New Catalytic DNA Fluorescent and Colorimetric Sensors for On-site and Real-time Monitoring of Industrial and Drinking Water

Yi Lu
Department of Chemistry
University of Illinois at Urbana-Champaign

RR-114
February 2009
www.istc.illinois.edu
New Catalytic DNA Fluorescent and Colorimetric Sensors for On-site and Real-time Monitoring of Industrial and Drinking Water

Yi Lu
Department of Chemistry
University of Illinois at Urbana-Champaign

February 2009
Submitted to the
Illinois Sustainable Technology Center
Institute of Natural Resource Sustainability
University of Illinois at Urbana-Champaign
www.istc.illinois.edu

The report is available on-line at:
http://www.istc.illinois.edu/info/library_docs/RR/RR-114.pdf

Printed by the Authority of the State of Illinois
Patrick J. Quinn, Governor
This report is part of ISTC’s Research Report Series (ISTC was formerly known as WMRC, a division of IDNR). Mention of trade names or commercial products does not constitute endorsement or recommendation for use.
I. Acknowledgments

We wish to thank the Illinois Sustainable Technology Center, a division of the Institute of Natural Resource Sustainability at the University of Illinois at Urbana-Champaign, for their support of this work under grant no. HWR04187.
CONTENTS

I. Acknowledgments ................................................................. iii

II. Tables .................................................................................. vi

III. Figures ................................................................................ vii

IV. Abstract ................................................................................ ix

1. Introduction .............................................................................. 1
   i. The need for metal sensor technology for water monitoring .......... 1
   ii. Designing metal sensors ........................................................... 1
   iii. Advantage of catalytic DNA as sensors ..................................... 2
   iv. Advantages of fluorescent sensors .......................................... 3
   v. Advantages of colorimetric sensors ......................................... 3

2. Results and discussion .............................................................. 3
   i. In vitro selection of catalytic DNA for sensing applications .......... 3
      1) In vitro selection scheme ....................................................... 4
      2) Uranium ........................................................................... 4
      3) Arsenic ........................................................................... 4
      4) Other metal ions ................................................................. 5
   ii. Design of fluorescent uranium biosensors: a catalytic beacon sensor with parts-per-trillion sensitivity and million-fold selectivity .................. 6
   iii. Design of fluorescent copper biosensors .................................... 9
   iv. Design of fluorescent mercury biosensors ................................ 11
   v. On-site Pb\(^{2+}\) detection using a DNAzyme biosensor coupled with a portable fluorometer ................................................................ 14
      In simulated water samples with various hardness ...................... 16
      Real sample test; Crystal Lake water in Urbana, IL ..................... 16
   vi. Colorimetric Pb\(^{2+}\) sensors based on DNAzyme-assembled gold nanoparticles .......................................................... 18
   vii. Towards more practical applications: simple “dipstick” tests ........ 19
   viii. The shelf-life and operation of the sensor in a real-world setting .... 20
3. Summary and Outlook ........................................................................................................ 21

4. References ........................................................................................................................ 23
II. Tables

1. Reaction time and detection limits calculated from each standard curve of fluorescence versus [Pb$^{2+}$] in the simulated water samples with different hardness and Crystal Lake water sample ......................................................................................................................... 18
III. Figures

1. *In vitro* selection protocol. See text for descriptions ........................................ 5

2. Design of a catalytic beacon to detect UO$_2^{2+}$ .................................................. 7

3. Sensitivity of the catalytic beacon-based UO$_2^{2+}$ sensor .................................... 8

4. Selectivity of the catalytic beacon-based UO$_2^{2+}$ sensor ..................................... 9

5. (A) The secondary structure of the Cu$^{2+}$ sensor; (B) Signal generation mechanism of the Cu$^{2+}$ catalytic beacon; (C) Fluorescence spectra of the sensor before and 10 min after addition of 20 µM Cu$^{2+}$; Inset: gel-based assay of the sensor DNAzyme ........................................ 10

6. (A) Kinetics of fluorescence increase over background at varying Cu$^{2+}$ levels. Inset: responses at low Cu$^{2+}$ levels; (B) The rate of fluorescence enhancement plotted against Cu$^{2+}$ concentration. Inset: rates at the low Cu$^{2+}$ region; (C) Sensor selectivity ................................................................. 11

7. (a) The secondary structure and modification of the Hg$^{2+}$ sensor DNAzyme; (b) Fluorescence spectra of the sensor in the absence and 8 min after addition of 0.5 µM Hg$^{2+}$; (c) Schematic presentation of the sensor design ................................................................. 12

8. Sensitivity of the Hg$^{2+}$ sensor ................................................................. 13

9. Sensor selectivity ................................................................. 14

10. (A) Secondary structure of the DNAzyme; (B) Design of a catalytic beacon sensor with a fluorophore and two quenchers ................................................................. 15

11. Fluorescence increases at different reaction time by Pb$^{2+}$ ions in Millipore water ..... 15

12. Standard curves of fluorescence enhancement versus Pb$^{2+}$ concentrations in simulated water samples with different hardness (a-e) and in Crystal Lake water (f) ....................... 17
13. (A) The secondary structure of the Pb\textsuperscript{2+}-specific DNAzyme; (B) In the presence of Pb\textsuperscript{2+}, the substrate is cleaved into two pieces; (C) Pb\textsuperscript{2+}-directed assembly of DNAzyme-linked nanoparticle aligned in a head-to-tail manner; (D) UV-vis spectra of disassembled (red) and assembled (blue) gold nanoparticles; (E) The assembly state or color of DNAzyme-linked nanoparticles in response to metal ions monitored by a spectrophotometer, or on a TLC plate (F) .............................................................. 19

14. Dipstick detection of toxic metal ions such as Pb\textsuperscript{2+} based on catalytic DNA and gold nanoparticles ............................................................. 20

15. (A) Stability study of the catalytic DNA-based lead sensor after drying at different temperatures. Stability at 45 °C (B) and 80 °C (C). (D) Stability of DNA attached to gold nanoparticles ............................................................. 21
IV. Abstract

We have developed new fluorescent and colorimetric sensor technologies for on-site, real-time detection and quantification of toxic metal ions such as lead, mercury and uranium in industrial and drinking waters. We used a combinatorial biology method called *in vitro* selection to obtain catalytic DNA with high specificity and selectivity for the metal ions. By labeling the DNA with either fluorophore/quencher pairs or gold nanoparticles, we have transformed the catalytic DNA into a highly sensitive and selective fluorescent or colorimetric biosensor, respectively. The presence of metal ions causes the catalytic DNA to cleave, resulting in either a dramatic increase of fluorescent signals or a distinctive change of colors. The sensors are highly sensitive (with detection limit as low as 11 ppt), and selective (with selectivity of over millions fold). The catalytic DNA fluorescent biosensors make it possible to analyze metal ions using simple portable fluorometers, and the catalytic DNA colorimetric biosensors can eliminate equipment altogether. This is possible because the toxic metal ions can be detected through simple color changes, just like pH paper.
1. Introduction

i. The need for metal sensor technology for water monitoring. According to publications from the Illinois Department of Public Health (IDPH), lead poisoning is the No. 1 environmental illness effecting children. In Illinois, ~25,000 children per year are identified with elevated blood lead levels. One of the main sources of lead in the environment is lead-based paint. About 75% of homes built before 1978 contain some leaded paint. In addition, IDPH has issued special mercury advisory, cautioning Illinois residents about the danger of eating certain fish species containing mercury in selected Illinois rivers and lakes. Finally, a recent report indicated that up to one-third of private wells in selected counties surrounding the Mahomet aquifer have elevated levels of arsenic. This aquifer is the main water source for central Illinois. Therefore, it is important to identify and quantify these and other toxic metal ions in our environment. New materials and technology have been developed to reduce or eliminate those type of ions. Assessing the success of the materials and technology in removing those ions from water requires new analytical techniques.

Analytical techniques, such as atomic absorption spectrometry, inductively coupled plasma mass spectrometry, anodic stripping voltammetry, capillary electrophoresis, x-ray fluorescence spectrometry and microprobes have been routinely used for metal ion analysis with high sensitivity (often ≤ ppb level). Many of them can quantify many metal ions simultaneously. However, it is generally believed that most of the above techniques require sophisticated equipment, sample pretreatments, or skilled operators, making it difficult to do on-site, real-time monitoring of metal ions. Due to the toxicity these metal ions may pose to operators, remote sensing devices are desirable. While important progress has been made to miniaturize many of the above analytical instruments, design of sensitive and selective metal sensors using either cost-effective and portable equipment or no equipment at all provides an effective alternative means of achieving the goal of industrial and drinking water monitoring.

ii. Designing metal sensors. Fluorosensors based on fluorescently-labeled organic chelators, proteins or peptides have emerged as powerful tools toward achieving the above goal. While remarkable progress has been made in developing fluorosensors for some metal ions such as Ca$^{2+}$ and Zn$^{2+}$, designing and synthesizing sensitive and selective metal ion fluorosensors remains a significant challenge. Perhaps the biggest challenge in fluorosensor research is the design and synthesis of a sensor capable of specific and strong metal-binding. Since knowledge about the construction of metal-binding sites is limited, searching for sensors in a combinatorial way is of significant value. For example, even though
Colorimetric sensors can allow onsite, real-time qualitative or semi-quantitative detection without complicated analytical instruments, the design of such sensors is not very advanced.\textsuperscript{45-48} In this regard, \textit{in vitro} selection of DNA/RNA from a library of $10^{14} - 10^{15}$ random DNA/RNA sequences offers considerable opportunity.\textsuperscript{49, 50} Compared with combinatorial searches of chemo- and peptidyl-sensors, \textit{in vitro} selection of DNA/RNA is capable of sampling a larger pool of sequences, amplifying the desired sequences by the polymerase chain reaction (PCR), and introducing mutations to improve performance by mutagenic PCR. For example, the \textit{in vitro} selection method has been used to obtain DNA/RNA aptamers\textsuperscript{51, 52} and aptazymes\textsuperscript{53, 54} that are responsive to small organic molecules. Similarly, catalytic DNA/RNAs that are highly specific for Pb\textsuperscript{2+},\textsuperscript{49, 55} Cu\textsuperscript{2+}\textsuperscript{56, 57} and Zn\textsuperscript{2+}\textsuperscript{58, 59} have been obtained. These results set the stage for the utilization of catalytic DNAs as metal ion sensors.

iii. Advantage of Catalytic DNA as sensors. Long considered as simply a genetic material, DNA was shown in 1994,\textsuperscript{49} through \textit{in vitro} selection,\textsuperscript{49, 60-63} to carry out catalytic functions, and thus became the newest member of the enzyme family (after proteins and RNA). Since then, the DNA molecules (called catalytic DNA in this paper, also called DNA enzymes, DNazymes or deoxyribozymes elsewhere) have been shown to catalyze many of the same reactions as catalytic RNA (ribozymes) or protein enzymes.\textsuperscript{64-67} Several features make catalytic DNA an excellent choice for sensing applications. The first and perhaps biggest advantage of choosing catalytic DNAs is that they can be subjected to \textit{in vitro} selection. When compared to other combinatorial methods based on organic chelators or peptides, \textit{in vitro} selection can sample a larger pool of different molecules (up to 100 trillion), amplify the desired sequences by the polymerase chain reaction (PCR), and introduce mutations to improve performance by mutagenic PCR. Second, the \textit{in vitro} selection can be carried out in short time and with limited cost (1-2 days and a few dollars per round of selection). Third, the synthesis of DNA is easier, and therefore less costly than the synthesis of RNA. Under physiological conditions, DNA is nearly 1,000-fold more stable to hydrolysis than proteins and nearly 100,000-fold more stable than RNA.\textsuperscript{66} As seen from a recent crystal structure,\textsuperscript{68} catalytic DNAs usually form compact globular-shaped proteins and are therefore not easily recognized by endo- or exonucleases, and thus are likely more resistant to nuclease attack than single or even double-stranded DNA/RNA.\textsuperscript{69} Fourth, unlike proteins, most catalytic DNA can be denatured and renatured many times without losing binding ability or activity. They can be used and stored under rather harsh conditions. Fifth, DNA is adaptable to fiber optic and microarray technology,\textsuperscript{70, 71} which is important for on-site or remote sensing of multiple metal ions simultaneously.
iv. Advantages of fluorescent sensors. We intend to convert the \textit{in vitro} selected catalytic DNAs into fluorescent sensors. Fluorescence provides significant signal amplification because a single fluorophore can absorb and emit many photons, which leads to strong signals even at very low concentrations of fluorescent probe or analyte. In addition, the fluorescence time-scale is fast enough to allow real-time monitoring of concentration fluctuations. Fluorescent properties only respond to changes related to the fluorophore and can be highly selective. Furthermore, portable fluorometers for use in the field are available.\textsuperscript{72-74} Fluorescence detection is also compatible with fiber-optic technology and well suited for remote sensing applications.\textsuperscript{27-29, 75-81} As demonstrated recently,\textsuperscript{82} there are three additional advantages of catalytic DNA fluorescent sensor systems. The metal sensing is achieved by both metal-binding and catalytic activity, allowing signal amplification through catalytic turnover. The fluorophores can be placed remotely from the binding and cleavage sites so that binding and sensing do not interfere with each other and can be optimized independently. Finally, the effective placement of the fluorophores can be accomplished with little knowledge of the three dimensional structure of the system.

v. Advantages of colorimetric sensors. While fluorescent sensors offer many advantages, a simple colorimetric detection method could eliminate or minimize most costs associated with the instrumentation and operation of fluorescence detection and thus can make on-site, real-time detection even easier. The colorimetric sensors described here combine cutting edge technologies in biology (in vitro selection of catalytic DNA) and nanotechnology (gold nanoparticles). Test results showed that the sensitivity and selectivity of this method rivals, and in many cases exceeds, other methods such as those based on fluorescence.

The results of our research demonstrate that the use of catalytic DNA for metal detection is an effective way to identify metal poisoning problems.

2. Results and discussion

i. \textit{In vitro} selection of catalytic DNA for sensing applications. Catalytic DNA molecules (also called DNAzymes) were first isolated by Breaker and Joyce in 1994\textsuperscript{49}. A combinatorial biology technique called \textit{in vitro} selection has been used to isolate DNAzymes that can perform a number of different catalytic functions such as RNA cleavage\textsuperscript{49}, ligation\textsuperscript{56, 83, 84}, phosphorylation\textsuperscript{85} and porphyrin metallation\textsuperscript{86}. In a typical \textit{in vitro} selection process, we start with a random DNA library containing \(10^{13} – 10^{16}\) sequences. The sequences that exhibit a desired function are separated by techniques such as column chromatography, gel based separation and more recently capillary electrophoresis\textsuperscript{87}. These active sequences are then amplified and subjected to additional rounds of selection, often increasing the selection
stringency. During each round, ‘winner’ sequences are amplified and the non active sequences are removed. The process is repeated to obtain the optimally active sequence. We are interested in obtaining RNA cleaving DNAzymes that cleave a single ribo-linkage embedded in a DNA sequence, only in the presence of a particular metal ion of interest. This forms the basis of DNAzyme based metal ion sensors.

1) **In vitro selection scheme** - In vitro selection has been carried out using a protocol shown in Figure 1. The template containing a 50 nucleotide random region is extended and then amplified to obtain the random pool containing a single riboadenosine cleavage site. In each round of selection, the DNA pool dissolved in selection buffer is incubated with the metal ion at a desired concentration. Some molecules in the initial pool will be catalytically active and will be able to catalyze the cleavage reaction at the riboadenosine site. The cleavage product is separated from the uncleaved pool by gel electrophoresis and extended to the full length of the random pool and amplified by PCR. The selection pressure is progressively increased so as to obtain the most efficient sequences that can catalyze the cleavage reaction in the minimum amount of time and metal ion concentration.

The selection scheme is presented in Figure 1. A total of three primers were used in two polymerase chain reactions (PCR). In the first PCR, P3 and P4 were used to generate a full-length pool; rA was introduced through P3, P4 contained a PEG spacer (Spacer-18, denoted as a green dot) that was incorporated into the negative strand without rA. The purpose of the spacer was to stop the PCR extension. As a result, two strands of unequal lengths were produced, and the positive strand was purified by denaturing polyacrylamide gel electrophoresis (PAGE). In the second PCR, based on the cleaved products, P2 and P4 were used to generate the selection pool for the next round of selection.

2) **Uranium** - UO$_2^{2+}$ was added to search for DNAzymes that can perform the self-cleavage reaction and the cleaved products were separated by PAGE to seed the next round of selection. Initially, 1 mM of UO$_2^{2+}$ with 5 hour reaction time was used, which gradually decreased to 0.1 mM and 15 minutes in round 11. The round 10 pool was cloned and 86 sequences were obtained. After performing activity assays on individual clones, clone 39 was chosen for uranium sensing. After truncation and rational design of substrate binding sequences, a trans-cleavage DNAzyme was constructed and converted into a fluorescent sensor with a detection limit of 45 pM (see section 2-ii for details).

3) **Arsenic** - Using a similar protocol as described above, we had carried out *in vitro* selection of As$^{3+}$ and As$^{5+}$-specific DNAzymes. Because As$^{3+}$ and As$^{5+}$ are in the form of oxyanions, it has
been difficult to obtain negatively-charged DNAzymes that have specific activity toward those metal ions. After three attempts of \textit{in vitro} selection at 10 rounds each, we obtained DNAzymes with no detectable activity in the presence of As$^{3+}$ or As$^{5+}$. We hypothesized that the DNAzyme templates used in the early selection tend to favor metal ions such as Pb$^{2+}$. Based on the work of Cruz et al., a new template that is less biased toward Pb$^{2+}$ was chosen, and used for in vitro selection. The 8\textsuperscript{th} round of \textit{in vitro} selection of As$^{3+}$ and As$^{5+}$-specific DNAzyme has been completed using a newly designed template.

4) \textbf{Other metal ions} - Protocols for \textit{in vitro} selection of Pb$^{2+}$\cite{49,55} and Cu$^{2+}$\cite{56,57} -specific DNAzymes have been reported in the literatures previously. Hg-specific allosterically controlled DNAzymes was modified from \textit{in vitro} selected UO$_2^{2+}$-specific DNAzymes (see section 2-iv for details).

\textbf{Figure 1.} \textit{In vitro} selection protocol. See text for descriptions.
ii. Design of fluorescent uranium biosensors: a catalytic beacon sensor with parts-per-trillion sensitivity and million-fold selectivity. We obtained a UO$_2^{2+}$-specific DNAzyme and demonstrated a highly sensitive and selective UO$_2^{2+}$ sensor based on the DNAzyme. A catalytic beacon sensor for uranyl (UO$_2^{2+}$) consists of a DNA enzyme strand with a 3’ quencher, and a DNA substrate with a ribonucleotide adenosine (rA) in the middle and a fluorophore and a quencher at the 5’ and 3’-ends, respectively (Figure 2). The presence of UO$_2^{2+}$ causes catalytic cleavage of the DNA substrate strand at the rA position and release of the fluorophore and thus a dramatic increase in fluorescence intensity. As shown in Figure 3A, the rate of fluorescence enhancement was faster in higher concentrations of UO$_2^{2+}$. F/F$_0$ in the y-axis is the fluorescence at a certain time over background fluorescence before addition of uranium, which thus represents the fold of fluorescence increase. The fold of fluorescence increase at different uranium concentrations was plotted in Figure 3B. The sensor’s performance was evaluated by conducting linear regression over the lower concentration portion of curve in Figure 3B: detection limit of 11 parts-per-trillion (45 pM), and dynamic range up to 400 nM. This sensor rivals the most sensitive analytical instruments for uranium detection. Its application in detecting uranium in contaminated soil samples is also demonstrated. This work shows those simple, cost-effective, and portable metal sensors have similar sensitivity and selectivity to much more expensive and sophisticated analytical instruments. With wide availability of portable fluorometers, such a highly sensitive and selective UO$_2^{2+}$ sensor will find wide applications in on-site and real-time environmental monitoring. Successes with the uranium sensor demonstrated that the DNAzyme sensing platform has an enormous potential for detection and quantification of many metal ions.
Figure 2. Design of a catalytic beacon to detect UO$_2^{2+}$. (A) The secondary structure of an *in vitro* selected DNAzyme specific to UO$_2^{2+}$, which contains a substrate (39S) and an enzyme (39E). (B) Design of a catalytic beacon with a fluorophore and two quenchers. Cleavage of the substrate in the presence of UO$_2^{2+}$ enhances the fluorescence. (C) Fluorescence signal in the absence and in the presence of 400 nM UO$_2^{2+}$ after 10 minutes. The UO$_2^{2+}$ sensor was in pH 5.5 MES buffer with 300 mM NaCl.
Figure 3. Sensitivity of the catalytic beacon-based UO$_2^{2+}$ sensor. (A) Kinetics of fluorescence increase over background fluorescence at varying UO$_2^{2+}$ concentrations. The DNAzyme sensor concentration was 60 nM, and the buffer contained 50 mM MES (2-(N-morpholino)ethanesulfonic acid) (pH 5.5) and 300 mM NaNO$_3$. (Inset) Sensor responses to low concentrations of UO$_2^{2+}$. F/F$_0$ in the y-axis is the fluorescence at a certain time over background fluorescence before addition of uranium, which thus represents the fold of fluorescence increase. (B) Plot of the initial rate of fluorescence enhancement (V$_{flu0}$, fold of fluorescence increase per minute) vs. UO$_2^{2+}$ concentration. (Inset) Low UO$_2^{2+}$ concentration range with linear responses.

To test the selectivity of the sensor for UO$_2^{2+}$ vs. other metal ions, the sensor response to 19 competing metal salts at concentrations of 10 µM, 200 µM, and 1 mM was tested. Most metals induced little fluorescence change, although some metals such as Cu$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Hg$^{2+}$, and Tb$^{3+}$ induced strong quenching to FAM (5-Carboxyfluorescein). Similarly, the rate of fluorescence change was calculated. As shown in Figure 4, at all three concentrations, none of the metals [except Th(IV)] showed a response higher than that of 1 nM UO$_2^{2+}$. Some Th(IV)-dependent fluorescence increase was observed. However, gel-based assays showed no Th(IV)-dependent cleavage even with 1 mM Th(IV) (see supplemental information in ref. 95). Therefore, Th(IV) interference is likely to be due to metal/fluorophore interactions, which can be removed.
by using other fluorophores. Overall, the sensor has over one million-fold selectivity for UO$_2^{2+}$ over any other metal ions.

![Selectivity of the catalytic beacon-based UO$_2^{2+}$ sensor](image)

**Figure 4.** Selectivity of the catalytic beacon-based UO$_2^{2+}$ sensor. Sensor responses to all competing metal ions at three concentrations (10 µM, 200 µM, and 1 mM) were tested.

### iii. Design of fluorescent copper biosensors.

Copper is a widely used metal that can enter the environment through various routes. At low concentration, copper is an essential nutrient to human beings. However, exposure to high level of copper even for a short period of time can cause gastrointestinal disturbance. Long term exposure causes liver or kidney damage. Therefore, regulations for copper in drinking water have been established. For example, the US Environmental Protection Agency (EPA) defines the limit of copper in drinking water to be 1.3 ppm (~20 µM).

We chose a Cu$_{2+}$-dependent DNA-cleaving DNAzyme reported by Breaker et al as a basis for the sensor design. Based on the original DNAzyme sequence, we designed a Cu$_{2+}$ sensor as shown in Figure 5A. The sensor contained two DNA strands that can form a complex. The substrate strand (in black) was labeled with a FAM fluorophore (6-carboxyfluorescein) on the 3'-end, and a quencher (Iowa Black FQ) on the 5'-end. The enzyme strand (in blue) contained a 5'-quencher. A dual-quencher approach was employed to suppress background fluorescence. The substrate and enzyme associate through two base pairing regions. The 5' region of the enzyme binds the substrate via Watson-Crick base pairs; while the 3' region binds through formation of a DNA triplex. Initially, the FAM emission was quenched by the nearby quenchers. In the presence of Cu$_{2+}$, the substrate was cleaved at the RNA site (the guanine in red). We hypothesized that the cleaved pieces were released due to decreased affinities to the enzyme, leading to increased distance between the FAM and the quenchers and increased fluorescence (Figure 5B). This hypothesis was supported by the observation that the FAM emission increased.
by ~13-fold after addition of 20 µM Cu²⁺ (Figure 5C). Such a signal generation method was termed “catalytic beacon” because of the involvement of catalytic reactions. Gel-based assays were carried out to confirm that the observed fluorescence increase was due to DNAzyme cleavage (inset of Figure 5C). Lane 1 contained the substrate alone incubated with Cu²⁺. Lane 2 had the DNAzyme complex but no Cu²⁺. Cleavage was observed only after addition of Cu²⁺ to the DNAzyme complex and the fraction of cleaved substrate increased over time (lanes 3-5), which supported the proposed mechanism in Figure 5B. In all the reactions, 50 µM of ascorbate was included. Although this DNAzyme has been shown to work in the absence of ascorbate, the reaction rates were significantly enhanced by ascorbate. Ascorbate was also useful in this system to suppress quenching of FAM by Cu²⁺. For example, FAM quenching was <15% with 50 µM Cu²⁺.

Figure 5. (A) The secondary structure of the Cu²⁺ sensor. F and Q denote fluorophore and quencher, respectively. The cleavage site is indicated by an arrow. (B) Signal generation mechanism of the Cu²⁺ catalytic beacon. (C) Fluorescence spectra of the sensor before and 10 min after addition of 20 µM Cu²⁺. Inset: gel-based assay of the sensor DNAzyme. Lane 1 is substrate plus Cu²⁺, Lane 2 is substrate plus enzyme, Lanes 3 to 5 are 5, 10, and 25 min after addition of Cu²⁺, respectively.

To test the sensitivity of the Cu²⁺ sensor, the kinetics of fluorescence increase at 520 nm in the presence of varying concentrations of Cu²⁺ were recorded on a fluorometer. As shown in Figure 6A, fluorescence enhancement rates were higher with increasing levels of Cu²⁺. The rates in the time window of 2 to 4 minutes were plotted in Figure 6B. A detection limit of 35 nM (2.3 ppb) was determined, which represents one of the most sensitive Cu²⁺ sensors. The sensor has a dynamic range up to 20 µM. This is very useful for detecting Cu²⁺ in drinking water because the US EPA has defined a maximum contamination level of 20 µM. In addition to being highly sensitive and “light-up”, the sensor response was also fast, so quantitative results can be obtained within several minutes.
To test selectivity of the sensor, 16 competing metal ions were assayed at three different concentrations: 1 mM, 100 µM, and 10 µM. The assay was performed in a 96-well plate and emission intensities at 12-min after addition of metal ions were compared. As shown in Figure 6C, besides Cu²⁺, only the spots with 1 mM Fe²⁺ and 1 mM UO₂²⁺ lit up and the intensities were lower than that with 0.5 µM of Cu²⁺. Therefore, the sensor selectivity for Cu²⁺ was at least 2,000-fold higher than these two metals, and >10,000-fold higher than any other metal ions. Fe²⁺ is known to cleave DNA through Fenton’s chemistry. For testing environmental samples, such as detection of Cu²⁺ in drinking water, Fe²⁺ is unlikely to interfere due to the oxidative environment. UO₂²⁺ is also unlikely to present in millimolar concentration in drinking water.

**Figure 6.** (A) Kinetics of fluorescence increase over background at varying Cu²⁺ levels. The arrow indicates the point of Cu²⁺ addition. Inset: responses at low Cu²⁺ levels. (B) The rate of fluorescence enhancement plotted against Cu²⁺ concentration. Inset: rates at the low Cu²⁺ region. (C) Sensor selectivity. The buffer contained 1.5 M NaCl, 50 mM HEPES, pH 7.0, and 50 µM ascorbate. Cu²⁺ concentrations were labeled on the left side of each well while others were on the right end. All concentrations are in µM.

**iv. Design of fluorescent mercury biosensors.** Mercury ions are known to bind in between T-T mismatches to stabilize such mismatches. Based on this knowledge, the catalytic DNA sensor for Hg²⁺ detection is shown in Figure 7a. This catalytic DNA was built on the basis of the uranium catalytic DNA shown in Figure 2A of this report. A fluorophore (FAM) was labeled on
the 5′-end of the substrate, a quencher was labeled on the 3′-end of the enzyme, and an additional quencher was labeled on the 3′-end of the substrate. Both quenchers were black hole quenchers. The DNAzyme was mixed with UO$_2^{2+}$ to become an Hg$^{2+}$ sensor (Figure 7b). In the absence of Hg$^{2+}$, the DNAzyme was incapable of binding UO$_2^{2+}$ because the active secondary structure cannot form. Addition of Hg$^{2+}$ quickly restored the stem loop structure and activated the DNAzyme to cleave the substrate, releasing the fluorophore-labeled piece and giving increased fluorescence. The fluorescence spectra of the sensor before and 8 min after addition of 500 nM Hg$^{2+}$ is shown in Figure 7c, and ~50-fold increase in the 520 nm peak was observed. This level of fluorescence increase is the highest in functional nucleic acid based sensors for metal ions.

**Figure 7.** (a) The secondary structure and modification of the Hg$^{2+}$ sensor DNAzyme. (b) Fluorescence spectra of the sensor in the absence and 8 min after addition of 0.5 µM Hg$^{2+}$. (c) Schematic presentation of the sensor design.

Given the very high fluorescence enhancement, the sensor performance was further tested. First, the sensor was titrated with varying concentrations of Hg$^{2+}$ and the kinetics of fluorescence enhancement at 520 nm was followed. As shown in Figure 8a, higher concentrations of Hg$^{2+}$ produced higher rates of emission enhancement. All the kinetic traces showed a roughly linear increase in 1-2 min after addition of Hg$^{2+}$. Therefore, the slope of fluorescence increase in this time window was calculated to quantify Hg$^{2+}$ concentrations (Figure 8b). The Hg$^{2+}$ dependent response had a sigmoid shape and was fit to a Hill plot with a Hill coefficient of 2.1. This result suggests that Hg$^{2+}$ binding to the sensor DNAzyme is a cooperative process. Although the DNAzyme has five Hg$^{2+}$ binding sites, the DNAzyme is stable enough in the time window to cleave its substrate after binding ~2 Hg$^{2+}$ ions. The detection limit was determined to be 2.4 nM (~0.5 ppb) based on 3σ/slope (inset of Figure 8b), which is a ~16-fold improvement compared to
Based on our results, among all the reported Hg$^{2+}$ sensors made from small molecules, oligonucleotides, peptides, and proteins, this catalytic beacon has the best detection limit. The US Environment Protection Agency (EPA) defined the toxic level of Hg$^{2+}$ in drinking water to be 2 parts-per-billion or 10 nM, which can be covered by the sensor.

![Image](image.png)

**Figure 8.** Sensitivity of the Hg$^{2+}$ sensor. (a) Kinetics of fluorescence increase in the presence of varying concentrations of Hg$^{2+}$. All the reactions were carried out at 24 °C in 10 mM MES, pH 5.5, 300 mM NaNO$_3$ with 100 nM DNAzyme sensor and 1 µM UO$_2$$^{2+}$. (b) Hg$^{2+}$ dependent fluorescence increase rate. Rates were calculated in the time window of 1-2 min in (a). Inset: responses at low Hg$^{2+}$ concentrations. The y-axis is the fluorescence counts increase per second.

To test selectivity, the sensor responses in the presence of 13 competing metal ions were assayed. These metal ions included Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, UO$_2$$^{2+}$, and Tb$^{3+}$. Each metal ion was tested at three concentrations (1, 20, and 1000 µM). None of the metal ions gave responses higher than half of that produced by 20 nM Hg$^{2+}$ (Figure 9), and the selectivity was determined to be at least 100,000-fold higher for Hg$^{2+}$ over any other metal ions (10 nM Hg$^{2+}$ versus 1 mM other metal ions).
Figure 9. Sensor selectivity. All competing metal ions were tested at 1, 20, and 1000 µM. For comparison, sensor responses to 20, 100, and 500 nM of Hg²⁺ were also presented.

v. On-site Pb²⁺ detection using a DNAzyme biosensor coupled with a portable fluorometer. After demonstrating individual fluorescent sensors for heavy metal detection, the use of the Pb²⁺ sensor for detecting Pb²⁺ in drinking water samples was carried out.

The secondary structure of the DNAzyme is shown in Figure 10. For the Pb²⁺ sensor, the substrate strand was labeled with a fluorophore (Fluorescein or Alexa fluor 488) at the 5'-end and a quencher (Dabcyl) at the 3'-end (FQ-17S). The enzyme strand was labeled with a quencher (Dabcyl) at the 3'-end (Q-17E). The Pb biosensor was 2 μM DNAzyme in 50 mM Tris-HCl (pH7.3) and 10 mM NaCl. Fluorescence enhancements were measured by using a portable fluorometer, Picofluor™ (Turner Designs). Measurements were made after the addition of 5 μL of 2 μM DNAzyme sensor into the 95 μL of Pb²⁺ solutions buffered with 50 mM Tris-HCl (pH7.3) + 30 mM NaCl. Reaction time varied depending on the samples’ ionic strength.
Figure 10. (A) Secondary structure of the DNAzyme. (B) Design of a catalytic beacon sensor with a fluorophore and two quenchers. The substrate strand, 17S contains a RNA base, rA, at the cleavage site denoted with an arrow in (A). It was labeled with a fluorophore, either fluorescein or Alex fluro 488, at the 5'-end and with a Dabcyl quencher at the 3'-end. The enzyme strand, 17E was labeled with a Dabcyl quencher at the 3'-end. Cleavage of the substrate in the presence of Pb$^{2+}$ enhances the fluorescence.

Figure 11. Fluorescence increases at different reaction time by Pb$^{2+}$ ions in Millipore water. Reactions were started by adding 5 μL of 2 μM DNAzyme into 95 μL of Pb$^{2+}$ in reaction buffer. The DNAzyme was annealed in 50 mM Tris-HCl(pH7.3) + 10 mM NaCl and the reaction buffer contained 50 mM Tris-HCl (pH7.3) + 30 mM NaCl. Each data point represents three replicates. The solid lines are linear regressions at each reaction time with all 7 points for 10 and 20 min, with first 6 points for 30 min, and with first 5 points for 60 min. Detection limits are indicated inside the brackets after each reaction time.
Figure 11 shows sensor response in Millipore water. At 10 minutes reaction time, the fluorescence showed linear increases over the entire region of 0-500 nM of Pb\(^{2+}\) with detection limit of \(~20\) nM. As the reaction time increased, the curves became steeper with lower detection limits, but the linearity was lost due to the saturated reaction time with high Pb\(^{2+}\) concentrations. For each reaction time, 50 nM of Pb\(^{2+}\) could be easily distinguished from the background. It was possible to detect 10 nM of Pb\(^{2+}\) after one hour of reaction time.

In simulated water samples with various hardness. Real world water sources contain various kinds of materials including minerals. It has been know that Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) are abundant in several hundreds micromolar ranges in most real world water sources while less than 1 \(\mu\)M of heavy metal ions are present.\(^{103}\) It was shown that the DNAzyme does not have activity in the presence of millimolar monovalent metal ions and has 1000-fold selectivity to Pb\(^{2+}\) over Ca\(^{2+}\) and Mg\(^{2+}\). However, it is most likely that real world water sources will contain more than 1000-fold excess of Ca\(^{2+}\) and Mg\(^{2+}\). Thus the DNAzyme’s performance was examined in the presence of highly concentrated Ca\(^{2+}\) and Mg\(^{2+}\).

Simulated water samples of various hardness were prepared by mixing MgCl\(_2\) and CaCl\(_2\) ions in 1:1 molar ratio and were spiked with different concentrations of Pb(OAc)\(_2\). Increasing hardness induced faster increase in fluorescence due to the reactions not only by Pb\(^{2+}\) but also by Ca\(^{2+}\) and Mg\(^{2+}\). Measuring fluorescence at short reaction time made it possible to distinguish [Pb\(^{2+}\)] in the low concentration region of 0–500 nM in the presence of the interferences by Ca\(^{2+}\) and Mg\(^{2+}\). Standard curves of fluorescence enhancements versus [Pb\(^{2+}\)] in the simulated water samples were obtained by reading the fluorescence at different reaction times (Figure 12). Fluorescence values were background-corrected (fluorescence at 0 nM Pb\(^{2+}\) in each hardness was used as the background signal). Linear curves were obtained in the fluorescence enhancement in the range of 0 to 500 nM Pb\(^{2+}\). Detection limits were calculated from the slopes of the curves and the standard deviations of the points at 0 nM of [Pb\(^{2+}\)] (Table 1). All cases except hardness 120 mg/L have detection limits lower than the EPA standard, 72 nM, and they were reproducible. For the case of hardness 120 mg/L, the linearity was poor and it was not easy to reproduce the same trend. These results show that it is possible to detect Pb\(^{2+}\) in the moderately hard (H 60 mg/L) water.

Real sample test; Crystal Lake water in Urbana, IL. In order to test the DNAzyme Pb\(^{2+}\) biosensor using a real water sample, we tested water from Crystal Lake in Urbana, IL. The water was filtered with a 0.2 \(\mu\)M syringe filter and analyzed with ICP-MS. The water contained 160 pM Pb\(^{2+}\), 100 \(\mu\)M Ca\(^{2+}\), and 44.3 \(\mu\)M Mg\(^{2+}\), which corresponds to a hardness of 14.43 mg/L. Since the Pb\(^{2+}\) concentration in the water is much lower than the level of concern and well below
the sensor detection limit, the water was spiked with Pb(OAc)$_2$ for the experiments. Fluorescence was measured at 5 minutes of reaction time. Linear fluorescence increase was shown in the range of 0-500 nM Pb$^{2+}$ with a detection limit of 44.2 nM (Figure 12f). This result implies that the DNAzyme biosensor can detect Pb$^{2+}$ in real world water sources that contain numerous minerals and organics.

**Figure 12.** Standard curves of fluorescence enhancement versus Pb$^{2+}$ concentrations in simulated water samples with different hardness (a-e) and in Crystal Lake water (f). Fluorescence was measured with a portable fluorometer, Picofluor$^{\text{TM}}$ (Turner Designs). The Fluorescence intensities were background (0 Pb$^{2+}$ point)-subtracted. The solid lines show the linear regression of the data. Reaction times were 10, 5, 5, 3, 2, and 5 minutes for the hardness values of 0, 15, 30, 60, 120 mg/L, and Crystal Lake water, respectively.
Table 1. Reaction time and detection limits calculated from each standard curve of fluorescence versus [Pb$^{2+}$] in the simulated water samples with different hardness and Crystal Lake water sample.

<table>
<thead>
<tr>
<th>Hardness (mg/L)</th>
<th>Reaction Time (min)</th>
<th>DL (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H 0</td>
<td>10</td>
<td>27.3</td>
</tr>
<tr>
<td>H 15</td>
<td>5</td>
<td>34.8</td>
</tr>
<tr>
<td>H 30</td>
<td>5</td>
<td>53.8</td>
</tr>
<tr>
<td>H 60</td>
<td>3</td>
<td>67.1</td>
</tr>
<tr>
<td>H 120</td>
<td>2</td>
<td>164</td>
</tr>
<tr>
<td>Crystal lake water</td>
<td>5</td>
<td>44.2</td>
</tr>
</tbody>
</table>

vi. Colorimetric Pb$^{2+}$ sensors based on DNAzyme-assembled gold nanoparticles. After demonstrating fluorescent sensors for metal detection, we aimed to further simplify the detection system to design colorimetric sensors. We employed a Pb$^{2+}$-specific RNA-cleaving DNAzyme (Figure 13A) to direct the assembly state of gold nanoparticles in response to Pb$^{2+}$. The Pb$^{2+}$-specific 8-17 DNAzyme was chosen as a model DNAzyme for metal sensor design. In the presence of Pb$^{2+}$, the enzyme cleaves the substrate into two pieces (Figure 13B). To allow the DNAzyme to bind DNA-functionalized gold nanoparticles, the substrate strand was extended on both ends and the extended substrate was named Sub$_{Au}$ (Figure 13C). The nanoparticles were aligned in a head-to-tail manner so that only one set of nanoparticles (5’DNA$_{Au}$) was used. The nanoparticles were pre-assembled by the DNAzyme to assure an optimal ratio between the DNAzyme and nanoparticles. The assembled nanoparticle aggregates can be used as colorimetric sensors for Pb$^{2+}$ detection. To detect Pb$^{2+}$, the sensor was heated to 50 °C to fully disassemble the aggregates. In the subsequent slow cooling process to room temperature (annealing), Pb$^{2+}$ can direct the color of the system. If Pb$^{2+}$ is present, the substrate is cleaved by the enzyme and assembly is inhibited, resulting in a red color. Otherwise, nanoparticles are re-assembled by the DNAzyme to form aggregated structures, accompanied by a red-to-blue color change due to surface plasmon coupling. Upon aggregation, the 522 nm plasmon peak decreases while the extinction in the 700 nm region increases (Figure 13D), with the extinction ratio at 522 nm over
700 nm used to quantify the nanoparticle assembly state. A higher ratio is associated with dispersed particles of red color, while a lower ratio is associated with aggregated particles of blue color. With increasing concentrations of Pb\(^{2+}\), the extinction ratio increases, suggesting the nanoparticles are in the disassembled state (Figure 13E). The detection limit was determined to be 100 nM. The color change was also conveniently observed by spotting the nanoparticle solution on a TLC plate (Figure 13F). A color progression from blue/purple to red can be observed with increasing Pb\(^{2+}\) concentrations, while competing metal ions resulted in only a background blue/purple color.

---

**Figure. 13.** (A) The secondary structure of the Pb\(^{2+}\)-specific DNAzyme. (B) In the presence of Pb\(^{2+}\), the substrate is cleaved into two pieces. (C) Pb\(^{2+}\)-directed assembly of DNAzyme-linked nanoparticle aligned in a head-to-tail manner. (D) UV-vis spectra of disassembled (red) and assembled (blue) gold nanoparticles. (E) The extinction ratio increases as the Pb\(^{2+}\) concentration increases. The detection limit of the system could be modulated by varying the ratio of the active enzyme (17E) and an inactivated version of the sensor (17Ec) so that the 17E was 100% or only 5% of the DNA in solution. (F) The assembly state or color of DNAzyme-linked nanoparticles in response to metal ions monitored by a spectrophotometer or on a TLC plate.

---

**vii. Towards more Practical Applications: Simple “Dipstick” Tests.** Although nanoparticle-based colorimetric sensors can eliminate the use of analytical instruments for detection, there is one limitation that prevents their practical application. The handling of
solutions, such as the transfer of microliter volumes of sensors and their subsequent mixing with target solutions, makes the sensors difficult for inexperienced people to use. One of the most useful methods to convert antibody-based assays to user-friendly test kits is lateral flow technology. A well-known example is the commercially available pregnancy test kit. Despite wide applications in antibody assays, nucleic acid-based lateral flow devices have been demonstrated only for DNA detection.\textsuperscript{101} We pursued the feasibility of using lateral flow devices to design DNazyme-based sensors that can be used as simple dip sticks. Figure 14 shows the design for a dipstick \( \text{Pb}^{2+} \) sensing device. The test strip is assembled by placing a carboxy ester membrane, glass fiber conjugate pad and adsorbent pad (Millipore Assembly kit) on a plastic adhesive backing. The chimeric substrate has a biotin moiety on the 3' and a thiol moiety on the 5' end. There are 2 capture areas on the membrane, called the control zone (with streptavidin) and the test zone (with DNA complementary to the 5' cleavage product). When the pad is dipped in a flow buffer, the gold nanoparticles conjugated to the catalytic DNA are rehydrated and they can migrate on the membrane. If the flow buffer contains \( \text{Pb}^{2+} \), a cleavage reaction can occur on the surface.

\textbf{Figure 14.} Dipstick detection of toxic metal ions such as \( \text{Pb}^{2+} \) based on catalytic DNA and gold nanoparticles.

The 5' product of the substrate (containing the gold nanoparticles) will be free to move forward and can be captured by a complementary DNA strand in the test zone, producing a red band to indicate presence of \( \text{Pb}^{2+} \). Any uncleaved substrate will be captured beforehand by streptavidin in the control zone. If there is no \( \text{Pb}^{2+} \) present, there will be no color in the test zone, but the control zone will have a red color. Thus, the color in the control zone can show if the test has been carried out properly. The main objective is to move all of the sophisticated sensing chemistry behind the scene and minimize user operation to a simple step. Preliminary results show this design can detect lead qualitatively in the lab (Figure 14).\textsuperscript{102}
viii. The Shelf-Life and Operation of the Sensor in a Real-World Setting. To apply the sensors for on-site, real-time detection and quantification, the sensor stability has to be high. It is well known that the stability of the DNA in solution is relatively low (no more than a few weeks). However, we normally store the sensors in dried (e.g., lyophilized) form and it has been shown in the PI’s lab and in other labs that the shelf lives of dried DNA sensors can be many months and even years. The data in this report were from dried DNA received from an oligonucleotide synthesis company. When we are ready to carry out our sensing application, aqueous solution is added to dissolve the DNA, mix the sensor components, and conduct the sensing. The sensing process completes in a few minutes, much shorter than the time required to degrade the DNA sensors under normal conditions.

![Figure 15](image-url)

**Figure 15.** (A) Stability study of the catalytic DNA-based lead sensor after drying at different temperatures. Stability at 45 °C (B) and 80 °C (C). (D) Stability of DNA attached to gold nanoparticles. The nanoparticles were stored at room temperature in a glass vial on a work bench.

The dried sensor has high stability at room temperature. To shorten the time needed for stability tests, we stored the sensor at extreme temperatures, such as -20 to 80 °C. The sensor did not show much difference in terms of lead response in all the storage conditions (Figure 15A), such as under -20°C (6 days), room temperature (2 months), 45 °C (21 days) and 60 °C (6 days). After storing at 45 °C for over 100 days, the sensor still maintained its activity (Figure 15B). Even after storage at 80 °C, at which most antibody-based sensors will lose their functions, the catalytic DNA-based sensor was still active (Figure 15C). This high stability allows the sensor to be used in field applications, where extreme temperature conditions may be encountered. These results demonstrate the excellent long-term stability of the dried sensors.
3. Summary and Outlook

In conclusion, we have demonstrated highly sensitive and selective fluorescent sensors for a number of heavy metals, including Pb$^{2+}$, UO$_2^{2+}$, Cu$^{2+}$, and Hg$^{2+}$. Each sensor was highly sensitive and selective towards its target metal ions. The use of the Pb$^{2+}$ sensor to detect Pb$^{2+}$ in field water samples with a portable fluorometer has been demonstrated. Incorporation of inorganic nanoparticles into DNAzyme nanostructures has proven to be a useful method to design highly sensitive and selective colorimetric and fluorescent sensors. Because nucleic acids can be selected for essentially any target molecule of choice, the methods of sensor design described in this report should be applicable to the detection of many other analytes of interest. Future work will be focused on increasing sensor sensitivity, selectivity, stability, and user-friendliness. These improvements can be achieved by: performing detailed characterizations to understand nucleic acid and nanoparticles interactions, introducing signal amplification mechanisms, and introducing novel sensing platforms such as lateral flow devices. The technologies demonstrated in this project have been licensed to DzymeTech, Inc., a local startup company that will commercialize the sensor products in the near future.
4. References


