Layer-by-Layer Films of Chitosan, Organophosphorus Hydrolase and Thioglycolic Acid-Capped CdSe Quantum Dots for the Detection of Paraoxon

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Layer-by-Layer Biosensor Assembly Incorporating Functionalized Quantum Dots

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Layer-by-layer (LbL) assembly has been utilized to fabricate an ultrathin film of polyelectrolytes. The architecture was composed of chitosan and organophosphorus hydrolase polycations along with thioglycolic acid-capped CdSe quantum dots (QDs) as the polyanion. The topography of the films was studied using epifluorescence microscopy imaging. The photoluminescence property of the functionalized QDs improved when sandwiched between the polycation layers. The enhanced optical property of QDs allowed easy monitoring of LbL growth and detection of paraoxon with high sensitivity. The presence of organophosphorus compounds was confirmed through UV−vis and emission spectroscopies.

1. Introduction

Thin film fabrication through immobilization of organic polymers on solid substrates has widespread applications in areas such as biosensing assemblies, biomedical devices, and light-emitting diodes.[1,2] There are many methods of thin-film development such as spin coating, Langmuir−Blodgett film deposition,[3] covalent attachment of polymers,[4,5] and layer-by-layer (LbL) assembly.[6,7]

Semiconductor nanocrystals often referred to as quantum dots (QDs) have received tremendous attention within recent years owing to their special physical and chemical properties.[8,9] QDs can be utilized as an emitting material in the preparation of ultrathin films.[10,11] The color emission can be tuned by size variation without altering their chemical properties.[12,13] High fluorescence quantum yields are achieved by introducing defects on the surface of the QDs.[14,15] The above-mentioned features of QDs can be employed to develop chemo-[16] or biosensing[17] assembly systems.

LbL assembly is used to fabricate films with molecular order and stability. One major advantage of this technique is its industrial application in the field of biosensing assemblies. The layer-by-layer deposition method is based on alternating adsorption of oppositely charged macromolecules such as polymers and biomacromolecules.[18−20] This leads to the fabrication of films 5−500 nm thick that possess a high strength.[21] Electrostatic forces, covalent bonding, and, to a lesser extent, hydrogen bonding and hydrophobic interactions hold the assembly of these ultrathin films together. LbL assembly leads to a wide range of combinations of charged species, which can be used for self-assembled ultrathin films[22−24] each having different functionalities.

Organophosphorus (OP) compounds are used quite extensively in pesticides and insecticides, but they are an environmental concern because of their structural similarity to nerve agents such as sarin and soman.[25] These compounds inhibit the enzyme acetylcholinesterase, which is responsible for the transmission of nerve impulses across synaptic junctions.[26] As a result of the acute toxicity of these OP neurotoxins, environmental monitoring of the presence of these compounds in food and groundwater is important to keep these compounds below the harmful level for humans and animals.[27] One such OP compound

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is paraoxon, which is poisonous if ingested, inhaled, or absorbed through the skin.[28]

Organophosphorus hydrolase (OPH) is an enzyme that catalyzes the hydrolysis of a large variety of organophosphorus compounds by producing harmless products such as *p*-nitrophenol (PNP) and diethyl phosphate.[29] OPH is very specific for the hydrolytic reaction in the P−O, P−S, P−F, and P−CN bonds in organophosphorus neurotoxins.[30] There are significant advantages associated with the utilization of enzyme-based sensing assemblies, and some of these advantages are the following: rapid response times, sensitivity, and reactivation of enzyme for continuous monitoring. Altering the pH of the enzyme solutions allows the enzyme to be charged. This facilitates its use as a polyelectrolyte in a LbL film.

This work presents the versatility of the LbL system where the polyelectrolytes each have a different functionality in the system. Chitosan (CS) is a polyglucosamine which has protonated amino groups at low pHs. This imparts polycation character to this structural polysaccharide. CS adsorbs strongly onto negatively charged surfaces, and the adsorbed CS layer adopts a flat conformation that provides a homogeneous film on which the thioglycolic acid (TGA)-capped CdSe QDs can be adsorbed. Five bilayers of CS and QDs were prepared to produce a stable supramolecular film and to ensure surface charge uniformity. The bilayer growth was monitored using UV−vis and emission spectroscopies. Our methodology shows that sandwiching TGA-capped CdSe QDs between polycation layers using the alternate LbL technique increases the photoluminescent properties of the QDs when compared to the ones in solution. This key characteristic allows QDs to be incorporated into a biosensing assembly system.

Multilayers of OPH/TGA-capped CdSe QDs were then integrated into the LbL system, so that on exposure to aqueous paraoxon solution, the hydrolytic product, *p*-nitrophenol (PNP), would be released and identified using UV−vis spectroscopy. Paraoxon was also detected using the change in the photoluminescent intensity of the QDs. This system shows increased sensitivity when compared to that of a similar sensing assembly[7] that was previously explored. Incorporating modified QDs into LbL films to be used as sensors is very promising.

2. Materials and Methods

CS was obtained from Biopolymer Engineering (Eagan, MN). A solution (1 mg/mL), pH 4.0, was prepared. OPH (85−90%) (E.C.3.1.8.1) was isolated, extracted, and purified by the U.S. Army Laboratory (Edgewood Chemical and Biological Center, MD). A stock solution of OPH (1.8 mg/mL) was prepared in 100 mM bis-tris-propane (BTP), pH = 7.3, containing 10 μM Co^{2+}. The stock solution was frozen at −4 °C. The stock solution was freshly thawed and diluted to a concentration of 0.18 mg/mL before use. The ionic strength of the above solution was adjusted with 0.5 M NaCl. Addition of NaCl aided in the adsorption of the polymer due to the relaxation of electrostatic repulsion in the enzyme.[31] Tetradecylphosphonic acid was acquired from Alfa Aesar (Ward Hill, MA). All other chemicals and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity available. The water used was purified with a Modulab 2020 water purification system (Continental Water Systems Corporation, San Antonio, TX). The pure water has a specific resistance of 18 MΩ·cm and a surface tension of 72.6 $mN \cdot m^{-1}$ at 20 ± 1 °C.

The photoluminescence spectra were recorded using a Spex Fluorolog 1680 spectrophotometer. A Perkin-Elmer UV/vis/NIR spectrometer Lamda 900, using a quartz cuvette of 1 cm path length, measured the UV−vis absorption spectra of solutions. An epifluorescence microscope (Olympus IX-FLA) was used for acquiring the epifluorescence micrographs. A thermoelectrically cooled Optronics Magnafire CCD camera detected the emission of the adsorbed layers.

2.1. Synthesis of TGA-Capped CdSe QDs. Trioctylphosphine oxide (TOPO)-capped CdSe QDs were prepared following a synthethic protocol that utilized CdO as precursor:[32] 0.0514 g of CdO, 0.2232 g of *n*-tetradecylphosphonic acid (TDPA), and 3.7768 g of TOPO were loaded into a 25 mL flask with a condenser assembly attached to it. The reaction mixture was heated to 310 °C under Ar flow and agitation. When a clear and colorless solution was obtained, the temperature was lowered to 270 °C followed by injection of 0.04108 g of selenium powder dissolved in 2 g of trioctylphosphine (TOP). Growth of QDs occurred at 250 °C until the desired particle size was reached. Immediately, the solution was cooled to 60 °C and purified by three cycles of precipitation/dispersion with anhydrous methanol/chloroform (7:1, v/v).

Finally, TOPO ligands were exchanged with thioglycolic acid (TGA) as follows: 150 μL of the acid was added to 2 mL of a QDs in chloroform solution and stirred for 2 h. Precipitate was collected and suspended in water with the help of concentrated NaOH. Purification was accomplished by three cycles of precipitation with acetone, followed by redissolving in water, followed by filtration. This procedure served to make the QDs water-soluble, since LbL assembly uses an aqueous medium.

The size (diameter) range determined by HRTEM[33] was 3.4 ± 0.5 nm. The λ_{abs} and λ_{em} were 560 and 630 nm, respectively.

2.2. Preparation of the Quartz Slide. The quartz slides were cleaned in a chromic solution to remove any impurities from the surface. This was followed by sonicating in pure water for 30 min. The slide was then made hydrophilic using the RCA method.[34]

2.3. Immobilization of Polycations/TGA-Capped CdSe QDs LbL Film. The charged substrate was alternately immersed in aqueous solutions of oppositely charged CS (positive) and TGA-capped CdSe (negative) QDs for 10 min. Each immersion was followed by washing with pure water. This procedure is shown in Figure 1. For each cycle, a bilayer film of CS/QDs was formed. The slide was dried with a cool stream of air, and the UV−vis and photoluminescence spectra of the growing layers were recorded in air after each assembly cycle. This cycle procedure was repeated five times until a stable film was obtained. On top of this stable bilayer system, two bilayers of OPH/TGA-capped CdSe QDs were deposited by alternately immersing in aqueous solutions of OPH and QDs each for 10 min. The OPH solution in PBS buffer had a pH of 7.3. The isoelectric point of OPH is 7.6, so at pH 7.3 the amino group of the enzyme was positively charged. On top of the OPH/TGA-capped CdSe QDs bilayers one last layer of OPH was adsorbed. This multilayer system of CS, TGA-capped CdSe QDs, and OPH from now on is referred to as the *sensing assembly* and was used for the detection of paraoxon.

3. Results and Discussion

3.1. UV−Vis Spectroscopic Studies of the LbL Adsorbed Films. The UV−vis spectrum of TGA-capped CdSe QDs in solution was recorded, and the well-defined λ_{max} at 560 nm indicated a highly monodisperse particle size (Figure 2, solid line). This λ_{max} was utilized to monitor the efficiency of the LbL bilayer growth. From the results

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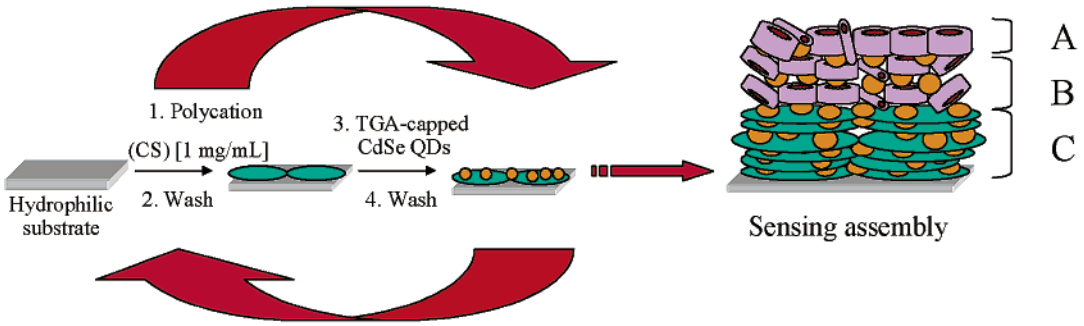

Figure 1. Sensing assembly: (A) top layer of OPH; (B) two bilayers of OPH/TGA-capped CdSe QDs; (C) 5 bilayers of CS/TGA-capped CdSe QDs.

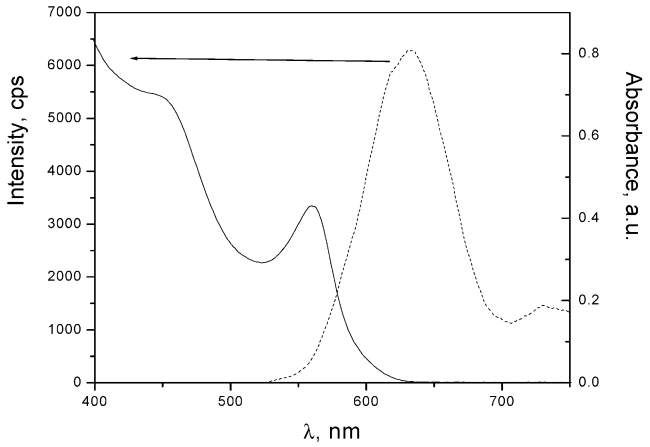

Figure 2. UV−vis (solid line) and luminescence spectra (dotted line) of TGA-capped CdSe QDs (3.4 nm) in solution.

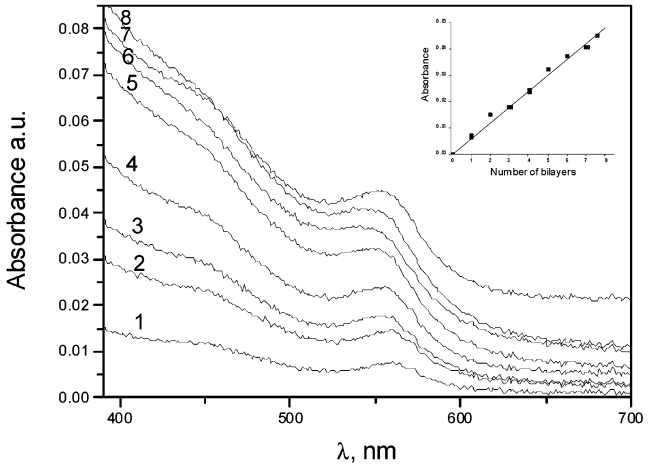

Figure 3. UV−vis spectra of growing CS/TGA-capped CdSe QD multilayers (1−5) followed by two bilayers of OPH/TGA-capped CdSe QDs (6, 7) and a top layer of OPH (8). The inset shows a plot of the λ_{max} versus the number of layers.

a continuous growth of the bilayer system (Figure 3) is observed. The absorbance at λ_{max} of 560 nm increased linearly with the adsorption of subsequent layers (inset). This suggests that equal amounts of QDs were adsorbed after each deposition cycle, which resulted in a buildup of a homogeneous film, as imaged using an epifluorescence microscope (shown in Figure 8).

UV−vis spectroscopy was used as an analytical tool to detect the presence of PNP produced by the OPH catalyzed hydrolysis. The *sensing assembly* was exposed to a paraoxon solution for different time intervals. This resulted in the OPH hydrolytic catalysis of paraoxon to

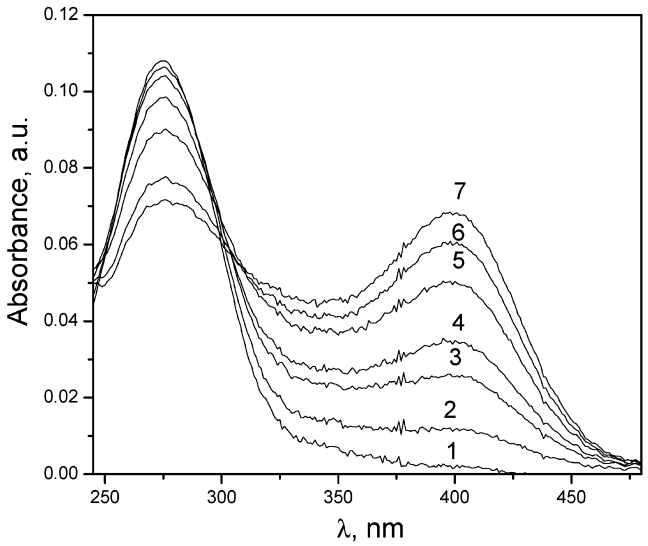

Figure 4. UV−vis spectra of the hydrolysis product PNP at different time intervals for the detection of a paraoxon solution: (1) paraoxon solution (1×10^{-7} M); (2−7) after the following incubation times of the substrate in paraoxon solution: (2) 30 s; (3−7) 1, 2, 5, 10, and 15 min.

PNP. Figure 4 shows that, at 0 min in the absence of the *sensing assembly*, the paraoxon solution had a λ_{max} at 275 nm. A new λ_{max} at 400 nm emerged after exposure of the *sensing assembly* to paraoxon solution for a period of time. This band is characteristic for *p*-nitrophenol. Figure 4 shows that the λ_{max} at 275 nm decreases with a corresponding increase in the λ_{max} at 400 nm as the exposure time is increased. The isobestic point at 303 nm at the beginning of the hydrolysis (Figure 4, curves 1−5), confirms that there are two states present in the system, that is, the transition from the paraoxon to the hydrolyzed product. Curves 6 and 7 do not pass through this isobestic point. As the concentration of the product increases, the absorption spectra show the predominance of the product. The shift of the isobestic point toward that of the product clearly indicates this. The rapid activity of the enzyme system is shown by the presence of the absorption band for PNP within as little as 30 s. UV−vis spectroscopy shows that paraoxon is successfully degraded by the immobilized OPH, releasing PNP.

Figure 5 shows additional analytical results. The *sensing assembly* was exposed to different concentrations of paraoxon solution for an incubation time of 10 min. A linear response to paraoxon solution between 1×10^{-8} and 1×10^{-5} M is shown in Figure 5. Within this range, PNP can be quantitatively estimated using the Beer−Lambert plot. Concentrations above and below this range were also detected* but showed a nonlinear correlation.

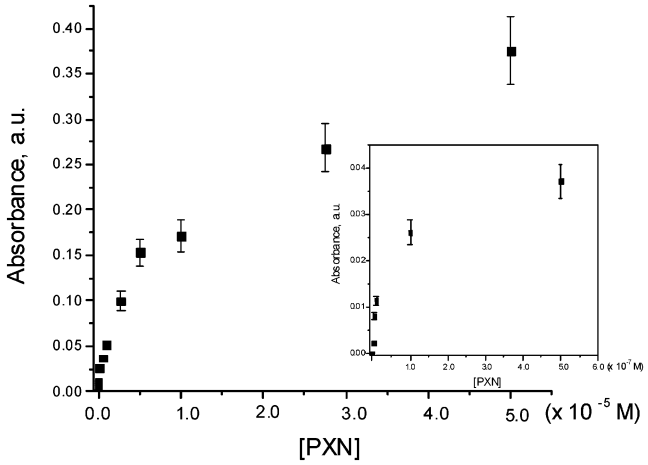

Figure 5. Absorbance maxima at 400 nm for different concentrations of paraoxon solution exposed to the *sensing assembly* for 10 min. The inset shows a plot of low concentrations of paraoxon vs the absorbance maxima.

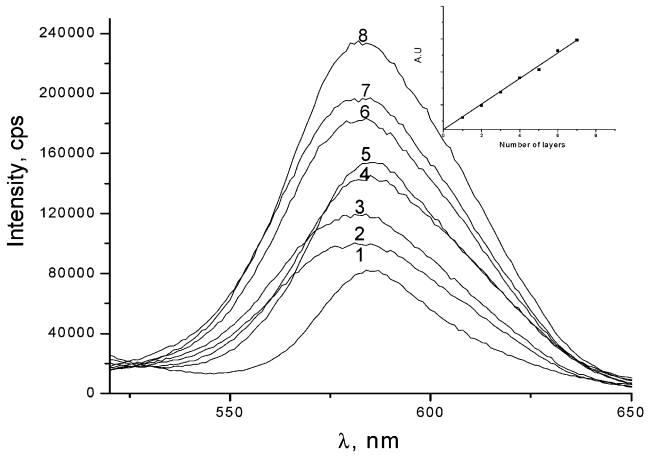

Figure 6. Photoluminescence spectroscopy of CS/TGA-capped CdSe QD multilayers (1−5) and two bilayers of OPH/TGA-capped CdSe QDs (6, 7) and a top layer of OPH (8).

(The limit of detection is the smallest concentration of paraoxon which can be detected positively.) This *sensing assembly* is very sensitive because concentrations as low as 10^{-9} M paraoxon were detected.

3.2. Photoluminescence Spectroscopic Studies for the Detection of Paraoxon. TGA-capped CdSe QDs demonstrated broad excitation spectra and a narrow emission band at 632 nm. The maximum emission intensity was observed when QDs were excited with 467 nm radiation (Figure 2). A narrow emission band reconfirms the high degree of size monodispersity. Figure 6 shows the use of photoluminescence spectroscopy to monitor the multilayer growth. QDs within the film showed an enhancement in the photoluminescence intensity when compared with QDs in colloidal solution. This can be explained by a higher degree of surface passivation of the QDs incorporated into the multilayer system relative to that of the QDs in solution. Water molecules are adsorbed on the surfaces of QDs, which passivates them and introduces surface traps, that is, electron and hole traps.[35] As a result of the electron−hole recombination process, this leads to photoluminescence. This is illustrated in Figure 7. Concentration changes of QDs also influence emission intensity as well as band

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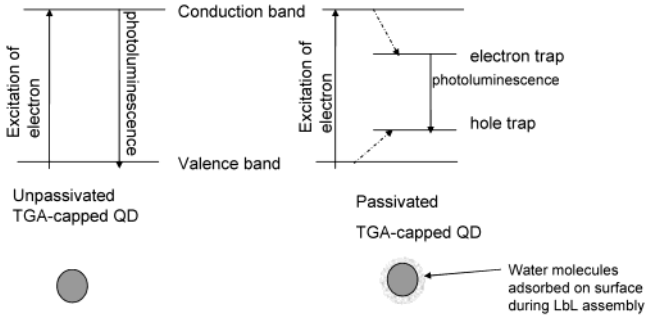

Figure 7. Photoluminescence arising from passivated quantum dots sandwiched between the polycation layers.

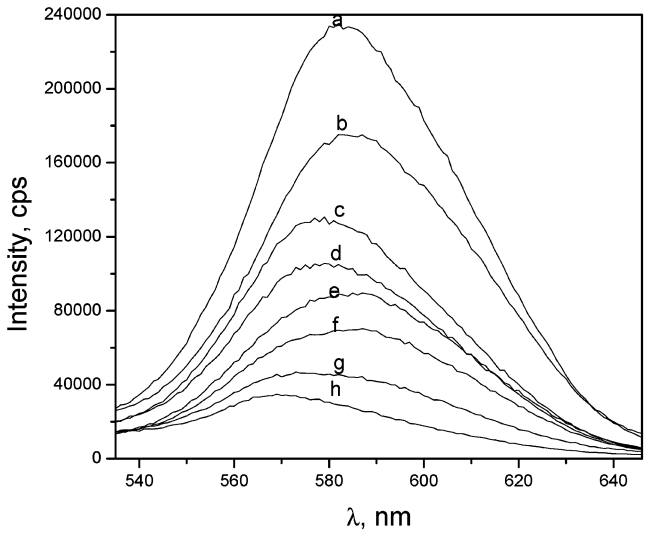

Figure 8. Photoluminescence spectroscopic measurements used in the detection of different concentrations of aqueous paraoxon solution (λ_{exc}= 467 nm): (a) luminescence of the *sensing assembly*; (b) 1×10^{-9} M; (c) 1×10^{-8} M; (d) 1×10^{-7} M; (e) 5×10^{-7} M; (f) 5×10^{-6} M; (g) 1×10^{-5} M; (h) 1×10^{-4} M.

spectral position as the result of interdot interactions. When the QDs are transferred from solution to the LbL film environment, this may alter the interactions among particles. All these may explain the observed blue shift of 52 nm on the emission spectrum when QDs were transferred from solution to a LbL film. In addition, water and oxygen molecules on the surface act to oxidize the surface of the QDs, which can also result in a blue shift in the emission spectra of the QDs. This increase in photoluminescence intensity plays an important role in the QD film being utilized in fluorescent detection applications such as the detection of paraoxon.

The *sensing assembly* was exposed to different concentrations of paraoxon solution (Figure 8). On exposure of the *sensing assembly* to paraoxon solution (1×10^{-9} M), there was an immediate decrease in the photoluminescence. As the concentration of paraoxon solution increased, the photoluminescence intensity decreased (b−h). OPH interacts with the substrate in solution, and the conformation is altered. This may act as a trigger for the observed changes in the photoluminescence property of the nanoparticles by influencing the degree of surface passivation. In addition, the photoexcited electron or hole interacts with OPH in a donor−acceptor charge transfer manner. This cascade of reactions ultimately leads to a decrease in photoluminescence. The *sensing assembly* works both to detect the presence of paraoxon solution and to detoxify it using OPH to catalyze the hydrolysis reaction.

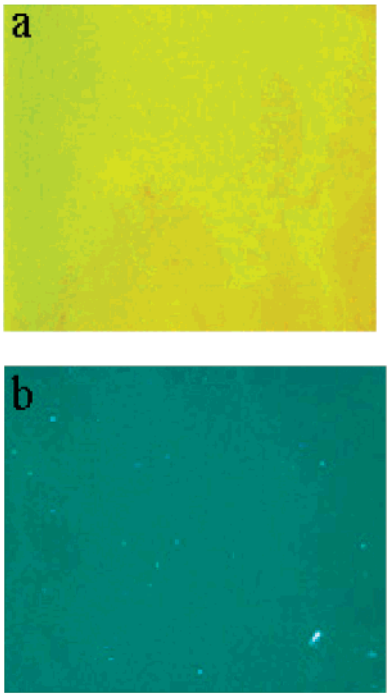

Figure 9. Epifluorescence microscope images of the *sensing assembly*: (a) before paraoxon exposure; (b) after paraoxon exposure in aqueous solution (5×10^{-6} M). The image size is 895 μm $\times$ 713 μm.

This *sensing assembly* was compared to another detection system used in previous work.[7] The half-bandwidths of the QDs were compared to that of poly(thiophene-3-acetic acid), which was utilized for its optical properties. It was quite evident that the TGA-capped CdSe QDs in this work had a narrow full width at half-maximum (fwhm) spanning 55 nm compared with that of poly(thiophene-3-acetic acid), which spanned ~100 nm. Many sizes of quantum dots may therefore be incorporated into the film, resulting in many emission colors that may be detected simultaneously.

3.3. Epifluorescence Microscopic Studies of the LbL Adsorbed Films. Epifluorescence imaging was utilized to examine the topography of the film. The images that were taken showed that the film was homogeneous with uniform quantum dots dispersal. As shown in Figure 2, the QDs emitted in the yellow region of the visible spectra. However, this image captured by epifluorescence showed a green film (Figure 9a) before paraoxon exposure. This green emission occurs because the emission from the film covered wavelengths that correspond to green in the visible spectrum. Although it is in the yellow region, it lies on the cusp of the green region, which ends at 570 nm. After paraoxon exposure, the QDs lose their photoluminescence, as shown in Figure 9b.

4. Conclusion

This work has presented results which show that TGA-capped CdSe QDs can be sandwiched into the LbL film, improving the photoluminescence of the QDs. As a result of the improved optical photoluminescence, the system was used as a sensor to detect the presence of paraoxon at different concentrations. The fabricated LbL thin film showed an attractive alternative to using polyelectrolytes with optical properties, for example, poly(thiophene-3-acetic acid), for biosensing assemblies due to a narrower fwhm. The problem of photobleaching was not encountered also. The system shows great potential for biosensor development.

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