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Peroxidase-Mimicking DNAzyme Modulated Growth of CdS Nanocrystalline Structures in Situ through Redox Reaction: Application to Development of Genosensors and Aptasensors

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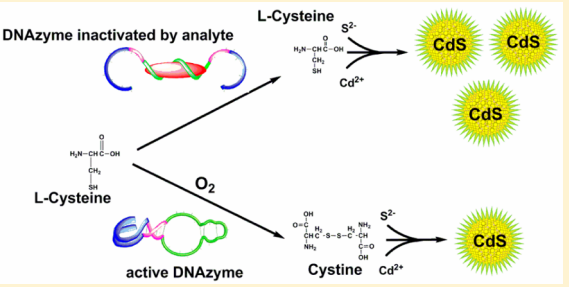
Supporting Information

ABSTRACT: This work demonstrates the use of the peroxidase-mimicking DNAzyme (peroxidase-DNAzyme) as general and inexpensive platform for development of fluorogenic assays that do not require organic fluorophores. The system is based on the affinity interaction between the peroxidase-DNAzyme bearing hairpin sequence and the analyte (DNA or low molecular weight molecule), which changes the folding of the hairpin structure and consequently the activity of peroxidase-DNAzyme. Hence, in the presence of the analyte the peroxidase-DNAzyme structure is disrupted and does not catalyze the aerobic oxidation of L-cysteine to cystine. Thus, L-cysteine is not removed from the system and the fluorescence of the assay increases due to the in situ formation of fluorescent CdS nanocrystals. The capability of the system as a platform for fluorogenic assays was demonstrated through designing model geno- and aptasensor for the detection of a tumor marker DNA and a low molecular weight analyte, adenosine 5′triphosphate (ATP), respectively.



The DNAzymes are nucleic acids with catalytic activity, isolated from combinatorial oligonucleotide libraries by in vitro selection.[1] Since the first DNAzyme was reported in 1994, many other DNAzymes with different catalytic activities have been described.[1,2] In comparison to conventional proteic enzymes, DNAzymes show clear advantages including higher stability and they can be denatured and renatured without losing catalytic activity. Their synthesis is cost-effective, and they can be massively produced by a DNA synthesizer with desirable modifications.[3] These unique characteristics make DNAzymes ideal biocatalysts to be applied for development of biosensors.

Application of peroxidase-mimicking DNAzymes (peroxidase-DNAzyme) to bioanalysis was pioneered by the group of Itamar Willner.[4,5] The common structural unit of such DNA showing peroxidase activity is a guanine-rich sequence that in the presence of hemin forms a catalytically active hemin/G-quadruplex complex. This hemin−DNA complex catalyzes oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonica-cid) ($ABTS^{2-}$) and luminol with H_2O_2 to yield colored product $ABTS^{\bullet-}$ and chemoluminescent oxidation products, respectively.[5−7] The oxidation of nonfluorescent precursors to fluorescent reporter molecules was also reported.[8,9] These catalytic activities were extensively used in bioanalytical assays to transduce and amplify the read-out signal of biorecognition events, such as DNA hybridization or aptamer−analyte complexation.[5,8,10] The group of Evgenii Katz pioneered application of hemin−DNA complexes to biocomputing security systems and biocatalytical multiplexers.[11] Hemin−DNA complexes have been used together with presynthesized fluorescent QDs for the detection DNA, low molecular weight analytes, and telomerase activity.[12,13] Semiconductor QDs are extensively used as labels for the optical and electrochemical sensing of biorecognition events.[14] They can be photoexcited to generate electron/hole couples which recombine to yield fluorescent emission of light.[15] Furthermore, their physico-chemical properties are defined by their submicrometer dimensions and differ significantly from those of the corresponding bulk material.[16] The advantages of QDs over traditional organic fluorophores, broadly used in detection of DNA, include higher quantum yield, reduced photobleaching, and higher extinction coefficient.[17] However, all reported assays were based on the conjugation between presynthesized QDs and peroxidase-DNAzyme.[12,13] Usually, they suffer from insufficient quenching of QDs by organic quenchers, non-specific adsorption on surfaces, and require complicated synthetic procedures to produce semiconductor QDs employing dangerous materials like Te.

Different unconventional routes have been introduced by our group to achieve in situ biocatalytical growth of CdS nanocrystals. It was demonstrated that S^{2-} ions generated through enzymatic reactions interact with exogenously added Cd^{2+} to yield CdS QDs.[18,19] Enzymes were also used to produce QDs capping agents, such as thiolated products

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(reduced glutathione and thiocholine) and orthophosphate that were able to affect the growth of fluorescent QDs.[20−22] It should be noted that these systems were successfully applied for the determination of clinically relevant enzymatic activities and inhibitors.[18−21,23]

Recently, a novel catalytic activity of the peroxidase-DNAzyme was reported: the aerobic oxidation of thiols to disulfides in the absence of exogenously added H_2O_2.[9] The present study reports on the use of this catalytic activity as a general and inexpensive platform for the development of label-free and enzyme-free homogeneous fluorogenic assays. For the first time the DNAzyme catalytic activity was used to modulate the fluorescence of the assay, through the control of the growth of CdS crystals. Furthermore, to prove the potential of the system for biosensors development, model fluorogenic genosensor and aptasensors were created for the detection of a tumor marker DNA and adenosine 5′-triphosphate.

EXPERIMENTAL SECTION

Materials. Adenosine 5′ diphosphate (ADP) and protoporphyrin IX containing different ions (Co^{3+}, Mn^{3+}, Sn^{4+}, Zn^{2+}, Cu^{2+}, Mg^{2+}) were purchased from Acros Organics (Belgium) and Frontier Scientific (Utah, U.S.A.), respectively. Adenosine 5′ triphosphate (ATP), uridine 5′-triphosphate (UTP), cytidine 5′-triphosphate (CTP), and guanosine 5′-triphopsphate (GTP), dimethyl sulfoxide (DMSO), hemin, and other chemicals were obtained from Sigma-Aldrich (Spain) and used as supplied. Sn(IV) protoporphyrin IX solutions in DMSO (5 mM) were freshly prepared prior to each experiment. The oligonucleotide sequences used in this study were synthesized by Integrated DNA Technologies (IDT, Belgium): **1**, 5′-TTTGGGTAGGGCGGGTTGGG-3′; **2**, 5′-GGGATGGGTCGCAAAGAACGAGAGTCTTTCTGCGA-GGGTTGGG-3′; **3**, 5′-CAGAAAGACTCTCGTTCTTT-3′; **4**, 5′-CAGAAAGACTCTCCTTCTTT-3′; **5**, 5′-CAGAAAGA-GTGTCGTTCTTT-3′; **6**, 5′-GGGATGGGCACCTGGGG-GAGTATTAGCGGAGGAAGGTTCCAGGTGGGGTTG-GG-3′.

Instruments. All assays were performed in black flat-well (330 μL) NUNC 96 wells microtiter plates, and the fluorescence spectra were recorded with a Varioskan Flash fluorimeter (Thermo Scientific). All water used was Milli-Q ultrapure grade (EMD Millipore).

Peroxidase-DNAzyme Catalyzed Oxidation of L-Cysteine to Cystine and Modulation of the Assay Fluorescence. To prove the operation mechanism of the proposed system control experiments were performed. Prior to the experiments oligonucleotides were dissolved in 10 mM phosphate buffer (pH 7.4). In a typical experiment 2×10^{-6} M **1** was incubated in HEPES buffer 5 mM containing 10 mM $NaNO_3$ (pH 7.5) in the presence of 3×10^{-6} M Sn(IV) protoporphyrin IX for 20 min at room temperature (RT). Afterward, 2×10^{-4} M L-cysteine was added to the assay, and the mixture was incubated for 10 min at RT, followed by the addition of 2.5×10^{-4} M Na_2S and 1.25×10^{-3} M $Cd(NO_3)_2$ necessary for the in situ formation of CdS QDs. The mixture was incubated for 5 min at RT, and the fluorescence emission spectra of the resulting suspension were recorded at λexc = 260 nm.

DNA Detection by the Peroxidase-DNAzyme Modulation of in Situ Growth CdS QDs. In a typical experiment, 2×10^{-6} M **2** was incubated in HEPES buffer 5 mM containing 10 mM $NaNO_3$ (pH 7.5) in the presence of variable concentrations of **3**. After 30 min of incubation at RT, 3×10^{-6} M Sn(IV) protoporphyrin IX was added and we let them interact for 20 min at RT. Afterward, 2×10^{-4} M L-cysteine was added to the assay, and the mixture was incubated for 10 min at RT, followed by the addition of 2.5×10^{-4} M Na_2S and 1.25×10^{-3} M $Cd(NO_3)_2$ necessary for the in situ formation of CdS QDs. The mixture was incubated for 5 min at RT, and the fluorescence emission spectra of the resulting suspension were recorded at λexc = 260 nm.

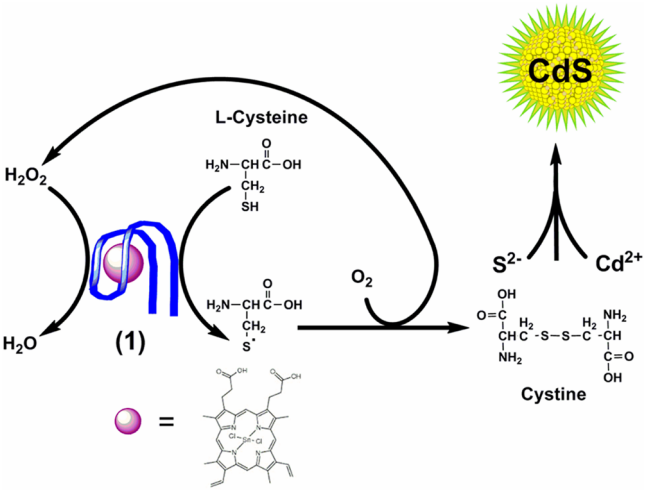
ATP Detection by the Peroxidase-DNAzyme Modulation of in Situ Growth CdS QDs. In a typical experiment 2×10^{-6} M **6** was incubated in HEPES buffer 5 mM containing 10 mM $NaNO_3$ (pH 7.5) in the presence of variable concentrations of ATP. After 30 min of incubation at RT, 3×10^{-6} M Sn(IV) protoporphyrin IX was added and we let them interact for 20 min at RT. Afterward, 2×10^{-4} M L-cysteine was added to the assay, and the mixture was incubated for 10 min at RT, followed by the addition of 2.5×10^{-4} M Na_2S and 1.25×10^{-3} M $Cd(NO_3)_2$ necessary for the in situ formation of CdS QDs. The mixture was incubated for 5 min at RT, and the fluorescence emission spectra of the resulting suspension were recorded at λexc = 260 nm.

Transmission Electron Microscopy Study. For CdS QDs characterization by transmission electron microscopy (TEM), L-cysteine-stabilized CdS QDs were produced in HEPES buffer 5 mM containing 10 mM $NaNO_3$ (pH 7.5) in the presence of 2×10^{-4} M L-cysteine, 2.5×10^{-4} M Na_2S, and 1.25×10^{-3} M $Cd(NO_3)_2$. Such prepared samples were freshly proceeded by desiccating a droplet of the solution on surfaces of hydrophilized, ultrathin carbon film coated copper grids or thin silicon oxide or silicon nitride membranes. Data was acquired in a TEM of type JEM-2100F-UHR (JEOL, Japan) equipped with a high-angle annular dark field (HAADF) detector, a digital camera of type F-216 (TVIPS, Germany), and an energy-dispersive X-ray spectroscopy (EDXS) system of type INCA (Oxford, U.K.).

RESULTS AND DISCUSSION

Scheme 1 illustrates the reported system. Briefly, the active peroxidase-DNAzyme (**1**), Sn(IV) protoporphyrin IX/peroxidase-DNAzyme complex, catalyzes the oxidation of the

Scheme 1. Oxidation of L-Cysteine to Cystine by Active Peroxidase-DNAzyme Results in Modulation of QD Growth



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thiolated molecule (L-cysteine) to its corresponding disulfide (cystine). It should be noted that the H_2O_2 required for the oxidation of L-cysteine is not exogenously added, as it is autocatalytically generated from O_2 during the disulfide formation.[9] The formed cystine cannot bind to the surface of growing CdS crystals generated via the interaction of Cd^{2+} with S^{2-}, due to the lack of the mercapto group. Thus, the oxidation of L-cysteine to cystine results in the decrease of the fluorescence of the assay, as cystine has low capacity to stabilize the growth of fluorescent CdS QDs. It should be noted that, in order to optimize the peroxidase-DNAzyme-catalyzed thiol oxidation, a number of protoporphyrin IX molecules containing different metal ions (Fe^{3+}, Co^{3+}, Mn^{3+}, Sn^{4+}, Zn^{2+}, Cu^{2+}, Mg^{2+}) were complexed with the peroxidase-DNAzyme. Afterward, the catalytic activity of the formed metal protoporphyrin IX/peroxidase-DNAzyme complexes was tested in terms of the fluorescent signal reduction capacity, as consequence of the depletion of the stabilizing agent (L-cysteine) and the disulfide (cystine) formation (Supporting Information Table S1). Taking into consideration these results, Sn(IV) protoporphyrin IX was selected for subsequent studies, instead of the commonly used hemin (Fe(III) protoporphyrin IX). CdS QDs formation time of 5 min was selected as the optimal incubation time to standardize the time period to perform reproducible fluorescent measurements. No further growth in fluorescence intensity was observed after 5 min of incubation. Optimum concentrations of Cd^{2+} and S^{2-} were found by comparing fluorescent signals arising from CdS QDs produced in assay mixtures containing inactive DNAzyme (DNAzyme only) and active DNAzyme (DNAzyme reconstituted with Sn(IV) protoporphyrin) (Supporting Information Table S2). The maximum ratio between fluorescence signals was observed at $[Cd^{2+}]$ = 1.25 mM and $[S^{2-}]$ = 0.25 mM; therefore, those concentrations were used for the subsequent experiments.

In order to attest the operating mechanism of our system, a number of control experiments were carried out (Figure 1). The highest emission peak attributed to QDs grown in situ was observed in the absence of Sn(IV) protoporphyrin IX in the reaction mixture (Figure 1, curves a and b). When only Sn(IV) protoporphyrin IX was present in the system, the fluorescent emission intensity was reduced (Figure 1, curve c) due to the intrinsic catalytic activity of Sn(IV) protoporphyrin IX. However, when both Sn(IV) protoporphyrin IX and peroxidase-DNAzyme were present in the system, the complex Sn(IV) protoporphyrin IX/peroxidase-DNAzyme was formed to catalyze the oxidation of thiol to disulfide. As a consequence, a detectable reduction of the fluorescent signal was recorded due to the depletion of the L-cysteine necessary for the stabilization of the in situ growing CdS QDs. To further demonstrate that the fluorescent signal was caused by the in situ formation of CdS QDs, experiments were performed in the absence of $Cd(NO_3)_2$, the metallic salt required for the formation of QDs (Figure 1, curve e), and in the absence of $Cd(NO_3)_2$ and Na_2S (Figure 1, curve f). Thus, we demonstrated that the peroxidase-DNAzyme catalyzing thiol oxidation could be used to modulate the fluorescent readout signal, through the consumption of the stabilizing thiolated agent. An affinity interaction between the peroxidase-DNAzyme bearing molecular hairpin and the corresponding analyte is able to change the peroxidase activity of the DNAzyme.

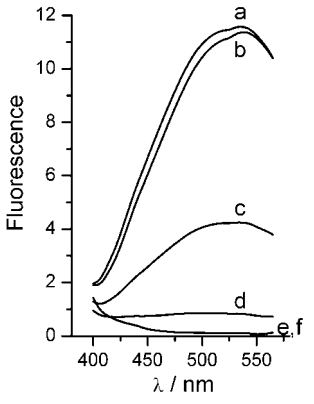

Figure 1. Fluorescence emission spectra of CdS QDs formed in (a) the absence of peroxidase-DNAzyme and Sn(IV) protoporphyrin IX, (b) peroxidase-DNAzyme only, (c) Sn(IV) protoporphyrin IX only, (d) the presence of peroxidase-DNAzyme and Sn(IV) protoporphyrin IX, (e) absence of $Cd(NO_3)_2$, (f) absence of $Cd(NO_3)_2$ and Na_2S.

In order to characterize the cysteine-stabilized CdS QDs, UV−visible (UV−vis) absorption and emission spectra were recorded in buffer solution (solid and dashed lines, respectively) as showed in Figure 2A. From the UV−vis spectrum, we observed an increased absorption below 500 nm and a shoulder at about 350 nm. The presence of this shoulder is explained by the excitonic transition between the electron 1S state and the hole 1S state, in semiconductor nanocrystals with a diameter about 3 nm.[24] On the other hand, the emission spectrum demonstrates a well-shaped peak at 550 nm which arises from excitonic emission of CdS nanocrystals. Furthermore, TEM was used to confirm the existence of stable CdS nanocrystals in the reaction mixture. Previously, it was reported in the literature that CdS nanoparticles produced from aqueous solutions using L-cysteine as stabilizer form aggregates on TEM grids,[25] contrary to separate nanoparticles produced with other stabilizing agents.[19,20] So the morphology and size of such CdS nanocrystals were evaluated on the basis of high-resolution TEM (HRTEM) images of CdS aggregates as shown in Figure 2B. The TEM-EDXS study on the produced material proves it to be composed of Cd and S in a 1:1 ratio. Images taken at STEM-HAADF and HRTEM conditions endorse that CdS nanocrystals of hexagonal phase have been formed (Supporting Information Figure S1).

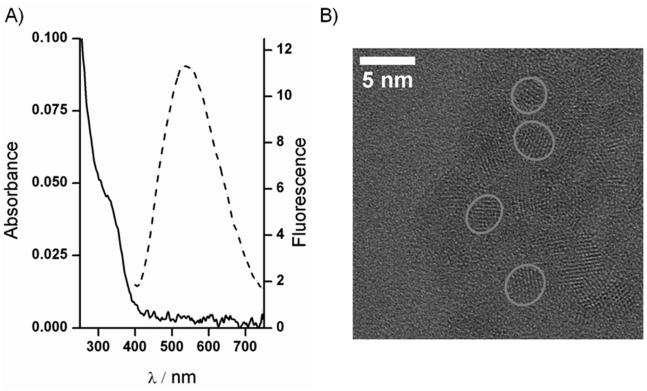

Figure 2. (A) Absorption (solid line) and emission (dashed line) of the formed CdS nanocrystals. (B) HRTEM image of CdS nanocrystals.

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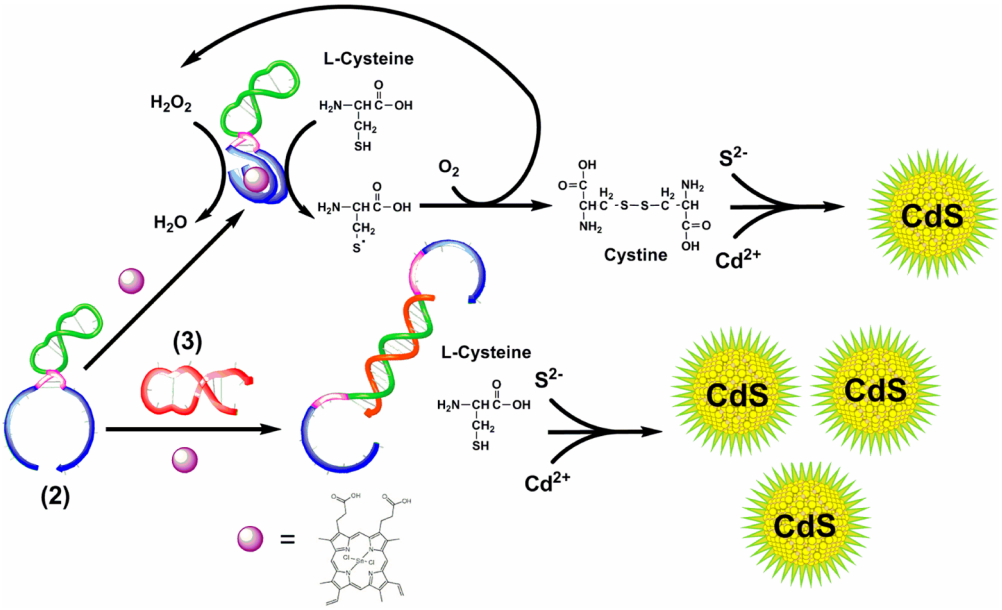
Scheme 2. DNA Detection through Peroxidase-DNAzyme Modulated Growth of CdS QDs in Situ



Scheme 2 shows the proposed system as a general platform for genosensors development based on the peroxidase-DNAzyme catalytic activity-mediated fluorescence intensity modulation. Briefly, a hairpin (**2**) consisting of three parts was designed: a single-stranded loop domain, complementary to the analyte DNA (green), a self-complementary sequence that stabilized the structure (pink), and the peroxidase-DNAzyme sequence (blue), split in two halves, placed at both ends of the construction. The free hairpin structure, in the absence of the target DNA and in the presence of Sn(IV) protoporphyrin IX, forms the peroxidase-DNAzyme sequences which self-assemble into functional Sn(IV) protoporphyrin IX/peroxidase-DNAzyme complexes to catalyze the oxidation of L-cysteine to cystine. The depletion of the thiol-bearing molecule (L-cysteine), required for the stabilization of the growing CdS crystals to form fluorescent CdS QD, results in inhibition of their growth and low fluorescent signal. On the other hand, when the analyte DNA is present in the assay, the affinity interaction between the loop domain and the target DNA disrupts the structure of the hairpin and the Sn(IV) protoporphyrin IX/peroxidase-DNAzyme complex cannot be formed in the presence of protoporphyrin IX. As a consequence, L-cysteine is not converted into cystine and binds to the surface of CdS crystals facilitating the growth of fluorescent NPs. Thus, the fluorescence intensity of the assay increases with the increase in concentration of the analyte DNA.

To prove the suitability of the proposed system for the detection of DNA, a model genosensor was developed for the detection of a DNA species related to oral cancer overexpressed 1 gene (ORAOV1) (**3**). ORAOV1 gene is overexpressed in many solid tumors and this fact makes it a potential candidate as tumor marker.[26] Figure 3A shows the fluorescence spectra obtained in the presence of 2 μM of the peroxidase-DNAzyme bearing molecular hairpin and different concentrations of the target tumor marker DNA. As can be seen, when the concentration of the analyte DNA increases, the fluorescence of the system increases because of the higher concentration of L-cysteine in the assay. Hence, when the analyte DNA is present in the system the switching of the molecular hairpin structure reduces the number of Sn(IV) protoporphyrin IX/peroxidase-DNAzyme complexes in the assay. Thus, L-cysteine is not removed from the system and can stabilize in situ growing CdS QDs to increase the detected fluorescence. The calibration curve (Figure 3B) was generated by plotting the fluorescence intensity, at 510 nm, generated by the in situ produced CdS QDs in the presence of variable concentrations of the tumor marker DNA. One can see that the system allows the analysis of the target DNA with a limit of detection of 100 nM, which is in the range of previously developed methods for DNA detection using enzymatic amplification free catalytic molecular hairpins.[5,27] In addition, the specificity of the proposed system was tested using one- (**4**) and two-base (**5**) mismatched target DNA sequences. Results showed that the system is specific only for the target DNA, and the presence of mismatched target DNA sequences (10 μM) yields a low fluorescence signal close to the background fluorescence of the system (Supporting Information Figure S2). Thus, we believe that with the development of this model system for the

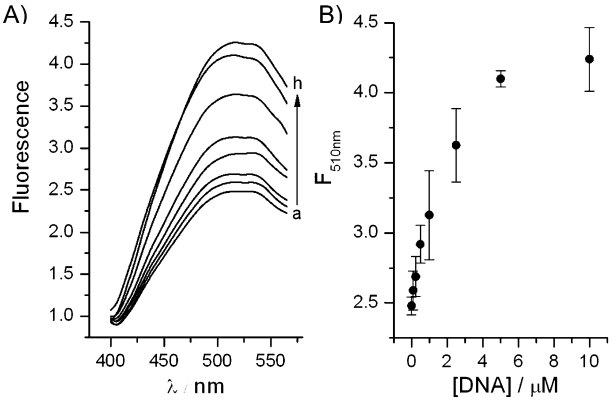


Figure 3. Fluorescence emission spectra of CdS QDs formed in the presence of variable concentrations of the DNA species related to ORAOV1 gene: (a) 0 M, (b) 1×10^{-7} M, (c) 2.5×10^{-7} M, (d) 5×10^{-7} M, (e) 1×10^{-6} M, (f) 2.5×10^{-6} M, (g) 5×10^{-6} M, (h) 1×10^{-5} M.

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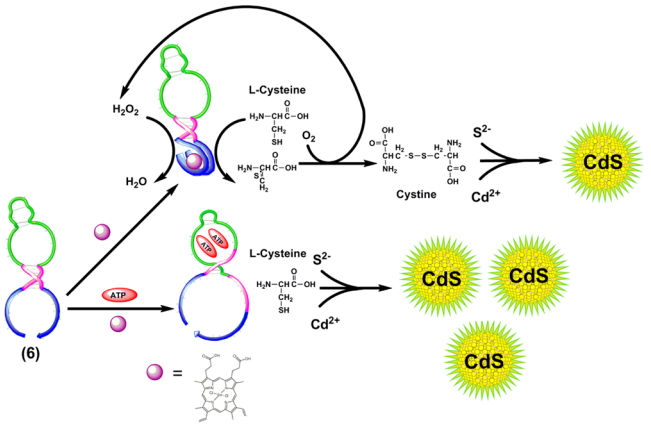
detection of a DNA sequence related with ORAOV1 gene, we demonstrate its capability as a general platform for genosensors development.

The peroxidase-DNAzyme catalytic activity mediated modulation of the in situ generation of QDs, was also applied to the development of a model fluorogenic aptasensors for low molecular weight analyte detection. The system was applied to the detection of ATP as depicted in Scheme 3. A catalytic DNA

Scheme 3. ATP Detection through Peroxidase-DNAzyme Modulated Growth of CdS QDs in Situ



hairpin was designed (**6**) consisting in an ATP aptamer sequence (green),[13] a self-complementary sequence that stabilized the structure (pink), and the peroxidase-DNAzyme sequence (blue), split in two halves, placed at both ends of the oligonucleotide. In the absence of the analyte and in the presence of Sn(IV) protoporphyrin IX, the sequence folds into the catalytically active peroxidase-DNAzyme structure to oxidize L-cysteine to cystine, which decreases the fluorescence of the assay as stated above. When ATP is present in the system, the affinity interaction between ATP and the aptamer sequence results in the disruption of the hairpin structure and the peroxidase-DNAzyme sequence cannot fold into the active conformation of peroxidase-DNAzyme even in the presence of Sn(IV) protoporphyrin IX. As L-cysteine is not removed from the assay solution, fluorescent CdS QDs can be formed via the stabilization of the in situ growing CdS nanocrystals with the thiolated product, enabling the fluorimetric detection of ATP molecules.

Figure 4A depicts the fluorescent emission spectra upon analyzing different concentrations of ATP in the presence of a fixed concentration of the peroxidase-DNAzyme bearing molecular hairpin. As one can see from the increasing intensity of the fluorescence peaks, the amount of formed CdS QDs depends on the ATP concentration. Thus, the increase in ATP concentration leads to the decrease in the number of active peroxidase-DNAzyme molecules, and hence, the fluorescence intensity in the assay mixture grows. Figure 4B shows the derived calibration curve corresponding to the fluorescence intensity, at 510 nm, generated in the assay mixture in the presence of different concentrations of ATP. According to the calibration plots the developed ATP detection system allows the detection of 5 μM of ATP, which is in the range of previously developed methods for ATP detection using enzymatic amplification free catalytic molecular hairpins.[27,28] To test the specificity of the reported system, the variation of the fluorescence intensity was tested in the presence of high

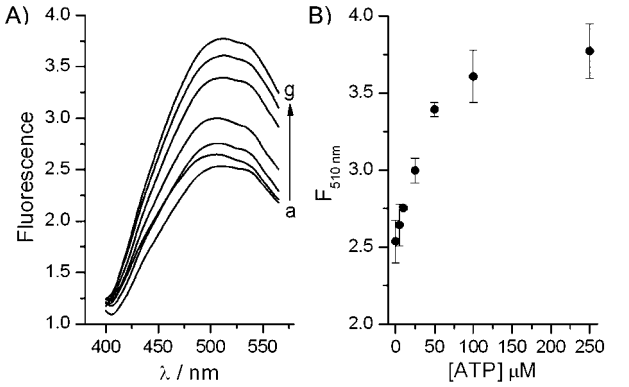


Figure 4. Fluorescence emission spectra of CdS QDs formed in the presence of variable concentrations of ATP: (a) 0 M, (b) 5×10^{-6} M, (c) 1×10^{-5} M, (d) 2.5×10^{-5} M, (e) 5×10^{-5} M, (f) 1×10^{-4} M, (g) 2.5×10^{-4} M.

concentration of adenosine diphosphate (ADP), uridine 5′-triphosphate (UTP), cytidine 5′-triphosphate (CTP), and guanosine 5′-triphopsphate (GTP) in the assay mixture. ADP, UTP, CTP, and GTP did not show any affinity interaction with the aptamer sequence leading to a fluorescent signal close to the background fluorescence, as demonstrated in Supporting Information Figure S3. Our system is able to distinguish between ATP and ADP. Such assay could find applications in biochemistry, because many biological pathways involve conversion of ATP to ADP and vice versa.

CONCLUSIONS

In this work we demonstrated the application of the peroxidase-DNAzyme catalyzed thiol oxidation to disulfides for the development of general and inexpensive fluorimetric geno- and aptasensors, based on the active peroxidase-DNAzyme mediated modulation of in situ produced QDs. It should be noted that the novelty of the reported sensing strategy lies on the use of inexpensive compounds, such as DNA and salts, for the development of fluorimetric bioanalytical systems. In comparison with other reported fluorogenic assays based on presynthesized QDs modified with recognition elements, our assays require neither any synthetic procedures for chemical modification of semiconductor NPs nor any organic fluorogenic enzymatic substrates. However, in order to reduce the background signal, improve the limit of detection, and finally to improve the signal to background ratio of a prospective assay, the present system will be coupled with analyte recycling DNA machinery operating by strand displacement. We believe that our system can serve as a model for new inexpensive tests for detection of relevant analytes in clinical diagnosis.

ASSOCIATED CONTENT

Supporting Information

TEM analysis of the CdS nanostructures, analysis of the influence of metal ions on the catalytic activity of protoporphyrin IX, and control experiments for DNA and ATP detection. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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