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# Engineering a Unimolecular DNA-Catalytic Probe for Single Lead Ion Monitoring

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***Abstract:*** The binding of proteins and small molecules by DNA is well established, but more recently, DNA molecules have been selected to catalyze biochemical reactions. These catalytic DNAs, or DNAzymes, can be activated by metal ions. In this paper, we take advantage of DNA molecular engineering to improve the properties of DNAzymes by designing a unimolecular probe for lead ion ($Pb^{2+}$)-catalyzed reaction, achieving in turn, the ability to monitor a single $Pb^{2+}$ in solution by fluorescence microscopy. Specifically, by applying a unimolecular design, a leaving substrate DNA strand labeled with a fluorophore is linked to a hairpin 8-17 DNAzyme sequence labeled with a quencher. The hairpin structure and the substrate are connected using poly T, which brings the quencher into close proximity with the fluorophore in the inactive state. The intramolecular linkage of the two strands assures efficient quenching of the fluorescence, generating almost zero background. In the presence of $Pb^{2+}$, however, the leaving substrate fragment is cleaved at the RNA site by the enzyme, releasing a fluorescent fragment for detection with repetitive cycling for signal amplification. The resulting high sensitivity with a quantifiable detection range from 2 nM to 20 $\mu$M was achieved with a high selectivity in excess of 80-fold for $Pb^{2+}$ over other metal ions. The limit of detection is about 167 times better than the previously reported similar probes (Liu, J; Lu, Y. *Anal. Chem.* **2003**, *75*, 6666−6672) and 1600 times better compared to the $Pb^{2+}$ detection limit obtained from atomic spectroscopy. Thus, this probe could provide a simple, yet rapid and sensitive measurement for $Pb^{2+}$. Furthermore, we used this probe to monitor single $Pb^{2+}$ reaction kinetics. Given this degree of sensitivity and selectivity, our new probe design may prove useful in the development of other nucleic acid-based probes for intracellular, toxicological, and environmental monitoring.

## Introduction

Nucleic acid probes represent a new class of detection tools that possess unique features.[2] The targets of nucleic acids can range from ions to small organic and inorganic molecules,[3−5] other nucleic acids,[6] peptides,[7] proteins,[8] and even whole living cells.[9] The selected sequences are generally very specific with excellent binding affinity to targets. Moreover, the ability to easily modify nucleic acids using highly advanced phosphoramidite chemistry allows the coupling of various signal transduction mechanisms for improved sensing properties. Examples include fluorescence, fluorescent anisotropy, fluorescent lifetime, fluorescence resonance energy transfer (FRET), electrochemistry, surface plasmon resonance (SPR), radiolabeling, and other signaling processes. Among the many studies demonstrating the potential of nucleic acid probes, those involving catalytic DNAs, termed DNAzymes, report especially interesting properties.

DNAzymes are DNA sequences, or deoxyribozymes, that catalyze chemical reactions, such as cleaving ribonucleic acid targets.[10] Among the DNAzymes attracting most attention are those that may be termed cation specific. Cation-specific DNAzymes are composed of two functional domains: a catalytic loop that recognizes specific ions employed as coenzyme and a binding arm that targets its complementary sequence, or substrate. When the DNAzyme is hybridized with its target substrate sequence and the desired cation binds to the catalytic loop, hydrolysis of the target substrate sequences is activated. Because of the lower affinity of the cleaved substrate, the DNAzyme can bind to yet another substrate so that it can be recycled for hydrolysis of multiple substrates in a manner similar to that of enzymes with their substrate target. Researchers have taken advantage of this unique enzyme-like feature of DNA probes by developing new classes of therapeutic agents to

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selectively down-regulate the translation of target mRNAs.[11] Still another application involves the development of 8-17 DNAzyme-based sensors for metal detection.[12] In fact, the design of fluorescent metal sensors and chemodosimeters has recently become one of the most active research areas because of the *in situ* and real-time information they provide in a variety of applications. Examples include monitoring environmental pollution,[13] metalloneurochemistry,[14] and biomedical diagnostics.[15] DNAzymes have also been shown to catalyze many of the same reactions as RNA or protein enzymes,[16] but DNAzymes are relatively less expensive to produce and possess more stable hydrolysis than RNA and protein enzymes.[17] Exceeding the capability of protein enzymes, most DNAzymes can be denatured and renatured many times without losing binding ability or activity.[17]

The development of lead ion sensors is an attractive research area, particularly since lead is an integral component of living systems and a common environmental contaminant.[18] Lead exposure can occur through a variety of sources, including air, bare soil, home remedies, drinking water, toy jewelry, and others.[19−21] Moreover, produce and other foods may be contaminated by dust exposure during growing or processing, and food containers add another source of contamination.[22] Low-level lead exposure is known to cause a number of adverse health effects.[23] For example, when exposed to lead, physical or mental development in infants and children might be delayed.[24] Furthermore, children can show slight deficits in attention span and learning abilities,[25] whereas adults may have kidney problems and high blood pressure.[26] As announced by the United States Environmental Protection Agency (EPA), the level of lead in the blood is considered toxic when it is ≥480 nM. In drinking water, lead is regulated by a treatment technique that requires systems to control the corrosiveness of their water. If more than 10% of tap water samples exceed the action level (0.015 mg/L),[27] water systems must take additional steps. Therefore, as a matter of public health, there is an urgent need

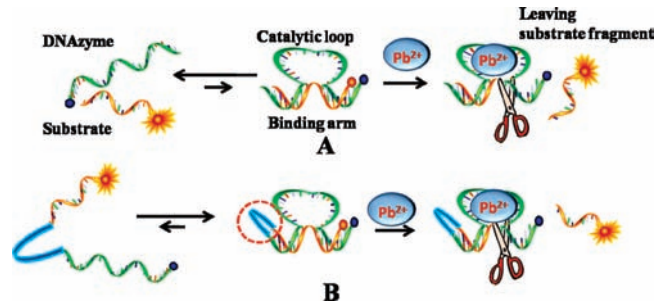


***Figure 1.*** Schematic of the hairpin-structured, DNAzyme-based $Pb^{2+}$ probe and its working principle. (a) the 8-17 DNAzyme strand, which is labeled with a quencher, and substrate, which is labeled with a fluorophore, hybridized together in Tris-acetate buffer. After lead-induced cleavage, a 10-mer ssDNA is released which induces the fluorescence enhancement. (b) The original substrate and enzyme were linked together as a hairpin structure by poly-T linker. The hairpin structure tends to maximize the hybridization efficiency.

to detect lead contaminants, either in solution *in situ* or in biological samples *in vivo*. To address this need, it has been reported that $Pb^{2+}$-specific 8-17 DNAzyme can be coupled to fluorescent signaling mechanisms to produce a metal biosensor with improved sensitivity and selectivity. However, it is also clear that such development still involves complicated modifications to 8-17 DNAzyme, with two problems posing particular challenges. First, as shown in Figure 1A, the 8-17 DNAzyme might not be close enough to its target substrate to achieve the best annealing of the DNAzyme and substrate strands. This results from the repulsive forces of steric interactions that prevent straightforward bonding and, therefore, decrease the hybridization efficiency otherwise required of the DNAzyme and its target substrate by bringing those two strands close together. Second, as a consequence of the catalytic loop sequence, where the DNAzyme is flanked in the middle, these two pieces do not form a stable duplex, but rather a loose and unstable one. Together, these two problems cause lower hybridization efficiency, resulting in a limited amount of active DNAzymes and minimized hydrolysis reaction. Under these conditions, a simple quenching mechanism is not sensitive enough to detect low concentrations of $Pb^{2+}$, even though the selectivity is superior.

To address these problems, we proposed to covalently link the DNAzyme and leaving substrate fragment with polythymine to create a strong intramolcular interaction. Specifically, the leaving substrate fragment is labeled with a fluorophore, the conventional enzyme fragment is labeled with a quencher, and these two pieces are linked together with poly T. The resulting intramolecular interaction tends to be strong enough to allow the hybridization of short base-paired sequences that could not otherwise form stable duplexes at room temperature via bimolecular interaction. By this intramolecular assembly method, a new type of probe is developed which possesses four functional domains: (1) a catalytic sequence which is a DNAzyme sequence, (2) a leaving substrate fragment that can be hybridized with the catalytic sequence, (3) a linker that ties together the 8-17 DNAzyme fragment and the leaving substrate fragment sequences, and (4) a signaling domain composed of fluorophore and quencher. In the absence of $Pb^{2+}$, the probe forms a stable hairpin structure such that fluorescence is completely quenched by intramolecular linkage. However, in the presence of $Pb^{2+}$ ion, the substrate sequence is cleaved, and the strand containing the fluorophore is released by lack of sufficient binding affinity that is otherwise required to maintain the duplex. It is the strong

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***Table 1.*** Names and Sequences of DNA Used in the Paper[a]

| name | sequences |
|---|---|
| $D_{10}$ | 5′-/Dabcyl/-TATCTCTTCTCCGAGCCGGTCGAAATAGTGAG-$(T)_{10}$ACTCACTATrAGGAAGAGATA-/FAM/-3′ |
| $D_7$ | 5′-/Dabcyl/-ATCTTCCGAGCCGGTCGAAATAGTGAG-$(T)_{10}$-ACTCACTATrAGGAAGAT-/FAM/-3′ |
| $D_5$ | 5′-/Dabcyl/-ATTCCCCGAGCCGGTCGAAATAGTGAG-$(T)_{10}$-ACTCACTATrAGGAAT-/FAM/-3′ |

[a] rA is the RNA monomer. All synthesis was done with ABI synthesizer. The DNA/RNA chimers were purified by HPLC.

quenching combined with high hydrolysis efficiency that allows the high sensitivity for the detection of $Pb^{2+}$. While this probe design utilizes intramolecular hybridization/dehybridization, it does not change the catalytic core of the DNAzyme, which maintains ribonucleotide cleavage efficiency and simplifies probe optimization. Theoretically, this probe design, which relies on intramolecular interaction, can therefore be used to engineer a prototype DNAzyme/substrate probe for any two given strands. In this paper, we report the design, molecular engineering, and optimization of the probe in terms of substrate length and linker, and we characterize the performance of the $Pb^{2+}$ probe, challenging its detection limit down to the single $Pb^{2+}$ ion.

## Experimental Section

**Chemicals and Reagents.** All DNA synthesis reagents, including 6-fluorescein (FAM) phosphoramidite, 5′-4-(4-dimethylaminophenylazo)benzoic acid (Dabcyl) phosphoramidite, and 2′-*O*-triisopropylsilyloxymethyl-protected RNA monomers were purchased from Glen Research. Lead acetate and all reagents for buffer preparation and HPLC purification were from Fisher Scientific. The buffer used for the experiment contained 50 mM Tris-acetate at pH 7.2 and 100 mM NaCl.

**Synthesis and Purification of Fluorescent-Labeled Oligonucleotides.** To optimize the design of the hairpin probe, multiple candidates were designed and prepared (Table 1). All of them were synthesized using an ABI 3400 DNA/RNA synthesizer (Applied Biosystems) at 1 $\mu$mol scale with the standard phosphoramidite chemistry.[28,29] After complete cleavage, deprotection, and ethanol precipitation, the precipitates were dissolved in 0.5 mL of tetrabutylammonium fluoride solution, with shaking for 6 h at 35 °C. Then, desalting was performed with the 0.1 M triethylammonium acetate (TEAA, pH 7.0) as elution buffer. The HPLC analysis was performed on a ProStar HPLC Station (Varian Medical Systems) equipped with a fluorescence detector and a photodiode array detector. A $C_{18}$ reverse-phase column (Alltech, C18, 5 $\mu$m, 250 mm × 4.6 mm) was used.

**Determination of the Melting Temperature.** Using a BioRad RT-PCR thermal cycler, thermal denaturizing profiles of each probe were measured to study the thermostability of the designed probes. The substrate and enzyme molecular beacon chimer was used in the fluorescence-based method to determine the melting temperature. $D_{10}$, $D_7$, and $D_5$ were dissolved to the final concentration of 50 nM each in 50 nM Tris-acetate buffer, pH 7.2, with 100 mM NaCl, then annealed by heating to 90 °C for 5 min and subsequently cooled to 4 °C in intervals of 1 °C. The sample was kept at target temperature for at least 1 min after the temperature was reached to ensure that the sample was at the stated temperature. Upon melting, the leaving substrate fragment was dissociated from the 8-17 DNAzyme strand, resulting in an increased fluorescence signal. The fluorescence intensity of each MB was measured and plotted against the temperature from 90 to 4 °C to generate the melting temperature curve.

**Hybridization Assay.** The mechanism of applying hairpin sequences to monitor DNA cleavage is shown schematically in Figure 1. $D_{10}$ was annealed to a final concentration of 200 nM using the same procedure performed in the melting temperature determination. The annealed sample was then taken to room temperature for subsequent assays. A 90-$\mu$L aliquot of the 100 nM hybridized DNAzyme/substrate solution was loaded into each well of a 96-well plate. A 10-$\mu$L aliquot of concentrated metal ion stock solution was then added to the DNA solution using an eight-channel pipet to initiate the cleavage reaction. The fluorescence intensity was recorded for 100 $\mu$L of buffer containing 50 mM Tris-acetate (pH 7.2), 100 mM NaCl, the 200 nM DNAzyme/substrate solution without lead, and the 100 nM DNAzyme/substrate solution with 100× concentrated lead contamination. The excitation and emission wavelengths were set to 473 and 520 nm, respectively. Signal enhancement was calculated using the equation $(F_{\text{cleaved}} - F_{\text{buffer}})/(F_{\text{annealed}} - F_{\text{buffer}})$, where $F_{\text{cleaved}}$ corresponds to fluorescence signals from the dissociation of this fluorescence strand in the presence of $Pb^{2+}$, $F_{\text{annealed}}$ stands for the fluorescence signals from the hairpin probes without cleavage, and $F_{\text{buffer}}$ represents the fluorescence signals of buffer.

**Data Acquisition and Quantification.** Substrate fluorescence detection was performed with a Tecan Safire microplate reader with 96-well plates. The excitation laser wavelength was set at 473 nm, and the emission wavelength was set at 520 nm, respectively, to monitor the fluorescence of FAM. Data quantification was performed in the XFluor program. For each experiment, 90 $\mu$L of the $D_{10}$ solution was placed in the well, and the fluorescence of the sample was measured immediately as $F_{\text{annealed}}$. Then, 10 $\mu$L of concentrated $Pb^{2+}$ solution was added, followed by a 10-min incubation at room temperature. The fluorescence was taken, and then the solution in the well was collected and heated to 90 °C for 5 min. The fluorescence was measured again as $F_{\text{cleaved}}$. RNase free water was used as the internal standard to minimize the difference between each scan. Other metal salts used included the following: $Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, and $Hg^{2+}$.

**Single $Pb^{2+}$ Reaction.** We applied Nuclepore polycarbonate membranes (Fisher Scientific), which are used in filtration, to form reactors of femtoliter volumes. We used membranes with pore diameters of 5 $\mu$m. These pore sizes were uniform, and the material was confirmed in bulk studies to be chemically inactive for the enzymatic reaction in this work. The thickness was ~6 $\mu$m, producing volumes between 110 and 200 fL for each vial. Liquid filling of nanoscopic volumes has been a difficult technological problem in many fields, including biological studies where micromanipulators coupled with microinjection have been employed. No less a problem in this study, it was impossible to fill the vials one-by-one since we were simultaneously studying more than 100 vials. Therefore, the techniques applied here combined ultrasonic vibration with vacuum degassing.[30] We used rhodamine 6G dye solution to test this procedure. From the fluorescence image, we concluded that this procedure ensured that this surface was filled with the desired solution. The membrane was put onto a glass slide, and a small drop of the $D_{10}/Pb^{2+}$ solution was added onto the top of the membrane. A very thin quartz coverslip (0.08 mm) was placed on the top to cover the liquid-filled vials. The polycarbonate was sandwiched between two quartz plates to create the vials. A tight seal was expected to form between the glass slides and the quartz coverslip. The quartz coverslip prevented any evaporation from these small vials and prevented mixing among the vials during the monitoring of the enzyme reactions.

## Results and Discussion

**Design and Optimization of Unimolecular Probe for Catalytic Reaction to Generate Fluorescent Product.** Lead-dependent, site-specific cleavage of RNA has been the focus

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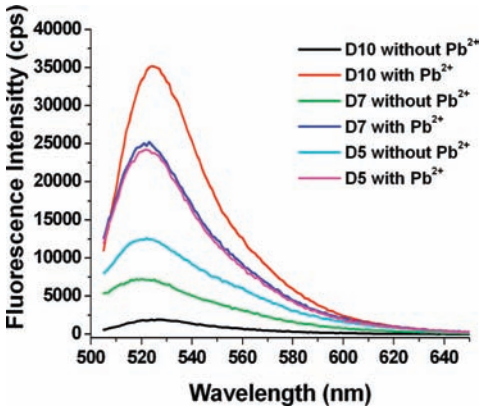


***Figure 2.*** Fluorescence signal in the absence and presence of 10 $\mu$M $Pb^{2+}$ after 10 min. The $Pb^{2+}$ sensor was in Tris acetate buffer (pH 7.2) with 100 mM NaCl; the image was scanned 10 min after addition of metal ions. The five base-paired $D_5$ (green) gave the highest background fluorescence; meanwhile, the $D_{10}$ (black) showed the lowest background. After a 10 min incubation, D10 showed a higher fluorescence signal (red) than either $D_7$ or $D_5$.

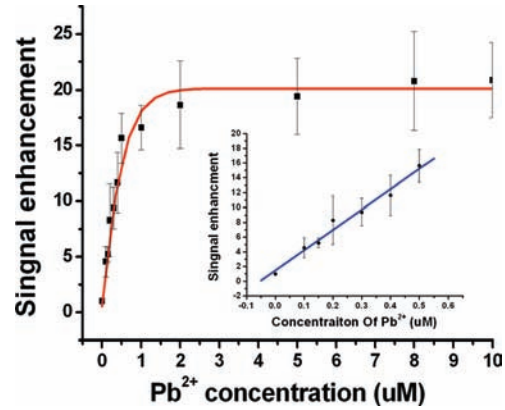


***Figure 3.*** Fluorescence increase over background fluorescence at varying $Pb^{2+}$ concentrations. The DNAzyme sensor concentration was 200 nM, and the buffer contained 50 mM Tris-acetate (pH 7.2) and 100 mM NaCl. (Inset) Probe responses to low concentrations of $Pb^{2+}$.

of many research endeavors.[31−33] We have designed a new probe for the $Pb^{2+}$-initiated catalytic reaction. We analyzed the 8-17 DNAzyme profile and designed a hairpin-structured $Pb^{2+}$ probe. This 8-17 DNAzyme preformed the catalytic cleavage reaction with a two-step mechanism. Product analysis by MALDI-MS demonstrated that $Pb^{2+}$ first catalyzed the cleavage reaction of the 8-17 DNAzyme by the formation of a product containing 2′,3′-cyclic phosphate. In a second catalytic reaction, $Pb^{2+}$ further hydrolyzed the 2′,3′-cyclic phosphate. To increase the cleavage reaction efficiency, we constructed a hairpin structure lead probe with the substrate in close proximity to the DNAzyme, ensuring hybridization efficiency. With this design structure, the lead binding pocket was well formed before the target $Pb^{2+}$ was added, thus increasing the apparent cleavage efficiency.

To maximize probe performance, the design of the probes needed to be optimized, including the length of linker between the catalytic and substrate domains and the design of the leaving substrate fragment. The design of the leaving substrate fragment is especially critical because it requires a sequence that is long enough to form a stable duplex with the 8-17 DNAzyme sequence domain, but short enough to be dissociated well after the hydrolysis reaction. To accomplish this, we investigated varying the substrate sequences by measuring background fluorescence and melting temperature of the hairpin structures to compare hybridization efficiency and by using gel electrophoresis to compare hydrolysis efficiency (see sup).

To implement this plan, we designed probes containing 5, 7, and 10 base-paired leaving sequences, termed $D_5$, $D_7$ and $D_{10}$, respectively. No probe was designed containing a longer substrate because dissociation with a base pair in excess of 10 would, most likely, not have been favorable by its strong binding effect.[34] As shown in Figure 2, the fluorescence background of the probes without $Pb^{2+}$ was significantly reduced with increasing numbers of base pairs. The low background is explained by the fact that probes can have quenched fluorescence signal only when they form stable hairpin structures. In the presence of $Pb^{2+}$, the stable hairpin structures bind the ions, switching into active state to catalyze the hydrolysis reaction. Thus, $D_{10}$, which had the lowest fluorescence background, still possessed the maximum efficiency in catalytic reaction, giving an approximate 16-fold signal enhancement when the $Pb^{2+}$ was added. This signal enhancement is outstanding and much higher than the previously reported 4-fold enhancement with single quencher design[35] or the 6-fold with a dual quencher design.[1] The reduced background can be easily explained by the enhanced duplex stability resulting from intramolecular hybridization. As noted above, such duplex stability is achieved if fluorophore and quencher are in close proximity, and, in our design, quenching efficiency is well correlated to duplex stability, as defined by thermo denaturing profile. The $T_m$ of D5 was only 45 °C, while that of $D_{10}$ is increased to 54 °C (see Supporting Information). The $T_m$ of unlinked DNAzyme and its substrate is only 34 °C, which is slightly above room temperature. Since, as just noted, our design correlates quenching efficiency to duplex stability, this enhanced stability is quite significant because, at room temperature, a stable duplex of DNA would not be formed with such a high percentage if the two DNA probes were not linked in this novel unimolecular design.

This enhanced stability of duplex also resulted in improved hydrolysis reaction. This was first demonstrated by the cleavage reaction which was detected within 2 min through fluorescence measurement, much faster than the previous design. Second, increased turnover numbers were calculated from the kinetics study. This result indicates the presence of a large amount of well-formed lead binding pockets, which, in turn, helped improve metal ion binding to $D_{10}$. Therefore, the increased hybridization efficiency which naturally accompanies intramolecular interaction positively correlates to the apparent increase in reaction rate. According to the result obtained from denaturing PAGE-gel electrophoresis (see Supporting Information), only $D_{10}$ could show an efficient cleavage reaction over the others.

**Characterizing the Probe with Analytical Parameters.** To further obtain the full profile of the performance of $D_{10}$, we determined the dose response, reaction kinetics, calibration curve, and limit of detection. Figure 3 shows the full profile of dose-dependent fluorescence signal enhancement and the linear response in the presence of a low concentration of $Pb^{2+}$.

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According to the full profile (Figure 2), $D_{10}$ can have up to a 20-fold signal enhancement with catalyzation of $Pb^{2+}$ ions over the background with no $Pb^{2+}$ catalyzation. This large signal enhancement allows us to detect $Pb^{2+}$ within a large dynamic range of nanomolar concentration with excellent detection limit, approximately 3 nM ($3\sigma$/slope). Such a low limit of detection is about 167 times better than that previously reported[1] and 1600 times greater compared to the $Pb^{2+}$ detection limit obtained from atomic spectroscopy, which was 4.8 $\mu$M.[36] Using the EPA standard for maximal lead contamination of drinking water at levels higher than 15 ppb $Pb^{2+}$, $D_{10}$, with its dynamic sensitivity range, can easily function well within that metric. In addition to the excellent sensitivity of $D_{10}$, this probe also showed fast kinetics in cleavage reaction (see Supporting Information). According to our investigation, it took much longer to reach this plateau (more than 30 min (data not shown)) in the case of the bimolecular approach (8-17 DNAzyme and its substrate). The superior performance of $D_{10}$ can be attributed to several factors associated with the molecular beacon-based sensing strategy, i.e., where an intramolecular interaction, as opposed to an intermolecular interaction, is brought between DNAzyme sequence and leaving substrate fragment. First, since $D_{10}$ mostly remains in the binding-active state, the probe itself binds $Pb^{2+}$ very strongly in a short period of time. Second, the probe design allows very low background fluorescence or background variation, which produces high signal increases. Finally, and probably most unique, the signal can be amplified through fast turnovers of $Pb^{2+}$ to react with multiple $D_{10}$, thus driving sensitivity up with fast dynamic response.

One of the most significant features of the DNAzyme-based metal probe is the excellent selectivity. This selectivity of artificial nucleic acid sequences to their targets derives from the unique secondary configurations enabling the probes to discriminate nontargets. 8-17 DNAzyme also possesses the selectivity needed to distinguish $Pb^{2+}$ out of nine competing bivalent ions. Thus, to demonstrate the excellent selectivity of $D_{10}$ for $Pb^{2+}$ against other metal ions, the fluorescence signal changes of nine competing divalent metals at concentrations of 10, 5, and 1 $\mu$M were obtained (Figure 4). As expected, they induced little to no fluorescence change, whereas some metals induced strong quenching to FAM, such as $Cu^{2+}$, $Fe^{2+}$, and $Hg^{2+}$. This result indicates that the intramolecular engineering of $D_{10}$ did not affect the selectivity of $Pb^{2+}$.

**Single $Pb^{2+}$ Ion Reaction.** According to the preliminary investigation, $D_{10}$ is a highly sensitive and selective probe for $Pb^{2+}$-participating reactions. To further challenge the limit of $D_{10}$'s detection capability, we designed a single $Pb^{2+}$ detection scheme using a femtoliter-well reactor. When a single $Pb^{2+}$ remains inside such a reactor long enough to catalyze the cleavage reaction, a significant amount of fluorescent product can be produced for sensitive detection. We used membrane holes of approximately 5 $\mu$m in diameter which contained a volume of ~120 femtoliter. When it contained a single $Pb^{2+}$, the concentration was about 14 pM, which was well below the limit of detection of $D_{10}$. We employed a $Pb^{2+}$ concentration (3.5 pM) by an amount statistically filling only 25% of the vials to minimize the chance that there would be more than 2 $Pb^{2+}$ per single vial. The fluorescence signal was recorded using $D_{10}$ only as a negative control (200 nM). We recorded the signal enhancement using $D_{10}$ with $Pb^{2+}$ as the sample. The obtained result is summarized in Figure 5. Single-ion reaction was monitored every 10 s for 1400 s. Since each image contained about 30−60 vials in most of the experiments, the fluorescence signal of each vial was analyzed, and a reaction rate curve was constructed. When the membrane vials were filled with only the $D_{10}$ solution, we did not observe any significant fluorescence intensity change during the monitoring. This means that the $D_{10}$ can form a stable hairpin structure without significant surface interaction. The probe was still stable, and no fluorescence increase was observed over a short period of time. After that, we added the desired amount of $Pb^{2+}$ and continued to monitor the fluorescence. In contrast to the blank sample containing only $D_{10}$, we could now observe three typical patterns of fluorescence increases from the $Pb^{2+}$-treated samples in membrane holes: negligible, slight, and large fluorescence changes (blue, red, and black, respectively, in Figure 5A). By the distribution of such different reaction rates, it was demonstrated that most fluorescence enhancement originated from the least number of membrane pores. However, most of the fluorescent pores showed only limited fluorescence enhancement. We therefore concluded that the differences in fluorescence enhancement must have been correlated to the presence or absence of $Pb^{2+}$ in the holes. We believe that the fluorescence increase was from hydrolysis reaction upon the addition of $Pb^{2+}$. Interestingly, despite the low concentration of $Pb^{2+}$ ion, $D_{10}$ was still able to bind the single target referring to 14 pM and, as a result, generate a detectable fluorescence signal under the microscope. In addition, the fluorescence signal reached a plateau in a relatively short amount of time (less than 15 min).

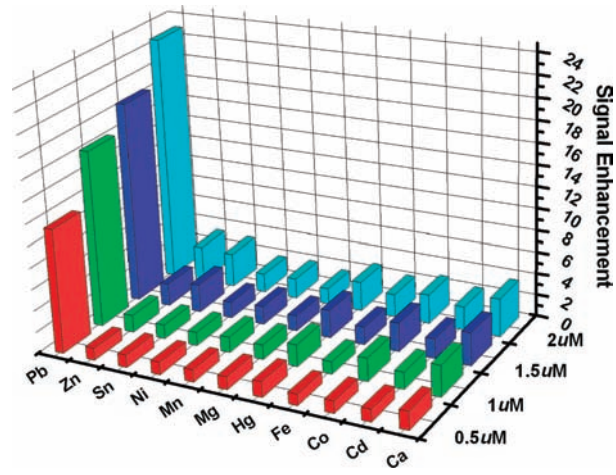


***Figure 4.*** Selectivity of the $Pb^{2+}$ probe sensor. Sensor responses to all competing metal ions at four concentrations (10, 5, 2, and 0.5 $\mu$M) were tested. The reaction time was 10 min.

## Conclusion

We have demonstrated a highly sensitive and selective DNAzyme-based $Pb^{2+}$ probe with DNA hairpin structure for $Pb^{2+}$-catalyzed reaction, leading to the ability to monitor a single $Pb^{2+}$ in solution by means of fluorescence detection. Specifically, in our system, the leaving substrate fragment is labeled with a fluorophore, and an 8-17 DNAzyme sequence is labeled with a quencher. The hairpin structure links these two strands using poly T, which brings the quencher into close proximity with the fluorophore in the

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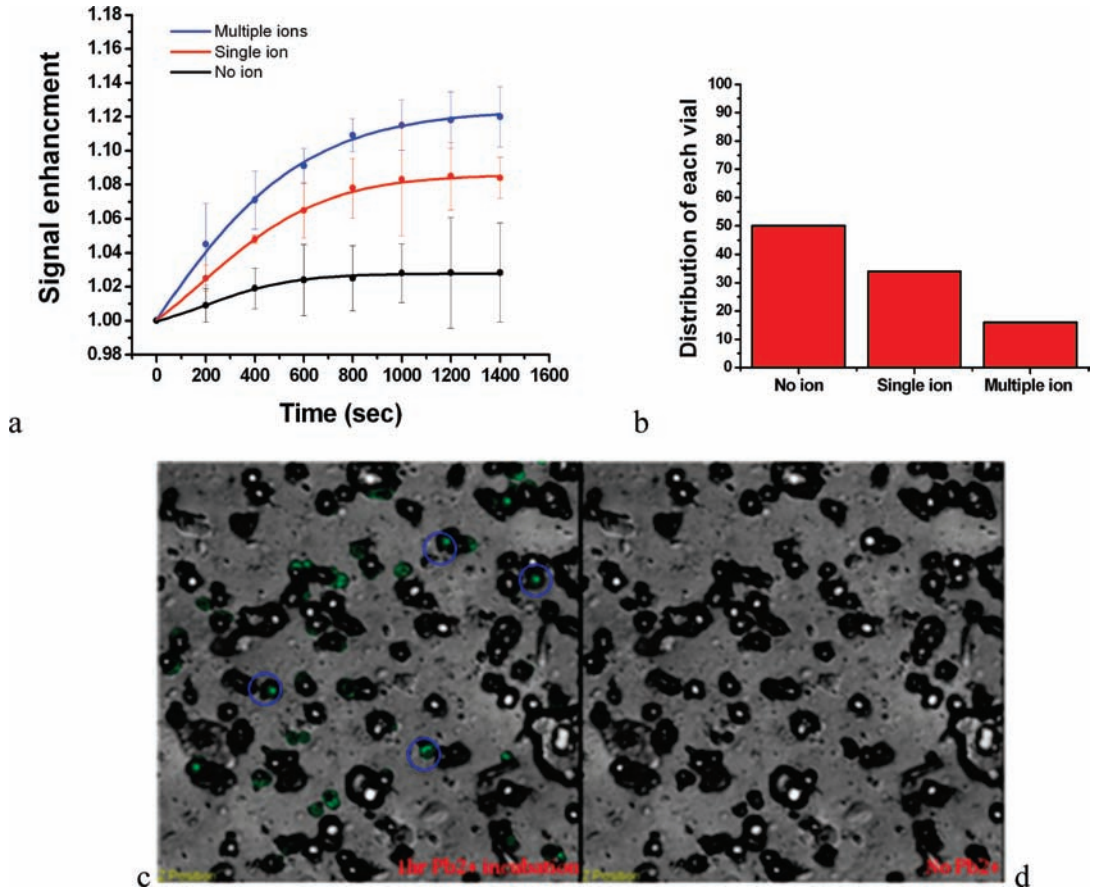


***Figure 5.*** Single-ion reaction kinetics inside polycarbonate membrane vials. (a and b) Individual $Pb^{2+}$ ions were trapped inside membrane holes with the $D_{10}$ solution. (c) The membrane picture after 1 h $Pb^{2+}$ incubation. (d) The membrane picture without $Pb^{2+}$ incubation. The blue line in (a) represents the dots in the blue circle in (c). All the other bright dots in (c) were recorded to the red line in (a). All the dark dots are represented by the black line in (a). The fluorescence enhancement was monitored over time. The background-corrected intensity in each vial has been normalized.

inactive state. The intramolecular linkage of the two strands assures efficient quenching of the fluorophore's fluorescence, surpassing the background. Upon reaction with $Pb^{2+}$, however, the leaving substrate fragment is cleaved at the RNA site by the enzyme, releasing a fluorescent fragment for detection. We have achieved a 16-fold increase of fluorescence intensity. This intramolecular design maintains a quantifiable detection range from 3 nM to 20 $\mu$M. We further demonstrated that this probe can be used to detect single $Pb^{2+}$ by having one $Pb^{2+}$ in a femtoliter reactor with sufficient substrate and about 15 min of reaction time. We believe that this probe could provide a simple and cost-effective, yet rapid and sensitive, measurement tool for $Pb^{2+}$ detection. Given this degree of sensitivity and selectivity, our molecular engineering design may prove useful in the future development of other nucleic acid−based probes for toxicological and environmental monitoring.

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**Supporting Information Available:** Additional figures and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org/.

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