CHINESE JOURNAL OF ANALYTICAL CHEMISTRY
Volume 40, Issue 8, August 2012
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Anal Chem, 2012, 40(8), 1236–1240.

RESEARCH PAPER

Amplified Fluorescence Detection of Pb^{2+} Using Pb^{2+}-dependent DNAzyme Combined with Nicking Enzyme-Mediated Enzymatic Recycling Amplification

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Abstract: A fluorescence sensing system was developed for the detection of Pb^{2+} with excellent sensitivity and selectivity based on Pb^{2+}-dependent DNAzyme (8-17E DNAzyme) with nicking enzyme (Nt.BbvCI)-assisted signal cascade amplification strategy. In the presence of Pb^{2+}, the 8-17E DNAzyme can catalyze the cleavage of its substrate. And subsequently, the partial substrate strand dissociated from DNAzyme could hybridize with molecular beacon (MB), resulting in the restoration of fluorescence signal as well as the formation of the double-stranded recognition site for nicking endonuclease (Nt.BbvCI). After the Nt.BbvCI mediated the cleavage of MB, the released partial substrate strand could hybridize with another MB probe again and be re-used for the second cycle of cleavage. Eventually, each target-induced partial substrate strand can trigger many cycles of cleavage to achieve the amplified fluorescence detection of Pb^{2+}. This new design avoids the modification on DNAzyme and substrate, and significantly improves the sensitivity with a detection limit down to 1.0×10^{-10} M. Moreover, it also exhibited satisfactory selectivity for Pb^{2+} detection, even in the presence of 2 times concentrations of Zn^{2+} and 5 times concentrations of each other interferential metal ions. Furthermore, this proposed method was successfully used for the determination of Pb^{2+} in river water samples with recoveries from 96.1% to 108%.

Key Words: Fluorescence assay; Lead ions; DNAzyme; Nicking enzyme; Enzymatic recycling amplification strategy

1 Introduction

With continuous urban expansion and rapid development of industrial production, a large amount of domestic sewage and industrial wastewater is discharged into the environment without extensive treatment, which results in the serious pollution problems. As a widespread heavy metal pollutant, Pb^{2+} is a highly and cumulative toxic element, and the trace amounts of Pb^{2+} can cause serious damage to various organs of the human body[1]. To effectively control environmental pollution of Pb^{2+}, an attention of government and scientific community has been focused on establishing a convenient, highly sensitive and highly selective method for detecting Pb^{2+} in water, food, air, soil and tobacco products, and so on. The traditional Pb^{2+} detection methods include atomic absorption/emission spectrometry[2,3], chromatography[4,5], and induced-coupling of plasma mass spectrometry[6,7]. However, these methods often involve large equipment and higher testing costs, as well as requiring professional operators and a time-consuming sample pretreatment process, all of which present some limitations for their practical implementation in the detection of Pb^{2+}.

Deoxyribozymes (DNAzymes) are a class of nucleic acids with enzymatic functions[8], and they are usually obtained via in vitro screening technology (the systematic evolution of ligands by exponential enrichment). The activity of these

Received 4 April 2012;accepted 3 June 2012
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This work was supported by the Research Project Foundation of Shaanxi Branch of China National Tobacco Corporation.

DOI: 10.1016/S1872-2040(11)60568-9

DNAzymes depends on the presence of certain metal ions[9,10]. In comparison with proteases, DNAzymes display high catalytic activity, good stability and are easy to synthesize and modify. In addition, DNAzymes are cheaper and environmentally-friendly. In the presence of Pb^{2+}, 8-17E DNAzyme can cleave a substrate DNA strand that has one ribonucleotide base[11], and based on that, many new Pb^{2+} detection techniques have been rapidly developed based on fluorescence[12–14], colorimetric[15] or electrochemical methods[16]. However, most of these methods require the 8-17E DNAzyme or its substrate chain to be tagged or modified, which increase the complexity and testing cost. In addition, the complex composition of the environmental sample can easily lead to a high background signal, thereby influencing the sensitivity of Pb^{2+} detection. The development of a novel and signal-amplified approach for further improving sensitivity is still an urgent demand. The combination of the existing isothermal signal amplification technique with the DNAzyme-based sensing system undoubtedly provides a promising alternative approach for simple and sensitive detection of Pb^{2+}.

In general, nicking enzyme-mediated isothermal signal amplification strategy is widely used in highly sensitive detection of DNA[17,18]. As a specific type of restriction endonuclease, the nicking enzyme recognizes a specific double-stranded sequence within the hybridized region between target DNA and probes particular nucleotide sequence, and then cleaves only probe strand to produce a detectable signal. This action frees the target DNA, allowing it to hybridize with the next probe. Finally, the detection signal is significantly amplified via the enzymatic recycling cleavage. In comparison with the traditional DNA polymerase based signal amplification techniques[19–22] that suffer from complex operation processes, high cost, and the non-specific signal amplification, the nicking enzyme-mediated amplification strategy can provide a simpler, faster and cost-effective technique for the development of the highly sensitive sensing platform. To introduce the nicking enzyme into the Pb^{2+} detection strategy and realize no labeling of 8-17E DNAzyme and its substrate, the substrate chain and MB are elaborately designed to contain the specific recognition sequence for Nt.BbvCI. In the presence of target Pb^{2+}, 8-17E DNAzyme will catalyze the cleavage of its substrate, and release a single-stranded DNA (ssDNA) complementary to the sequences of MB. Then, a recognition site for Nt.BbvCI is generated upon the formation of a dsDNA, triggering the nicking enzyme-mediated recycling cleavage of MBs. As a result, the Pb^{2+}-mediated cleavage was efficiently and specifically converted into the amplification of the fluorescence signal, significantly enhancing the sensitivity of Pb^{2+} detection.

2 Experimental

2.1 Instruments and reagents

The sterization were executed by SX-500 automatic high-pressure steam sterilizing pot (Tomy Digitial Biology, Japan). The reaction was carried out in HH-2-type digital constant temperature water bath (Ronghua Instrument, Jiangsu, China). Fluorescence measurements were performed on FluoroMax-4 fluorescence spectrometer (Horiba Jobin Yvon, Longjumeau, France). The emission spectra were collected from 505 nm to 580 nm with an excitation wavelength of 495 nm at room temperature. The fluorescence intensity at 518 nm was chosen as the optimal experimental conditions to evaluate the performance of the proposed sensing system. Both the excitation and emission bandpass widths were set at 5 nm.

Oligonucleotides used in this study were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd (Shanghai, China). Their sequences were shown as follows:

8-17E DNAzyme:
5'-CATCTCTTCTCCGAGCCGGTCGAAATAGGCCTCA-3'

Substrate: 5'-CGCTGAGGCCTAT***rA***GGAAGAGATG-3'

Molecular Beacon:
5'-FAM-CACGCCACGAGTTATAGGCCTCAGCGGGCGTG-DABCYL-3'

The underlined letters in Substrate and Molecular Beacon represent the recognition site of Nt.BbvCI.

$Pb(NO_3)_2$ and other metal salts ($Hg(NO_3)_2$, $Cd(NO_3)_2$, NaCl, KCl, $MgCl_2$, $CaCl_2$, $AlCl_3$, $FeCl_2$, $FeCl_3$, $ZnCl_2$, $CrCl_3$, $CoCl_2$, and $SnCl_2$) of analytical-reagent grade were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Nt.BbvCI was purchased from New England Biolabs Ltd (Beijing China). The DEPC-treated Milli-Q water (resistance > 18.2 MΩ) used for solution preparation and reaction was RNase free.

2.2 Sample pretreatments

All of the laboratory glassware were cleaned with DEPC-treated Milli-Q water and dried at 150 ^{o}C for 5 h before use. Other supplies such as Eppendorf tubes were pretreated by high-pressure sterization at 121 ^{o}C. All metal ions solutions were prepared and diluted with DEPC-treated Milli-Q water.

2.3 Amplified fluorescence detection of Pb^{2+}

The Pb^{2+} detection was performed in the solution prepared by mixing 2 μL 8-17E DNAzyme (2 μM), 2 μL substrate (2 μM), 20 μL 10 × NEB buffer 4 (200 mM Tris-Ac, 100 mM $Mg(Ac)_2$, 500 mM KAc, 10 mM DTT, pH 7.9) and then diluting with DEPC-treated Milli-Q water to make the total volume of 195 μL. Then, 2 μL Pb^{2+} of different concentrations were added and the solution was incubated at room temperature for 15 min. Subsequently, 1 μL 8 U Nt.BbvCI and 2 μL MB probes (10 μM) were added into the solution (the

final concentrations of Pb^{2+} were 0.0, 1.0×10^{-10}, 2.0×10^{-10}, 4.0×10^{-10}, 8.0×10^{-10}, 1.6×10^{-9}, 3.2×10^{-9}, 6.4×10^{-9}, 1.3×10^{-8}, 2.5×10^{-8}, 5.0×10^{-8}, 1.0×10^{-7}, 2.0×10^{-7}, 4.0×10^{-7}, 8.0×10^{-7} and 1.6×10^{-6} M). After incubating for 30 min at 37 °C, the samples were measured by fluorescence spectrometer.

For the investigation of selectivity, the same protocol was used by adding 2 μL Zn^{2+} or other metal ions into the 195 μL solution above, respectively. Subsequently, 1 μL 8 U Nt.BbvCI and 2 μL MB probes (10 μM) were added into the solution (the final concentration of Zn^{2+} and each other metal ions were 2.0×10^{-8} and 5.0×10^{-8} M, respectively). The river water samples were firstly filtered through a 0.2 μm membrane to remove the insoluble substance, and then the target Pb^{2+} were spiked into the river water samples with a series of concentrations. Finally, 2 μL of the samples was added into the 195 μL solution (the final concentrations of Pb^{2+} were 5.0×10^{-9}, 2.0×10^{-8} and 1.0×10^{-7} M).

3 Results and discussion

3.1 Principle of amplified fluorescence sensing system for detection of Pb^{2+}

The principle of the cyclic amplified fluorescence detection of Pb^{2+} is shown in Fig.1. The single-stranded recognition sequence of Nt.BbvCI (3' ... GGAGTCG ... 5') is introduced in the 5' end of the substrate strand, while the loop sequence of MB is designed to be complementary with the sequence at the 5' end of substrate. The 3-base overhang at the 5' end of substrate will effectively impel the competing hybridization between MB and the substrate strand, affording a low fluorescence background. In the presence of Pb^{2+}, the cleavage reaction is catalyzed to cut this substrate into two portions. Due to the low Tm values, the two portions will be separated into two independent ssDNA fragments at the reaction temperature (37 °C). Subsequently, the portions with 3-base overhang at the 5' end can hybridize with the MBs in accordance with the ratio of 1:1 and form the double-stranded recognition site for Nt.BbvCI, resulting in the spatial separation of the FAM at the 5' end of the MB, and DABCYL modified at the 3' end of the MB generating the detectable fluorescence signal. At the same time, MB is nicked by Nt.BbvCI, the cleaved strands are dissociated from the sensing system, and the fluorescence signal is completely restored because of the spontaneous separation of FAM and DABCYL. And then the released substrate portion hybridizes with another MB and triggers the second cycle of cleavage. Finally, per Pb^{2+}-mediated cleavage event can go through the cleavage of many MBs (enzymatic recycling amplification), thereby providing a dramatically amplified fluorescence signal for target Pb^{2+}. In contrast, in the absence of Pb^{2+} ions, 8-17E DNAzyme only forms a complex with its substrate and cannot hybridized with MB, which prevents the formation of double-stranded recognition site for Nt.BbvCI. Thus, no fluorescence signal amplification is observed.

3.2 Feasibility verification

As shown in Fig.2, in order to estimate the amplification efficiency of the proposed sensing system, Pb^{2+}-triggered fluorescence enhancement in the presence (a and c) and absence (b and d) of Nt.BbvCI were recorded, respectively. It can be seen that a slightly increased background fluorescence in the presence of Nt.BbvCI was observed (Fig.2c), which might be caused by the partially hybridization of the substrate strand with MB as well as subsequent cleavage of some MBs in the absence of target Pb^{2+}. However, upon addition of 2.0×10^{-7} M Pb^{2+}, an obvious fluorescence signal increase (502%) was observed (Fig.2a). In contrast, in the absence of Nt.BbvCI, the corresponding signal enhancement was only 284%. The results show that the proposed sensing strategy could provide a significantly amplified signal for the detection of Pb^{2+} by nicking enzyme-mediated enzymatic recycling amplification.

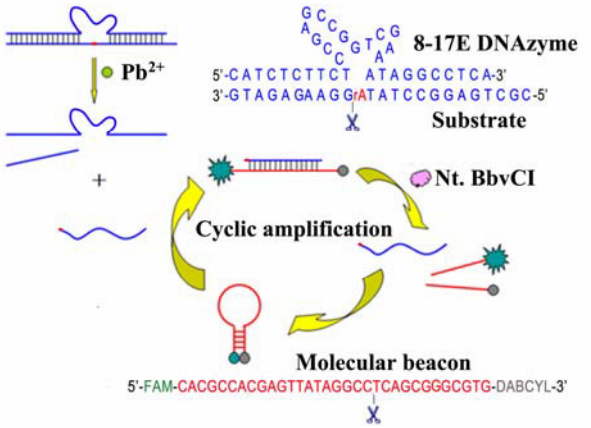

Fig.1 Schematic diagram of cyclic amplified fluorescence detection of Pb^{2+} based on Pb^{2+}-dependent DNAzyme and nicking endonuclease

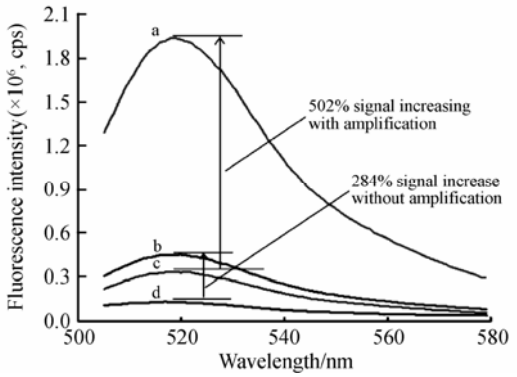

Fig.2 Fluorescence responses of sensing system to 2.0×10^{-7} M of target Pb^{2+} in the presence (a) and absence of nicking enzyme (b), with corresponding backgrounds (c and d)

a. DNAzyme + substrate + MB + Nt.BbvCI + Pb^{2+}; b. DNAzyme + substrate + MB + Pb^{2+} (2.0×10^{-7} M); c. DNAzyme + substrate + MB + Nt.BbvCI; d. DNAzyme + substrate + MB

3.3 Optimization of experimental condition

The full hybridization equilibrium between 8-17E DNAzyme and its substrate is a key factor for ensuring efficient signal amplification. In order to achieve the best performance of this Pb^{2+} sensing system, the effect of 8-17E DNAzyme to its substrate was examined using a fixed concentration of 8-17E DNAzyme and Pb^{2+}, namely 2.0×10^{-8} and 2.0×10^{-7} M, and various concentrations of the substrate (1.0×10^{-8}, 1.5×10^{-8} and 2.0×10^{-8} M). The results of these optimization experiments are shown in Fig.3. It was observed that with the increasing concentrations of substrate, 2.0×10^{-7} M of Pb^{2+} produced a corresponding increase in fluorescence signal intensity. Therefore, the endonuclease activity of Nt.BbvCI on the MB was greatly inhibited, leading to only a slight increase in the intensity of background fluorescence signal. Finally, owing to the maximal fluorescence enhancement ($(F - F_0)/F_0$), the molar ratio of 8-17E DNAzyme to its substrate of 4:3 was selected for the subsequent investigation.

3.4 Quantitative analysis of Pb^{2+}

Based on the optimized ratio of 8-17E DNAzyme to substrate, the sensitivity of this proposed sensing system was evaluated by varying the concentration of Pb^{2+} and measuring the corresponding fluorescence intensity at 518 nm. Figure 4A illustrates the relationship between fluorescence enhancement and the different concentrations of target Pb^{2+} (0.0, 1.0×10^{-10}, 2.0×10^{-10}, 4.0×10^{-10}, 8.0×10^{-10}, 1.6×10^{-9}, 3.2×10^{-9}, 6.4×10^{-9}, 1.3×10^{-8}, 2.5×10^{-8}, 5.0×10^{-8}, 1.0×10^{-7}, 2.0×10^{-7}, 4.0×10^{-7}, 8.0×10^{-7} and 1.6×10^{-6} M). Figure 4B shows that the linear relationship between fluorescence enhancement and the logarithm of the concentrations of Pb^{2+} is from 8.0×10^{-10} to 1.0×10^{-7} M. This linear relationship can be described as $Y = 1.745\log[Pb^{2+}] + 1.386$ with a correlation coefficient of $R^2 = 0.9989$, where Y is the fluorescence enhancement, $[Pb^{2+}]$ represents the concentration of Pb^{2+}. And the value of relative standard deviations (RSD) of each concentration of Pb^{2+} in the linear range was below 3% for three repeated measurements, indicating its good reproducibility. Moreover, the detection limit of Pb^{2+} was estimated at 1.0×10^{-10} M from three times of the standard deviation corresponding to the background fluorescence signal, which was much more sensitive than that of Pb^{2+} fluorescence sensors previously reported[8–10].

3.5 Investigation of specificity

In addtion to the sensing sensitivity, selectivity is another vital factor to evaluate the performance of a sensing system. The specificity of the sensing strategy was investigated by comparing the fluorescence response of 1.0×10^{-8} M Pb^{2+} to that of 2.0×10^{-8} M Zn^{2+} and 5.0×10^{-8} M other metal ions such as Na^+, Mg^{2+}, K^+, Ca^{2+}, Sn^{2+}, Fe^{3+}, Fe^{2+}, Cd^{2+}, Al^{3+}, Cr^{3+}, Hg^{2+} and Co^{2+}. These ions are the most common metal ions present in real samples. As shown in Fig.5, except for a little fluorescence enhancement generated by Zn^{2+} and Cd^{2+}, the other interferential metal ions exhibited almost the same fluorescence response as the blank solution without Pb^{2+} (data not shown) and did not induce any obvious fluorescence signal enhancement. The results demonstrated that the developed sensing strategy exhibited satisfied selectivity for Pb^{2+}, which was attributed to the high specificity of 8-17E DNAzyme toward Pb^{2+}. To further eliminate the interference of Zn^{2+} and Cd^{2+}, we will pay more attention on the GR-5 DNAzyme that can dramatically improve the specificity to Pb^{2+} than 8-17E DNAzyme[22] for future study.

It is well-known that the practical applicability of a sensing system in real or complex samples is very important. To further verify the feasibility of this proposed Pb^{2+} sensing system in practical use, we tested the Pb^{2+} in Xi'an Chan River with a variety of interferences from different locations. The river water samples were simply filtered through a 0.2 μm membrane to remove the insoluble substance, and then spiked with target Pb^{2+} at three different final concentration levels of 5.0×10^{-9}, 2.0×10^{-8} and 1.0×10^{-7} M. As shown in Table 1, the recoveries of Pb^{2+} of the three concentrations were from 96.1% to 108%. The results indicates that the proposed sensing system allows the detection of Pb^{2+} in real samples without obvious interference from other potentially coexisting metal ions.

In summary, a fluorescence sensing strategy for sensitive and selective detection of Pb^{2+} in aqueous solution was developed in this study. The approach took advantages of the high specificity of 8-17E DNAzyme toward Pb^2 and the powerful signal amplification capability of nicking enzyme-mediated recycling cleavage of MBs. By exploiting the strategy, an impressive detection limit as low as 1.0×10^{-10} M

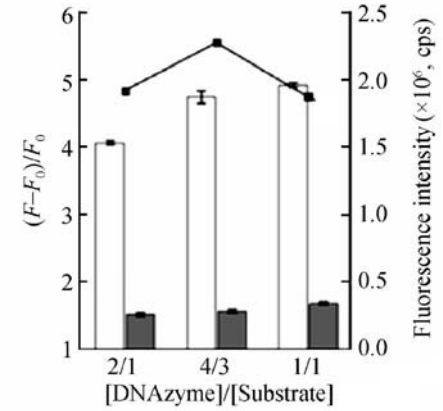

Fig.3 Effect of ratio of DNAzyme to substrate on amplified fluorescence detection of 2.0×10^{-7} M Pb^{2+}

White and gray bars represent the fluorescence intensity at 518 nm in the presence (F) and absence (F_0) of 2.0×10^{-7} M Pb^{2+}, respectively. The curve represents the fluorescence enhancement ($(F - F_0)/F_0$) of Pb^{2+} compared with background fluorescence intensity

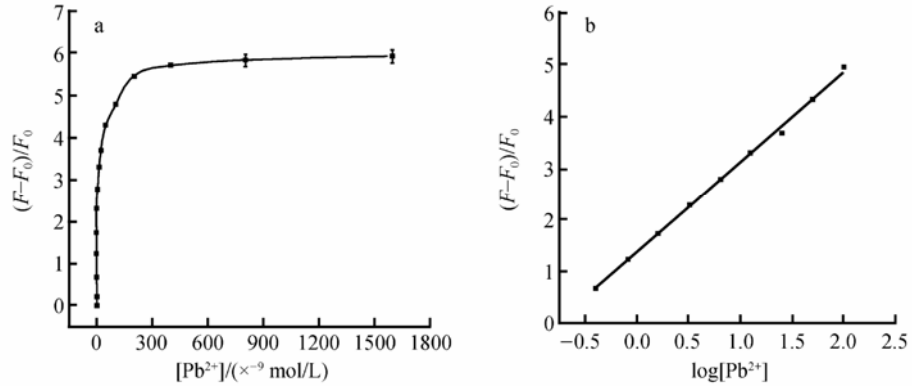

Fig.4 (A) Relationship between fluorescence enhancement and the concentrations of Pb^{2+} and (B) linear relationship between fluorescence enhancement and the logarithm of the concentrations of Pb^{2+}

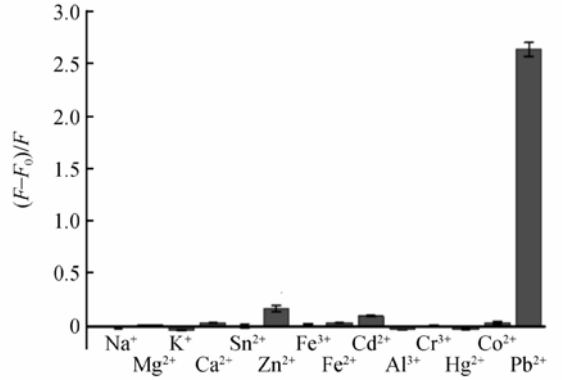

Fig.5 Selectivity of sensing strategy for Pb^{2+}detection over other common interference metal ions

Concentration of Pb^{2+} and Zn^{2+} is 1.0×10^{-8} and 2.0×10^{-8} M, respectively. Concentration of other metal ions is 5.0×10^{-8} M each. Error bars show the standard deviation of three experiments

Table 1 Recovery of Pb^{2+} detection in Xi'an Chan River water samples

Sample No.	Pb^{2+} spiked (nM)	Pb^{2+} recovered (nM)	Recovery (%)
1	5.0	5.4 ± 0.3	108.0
2	20.0	21.3 ± 2.2	106.5
3	100.0	96.1 ± 5.6	96.1

was achieved, which was much lower than those of previously reported Pb^{2+} fluorescence sensor. At the same time, the proposed sensing system was able to readily discriminate Pb^{2+} from other interference metal ions and even detect the target in complex river water samples. In addition, it was worthwhile to point out that the no modification is required for 8-17E DNAzym and its substrate. Given simple operations, rapid analysis time, excellent sensitvity and seltectivity, the approach might be implemented for many on-site Pb^{2+} sensing applications, ranging from environmental samples analysis to biological monitoring.

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