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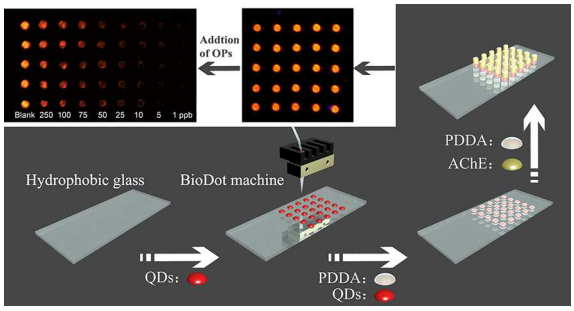

A fluorimetric assay for high-throughput screening of organophosphorous pesticides was developed based on the CdTe QDs/AChE microarrays via inkjet-assisted LbL printing techniques.

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Inkjet-assisted Layer-by-layer Printing of Quantum Dot/Enzyme Microarrays for Highly Sensitive Detection of Organophosphorous Pesticides

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†Electronic Supplementary Information (ESI) available: Synthesis of QDs, Figure S1, S2, S3, S4, S5, S6 and S7.

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Abstract

We present a facile fabrication of layer-by-layer (LbL) microarrays of quantum dots (QDs) and acetylcholinesterase enzyme (AChE). The resulting arrays had several unique properties, such as low cost, high integration and excellent flexibility and time–saving. The presence of organophosphorous pesticides (OPs) can inhibit the AChE activitity and thus changes the fluorescent intensity of QDs/AChE microscopic dot arrays. Therefore, the QDs/AChE microscopic dot arrays were used for the sensitive visual detection of OPs. Linear calibration for parathion and paraoxon was obtained in the range of 5-100 µg L^{-1} under the optimized conditions with the limit of detection (LOD) of 10 µg L^{-1}. The arrays have been successfully used for detection of OPs in fruits and water real samples. The new array was validated by comparison with conventional high performance liquid chromatography-mass spectrometry (HPLC-MS).

Keywords: Quantum Dot, Acetylcholinesterase, Biosensor, Organophosphorus pesticide, Layer-by-layer assembly, Inkjet-printing

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1. Introduction

Organophosphorous pesticides (OPs) are largely used in agriculture and fish hatcheries in the past several decades. However, their uncontrolled use has caused serious health and environmental problems throughout the world. Due to their high toxicity and potential to cause neurological disorders in humans [1-3], the development of rapid and reliable quantification of OPs in the food and environment has become imperative for the protection of public health and ecological systems. Although there are many conventional methods available for the detection of OPs, such as gas chromatography (GC), high performance liquid chromatography (HPLC), gas/liquid chromatography–mass spectrometry (GC/LC–MS) and capillary electrophoresis [4-5], which have high accuracy and sensitivity, they are not always convenient to employ such established analytical methods due to their high cost, long analysis duration and complicated operation procedures. Therefore, the development of a rapid, low cost, portable detection method is necessary to facilitate routine analysis with more convenience. Fortunately, biosensors based on acetylcholinesterase (AChE) or butyryl cholinesterase inhibition offer remarkable advantages over chromatographic methods, mainly in terms of simplicity, rapidly, reliability, low detection limits, and cost-effectiveness [6-13]. Thus it has become a simplified and portable alternative for OPs detection. In this type of biosensors, the enzyme inhibition-based colorimetric [14-15] or fluorimetric [16] screening methods for OPs determination have attracted increasing attention because of

its low cost, simplicity and practicality. Since the colour of the assays can be observed directly with the naked eyes or analyzed through a digital camera or typical DNA microarray scanners, colorimetric or fluorimetric screening methods does not require expensive or sophisticated instrumentation, and can be applied to field analysis and point-of-care diagnosis [17-19]. There are a number of enzyme inhibition-based colorimetric [14, 15, 20] or fluorimetric [16, 21, 22] assays available, however, these assays suffer from some limitations such as low sensitivity, the need for control of reaction conditions (pH and ionic strength) and utilization specific excitation wavelength etc.

To overcome these issues, we used semiconductor nanoparticles known as quantum dots (QDs) in place of organic dyes because QDs have unique optical properties including broad absorption with narrow photoluminescence spectra, high quantum yield, low photobleaching, resistance to chemical degradation, and are not sensitive to pH [23-24]. In this study, we report on the fabrication of large scale microarrays of CdTe QDs and cationic polyelectrolyte (poly(dimethyldiallyl ammonium chloride), PDDA) via facile inkjet-assisted layer-by-layer assembly (LbL) printing technique without a rinsing step [25-27]. The LbL microarrays of QDs/PDDA with relatively uniform surface morphology and red fluorescence were fabricated on hydrophobic surfaces. By further overprinting AChE/PDDA bilayers, the QDs/PDDA microarrays were developed to a robust fluorimetric assay for detection of OP residues. The fluorimetric assay is based on the reaction between the holes of CdTe QDs and

thiocholine (TCh) generated by the AChE-catalyzed hydrolysis of acetylthiocholine (ATCh), which resulted in the fluorescence quenching of QDs/PDDA LbL microarrays. In the absence of OPs, AChE catalyzes the hydrolysis of ATCh, yielding TCh, which then quench the fluorescence of QDs through dissociation of photogenerated electron-hole pairs in QDs [28-29]. However, OPs can inhibit the enzyme activity, prohibiting the production of TCh. By analyzing the difference in fluorescence signal in the absence or presence of OPs, we are able to determine the concentration of OPs rapidly and accurately.

2. Experimental section

2.1. Materials

$Cd(ClO_4)_2 \cdot 6H_2O$, 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^{-1}), acetylthiocholine chloride (ATCh), poly(dimethyldiallyl ammonium chloride) (PDDA, MW 478.9) and trimethoxy(octadecyl)silane (MW 387.93) were purchased from Sigma-Aldrich. The standard samples of paraoxon and parathion (100 μg mL^{-1} in acetone) were purchased from Beijing (WeiyekechuangKeji CO., LTD, China). MPA-capped CdTe QDs with PL peaks at 602 nm were synthesized according to our previous report (Figure S1 of the Supporting Information) [30]. Other chemicals of analytical grade were obtained from commercial sources and used as received.

All solutions were prepared with ultrapure water from a Milli-Q water purification system (Billerica, MA).

2.2. Methods

The UV-vis absorption spectra were measured on a U-4100 UV-vis-NIR spectrometer (Hitachi, Japan), while the fluorescence spectra were recorded with a Fluoromax-4 fluorescence spectrophotometer (Horiba JobinYvon, Japan). An AD1510 (BioDot, America) printer with 15 μL printing cartridges was used for inkjet printing of all materials. A digital camera EOS 550D (Canon, Japan) was used to record images of the sensing arrays.

2.3. Fabrication of inkjet-assisted LbL microarrays of QDs/AChE

Inkjet-assisted LbL printing was conducted on different hydrophilic and hydrophobic substrates including glass, filter paper and polydimethylsiloxane (PDMS). The glass substrates was treated in hydrophilic or hydrophobic nature according to the following procedures: (1) The hydrophilic glass can be obtained by cleaning with Piranha solution (3:1 concentrated sulfuric acid and hydrogen peroxide mixture) according to the normal procedure used in our laboratory (Caution! Piranha solution is highly corrosive and toxic) [31]. (2) The hydrophobic glass was then obtained by functionalizing the cleaned glass with octadecyltrichlorosilane to yield an octadecyl-functionalized surface. Filter paper was treated with organic solvents to remove the intrinsic fluorescent species. For the preparation of hydrophobic substrates, a 90 wt.% PDMS solution was prepared by dissolving the silicone elastomer in toluene and

shaking for 12 hours and spun cast onto the silicon substrate. The coated films were subsequently cured in a vacuum oven at 80 ^{o}C for 12 hours and carefully released from the silicon substrate.

As illustrated in Figure S2, the inkjet printing of the QDs/PDDA multilayer arrays was performed by alternatively printing 1 mg mL^{-1} positively charged PDDA solutions (pH 7.5, 0.5 M NaCl) and negatively-charged CdTe QD solution at the same position until the desired bilayer number was obtained. Subsequently, 1 mg mL^{-1} PDDA (pH 8.0, without NaCl) and 0.5 mg mL^{-1} negatively-charged AChE in PBS solution (20 mM) were alternatively printed at the same position in order to form three bilayers of AChE/PDDA on top of the QDs/PDDA multilayer microarray.

2.4. OP determination

The multilayer with a configuration of $(QDs/PDDA)_3(AChE/PDDA)_3$ was used for the detection of OPs. The procedure for OPs determination was as follows: (1) Different concentrations of OPs was first overprinted on the top of $(QDs/PDDA)_3(AChE/PDDA)_3$ microarrays and incubated at 38 ^{o}C for 15 min; (2) 20 mM ATCh was dropped onto the microarrays, and the fluorescence signal was then recorded after 5 min. The inhibition of OPs was calculated as following equation (1):

$$Inhibiton(\%) = (F_{with} - F_{without}) / (F_0 - F_{without}) \times 100 \quad (1)$$

Where F_0, $F_{without}$ and F_{with} were the fluorescence intensity of $(QDs/PDDA)_3(AChE/PDDA)_3$ microarrays before detection, in the absence and

presence of OPs inhibition, respectively.

2.5. OPs detection in real water/fruit samples

The procedure for OPs determination in real samples was as follows: (1) The fruit sample was first chopped and ultrasonically extracted with 20 mL ddH_2O for 15 min; (2) The extracted solution was then printed at the same position of $(QDs/PDDA)_3(AChE/PDDA)_3$ microarrays; (3) 15 min later, 20 mM ATCh was dropped onto the microarrays, and the fluorescence signal was then recorded after 5 min. The results were compared with the calibration curve, and the OPs concentration could be obtained.

3. Results and Discussion

3.1. Inkjet printing of QDs/AChE LbL microarrays

The fabrication of QDs/AChE LbL microarrays necessarily satisfies three basic requirements: 1) The substrate materials should not have any intrinsic fluorescence under UV lamp; 2) The QDs/AChE multilayers can firmly absorb on the substrate through inkjet-printing and should not disassociate upon rinsing or dipping into aqueous solution; 3) The substrate materials have good biocompatibility. Based on above considerations, we choose hydrophilic substrates (glass and filter paper) and two hydrophobic substrates (octadecyltrichlorosilane-functionalized glass and PDMS) for inkjet printing of QDs/AChE LbL microarrays. In the case of filter papers, it demonstrates strong background fluorescence under UV lamp, which may seriously interfere the

colorimetric observations, thus the filter papers were ultrasonically treated in acetone solvents for 30 min to remove the fluorescent dyes before printing. PDMS and glass substrates were used as prepared.

First, the desired bilayer number of QDs/PDDA microarrays was prepared by alternatively printing PDDA and MPA-capped CdTe QDs on different substrates. In order to keep the microarray size reasonably small and the use of sensing reagents as low as possible, a spot diameter of 0.8 mm with a spacing of 1.2 mm between spots was selected. Fluorescence optical microscopy characterization indicated that very clear and periodic microarrays are printed on glass, filter paper and PDMS substrates, as shown in Figure 1. Under the observation of fluorescence microscope, we found that the dried microarrays showed a strong red fluorescence under ultraviolet excitation and the brightness of the QDs/PDDA LbL microarrays is easily tunable by stacking more layers with inkjet printing process (Figure S3). However, the nature of different substrate surfaces shows obvious influence on the morphology of the printed QDs/PDDA LbL microdots: (1) when printing on hydrophilic glass, we observe coffee-ring structure where the height of the edge of the dot is higher than the center of the structure (Figure 1a). The filter paper has a larger number of micrometer-sized pores, therefore obvious diffusion from the printed spots on the filter paper was observed and thus result in smaller inter-drop distance (Figure 1b). However, on the hydrophobic octadecyltrichlorosilane-functionalized glass and PDMS substrates, the edges

of dot microarray and their fluorescent brightness are quite uniform (Figure 1c and 1d). (2) The average diameter of the QDs/PDDA LbL microdots on filter paper and hydrophilic glass is around 1.23±0.17 mm and 1.15±0.11 mm due to the rapid spreading behavior of the water solution on the hydrophilic substrates, larger than that on octadecyltrichlorosilane-functionalized glass (0.71±0.03 mm) and PDMS (0.86±0.06 mm) substrates, respectively. (3) Moreover, it can be found that the increase in overprints did not almost affect the overall diameter of the QDs/PDDA LbL microdots on PDMS and octadecyltrichlorosilane-functionalized glass (Figure S3a), while in the case of hydrophilic glass and filter paper, some degree of increase occurred. These results indicate that the hydrophobic surfaces prevent the spreading of the liquid droplets and allow a higher resolution printing process. However, the printed QDs/PDDA LbL microdots on PDMS automatically exfoliate from PDMS substrates upon contacting aqueous solution, which is due to weak interaction of QDs/PDDA multilayer with PDMS substrate. Therefore, the octadecyltrichlorosilane-functionalized glass is the best for supporting for QDs/PDDA LbL microarrays.

In the next stage, the AChE/PDDA bilayers were printed onto $(QDs/PDDA)_3$ LbL microdots. As shown in Figure 2b, the average diameter of the printed dots is 0.90±0.05 mm and remains virtually unchanged even as the number of the AChE/PDDA bilayers exceeds up to 4. Moreover, compared to $(QDs/PDDA)_3$ LbL microarrays (Fig. 2a), the deposition of the AChE/PDDA

bilayers results in a minor decrease in the brightness of $(QDs/PDDA)_3$ LbL microdots (Figure 2d), presumably because the thicker enzyme film may decrease the luminescence efficiency.

To determine if the printing process retains the enzyme activity, we took a wafer printed with 5×5 $(QDs/PDDA)_3(AChE/PDDA)_n$ microarrays and exposed it to 20 mM PBS containing 20 mM ATCh for 5 min to perform AChE activity assay. After washing with 20 mM PBS solution, the substrate was investigated by fluorescence microscopy. The microdots still kept uniform size and shape and adhered well to the substrates and no obvious exfoliation or cracking could be observed, confirming the stability of the printed microdots upon the enzyme reaction. Further, the fluorescence intensity of $(QDs/PDDA)_3(AChE/PDDA)_n$ microdot arrays before and after performing AChE activity assay was analyzed by using Image J. As shown in Figure 2d, the fluorescence intensity of $(QDs/PDDA)_3$ microdots has negligible reduction, while the fluorescence intensity of the $(QDs/PDDA)_3(AChE/PDDA)_n$ (n=1-4) microdots significantly decreased. The decrease in the fluorescent intensity is attributed to the AChE-catalyzed hydrolysis of ATCh, which produces acetate and TCh. TCh could act as electron donor to the holes of QDs and thus leads to the quenching of the fluorescence of QDs (Figure S3b and S3c) [28-29]. Therefore, we can conclude that AChE was already incorporated in the microdots and the inkjet printing-assisted LbL technique could support enzyme activity. Additionally, the decrease degree of the fluorescence intensity of the

$(QDs/PDDA)_3(AChE/PDDA)_n$ increases with the increasing of the AChE/PDDA bilayers, and the $(QDs/PDDA)_3(AChE/PDDA)_3$ microdots had the highest ratio of the fluorescent intensity before and after the addition of 20 mM ATCh, thus it was chosen as the optimal microarrays. Michaelis-Menten plots for determination of K_M and V_{max} values of AChE-catalyzed hydrolysis of ATCh were further obtained for $(QDs/PDDA)_3(AChE/PDDA)_3$, as shown in Figure 3. The apparent Michaelis–Menten constant K_M and V_{max} values are 0.44±0.08 mM and 2.38±0.26 mM min^{-1}, respectively. The apparent K_M is slightly higher than that of enzyme in solution, 0.14 mM, which may be due to the internal diffusion limitation [32-33].

Additionally, AChE activity of the microarrays can be maintained during the storage. The $(QDs/PDDA)_3(AChE/PDDA)_3$ LbL microarrays were stored at 4 ^oC, -20 ^oC and room temperature, respectively, and the enzyme activity was then tested after different period. As shown in Fig.S5, arrays stored at 4 ^oC, room temperature and -20 ^oC showed 75%, 48% and 94% activity after a week, respectively. After one-month storage, the microarrays stored at -20 ^oC conditions still kept above 92% activity. This result indicates that the multilayer structure can maintain AChE stability during storage.

3.2. Detection of OPs

The detection mechanism of OPs is based on the irreversible inhibition of the activity of AChE. In the absence of OPs, AChE catalyzes the hydrolysis of

ATCh, yielding TCh which then reacts with the holes of CdTe QDs to quench the fluorescence of QDs. However, in the presence of OPs the enzyme activity was inhibited since they covalently block the active site of AChE. This irreversible inactivation prohibits the production of TCh and thus leads to an increase in fluorescence emission. The difference in fluorescence signal can be correlated to the concentration of OPs. In order to obtain good signal-to-background levels, the first experiments were carried out to determine the effect of substrate concentration, substrate volume, pH, reaction time and reaction temperature on the microarrays (see Supporting Information, Figures S6). Furthermore, we investigated the effect of incubation time of $(QDs/PDDA)_3(AChE/PDDA)_3$ LbL microarrays with OPs on the inhibition rate. As shown in Fig. S7, a high inhibition rate (ca. 75%) can be obtained at 15 min, while further extending the incubation time only causes slight increase. Therefore, considering the detection time, we chose 15 min as incubation time. The optimal conditions were determined as follows: 15 min incubation time, 20 mM ATCh, 100 nL, pH 8.0, 5 min reaction time and 38^{o}C. Therefore, the determination of OPs on $(QDs/PDDA)_3(AChE/PDDA)_3$ LbL microarrays was performed in the following procedures: the microarrays of 90 $(QDs/PDDA)_3(AChE/PDDA)_3$ microarrays in a 18×5 spot pattern were first printed onto the octadecyltrichlorosilane-functionalized glass and then overprinted with 100 nL different concentration of OPs. After incubation for 15 min, the spots were then overprinted with a 20 mM phosphate buffer solution (pH 8.0) containing 20

mM ATCh. The microarrays were imaged and analyzed after 5 min reaction time.

With the above procedures, we then tested the microarrays for the quantitation of two typical OP compounds: parathion and paraoxon. Fig. 4a and Fig. 5a shows the fluorescence image of $(QDs/PDDA)_3(AChE/PDDA)_3$ LbL microarrays with parathion and paraoxon concentrations increasing from 1 to 250 μg L^{-1} (right to left). It is clear that the red fluorescence emission increases gradually as the concentration of parathion increases. The inhibition of OPs was calculated according to the following equation (2):

$$Inhibiton(\%) = (F_{with} - F_{without}) / (F_0 - F_{without}) \times 100 \quad (2)$$

Where F_0, $F_{without}$ and F_{with} were the fluorescence intensity of $(QDs/PDDA)_3(AChE/PDDA)_3$ microarrays before detection, in the absence and presence of OPs inhibition, respectively. As shown in Figure 4b and 5b, the inhibition rate is proportional to the concentration of parathion and paraoxon in the range of 5 μg L^{-1} to 100 μg L^{-1}. The regression equation is I(%)=0.436[parathion]+7.640 (R^2=0.994, n=5) for parathion and I(%)=0.486[parathion]+8.393 (R^2=0.992, n=5) for paraoxon, respectively. The detection limit is set to be the concentration of OP that causes 10% inhibition [34]. It should be noted that the minimum concentration examined in our experiment was as low as 10 μg L^{-1} which is much lower than levels specified by standard food safety tests and other colorimetric detection methods [15].

The reproducibility of the microarray-based assay was evaluated by

assaying same concentration of paraoxon with six batches in an 18×5 spot pattern printed on different days by the same procedures independently. The relative standard deviation (RSD) among six detection of the same concentration of OPs is found to be less than 10%, indicating a good reproducibility.

3.3. Real sample testing and comparison with traditional methods

To evaluate this method for the detection of pesticide residues in real samples, apple and tap water samples were sprayed with different concentrations of parathion and paraoxon. The OPs extracted from the apple and tap water samples was quantitated by both the QDs/AChE microarrays and spectroscopic method. As shown in Table 1, the quantification of the three concentrations of OPs correlates well with the spectroscopic method. The recoveries of the spiked OPs are from 92.0% to 117.0%.

4. Conclusion

In this study, we present inkjet-assisted LbL printing of large-scale arrays of CdTe QDs and cationic polyelectrolytes. Microscopic dots with relatively uniform surface morphology and fluorescent brightness can be formed on the hydrophobic substrates. By overprinting AChE/PDDA bilayers on top of these fluorescent LbL dots, a novel fluorimetric AChE activity assay has been

developed. We also demonstrated that the microarray-based assay could be used quantitatively and rapidly for high-throughput screening of OP residues compared to the reported methods. The red fluorescence emission increases gradually as the concentration of parathion increases. A detection limit of 10 μg L^{-1} was achieved, much lower than levels specified by standard food safety tests and other colorimetric detection methods. The low cost, short processing time, sufficient sensitivity and ease of use makes it for a facile platform for on-site screening.

Supporting Information

Supporting Information is available online from or from the author.

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Table 1 Recovery ratios of OPs in real samples

Sample	Added (μgL^{-1})	The proposed biosensor			HPLC	
		Inhibition (I%)	Measured value (μgL^{-1})	Recovery (%)	Measured value ($\mu g\ L^{-1}$)	Recovery (%)
Apple		1.6%	N/A[a]	N/A[a]	N/A[b]	
Parathion	10	12.1±1.1%	10.2±2.5	102.0%	11.57	115.7%
	25	19.3±3.1%	26.7±7.1	106.8%	26.27	105.1%
	75	39.8±2.4%	73.6±5.5	98.1%	77.31	103.1%
Paraoxon	10	12.7±1.3%	11.7±3.0	117.0%	12.38	123.8%
	25	18.8±1.9%	25.5±4.4	102.0%	27.17	108.7%
	75	40.0±2.1%	74.1±4.7	98.8%	73.57	98.1%
Water		2.3%	N/A[a]	N/A[a]	N/A[b]	
Parathion	10	12.9±2.0%	9.2±3.9	92.0%	12.20	122.0%
	25	19.7±1.1%	23.3±2.2	93.2%	23.45	93.8%
	75	45.7±2.1%	76.8±4.3	102.4%	70.72	94.3%
Paraoxon	10	13.4±2.2%	10.2±4.6	102.0%	11.53	115.3%
	25	21.1±1.6%	26.1±3.3	104.4%	22.61	90.4%
	75	46.0±0.5%	77.4±1.0	103.2%	72.23	96.3%

[a] Since the inhibition rate is lower than 10%, we assume that OP residue is not detected.

[b] Total OP content in natural vegetable and fruit samples is lower than the limit of detection by HPLC-MS, so the OPs cannot be detected out.

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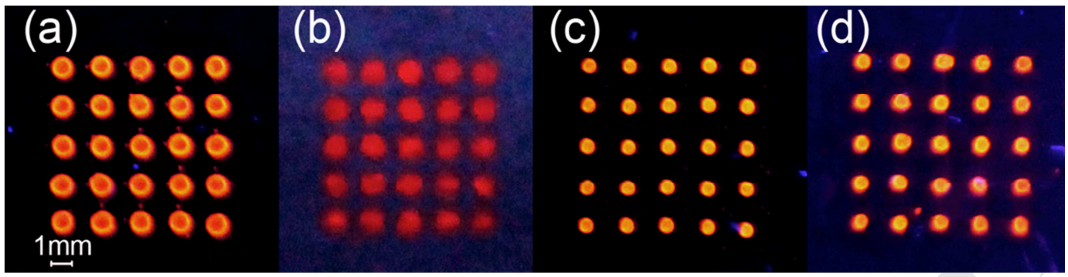

Figure 1. The fluorescence images of three QDs/PDDA bilayers printed on glass slide (a), filter paper (b), octadecyltrichlorosilane-functionalized glass (c) and PDMS (d).

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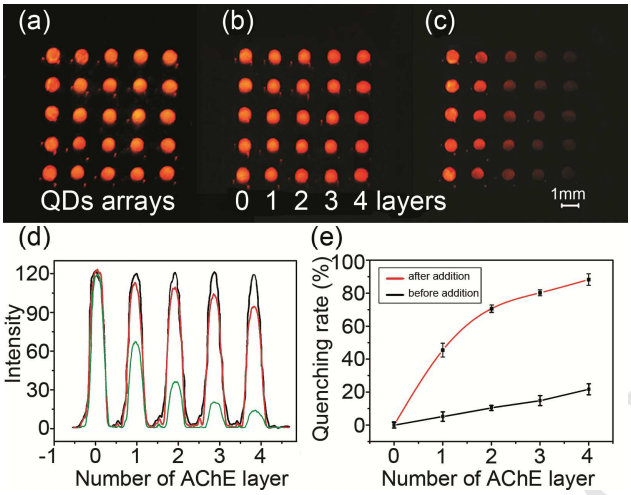

Figure 2. The fluorescence images of $(QDs/PDDA)_3$ LbL microarrays (a), $(QDs/PDDA)_3(AChE/PDDA)_n$ LbL microarrays (b), after exposure to 20 mM ATCh (c). (d) Comparison of the fluorescent intensity of $(QDs/PDDA)_3$(black curve), $(QDs/PDDA)_3(AChE/PDDA)_n$ (red curve), and $(QDs/PDDA)_3(AChE/\ PDDA)_n$ after exposure to ATCh (green curve). (e) The ratio of the fluorescent intensity of $(QDs/PDDA)_3(AChE/PDDA)_n$ LbL microarrays before and after the addition of 20 mM ATCh.

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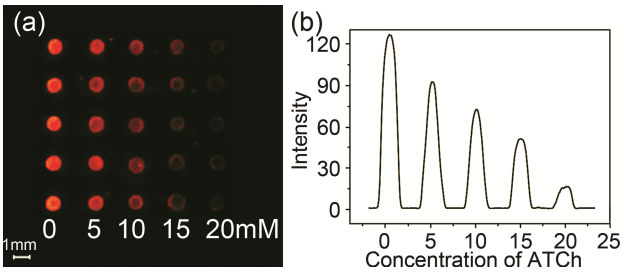

Figure 3. (a) The fluorescence images of $(QDs/PDDA)_3(AChE/PDDA)_3$ LbL microarrays after exposure to different concentration of ATCh. (b) The dependence of fluorescent intensity on the concentration of ATCh.

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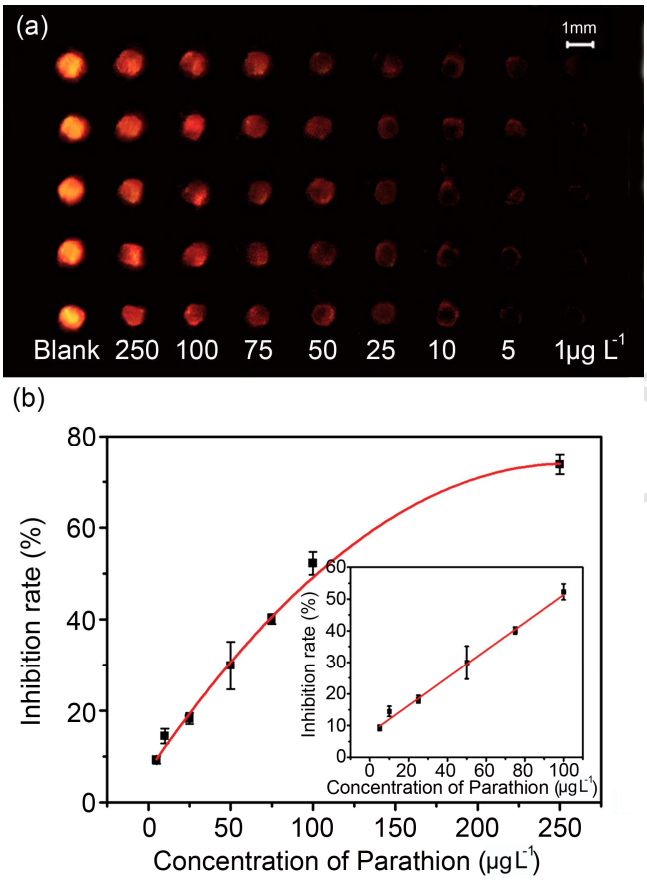

Figure 4. (a) The fluorescence images of $(QDs/PDDA)_3(AChE/PDDA)_3$ LbL microarrays after exposure to different concentration of parathion. (b) Relationship between the inhibition rate and the concentration of parathion.

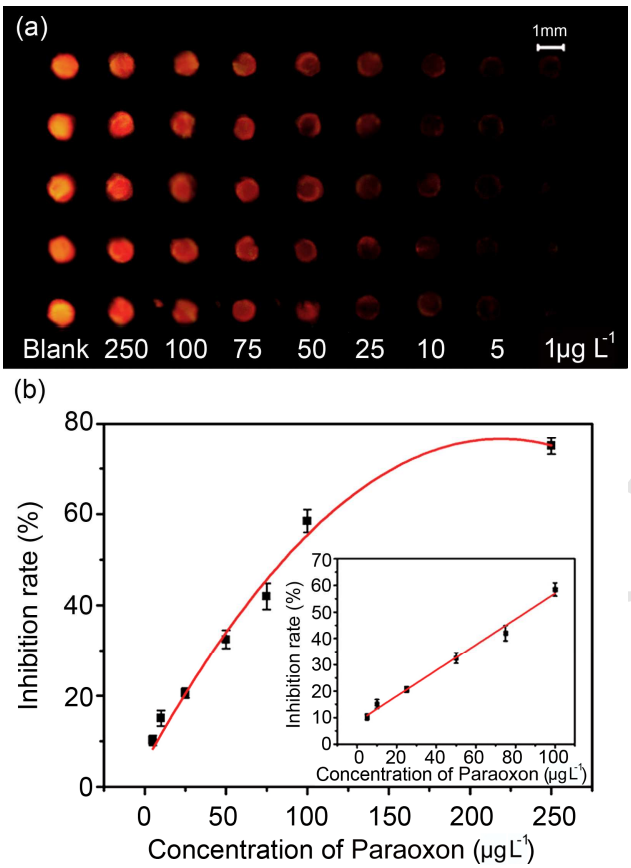

Figure 5. (a) The fluorescence images of $(QDs/PDDA)_3(AChE/PDDA)_3$ LbL dot microarrays after exposure to different concentration of paraoxon. (b) Relationship between the inhibition rate and the concentration of paraoxon.

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Inkjet-assisted Layer-by-layer Printing of Quantum Dot/Enzyme Microarrays for Highly Sensitive Detection of Organophosphorous Pesticides

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†Electronic Supplementary Information (ESI) available: Synthesis of QDs, Figure S1, S2, S3, S4, S5, S6 and S7.

Highlights

1. The large scale microarrays of CdTe QDs and acetylcholinesterase enzyme (AChE) with relatively uniform surface morphology and fluorescent brightness were fabricated by facile inkjet-assisted layer-by-layer assembly (LbL) printing technique.
2. The QDs/AChE microscopic dot arrays could be used quantitatively and rapidly for the sensitively visual detection of OPs.
3. A detection limit of 10 μg L^{-1} was achieved, which is much lower than levels specified by standard food safety tests and other colorimetric detection methods.
4. The low cost, short processing time, sufficient sensitivity, good stability and ease of use make it for a facile platform for on-site screening.

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