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Spinach RNA aptamer detects lead (II) with high selectivity†

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Abstract

Spinach RNA aptamer contains a G-quadruplex motif that serves as a platform for binding and fluorescence activation of a GFP-like fluorophore. Here we show that Pb^{2+} induces formation of Spinach's G-quadruplex and activates fluorescence with high selectivity and sensitivity. This device establishes the first example of an RNA-based sensor that provides a simple and inexpensive tool for Pb^{2+} detection.

Heavy metals are among the most dangerous pollutants in our environment, and lead toxicity has been a major concern in recent times.[1-3] The traditional methods to detect Pb^{2+} such as, Atomic Absorption Spectroscopy (AAS), Atomic Emission Spectroscopy (AES) and Inductive Coupled Plasma-Mass Spectrometry (ICP-MS) involve sophisticated equipment and are not suitable for on-site detection.[1] During the last decade, DNA has been used extensively as a biomolecular Pb^{2+}sensor.[3] Most DNA-based devices fall broadly into two classes based on their mechanisms of action- first, Pb^{2+}-dependent DNAzyme-catalyzed RNA cleavage,[4a-f] and second, Pb^{2+}-dependent formation of DNA G-quadruplex (Table S1†).[3,5a-c,6a,b] In the first class of sensors, the rates of signal enhancement need to be monitored for quantitative detection, requiring substantial data processing.[4a-f] The second class exploits the potential of Pb^{2+} to induce formation of G-quadruplexes from single or double-stranded G-rich DNA. This class of sensors usually needs covalent incorporation of fluorophores[6a,b] or involves chemical reactions that generate colored/chemiluminiscent products,[3,5a-c] which limit the use of these sensors to certain favourable conditions of pH and ionic strength. DNA based electrochemical sensors, most of which use one of the above mechanisms, have been used for sensitive Pb^{2+} detection. These sensors involve elaborate assemblies, usually requiring immobilization of DNA molecules to gold electrodes.[2,4d,5c,6c,d] Beyond these potential limitations, the high costs of these devices complicate their practical use.[6b] Inspired by the fact that Pb^{2+} could induce/stabilize G-quadruplex formation, we report the use of the Spinach RNA aptamer as the first example of an RNA-based device for detection of Pb^{2+} with high sensitivity and selectivity.

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Spinach, an RNA aptamer that was obtained by *in-vitro* selection from a random sequence pool of RNAs, bind to 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), an analogue of the fluorophore in GFP, and activates its fluoresence.[7] Fusion of Spinach to other aptamers[8a] and hybridization sequences[8b,c] has enabled detection of metabolites and proteins *in-vivo* and oligonucleotides *in vitro*, respectively. In these Spinach coupled sensors binding of a specific analyte induces the active conformation of Spinach, thereby triggering DFHBI fluorescence. Spinach has also been used to monitor *in-vitro* transcription efficiency in real time.[8d] Crystal structures of Spinach reveal the presence of a two-layer G-quadruplex motif that serves as a stacking platform for DFHBI binding.[9a,b] Formation of this quadruplex motif requires millimolar concentrations of K^+, Na^+, or NH_4^+. Micromolar concentrations of Pb^{2+} induce the formation of DNA G-quadruplexes even in the absence of K^+ or Na^+, the two most common cations associated with G-quadruplexes. Pb^{2+}-stabilized G-quadruplexes adopt a more compact structure than those stabilized by monovalent cations due to the smaller size and high charge density of Pb^{2+}.[6a,10,11,12a,b] We wondered whether these observations extend to the RNA G-quadruplex in Spinach. If Pb^{2+} could support formation of the RNA G-quadruplex in Spinach, the high quantum yield and low photo-bleaching of DFHBI[7] would make Spinach an attractive candidate for a sensitive and selective Pb^{2+} sensor. We used a truncated form of Spinach (Fig. 1, Fig. S1†) that shows equivalent fluorescence to the wild-type RNA.[9a] In the absence of cations at concentrations sufficient to support G-quadruplex formation, no fluorescence occurred when Spinach was incubated with DFHBI. However, addition of sub-micromolar quantities of Pb^{2+} resulted in fluorescence, suggesting that Pb^{2+} can support formation of the RNA G-quadruplex in Spinach, thereby allowing the RNA to bind the fluorophore and activate its fluorescence. Even at 1 μM concentration of Pb^{2+} we observed a strong fluorescence signal that was visible under a hand-held UV lamp (Fig. S2†). An enhancement in the 264 nm peak in the Circular Dichroism (CD) spectrum of Spinach upon addition of 10 μM Pb^{2+} suggested the formation of a G-quadruplex in presence of Pb^{2+} (Fig. 2). The relatively low peak enhancement presumably reflects the fact that only about 14% (8 out of the total of 57 nucleotides) of the RNA folds into a G-quadruplex. Mutating the quadruplex guanines to disturb the formation of the G-quadruplex completely abrogates Pb^{2+}-induced Spinach fluorescence, providing further evidence that Pb^{2+} supports formation of the G-quadruplex in Spinach (Fig. S2†). The short incubation time for this sensor (15 minutes) makes detection fast compared to most Pb^{2+} sensors reported in literature (Table S1†).[3,4c-e,5a-c] In addition, short incubation times limit any significant RNA cleavage induced by Pb^{2+}, RNase A or pH variations that might be encountered in real life applications and thus attenuate sensitivity (Fig. S4†).

For optimum performance, the sensor must be sensitive enough to detect Pb^{2+} at nanomolar concentrations and selective for Pb^{2+} over other cations. Fluorescence was measured at different concentrations of Pb^{2+} and signal enhancement was observed up to 10 μM (Fig. 3), consistent with increased folding of the RNA into a quadruplex with increased concentrations of Pb^{2+}. Increasing concentrations of Pb^{2+} in the presence of DFHBI (without the Spinach aptamer) showed no fluorescence enhancement, underscoring the role of the RNA in fluorescence activation of DFHBI (Fig. S5†). The decrease in fluorescence after 10 μM Pb^{2+} might reflect non-specific quenching by Pb^{2+} (Fig. S6†).[5d] The signal response was linear in the range 5 nM-500 nM Pb^{2+} (inset Fig. 3) and the detection limit for Pb^{2+} was 6 nM (based on 3σ/slope method, where σ is the standard deviation of blank), which is well below the maximum permissible level for Pb^{2+} concentration in drinking water (72 nM or 15 ppb).[1] The linear range and sensitive detection limit make our Spinach sensor suitable for quantitative detection of Pb^{2+} at low to moderate concentrations that are likely to be encountered in real life samples.

We tested the Spinach sensor with different environmentally relevant cations and found it to be highly selective towards Pb^{2+} (Fig. 4, Fig. S7†). Cations known to stabilize G-quadruplexes, Na^+, K^+ and Ca^{2+} [12a,13] showed fluorescence signals that were only a little above background even when present in 30-fold (Na^+) and 3-fold (K^+, Ca^{2+}) excess over Pb^{2+}. In fact the Spinach sensor is more than 17000-fold selective for Pb^{2+} compared to the most competing ion Ca^{2+} (inset Fig. 4), which to our knowledge makes it the most selective nucleic acid-based sensor for Pb^{2+}(Table S1†). Spinach signal due to the presence of Pb^{2+} was virtually unaffected in a metal soup that contained Ag^+, Ca^{2+}, Co^{2+}, Cr^{3+}, Cd^{2+}, K^+, Na^+, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} (Fig. 4). These observations highlight the high selectivity of the sensor and demonstrate its potential utility for analysis of Pb^{2+} in samples containing other metal ions. The apparent K_D values for metal ion binding to Spinach RNA were 0.0013±0.0004 mM (Hill coefficient n=1.5±0.4), 2.3±1.1 mM (Hill coefficient n=0.7±0.3) and 9.6±0.4 mM (Hill coefficient n=1.6±0.1)[9a] for Pb^{2+}, Ca^{2+} and K^+, respectively, indicating that Pb^{2+} bound to Spinach with an affinity that was three orders of magnitude greater than did K^+ or Ca^{2+}, further emphasizing the selectivity of the sensor towards Pb^{2+} (Figs S8†, S9†). Consistent with these observations, at micromolar concentrations of Pb^{2+}, K^+ or Ca^{2+}, significantly lower concentrations of DFHBI were required to show fluorescence in the presence of Pb^{2+} compared to K^+ or Ca^{2+} (Figs S10†, S11†), reflecting the formation of G-quadruplex in micromolar concentrations of Pb^{2+} but not in micromolar concentrations of K^+ or Ca^{2+}.[12a]

Detection methods based on Pb^{2+}-induced G-quadruplex formation tend to yield low signal to noise ratios due to presence of high concentrations (in the millimolar range) of metals like K^+, Na^+ and Ca^{2+} that induce G-quadruplex formation. High background signals make their use in practical samples potentially problematic.[14] To assess this possible limitation in the Spinach sensor, we tested its performance using samples of tap water. First, we examined the sensor's stability in tap water and observed no degradation over the course of the measurement including after incubation at 37°C overnight in presence of 10 µM Pb^{2+} (Fig. S4a†). The sensor was stable to degradation between pH 3 and pH 11 in the time frame of detection (Fig. S4b†) and fluorescence signal was optimal between pH 6 and pH 8 (Fig. S12†). ICP-MS analysis of tap water samples showed that Ca^{2+}, Na^+ and K^+ were present in 568 µM (22.7 ppm), 350 µM (8.1 ppm) and 28 µM (1.1 ppm) concentrations, respectively. The absence of significant fluorescence in the presence of K^+ and Na^+ at these concentrations (Fig. 4) indicated that the presence of Ca^{2+} caused the observed background signal when the Spinach sensor was used in tap water. To identify conditions that enable improved selectivity for detection of Pb^{2+} over Ca^{2+}, we analyzed fluorescence signal as a function of increasing DFHBI concentration in the presence of a constant concentration of Pb^{2+} or Ca^{2+} (Fig. S11†). We found that Spinach required significantly higher concentrations of DFHBI to give the same intensity of fluorescence signal in the presence of

Ca^{2+} compared to the presence of Pb^{2+}. For example, in the presence of 50 mM Ca^{2+}, Spinach did not show substantial signal above background when the concentration of DFHBI was below 10 μM, (Fig. S11b†) whereas significant signal was obtained in the presence of 10 μM Pb^{2+}, even at concentrations as low as 0.5 μM (Fig. S11a†). Thus, the background signal due to Ca^{2+} was minimized by lowering the concentration of DFHBI used from 20 μM (used with 5 μM RNA for most assays) to 1 μM (used with 100 nM RNA). These conditions rendered the sensor completely insensitive to the presence of Ca^{2+}. Using these conditions, we then tested the performance of our sensor for Pb^{2+} detection in tap water. We spiked tap water with different concentrations of Pb^{2+}. Fluorescence signal increased linearly with Pb^{2+} concentration in the range of 100 nM-5 μM (Fig. S13†). These results demonstrate a possible real-life application of the Spinach sensor for detecting Pb^{2+} in tap water. Other potential applications of our sensor could include measuring Pb^{2+} in paint, which contains no interfering G-quadruplex stabilizing cations.[15] A DNA version of the Spinach-sensor could be potentially a cheaper and more robust alternative for these practical applications. An effective DNA-Spinach could be obtained by carrying out an *in-vitro* selection for DFHBI binding from a random pool of DNAs, possibly enriched in guanines to bias toward the possibility of the molecule having a G-quadruplex, which is the chemical basis for Pb^{2+} sensing.

In conclusion, we have reported the first example of an RNA-based sensor as a selective and sensitive tool for detection of Pb^{2+}, thus expanding the repertoire of nucleic acid based sensors for heavy metals to include RNA (Table S1†). Our Spinach sensor offers the following distinct advantages: it does not need fluorophore-quencher pairs that require covalent attachment to the nucleic acid, does not involve expensive nanomaterials or complicated detection systems that have been used for some previous DNA-based Pb^{2+} sensors, [16] and has a very simple design and operation that is cost effective and easy to use for ‘on the spot’ detection of Pb^{2+}.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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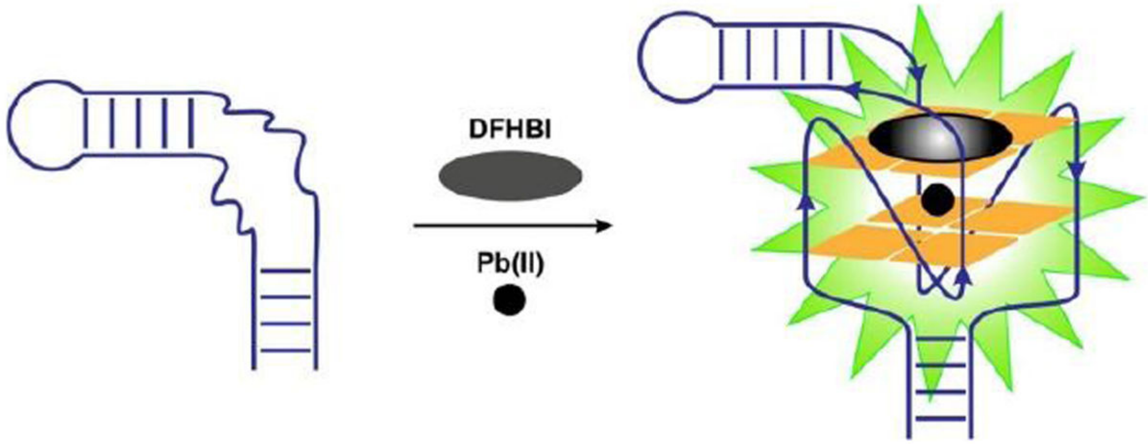

Fig.1.

Schematic illustration of the Spinach sensor. The unstructured region (shown by curved lines) in the Spinach RNA aptamer folds into a two-layered G-quadruplex in the presence of Pb^{2+} and binds DFHBI fluorophore, activating fluorescence.

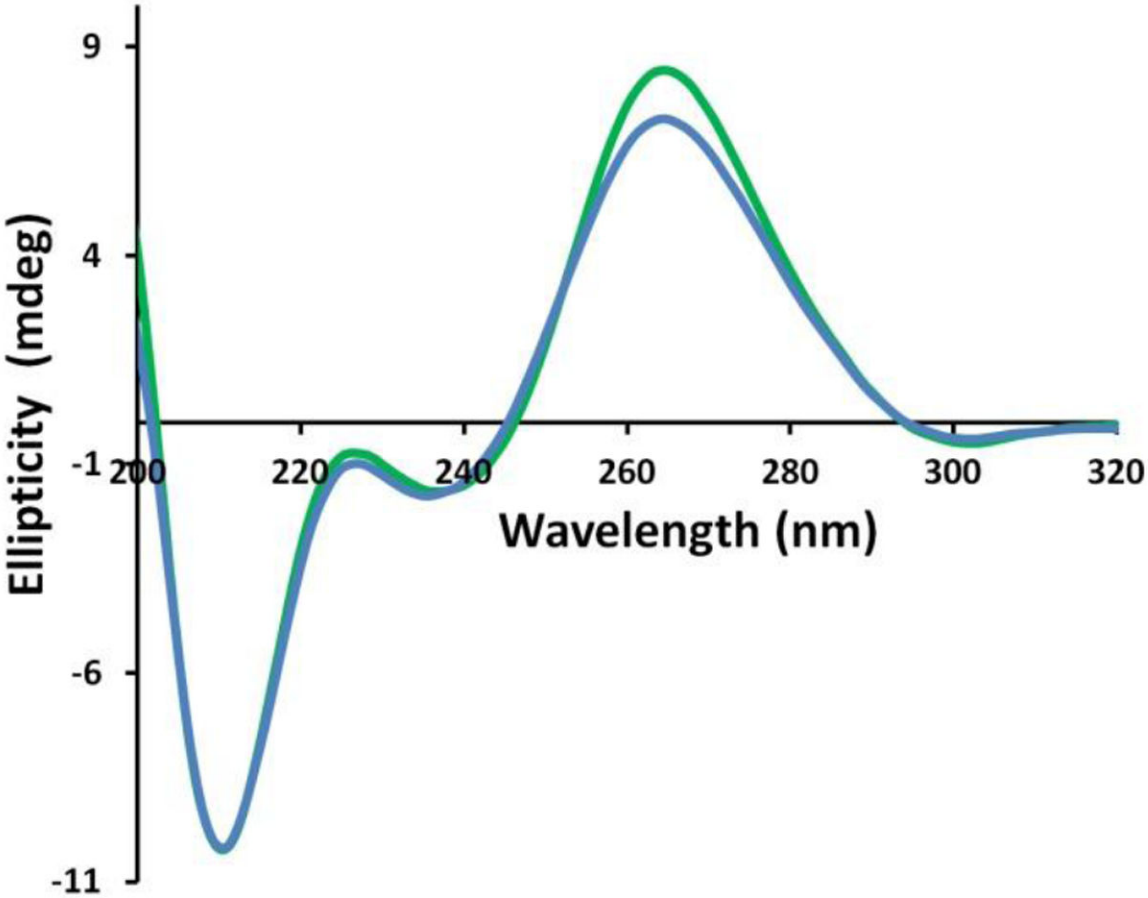

Fig. 2.

CD spectra of Spinach-DFHBI complex. A distinct enhancement of the positive peak at 264 nm after addition of 10 μM Pb^{2+} (green) indicates formation of G-quadruplex in Spinach RNA in the presence of Pb^{2+} at this concentration. The spectrum in blue is obtained without Pb^{2+}. Experiments were performed at a pH 7.5 and 5 mM Mg^{2+}. RNA and DFHBI concentrations were 5 μM and 20 μM, respectively.

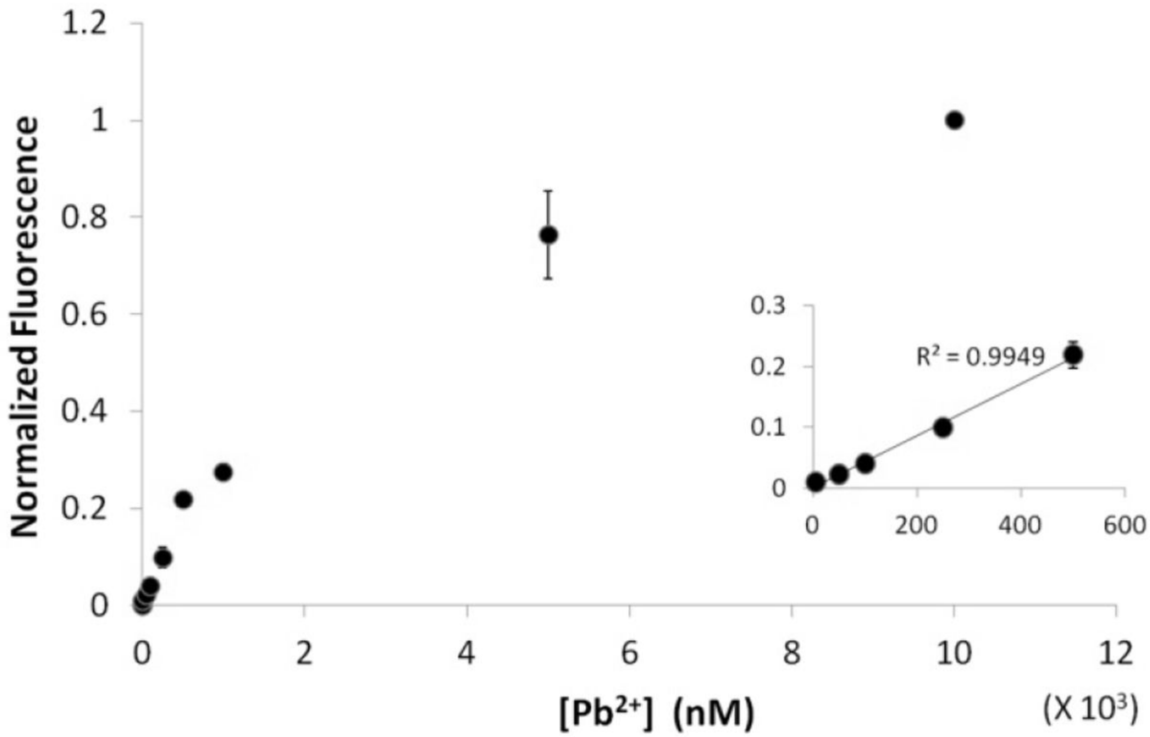

Fig.3.

Spinach fluorescence versus the concentration of Pb^{2+}. Inset: signal increases linearly (R^2=0.9949) with Pb^{2+} concentration in the range of 5 nM-500 nM. Experiments were performed at a pH 7.5 and 5 mM Mg^{2+}. RNA and DFHBI concentrations were 100 nM and 1 μM, respectively.

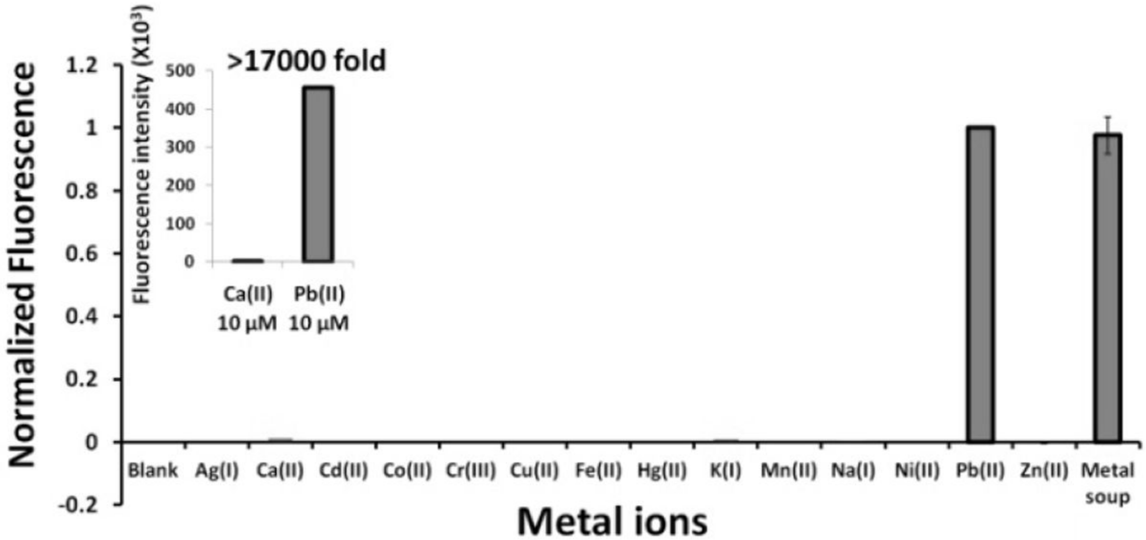

Fig.4.

Spinach sensor detects Pb^{2+} with greater than 17000-fold selectivity relative to the most interfering cation Ca^{2+} (inset). Pb^{2+} was assayed at 10 µM, Ca^{2+} and K^{+} at 30 µM and the remaining metal ions at 300 µM. Experiments were performed at a pH 7.5 and 5 mM Mg^{2+}. RNA and DFHBI concentrations were 5 µM and 20 µM, respectively.