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PII: S0039-9140(15)30369-6
DOI: http://dx.doi.org/10.1016/j.talanta.2015.10.004
Reference: TAL16012

To appear in: *Talanta*

Received date: 9 June 2015
Revised date: 28 September 2015
Accepted date: 1 October 2015

Cite this article as: Ting Fu, Songlei Ren, Liang Gong, Hongmin Meng, Liang Cui, Rong-Mei Kong, Xiao-Bing Zhang and Weihong Tan, A label-free DNAzyme fluorescence biosensor for amplified detection of Pb^{2+}-based on cleavage-induced G-quadruplex formation, *Talanta* http://dx.doi.org/10.1016/j.talanta.2015.10.004

A Label-free DNAzyme Fluorescence Biosensor for Amplified Detection of Pb^{2+}-based on Cleavage-Induced G-quadruplex Formation

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Abstract

DNAzyme-based catalytic beacons have been widely studied for both in vivo and in vitro molecular detection. However, only a few label-free catalytic beacons with excellent analytical performance have been reported so far. In this work, by combining a catalytic DNAzyme for amplified sensing through enzymatic turnover with cleavage-induced G-quadruplex formation, a label-free DNAzyme biosensor was developed for amplified "turn-on" fluorescence detection of Pb^{2+} with a detection limit of 3 nM. The method is very competitive compared to many other labeled or label-free methods with or without signal amplification. Due to the inherent specificity of the GR-5 DNAzyme, the method also exhibits excellent selectivity. This biosensor successfully detected Pb^{2+} in river water samples with high sensitivity and

selectivity. Such a method might provide a universal DNAzyme-based sensing platform for sensitive detection of various targets both in environmental and biomedical fields.

Keywords: DNAzyme; label-free; G-quadruplex; biosensor; fluorescence

1. Introduction

Sensing and detection of low concentrations of metal ions is very important in many fields including environmental, biological, and medical sciences [1-5]. Lead ions (Pb^{2+}), as a major environmental pollutant, is quite harmful to human health, and a very small amount of Pb^{2+} could cause serious damage to the brain and central nervous system [6, 7]. Thus, highly sensitive and selective sensors for Pb^{2+} detection in environmental and biological samples are of great interest [8-11]. Traditional analytical techniques such as atomic absorption spectrometry, inductively coupled plasma mass spectrometry and inductively coupled plasma-atomic emission spectrometry can detect Pb^{2+} with extremely high sensitivity [12-15]. However, these techniques are limited by a series of drawbacks such as expensive and bulky instrumentation, and complicated pretreatment procedures, making them inappropriate for on-site and real-time analysis.

DNAzymes, which are single stranded DNA or RNA, have been applied as a platform for sensing a wide range of metal ions due to their highly catalytic activity, low cost, non-sample-destruction or less cell damage, and the ability to provide in situ and real-time information [5, 16-18]. For example, by combining inter- and intramolecular quenchers, Lu's group developed a series of highly sensitive DNAzyme catalytic beacon sensors for metal ions detection in aqueous solution with low background [8, 19-21]. By taking advantage of molecular beacons (MBs) and multiple enzymatic turnover properties of catalytic beacon, our group has reported a catalytic and molecular beacon (CAMB) for amplified sensing of Pb^{2+} and adenosine with improved sensitivity [22]. By replacing the quencher with graphene oxide, the DNAzyme-based sensing system exhibited lower background and higher sensitivity [23]. Although DNAzyme-based catalytic beacons have been widely applied in both in vitro and in vivo detection [5, 24], multi-fluorophore/quencher covalent labeling

often leads to complicated synthetic routes, high cost and low synthetic yield [25]. Worse, the label may also interfere with the binding of DNAzyme to its substrates or metal ions, thus inhibiting the catalytic activity of the DNAzyme [5, 26]. Therefore, a label-free fluorescent biosensor has become an effective approach to solve these problems [27]. For example, based on DNA-intercalating dyes, such as Picogreen and SYBR Green I, which exhibit distinct fluorescence signal between DNAzyme-substrate double-stranded DNA and metal-catalyzed substrate-cleavage single-stranded DNA, simple and low consumption label-free biosensors have been proposed for metal ion sensing with high sensitivity [28-30]. However, there are some drawbacks for these label-free biosensors, such as high background fluorescence induced by DNA-intercalating dyes themselves for the discrimination of single-stranded DNA and double-stranded DNA, and the fact that each target can react with only a single copy of the probe, thereby limiting the detection sensitivity. DNAzyme biosensors give multiple turnovers and can therefore be used to design new probes with amplified output signal and low fluorescence background.

Herein, by combining catalytic turnover inherent to DNAzymes and subsequent cleavage-induced G-quadruplex formation, a label-free DNAzyme biosensor for amplified "turn-on" fluorescence detection of Pb^{2+} is presented. Compared to 8-17 DNAzyme, GR-5 DNAzyme-based sensing system exhibited obvious improved selectivity to Pb^{2+} detection with no interference from Zn^{2+} and other common metal ions. Therefore, GR-5 DNAzyme was chosen as the catalytic unit and zinc(II)-protoporphyrin IX/G-Quadruplex system as the label-free signal reporter unit [21][11]. The results demonstrate that the proposed DNAzyme sensor can achieve high selectivity and sensitivity toward Pb^{2+}, with a detection limit of 3 nM. Moreover, the label-free design endows the sensor with many advantages including ease of use, rapid Pb^{2+} response and low-cost.

2. Experimental

2.1 Reagents and Apparatus

The substrate DNA labeled with rA (S(r)) [5'- GGG TTG GGC GGG ATG GGT ATC ATT ACT AT rA GGA AGA GAG ATT ACT AAA CGA TCG CCC A-3']

used in this work was purchased from Takara Biotechnology Co. Ltd. (Dalian, China). GR-5 DNAzyme（5'- AAT CTC TCT GAA GTA GCG CCG CCG T ATA GTA ATG -3') was purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). Zinc(II)-protoporphyrin IX (Zn PPIX) was purchased from Alfa Aesar Co. Ltd. (Tianjin, China). $Pb(CH_3COO)_2$, NaCl, $Hg(NO_3)_2$, $NiCl_2$, $CoCl_2$, $Cd(NO_3)_2$, $Cu(NO_3)_2$, $Fe(NO_3)_3$, $Mg(NO_3)_2$, $MnCl_2$, $CaCl_2$, $FeCl_2$, $Ba(NO_3)_2$, HEPES were purchased from Xilong Co. Ltd. (Shantou, China). DMSO (analytical reagent grade) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All reagents were used without further purification. The buffer involved in this work was composed of 25 mM HEPES, 100 mM NaCl, and 2.5 mM $MgCl_2$ (pH 7.0). All the reagents except ZnPPIX involved in the experiment were dissolved in Milli-Q water (resistance >18 MΩ cm at 25 °C), prior to use. The ZnPPIX was dissolved in DMSO.

All fluorescence measurements were carried out on a Hitachi F-4500 Fluorescence Spectrometer (Tokyo, Japan) with both excitation and emission slits set at 10.0 nm. All measurements were carried out at room temperature unless otherwise stated.

2.2 Procedure for fluorescence detection

The reaction was conducted in final volume of 100μL buffer solution (25 mM HEPES, 100 mM NaCl, and 2.5 mM $MgCl_2$, pH 7.0) at 37 °C. To detect target Pb^{2+}, the hybridization between S(r) (5μL, 10 μM) and GR-5 DNAzyme (10 μL, 1μM) were performed in buffer solution for 30 min. Then, varying concentrations of Pb^{2+} (in the range of 0-10 μM) were added into the above solution and incubated for 100 min. Finally, upon the addition of 10 μM ZNPPIX for 60 min, the fluorescence of the mixture was measured. The emission spectra were obtained by exciting the samples at 420 nm and scanning the emission from 550 to 670 nm in step of 1 nm. The PMT detector was set at 950 V. The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter. To get the best performance of the DNAzyme activity, the concentrations of DNAzyme and ZnPPIX, the catalytic reaction time and temperature were optimized. The detailed data are shown in the part of Results and Discussion.

3. Results and discussion

3.1. The design principle of the biosensor

The design of the DNAzyme-based label-free Pb^{2+} biosensor is shown in Scheme 1. The biosensor is consisted of three parts: a single-strand DNAzyme, a single-strand substrate and a free ligand. In the presence of Pb^{2+}, the hairpin-shaped substrate DNA can be cleaved by DNAzyme into two segments, thus zinc(II)-protoporphyrin IX (ZnPPIX) can selectively insert into G-quadruplex assembled by one G-rich segment and take a remarkable fluorescence enhancement [31]. In this work, GR-5 DNAzyme was employed because of its high selectivity towards Pb^{2+}. The substrate strand of the DNAzyme was designed to be converted into a hairpin structure by extending the original DNAzyme substrate strand at both ends, which consist of a G-rich sequence and its partial complementary sequence to the G-rich sequence. In the absence of Pb^{2+}, the intramolecular hybridization between G-rich sequence and complementary sequence formed stable hairpin structure, thus the G-rich sequence lost ability to form the G-quadruplex structure and no fluorescence signal was observed. In the presence of Pb^{2+}, the DNAzyme is activated to catalyze the cleavage of the hairpin substrate strand. The cleavage disrupts the stability of the hairpin structure and frees the G-rich sequence out of the cage, resulting in the self-assembly of the G-quadruplex and the association of ZnPPIX to it, and thus produces an increased fluorescence signal and a free DNAzyme strand. More importantly, the released DNAzyme strand can bind to another hairpin substrate strand to initiate the next round of cleavage in the presence of Pb^{2+}. Eventually, each released DNAzyme strand can undergo many cycles to trigger the cleavage of many hairpin substrate strands and the formation of G-quadruplex, thus providing a significant fluorescence signal. Therefore, by this method, a signal amplified biosensor could be developed for Pb^{2+} with high sensitivity and selectivity. Meanwhile, the label-free design makes the biosensor simple and cost-effective.

3.2.Optimization of assay conditions

To achieve the best sensing performance, the concentrations of DNAzyme and ZnPPIX were first optimized. Various concentration of GR-5 DNAzyme was investigated in the presence of 500 nM S(r), 5 μM Pb^{2+} and 10 μM ZnPPIX at 37 °C.

As shown in Fig. 1A, the signal-to-background ratio (SBR) of the fluorescence signal increased significantly with the increase of the concentration of GR-5 DNAzyme. When the DNAzyme concentration reached 100 nM, the system exhibited the best sensing performance with the SBR achieved 3.6. However, with increasing concentration, the SBR of the fluorescence signal decreased because highly concentrated GR-5 DNAzyme may disturb the dissociation of the cleaved hairpin substrate from it to form the G-quadruplex structure. Therefore, a concentration of GR-5 DNAzyme at 100 nM was chosen for further Pb^{2+} sensing experiments. Similarly, the effect of ZnPPIX concentration was investigated in the presence of 500 nM S(r), 100nM GR-5 DNAzyme and 5 μM Pb^{2+}at 37 °C. The experimental results showed that the SBR of the fluorescence signal increased with increasing concentration of ZnPPIX, and reached the maximum SBR at 10 μM ZnPPIX(Fig. 1B). Therefore, 10 μM ZnPPIX was selected for the sensing system.

The effect of the reaction temperature and catalytic reaction time on the performance of the sensing system was also investigated. As shown in Fig. 2A, the SBR of the fluorescence signal increased with the increase of the reaction temperature and the maximum SBR of the fluorescence signal was reached at 37°C. However, the higher temperature resulted in the decrease of the SBR due to the instability of the hybridization between the substrate strand and the DNAzyme strand. In addition, the results indicated that the sensing system exhibited the best performance when the catalytic reaction time reached 100 min (Fig. 2B). Therefore, the further experiments were all carried out at 37 °C for 100 min for the detection of Pb^{2+}. Moreover, the effect of pH on fluorescence response of probe was also investigated (Fig. 3). The results show that the change of pH value did not show obvious effect on the fluorescent response of the probe.

3.3. The sensitivity of the sensing system

Titration experiments were carried out to test whether the proposed method can be used to quantitatively detect Pb^{2+}. As shown in Fig. 4 (A), the fluorescence spectra of the sensing system upon the addition of different concentration of Pb^{2+}were recorded

under the optimized conditions. In the absence of Pb^{2+}, the fluorescence intensity of the sensing system is very low, indicating that no G-quadruplex was formed, that is, the stable intra-molecular hairpin structure could not be cleaved in the absence of Pb^{2+}. However, with the addition of Pb^{2+}, the fluorescence intensity significantly increased, suggesting that the DNAzyme was activated and catalyze the cleavage of the hairpin substrate strand into inter-molecularly hybridized double-strand DNA with much less stability, resulting in the self-assembly of the G-quadruplex and the association of ZnPPIX to it, thus producing an increased fluorescence signal. As a result, an activated DNAzyme can catalyze the cleavage of many hairpin substrate strands with releasing G-rich sequences to form G-quadruplex nanostructures, resulting in significantly fluorescence enhancement. The fluorescence intensity was increased with the increase of Pb^{2+} concentration. It reached a plateau when the Pb^{2+} concentration increased to 5 μM (Fig. 4A). The low background fluorescence of the caged G-rich sequence in the hairpin substrate strand and the multiple turnover capability of the DNAzyme endow great sensitivity to the sensing system. Fig.4B depicts the relationship between the fluorescence intensity changes at 580 nm and Pb^{2+} concentration. The analytical performance characteristics of the developed biosensor were shown in Table 1, with a detection limit of 3 nM (based on 3σ/slope) observed for Pb^{2+}. The high sensitivity indicates the success of the developed DNAzyme-based amplified design for "turn-on" fluorescence detection of Pb^{2+}.

3.4. The selectivity of the sensing system

Based on the inherent specificity of GR-5 DNAzyme [21], the sensing system is found to be highly selective for Pb^{2+} detection. The selectivity test was carried out by measuring and comparing the fluorescence responses of Pb^{2+} with other metal ions, including Cu^{2+}, Hg^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Fe^{3+} , Ca^{2+} , Ba^{2+} and Fe^{2+} under the optimized conditions. As shown in Fig. 5, 100 nM Pb^{2+} could induce a significant fluorescence enhancement, while all the other metal ions resulted in very weak signal changes, even at 100 times concentration of Pb^{2+}(10 μM), indicating that our sensing system exhibits an excellent selectivity for Pb^{2+} detection.

As shown in Table 2, the selectivity of the biosensor is improved than those based on 8-17 DNAzyme [8, 22, 23], while the sensitivity of the biosensor is competitive with many other labeled and label-free methods [8, 21, 32]. Moreover, the label-free design makes the biosensor more simple and cost-effective than the fluorophore-labeled biosensors [8, 21, 22, 23].

3.5. Detection of Pb^{2+} in Synthetic Samples

The practical application of the sensing system was then evaluated by determination of Pb^{2+} in synthetic samples from river water. The river water samples were collected from Xiang River (Changsha, China). All the collected samples were simply filtered and showed that no Pb^{2+} was present. Pb^{2+} stock solution at different concentrations was spiked in these samples, and the label-free DNAzyme biosensor was then employed to detect the Pb^{2+} concentration. The analytical results were shown in Table 3. It was observed that the results measured for river water samples showed good recovery values, which confirmed that the proposed probe was applicable for practical Pb^{2+} detection in real samples with other potentially competing species coexisting.

Conclusions

In conclusion, a label-free DNAzyme biosensor was developed for amplified fluorescence "turn-on" detection of Pb^{2+} with high sensitivity and selectivity. The substrate strand is designed as a hairpin structure which includes G-rich sequence. In the presence of Pb^{2+}, the caged G-rich sequence can be released from the hairpin substrate strand by enzymatic cleavage, and form the G-quadruplex nanostructure based on the large difference in the melting temperatures between the intramolecular hairpin structure and the enzymatic intermolecular products of identical sequences. The label-free signal transduction mechanism is based on the different fluorescence output of ZnPPIX fluorophore before and after binding to G-quadruplexes, which is produced and released by the target-induced cleavage. Moreover, all DNAzyme can be released to catalyze a new round reaction, take this advantage small amount of Pb^{2+} can result significantly amplified fluorescence enhancement. Our proposed method exhibits high sensitivity towards Pb^{2+} with a detection limit of 3 nM, which is competitive with many labeled and label-free methods with and without signal

amplification. Due to the inherent specificity of the GR-5 DNAzyme, the method also exhibits excellent selectivity. Therefore, with the excellent performance such as high sensitivity and selectivity, simple processing and cost-effective, the proposed sensing system can provide a universal DNAzyme-based sensing platform for sensitive detection of various targets.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (21205068), Science and Technology Plan of Hunan province (2013WK3015).

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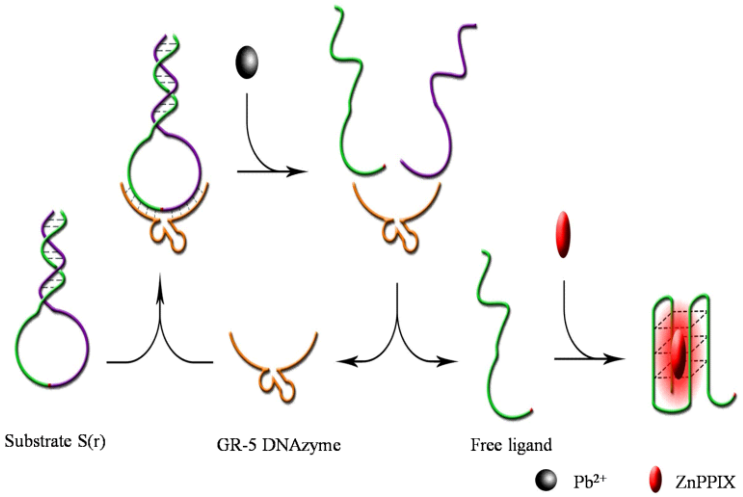

Scheme 1. Schematic illustration of the label-free DNAzyme biosensor for amplified Pb^{2+} detection.

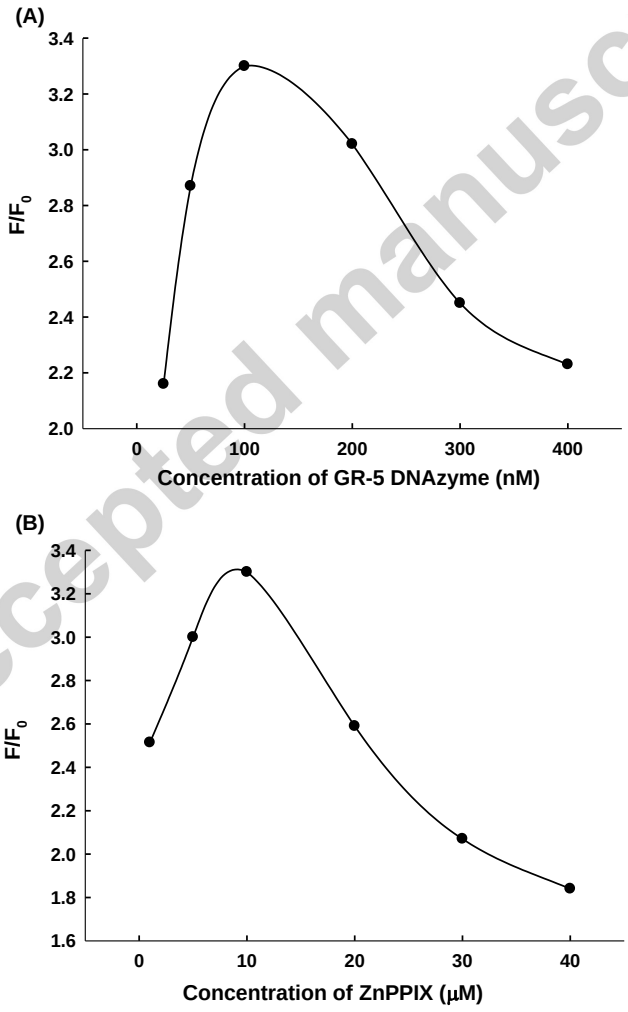

Fig. 1. (A) The effect of the concentration of GR-5 DNAzyme on the performance of the sensing system. The concentration of S(r), Pb^{2+} and ZnPPIX were 500 nM, 500

nM and 10 μM, respectively. (B) The effect of the concentration of ZnPPIX on the performance of the sensing system. The concentration of S(r), Pb^{2+} and GR-5 were 500 nM, 5 μM and 100 nM, respectively.

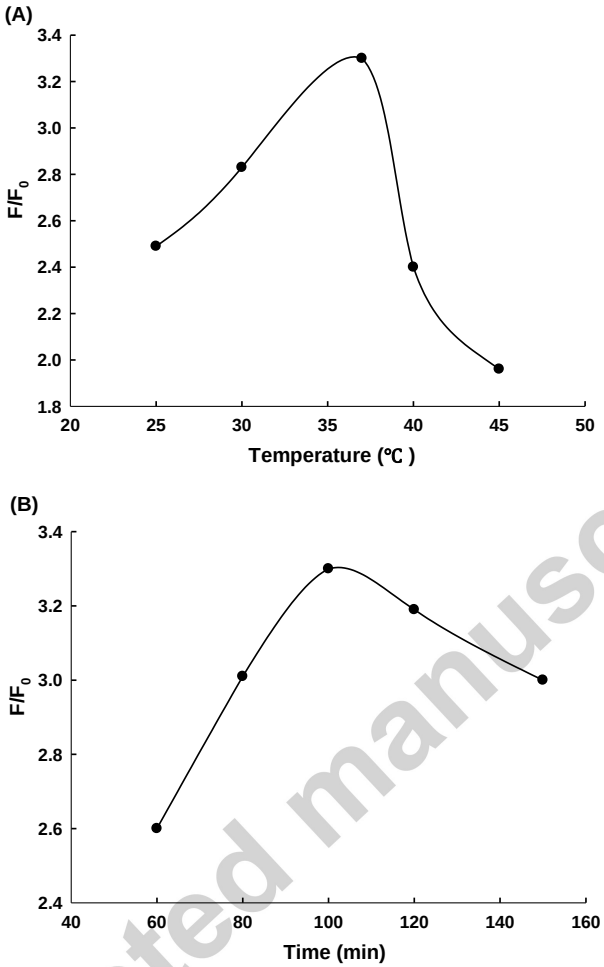

Fig. 2. The effect of the reaction temperature (A) and time (B) on the performance of the sensing system. The concentration of S(r), GR-5 DNAzyme, $Pb^{2+}$and ZnPPIX were 500 nM, 100 nM, 500 nM and 10 μM, respectively.

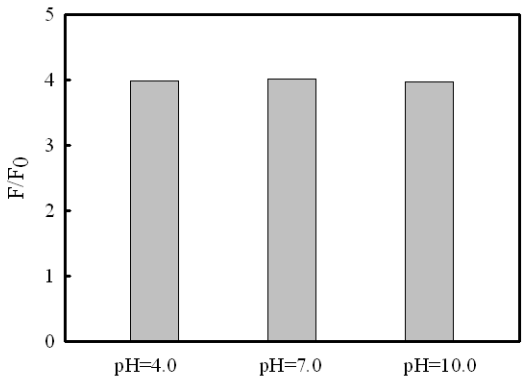

Fig. 3. Effect of pH on fluorescence response of probe in the presence of Pb^{2+} (5 μM).

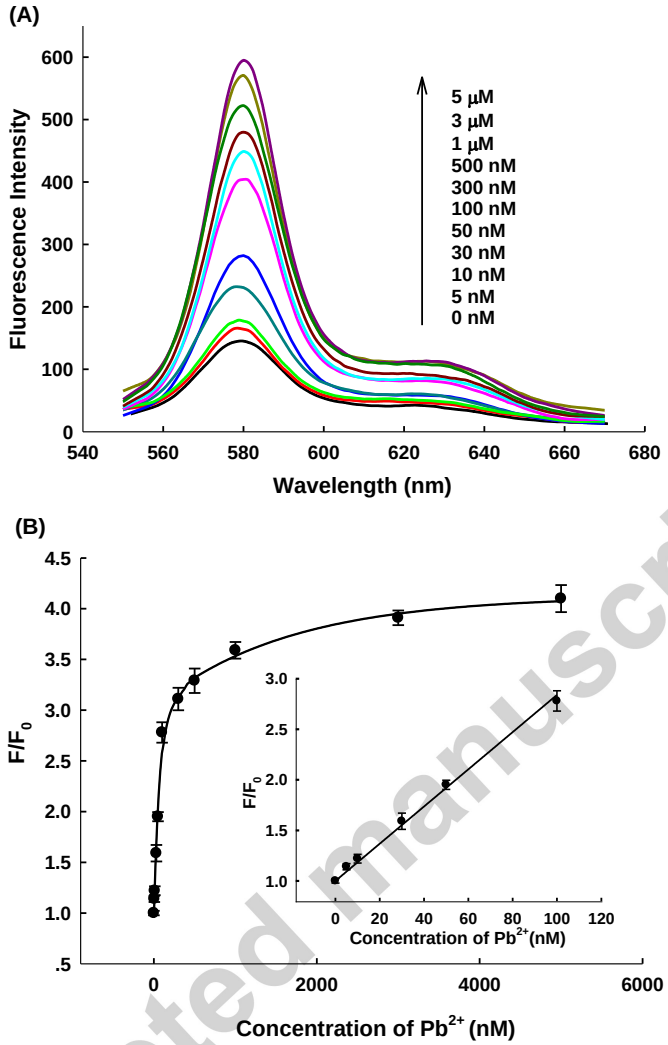

Fig.4. (A) The fluorescence emission spectra of the sensing system upon the addition of different Pb^{2+} concentrations: 0; 5 nM; 10 nM; 30 nM; 50 nM; 100 nM; 300 nM; 500 nM; 1μM; 3 μM; 5 μM. (B) Fluorescence increase over background fluorescence at varying concentrations of Pb^{2+}. F and F_0 are the fluorescence intensity in the presence and absence of Pb^{2+}.

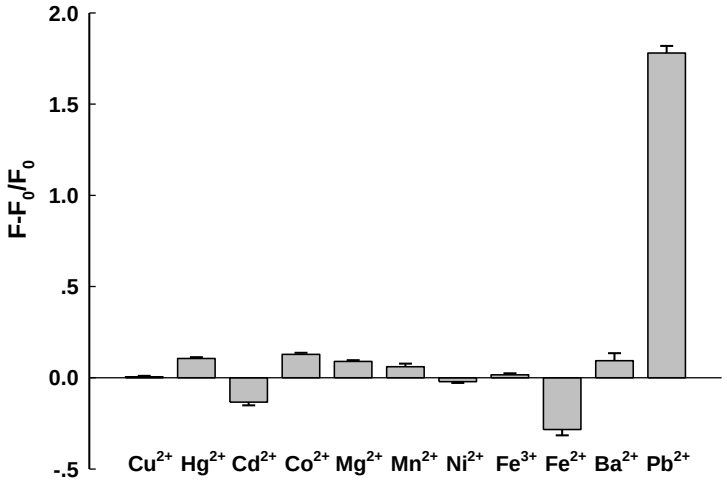

Fig.5. Selectivity of the DNAzyme-based biosensor for Pb^{2+}over other competing metal ions. The concentration was 100 nM for Pb^{2+}, 1 μM for Cu^{2+}, and 10 μM for other tested metal ions.

Graphical abstract

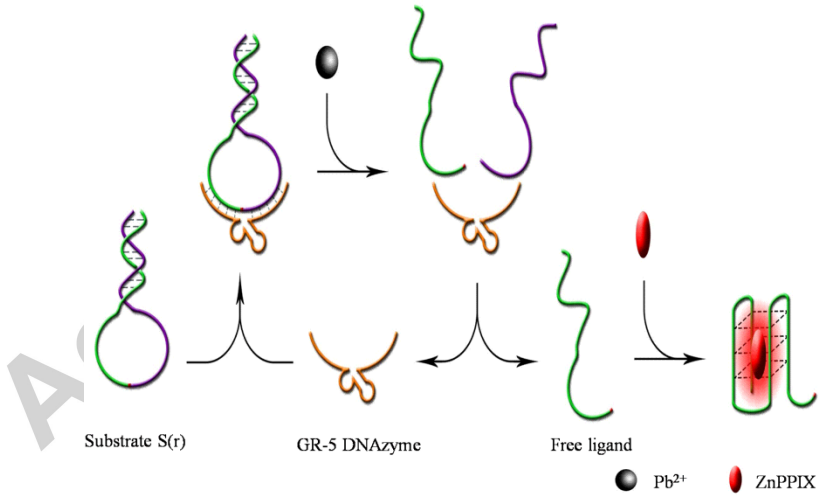

Table 1. The analytical performance characteristics of the developed biosensor

Analytical parameter	Result
Linearity domain	5-100 nM
Limit of Detection	3 nM

Regression equation	y=1.084+0.018x
R2	0.996

Table 2. Comparison of the present biosensor with previously reported DNAzyme-based fluorescent Pb^{2+} biosensors

DNAzyme	LOD	Interferences	Label	Ref.
8-17	10 nM	Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}	Labeled with FAM	[8]
8-17	600 pM	Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}	Labeled with FAM	[22]
8-17	4 nM	Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}	Label free	[32]
GR-5	3.7 nM.	/	Labeled with FAM	[21]
GR-5	200 pM	/	Labeled with FAM	[23]
GR-5	3 nM	/	Label free	This work

Table 3. The concentration of Pb^{2+} in synthetic samples detected using the biosensor

River water	Pb^{2+} spiked (nM)	Pb^{2+} recovered (nM) mean[a]± SD[b]	Recovery (%)
1	10.00	9.89±1.12	98.90
2	30.00	29.80±3.57	99.33
3	50.00	49.70±5.44	99.40

[a] Mean value of four determinations. [b] Standard deviation.

Highlights

A label-free biosensor was developed for amplified fluorescence detection of Pb^{2+}.

The strategy provided a universal label-free platform for DNAzyme-based biosensors.

The biosensor was successfully applied in detection of Pb^{2+} in complex samples.