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

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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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ sensor. 3 Most DNA-based devices fall broadly into two classes based on their mechanisms of action- first, Pb²⁺-dependent DNAzyme-catalyzed RNA cleavage 4a,d and second, Pb²⁺-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,d The second class exploits the potential of Pb²⁺ to induce formation of G-quadruplexes from single or double-stranded G-rich DNA. This class of sensors usually needs convenient incorporation of fluorophores 4a,b or involves chemical reactions that generate colored/chemiluminescent 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 Pb²⁺ detection. These sensors involve elaborate assemblies, usually requiring immobilization of DNA molecules to gold electrodes 2,4d,5c,6a,d Beyond these potential limitations, the high costs of these devices complicate their practical use. 6c Inspired by the fact that Pb²⁺ 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 Pb²⁺ 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 fluorescence. 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. 6d 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 NH₄⁺. Micromolar concentrations of Pb²⁺ induce the formation of DNA G-quadruplexes even in the absence of K⁺ or Na⁺, the two most common cations associated with G-quadruplexes. Pb²⁺-stabilized G-quadruplexes adopt a more compact structure than those stabilized by monovalent cations due to the smaller size and high charge density of Pb²⁺ 6a,10,11,12a,b. We wondered whether these observations extend to the RNA G-quadruplex in Spinach. If Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ resulted in fluorescence, suggesting that Pb²⁺ can support formation of the

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 Pb²⁺ and binds DFHBI fluorophore, activating fluorescence.

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enhancement in the 264 nm peak in the Circular Dichroism (CD) spectrum of Spinach upon addition of 10 μM Pb²⁺ suggested the formation of a G-quadruplex in presence of Pb²⁺ (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 Pb²⁺-induced Spinach fluorescence, providing further evidence that Pb²⁺ supports formation of the G-quadruplex in Spinach (Fig. S3†). The short incubation time for this sensor (15 minutes) makes detection fast compared to most Pb²⁺ sensors reported in literature (Table S1†, 3.3c-5a-c). In addition, short incubation times limit any significant RNA cleavage induced by Pb²⁺, 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 Pb²⁺ at nanomolar concentrations and selective for Pb²⁺ over other cations. Fluorescence was measured at different concentrations of Pb²⁺ 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 Pb²⁺. Increasing concentrations of Pb²⁺ 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 Pb²⁺ might reflect non-specific quenching by Pb²⁺ (Fig. S6†). The signal response was linear in the range 5 nM-100 nM Pb²⁺ (inset Fig. 3) and the detection limit for Pb²⁺ 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 Pb²⁺ concentration in drinking water (72 nM or 15 ppb). The linear range and sensitive detection limit make our Spinach sensor suitable for quantitative detection of Pb²⁺ 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 Pb²⁺ (Fig. 4, Fig. S7†). Cations known to stabilize G-quadruplexes, Na⁺, K⁺ and Ca²⁺ showed fluorescence signals that were only a little above background even when present in 30-fold (Na⁺) and 3-fold (K⁺, Ca²⁺) excess over Pb²⁺. In fact the Spinach sensor is more than 17000-fold selective for Pb²⁺ compared to the most competing ion Ca²⁺ (inset Fig. 4), which to our knowledge makes it the most selective nucleic acid-based sensor for Pb²⁺ (Table S1†). Spinach signal due to the presence of Pb²⁺ was virtually unaffected in a metal soup that contained Ag⁺, Ca²⁺, Co²⁺, Cu²⁺, Zn²⁺, K⁺, Na⁺, Cu²⁺, Fe²⁺, Mn²⁺, Ni²⁺, Zn²⁺ (Fig. 4). These observations highlight the high selectivity of the sensor and demonstrate its potential utility for analysis of Pb²⁺ in samples containing other metal ions. The apparent Kₒ 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) for Pb²⁺, Ca²⁺ and K⁺, respectively, indicating that Pb²⁺ bound to Spinach with an affinity that was three orders of magnitude greater than did K⁺ or Ca²⁺, further emphasizing the selectivity of the sensor towards Pb²⁺ (Figs S8†, S9†). Consistent with these observations, at micromolar concentrations of Pb²⁺, K⁺ or Ca²⁺,
significantly lower concentrations of DFHBI were required to show fluorescence in the presence of Pb²⁺ compared to K⁺ or Ca²⁺ (Figs S10†, S11†), reflecting the formation of G-quadruplex in micromolar concentrations of Pb²⁺ but not in micromolar concentrations of K⁺ or Ca²⁺.¹² swore

Detection methods based on Pb²⁺-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 Ca²⁺ 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 Pb²⁺ (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 Ca²⁺, 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 Ca²⁺ caused the observed background signal when the Spinach sensor was used in tap water. To identify conditions that enable improved selectivity for detection of Pb²⁺ over Ca²⁺, we analyzed fluorescence signal as a function of increasing DFHBI concentration in the presence of a constant concentration of Pb²⁺ or Ca²⁺ (Fig. S11†). We found that Spinach required significantly higher concentrations of DFHBI to give the same intensity of fluorescence signal in the presence of Ca²⁺ compared to the presence of Pb²⁺. For example, in the presence of 50 mM Ca²⁺, 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 Pb²⁺, even at concentrations as low as 0.5 μM (Fig. S11a†). Thus, the background signal due to Ca²⁺ 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 Ca²⁺. Using these conditions, we then tested the performance of our sensor for Pb²⁺ detection in tap water. We spiked tap water with different concentrations of Pb²⁺. Fluorescence signal increased linearly with Pb²⁺ 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 Pb²⁺ in tap water. Other potential applications of our sensor could include measuring Pb²⁺ 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 Pb²⁺ sensing.

In conclusion, we have reported the first example of an RNA-based sensor as a selective and sensitive tool for detection of Pb²⁺, 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 Pb²⁺ sensors,¹⁶ and has a very simple design and operation that is cost effective and easy to use for ‘on the spot’ detection of Pb²⁺.

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Notes and references