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Temperature-robust DNAzyme biosensors confirming ultralow background detection

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ABSTRACT: Catalytic DNA/RNA, such as DNAzyme, has been widely adopted to construct biosensors, especially for metal ion analysis. However, traditional DNAzyme biosensors still suffer from fluctuating and relatively high background. Herein, we proposed a temperature-robust DNAzyme, conferring ultralow background in various temperatures, thus leading to highly sensitive and robust detection of metal ions. Instead of labelling substrate to directly output fluorescence signal, our proposed DNAzyme biosensor utilized a sequential detection process with a couple of proximity fluorescent probes, confirming very low background regardless of the conditions of cleavage reaction. This sequential DNAzyme biosensor conferred a signal to background ratio over 20 when the temperature of catalytic reaction ranged from 20 to 41 ℃. Benefited from its ultralow-background, it could confer a detection limit of 0.22 nM, which ranked one of the highest sensitivity levels among DNAzyme-based fluorescent biosensors. This DNAzyme biosensor was over 6000 times more selective for Pb^{2+} against the most active interfering metal ions, Zn^{2+}. Further, it has been successfully applied for analyzing lead pollution in tap water and eggs, with total recoveries ranging from 87% to 114%. This facile, simple and effective design strategy would significantly improve the detection performance of DNAzyme biosensors, thus facilitating its practical applications for both food safety analysis and environment monitoring.

KEYWORDS: DNAzyme; DNA nanotechnology; metal ions; fluorescent biosensor; functional nucleic acids

Functional nucleic acids,[1] such as DNAzyme[2-4] and aptamer,[5-9] have been widely explored as molecular tools for constructing biosensors. Considering its excellent specificity in molecular recognition and catalytic capacity,[2-4] DNAzyme is especially expected to act as both versatile recognition elements and outstanding signal amplifiers for biosensing. It can be created to recognize a wide range of analytes including amount of metal ions through *in vitro* selection.[10-12] Benefited from the nucleic acid nature which could be utilized to construct programmable and structure-switching DNA probes,[13-16] DNAzyme-based biosensors have been developed to detect a wide range of targets, including metal ions Cu^{2+}, UO_2^{2+}, Na^+ and Ca^{2+} with high selectivity and sensitivity.[4,17-20] Among them, fluorescent DNAzyme biosensors occupy a central niche because of their appealing attributes such as high sensitivity and fast kinetics, and recently even being applied for *in situ* detection and imaging of metal ions.[21,22]

In fact, it has long been paid myriad effects to develop robust fluorescent DNAzyme with suppressed background and background fluctuations. The basic rule for developing fluorescent DNAzyme biosensors is based on the differential stability between completely hybridized DNAzyme-substrate strand and the cleaved products.[23] The pioneer work for constructing DNAzyme biosensors has been done by Lu group.[24] A singly-labelled substrate strand with a fluorophore and a DNAzyme strand with a quencher were designed to construct this initial DNAzyme biosensors. The cleavage event led to the unstabilized binding of cleaved substrate to DNAzyme strands, resulting in an increase in the fluorescent intensity. Yet, the performance of the biosensor decreased considerably when the reaction temperature was raised to room temperature, conferring a high background and only 60% increase of fluorescence intensity in the presence of target metal ions.[25] This was caused by the partial dissociation of the substrate

strand-DNAzyme hybridization in the elevated temperature. Therefore, a dually labelled substrates with the introduction of a second quencher on its other end was designed, leading to both inter- and intra- molecular quenching effects.[25,26] And the DNAzyme biosensors using a dually-labelled substrates could be performed at room temperature with a suppressed background, conferring a signal to background (S/B) of 660%. Furthermore, the substrate has been designed to be a molecule beacon structure with improved quenching efficiency,[27] to further low down the background, conferring a S/B of 1300%. However, since DNA dehybridization and hybridization play a critical role in fluorescent DNAzyme biosensor design, to this end, almost all DNAzyme-based fluorescent biosensors are temperature dependent.[23,28] Thus, all these methods still suffer from the temperature-dependent fluctuations in background, which may seriously hamper the reliable detection, in turn, preventing their wide range applications for on-site and real-time detection.

To circumvent the long-term bothered issue, instead of using labelled substrate to directly output signals, we designed a DNAzyme biosensors based on a sequential detection process using a couple of proximity fluorescent probes, confirming ultra-low background regardless of the conditions of cleavage reaction. The sequential design strategy would circumvent the temperature-dependent background of DNAzymes. Benefited from the highly proximity designed fluorescent probes, this sequential DNAzyme biosensors could confer ultra-low background. Thus, it presented much higher sensitivity for metal ion detection. Besides, the temperature-robust feature would benefit the precise and reliable detection. This facile, simple and effective design strategy would significantly improve the detection performance of

DNAzyme biosensors, thus facilitating its practical applications for both food safety analysis and environment monitoring.

EXPERIMENTAL SECTION

Materials and reagents. Oligonucleotide sequences of DNAzyme biosensors for detecting lead ions are listed in Table S1. Oligonucleotide sequences with the incorporation of RNA bases were purchased from Takara Bio-technology Co. Ltd. (Dalian, China), and were HPLC purified. Other sequences were bought from Sangon Biotechnology Co. Ltd. (Shanghai, China), and HPLC purified. $Pb(CH_3COO)_2$, NaCl, $MgCl_2$, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES) were purchased from Alfa Aesar (Shanghai, China). $NiCl_2$, $Cd(NO_3)_2$, $Co(NO_3)_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$ (analytical reagent grade) were purchased from Sichuan Kelun Pharmaceutical Co., Ltd. Agarose, 6×Loading buffer, 50×TAE and Gelred dye were bought from Beijing DingGuo Biotechnology Co., Ltd. (Beijing, China). Molecular biology grade water was purchased from Corning Incorporated (New York, USA). All the reagents were dissolved in molecular biology grade water.

The procedure for metal ions detection. The detection of metal ions using DNAzyme biosensors was carried out in a volume of 20 μL containing 2 μL 10 × buffer (50 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$, pH = 7.26), 3 μL **Sub 1** (2 μM), 6 μL DNAzyme (0.5 μM), and 7 μL H_2O. Then, the mixture was heated to 80 ℃ for 3 min and incubated at room for 40 min to allow the hybridization of DNAzyme-substrate. 2 μL different concentrations of Pb^{2+} solution were introduced into the above solution and incubated at 25 ℃. Next, 2 μL **Sub F** (2 μM) and 4 μL **Sub Q** (2 μM) were added to stop the reaction. The fluorescence detection of the mixture

was carried on fluorescence microplate reader Synergy H1 (BioTek, USA). The excitation wavelength was set at 480 nm, and the emission was ranged from 510 to 650 nm.

Gel electrophoresis analysis. The mixtures of DNAzyme biosensor sequences or other sequences were mixed in 6 μl 1 × gel loading buffer, and used for gel electrophoresis. 3% agarose was prepared with 1 × TAE and 1 × Gelred dye. Gel electrophoresis was performed on the prepared gel in TAE at 150 V for 30 min. After electrophoresis, the gel was visualized via Gel Doc XR+ system (Bio-Rad, USA).

Real-time fluorescence analysis. A 20 ul volume of DNAzyme biosensor sequences or other sequences were used for real-time fluorescence analysis, and the concentrations of these sequences were the same as those used for metal ions detection procedures. The real-time fluorescence monitoring was performed using fluorescence microplate reader Synergy H1 (BioTek, USA). The excitation wavelength was 485 nm, and the emission wavelength was 522 nm.

Detecting Pb^{2+} in tap water and egg extract samples. The tap water samples were collected from the campus of Sichuan University. Fresh eggs were purchased from a local supermarket. For water sample, different amounts (0 nM, 0.4 nM, 0.8 nM, 4 nM and 8 nM) of Pb^{2+} were spiked in water samples, and the spiked samples were ready for further analysis. For the egg sample, firstly, the fresh egg samples (including white and yolk) were homogenized, then 1 g of the sample was mixed with 10 mL concentrated nitric acid solution in a fume hood for 2 h at room temperature. And different amounts (0 nM, 0.4 nM, 0.8 nM, 4 nM and 8 nM) of Pb^{2+} were spiked in (volume as 10 mL). After that initial nitrification, 0.5 ml $HClO_4$ was added and the mixture was heated for digestion until the solution became colorless, and finally heated to dry.

The remaining material was dissolved in water, and adjusted the pH to 7.26 with NaOH with a final volume of 10 mL, to be used for later analysis.

RESULTS AND DISCUSSION

Principles of sequential DNAzyme biosensor. The principle for sequential DNAzyme biosensor lies on the separation of catalytic reactions from the signaling process: cleavage step for recognizing Pb^{2+}, following hybridization step for outputting signals (Figure 1). Sequential DNAzyme biosensor is constituted with four strands. Besides the DNAzyme strand, there are two sets of substrates: unlabeled substrate 1 (**Sub 1**) and proximate singly-labelled substrates **Sub F** and **Sub Q** (labelled with a fluorophore and a quencher, respectively). The addition of target metal ions would specifically trigger the catalytic cleavage of **Sub 1**. And the extent of cleavage of **Sub 1** would then be monitored by the following addition of **Sub F** and **Sub Q**. Uncleaved **Sub 1** would result in the closely proximity of **Sub F** and **Sub Q** (this is no gap of nucleotides between bound **Sub F** and **Sub Q**), thus leading to an ultralow background. While DNAzyme catalysis would lead to the split of **Sub F** and **Sub Q,** resulted in the enhancement of fluorescence signals. The second set of substrates would contribute to one-substrate-one fluorophore light-up process to precisely output the signals for cleavage reaction. The sequential design strategy would lead to the separation of cleavage reaction and fluorescence detection process. Thus, sequential DNAzyme biosensors would obviate the disturbance of reaction conditions on background signal output, leading to temperature-robust and ultra-low background, which would benefit DNAzyme biosensors for the precise and reliable detection.

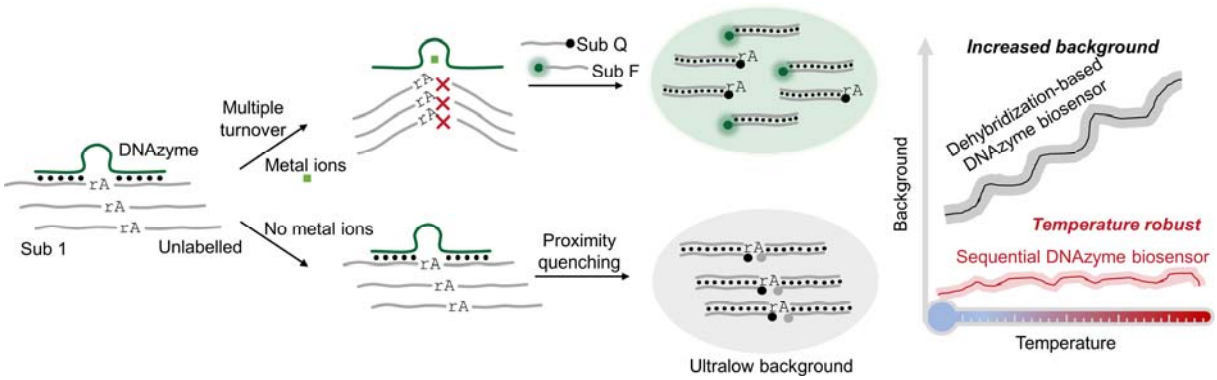

Figure 1. Working principles of sequential DNAzyme biosensor. Sequential DNAzyme biosensor is constituted with four strands: DNAzyme strand, unlabeled **Sub 1** and proximate singly-labelled substrate **Sub F** and **Sub Q**. The extent of cleavage of **Sub 1** would be monitored by the following addition of **Sub F** and **Sub Q**. Uncleaved **Sub 1** would result in proximity quenching of **Sub F**, thus leading to an ultralow background. The sequential design strategy would lead to the separation of cleavage reaction and fluorescence detection process, thus obviate the disturbance of reaction conditions on background signal output.

As a proof of principle, GR-5 DNAzyme which has been demonstrated to be highly active and specific for Pb^{2+} ion was chosen for testing sequential DNAzyme biosensors. The sequential detection process of sequential DNAzyme biosensors was firstly confirmed by electrophoresis analysis (Figure 2A). The GR-5 DNAzyme presented high catalysis activity in the presence of Pb^{2+} ions, leading to the total cleavage of **Sub 1** (line 7 and 8). The presence of **Sub 1** would lead to the proximate hybridization of **Sub F** and **Sub Q**, while spliced **Sub 1** subunits cleaved by GR-5 DNAzyme would separately be hybridized with **Sub F** and **Sub Q** (line 11 and line 12). These results manifest the success of the sequential detection processes.

These processes were also demonstrated by fluorescence analysis (Figure 2B). The binding of **Sub F** and **Sub Q** to **Sub 1** would lead to very low fluorescence signals, indicating the

proximity of these two probes and the high quenching efficiency of substrate sets. The addition of Pb^{2+} ion would lead to a sharp increase of fluorescence intensity, reaching to that of unbinding **Sub F** and **Sub Q**. While in the absence of Pb^{2+} ion, the fluorescence intensity of these substrates remained close to that of proximate **Sub F** and **Sub Q,** resulted a signal to background ratio of 20.19. The high signal to background ratio may potentially contribute high detection sensitivity of sequential DNAzyme biosensors.

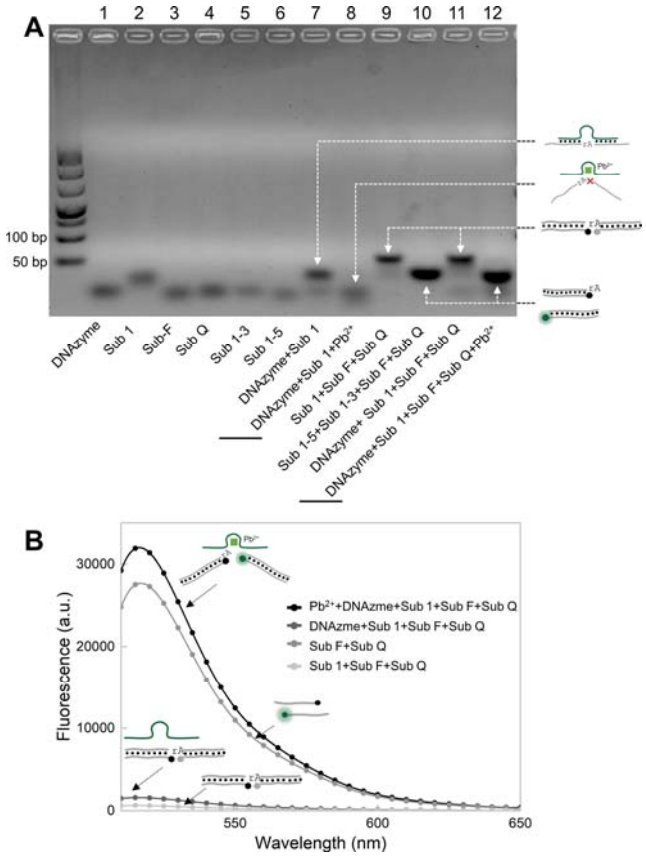

Figure 2. Validation of sequential DNAzyme biosensors for metal ion detection. (A) Electrophoresis analysis of the detection process of sequential DNAzyme biosensors; (B) Fluorescence analysis for the detection process of sequential DNAzyme biosensors. The concentrations of Pb^{2+} ion, DNAzyme, **Sub 1**, **Sub F**, and **Sub Q** were 2 μM, 0.15 μM, 0.3 μM, 0.2 μM and 0.4 μM, respectively. The excitation wavelength was set at 480 nm, and the emission was ranged from 510 to 650 nm.

The ultra-low background of sequential DNAzyme biosensor would contribute to its detection sensitivity, we then set to test its robustness which may benefit precise and accurate detection. As a comparison, dehybridization-based DNAzyme biosensors using singly-labelled substrate (singly-labelled DNAzyme) and dually-labelled substrate (dually-labelled DNAzyme) were also tested. The temperature for catalysis reaction was set to be ranging from 20 to 41 ℃ (with an interval of 3 ℃), which would cover most occasions using DNAzyme biosensors (Figure 3). Sequential DNAzyme biosensors outputted backgrounds of fluorescence intensity lower than 2800 and S/Bs over 20 when the temperature of cleavage reaction ranging from 20 to 41 ℃. It indicated that sequential DNAzyme biosensor would confer a background remained at very low levels regardless of increased temperature of catalysis reaction. This was because the adoption of sequential detection process would lead to the separation of cleavage reaction and fluorescence detection process, thus circumvent the disturbance of conditions of catalytic reaction on background, and the fluorescence intensity was only proportional to the amount of cleaved substrates. However, the background of singly-labelled DNAzyme and dually-labelled DNAzyme raised with the enhanced reaction temperatures. The background fluorescence of singly-labelled DNAzyme would lead to an increasement of about 50%, and that of dually-labelled DNAzyme was with an increasement of about 39% when the temperature of catalysis reaction altered from 20 ℃ to 41 ℃. This further implied the unstable binding of DNAzyme-substrate caused by temperature variation may possibly lead to disturbance of background. The temperature robust ultra-low background may render the sequential DNAzyme biosensors well fit for sensitive and precise detection of metal ions.

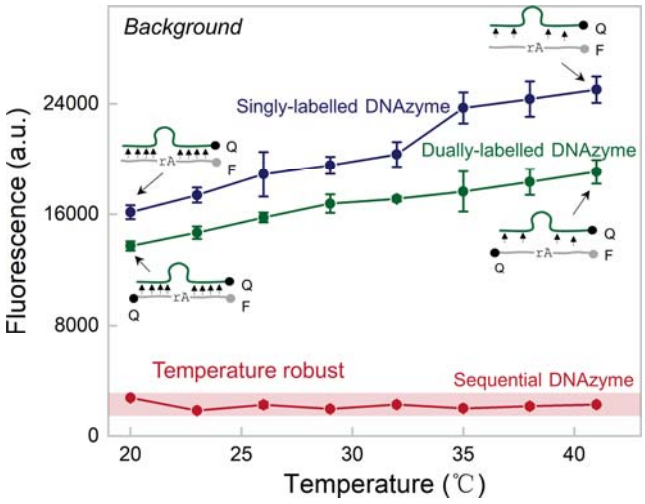

Figure 3. Temperature robustness test for DNAzyme biosensors. The background fluorescence intensity for sequential DNAzyme biosensors, dehybridization DNAzyme biosensors using singly-labelled substrate (singly-labelled DNAzyme) and dually-labelled substrate (dually-labelled DNAzyme) when the temperature ranged from 20 to 41 ℃. The concentrations of Pb^{2+} ion and DNAzyme were 2 μM and 0.15 μM, respectively. The excitation wavelength was 480 nm, and the emission was 518 nm.

Optimization of sequential DNAzyme biosensor. The catalysis activity of DNAzyme is dependent on the multiple turnover of cleavage reactions, and has been demonstrated highly affected by the stability of DNAzyme-substrate hybridization [27]. Eight GR-5 DNAzymes with different arms according to Lu group' work were designed. The melting temperature of DNAzyme-substrate hybridization fell down from 49.3 ℃ to 12.9 ℃ when arm lengths were shortened from 15-9 nucleotide (nt) to 7-7 nt. And for DNAzymes with 6-6 nt and 5-5 nt, they could hardly form DNAzyme-substrate complex (Figure S1). Interestingly, we found that shortening the arm length to 7-7 nt would lead to higher catalytic activity of DNAzyme (Figure 4A). This indicated that less stable binding of DNAzyme to substrate may increase the turnover of the cleavage reaction. For less stable DNAzyme-substrate hybridization, cleavage substrate

would be rapidly released, and new uncleaved substrates could be easily exchanged to bind with DNAzyme, thus facilitating the reaction kinetics and catalysis activity of DNAzyme.

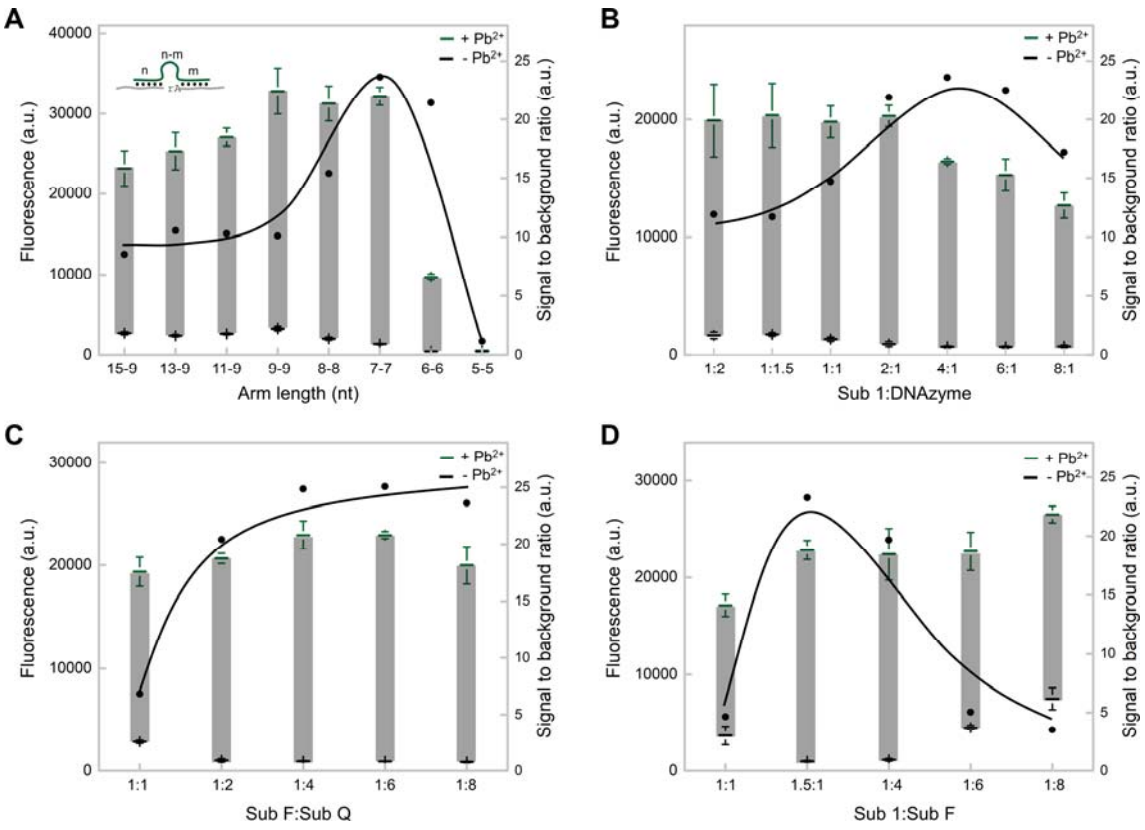

Figure 4. Optimization of sequential DNAzyme biosensor. The fluorescence intensity using different DNAzyme sequence (A), ratio of **Sub 1** to DNAzyme (B), ratio of **Sub F** to **Sub Q** (C) and ratio of **Sub 1** to **Sub F** (D) with or without the addition of Pb^{2+} ion. The signal to background ratio indicates the ratio of fluorescence intensity with the addition of Pb^{2+} ion to that without the addition of Pb^{2+} ion. The concentration of Pb^{2+} ion was 2 μM. The excitation wavelength was 480 nm, and the emission was 518 nm.

As the catalysis activity and the background of sequential DNAzyme biosensors were highly dependent on the constitution of substrates, the ratios between DNAzyme, different substrates were optimized. Reduced amount of DNAzyme would both result in decreased background and

catalysis activity (Figure 4B), and the catalysis activity would remain at a high level a ratio of **Sub 1** to DNAzyme of 2:1. Thus the ratio of **Sub 1** to DNAzyme was used for the further detection of Pb^{2+} ion. For the adoptions of **Sub Q** to **Sub F** with different ratios, excess **Sub Q** would low down the background of DNAzyme biosensors, while had relatively low influence on the signal with additions of Pb^{2+} ion (Figure 4C). Considering the cost of labelled probe, the **Sub Q** for sequential DNAzyme biosensors was used with the amount of two times of F-sub to obtain a relatively high S/B. Further, the optimized DNAzyme biosensors was achieved using a ratio of **Sub 1** to **Sub F** of 1.5:1 based on the S/B output (Figure 4D). Under the optimized conditions, the catalytic cleavage reaction could be finished within 10 min, and the binding of **Sub Q** and **Sub F** to **Sub 1** would reach equilibrium in 10 min, and would lead to very low background fluorescence signal (Figure S2).

Sequential DNAzyme biosensor for metal ion detection. The quantification performance of DNAzyme biosensors was validated by using a series of Pb^{2+} solutions with different concentrations. The fluorescence signals would be linearly related to the concentrations of Pb^{2+} ion when the concentration of Pb^{2+} ion was ranging from 0.1 to 10 nM, while would be reaching saturation when the concentration of Pb^{2+} ion was above 40 nM (Figure 5). Benefited from its low background, sequential DNAzyme biosensors could confer a highly sensitive detection of Pb^{2+} ion, with a limit of detection (LOD) of 0.22 nM. LOD was defined as the concentration corresponding to the fluorescence signal at three times standard deviation of blank without Pb^{2+} ion. Sequential DNAzyme biosensor was one of the most sensitive DNAzyme-based fluorescent biosensors (Table S2). Besides, the low background of sequential DNAzyme would contribute to a broad range of fluorescence signals for Pb^{2+} ion detection, benefiting the precise

quantification. As for DNAzyme biosensors with higher background (singly-labelled and dually-labelled DNAzymes), the signal would present crossed error bars in the low concentration range of Pb^{2+} ion (Figure S3), which may exert uncertainty for quantification.

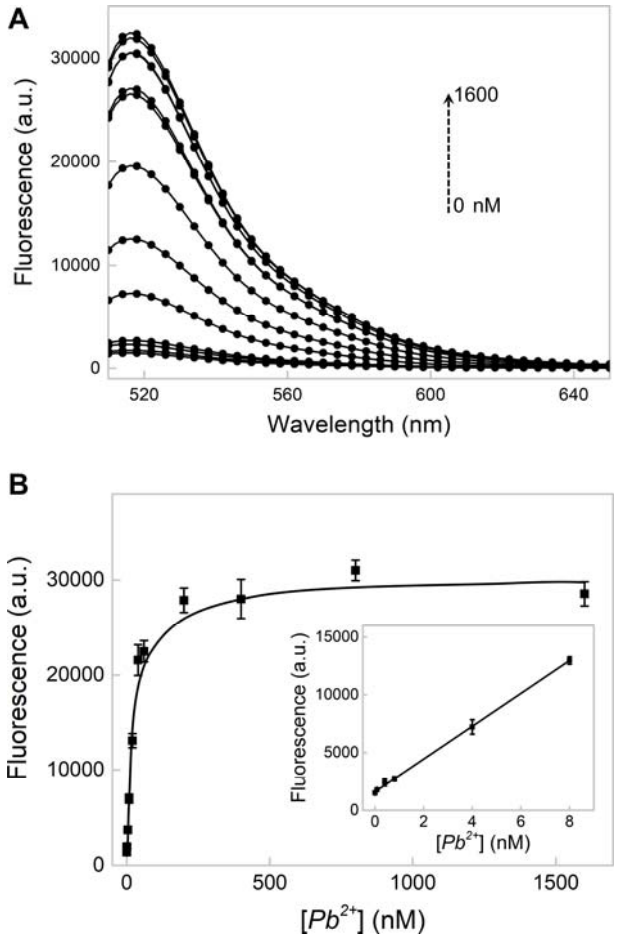

Figure 5. (A) Typical fluorescence spectra of sequential DNAzyme biosensor upon addition of different concentrations of Pb^{2+} ion ranging from 0 to 1600 nM; (B) The relationship between target concentration and fluorescence response using sequential DNAzyme biosensor; Inset: The linear fitting for quantification of Pb^{2+} ions. The concentrations of DNAzyme, **Sub 1**, **Sub F**, and **Sub Q** were 0.15 μM, 0.3 μM, 0.2 μM and 0.4 μM, respectively. The excitation wavelength was set at 480 nm, and the emission was ranged from 510 to 650 nm.

The GR-5 DNAzyme has been demonstrated to be highly specific for Pb^{2+} ion [26]. The specificity of sequential DNAzyme biosensors was examined by detecting fluorescence signal of Pb^{2+} ion and other metal ions (Ni^{2+}, Cd^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+}) in different concentrations. No metal ions would lead to a nonnegligible signal change besides Pb^{2+} ion (Figure 6A), and outputted signal the same as that of blank control in all these four concentrations (10 nM, 50 nM, 100 nM and 200 nM).

To further confirm its specificity, most active interfering metal ions Zn^{2+}, Mn^{2+} and Ca^{2+} with 5 times, 20 times, 100 times and 500 times concentrations of Pb^{2+} ions were added. Remarkably, these competing metal ions displayed little increasement of fluorescence of sequential DNAzymes. And this DNAzyme biosensor was over 6000 times more selective for Pb^{2+} ions against Zn^{2+}, Mn^{2+} and Ca^{2+}. What' more, the fluorescence response to Pb^{2+} ions in the presence of a "metal soup" (Ni^{2+}, Cd^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+}) containing 500 times concentration of Pb^{2+} ion was tested. No obvious fluorescence increasement was observed with the metal soup in the absence of Pb^{2+} ions. While the addition of 50 nM Pb^{2+} ion to the "metal soup" would resulted in 7-fold fluorescent enhancement. Therefore, sequential DNAzyme biosensors could strictly identify Pb^{2+} ion, potentially accommodating it for the application of detecting Pb^{2+} ion in real sample which may contain high concentrations of other metal ions, such as waters with high content of calcium.

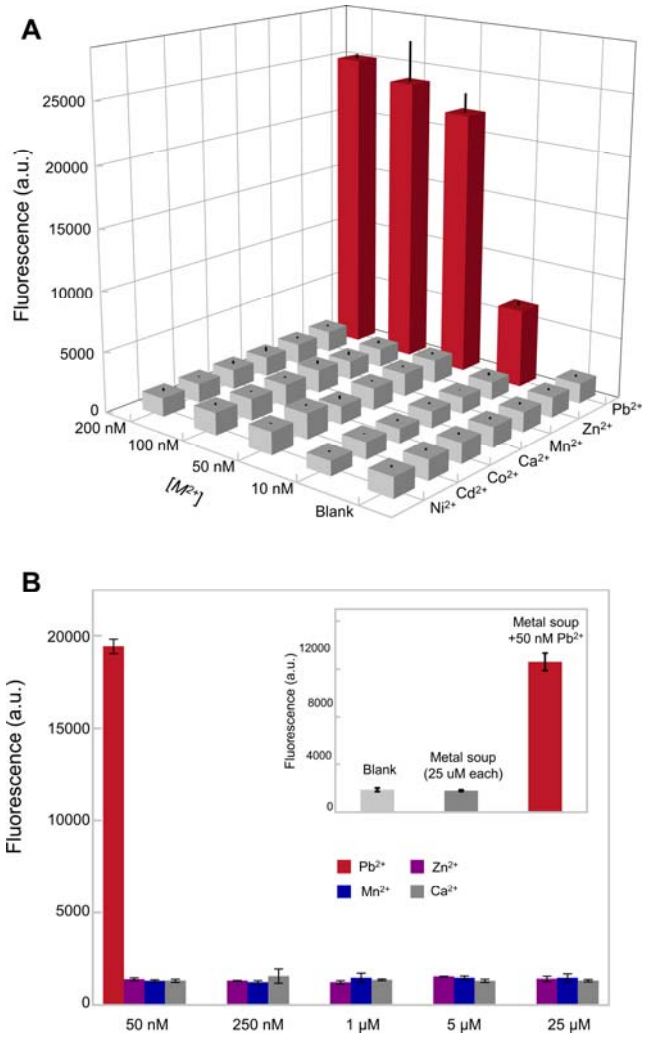

Figure 6. Specificity test of Pb^{2+} ion detection. (A) The fluorescence intensity with the addition of Pb^{2+} and other metal ions (Zn^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+}) with concentrations of 10, 50, 100 and 200 nM; (B) The fluorescence intensity with the addition of mostly interfering metal ions Zn^{2+}, Mn^{2+} and Ca^{2+} with 5, 20, 100, 500 times concentration of Pb^{2+} ion (50 nM, 250 nM, 1 μM, 5 μM and 25 μM). Inset: fluorescent response of sequential DNAzyme biosensors in the presence of other six metal ions with 500 times concentration. The concentrations of Pb^{2+} ion, DNAzyme, **Sub 1**, **Sub F**, and **Sub Q** were 2 μM, 0.15 μM, 0.3 μM, 0.2 μM and 0.4 μM, respectively. The excitation wavelength was set at 480 nm, and the emission was ranged from 510 to 650 nm.

Finally, to assess the feasibility of DNAzyme biosensors for metal ion detection in complex matrixes such as fluid sample or liquid sample, we applied sequential DNAzyme biosensors to detect spiked Pb^{2+} ion in tap water and eggs. For targets with a concentration falling in the detection dynamic range of DNAzyme biosensors, the recovery ratios were fall in 87% to 114% (Table 1, Figure S4) for detection in both tap water and eggs. Thus, this DNAzyme biosensors holds great potential for on-site detection of metal ion pollution both benefiting from its low cost, robustness and highly sensitivity.

Table 1. Determination of Pb^{2+} spiked in the tap water and egg samples

Samples	Added (nM)	Found (nM)	Recovery (%)	RSD (%) n=3
Tap water	0.4	0.436	108.93	5.31
	0.8	0.913	114.16	2.74
	4	3.596	89.91	2.04
	8	7.542	94.28	3.37
Egg	0.4	0.348	87.09	6.64
	0.8	0.703	87.83	1.02
	4	3.614	90.35	9.01
	8	8.099	101.24	11.56

CONCLUSION

In summary, a temperature-robust DNAzyme biosensor has been developed for achieving ultralow-background detection, allowing highly sensitive and robust detect metal ions. The key difference for DNAzyme lies on the sequential detection process using a couple of proximity fluorescent probes, rather than directly output fluorescence signal using labelled substrate, thus confirming very low background regardless of the conditions of cleavage reaction. This DNAzyme biosensor presents novel features: 1) temperature-robust; 2) ultralow background; 3) super sensitivity. 4) one-tube homogenous detection. Benefited from its general and simple

design, the DNAzyme biosensors may potentially been used as a universal platform for various metal detection, such as Cu^{2+}, UO_2^{2+}, Na^{+}, Mg^{2+} and Ca^{2+}; Thus, this simple, while the well-performed DNAzyme biosensors would greatly facilitate the application of DNAzyme for on-site detection, leading it to be competitive tools for food safety analysis and environment monitoring.

Supporting Information

Supporting Information Available: The following files are available free of charge.

Kinetics of sequential reaction process, secondary structures of DNAzyme-substrate hybridization, quantification performance of singly-labelled and dually-labelled DNAzyme, Recovery of Pb^{2+} in the tap water and egg samples

Notes

The authors declare no competing financial interest.

Acknowledgement

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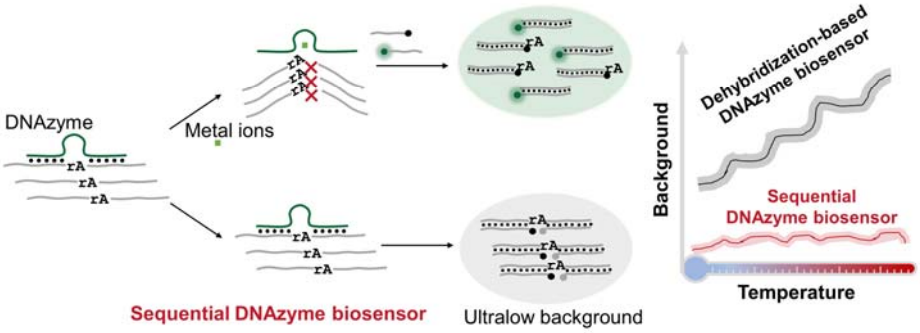

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