

Analytical Methods

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Analytical Methods

PAPER

Label-free fluorescent sensor for detection of Pb²⁺ and Hg²⁺Hanchu Xu,^a Shenshan Zhan,^a Dongwei Zhang,^b Bing Xia,^b Xuejia Zhan,^a Lumei Wang^a and Pei Zhou^{a*}Received 00th January 20xx,
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A label-free fluorescent DNA-based sensor for detection of Pb²⁺ and Hg²⁺ was reported in this paper. A single-strand DNA named modified T30695 was used as a recognition probe and SYBR Green I (SG) was used as a signal reporter. This sensor consisted of two interaction sections: Pb²⁺ interacts with modified T30695 to form G-quadruplex and Hg²⁺ interacts with modified T30695 to form T-Hg(II)-T hairpin conformation. Circular dichroism confirmed the interactions between modified T30695 and Pb²⁺ or Hg²⁺. Based on this, a sensor for detection of Pb²⁺ and Hg²⁺ with a limit of detection of 2.09 ppb and 1.14 ppb was constructed.

Introduction

Lead ion (Pb²⁺) and mercury ion (Hg²⁺) are two of the most toxic heavy metal ions in the environment. They pose various severe risks to human health and the environment.¹ Moreover, they can cause a number of serious health problems, such as damaging nervous, motion, renal, immune and cardiovascular systems, even at low concentrations.^{2,3} So it is highly necessary to develop sensitive and selective method for Pb²⁺ and Hg²⁺ detection. Traditional methods for the detection of Pb²⁺ and Hg²⁺ include inductively coupled plasma mass spectrometry (ICP-MS), anodic stripping voltammetry (ASV), atomic absorption spectrometry (AAS) and atomic fluorescence spectrometry (AFS).^{4,5} Though these methods have prominent accuracy and sensitivity, most of them have drawbacks such as needing expensive sophisticated instruments, complicated sample pre-treatment and well-trained operators, failing to meet the requirement of real-time detection.^{6,7} Therefore, it is very important to develop simple and effective approaches to detect Pb²⁺ and Hg²⁺.

In recent years, much effort has been devoted to design DNA-based sensors to detect heavy metal ions.⁸ For Pb²⁺ detection, most of these sensors were based on Pb²⁺-specific DNAzyme^{9,10} and G-quadruplex that formed by folding a G-rich random coil single-stranded DNA in the presence of metal ion.^{11,12} For Hg²⁺ detection, it has been reported that Hg²⁺ can specifically interact with T-T mismatch to form the T-Hg(II)-T hairpin conformation.^{13,14} Based on these features, various DNA

sensors coupled with colorimetric, chemiluminescent, fluorescent and electrochemical as the signal output have been developed for the detection of Pb²⁺¹⁵⁻¹⁸ or Hg²⁺¹⁹⁻²¹. Although these methods have demonstrated high sensitivity and selectivity, most of them were performed for the determination of individual metal ion in samples, which restrain the frequency and efficiency of utilization. So it is a high demand to construct a method for detection of two or more metal ions with only one sensor.

During the past few years, there are a few sensors designed for detection of two or more metal ions. Kang et al. reported an alignment-addressed Au nanowires-on-chip surface-enhanced Raman scattering sensor for multiplex detection of Hg²⁺, Ag⁺ and Pb²⁺.²² Zhang and co-workers proposed a simple, rapid and label-free assay for the highly selective detection of Hg²⁺ and Ag⁺ by using the double-strand-chelating dye SG as the optical probe.²³ Although these assays were effective, the use of labeled-DNA and different DNA probes for different metal ions detection bring out the drawbacks such as expensive cost, complicated and time-consuming operation. Liu et al. adopted a fluorescence-labeled thrombin binding aptamer as a probe to detect Pb²⁺ and Hg²⁺ simultaneously.²⁴ While this approach may suffers from relatively poor detection limit and using poisonous masking agent. As these aforementioned methods are complicated, it is much desired to simplify the constitutes of the sensing system.

In this study, a label-free fluorescent DNA sensor for the detection of Pb²⁺ and Hg²⁺ was reported. Modified T30695 was used as the recognition probe and SYBR Green I (SG) as the signal reporter. The schematic of the sensor is shown in Fig. 1. The single-stranded modified T30695 will stay in random-coil status in the absence of Pb²⁺ and Hg²⁺. When SG adds into the system and binds to the modified T30695 through electrostatic interactions, it would exhibit high fluorescence. Modified T30695 will form into a G-quadruplex through Hoogsteen hydrogen bonds in the presence of Pb²⁺, leads to the

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fluorescence intensity of SG changing from high to low.¹⁵ And modified T30695 will form a T-Hg(II)-T hairpin conformation through both intercalation and minor groove binding in the presence of Hg²⁺, inducing the SG presenting higher fluorescence.²⁵ By taking advantage of these fluorescence changes, a label-free fluorescent method for Pb²⁺ and Hg²⁺ detection was constructed.

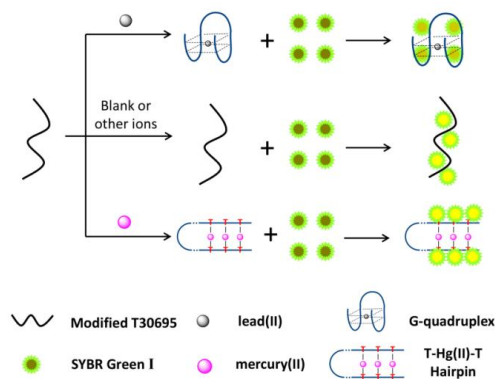


Fig. 1 Schematic representation of label-free sensor based on lead(II)-stabilized G-quadruplex and T-Hg(II)-T hairpin conformation.

Materials and Methods

Reagents and apparatus

A single-strand DNA named modified T30695 was designed by adding six thymines (TTTTTT) at each end of T30695, thus its sequence is 5'-TTTTTTGGGTGGGTGGGTGGGTTTTTT-3' (28 mer). The modified T30695 was supposed to bind with Pb²⁺ to form G-quadruplex²⁶ and Hg²⁺ to form T-Hg(II)-T hairpin conformation²⁷ according to previous reports, and it was synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

Tris (Tris-(hydroxymethyl)aminomethane), acetic acid, cationic compounds such as nitrates of Ag⁺, K⁺, Fe³⁺, Ni²⁺, Mg²⁺, Zn²⁺, Ca²⁺, Na⁺ and sulfates of Cu²⁺, Fe²⁺, Mn²⁺, and organic compounds were obtained from commercial sources and used without further purification. Standard solution (1 mg mL⁻¹, 1000 ppm) of Pb²⁺, Hg²⁺ and Cd²⁺ were purchased from Merck Co., Inc. (Germany) and used after diluted to appropriate concentration with ultrapure water. SYBR Green I (shortly called SG, 10000× concentrate in dimethyl sulfoxide) was purchased from Shanghai DoBio Biotech Co., Ltd. (Shanghai, China) and was diluted to 50× and 400× with ultrapure water before using and stored at 4 °C. Ultrapure water that utilized to prepare all aqueous solutions was from a Millipore-MilliQ (Milli-Q plus, Millipore Inc, Bedford, MA, USA) system.

An F-4500 fluorescence spectrophotometer (Hitachi, Japan) was used to record the fluorescence intensity, with the response time of 0.5 s, PMT voltage of 700 V, scan speed of 1200 nm min⁻¹, excitation wave length of 490 nm and excitation and emission slits of 10 nm. A J-815 circular dichroism (CD) spectrometer (Jasco, Japan) was employed to characterize the structural changes of the oligonucleotides.

The optical chamber (1 cm path length, 1 mL volume) was deoxygenated with dry purified nitrogen (99.99%) before use and kept the nitrogen atmosphere during experiments. Three scans (100 nm min⁻¹) from 230 to 310 nm at 1 nm intervals were accumulated and averaged. The background of the buffer solution was subtracted from the CD data.

Determination of the SYBR Green I concentration

First of all, the amount of modified T30695 used in each sample in all the following experiments was fixed at 50 nM. The concentration of the applied SG was optimized as follows. Pb²⁺ or Hg²⁺ with a final concentration of 250 ppb and 5 μL of 5 μM modified T30695 were firstly added into 1.5 mL plastic tubes, and then Tris-acetate buffer (10 mM, pH 8.0) with appropriate volume was introduced into the above solutions. After incubated at room temperature for 30 min, different volumes of 400× SG was added into the Pb²⁺ samples and 50× SG was added into Hg²⁺ samples. The final mixed solution with a total volume of 500 μL was kept in darkness at room temperature for 15 min and then the fluorescence intensity of each sample was measured. Control experiments were carried out by replacing Pb²⁺ or Hg²⁺ with ultrapure water. The fluorescence intensity of the control was recorded as F_0 and the fluorescence intensity of the ion or organic compound treated one was recorded as F , and the relative fluorescence intensity was calculated as $(F_0 - F)/F_0 \times 100\%$ for Pb²⁺ detection and $(F - F_0)/F_0 \times 100\%$ for Hg²⁺ detection. The SG concentration which corresponds to the maximum relative fluorescence intensity was chosen as the optimized concentration.

Sensitivity and selectivity of the detection of Pb²⁺ and Hg²⁺

Various concentrations of Pb²⁺ (from 0 to 500 ppb) or Hg²⁺ (from 0 to 300 ppb) and 5 μL of 5 μM modified T30695 were mixed in 1.5 mL plastic tubes and then Tris-acetate buffer (10 mM, pH 8.0) with appropriate volume was added into the above solution. After incubated at room temperature for 30 min, 6 μL of 400× SG was added into Pb²⁺ samples and 4 μL of 50× SG was added into Hg²⁺ samples. The final mixed solution with a total volume of 500 μL was kept in darkness at room temperature for 15 min, and then the fluorescence intensity of each sample was measured.

To test the selectivity of this sensor for Pb²⁺ and Hg²⁺ ions, different environmentally relevant metal ions, including Cu²⁺, Cd²⁺, Ag⁺, Fe²⁺, Mn²⁺, K⁺, Fe³⁺, Ni²⁺, Mg²⁺, Zn²⁺, Ca²⁺ and Na⁺, at a concentration of 250 ppb, and different organic compounds with a concentration of 2.5 ppm were added into the sensor solution individually under the same conditions and the differences in the fluorescence intensity were recorded.

Results and Discussion

Sensing mechanism

Proof-of-concept experiments were carried out to demonstrate the mechanism of this sensor. As shown in Fig. 2, the free SG presented almost no fluorescence because the quantum yield of it is close to zero in the absence of DNA.²⁸ After it intercalated with the modified T30695, the

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fluorescence intensity increased. However, a significant decrease of the SG fluorescence intensity was observed in the condition when the modified T30695 was mixed with Pb^{2+} . According to previous reports, the formation of Pb^{2+} -stabilized G-quadruplex can cause an effective stack of fluorophore on the G-quadruplet; obviously intensify the quenching efficiency of fluorophore,¹⁵ thus lead to weak fluorescence. In the presence of Hg^{2+} , modified T30695 formed into T-Hg(II)-T hairpin construction, which similar to double-strand DNA.²⁵ Consequently, SG embedded into the grooves of the duplex smoothly, leading to a strong enhancement of the fluorescence intensity due to a dampening of its intramolecular motions.

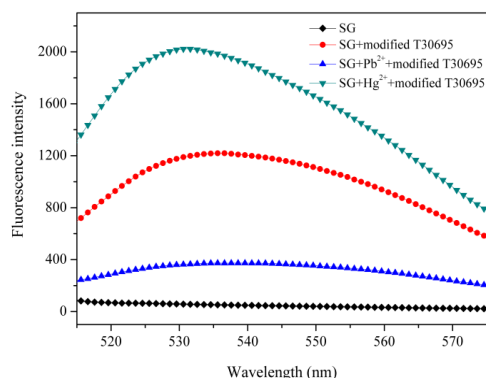


Fig. 2 Variation of the fluorescence intensity of SG in the absence and presence of 50 nM modified T30695, 250 ppb Pb^{2+} or 80 ppb Hg^{2+} .

Characterization of the oligonucleotide structure

In order to further confirm the interactions between the modified T30695 and Pb^{2+} or Hg^{2+} , circular dichroism (CD) spectrum was applied in our study. As is shown in Fig. 3, the free modified T30695 had a positive peak around 265 nm, after interacting with Pb^{2+} , there was an enhancement on the positive peak around 265 nm and on the negative peak around 240 nm. As it has been reported that typical CD spectrum of a "parallel" G-quadruplex structure has a positive peak around 260 nm and a negative peak around 240 nm,²⁹ the change of the peaks appear here confirmed the formation of the parallel G-quadruplex. In the presence of Hg^{2+} , a new negative peak at 280 nm appeared, which indicated the formation of T-Hg(II)-T hairpin conformation.¹

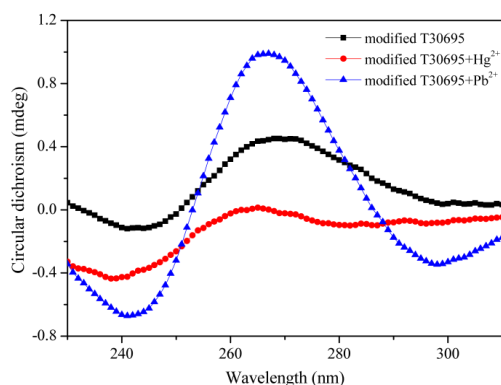


Fig. 3 CD spectra of 1 μM modified T30695 in the absence (black) and presence of 1 ppm Pb^{2+} (blue) or 1 ppm Hg^{2+} (red).

Determination of the SYBR Green I concentration

To obtain a highly sensitive response for the detection of Pb^{2+} and Hg^{2+} , the concentration of SG applied was optimized. Fig. 4A shows that the relative fluorescence intensity of the solution containing 50 nM modified T30695 increased firstly and decreased subsequently with the increase of SG volume for the detection of Pb^{2+} . The relative fluorescence intensity reached its peak at 6 μL of 400 \times SG, so 6 μL of 400 \times SG was chosen as the optimized SG concentration to detect Pb^{2+} . In a similar way, the optimized concentration of SG for the detection of Hg^{2+} was also determined. As is shown in Fig. 4B, the relative fluorescence intensity achieved maximum at 4 μL of 50 \times SG, so 4 μL of 50 \times was the optimized SG concentration for Hg^{2+} detection.

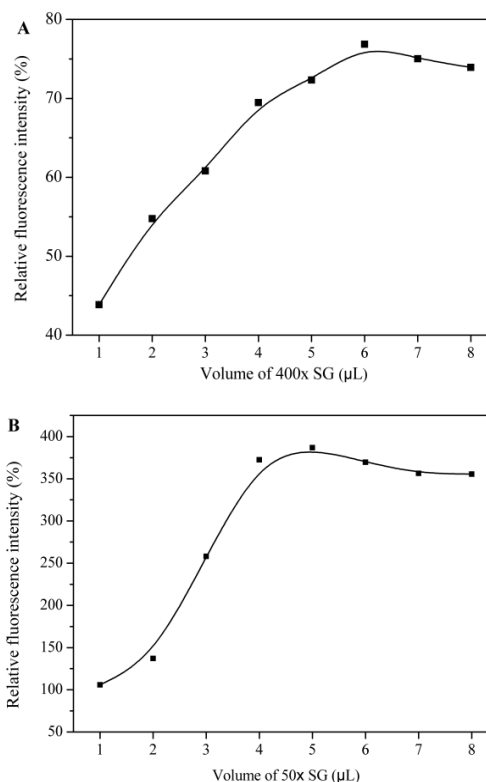


Fig. 4 (A) Determination of the SYBR Green I concentration for the detection of Pb^{2+} . The solution contains 50 nM modified T30695, 250 ppb Pb^{2+} and different volumes of 400 \times SYBR Green I. (B) Determination of the SYBR Green I concentration for the detection of Hg^{2+} . The solution contains 50 nM modified T30695, 250 ppb Hg^{2+} and different volumes of 50 \times SYBR Green I.

Sensitivity and selectivity

After the optimal pH of the buffer was selected as 8.0 (Fig. S1), the optimal temperature was determined as 25 $^{\circ}\text{C}$ (Fig. S2), and the incubation time and reaction time was chose as 30 min and 15 min, respectively (Fig. S3), the sensitivity of this sensor was investigated by adding different concentrations of Pb^{2+} and Hg^{2+} into the sensing solutions individually and measuring the fluorescence signals. In the presence of Pb^{2+} , the binding of Pb^{2+} with modified T30695 resulting a significant fluorescence intensity decrease of SG. As shown in Fig. 5A, the

fluorescence intensity decreased with the Pb^{2+} concentration increased from 0 to 500 ppb. Fig. 5B depicts the relative fluorescence intensity plotted against the concentration of Pb^{2+} by fitting to a Hill plot with a correlation coefficient of 0.996. The inset shows the linear relationship between the relative fluorescence intensity and the low concentrations of Pb^{2+} . The linear regression equation was $y = 0.82x + 3.05$ with a correlation coefficient of 0.991. Based on a previous report,³⁰ $3\sigma/\text{slope}$ was used to determine the limit of detection (LOD) as 2.09 ppb (~ 10 nM) for Pb^{2+} , which is much lower than the United States Environmental Protection Agency (EPA) defined toxicity level of Pb^{2+} (72 nM, ~ 15 ppb) in drinking water.³¹ Therefore, this sensor can be used to detect Pb^{2+} in the aqueous solution. As for Hg^{2+} detection, Hg^{2+} interacted with the modified T30695 to form T-Hg(II)-T hairpin construction, resulting in a significant fluorescent increase of SG. Fig. 5C shows that the fluorescence intensity increased with the Hg^{2+} concentration increased from 0 to 300 ppb. As the concentration of Hg^{2+} was higher, the fluorescence intensity of SG tended to steady. Fig. 5D shows the relative fluorescence intensity plotted against the concentration of Hg^{2+} by fitting to a Hill plot with a correlation coefficient of 0.985. The inset shows the linear response at low Hg^{2+} concentrations. The linear regression equation was $y = 1.5x + 1.09$, with a correlation coefficient of 0.987. Based on the above-mentioned method, $3\sigma/\text{slope}$ was used to determine the limit of detection as 1.14 ppb (~ 5.7 nM) for Hg^{2+} , which is lower than EPA defined toxicity level of Hg^{2+} (10 nM, ~ 2 ppb).³² Therefore, this sensor can also be used to detect Hg^{2+} in the aqueous solution.

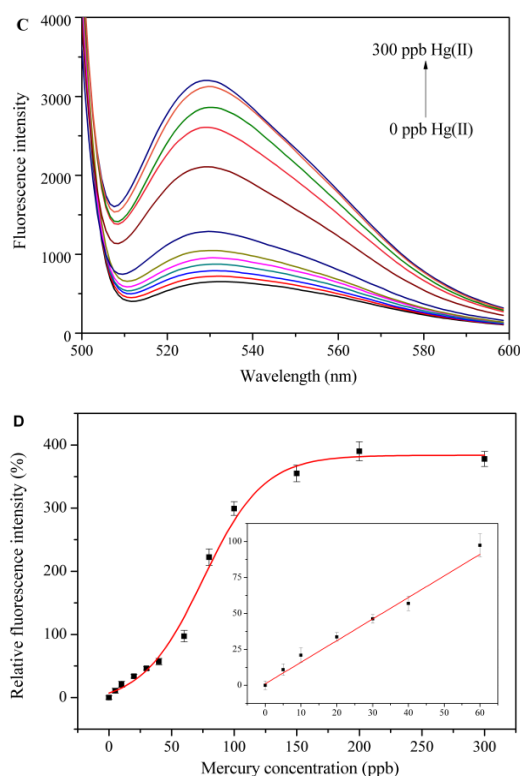
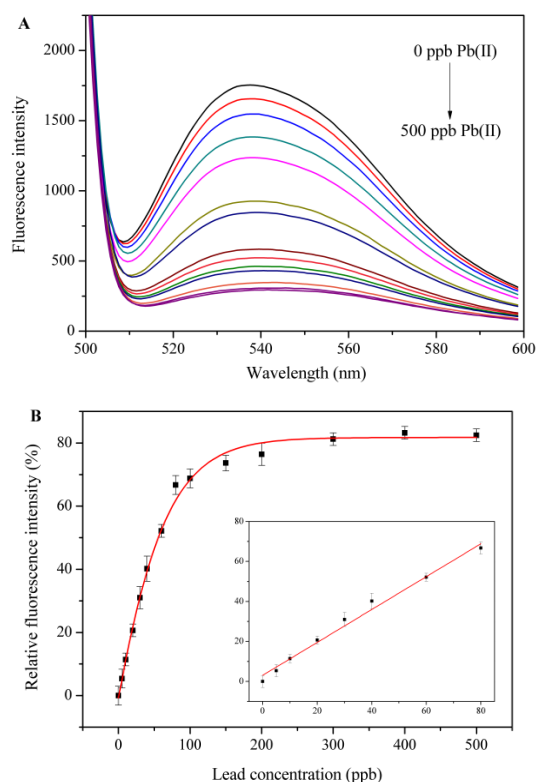


Fig. 5 Sensitivity of this sensor for Pb^{2+} and Hg^{2+} detection: (A) Fluorescence spectra of a solution containing 50 nM modified T30695, 6 μL of 400 \times SG and 0, 5, 10, 20, 30, 40, 60, 80, 100, 150, 200, 300, 400, 500 ppb Pb^{2+} . (B) Calibration curve was fitted to a Hill plot with a correlation coefficient of 0.996. Inset: the relative fluorescence intensity increased at low Pb^{2+} concentrations. The linear regression equation was $y = 0.82x + 3.05$, with a correlation coefficient of 0.991. (C) Fluorescence spectra of a solution containing 50 nM modified T30695, 4 μL of 50 \times SG and 0, 5, 10, 20, 30, 40, 60, 80, 100, 150, 200, 300 ppb Hg^{2+} . (D) Calibration curve of the sensing system for Hg^{2+} detection. Inset: the relative fluorescence intensity increased at low Hg^{2+} concentrations.

The selectivity of the sensor was also investigated. Different relative fluorescence intensity were obtained by adding other metal ions such as Cu^{2+} , Cd^{2+} , Ag^+ , Fe^{2+} , Mn^{2+} , K^+ , Fe^{3+} , Ni^{2+} , Mg^{2+} , Zn^{2+} , Ca^{2+} and Na^+ instead of Pb^{2+} and Hg^{2+} into the sensing solutions. What's more, a mix solution which contains equal concentration of Pb^{2+} and Hg^{2+} was also being tested. The results in Fig. 6 (A) (B) indicate that only Pb^{2+} and Hg^{2+} caused a considerable increase in the relative fluorescence intensity in their own sensing system while other ions had no apparent fluorescence signal changes. These results clearly showed that this sensor for detection of Pb^{2+} and Hg^{2+} was highly selective to their targets over the other metal ions. The selectivity of the sensor over other organic compounds were also studied (Fig. 6 (C) (D)). It revealed that all the testing organic compounds caused no interference even at high concentration. Moreover, compared with some previous reports about the detection of two or more metal ions (Table S1, ESI[†]), the sensing system and the operation process of this sensor were much simpler.

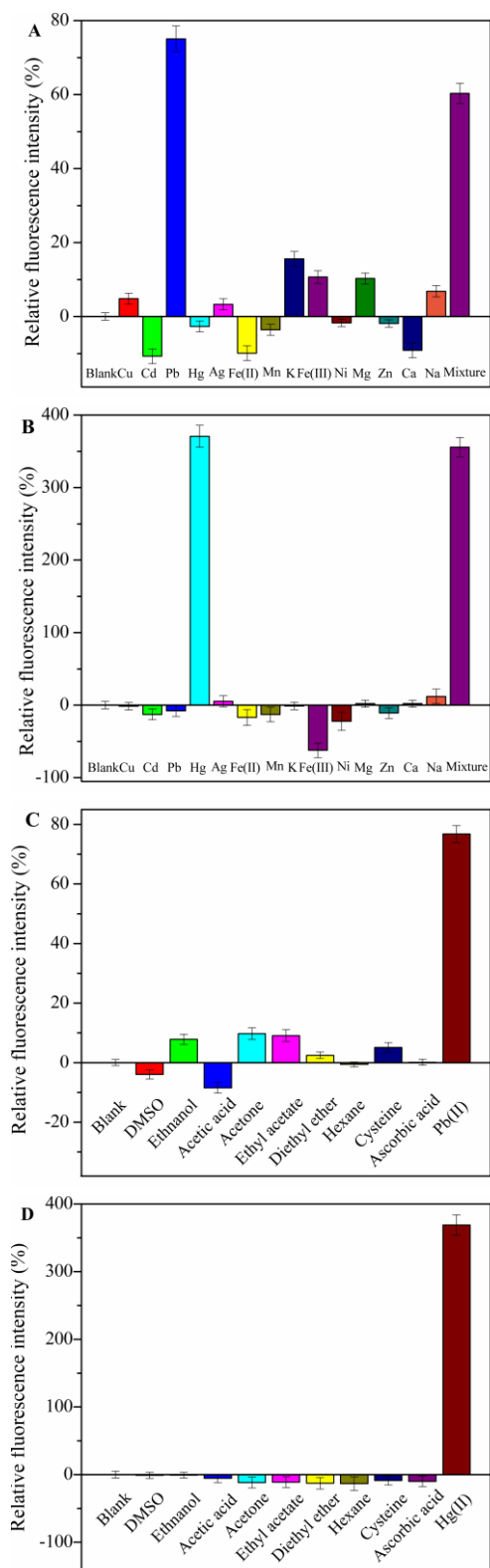


Fig. 6 Selectivity of the sensor for Pb²⁺ (A) and Hg²⁺ (B) detection over other metal ions. The concentration of all metal ions was 250 ppb. The mixture contains 250 ppb Pb²⁺ and 250 ppb Hg²⁺. Selectivity of the sensor for Pb²⁺ (C) and Hg²⁺ (D) detection over other common organic compounds. The concentration of Pb²⁺ and Hg²⁺ is 250 ppb, other organic compounds' concentration is 2.5 ppm.

Detection of Pb²⁺ and Hg²⁺ in water samples

The application of this sensor was evaluated by testing Pb²⁺ and Hg²⁺ in mixtures containing different metal cations and anions. The results were summarized in Table 1 and Table 2, which showed that the mean recovery of samples was in the range of 95.2–113.6 % and the relative standard deviation (RSD) was between 3.51–6.36 %. The results confirmed the potential application of this sensor for detection of Pb²⁺ and Hg²⁺ in water samples.

Table 1 Determination of Hg²⁺ in water samples.

Samples	Mean found (ppb)	Mean recovery (%)	RSD (%)
Hg ²⁺ (50), [§] Pb ²⁺ (60), Na ⁺ (100), Mg ²⁺ (60), Fe ²⁺ (60), Cu ²⁺ (40), NO ₃ ⁻ (646.8), ^{§§} SO ₄ ²⁻ (162.9)	47.6	95.2	3.51
Hg ²⁺ (100), Cd ²⁺ (100), K ⁺ (150), Ca ²⁺ (150), Ni ²⁺ (60), Mn ²⁺ (100), NO ₃ ⁻ (1002), SO ₄ ²⁻ (174.5)	108.3	108.3	5.66
Hg ²⁺ (200), Ag ⁺ (200), Pb ²⁺ (100), Mg ²⁺ (300), Na ⁺ (200), Ca ²⁺ (200), NO ₃ ⁻ (3007.3)	193.7	96.9	4.23

Table 2 Determination of Pb²⁺ in water samples.

Samples	Mean found (ppb)	Mean recovery (%)	RSD (%)
Pb ²⁺ (50), Mg ²⁺ (40), Ni ²⁺ (100), Zn ²⁺ (100), Cu ²⁺ (80), Mn ²⁺ (80), NO ₃ ⁻ (638.1), SO ₄ ²⁻ (259.6)	56.8	113.6	5.73
Pb ²⁺ (100), Cd ²⁺ (100), Fe ³⁺ (100), Ca ²⁺ (300), Na ⁺ (300), Mn ²⁺ (200), NO ₃ ⁻ (224.5), SO ₄ ²⁻ (349)	97.1	97.1	3.81
Pb ²⁺ (200), Hg ²⁺ (100), Mg ²⁺ (300), Ni ²⁺ (200), Na ⁺ (300), Fe ²⁺ (200), NO ₃ ⁻ (2962.6), SO ₄ ²⁻ (343)	215.6	107.8	6.36

Conclusions

In this study, a label-free fluorescent DNA sensor with high sensitivity and selectivity for detection of Pb²⁺ and Hg²⁺ has been constructed. The design was based on the formation of

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lead (II)-stabilized G-quadruplex and T-Hg(II)-T base pairs. In the presence of Pb^{2+} , modified T30695 folds into a G-quadruplex structure, which leading to a weak fluorescence intensity of SG. While in the presence of Hg^{2+} , modified T30695 forms into a T-Hg(II)-T hairpin conformation which similar to double-strand DNA, leading to a high fluorescence intensity of SG. Through this study, Pb^{2+} and Hg^{2+} in aqueous solution can be detected as low as 2.09 ppb and 1.44 ppb, respectively. This sensor is easy, reliable and convenient for detection of Pb^{2+} and Hg^{2+} in aqueous solution. Compared with some previous reports on detection of two or more heavy metal ions, this sensor shows an obvious advantage on simplifying the sensing system.

Acknowledgements

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Notes and references

§ Final concentration (ppb) of ions added.

§§ Concentration of anion was calculated from that of corresponding cation.

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