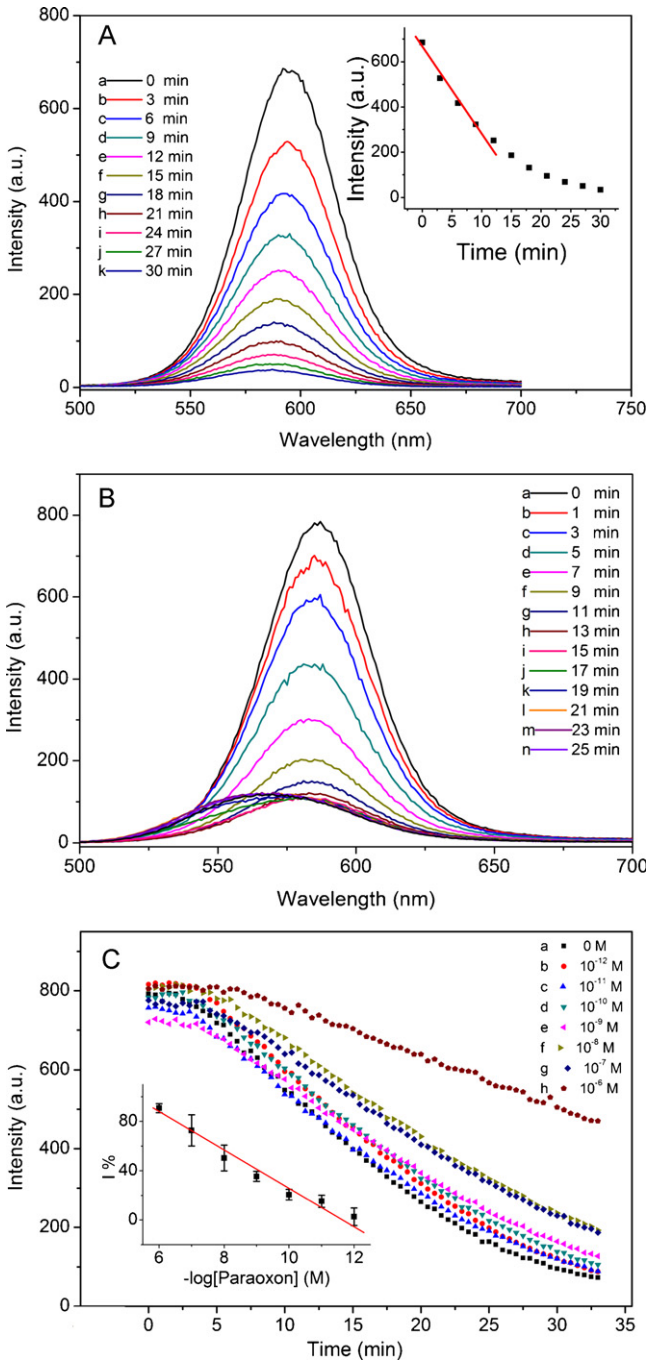

Fig. 1. (A) Time-dependent PL changes upon the interaction of $(PAH/CdTe)_8(PAH/PSS)_3(PAH/AChE)_3$ multilayers with 4 mM acetylthiocholine solution at 38 °C. Inset presents the time-dependent PL change of multilayers during the first 30 min of reaction. (B) Time-dependent PL changes upon the interaction of 8 bilayers of PAH/CdTe QDs in the absence (a) and presence (b–n) of 2 mM thiocholine at room temperature. (C) PL response of the $(PAH/CdTe)_8(PAH/PSS)_3(PAH/AChE)_3$ multilayers to 4 mM acetylthiocholine after incubation with variable concentrations of paraoxon: (a) 0, (b) 10^{-12}, (c) 10^{-11}, (d) 10^{-10}, (e) 10^{-9}, (f) 10^{-8}, (g) 10^{-7}, and (h) 10^{-6} M. The inset shows inhibition rate as a function of paraoxon concentration. Each data point is an average of six measurements. All measurements were performed in a 20 mM PBS solution, pH = 8.0.

where F_0 and F_{10} are the absolute PL intensity recorded at 592 nm before and the first 10 min after addition of acetylthiocholine, respectively. Using K_{10} as a signal, the experimental conditions including the concentration of acetylthiocholine and Mg^{2+}, pH value, and temperature, are optimized for determination of the concentration of enzyme inhibitors (see detailed discussion in Part S4 and Fig. S6). The optimal conditions for the rest of study on the $(PAH/CdTe)_8(PAH/PSS)_3(PAH/AChE)_3$ biosensor are explored to be 4 mM acetylthiocholine, pH 8.0, 38 °C, and 2 mM Mg^{2+}.

Immobilization of AChE into polyelectrolyte multilayers can effectively stabilize enzymes against unfolding forces. This effect can be observed by testing the long term stability of the sensors (Fig. S7). A stock of $(PAH/CdTe)_8(PAH/PSS)_3(PAH/AChE)_3$ multilayers were prepared and stored at −20 °C. At defined time intervals, the samples were taken and the remaining activity of enzyme was analyzed under the optimal conditions. Evidently, even after a storage period of 35 days, the immobilized enzymes into polyelectrolyte multilayers retained all of their bioactivity. The slight increase of the AChE activity might be attributed to changes of the enzymes' three-dimensional structure inside the multilayers, which was already observed in many biosensor systems (Khan and Wernet, 1997; Vamvakaki and Chaniotakis, 2007). The long-term stability of enzymes in the nanostructured film will benefit application of biosensors.

3.2. Detection of OPs by PAH/CdTe QDs/AChE nanostructured film

The PAH/CdTe QDs/AChE nanostructured film is further used for fluorescent detection of OPs. When OPs are introduced in solution, they can interact with the active centers of AChE and decrease the enzyme activity. This leads to the decrease of the thiocholine production and then the PL quenching rate of QDs. By means of measuring the quenching rate before and after an incubation step with the pesticide, one can calculate the concentration of OPs.

Four commonly-used OPs, paraoxon, parathion, dichlorvos, and omethoate, were detected with the proposed biosensor. Their molecular structures are shown in Fig. S8. The detection procedure involved two separate steps: (1) incubation in pesticide solution and (2) exposure to acetylthiocholine substrate. In the first step, the incubation time in OP solution influenced the degree of inhibition, and 15 min was found to maximize the inhibition effect. Fig. 1C shows the typical PL response of the $(PAH/CdTe)_8(PAH/PSS)_3(PAH/AChE)_3$ multilayers to 4 mM acetylthiocholine after incubation with variable concentrations of paraoxon for 15 min. Increase of the concentration of paraoxon decreases the quenching rate.

The percentage of inhibition can be calculated from $I(\%) = (K_{10\ \text{without}} - K_{10\ \text{with OP}})/K_{10\ \text{without}} \times 100$, where $K_{10\ \text{without}}$ and $K_{10\ \text{with OP}}$ are the absolute quenching rates without inhibition and with inhibition at a certain concentration of OPs, respectively. The detection limit (LOD) is set to be the concentration of pesticide that causes 10% inhibition (Skladal et al., 1997). Under the optimal conditions established in the above studies, calibration plots were generated for four OPs. The percent inhibition is proportional to the logarithm of paraoxon within the concentration range of 10^{-12} to 10^{-6} M (the inset of Fig. 1C), with a LOD down to 1.05×10^{-11} M (2.89×10^{-3} μg/L). The regression equation is $I(\%) = 180.88 + 15.55$ log[paraoxon] ($R^2 = 0.99$, $n = 6$). The relative standard deviation (RSD) among six different biosensors with equal concentration of paraoxon is found to be less than 15%, indicating a reasonably good reproducibility for most measurements.

Similar calibration curves for different OPs are shown in Fig. S8 and S9. The regression equation is $I(\%) = 140.38 + 11.49$ log[parathion] ($R^2 = 0.99$, $n = 6$), $I(\%) = 165.03 + 17.30$ log[dichlorvos] ($R^2 = 0.99$, $n = 6$), and $I(\%) = 69.25 + 5.79$ log[omethoate] ($R^2 = 0.96$, $n = 6$), respectively. The LOD is 4.47×10^{-12} M (1.30×10^{-3} μg/L) for parathion, 1.10×10^{-9} M (0.243 μg/L) for dichlorvos and 5.89×10^{-11} M (1.26×10^{-2} μg/L) for omethoate, respectively.

The proposed method shows excellent sensitivity for the detection of OPs. For example, the LOD of 1.05×10^{-11} M for paraoxon and 4.47×10^{-12} M for parathion is 10^3 times lower than that of

Table 1
A correlation of the concentrations of total OP content determined directly by the proposed biosensor with that determined by GC/MS.

Sample	The proposed biosensor			GC/MS	
	Measured value (I%)	Average value (μg/mL)	Relative recovery	Average value (μg/mL)	Relative recovery
Paraoxon[a]		21.92 ± 2.14 (RSD = 9.76%)	110.0	17.88 ± 0.37 (RSD = 2.0%)	89.4
Apple sample, 2.19×10^{-2} μg/mL paraoxon added	70.71 ± 69	2.28 ±0.23 (RSD = 10.2%, $n = 6$)	104.6	N/A[c] ($n = 3$)	
Apple sample, 2.29×10^{-3} μg/mL paraoxon added	56.12 ± 0.54	2.4 ± 0.67 (RSD = 8.15%, $n = 6$)	115.0	N/A[c] ($n = 3$)	
Apple	8.32 ± 2.80	N/A[b] ($n = 6$)		N/A[c] ($n = 3$)	
Bean	1.00 ± 0.96	N/A[b] ($n = 6$)		N/A[c] ($n = 3$)	
Tap water	3.76 ± 2.53	N/A[b] ($n = 6$)		N/A[c] ($n = 3$)	

[a] Since the limit of detection by the proposed optical biosensor is approximately 10^3 times lower than that by GC/MS method, and the standard samples for GC/MS were diluted before detection with the proposed biosensors.
[b] Since the inhibition rate is lower than 10%, we assume that OPs are not detected.
[c] Total OP content in natural vegetable and fruit samples is lower than the limit of detection by GC/MS, so the OPs cannot be detected out.

the GC/MS method (1 μg/L) (Amendola et al., 2002), 3 orders of magnitude lower than that from the fiber-optic AChE-inhibition biosensor (Mulchandani et al., 1999), 2 orders lower than that from a reagentless bioactive paper-based solid-phase biosensor (Hossain et al., 2009), and 400 times lower than that with the amperometric biosensor based on chemically modified electrode (Joshi et al., 2005). Since the enzyme loading of the biosensor influences the upper and lower limits of detection, further experiments with tunable enzyme loadings can provide an inhibitor biosensor with even better detection limits. Moreover, both LOD and RSD of the nanostructured biosensors for all the four types of OPs are enough to satisfy current detection requirement with high precision (According to the pesticide residue standard of the European Union (EC 149-2008), the detectable pesticide concentration in imported fruits and vegetables should be lower than 20 μg/L for paraoxon, 50 μg/L for parathion, 100 μg/L for dichlorvos, and 200 μg/L for omethoate, respectively).

Fig. 2 further compares the inhibition efficiencies of four OPs in the range of 10^{-6} M and 10^{-7} M against the biosensors. One can see that, at the same concentration of 10^{-7} M, the percentages of inhibition are only 31 ± 4% and 27 ± 8% for dichlorvos and omethoate, respectively, which are considerably smaller than those of parathion (56 ± 9%) and paraoxon (73 ± 10%). Our experimental results are inconsistent with previous investigation that dichlorvos and paraoxon have higher inhibition capacity toward AChE. The contact angle measurements (Fig. S11) demonstrate that on top

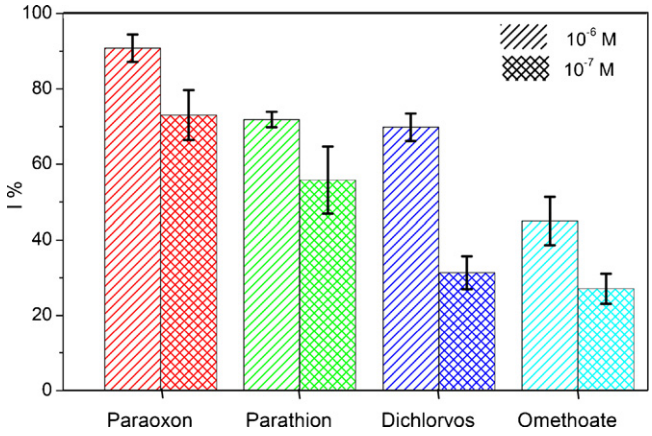

Fig. 2. Inhibition rate of 10^{-6} M and 10^{-7} M of different OPs toward $(PAH/CdTe)_8(PAH/PSS)_3(PAH/AChE)_3$ multilayers. (Each data point is an average of six measurements.).

of positively-charged PAH layer, the active center gorge of AChE mainly faces upward due to the reciprocal repulsion between the hydrophobic areas of AChE and hydrophilic PAH layers. Thus, the lower inhibition capacity of dichlorvos and omethoate is attributed to their more hydrophobic nature (see the detailed discussion in Part S5). Nevertheless, above results suggest that tuning the structures of the biosensors could allow manipulation of their selectivity toward different types of pesticides.

3.3. Determination of total OP content in vegetable and fruit samples

It is noted that many publications are devoted to determination of pesticides in clean distilled or deionized water, while the corresponding applications in vegetables and fruits are rare (Schulze et al., 2002). The unique advantages of the nanostructured biosensors, e.g. specific recognition with OPs and small influence of interferents, encourage us to apply them to determine total OP content in natural vegetable and fruit samples. Different from the conventional GC/MS method that needs the complex multiple-step sample pretreatments to avoid possible interference (Part S1 in Supporting Information), the preparation process of samples is much simpler in our method: The vegetable/fruit samples are chopped and extracted with 20 mL PBS, and then the biosensors are incubated with the extracted solution at 38 °C for 15 min before measurement. Table 1 depicts the correlation between the analysis of five vegetable and fruit samples by the proposed biosensor and GC/MS method. The amount of total OP content analyzed in most samples by the optical biosensor correlates well with that of GC/MS. The standard deviation of 6 proposed biosensors is less than 12%, and the recoveries of paraoxon added to apple samples are from 104.6% to 115% at the two concentration levels, indicating an acceptable accuracy for the total OP content in vegetable/fruit samples with the proposed biosensor. Furthermore, it is evident in Table 1 that the detection limits of the biosensors are lower than those of GC/MS methods, in which the OPs with concentration lower than 10 μg/mL cannot be detected out. Both fast sample preparation and low detection limits are very important for development of new sensing techniques with low cost and high-throughput screening.

4. Conclusion

In this communication we have demonstrated a highly-sensitive optical biosensor for detection of picomolar concentrations of paraoxon and parathion. The detection limits of the developed

biosensors are much lower than that of reported detection methods. With the developed biosensor we successfully detect OPs in the real samples of vegetables and fruits. To our knowledge this is the first report on employing the biosensors to detect OPs in the real samples of vegetables and fruits with satisfactory reproducibility and accuracy. The proposed method could also be used for visual monitoring of OPs (see the detailed discussion in Part S6). The possible disadvantages of this biosensor are: (1) the quenching of QD multilayers is not fully reversed and its fluorescence partly recovers (about 60% of the original fluorescence intensity is recovered by keeping it overnight under ambient condition), and the sensor is designed for one-time use. (2) The sensors show different response characteristics in presence of different types of OPs, so the measurement may be not accurate in the mixture of different OPs. Nevertheless, many advantages provide by the sensors, such as easy sample pretreatment, high sensitivity, and mass production with low cost, could facilitate future development of rapid, high-throughput screening of OP residues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.12.021.

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