# Highly Sensitive Multiplexed Heavy Metal Detection Using Quantum-Dot-Labeled DNAzymes

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**ABSTRACT** We developed highly sensitive and specific nanosensors based on quantum dots (QDs) and DNAzyme for multiplexed detection of heavy metal ions in liquid. The QDs were coated with a thin silica layer for increased stability and higher quantum yield while maintaining a relatively small size for highly efficient energy transfer. The QD−DNAzyme nanosensors were constructed by conjugating quencher-labeled DNAzymes onto the surface of carboxyl-silanized QDs. In the presence of metal ions, the emission is restored due to the cleavage of DNAzymes. The detection could be completed within 25 min with a single laser excitation source. The detection limit of 0.2 and 0.5 nM was experimentally achieved for $Pb^{2+}$ and $Cu^{2+}$, respectively, which is a 50- and 70-fold improvement over the recent results obtained with dye molecules. Multiplexed detection was also demonstrated using two different colors of QDs, showing negligible cross-talk between the $Pb^{2+}$ detection and $Cu^{2+}$ detection.

Heavy metal contamination is one of the most serious concerns to human health because these substances are toxic and retained by the ecological system.[1] The presence of heavy metals in ambient environment has been increased dramatically during the last few decades.[2] The combustion of immense quantities of coal and other fossil fuels introduces a large amount of industrial heavy metals into atmosphere and the aquatic environment. Although some of the heavy metals such as copper (Cu),[3,4] iron (Fe),[4,5] and zinc (Zn)[6] are biologically essential and required by some organisms,[4] they can lead to toxicity at higher concentrations. On the other hand, some other heavy metals such as lead (Pb),[7] mercury (Hg),[8] and cadmium (Cd)[9] are not biologically essential and are harmful to organisms even at very low concentrations because they lead to excessive free-radical proliferation.[10]

To minimize the pollution of heavy metals, one way is to develop analytical tools for heavy metal detection and prevent the damaging effects of pollution in the very beginning stage. Conventional analytical techniques for heavy metals, such as atomic absorption spectrometry,[11] inductively coupled plasma mass spectrometry,[12] capillary electrophoresis,[13] X-ray fluorescence spectrometry,[14] and microprobes,[15] have routinely been used for metal ion analysis with high sensitivity. However, most of these techniques suffer from the disadvantages of high costs, sophisticated instruments, and requirements for trained personnel and sample pretreatment, making it difficult for real-time and on-site monitoring of metal ions.[16] Therefore, the development of a highly sensitive and portable sensor system to detect toxic heavy metal ions has long been a focus of research.

DNAzymes, a new category of enzymes that are highly specific and sensitive for metal ions such as Pb(II), Cu(II), and Zn(II), have increasingly been used as metal sensors.[17,18] Recent studies by Lu *et al.* have demonstrated that the combinatorial selection of DNAzymes can be employed for heavy metal detection.[18] For instance, they reported functional DNAzyme biosensors labeled with fluorophores and quenchers that used DNAzyme-catalyzed ligation reaction for $Pb^{2+}$ detection with a detection limit of 10 nM.[19] By using *in vitro* selection methods,[18] DNAzymes with high specificity for any metal ion such as $Pb^{2+}$, $Hg^{2+}$, $Cu^{2+}$, and $UO_2^{2+}$ can be obtained, and by modifying fluorophore/quencher pairs attached to those DNAzymes, different types of heavy metal sensors can be developed.[18−22] However, applications of the organic fluorophore have been limited due to several problems such as low photostability, low quantum yield, and the requirement for multiple excitation sources.[23]
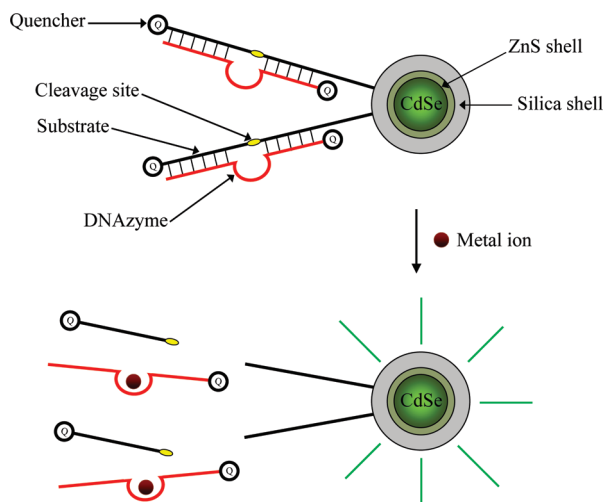
**Figure 1. Schematic of QD-based catalytic DNAzymes. The ZnS-capped CdSe QD is embedded in a siloxane shell and covalently coupled to DNAzymes. Each DNAzyme is composed of two quenchers. In the absence of the target metal ions, the fluorescence from the QD is quenched. Once the target metal ion binds to the DNAzyme, the fluorescence from the QD is restored due to the cleavage of the DNAzyme substrate.**

Semiconductor quantum dots (QDs) represent a revolution in the application of fluorophores in fluorescent labeling due to their high photo- and chemical stability and high quantum yield, as compared to traditional organic dyes. In addition, different QD fluorescence emissions can be controlled by simply adjusting the QD size. Furthermore, all colors of QDs can be excited by a single excitation source, which makes it much easier to achieve multiplexed detection than traditional dyes that require the excitation light source be tuned into their respective narrow absorption bands.[24−26] Therefore, QDs are increasingly adopted as an alternative for organic dyes in biosensing,[27,28] imaging applications,[3,27,29,30] and ion detections.[31,32] In this study, we combined the benefits of DNAzymes and QDs to develop a highly sensitive and selective sensor that is capable of detecting sub-nanomolar heavy metal ions in less than half an hour in a multiplexed format with a single excitation source.

## RESULTS AND DISCUSSION

As illustrated in Figure 1, the DNAzyme heavy metal sensor relies on fluorescence resonance energy transfer (FRET) to carry out the detection. To reach the highest possible quenching efficiency, the dual quenchers on both substrate and DNAzyme segments were designed and introduced in this study.[19] In the absence of the target metal ions, DNAzymes (and hence quenchers) are bound to the QD surface. The quencher on the DNAzyme can efficiently quench fluorescence of the QDs due to its close proximity. The quencher attached to the substrate is used to reduce the background fluorescence from the free QDs in the solution when the DNAzyme is detached from the substrate due to the low stability of the DNAzyme−substrate complex at

room temperature. In the presence of the target metal ions, the substrate and enzyme are cleaved off the QD and the QD emission is restored.

FRET is highly dependent upon the distance between the donor (QD) and the acceptor (quencher). In order to maximize the quenching efficiency, the smallest possible QDs are preferred. In our previous work, we have successfully synthesized a very compact QD−DNA probe (2.8 nm for green QDs and 3.3 nm for red QDs) that enabled highly efficient energy transfer.[3] Therefore, they seem to be promising candidates for the development of QD−DNAzyme-based heavy metal sensors. However, we found that while the QD emission was initially restored upon the addition of the target metal ion and the subsequent cleavage of the DNAzymes, the emission dropped ~50% in 5 min, which prevented us from carrying out sensitive and quantitative measurement. This quenching effect of metal ions on the fluorescence of QDs was also reported recently by other groups[33−35] and was attributed to the metal ions nonspecifically bound to the QD surface that facilitate the nonradiative electron/hole recombination.[36−38]

To overcome the quenching effect, we introduced surface silanization onto QDs to isolate and protect the QDs, which has previously been applied to generate higher quantum yield and more stable QDs in aqueous solution.[39,40] The thickness of the silica shell could be controlled by adjusting the reaction time and the silane compound concentration. The method for synthesis of carboxyl-silanized QDs is shown in Figure 2a. The hydroxyl group was first introduced using 2-mercaptoethanol to replace the TOPO Trioctylphosphine oxide group on the QDs' surface. Then robust QDs could easily be established *via* a silanization reaction in organic solvent. In this article, 3-APS 3-aminopropyltrimethoxysilane was used to form a silica shell and introduce the primary amine group to the QDs, which was subsequently converted to the carboxyl group. The carboxyl-silanized QDs could readily be coupled to DNAzymes by using well-established cross-linking methods and commercially available cross-linkers.

In Figure 2b−e, we used dynamic laser scattering (DLS) to characterize the size of the before and after silanization of OH-capped QDs and found that the thickness of the silane layer on the QDs was 3.1 nm (3.3 nm) for QD530 (QD625), corresponding to the final QD size of approximately 9.6 nm (12.7 nm) in diameter. Since the Förster distance for QD and dye is approximately 7 nm,[41] the above size ensures the efficient energy transfer between the QD and the quencher, as shown later. To test their stability, the silanized QDs were challenged with high concentrations of $Pb^{2+}$ and $Cu^{2+}$ solution. As shown in Figure 3, the QD fluorescence intensity shows virtually no change even after 30 min of incubation, attesting to high resistance of the silanized QDs to the metal quenching effect.

To form the QD−DNAzyme complex, we used the zero-length cross-linkers, EDC and sulfo-NHS, to attach
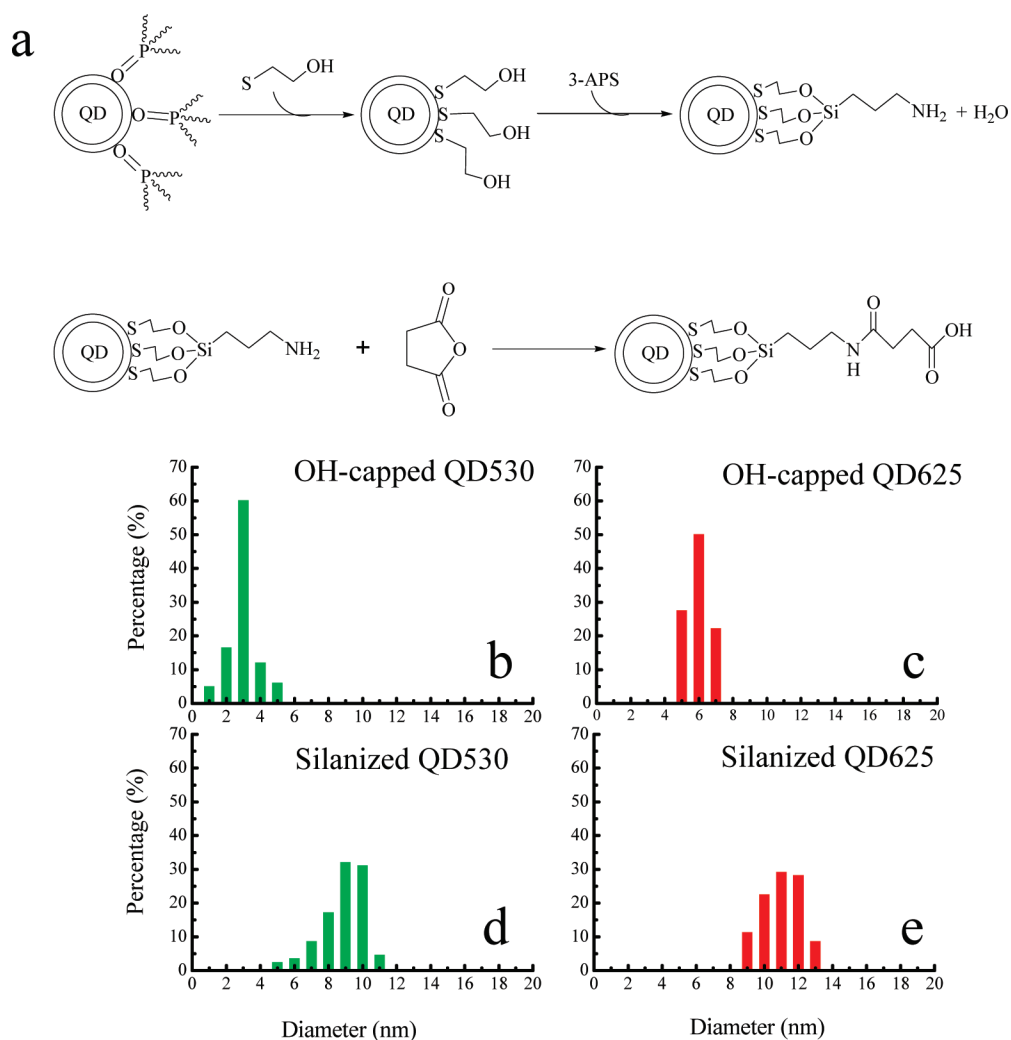
**Figure 2. (a) Reaction scheme of the silanization and functionalization of QDs. Chemically synthesized TOPO-capped QDs were replaced by mercaptoethanol using thiol exchange reaction to introduce hydroxyl groups. Then, the QDs were silanized with 3-APS. The ethoxysilane (Si-OCH₂CH₃) groups hydrolyze into hydroxyl groups and release water molecules. Finally, functional exchange reaction of the amine functions by ring opening of succinic anhydride. (b−e) Size distributions of OH-capped QDs and silanized QDs measured by DLS. After silanization, the QD size is ~9.6 nm (12.7 nm) in diameter for QD530 (QD625).**

the aminated quencher-labeled DNAzyme to the carboxyl-silanized QD surface. The quenching effect of the DNAzyme was investigated in Figure 4a,b by incubating 500 μL of QDs at 50 nM with 500 μL of DNAzymes with different concentrations. A drastic increase in the QD emission quenching efficiency from 64 to 97.5% for the $Pb^{2+}$-specific QD530−DNAzyme and from 65 to 95% for the $Cu^{2+}$-specific QD625−DNAzyme was observed when the DNAzyme-to-QD molar ratio was increased from 8:1 to 16:1. To further verify that the DNAzymes were successfully conjugated with carboxyl-silanized QDs and that the decreased emission was indeed from the quenchers on the DNAzymes, we used negative control DNAzymes without any quenchers attached and attached them to the corresponding QDs under the same cross-linking reaction conditions. Figure 4c (4d) shows the emission of the silanized QD530 (QD625) linked with nonquencher DNAzymes, control DNAzyme1 (2), prepared with NPb-Sub (NCu-Sub) and

NPb-Enz (NCu-Enz) (see Table 1). The nearly identical spectrum was observed as compared to the original QDs before DNAzyme attachment, thus confirming the successful assembly of QD−DNAzyme sensors. In the subsequent experiments, we used QD−DNAzyme sensors prepared with the DNAzyme-to-QD molar ratio of 16:1. Under this condition, the size of QD530−DNAzyme and QD625−DNAzyme became 15.8 and 16.2 nm in diameter, respectively, according to DLS results, indicating that there were on average 11 DNAzymes on each QD530 and 10 DNAzymes on each QD625.

To characterize the performance of the QD−DNAzyme sensors, various concentrations of $Pb^{2+}$ and $Cu^{2+}$ were added to the QD−DNAzyme solutions. Figure 5 shows the time-dependent fluorescence with various concentrations of metal ions. The higher reaction rate is associated with the higher ion concentration. Saturation was reached in approximately 25 min,
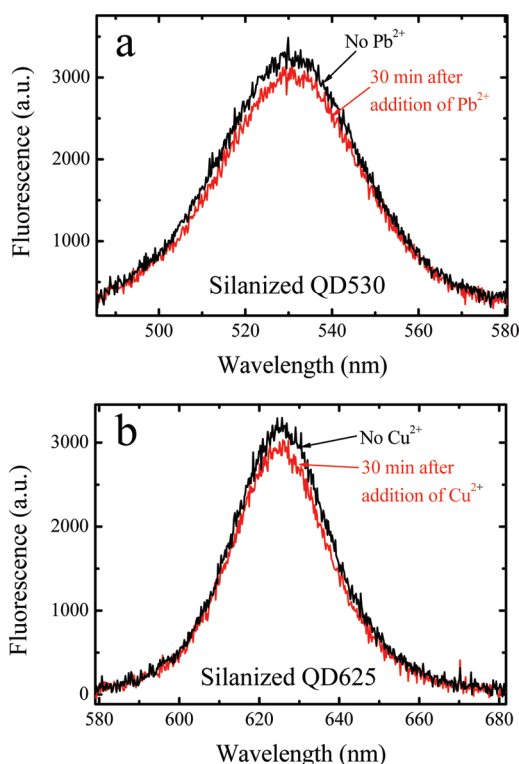
**Figure 3. Stability test of the silanized QDs with high concentrations of heavy metal ions. The control spectra were obtained by diluting the 25 nM silanized QD solutions with deionized H$_2$O at the ratio of 1:1. The 30 min spectra were obtained 30 min after the addition of 50 µL of 3 µM metal ion solution to 50 µL of 25 nM silanized QDs.**
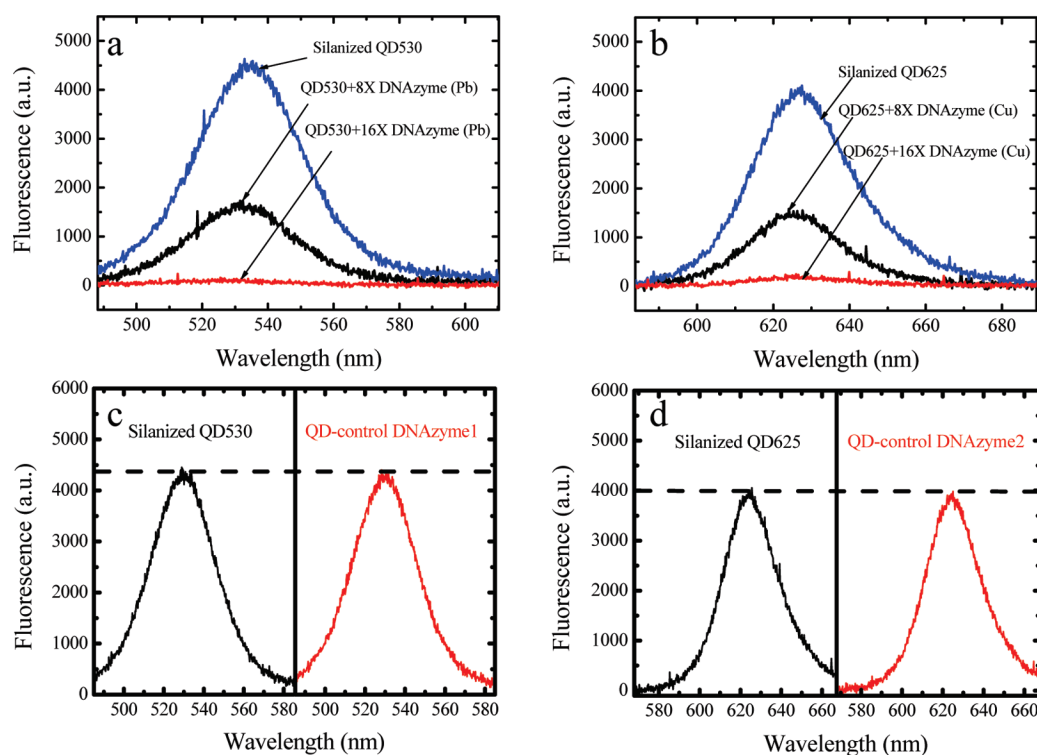
**TABLE 1. Sample Names and DNA Sequences**

| sample name | sequence |
|---|---|
| Pb-Sub | NH$_2$C$_6$-5′-ACTCACTAT**rA**GGAAGAGATG-3′-Iowa black FQ |
| Pb-Enz | 5′-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3′-Iowa black FQ |
| Cu-Sub | Iowa black RQ-5′-AGCTTCTTTCTAATACGGCTTACC-3′-C$_6$NH$_2$ |
| Cu-Enz | Iowa black RQ-5′-GGTAAGCCTGGGCCTCTTTCTTTTTAAGAAAGAAC-3′ |
| NPb-Sub | NH$_2$C$_6$-5′-ACTCACTAT**rA**GGAAGAGATG-3′ |
| NPb-Enz | 5′-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3′ |
| NCu-Sub | 5′-AGCTTCTTTCTAATACGGCTTACC-3′-C$_6$NH$_2$ |
| NCu-Enz | 5′-GGTAAGCCTGGGCCTCTTTCTTTTTAAGAAAGAAC-3′ |

and no degradation in fluorescence was observed thereafter. Figure 6a,c shows the fluorescence spectra taken 25 min after sample injection. The corresponding fluorescence intensity increase with respect to the background is presented in Figure 6b,d and is found to be linearly proportional to the heavy metal ion concentration in the range of 1 to 50 nM. The lowest concentration experimentally obtained is 0.2 and 0.5 nM for Pb$^{2+}$ and Cu$^{2+}$, respectively, which is a 50- and 70-fold improvement as compared to the recently reported results using DNAzymes.[19,20]

One of the advantages of using QDs in replacement of conventional dye molecules is the multiplexed detection capability with a single laser excitation. To demonstrate such capability, Pb$^{2+}$-specific QD530−DNAzymes were mixed with Cu$^{2+}$-specific QD625−DNAzymes before the mixture of heavy metal



**Figure 4. Fluorescence emission spectra evolution of the QD−DNAzymes. (a) Pb$^{2+}$-specific QD530−DNAzyme complex at a DNAzyme-to-QD molar ratio of 0:1, 8:1, and 16:1. (b) Cu$^{2+}$-specific QD625−DNAzyme complex at a DNAzyme-to-QD molar ratio of 0:1, 8:1, and 16:1. (c) Negative control experiment. The DNAzyme1-to-QD530 molar ratio was 16:1. (d) Negative control experiment. The DNAzyme2-to-QD625 molar ratio was 16:1.**
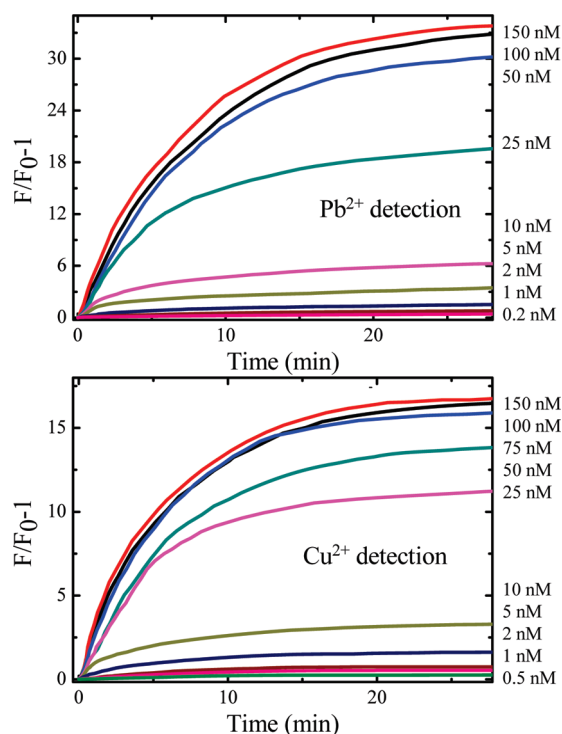
**Figure 5. Time-dependent spectrally integrated emission intensity of QD−DNAzyme sensors over the background at different metal ion concentrations.** $F_0$ ($F$) is the QD emission intensity in the absence (presence) of target heavy metal ions. (Top) $Pb^{2+}$-specific QD530−DNAzyme. Spectral integration takes place from 490 to 600 nm. (Bottom) $Cu^{2+}$-specific QD625−DNAzyme. Spectral integration takes place from 580 to 700 nm.

ions was added. The excitation was set at 480 nm, and emission centered around 530 nm for $Pb^{2+}$ and 625 nm for $Cu^{2+}$ was monitored. The increase of the fluorescence intensity in the 530 nm channel and 625 nm channel relative to the respective background reflects the concentration of the corresponding metal ions. Figure 7 shows that the $Pb^{2+}$ and $Cu^{2+}$ can be simultaneously and quantitatively detected with virtually no cross-talk between the 530 nm channel and the 625 nm channel.

## CONCLUSION

We have developed the QD−DNAzyme sensors for ultrasensitive and selective and multiplexed detection of heavy metal ions in liquid by combining the highly stable silanized QDs with high ion-specific DNAzymes. The detection could be completed within half an hour, and the sub-nanomolar detection limit was achieved for $Pb^{2+}$ and $Cu^{2+}$, nearly 2 orders of magnitude better than the state-of-the-art. Future work will involve using other types of quenchers such as gold nanoparticles and optimizing the salinization method to better control the saline layer thickness, both of which will further improve the quenching efficiency and result in an even better detection limit. Our sensors will have broad applications in monitoring of waste treatment, industrial drain control, medical diagnostics, and homeland security.

## EXPERIMENTAL SECTION

**Materials.** 2-Mercaptoethanol (99.0%), sodium hydroxide (NaOH, 98%), methanol (99.8%), ethanol (99.8%), 3-aminopropyltrimethoxysilane (3-APS, 97%), chlorotrimethylsilane (99%), TMAH pentahydrate (97%), chloroform (99%), succinic anhydride (99%), tetrahydrofuran (THF, 99.9%), sodium ascorbate, phosphate buffered saline (PBS), isacetate buffer, 0.2 $\mu$m syringe filter, 0.45 $\mu$m syringe filter, 30 000 MWCO Nanosep centrifugal filter, and 100 000 MWCO Nanosep centrifugal filter were obtained from Sigma-Aldrich (St. Louis, MO). Sodium chloride (NaCl), lead(II) acetate trihydrate (99.999%), cupric chloride (1 M), HEPES, pH 7.0 buffer, and phosphate buffer (50 mM, pH 6.5) were obtained from Fisher Scientific (Pittsburgh, PA). 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) were obtained from PIERCE (Rockford, IL). NAP-25 columns were obtained from GE Healthcare (South Burlington, VT).

All DNAs used in experiments were HPLC grade and were obtained from IDT (Coralville, IA). The DNA sample names and the corresponding sequences are given in Table 1. Briefly, Pb-Enz (33-base labeled with Iowa black FQ) and Cu-Enz (35-base labeled with Iowa black RQ) served as the $Pb^{2+}$ and $Cu^{2+}$ enzyme sequence. Pb-Sub with a ribo-adenosine (rA) (19-base labeled with Iowa black FQ) and Cu-Sub (24-base labeled with Iowa black RQ) served as the substrates for $Pb^{2+}$ and $Cu^{2+}$ enzyme sequences, respectively. NPb-Enz and NCu-Enz were $Pb^{2+}$ and $Cu^{2+}$ enzyme sequences without quenchers; NPb-Sub and NCu-Sub were $Pb^{2+}$ and $Cu^{2+}$ substrate sequences without quenchers. They were used to form the negative control DNAzymes.

**Preparation of OH-Capped QDs.** TOPO-capped QD synthesis was carried out by following the protocol that we developed previously.[3] To obtain OH-capped QDs, TOPO-capped QDs were dried by evaporation, then 100 mg of the QDs in 1 mL of 2-mercaptoethanol and 0.5 mL of methanol was added and pH was adjusted to 9 with 0.2 M NaOH. This mixture was stirred overnight at 85 °C, and the resulting OH-capped water-soluble QDs were then precipitated by adding 3 mL of ethanol. The residue was washed three times with ethanol, and then the resulting suspension was centrifuged at 14 000 rpm for 5 min. The supernatant was discarded, and the QDs were dissolved in 1 mL of deionized $H_2O$ and filtered through a 0.2 $\mu$m syringe filter to obtain OH-capped QDs in clear solution. During the experiment, we prepared two types of OH-capped QDs with the emission peak at 525 and 619 nm, respectively.

**Silanization of QDs and Surface Functional Modification.** To obtain the silanized QDs, 0.5 mL of 3-aminopropyltrimethoxysilane (3-APS) diluted with 2 mL of methanol was added to 1 mL of OH-capped QDs (15 $\mu$M) slowly at 0 °C with vigorous mixing. This mixture became cloudy immediately and then turned clear within a few minutes. After 10 min of stirring, the solution was heated to ∼60 °C for 30 min and then cooled to ∼30 °C. To quench the silanization reaction, a mixture of 15 mL of methanol and 1.5 mL of chlorotrimethylsilane basified with 2.3 g of solid TMAH pentahydrate was added at room temperature. After ∼2 h of stirring, the solution was heated to ∼60 °C for 30 min and then stirred slowly under argon at room temperature for 2 days. Methanol was removed *in vacuo* for 24 h, and the solution was filtered through a 0.45 $\mu$m syringe filter and free silane was removed *via* size-exclusion chromatography using a NAP-25 column with deionized $H_2O$. Then the solution was concentrated twice with a centrifuge (8000 rpm) using a 30 000 MWCO Nanosep centrifugal filter. Finally, the silanized QD solution was reduced to ∼1 mL and stored in a 4 °C dark room. The emission peak of the amino-silanized QDs was found to slightly red shift to 530 nm (QD530) and 625 nm (QD625) with respect to their un-
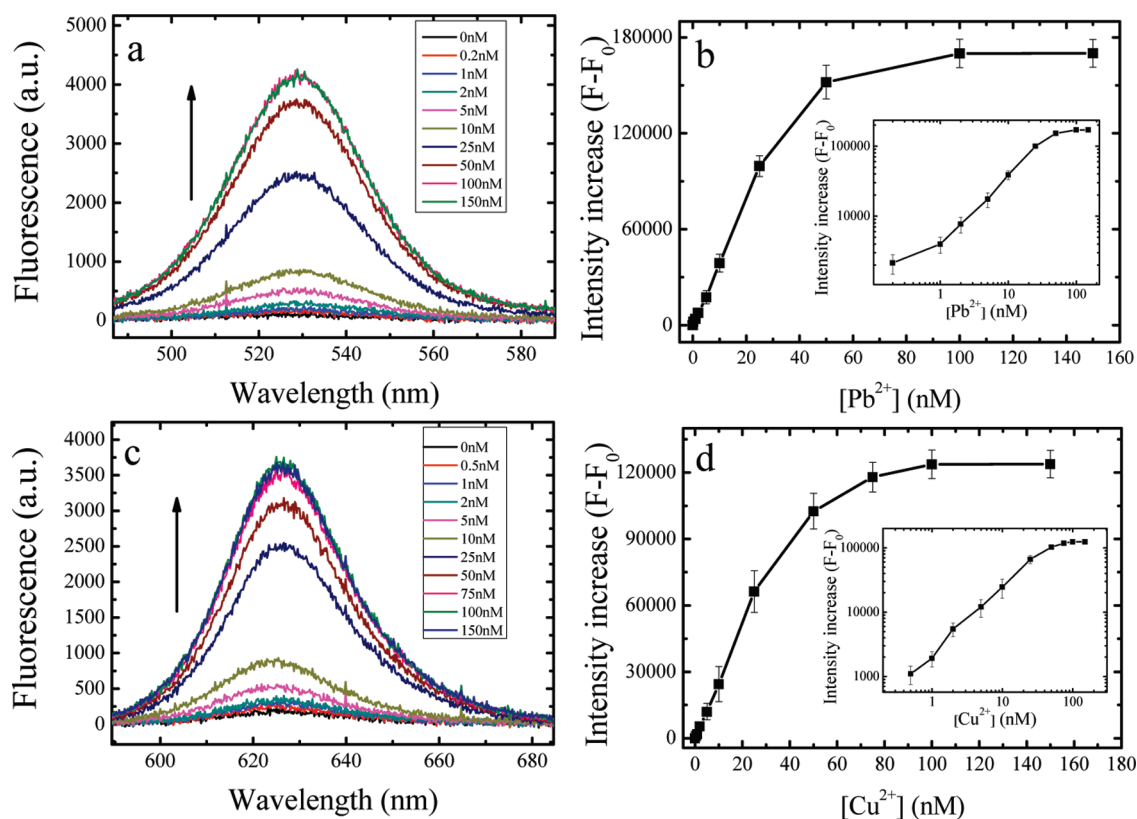
**Figure 6. (a)** Fluorescence spectra of $Pb^{2+}$-specific QD530−DNAzyme at various $Pb^{2+}$ concentrations taken after 25 min of sample incubation. **(b)** QD530 emission intensity increase ($F-F_0$) based on the spectra in (a) as a function of the $Pb^{2+}$ concentration. Error bars indicate variations from three measurements. Inset: the corresponding log−log plot. **(c)** Fluorescence spectra of $Cu^{2+}$-specific QD625−DNAzyme at various $Cu^{2+}$ concentrations taken after 25 min of sample incubation. **(d)** QD625 emission intensity increase ($F-F_0$) based on the spectra in (c) as a function of the $Cu^{2+}$ concentration. Error bars indicate variations from three measurements. Inset: the corresponding log−log plot.

salinized counterparts (*i.e.*, OH-capped QDs). The quantum yield was approximately 30% using rhodamine 6G in ethanol as a standard.

Carboxyl-silanized QDs were generated by the following procedures described in the literature.[42] The amino-silanized QD solution was precipitated using anhydrous chloroform. The wet precipitate (100 mg) was suspended in 5 mL of THF, and 10 mg of succinic anhydride was added under sonication. The suspension was stirred for 16 h at room temperature, and then THF was removed by a rotary evaporator. The residue was dissolved in 2 mL of deionized $H_2O$ and then filtered through a 0.2 μm syringe filter. The excessive succinic anhydride was removed by a NAP-25 column with deionized $H_2O$. The carboxyl-silanized QDs were concentrated with a centrifuge (8000 rpm) using a 100 000 MWCO Nanosep centrifugal filter.
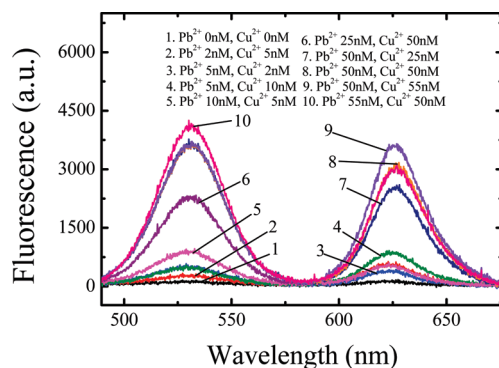


**Figure 7. Multiplexed detection of heavy metal ions using QD−DNAzyme sensors.**

**Preparation of DNAzymes and QD−DNAzyme Sensors.** The names and the modifications of the DNA sequences are given in Table 1. The sequence selection was based on the work by Lu *et al.*[19,20] Each DNAzyme was designed with two quenchers to efficiently quench the QD fluorescence. For the $Pb^{2+}$-specific DNAzyme, the substrate strand was 5′-end labeled with the amino group to couple with the carboxyl group on QDs and the 3′-end was labeled with Iowa black FQ. The enzyme strand was 3′-end labeled with Iowa black FQ. For the $Cu^{2+}$-specific DNAzyme, the substrate strand was 5′-end labeled with Iowa black RQ and the 3′-end was labeled with the amino group. The enzyme strand was 5′-end labeled with Iowa black RQ.

To form the $Pb^{2+}$-specific DNAzyme, a total of 0.8 μM enzyme (Pb-Enz) and 0.8 μM corresponding substrate (Pb-Sub) were annealed in 50 mM trisacetate buffer, pH 7.2 with 25 mM NaCl, in a 95 °C water bath for 5 min and subsequently cooled to 4 °C slowly. For $Cu^{2+}$-specific DNAzyme, 0.8 μM enzyme (Pb-Enz) and 0.8 μM corresponding substrate (Pb-Sub) were annealed in 50 mM HEPES, pH 7.0 buffer, with 1.5 M NaCl by heating in a 95 °C water bath for 5 min and then cooled to 4 °C slowly. The annealed DNAzyme (0.8 μM, 500 μL) was mixed with carboxyl-silanized QDs (50 nM, 500 μL), and then EDC (0.2 mg) and sulfo-NHS (0.1 mg) dissolved in 50 μL of phosphate buffer (25 mM, pH 6.5) were added. After 12 h of incubation at 4 °C, unreacted EDC/sulfo-NHS and excessive DNA were removed by NAP-25 column with 1× PBS, and the purified QD−DNAzyme conjugates were stored in a 4 °C dark room. The QD−DNAzyme was concentrated with a centrifuge (8000 rpm) using a 100 000 MWCO Nanosep centrifugal filter with the final concentration of 25 nM.

**Spectroscopy of Heavy Metal Detection.** Detection of heavy metal ions took place by mixing 90 μL of the 25 nM QD−DNAzyme and 10 μL of different concentrations of metal ion stock solutions in a microquartz cuvette at room temperature. The metal

ion solutions were prepared by diluting lead(II) acetate trihydrate and cupric chloride with deionized $H_2O$. For the multiplexed heavy metal ion detection, 180 μL of the mixture of $Pb^{2+}$-specific QD530−DNAzyme and $Cu^{2+}$-specific QD625-DNAzyme (25 nM each) was mixed with a 20 μL mixture of $Pb^{2+}$ and $Cu^{2+}$ solutions in a quartz cuvette at room temperature. For the $Cu^{2+}$ and multiplexed ion detection, ascorbate was added to a final concentration of 50 μM. A 480 nm laser was used for QD excitation. The fluorescence spectra were recorded by a USB 4000 miniature fiber optic spectrometer (Ocean Optics, FL) at a scan rate of 300 ms for post-analysis.

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