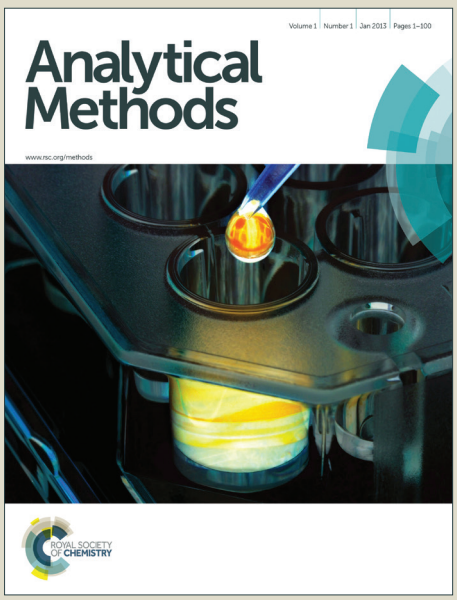
Analytical Methods

Accepted Manuscript



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Ultrasensitive detection of lead ions based on a DNA-labelled DNAzyme sensor

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Abstract

Here, we report a facile approach for highly sensitive and selective detection of aqueous lead ions that uses real-time quantitative polymerase chain reaction technology and a lead-dependent DNAzyme, termed GR-5. In this method, substrate DNA is cleaved at the site of the adenosine ribonucleotide by GR-5 DNAzyme in the presence of lead ions, resulting in a decrease in template DNA available for PCR and a consequent change in signal detection (cycle threshold (Ct) value). This novel approach takes advantage of the exponential amplification of PCR and the specific recognition of the GR-5 lead-dependent DNAzyme to provide Pb^{2+}-specific detection with an excellent linear relationship between Ct value and Pb^{2+} concentration within a range of 1–500 nM. The correlation coefficient of the standard curve was 0.9898, and the limit of detection was 0.7 nM. Moreover, this sensor showed good selectivity for Pb^{2+} ions over other metal ions.

Keywords: RT-qPCR**,** GR-5 DNAzyme, lead ion, sensitive detection

1. Introduction

Lead ions (Pb^{2+}) are major environmental pollutants that exist widely in soil and water. Lead ion pollution has attracted increasing attention, as exposure to even very low levels of lead can have severe adverse effects on the environment and on human health by causing neurological, cardiovascular, and developmental disorders.[1, 2] However, no facile method for monitoring lead ions at low concentrations has yet been established. Many analytical methods have been developed for the determination of Pb^{2+} concentration, including traditional instrumental analysis. In general, graphite furnace atomic absorption spectroscopy (GFAAS), anodic stripping voltammetry (ASV), inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), and X-ray fluorescence spectrometry are commonly used for Pb^{2+} analysis.[3-8] Although these methods are highly sensitive and accurate, they often

require a special experimental environment, expensive and complex experimental equipment, prolonged pre-treatment and skilled operators. Therefore, the development of a field-applicable, inexpensive, and sensitive Pb^{2+} ion detection method would be highly beneficial.

One possible approach to Pb^{2+} ion detection is the use of DNAzymes, which are specific DNA molecules that possess catalytic activity such as DNA/RNA cleavage.[9-15] The catalytic activity of DNAzymes is often activated by cofactors and induced chemical transformations. DNAzymes are easily synthesized and chemically stable and have been widely used for analytical purposes. Lead-specific DNAzymes are known to cleave at adenosine ribonucleotide (rA) sites on partially complementary DNA substrates in the presence of lead ions.[16] Typical Pb^{2+}-specific DNAzyme-based sensors utilize the enzymatic activity of the GR-5 and "8-17" DNAzymes on a target element to produce a fluorescent or colorimetric output.[17-20] Although these methods are sensitive and versatile, they have inherent drawbacks such as high detection limits and high background signals.

The inherent limitations of DNAzyme-based sensors could possibly be overcome by using real-time quantitative polymerase chain reaction (RT-qPCR) technology. RT-qPCR has been widely used for exponential DNA amplification and provides an accurate and sensitive method for the detection of specific DNA fragments. Furthermore, differences in the initial DNA concentration are reflected in the cycle threshold (Ct) values, which can be used as a quantitative indicator to determine DNA concentration. The RT-qPCR technique was initially used for the measurement of

gene expression and has since been applied to the quantification of microorganisms, including clinical bacteria and viruses.[21-23] RT-qPCR-based sensors are sensitive and specific, show a broad quantification range, and show a good linear relationship between the Ct value and target DNA concentration.

In this study, a novel biosensor for lead ions was developed using the GR-5 DNAzyme. The GR-5 DNAzyme was immobilized on the inner wall of a PCR tube, and the template DNA was bound to the GR-5 DNAzyme via base pairing. When Pb^{2+} ions were added, the template DNA was cleaved at the rA site by the GR-5 DNAzyme, leading to a decrease in template DNA concentration and a consequent increase in signal (Ct value) detection. Importantly, the Ct value was found to have a linear relationship with Pb^{2+} concentration. To the best of our knowledge, this is the first example of a lead ion sensor that combines DNAzyme activity with RT-qPCR technology.

2. Experimental and methods

2.1 Materials and reagents

GR-5 DNAzyme, substrate DNA and primers were synthesized and purified by Shengon Biotechnology Co. Ltd. (Shanghai, China). Their sequences were as follows:

GR-5 DNAzyme: 5′-biotin-GGCTACGAGGGAAATGCGGTAATCATCTCTGAAG TAGCGCCGCCGTAGTG-3′;

Substrate DNA: 5′-AATCTGGTTTAGCTACGCCTTCCCCGTGGCGATGTTTCTTA GCGCCTTACCACTrAGGAAGAGATGATT-3′;

Upstream primer: 5′-AATCTGGTTTAGCTACGCCTTC-3′;

Downstream primer: 5′-GTAAGGCGCTAAGAAACATCG-3′.

iTaq™ Universal SYBR® Green Supermix was purchased from Bio-Rad (Shanghai, China). Streptavidin was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Lead ions, mercury ions, cupric ions, cadmium ions, and nickel ions were obtained from the National Standard Substances Center (Beijing China). All other chemicals were of analytical grade. Ultrapure water was used to prepare aqueous solutions.

2.2 RT- qPCR assay for Pb^{2+} detection

First, the PCR tubes were incubated with 20 μL of 0.8% glutaraldehyde solution at 37°C for 5 h to improve their adsorption capability. After three washes with ultrapure water, 20 μL of streptavidin dissolved in 0.01 M carbonate buffer solution was added, followed by incubation at 37°C for 2 h to fix the streptavidin to the PCR tubes. Each PCR tube was then washed with PBST (10 mM PBS, pH 7.2, 0.05% Tween-20). After washing, 20 μL of the appropriate concentration of the mixture of GR-5 DNAzyme and substrate DNA was added to the PCR tubes. GR-5 DNAzyme was fixed to the PCR tubes by specific binding between biotin and streptavidin, and the substrate DNA was hybridized by base-pairing with the GR-5 DNAzyme through incubation at 37°C for 40 min. After each tube was washed with hybridization buffer (750 mM NaCl, 75 mM $C_6H_5Na_3O_7$, pH 8.0), Pb^{2+} was added to each tube at the indicated concentration and incubated at 37°C for 40 min. Then, each tube was washed three times with hybridization buffer to remove the free DNA fragment, leaving the biotin-labelled GR-5 DNAzyme or dsDNA on the surface of the PCR

tubes.

Finally, the PCR mixture (total volume, 20 μL) was added to the washed tubes. The PCR mixture was composed of 2 μL upstream and downstream primers at 500 nM, 10 μL of iTaq Universal SYBR Green Supermix, and 6 μL of water. RT-qPCR was performed using the CFX-96 real-time PCR system. The PCR cycling parameters were as follows: an initial denaturation for 30 s at 95°C, followed by 39 cycles of denaturation for 5 s at 95°C and annealing for 30 s at 59°C. Fluorescence was measured after each annealing step. A melting curve was obtained from 65°C to 95°C to detect potential nonspecific products, with signal measurement every 0.5°C.

2.3 Specificity analysis

Specificity is an important factor for a new proposed method. To evaluate the specificity of this method for Pb^{2+} detection, other common metal ions (mercury, copper, cadmium, and nickel ions) were used in place of Pb^{2+}. These metal ions were tested at a concentration of 1 μM. All other detection conditions were the same as those used in the Pb^{2+} detection procedure.

2.4 Recovery in tap water samples

To demonstrate the practicality of our proposed method, the negative tap water sample was spiked with Pb^{2+} (1 nM, 5 nM, 10 nM, and 50 nM). After simple filtration, these samples were tested with the same procedure used for Pb^{2+} detection. The recovery ratio was calculated based on the PCR signals.

3. Results and discussion

3.1 The principle of the DNAzyme sensing system

To develop a novel method for aqueous Pb^{2+} detection, we employed the combination of GR-5 DNAzyme catalytic activity and qPCR (see scheme 1). In this method, GR-5 DNAzyme is hybridized to a substrate strand through complementary base pairing. The substrate strand contains a single RNA base that is cleaved specifically by the classic Pb^{2+}-dependent GR-5 DNAzyme as the catalytic unit and a 50-base substrate strand extension that is required as a template for PCR amplification. Biotin-modified GR-5 DNAzyme is fixed on the inner wall of a streptavidin-coated PCR tube via biotin-avidin interactions.[24] The addition of aqueous Pb^{2+} to the PCR tube leads to cleavage of the substrate DNA at the rA site by the GR-5 DNAzyme, resulting in release of the template DNA. Consequently, the PCR amplification signal (Ct value) varies according to the amount of template available for amplification, which in turn depends on the concentration of aqueous Pb^{2+}. Thus, the concentration of the Pb^{2+} target can be quantified using the Ct value.

3.2 Optimization of assay conditions

Before applying this new approach for Pb^{2+} detection, we optimized the experimental conditions, including streptavidin concentration and reaction time. Figure 1 shows the relationship between streptavidin concentration and Ct value that was observed when the GR-5 DNAzyme and substrate DNA concentrations were both fixed at 100 nM. The Ct value gradually decreased with increasing streptavidin concentration in the range of 1–10 ng/mL, whereas the Ct value increased notably with increasing streptavidin concentration in the range of 10–20 ng/mL. The minimum Ct value (corresponding to maximum DNA template level) was observed

when the concentration of streptavidin was 10 ng/mL; thus, 10 ng/mL streptavidin was selected for the subsequent assays.

The sensitivity of the sensor is affected by the reaction time, which ideally permits a complete reaction. Therefore, the time allowed for binding of the substrate DNA to the GR-5 DNAzyme and for substrate cleavage in the presence of Pb^{2+} was also optimized (Figures 2 and 3). The Ct value decreased with increasing substrate-binding time until 40 min. When the time exceeded 40 min, the Ct value only changed slightly. As a reduction in Ct value reflects an increase in the amount of template DNA available, the results indicate that a sufficient amount of GR-5 DNAzyme binding occurred within 40 min. The optimization of substrate cleavage time showed that in the presence of Pb^{2+}, the Ct values increased with increasing cleavage time until 40 min. Based on these results, the time for hybridization of the GR-5 DNAzyme to the substrate DNA and the time for substrate cleavage were both set at 40 min for subsequent experiments.

3.3 Sensitivity and selectivity of Pb^{2+} detection

The amplification curves of the optimized detection system for different Pb^{2+} concentrations (0.5 nM to 1 μM) were obtained by RT-qPCR (Figure 4). The Ct value increased with increasing Pb^{2+} concentration over the entire 0.5–1000 nM range, and a good linear relationship was observed in the range of 1–500 nM Pb^{2+} (Figure 5). The linear correlation equation was determined to be $Ct = 11.79516 + 2.99978\lg C$, where C is the concentration of Pb^{2+} in nM. The correlation coefficient of the standard curve was 0.9898, and the limit of detection was 0.7 nM (S/N ratio = 3). Relative to other

detection sensors,[25-31] the sensor system described herein was considerably more sensitive.

To evaluate the selectivity of this method, several common metal ions (mercury, nickel, copper, and cadmium ions; each at a concentration of 1 μM) were assessed. In contrast to that observed in the presence of lead ions, the Ct values in the presence of these four metal ions did not notably change, indicating that the substrate DNA was not cleaved (Figure 6). These results clearly demonstrate the selectivity of the sensor.

3.4 Real sample analysis

To test the practicality of the proposed method, we applied this assay to determine the lead ion concentration in tap water. Blank tap water samples spiked with different concentrations of lead ions (1, 5, 10, and 50 nM) were evaluated (Table 1). The recovery for the tap water samples was in the range of 97.1–106.0%, with a relative standard deviation lower than 5.2%. These results show that the Pb^{2+} sensor has good recovery values and is suited for practical application.

4. Conclusion

In this paper, we have introduced a novel lead ion sensor based on RT-qPCR technology. In the presence of lead ions, substrate DNA was cut at the rA site by the GR-5 DNAzyme. Under optimized conditions, a good linear relationship between the concentration of lead ions and the Ct value was observed, and a low detection limit (0.7 nM) was noted, indicating that a sensitivity higher than that of several new sensors[27, 29, 32] was achieved. To our knowledge, this is the first sensor to combine RT-qPCR technology and GR-5 DNAzyme for the detection of lead ions. This novel

sensor showed high sensitivity and specificity and demonstrated satisfactory recovery of lead ions from tap water samples. Thus, this new sensor is a potential practical tool for the detection of lead ions in environmental samples.

Acknowledgments

This work is financially supported by the Key Programs from MOST (2012AA06A303), NSF of Jiangsu Province (BK20130379, 13KJB550001), and the Suzhou Science and Technology Committee Program (SS201335)

Notes

† These authors contributed equally to this work.

The authors have no competing financial interests to declare.

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Legends to Figures

Scheme 1. (A) Schematic illustration of the developed method using RT-qPCR technology. (B) The reaction diagram of GR-5 DNAzyme in the presence of lead ions.

Figure 1. Effect of streptavidin concentration on the Ct values of the sensor system.

Figure 2. Effect of the time for hybridization of the substrate DNA and GR-5 DNAzyme on the Ct values of the sensor system.

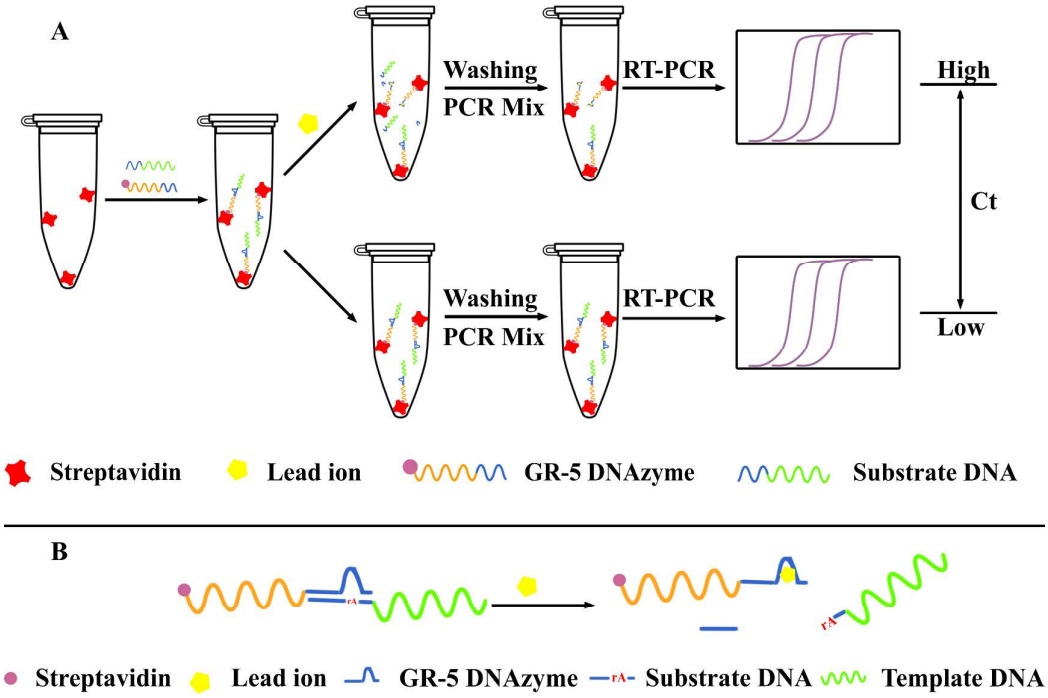
Figure 3. Effect of the time for substrate cleavage on the Ct values of the sensor system.

Figure 4. Amplification curves at different concentrations of Pb^{2+} in the range of 0.5–1000 nM.

Figure 5. Ct value as a function of Pb^{2+} concentration. Inset: Linear relationship between Pb^{2+} concentration and Ct value in the range of 1–500 nM.

Figure 6. Selectivity of the RT-qPCR-based sensor for Pb^{2+} ions over other metal ions.

Table 1. Recovery experiments for Pb^{2+} ions in tap water samples.



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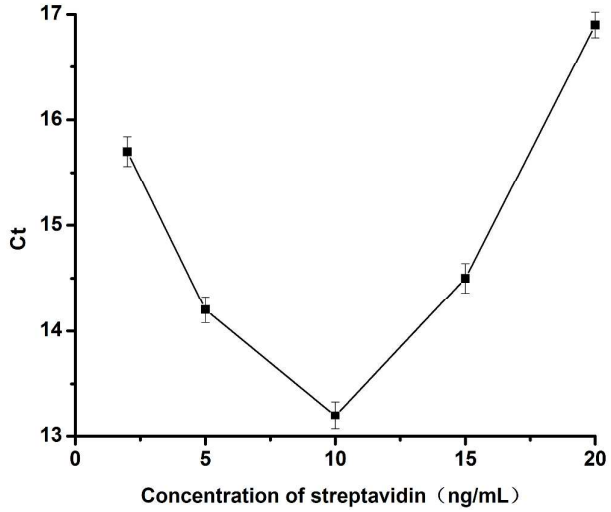

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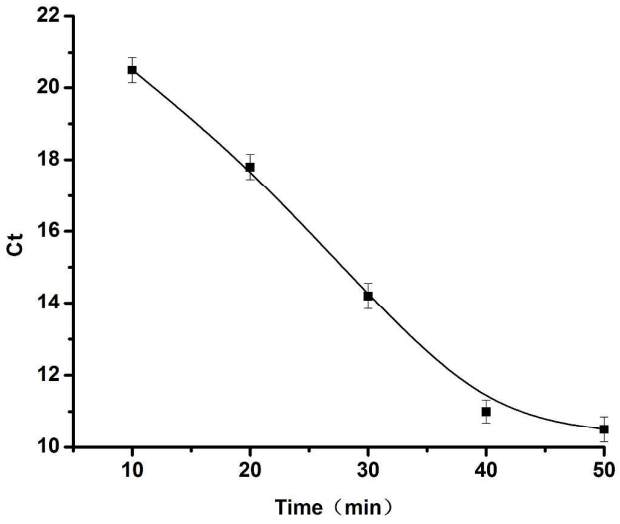


Figure 2. Effect of the time for hybridization of the substrate DNA and GR-5 DNAzyme on the Ct values of the sensor system.

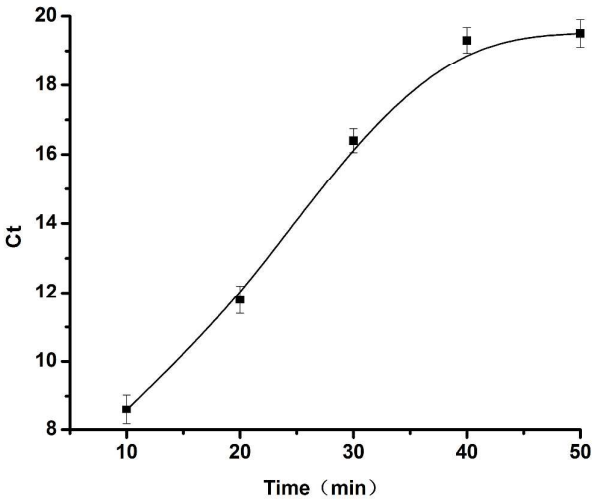


Figure 3. Effect of the time for substrate cleavage on the Ct values of the sensor system.

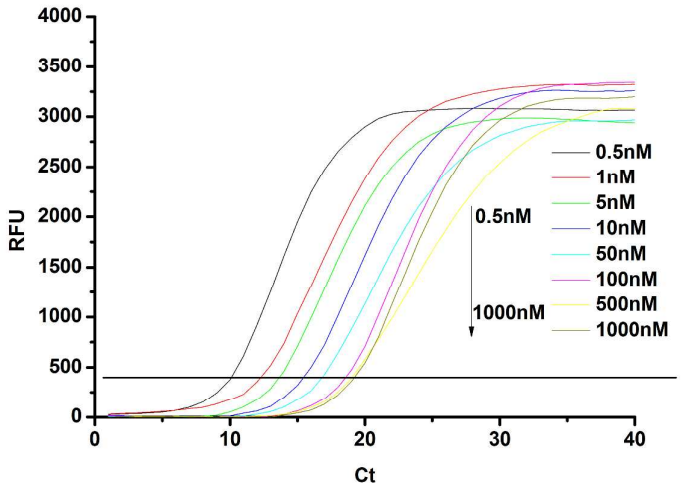
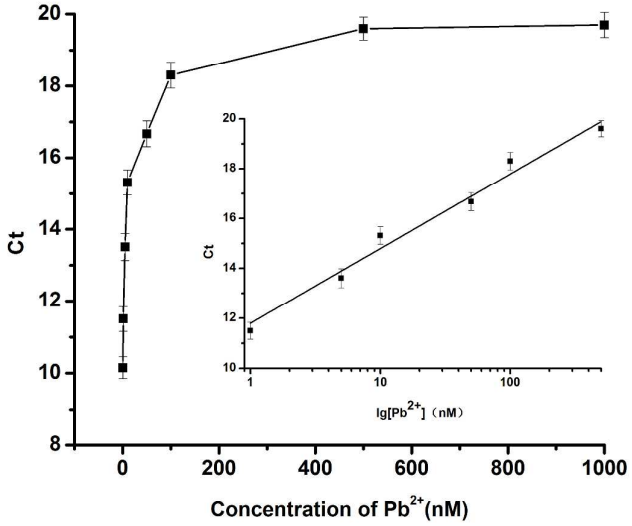


Figure 4. Amplification curves at different concentrations of Pb^{2+} in the range of 0.5–1000 nM.



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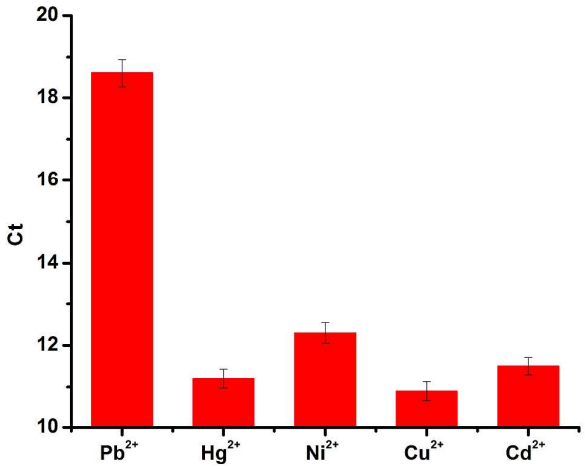

Figure 6. Selectivity of the RT-qPCR-based sensor for Pb^{2+} ions over other metal ions.

Table 1. Recovery experiments for Pb^{2+} ions in tap water samples.

Sample	Spiked Pb^{2+}（nM）	Measured Pb^{2+}(nM)	Recovery (%)
Water sample	1	1.06±0.03	106.00±4.74
	5	4.97±0.23.	99.40±3.32
	10	10.17±0.34	101.70±5.16
	50	48.55±2.10	97.10±3.56