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ORIGINAL PAPER

Bienzyme-based visual and spectrophotometric aptamer assay for quantitation of nanomolar levels of mercury(II)

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Abstract The article describes a bienzyme visual system for aptamer-based assay of Hg(II) at nanomolar levels. The detection scheme is based on the finding that Hg(II) ions captured by aptamer-functionalized magnetic beads are capable of inhibiting the enzymatic activity of uricase and thus affect the formation of H_2O_2 and the blue product, i.e., oxidized tetramethylbenzidine. This strategy allows for a visual detection of Hg(II) at nanomolar levels without additional amplification procedure. Measuring the absorbance at 650 nm, the logarithmic calibration plot is linear in the concentration range of 0.5–50 nM and the limit of detection (LOD) is 0.15 nM. This is as low as the LOD obtained by atomic fluorescence spectrometry (AFS). The ions K^+, Mg^{2+}, Na^+, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Co^{2+}, AsO_2^-, Ni^{2+}, Cd^{2+} and Pb^{2+} do not have a significant effect on color formation. The method was applied to the analysis of (spiked) river water, lake water, mineral water, tap water and certified reference water samples, and the results agreed well with those obtained by AFS or certified values, with recoveries ranging from 97% to 109%. The relative standard deviation for five parallel detections at a 10 nM Hg(II) level is 5.2%.

Keywords Visual assay · Uricase · Peroxidase · Enzyme inhibition · Hydrogen peroxide · AFS · Water analysis · Tetramethylbenzidine

Introduction

Hg^{2+} can result in DNA mutation, disruption of biological events at the cellular level, damage of the liver and kidney, and even death, and thus it was considered to be an important environmental pollutant. The upper limit of Hg^{2+} mandated by United States Environmental Protection Agency (EPA) guidelines is 10 nM (2 ppb) in drinking water [1]. Additionally, indirect exposure caused by eating Hg^{2+}-tainted fish or other aquatic products has also been considered as a common route that leads to the toxic effects of Hg^{2+}. Therefore, it is highly desirable to develop a sensitive and selective Hg^{2+} detection method that can provide simple, practical, and high-throughput routine determination of trace levels of Hg^{2+} ions in water samples.

Currently, the widely used methods for Hg^{2+} detection are atomic spectrometry-based approaches, such as atomic absorption spectroscopy, cold vapor generation atomic fluorescence spectrometry (CVG-AFS), and inductively coupled plasma atomic emission spectrometry (ICP-OES) and mass spectrometry (MS) [2]. These methods, although offered the advantages of high accuracy and selectivity, required sophisticated and expensive instrumentation and skilled personnel, which are inappropriate for point-of-use applications. To overcome these drawbacks, much effort has been devoted towards the design of a variety of sensing systems, such as organic chromophores or fluorophores [3, 4], conjugated polymers [5], gold or silver nanoparticles [6–9], upconverting nanoparticles [10], magnetic fluorescence probe [11], etc. for detection of Hg^{2+} ions. However, most of these methods suffered

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from limitations such as poor selectivity with interference from closely related metals, insufficient sensitivity, etc.

Aptamer-assay has been considered as a new emerging approach for selective detection of mercury because it can specifically interact with thymine bases to form strong and stable thymine-Hg^{2+}-thymine complexes (T-Hg^{2+}-T) [12]. The high stability of T-Hg^{2+}-T base pair have boosted a large number of fluorescent [13, 14], chemiluminescent [15, 16], electrochemical [17, 18] and colorimetric assays [19, 20]. Among these Hg^{2+} sensors, colorimetric aptasenosors have attracted particularly much attention for point-of-use applications, since the target recognition event can be determined visually. The reported colorimetric method for Hg^{2+} detection is mainly based on gold nanoparticles (AuNps), since the color was readily changed by aggregation or deaggregation of AuNps during the target recognition [20–24]. The visual process was can be also realized by inhibition of the G-quadraplex DNAzyme function via T-Hg^{2+}-T [19, 25]. Besides, we also tried to use photocatalytic oxidation of TMB to visual assay of Hg^{2+} [26]. Although these colorimetric Hg^{2+} sensors showed the obvious advantage of simplicity, it is difficult for them to distinguish the color change of 10 nM Hg^{2+} (upper limit of Hg^{2+} in drinking water) except the use of an extra amplification step [24, 27].

Bienzyme reaction system has attracted much attention because the substrate of the latter enzymatic reaction can be produced on-line by the former enzymatic reaction [28, 29]. By utilizing the efficient bienzyme reaction system, we also have developed ultrasensitive chemiluminescence resonance energy transfer (CRET) biosensor for detection of glucose, cholesterol, and benzylamine [30]. Thus, the use of bienzyme catalytic coloration is also expected to be a promising visual detection scheme for aptamer-assay. We found the coloration of the bienzyme (i.e., uricase and HRP)-TMB system to be inhibited by Hg^{2+}. This effect can be used for aptamer-based assay of Hg^{2+} at nanomolar levels without an extra amplification (Fig. 1).

Experimental

Reagents

3,3′,5,5′-Tetramethylbenzidine (TMB), uric acid and urea-formaldehyde magnetic microspheres (10 mg·ml^{-1}, 1–2 μm in diameter) were purchased from Aladdin (Shanghai, China, www.aladdin-e.com). Sodium hydroxide, hydrochloric acid, dimethyl sulfoxide (DMSO) and phosphate (KH_2PO_4) were obtained from Kelong Reagent Co. (Chengdu, China, kelonghg.51pla.com). Uricase and horseradish peroxidase(HRP) were provided by Sangon Biotech (Shanghai, China, www.sangon.com). Mercury standard sample (GSBZ50016–90) was obtained from National Research Center for Standard Materials (Beijing, China, www.ncatn.com). The Oligonucleotides (5′-NH_2-TTCTTTCTTCCCCTTGTTTGTT-3′) for recognition of Hg^{2+} were also provided by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China, www.sangon.com).

Preparation of aptamer-functionalized magnetic beads

First, 200 μL of magnetic beads (MBs) were diluted to 1 mL in phosphate buffer. 100 μL of 100 μM amine modified Hg^{2+} aptamer was added to the diluted MBs and vortexed briefly. Ten milligrams of EDC was then added to the MBs/aptamer and vortexed for overnight. The particles were then washed three times using the phosphate buffer, and resuspended in 2 mL of the phosphate buffer. The aptamer-functionalized magnetic beads (AFMBs) were stored at 4 °C prior to use.

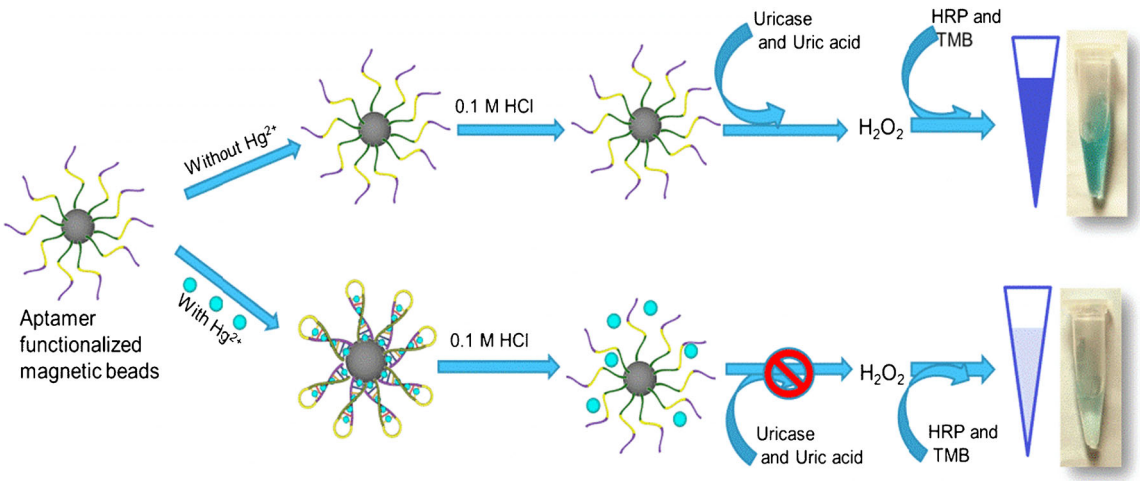

Fig. 1 Principle of bienzyme-based visual assay of mercury by aptamer

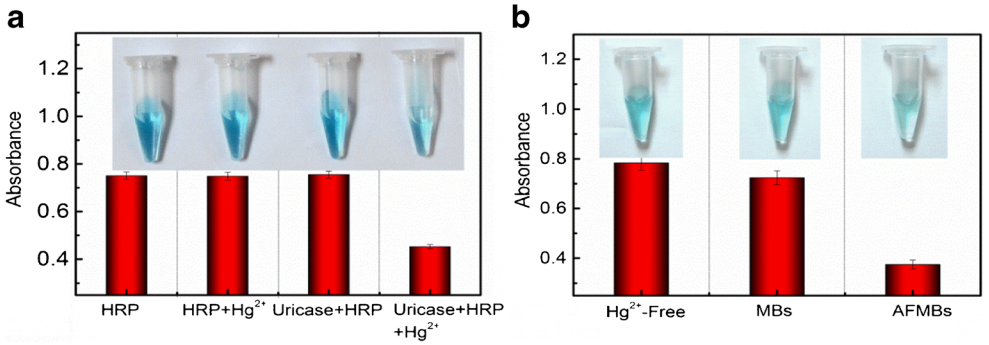

Fig. 2 **a** The inhibition of uricase enzymatic activity by Hg2+; and **b** the color read-out of bienzyme-based visual assay. Experiment conditions: **a** Hg2+ concentration, 10 nM; solution pH, 4.5; H_2O_2 generation time, 20 min; Uric acid amount, 6.72 μg; TMB concentration: 0.01 mg·mL^{-1}; and **b** sample solution pH, 7.0; amount of AFMBs, 40 μg; Hg2+ capture time, 10 min; and other conditions were the same as in **a**

Analytical protocol

40 μL of 40 μg·mL^{-1} AFMBs were added to 1.5 mL of Hg^{2+} standard solutions or samples and incubated for 60 min to recognition of Hg^{2+}; then, the AFMBS were deposited with a magnet and washed twice by phosphate buffer; the Hg^{2+} ions were desorbed by addition of 100 μL 0.1 M HCl; after 20 min, 330 μL of 0.08 mg·mL^{-1} uricase in phosphate buffer (0.1 M, pH 7.0) were added and the enzymatic activity of uricase was inhibited for 30 min by desorbed Hg^{2+}; 40 μL Uric acid (0.168 mg·mL^{-1} or 1.68 U·mL^{-1}) were added to the solution for generation of H_2O_2 under the catalysis of the uninhibited uricase; the 160 μL of 0.1 M HCl, 40 μL of 0.8 μg·mL^{-1} (or 0.2 U·mL^{-1}) HRP and 30 μL of 0.1 mg·mL^{-1} TMB were added for coloration. The absorbance was measured at 650 nm. Here, it is worthy of noting that the uricase solution should be freshly prepared daily.

Results and discussion

Design of bienzyme visual aptamer-assay for detection Hg^{2+}

At first, monoenzyme, i.e., HRP was used to catalyze the coloration of TMB. However, the HRP catalytic activity did not changed in the present of Hg^{2+} (Fig. 2a), and thus was unable to be applied for the visual readout of Hg^{2+}. Further experiments showed that Hg^{2+} ions in 10 nM concentration inhibit the enzymatic activity of uricase. This effect leads to a retarded rate of H_2O_2 formation in the presence of uricase substrate and slower rate of the chromogenic reaction of TMB as can be seen in Fig. 2a. Thus, the uricase-HRP-TMB system can be used for visual assay of Hg^{2+}. It has been proved that Hg^{2+} was be readily react with –SH or $–NH_2$ contained compounds [31]. Hence, we infer that Hg^{2+} inhibited the catalytic activity of uricase via interacting with –SH or $–NH_2$ in amino acid residues, but the specific interaction mechanism remains to be further investigated.

Figure 2b shows that the Hg^{2+} ions specifically captured and separated by AFMBs inhibit the coloration of the system; the small amount of Hg^{2+} ions adsorbed by MBs, in contrast, lead to a much lesser inhibition. This bienzyme-based aptamer assay possessed the advantages of high selectivity (aptamer recognition) and satisfying sensitivity (signal amplified by bienzyme).

Parameters affecting visual assay of Hg^{2+}

The following parameters were optimized: (a) time for H_2O_2 generation; (b) sample pH value; (c) amount of AFMB; (d) time for capturing Hg^{2+} and (e) inhibition time by Hg^{2+}. Respective data and Figures are given in the Electronic Supporting Material (Figs. S1–S5). We found the following

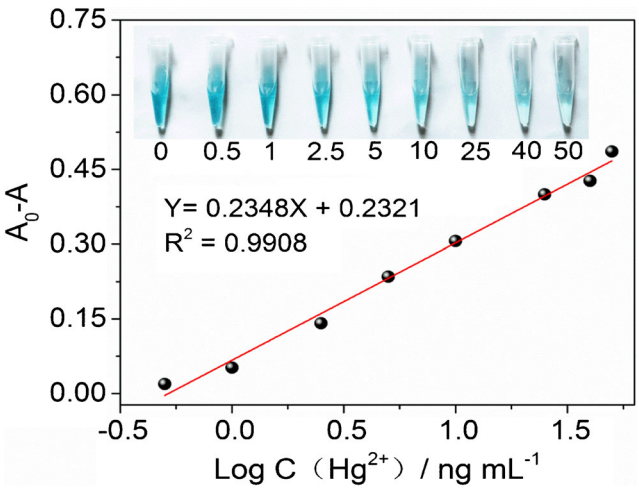

Fig. 3 The visual performance and linearity of bienzyme-AFMBs system for detection of Hg^{2+}. Experiment conditions: H_2O_2 generation time, 20 min; Uric acid amount, 6.72 μg; TMB concentration, 0.01 mg·mL^{-1}; sample solution pH, 7.0; amount of AFMBs, 40 μg; and Hg^{2+} capture time, 60 min; analytical wavelength, 650 nm

Table 1 Comparison of colorimetric aptamer-assay and atomic spectrometry for detection of Hg^{2+}

Strategy	Label	Amplification/ enrichment	Detection limit (nM)	Ref.
Bienzyme-based coloration			0.15	This work
G-quadruplex-basedDNAzyme			100	[19]
AuNPs& conjugated polyelectrolyte			50	[21]
G-quadruplex-basedDNAzyme			50	[25]
AuNPs			250	[32]
AuNps			0.6	[22]
Methylene Blue		hybridization chain reaction	0.7	[26]
Au NPs & LFSB	Labeled by digoxin, biotin or Au NPs	Exonuclease III-assisted signal amplification	0.001	[24]
CVG-AFS			0.025	[33]

LFSB lateral flow strip biocomponent, *CVG* chemical vapor generation, *AFS* atomic spectrometry

experimental conditions to give best results: (a) 30 min for H_2O_2 generation; (b) sample pH value of 7.0; (c) 40 μg of AFMB; (d) 60 min for capturing Hg^{2+}; and 30 min for inhibition time by Hg^{2+}.

Analytical performance of aptamer-assay for detection of Hg^{2+}

A series of Hg^{2+} standard solutions were captured by AFMBs, and then detected by bienzyme-TMB coloration system. The aptamer-assay system permitted color discrimination with a minimal concentration of 2.5 nM (inset of Fig. 3). It should be noted that the toxic level for Hg^{2+} defined by the US Environmental Protection Agency in drinkable water is below 10 nM. Hence, the resultant color change enables a differentiation between target-containing and target-free samples via visual inspection. Further using spectrophotometry, Fig. 3 shows that the absorbance increases linearly with the logarithm of Hg^{2+} concentration in the range of 0.5–50 nM, and the limit of detection (3σ) can be calculated to be 0.15 nM (about 0.03 ng·mL^{-1}). Table 1 shows that this approach is much more sensitive than the reported colorimetric aptamer-assay without an amplification/enrichment procedure and even comparable to AFS methods. Besides, this assay eliminates the tedious procedure of labeling. The reproducibility was also examined using 10 nM of Hg^{2+}, and the relative standard deviation (RSD) for five parallel detections was 5.2%.

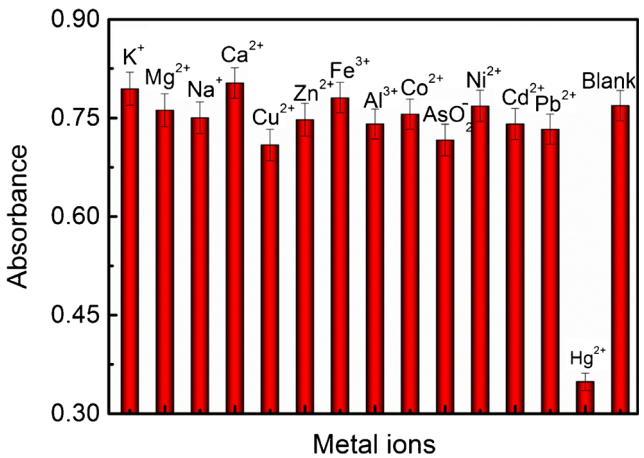

Fig. 4 The specificity of the bienzyme-AFMBs system. The experimental conditions were the same as Fig. 3

Interference study

The specificity of the assay was investigated by using other metal ions in place of Hg^{2+}. The potentially interfering ions such as K^+, Mg^{2+}, Na^+, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Co^{2+}, AsO_2^-, Ni^{2+}, Cd^{2+} and Pb^{2+} were used at concentrations of 1000 nM, and the concentration of Hg^{2+} was chosen to be 10 nM (100 times lower than the interfering ions). Hg^{2+} (10 nM) led to an obvious absorbance decrease (more than 50%), while other metallic ions (1000 nM) had no significant effects (Fig. 4 and Fig. S6). It demonstrated good specificity of this bienzyme-based assay, which has more selective for the recently reported aptamer-assay for Hg^{2+} detection [34].

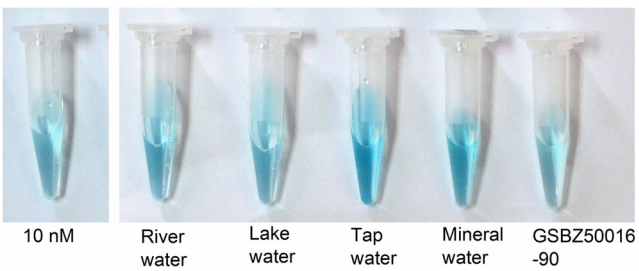

Fig. 5 The pictures of analyzing water samples by the visual assay. The experimental conditions were the same as Fig. 3

Table 2 Analytical results of real samples by the visual assay

Sample	This assay (nM)	AFS (nM)
River water	<10	3.95 ± 0.27
Lake water	<10	4.25 ± 0.39
Tap	<10	0.31 ± 0.04
Mineral	<10	0.35 ± 0.05
GSBZ50016–90[a]	>10	25.10 ± 2.55

[a] certified value

Sample analysis

To estimate its real application, this assay was applied for analysis of various water samples, i.e., river water, lake water, mineral water, tap water and certified water (GSBZ50016–90) samples, and the color change for these samples were shown in Fig. 5. The Hg^{2+} concentrations of mineral, tap, lake and river waters were found to be <10 nM (the toxic level for Hg^{2+} defined by the EPA in drinkable water) and the Hg^{2+} concentration in certified water sample (GSBZ50016–90) was higher than 10 nM (Table 2). The results coincided with those obtained by AFS or certified value.

Using spectrometry, a more quantitative analysis of Hg^{2+}can be made, and the results were in good agreement with those obtained by AFS or certified value (Table S1). The recoveries for the river water, lake water, mineral water, tap water were in the range of 97–109% (Table S2). These results indicated that this system might be a promising tool for fast and convenient detection of Hg^{2+} in water samples.

Conclusion

We have developed a bienzyme aptamer-assay for ultrasensitive visual detection of Hg^{2+} in water samples. The efficient inhibition of uricase activity by Hg^{2+} provided the assay with high sensitivity, allowing detection of Hg^{2+} at nanomolar level without an extra amplification procedure. The capture of Hg^{2+} by AFMBs contributed greatly to the high specificity of the system. As a result, 100-fold of potential coexisting metal ions did not yield obvious interference. Simplicity, high sensitivity and selectivity were the main benefits of our assay. By using aptamers selective for other metal ion, this detection scheme may be applied to ions such as Pb^{2+}, Ag^{+} and the like. Therefore, the bienzyme-AFMBs assay was an appealing tool for fast detection of metal ion pollutants in water samples.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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