Final Report on
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New Catalytic DNA Biosensors for Radionuclides and Metal Ions

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I. SUMMARY

We aim to develop new DNA biosensors for simultaneous detection and quantification of bioavailable radionuclides, such as uranium, technetium, and plutonium, and metal contaminants, such as lead, chromium, and mercury. The sensors will be highly sensitive and selective. They will be applied to on-site, real-time assessment of concentration, speciation, and stability of the individual contaminants before and during bioremediation, and for long-term monitoring of DOE contaminated sites.

To achieve this goal, we have employed a combinatorial method called "*in vitro* selection" to search from a large DNA library (~ 10^{15} different molecules) for catalytic DNA molecules that are highly specific for radionuclides or other metal ions through intricate 3-dimensional interactions as in metalloproteins. Comprehensive biochemical and biophysical studies have been performed on the selected DNA molecules. The findings from these studies have helped to elucidate fundamental principles for designing effective sensors for radionuclides and metal ions. Based on the study, the DNA have been converted to fluorescent or colorimetric sensors by attaching to it fluorescent donor/acceptor pairs or gold nanoparticles, with 11 part-per-trillion detection limit (for uranium) and over million fold selectivity (over other radionuclides and metal ions tested). Practical application of the biosensors for samples from the Environmental Remediation Sciences Program (ERSP) Field Research Center (FRC) at Oak Ridge has also been demonstrated.

II. Work accomplished

The PI's group has been supported by the DOE since 2001 (2001-2004 under the NABIR program, $489,998 total for three years, and then 2004-2007 under ESRP (grant #DE-FG02-01ER63179), $499,216 total for three years). Thanks to the generous support by DOE, we have made significant progress toward the objectives and proving the working hypotheses of this project. We demonstrated the use of *in vitro* selection method to obtain highly efficient catalytic DNA with strong binding affinities for metal ions such as lead[1,2] and uranium.[3] Work is in progress to obtain catalytic DNA specific for radionuclides such as plutonium and metal ions such as cadmium, mercury, and chromium (see section A below). In addition, we have demonstrated that the catalytic DNA can be selected for not only a particular metal ion (e.g., chromium), but also a particular oxidation state of the same metal ion (Cr(VI) instead of Cr(III), U(VI) instead of U(IV),[3] see section A). To further improve the selectivity, we have developed a "negative selection" strategy for *in vitro* selection of catalytic DNA to further improve selectivity toward metal ions of choice;[4] Furthermore, we carried out detailed biochemical and biophysical studies of the *in vitro* selected catalytic DNA to provide insight into designing metal sensors (see section B). Finally, we became the first to report the use of catalytic DNA as a fluorescent sensor for metal ions such as Pb(II) and UO_2^{2+} (see section C). The detection limit for Pb(II) and U(VI) is 200 and 11 ppt, respectively, much lower than the Maximum Contaminant Levels (MCL) in drinking water defined by the EPA (15 ppb and 30 ppb for Pb(II) and U(VI) respectively). Even in the presence of other metal ions, the biosensors display a remarkable sensitivity and selectivity (e.g., the U(VI) sensor has more than a millions fold

selectivity over ~ 20 other competing metal ions tested). Recently, we had a breakthrough in the sensor development by converting the catalytic DNA into colorimetric metal sensors, making on-site, real-time detection even more affordable and achievable because no equipment is needed in the operation.[5-8] A novel approach of using an inactive variant of catalytic DNA in fine-tuning the detection limits and ranges of the sensors has also been established.[5] More excitingly, in collaboration with Drs. David B. Watson and Jonathan D. Istok, we have demonstrated detection and quantification of uranium in ERSP's FRC samples with the same accuracy and detection limit as ICP-MS and kinetic phosphorescence, but with much lower costs and simpler operation.[3]

The sensor work described above has produced 33 peer-reviewed publications,[1-33] including two in *Proc. Natl. Acad. Sci (USA)*, seven in *J. Am. Chem. Soc.*, three in *Angew. Chem., Int. Ed.*, four in *Anal. Chem.* and one in *Environ. Sci. Technol.*. It also resulted in ten US and International patent applications and has won the 2002 *Biosensors and Bioelectronics* Award. The DNA colorimetric sensors developed in our lab were recognized as one of the Chemistry Highlights of 2003 as selected by the *Chemical and Engineering News*, December 22, 2003 issue.[34] The uranium sensor work published in PNAS in Jan of 2007[3] was highlighted by the PNAS editor in a news release, which was then picked up and reported in a number of news media, including the *Chemical and Engineering News*, *Science Daily*, and *Biophotonics International*. This work was also mentioned by Dr. Michael Kuperberg, the ERSD Acting Director, as one of the highlighted achievements of ERSP at the 2nd Anural DOE-ERSP PI meeting. We are now poised to expand the methodology to other radionuclides and metal ions, and to its applications in field research.

A. *In vitro* selection of catalytic DNA with high specificity and selectivity for radionuclides and metal ions

A group of highly efficient catalytic DNA molecules that depend on Pb(II) or U(VI) have been selected and published using *in vitro* selection,[1,3,4] the protocol of which is described in the Research Plan Section (V-A-i Fig. 9). In the process, we have developed a "negative selection" strategy for *in vitro* selection of catalytic DNA to further improve selectivity toward metal ions of interest.[4] We have now applied the above *in vitro* selection methods to obtaining catalytic DNA for Cd(II) and Cr(VI).

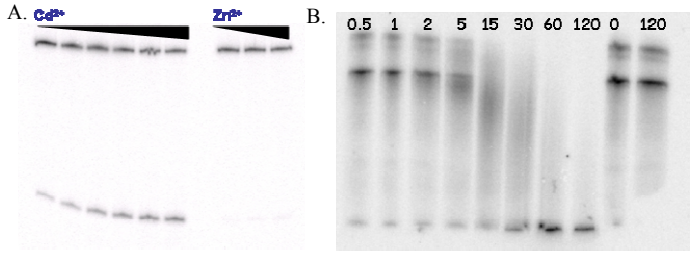

Figure 1. In vitro selected catalytic DNA with Cd(II) activity (A), and with Cr(VI) activity (B). In (A), the time increased from left to right (longest time: 5 h). Cd(II) induced cleavage, but not Zn(II). In (B), The unit above each lane is minutes. The last two lanes are controls where no metal ions were added (and no cleavage was observed). Adding Cr(III) also did not result in any cleavage, demonstrating that the DNA is selective toward Cr(VI).

B. Biochemical and biophysical study of the *in vitro* selected catalytic DNA molecules

Biochemical and biophysical studies of the catalytic DNA are required to learn the principles of metal-binding affinity and specificity in the catalytic DNA and to aid in design of better metal sensors. We have obtained the following exciting results:

i. Biochemical studies of in vitro selected catalytic DNA.

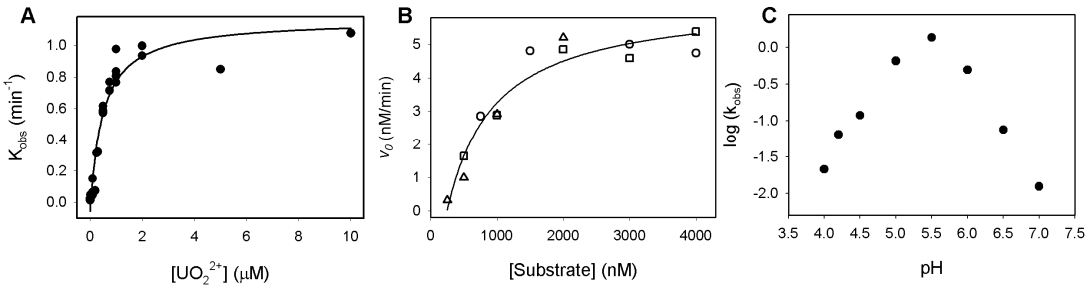

Figure 2. Biochemical assays of the uranium specific catalytic DNA. (A) UO_2^{2+}-dependent single turnover reactions. (B) Multiple turnover reactions. (C) pH profile.

Two detailed biochemical studies of Pb(II)-specific DNA have elucidated conserved sequence for Pb(II)-binding.[1,2,4] Biochemical assays have also been carried out on the newly selected UO_2^{2+}-dependent catalytic DNA called 39E. The enzyme cleaved its substrate faster with high concentrations of UO_2^{2+} and an apparent K_d of 469 nM was determined (Figure 2A). The enzyme was also capable of performing multiple turnover reaction (Figure 2B) and a K_m of 470 nM was obtained. Unlike the Pb^{2+}-dependent catalytic DNA, the UO_2^{2+} DNA showed a bell shaped pH profile with the optimal pH being 5.5. The activity of the enzyme dropped on either side. This is not surprising, as the in vitro selection was carried out under pH5.5. To obtain a pH optimum at another pH, we should be able to select another class of catalytic DNA using buffers with desired pH. This catalytic DNA worked only in the presence of uranium, while no other metal ion gave cleavage products. A manuscript for publication is under preparation.

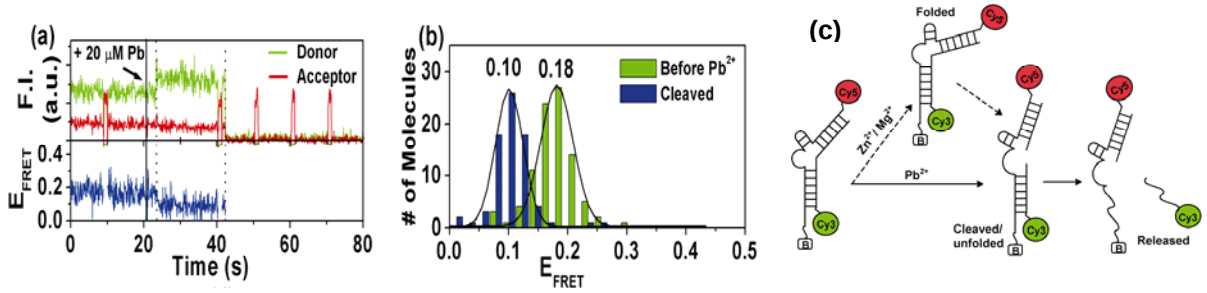

Figure 3. Single molecule FRET study of the catalytic DNA sensor. (a) Time traces of the FRET signals and changes upon injection of 20 μM Pb^{2+} at 21 s. (b) FRET histograms obtained by measuring the FRET values at the two states; (c) Proposed reaction pathways of the 8-17 catalytic DNA in the presence of Zn^{2+}/ Mg^{2+} and Pb^{2+}.

ii. Biophysical studies of in vitro selected catalytic DNA. Despite demonstrated applications such as metal sensing, a fundamental understanding on metal-binding sites in catalytic DNA has not yet been achieved, largely due to a lack of structural information. We first published a novel FRET methodology using a trifluorophore-labeled catalytic DNA.[12] We have then carried out the first comprehensive study on metal induced folding of the Pb(II)-specific catalytic DNA. By labeling the three stems of the DNAzyme with the Cy3/Cy5 FRET pair two stems at a time, FRET results indicated that, in the presence of Zn(II) and Mg(II), the DNA folds into a compact structure. Interestingly, no Pb^{2+}-dependent global folding was observed. These results suggest that for Pb^{2+}, global folding of the DNAzyme may not be a necessary step in its function, which may contribute to the DNAzyme having the highest activity in the presence of Pb^{2+}. A manuscript on this research is in press in *J. Am Chem. Soc.*. To offer deeper insight into this system, we carried out single molecule FRET using active catalytic DNA. On addition of Zn^{2+} and Mg^{2+}, the reaction proceeded through a folding step followed by cleavage reaction, suggesting that the DNAzyme may requires metal-dependent global folding for activation (Figure 3C). In the presence of Pb^{2+}, however, the cleavage reaction occurred without a precedent folding

step, suggesting that the DNAzyme may be prearranged to accept Pb^{2+} for the activity. This feature, not reported in catalytic RNA but observed only in metalloprotein enzymes, may contribute to the remarkably fast Pb^{2+}-dependent reaction of the catalytic DNA. These results suggest that catalytic DNA can use both lock-and key and induced fit modes of activation that metalloproteins use. A manuscript on this research has been submitted to *Nature Chem. Biol.*

C. Design and demonstration of highly sensitive and selective catalytic DNA biosensors for radionuclides and metal ions.

i. Fluorescent sensors for Pb(II), U(VI), and Hg(II). We are the first to report a new application for catalytic DNA molecules as biosensors for metal ions, such as Pb(II).[11,13] This catalytic DNA biosensor is highly sensitive for Pb(II), with a detection limit of 10 nM, less than 75 nM MCL defined by EPA. Even in the presence of other metal ions (Mg(II), Ca(II), Mn(II), Co(II), Ni(II), Zn(II), Cd(II), and Cu(II)) and under simulated physiological conditions, this biosensor displays a remarkable sensitivity and selectivity. Recently, we have also selected a catalytic DNA for UO_2^{2+} (Figure 4A (left inset)).[3] By attaching a fluorophore on the 5'-end of the substrate strand, a quencher on the 3'-end of the substrate and another quencher on the 3'-end of the enzyme, a fluorescent sensor for UO_2^{2+} was constructed (middle inset of Figure 4A). Initially, the fluorescence was quenched. Upon addition of UO_2^{2+}, increased fluorescence signal was observed (right inset of Figure 4A). The detection limit (45pM or 11 ppt) of the sensor rivals the most sensitive analytic instruments for uranium detection (Figure 4B, C). The EPA defined toxic level of UO_2^{2+} in drinking water is ~130 nM, which is well matched by the sensor. The sensor response to competing metal ions was also studied. For the tested 18 competing metal ions at 10 μM, 200 μM and 1 mM concentration, the sensor response was less than that in the presence of 1 nM UO_2^{2+} (Figure 4A). Therefore, the sensitivity was at least one million fold higher for UO_2^{2+}.

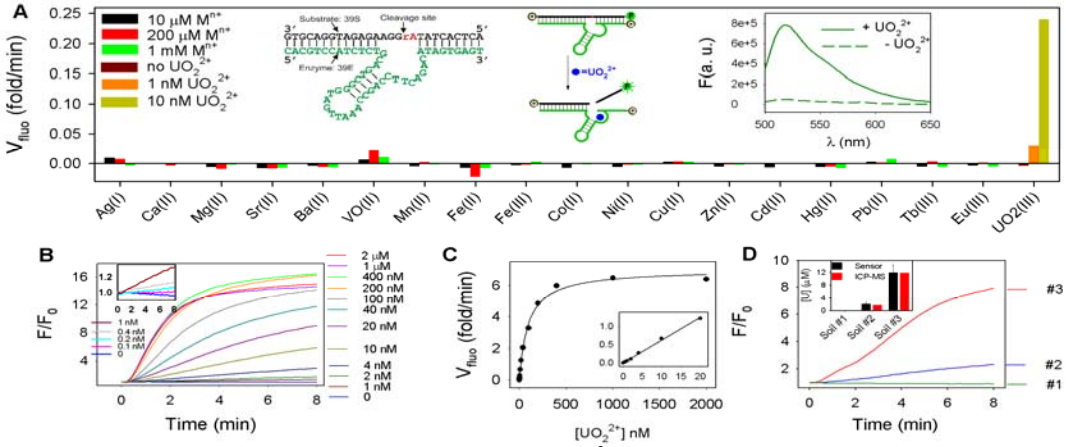

Figure 4. Design and performance of catalytic DNA-based UO_2^{2+} sensor. (A) metal selectivity. Competing metals were tested from 10 μM to 1 mM, and UO_2^{2+} (the last three bars) was tested from 0 to 10 nM. Inset: schematics of sensor design. (B) sensor fluorescence before and 10 minutes after addition of 1 μM UO_2^{2+}. (C) Kinetics of fluorescence increase with various UO_2^{2+} concentrations. (D) UO_2^{2+} quantification in field soil samples.

With the high sensitivity and selectivity, the performance of the sensor for UO_2^{2+} detection in contaminated soil samples was tested. In a collaboration with David Watson and Jonathan Istok, we tested three different samples from the Field Research Center at the Department of Energy Y-12 National Security Complex in Oak Ridge, TN. UO_2^{2+} was extracted by literature procedures using $NaHCO_3$ and Na_2CO_3.[35] The sensor response of the three extracted solutions (after diluting 300 fold) is shown in Figure 4D. The UO_2^{2+} concentrations in the original solutions were calculated to be 9.8, 2.0 and 0 for the three samples, respectively. These numbers were in good agreement with the values obtained from inductively

coupled plasma mass spectrometry (ICP-MS) analysis (within 20% difference). Therefore, the catalytic DNA-based sensor is capable of quantitative analysis of real-world samples with high accuracy.

Recently, we also obtained a highly sensitive Hg(II) sensor based on rational design. The sensor is based on the uranium catalytic DNA and several T-T mismatches were introduced in the stem loop region (Fig. 5A). The catalytic DNA is active only in the presence of Hg(II). Over 50-fold increase in fluorescence was observed (Fig. 5B). The rate of fluorescence enhancement increased with increasing Hg(II) concentration (Fig. 5C) and a detection limit of 2.4 nM was determined (Fig. 5D), less than the 10 nM MCL defined by EPA.

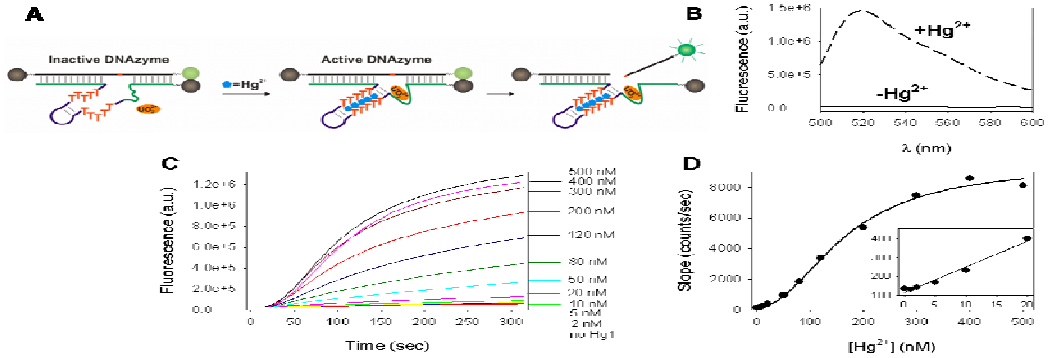

Figure 5. (A) Rational design of a highly sensitive and selective Hg(II) sensor. (B) Fluorescence spectra of the sensor in the absence and presence of Hg(II). (C) Hg(II) dependent kinetics of fluorescence increase. (D) Quantification of Hg(II) concentration with this sensor.

ii. Colorimetric biosensors. A simple colorimetric sensor could eliminate or minimize most costs associated with instrumentation and operation in fluorescence detection and thus can make on-site, real-time detection easier. Furthermore, a metal ion sensor with a tunable dynamic range is desirable for applications under widely different concentration ranges. However, few general strategies have been reported to vary the dynamic range without having to design new sensors. We have developed new highly sensitive and selective colorimetric metal sensors based on catalytic DNA-directed assembly of gold nanoparticles (Figure 6A, B).[5] The detection level can be tuned to several orders of magnitude, from 100 nM to 200 μM, through addition of an inactive variant of the catalytic DNA (Figure 6C). Through a series of careful studies and optimization, we can now perform the sensing at room temperature in <5 minutes.[6-8]

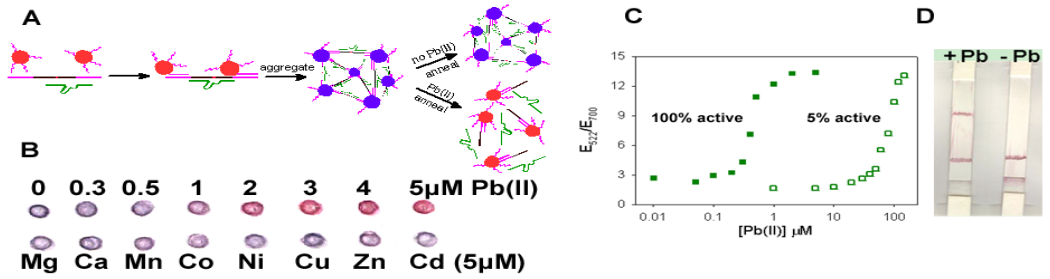

Figure 6. (A) Design of a colorimetric Pb(II) sensor. (B) Color of the sensor in the presence of Pb(II) or other divalent metal ions. (C) Tunable Pb(II) detection level of the sensor. When the enzyme strand is the active 17E only, the Pb(II) detection range is from 0.1 μM to 4 μM (solid green squares). When the ratio of 17E and 17Ec (inactive DNAzyme) is 1:20, the Pb(II) detection range is from 10 μM to 200 μM (open green squares). (D) Litmus paper-like devices for Pb^{2+} sensing.

With the experience gained from the design of colorimetric lead sensors, colorimetric uranium sensors have been demonstrated in a similar manner by using DNA-functionalized gold nanoparticles as the signaling method. Some preliminary data are presented in Figure 7. With increasing UO_2^{2+}

concentration, the rate of color change from blue to red increased (Figure 7A). At high UO_2^{2+} concentrations, the color change from blue to red was complete in 3 minutes. A photograph of UO_2^{2+} dependent color change of the sensor is shown in the inset of Figure 7B. A detection limit of 15 nM was determined, which was much lower than the 130 nM MCL defined by EPA. High selectivity of the sensor was also demonstrated and only UO_2^{2+} produced a color change (Figure 7C).

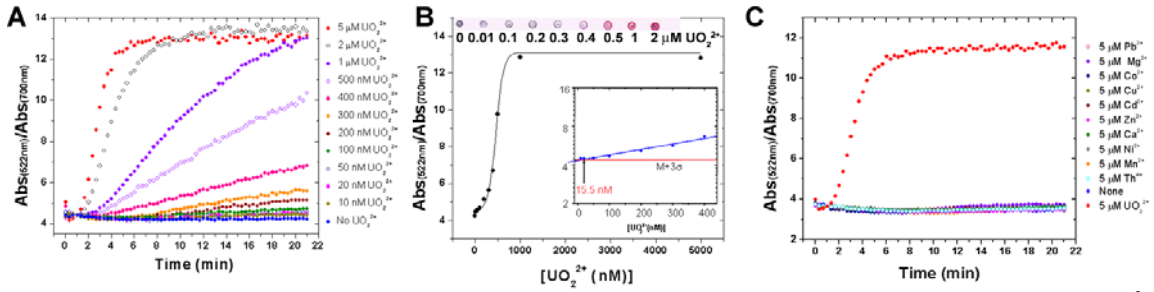

Figure 7. Kinetics of color change of the uranium sensor in the presence of varying concentrations of UO_2^{2+}. (B) UO_2^{2+} quantification. An image of UO_2^{2+} dependent color change is also shown. (C) Color change produced by only UO_2^{2+} and not other metal ions, shows high selectivity of the sensor.

iii. Dipstick colorimetric test. We have performed preliminary studies on the use of litmus paper-like devices for lead detection as proposed in Figure 13 on page 17. As shown in Figure 6D, two red lines are observed in the presence of lead, while only one red line in the control zone is observed in the absence of lead. This test format allows the elimination of solution transfer steps in detection, and therefore people without chemistry laboratory experience can also perform the test.

D. Stability and cost associated with catalytic DNA-based sensors.

i. Shelf life: The stability of the DNA sensors in solution is relatively low (no more than a few weeks). However, we and others in the field 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, if the DNA sensors are stored in dried form, the shelf lives can be many months and even years. The dried sensor has very high stability at room temperature. To shorten the time needed for stability assays, 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 8A), such as under -20ºC (6 days), room temperature (2 months), 45 ºC (21 days) and 60 ºC (6 days). After storing at 45 °C for over 100 days, the sensor still maintained its activity (Figure 8B). Even after storage at 80 °C, at which temperature most antibody-based sensors lose their function, the catalytic DNA-based sensor is still active (Figure 8C). 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.

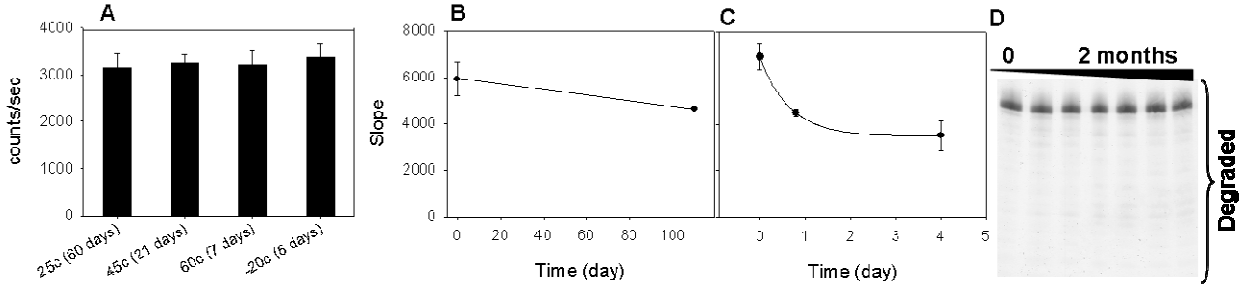

Figure 8. (A) Stability study of the catalytic DNA-based lead sensor after drying at different temperatures. Stability at 45 °C (B) and 80 °C (C). (D) Stability of DNA attached to gold nanoparticles. The nanoparticles were stored at room temperature in a glass vial on a work bench.

In addition, preliminary results from the PI's lab indicate that the gold nanoparticles in our colorimetric sensors can provide extra stability enhancement for the DNA attached on the particles as compared to the DNA not attached, even in solution. We prepared gold nanoparticles functionalized with fluorescently labeled DNA. At designated time points, an aliquot of the nanoparticle solution was centrifuged to separate the nanoparticles from supernatant. The DNA attached to the nanoparticle was released by adding an excess of a small alkanethiol ligand ($HSCH_2CH_2COOH$), and analyzed by gel electrophoresis. The DNA in the supernatant solution was also analyzed by gel electrophoresis. As can be observed in Figure 8D, no significant degradation of the DNA was observed over two months for the DNA attached to nanoparticles in solution.

ii. Cost. It takes only several dollars to carry out each round of selection, and the method for converting selected DNAzymes to sensors is well-established. Therefore, the development of the sensor is highly cost effective. The catalytic DNA-based sensors consume minimal materials, which can be attributed to the highly sensitive fluorescence detection, and the high extinction coefficients of metallic nanoparticles. The estimated cost for each detection is on the order of < $0.5 for both fluorescent and colorimetric sensors.

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