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Computer-Readable DNAzyme Assay on Disc for ppb-Level Lead Detection

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ABSTRACT: A method for the convenient detection of lead at the parts-per-billion (ppb)-level has been developed; it uses a conventional compact disc (CD) as the platform for preparing DNAzyme assays and an unmodified optical drive of ordinary desktop/laptop computers as the readout device. In particular, by immobilization of Pb^{2+}-specific DNAzyme sensing constructs on the "transparent side" of a conventional CD-R via mild surface reactions, the Pb^{2+} concentration can be determined by a free diagnostic program that checks the error distribution on the CD (i.e., it extracts the number of errors in a prerecorded audio file). The reading errors increase monotonically over a wide range of Pb^{2+} concentrations (from 10 nM to 1 mM), and the selectivity is confirmed by testing several other divalent cations (Zn^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, and Hg^{2+}).

Because of its accumulative biological effects, lead is considered a major environmental pollutant.[1] Fertilizers and pesticides are often contaminated with lead during inappropriate manufacturing processes or accidents.[2] Many health problems arise from the use of gasoline antiknock products and paint pigments.[3] Ingestion of lead can result in a wide variety of symptoms such as memory loss, irritability, anemia, and muscle paralysis.[4] Lead is also a potential neurotoxin that can be accumulated in and ultimately damage bones and kidneys. It can cause high blood pressure in adults and physical/mental developmental delays in children.[5] Nowadays, legal restrictions limit the lead level in drinking water and in the environment. The U.S. Environmental Protection Agency (EPA) has classified lead as a group B2 (probable) human carcinogen.[3] The "action level" of lead is 15 ppb in drinking water,[6] and 400−1200 ppm in soil.[7] According to the U.S. Food and Drug Administration (FDA), the lead action level is 0.5 ppm in infant and children products.[7]

Several analytical techniques have been developed for the determination of lead concentrations at ultralow levels including reversed phase-high-pressure liquid chromatography (RP-HPLC),[8] flame atomic absorption spectrometry (FAAS),[9] and X-ray fluorescence spectrometry.[10] For instance, FAAS has a detection limit as low as 8 ppb, which is not easily topped by other methods.[11] Despite the high sensitivity of these well-established analytical techniques, they require special laboratories, expensive and sophisticated equipment, time-consuming sample preparation, and experienced operators. The development of an on-site, inexpensive, and rapid method for lead detection would be desirable.

DNAzymes are specific DNA sequences that possess catalytic activities such as DNA/RNA ligation,[12] cleavage,[13] porphyrin metalation,[14] thymine dimer photoreversion, and nucleopeptide formation.[15] During the in vitro selection process,[16] a metal ion is typically used as a cofactor for a specific DNAzyme sequence, and this feature can be utilized to design a sensor for that ion.[17−24] Recently, several DNAzyme-based biosensors have been developed for lead detection by fluorescence, colorimetric, or electrochemical methods.[18] Lu and co-workers designed a colorimetric biosensor for lead(II) with gold nanoparticles modified with DNAzyme substrate strands. Partial hybridization induces their aggregation in solution (blue color); in the presence of lead(II), the DNAzyme-modified gold nanoparticles separate and the color changes to red.[18b] Another version of a DNAzyme biosensor detects lead ions electrochemically.[18e] When methylene-blue (MB) is tethered to the end of a surface-bound DNAzyme strand, the distance between the surface of a gold electrode and MB is far enough to inhibit electron transfer from MB to gold. In the presence of lead ions, DNA cleavage "destroys" the rigid structure which allows the MB to move closer to the electrode surface to produce a strong electrochemical signal.[18e]

Herein we describe a novel lead(II) detection method by using a conventional compact disc (CD) as the platform to prepare DNAzyme assays and a standard computer drive as the readout device. After the Pb^{2+}-specific DNAzyme sensing construct is immobilized on a CD-R surface via mild surface reactions, the Pb^{2+} concentration is determined by a diagnostic program (Plextools Professional LE V3.13 software, C1/C2 error test model, 10 × 24CAV) that checks the error distribution on the CD (i.e., it extracts the error numbers of prerecorded audio files). Besides the confirmation of the assay design with gel electrophoresis measurements, its sensitivity, selectivity, and dynamic range are thoroughly examined.

RESULTS AND DISCUSSION

Assay Design. The lead(II)-specific "8-17" DNAzyme sequence employed in this work has been previously investigated for the fabrication of fluorescent and colorimetrical sensors.[18a,18b] Our DNAzyme sensing construct to be immobilized on disc

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was designed to contain three single stranded DNA (Figure 1). The substrate (17DS) is a 32-mer oligonucleotide containing the single, sessile ribonucleoside adenosine (rA) and modified at the 5′-end with an amine group (for immobilization on the polycarbonate surface of a CD-R via amide coupling). The DNAzyme strand (17E) is a 33-mer oligonucleotide; a 5′-biotin-modified 12-mer DNA strand (biotin-DNA) was designed to hybridize with the 17DS strand at the 3′-end and serve as the reporter.

A conventional CD-R with prerecorded audio files was irradiated with UV light in the presence of ozone to generate carboxylic acid groups on the polycarbonate surface (the "transparent side"). It has been demonstrated previously that such a mild surface reaction does not alter the optical properties of the disk; the CD-R is still readable with a standard optical drive.[25a] Figure 2a shows the surface reactions involved in running disk-based DNAzyme assays for Pb^{2+} detection. The DNAzyme strand 17E was hybridized with the substrate strand 17DS in solution first. The half-duplex thus formed was immobilized on the CD surface via an amide coupling reaction between the 5′-amino and the surface carboxy groups. In the absence of Pb^{2+}, the 17DS was not cleaved and the entire strand remained on the CD surface (Figure 2a, top middle) upon denaturing the sensing construct by heating/washing. After addition of the biotin-labeled reporter strand, it hybridized with the 3′-end of the surface-attached 17DS. In the presence of Pb^{2+}, the substrate strand 17DS was cleaved into two fragments at the rA position (Figure 2a, bottom left). In this case, the denatured DNAzyme sensor construct was removed leaving only its 5′-end piece on the CD surface (Figure 2a, bottom middle) and a biotin-DNA strand was not able to hybridize with this short strand. In the presence of biotin-DNA strands on the surface (without Pb^{2+}), the streptavidin/gold nanoparticle conjugates were able to bind to the surface (Figure 2b, left). After the silver-staining reaction (the gold-nanoparticle-promoted reduction of Ag^{+}), the CD surface was covered with silver particles that interfere with the laser reading of the optical drive. The reading errors detected by the CD-quality control software corresponding to each of the assay "strips" on the CD[25b] were used to quantitate the concentration of Pb^{2+} in the testing solution.

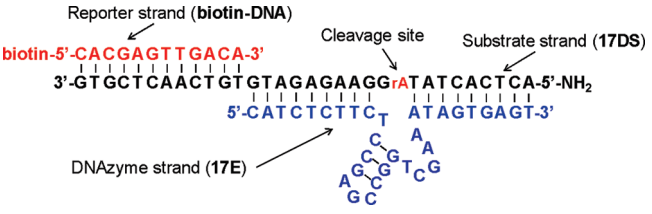

Figure 1. Design of the Pb^{2+}-specific DNAzyme sensing construct and the DNA strand sequences.

Confirmation and Optimization of the DNAzyme Sensing Construct. Figure 3a shows the gel electrophoresis results of

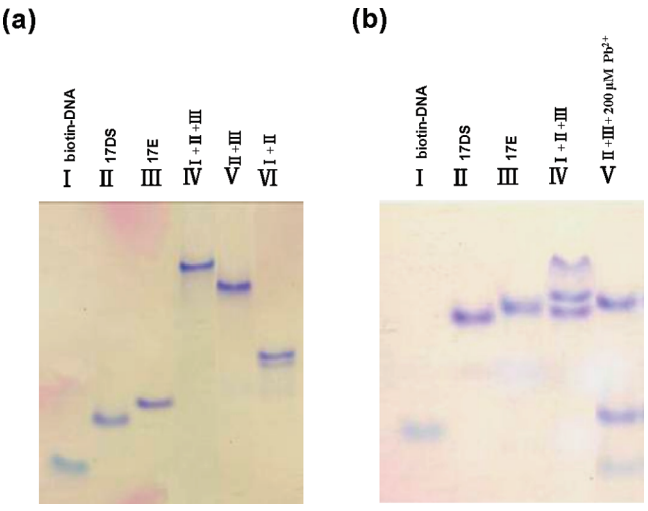

Figure 3. Gel electrophoresis assays of the DNAzyme sensors. (a) At 4 °C, lane I, 3′-biotin-modified DNA strand (biotin-DNA); lane II, 5′-NH_2-modified substrate strand (17DS, 32 mer); lane III, DNAzyme strand (17E, 33 mer); lane IV, DNAzyme sensor construct consisting of all three DNA strands; lane V, partial dsDNA of 17DS-NH_2 and 17E; lane VI, partial dsDNA of biotin-DNA and 17DS. (b) At room temperature (25 °C), lanes I−IV are the same as those in part a; lane V, a solution of 200 μM Pb^{2+} was added to the partial dsDNA (17DS-NH_2/17E); this lane confirms the structure shown in Figure 2a.

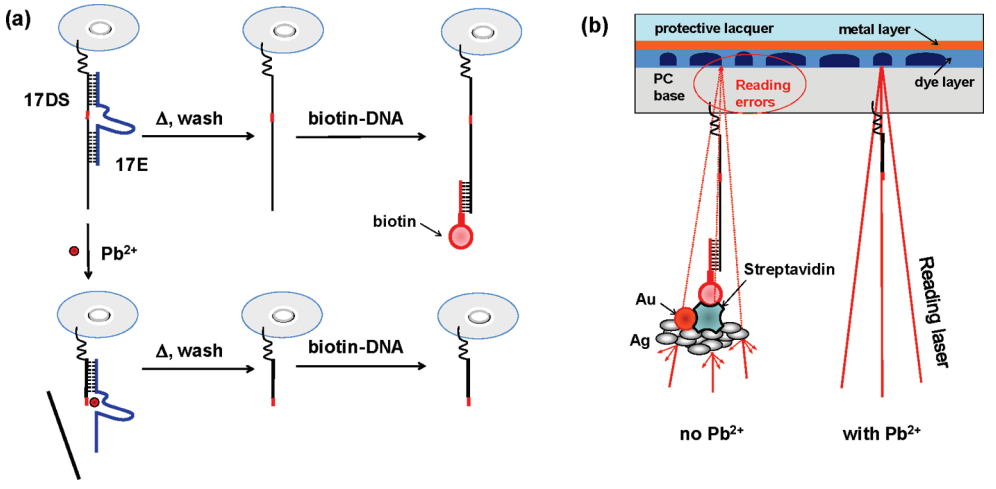

Figure 2. (a) Surface immobilization and reaction of DNAzyme sensors in the presence/absence of Pb^{2+}. (b) Digital reading with a conventional optical drive: the nanoparticles accumulated at the binding sites block the reading laser and generate significant errors at zero or low Pb^{2+} concentrations.

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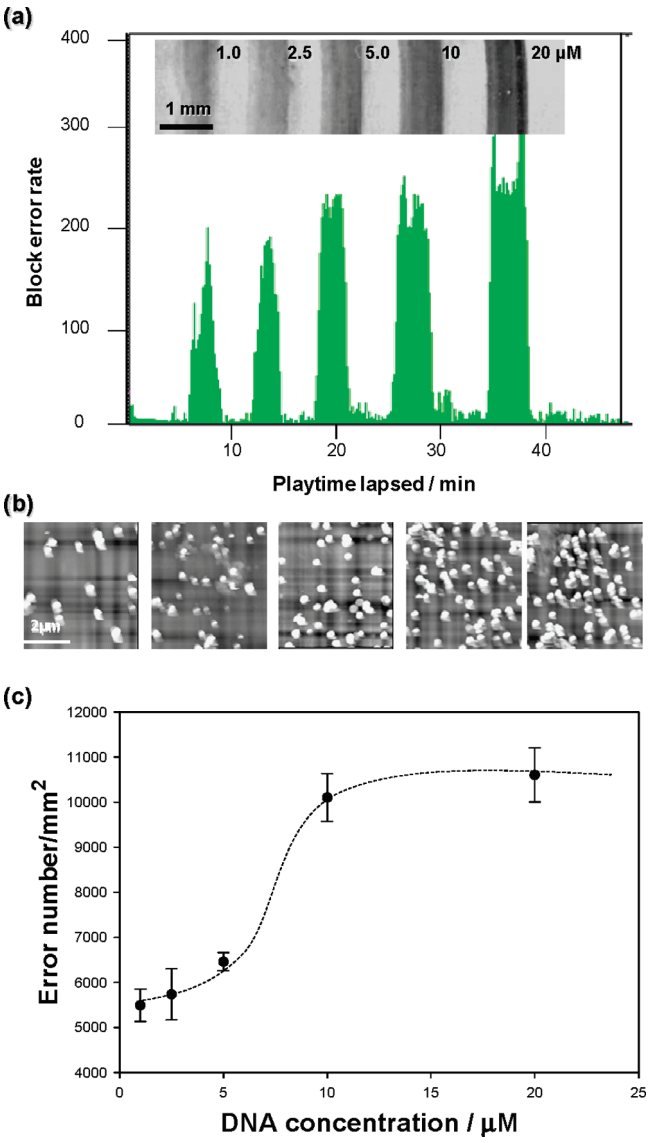

Figure 4. (a) Quantification of DNA binding assay: block error rates on a CD modified with five testing "strips" after silver staining for 75 min. The top inset is an optical image of the assay strips. (b) AFM images of the assay strips showing the size and density of silver particles/aggregates on the surface. (c) Error number as a function of the DNA (17DS) concentration used to prepare the assay disk; the dashed line is to direct the eyes only.

several combinations of the three DNA strands for which the gel was run at 4 °C. Lanes I, II, and III correspond to the biotin-DNA (12 mer), the substrate strand (17DS, 32 mer), and the DNAzyme strand (17E, 33 mer), respectively. Lane IV confirms the formation of the DNAzyme sensing construct (the single band at the top), lane V the partial double-stranded DNA structure when 17E is hybridized with 17DS (as shown in Figure 2a), and lane VI the other partial dsDNA structure (biotin-DNA/17DS). On the basis of these results, we can conclude that the three DNA strands (17E, 17DS, and biotin-DNA) can "hybridize" together to form the DNAzyme sensing construct.

When the assay was run at room temperature (25 °C) (Figure 3b), the DNAzyme sensing construct partially denatured, yielding the three bands shown in lane IV. We believe that

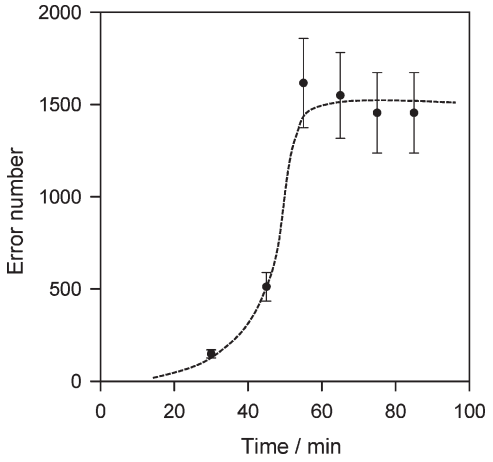

Figure 5. Block error rates versus the silver staining time. The DNA (17DS) concentration used to prepare the assay was 5.0 μM, and no Pb^{2+} was added to the system (maximum signal).

the denaturation was caused by the electric current, which generated sufficient heat to raise the temperature above the DNA melting point. Upon adding Pb^{2+} (200 μM) to the solution containing 17E/17DS, the 17DS strand was cleaved (lane V), demonstrating the feasibility of the DNAzyme sensor design shown in Figure 1.

It is important to optimize the surface density of DNAzyme sensing constructs on the disc to achieve the best detection results, and this can be done by changing the concentration of the 17DS strands in the deposition solution. Figure 4a shows the relationship between the concentration of 17DS (used to prepare the assay disk) and the block error rate, along with an optical image (inset) of the CD surface after silver staining. The CD exhibited a characteristic error distribution with five distinct bands whose digital positions (playtime) can be mapped to the physical locations of the assay strips. Atomic force microscopy (AFM) images (Figure 4b) revealed that these strips were composed of large silver aggregates (100−400 nm) with different densities which increased with increasing 17DS concentrations. The silver particle/aggregate size variations can be attributed to the differences in the number of gold nanoparticle "seeds" and the effects of competitive growth.[26]

Figure 4c shows the effect of DNA concentration (which in turn dictates the surface density of the DNAzyme sensing construct) on the block error rates of the assay strips. At lower concentrations, the block error rate is approximately proportional to the 17DS concentration. At higher concentrations, it reaches a plateau due to signal saturation. In the AFM images (Figure 4b), the surface density of silver particles shows the same trend.

The silver-staining time was another important parameter requiring optimization. Five different concentrations of 17DS (1.0, 2.5, 5.0, 10, and 20 μM) and 20 μM of the biotin-DNA solution were used for the test. Figure 5 shows that the error reading signals increased initially with time and then reached saturation. A shorter time was required to reach the saturation level with higher DNA concentrations. When the concentration exceeded 5.0 μM, the signals were saturated within 65 min of silver staining. On the basis of these results, we selected 5.0 μM as the concentration for 17DS and 65 min as the silver staining time for Pb^{2+} detection.

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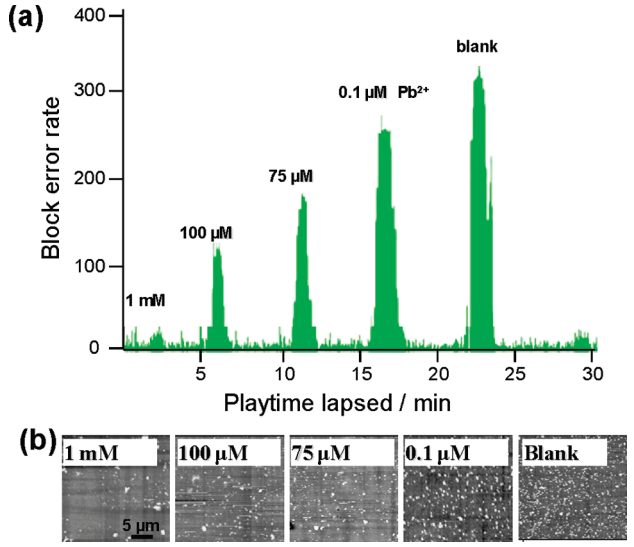

Figure 6. (a) Error reading plot of a Pb^{2+}-specific DNAzyme assaying disc. The Pb^{2+} concentration of each binding strip is listed at the top of each peak. The concentration of the 17DS solution used to prepare the assay was 5.0 μM and the time for silver staining was 75 min. (b) AFM images of the binding strips of four Pb^{2+} samples and a blank.

Quantification of Pb^{2+}. To illustrate the digitized signal reading protocol, we prepared a DNAzyme assay disk with four different concentrations of Pb^{2+} (from nanomolar to millimolar). Figure 6 shows the resulting error-reading plot, including a blank sample for comparison. As shown in Figure 6a, five distinct peaks whose playtimes could be traced back to the physical location of each sensing strip appeared.[25b] At a high concentration (1.0 mM) of Pb^{2+}, the reported errors are very small as the detachment of the 3′-ends of the substrate strands (17DS) significantly reduces the binding of the reporter strands (biotin-DNA). Consequently, the silver enhancement reaction is not occurring. The AFM image of the binding strips with four different Pb^{2+} concentrations (Figure 6b) confirmed our observation. The density of silver particles on the surface decreased significantly with increasing Pb^{2+} concentration, although the aggregate size increased slightly. With an increase in the Pb^{2+} concentration and the subsequent cleavage of 17DS, fewer biotin-DNA strands were able to attach to the surface. As the amount of streptavidin-nanogold conjugates depositing at the binding sites was reduced, silver particle accumulation occurred to a smaller extent. This leads to a smaller number of reading errors. These results demonstrate a direct correlation between the Pb^{2+} concentration and the error reading signal, which can be applied to the quantitative determination of ultralow levels of Pb^{2+}.

To evaluate the sensitivity and dynamic range of the disk-based DNAzyme assay method, various concentrations of Pb^{2+} (10 nM to 1 mM) were examined. Figure 7a shows the change in error numbers as a function of the Pb^{2+} concentration for the entire range of concentrations tested. Figure 7b shows the response over a smaller range of concentrations (0.01−10 μM), for which a linear calibration curve was obtained by using a log scale on the concentration axis. The error number per millimeter^2 for a Pb^{2+} concentration of 10 nM decreased by 9% with respect to the blank, while that for a concentration of 1.0 mM changed as much as 94%. The average uncertainty associated with error detection after repeated

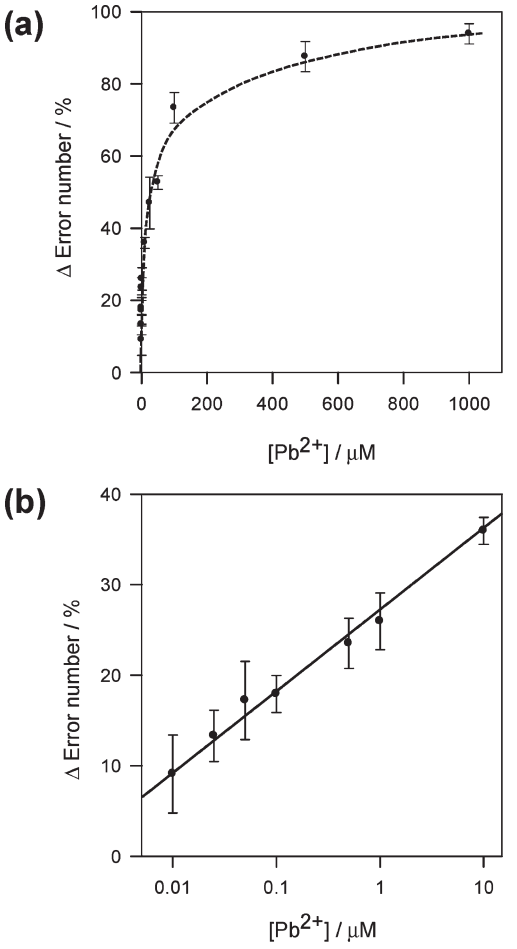

Figure 7. Dependence of the reading error number change on the concentration of Pb^{2+}. (a) The entire concentration range being tested (up to 1 mM). The dashed lines are to direct the eyes only. (b) A semilog calibration curve in the range of 10 nM to 10 μM. The solid line is a linear fit, $\Delta Er = 2.67 + 8.54 \log[Pb^{2+}]$, with $R^2 = 0.99$.

experiments is approximately 3%. Considering this uncertainty of ±3%, a change of 9% is an unambiguous signal that can be used to quantify the Pb^{2+} concentration. In other words, the detection limit of the disk-based DNAzyme assay is around 10 nM.

By obtaining the mean signal for blanks plus 3 times the standard deviation, we note that the detection limit (10 nM, which corresponds to 2 ppb by weight) is more than sufficient for the routine monitoring of lead levels in food and environmental samples. As mentioned previously, the toxic level of Pb^{2+} for human beings is 96 ppb according to the standard set by the U.S. Department of Health and Human Services. The FDA set the level at 0.5 ppm (0.5 μg/mL) in food products intended for infants and children.[7] The level of lead in drinking water should be less than 15 ppb, although the acceptable range in soils is much higher (400−1200 ppm) according to the Environmental Protection Agency's standard. The above comparison indicates that the disk-based DNAzyme assaying method is promising for on-site monitoring and testing with it superior detection limit and wide dynamic range.

Verification Using Optical Means and Selectivity Validation. The concentration dependence was further confirmed by colorimetric measurements, i.e., by determining the optical darkness ratios (ODR) of the Pb^{2+} assay strips with a flatbed

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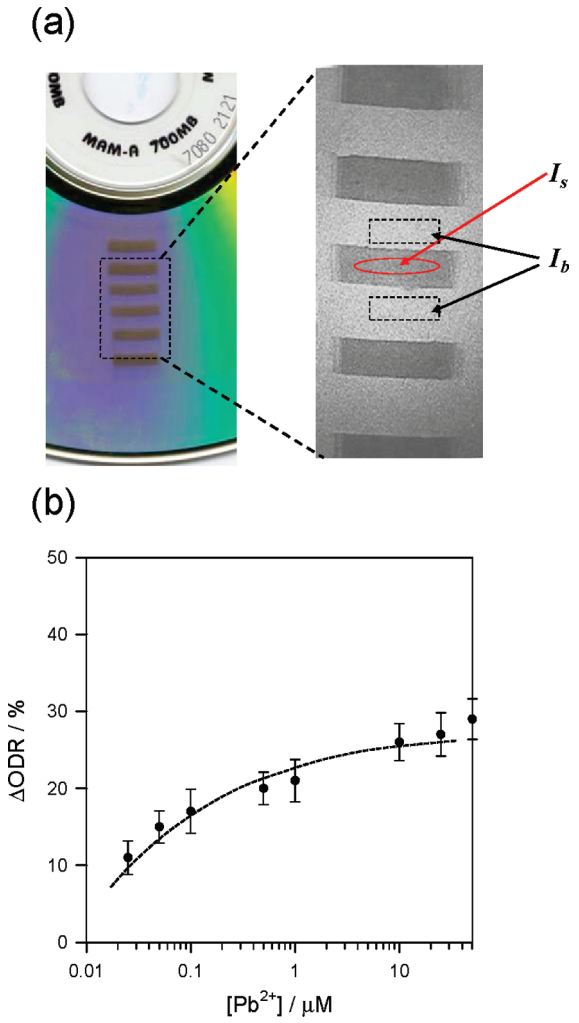

Figure 8. (a) The DNAzyme assay strips on the CD surface (left) and their corresponding optical images (right). (b) Dependence of the ODR (optical darkness ratio) change on the concentration of Pb^{2+} (ranging from 10 nM to 50 μM). On the basis of four replicates, the ODR changed by 10−28% in this range. The dashed line is to guide the eyes only.

scanner and the Photoshop software using the formula:[27]

$$\mathrm{ODR} = (I_b - I_s)/I_b \quad (1)$$

where I_b is the average luminosity of the background and I_s is that of a binding strip (Figure 8a), which is a function of Ag/Au particle size and density. Figure 8b shows the dependence of ODR values on the Pb^{2+} concentration. It is expected that the ODR values decrease upon increasing Pb^{2+} concentrations: it decreased by 10% to 28% with a change in the Pb^{2+} concentration from 10 nM to 50 μM. The digital readout method (i.e., reading disc-based DNAzyme assays with a computer drive) to determine error number rate is better than the colorimetric protocol for the following two reasons. First, it can be read directly from the disk with a CD-quality diagnostic software. In contrast, to obtain the ODR values, we had to take images of the binding assays with an optical microscope/high-resolution flatbed scanner and calculate the values manually. Second, the digital method to determine error numbers (Figure 7) is more sensitive to low Pb^{2+} concentration changes than the ODR approach (Figure 8b).

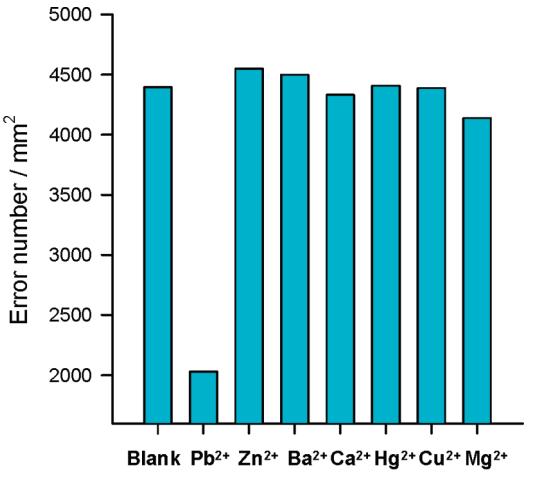

Figure 9. Error numbers of DNAzyme sensing strips on a CD treated with 50 μM of different divalent metal ions. No significant error number changes were observed with divalent metal ions other than Pb^{2+}.

In addition to the unprecedented sensitivity, the specificity of this method was evaluated by testing several other divalent metal ions (Zn^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}) at a concentration of 50 μM (Figure 9). A significant error number decrease was evident only for Pb^{2+}, not for any other divalent metal ions. This high selectivity for lead can be attributed to the selection process of the DNAzyme.[17,18a,18b] We also note that in testing water samples, the dissolved organic carbon (DOC) levels should not affect the detection; the cleavage of the substrate strand in the DNAzyme sensor construct only takes place in the presence of the specific metal ion.[18]

With the standard optical drive being the only instrument required, the cost of the analysis is significantly reduced. Although the DNAzyme is relatively expensive, the amount of it being used for each test is little and the overall cost would not be significant. In comparison with the conventional test in which an atomic spectrometer is used, a typical test costs less but the instrument is expensive, not to mention regular maintenance required for such an instrument. Despite the fact that digestion of the environmental sample is still required,[28] this disc-based analytical method for certain does aid in making the analysis more accessible to peripheral laboratories rather than central laboratories. In doing so, the time and cost for the test would be further reduced.

CONCLUSIONS

We have developed a disk-based DNAzyme assay for Pb^{2+} detection with high sensitivity and specificity. The signal readout is performed with a conventional optical drive, and the assay is prepared on a regular compact disk. The detection limit of this method is as low as 10 nM (2 ppb), which is adequate for the analysis of drinking water and environmental samples. It should be emphasized that a standard CD drive is the only instrument required. More importantly, this detection method offers a platform technology for the fabrication of DNAzyme sensors to analyze other metal ions because DNAzymes specific for Hg^{2+}, Cu^{2+}, UO^{2+}, and Zn^{2+} have also been obtained by in vitro selection.[17]

EXPERIMENTAL SECTION

Materials and Chemicals. The DNA oligonucleotides were purchased from the University Core DNA Services, University of

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Calgary (Calgary, CA). CD-Rs (80 min, 700 MB) were purchased from MAM-A Inc. (Colorado Springs, CO). *N*-Hydroxysuccinimide and *N*-(3-dimethylaminopropyl)-*N*′-ethylearbodiimide hydrochloride were purchased from Sigmal-Aldrich. The silver enhancement kit was purchased from Nanoprobes Inc. (New York). Acrylamide−Bis solution 40% was obtained from EMD Chemicals Inc. (Gibbstown, NJ). Deionized water (>18.3 MΩ cm) was produced in a Barnstead Easy Pure UV/UF compact water system (Dubuque, IA).

Gel Electrophoresis. To prepare the DNAzyme sensor for Pb^{2+} detection, 5.0 μM of each of the DNA strands (17E, 17DS) were mixed in a 25 mM tris-acetate buffer containing 300 mM NaCl (pH 7.2). The solution was heated to 75 °C for 5 min and allowed to cool slowly to room temperature in a water bath. Gel electrophoresis experiments were carried out using a 15% nondenaturing gel containing 10% ammonium persulfate (APS) and 4% tetramethylethylenediamine (TEMED). The gel was run in a SE600 series standard vertical electrophoresis unit, either at 140 V for 5 h at 4 °C or at 200 V for 2.5 h at 25 °C. After running, the gel was stained with the "stains all" solution for 1 h and destained by exposure to room light.

Assay Preparation. Before surface activation, audio data were recorded onto a blank CD-R. The CD surface was then cleaned with ethanol and deionized water and irradiated with UV light in the presence of ozone for 20−40 min to produce a hydrophilic surface with a high density of carboxylic acid groups. The disk was immersed in a 0.1 M phosphate buffer containing 5 mM 1-ethyl-3-(3′-dimethyl aminopropyl)-carbodiimide (EDC) and 0.33 mM *N*-hydroxysuccinimide (NHS) at pH 6.0 for 4 h.[25b] A polydimethylsiloxane (PDMS) plate with embedded microfludic channels was placed onto the CD surface. The hybridized 17DS/17E samples were injected into the channels for immobilization. Unbound partial dsDNAs in the channels were removed by washing with the phosphate buffer saline (PBS), and the unreacted carboxy groups were blocked with bovine serum albumin (BSA). The Pb^{2+} solution was added into the channels and incubated for 45 min which led to the cleavage of the DNAzyme substrate strands (17DS) at the rA position. After placing the CD in an oven at 75 °C for 10 min, the denatured dsDNA constructs were removed by washing with the PBS buffer, leaving only the 5′-end of the 17DS strand on the surface. The surface was passivated by treating with 20 mM phosphate buffer containing 150 mM NaCl, 4% bovine serum albumin (BSA), and 2 mM sodium azide (NaN_3) (pH 7.40) for 30 min at room temperature, followed by the addition of the biotin-DNA strands (reporter) for 1 h at room temperature. The CD was then rinsed with the same buffer (3 × 10 min).

Signal Amplification and Signal Readout. Streptavidin-gold nanoparticle conjugates in the 20 mM phosphate buffer containing 0.8% BSA and 0.1% gelatin were added to bind to the biotin groups on the surface. The CD was then rinsed sequentially with the phosphate buffer (5 min), deionized water (2 × 5 min), and 0.05 M EDTA at pH 4.5 (5 min). The removal of the PDMS stamp was followed by silver enhancement reaction with a mixture of equal volumes of the initiator and enhancer solutions provided by the manufacturer (Nanoprobes Inc.; LI Silver Kit). The signal-enhanced CD was washed with deionized water and dried under nitrogen. As mentioned above, a free diagnostic program (Plextools Professional LE V3.13 software, C1/C2 error test model, 10 × 24CAV) was used to check the error distribution on the DNAzyme disk with a standard optical drive.

Optical Imaging and Analysis of Pb^{2+}-Specific DNAzyme Assays on Disc. The optical images of the DNAzyme assay strips on the CD surface were taken with a Motic digital microscope (DM143, Micro-Optic Industrial Group Co.) and analyzed with the Adobe Photoshop CS software. The surface topographies of the assay "strips" were examined using an MFP-3D-SA atomic force microscope (Asylum Research, Inc.) in the tapping mode with a rotated monolithic silicon tip (Innovative Solutions Bulgaria Ltd., resonance frequency 13 kHz and force constant 0.2 N/m). The number, size, and morphology of the particles after silver enhancement were analyzed with the software IGOR Pro 4.

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