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Mercury detection based on label-free and isothermal enzyme-free amplified fluorescence platform

Fanbin Meng [a], Hui Xu [a,*], Xue Yao [a], Xuan Qin [b] Tingting Jiang [c], Shanmin Gao [a], Yahui Zhang [a], Di Yang [a], Xia Liu [a]

[a] *School of Chemistry and Materials Science, Ludong University, Yantai 264025, China*

[b] *School of Materials Science and Engineering, Lanzhou University of Technology, Lanzhou 730050, China*

[c] *School of life sciences, Ludong University, Yantai 264025, China*

*Corresponding authors. *E-mail address*: xuhui235@163.com (H. Xu)

Abstract

A novel and convenient biosensor for Mercury (II) detection was developed based on toehold-mediated strand displacement isothermal enzyme-free amplification (EFA) technology and label-free fluorescence platform using Sybr Green I (SG) and graphene oxide (GO). The method is highly sensitive and selective, and the logarithmically related Hg^{2+} linearity range is from 0.1 nM-50 nM with a detection limit 0.091 nM. Moreover, our strategy is simple, enzyme-free, and inexpensive and can be applied to detect spiked Hg^{2+} in environmental water samples with good recovery and accuracy, which demonstrates that the biosensor has a good potential in the environment surveys in the future.

Keywors: isothermal enzyme-free amplification, label-free fluorescence platform, Hg^{2+}

1. Introduction

Mercury ion is a highly toxic pollutant to human being [1, 2]. Hg^{2+} exposure can lead to severe effects to the brain, immune system, nervous system and many other organs [1, 3]. Mercury contamination could be easily found in surrounding environment such as ambient air, soil, water and even food due to the wide use of mercury and mercuric salts in industry. Therefore, it is indispensible to develop new approaches to detection mercury with great simplicity, high sensitivity and selectivity.

Until now, various methods have been used to detect Hg^{2+}, including atomic absorption/emission spectroscopy[4, 5], inductively coupled plasma mass spectrometry (ICPMS)[6], selective cold vapor atomic fluorescence spectrometry [7, 8], and optical and electrochemical sensing devices [9-12]. Among these, fluorescence measurement is a widely adopted technique due to its simple operation and low cost. To date, several protocols [9-11, 13] have been reported for detection of Hg^{2+} using a mercury specific DNA (MSD) based on T-Hg^{2+}-T (T=thymine) coordination chemistry. The detection limit has dropped to as low as 1.33 nM [11]. However, these strategies were still limited by the relatively high detection limit (1.33 nM). Recently, Cui et al developed a highly sensitive electrochemical method for the detection of Hg^{2+} via single T-Hg^{2+}-T coordination-induced three-way junction of DNA [12]. However, it needs ferrocene labeled electrochemical probes as signal probes. Some enzyme-dependent amplification technologies such as polymerase, DNAzyme,

nicking endonuclease and exonuclease have been widely used for the sensitive detection of metal ions[14-17], small molecules[14, 18-20] and DNA[21-23]. However, polymerase is primer-dependent and DNAzyme is specific metal ion-dependent; nicking endonuclease is sequence-specific, and exonuclease is terminal and direction-dependent. They need particular reaction procedures and are not cheap, limiting their wide applications for signal amplification. In contrast, recently, isothermal enzyme-free amplification (EFA) technology [17, 24-29] has been widely combined with fluorescence and electrochemiluminescence (ECL) detection platform. They were realized by target-triggered circulatory interaction of two hairpin probes, which is inspired by programming biomolecular self-assembly pathways [27, 28]. However, most of them needed luminophore labeled probes as signal probes. Detection of DNA or small molecules using a DNA intercalator is a label-free method. It is simple and cost-effective, which has been widely used recently [11, 30, 31]. Some groups have used DNA intercalator to detect mercury ion based on the formation of T-Hg^{2+}-T structure. On the other hand, label-free strategies combining a DNA intercalating dye and nanomaterial such as graphene oxide (GO)[31] have attracted great interest in the biosensor fields due to the high fluorescence quenching capability of GO and different affinity towards single-strand DNA (ssDNA), hairpin DNA and double-strand DNA (dsDNA) [32, 33]. Inspired by the above researches, a highly sensitive, selective and label-free method for the detection of Hg^{2+} was developed based on toehold-mediated strand displacement isothermal EFA technology, GO and a DNA intercalator Sybr Green I (SG). The

enhanced signals were obtained by the recycling of Hg^{2+}, which is sensitive, simple and cost-effective.

2. Experimental section

2.1. Materials and reagents

All oligonucletides were synthesized and high-performance liquid chromatography (HPLC) purified in Takara Biotechnology Inc, as shown in Table 1. The concentration of DNA was determined by measuring the absorbance at 260 nm in 1 cm quartz cuvette. SG (10,000×) was purchased from Invitrogen Inc. All solutions were prepared and diluted using ultrapure water (18.2 MΩ•cm). All other chemicals were purchased from China National Pharmaceutical Group Corporation as analytical grade and used as received.

2.2. Preparation of probes

All the tubes and cuvettes used were completely cleaned by soaking in 1:1 HNO_3 for 4 hours and then washed using ultrapure water many times in order to get rid of possible contamination from equipment and environment. The standard stock solution of Hg^{2+} was prepared by dissolving mercuric acetate with 0.5 % acetic acid. Various concentrations of Hg^{2+} solutions were obtained by serial dilution of the stock solution with ultrapure water. Two hairpin probes (H1 and H2) were designed according to the principles of isothermal EFA [26-28]. Assistant DNA is complementary to the part of the H1 except several T-T mismatches, which can serve as specific recognition elements for Hg^{2+} binding. The sequences of the two hairpin probes and assistant

DNA are listed in Table 1. Before experiment, both H1 and H2 were denaturized at 95 °C for 5 min and then cooled to the room temperature slowly within two hours, to form a complete hairpin structures. The probes were then stored at 4 °C for further use.

2.3. Enzyme-free amplification

50 nM H1 and 50 nM H2, 10 nM T and different concentrations of Hg^{2+} were added to 20 mM Tris-HAc Buffer (containing 0.75 M $NaNO_3$ and10 mM $Mg(Ac)_2$, pH=7.4) to make a final volume of 600 μL. The mixture was incubated at 37 °C for 2 h for amplification. The incubation temperature was optimized by changing the incubation temperature in the absence or presence of 10 nM Hg^{2+}.

2.4. Polyacrylamide gel electrophoresis (PAGE)

The isothermal EFA products were analyzed by 10 % native PAGE in 1× TBE buffer (90 mM Tris, 90 mM boric acid, 3 mM EDTA, 6.25 mM $Mg(Ac)_2$, pH 8.4). Five microlitre of different samples were loaded and run at 120 V for 80 min at room temperature, and then dyed by Ethidium bromide (EB) and photographed by Biosens SC750 digital imaging system.

2.5. Linearity of detection for Hg^{2+} by GO-based SG fluorescence platform

SG and GO were added to the above mixture with different concentrations of Hg^{2+}, the final concentrations of them are 1× and 5 μg/mL, respectively. After incubation at 37 °C for 10 min, the fluorescence of SG was measured by Perkin-Elmer LS55 luminescence spectrometer (USA). The excitation wavelength was set at 490 nm and

the emission spectra were collected from 500 nm to 600 nm with the excitation and emission slits of 8 nm and 7 nm, respectively. The optimization was performed at constant Hg^{2+} concentration of 0 and 10 nM with different concentrations of salt, SG or GO, respectively.

2.6 Selectivity experiment

The selectivity experiment was studied under the same conditions as Hg^{2+} detection except by using 1 μM other metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Ag^+, Ni^{2+}) instead of 50 nM Hg^{2+}.

2.7 Precision

Every assay was performed for three times and reported as average. Error bar represents the standard deviation of the results.

2.8 Analysis of real environmental water sample

Tap water was obtained from our laboratory. The tap water was collected after discharging tap water for 20 min and boiled for 5 min to remove chlorine. Aliquots of the tap water samples were spiked with different concentrations of Hg^{2+} (10 nM and 50 nM) and diluted 5 times with 20 mM Tric-HAc buffer (0.75 M $NaNO_3$, 10 mM $Mg(Ac)_2$, pH=7.4) containing equivalent amounts hairpin DNA (50 nM) and assisted DNA (10 nM) above mentioned. The other procedures were the same as described above. The spiked samples were then analyzed using the proposed strategies. To evaluate the accuracy the method, the concentration of Hg^{2+} (10 nM and 50 nM) in the spiked water samples was further analyzed by the standard inductively coupled plasma-atomic emission spectrometry (ICP-AES, Shimadu, ICPE-9000) method.

3. Results and discussion

3.1. Design Principle of the Biosensor

The design principle of the biosensor for Hg^{2+} detection is schematically illustrated in Scheme 1. Domains of DNA are named here by numbers and the asterisk of superscript denotes the complementarity between numbered domains (for example, domain 1* is complementary to domain 1). Two hairpin probes (H1 and H2) are carefully designed according to the EFR and the assistant DNA sequence. The domain 1* in the hairpin DNA H1 is complementary to the domain 1 in the assistant DNA except the designed T-T mismatches, which can recognize Hg^{2+} specifically by forming T-Hg-T structure. In the presence of Hg^{2+}, the assistant DNA (domain 1, 2 and 3) can initiate strand displacement reaction to open the hairpin DNA H1 by complementary 1 and 1*, 2 and 2*, and 3 and 3* domains with the help of T-Hg^{2+}-T base pairs. Therefore, Hg^{2+}-triggered toehold binding can lead to the formation of a partially double-stranded structure. H2 contains five domains named as 3*, 4*, 3, 2, 4. Among them, 3*, 4*, 3, and 2 domains are complementary to the 3, 4, 3*, and 2* in hairpin H1. When adding H2, the existence of outshoot 3* in H2 can help open up the hairpin structure [28] to hybridize with the 3, 4, 3*, and 2* in hairpin H1, setting the assistant DNA and target Hg^{2+} free from the H1-H2 complex. Then the assistant DNA and Hg^{2+} can initiate the next cycle. So, one target Hg^{2+} can trigger many cycles of EFA and lead to a large amount of hybridized dsDNA H1-H2 complex. At last, the intercalator SG and GO were added to realize the label-free fluorescence detection. The different affinities of GO to ssDNA, hairpin DNA and dsDNA[32] and the

super-quenching capacity of GO to all kinds of dye [34, 35] lead to different fluorescence intensity in the absence and presence of Hg^{2+}. In the absence of Hg^{2+}, two hairpins DNA representing mainly ssDNA and assistant DNA T were easily adsorbed on the surface of GO and the fluorescence of SG was quenched, which combined with partly dsDNA stem of the hairpin probes. On the contrary, upon addition of Hg^{2+}, the rigid double helix structure H1-H2 complex is not easily adsorbed on the surface of GO, the intercalator SG combined with the dsDNA structure and led to enhanced fluorescence emission.

3.2. Polyacrylamide gel electrophoresis

To confirm the validity of the dual chain displacement strategies, target Hg^{2+} was amplified by our designed EFA system. Hairpin probe H1, H2, negative control and the amplified products were analyzed by 10% PAGE. As shown in Fig. 1, hairpin probe H1, H2, and negative control without Hg^{2+} show bands of their hairpin structures. While for the amplified EFA products, the new bands representing the generation of hybridized dsDNA H1-H2 complex structure are obvious (lane 1, 2, 3), indicating the validity of the amplification system.

3.3. Optimization of the experimental parameters

The experimental parameters such as the concentration of salt, GO or SG, and incubation temperature that could affect the analytical performance and results of the above sensing system were optimized.

Since ionic strength can affect the hybridization of dsDNA, higher ionic strength

will stabilize the hairpin structure, leading to higher specificity but lower sensitivity. In contrast, lower ionic strength makes the dsDNA instable and increases the sensitivity but sacrifices the specificity. In addition, GO/DNA interaction [36, 37] was considered to be closely related to salt concentration. To obtain an appropriate ionic strength, we investigated the effect of different concentrations of $NaNO_3$ in Tris-HAc buffer on the signal to noise ratio of the method (F represents the fluorescence intensity upon addition of 10 nM Hg^{2+}, F0 represents the fluorescence intensity without Hg^{2+}). As indicated in Fig. 2a, the signal to noise ratio first increases with the increase of ionic strength, then keeps at a stable level. Further increase of concentration from 0.75 mol/L to 1.2 mol/L could not get an equivalent increase in the signal to noise ratio. So 0.75 mol/L $NaNO_3$ was chosen as the optimum concentration for the platform.

The concentration of GO is another important factor to affect the signal to noise ratio because GO can eliminate the background fluorescence due to the different absorption capacity of stem-loop hairpin DNA and dsDNA to GO [32]. Whether the fluorescence of H1, H2 and T without Hg^{2+} could be effectively quenched by GO was crucial for the design of the EFA/GO/SG assay. When the concentrations of H1 H2, T and SG were fixed, the quenching efficiency could be strongly influenced by GO concentration. The effect of GO concentration on the fluorescence quenching efficiency was evaluated by setting different concentrations of GO as 0，2，5，7，and 10 ug/mL GO, respectively. We can find from Fig. 2b that the fluorescence quenching efficiency (represented as the ratio of the decreased fluorescence intensity

after addition of GO to the fluorescence intensity before addition of GO) first increases from 0 to 5 μg/mL then keeps almost constant. Further increase of the GO concentration could not lead to a continuous increase of quenching efficiency. So 5 μg/mL GO was selected as the optimum concentration in our detection.

It is well known that temperature influences the binding kinetics between assistant DNA and hairpin DNA and subsequent strand displacement. So the incubation temperature was optimized by detecting 10 nM Hg^{2+} and blank sample at different temperatures (Figure S1 in Supporting Information). The signal to noise ratio first increases with increasing incubation temperature, and then decreases. The optimal incubation temperature is 37 °C.

Other factor taken into account for the parameter optimization is the concentration of SG because it also influences the performance of the developed biosensor. The optimization of SG concentration was illustration in Supporting Information (Figure S2 in Supporting Information). A peak value of signal to noise ratio was obtained at 1× in the range detected. Hence, the optimal concentration of SG is 1×.

3.4. Sensitivity

To demonstrate the performance of this label-free and isothermal enzyme-free amplified fluorescence sensor in the quantitative analysis of Hg^{2+}, the sensitivity analysis was investigated. Several calibration samples with different concentrations of Hg^{2+} (three measurements for each concentration) were analyzed, and fluorescence emission spectra were collected. A significant fluorescence enhancement was observed along with increased concentrations of standard Hg^{2+} in the range of 0.1-50

nM (Fig. 2c). The fluorescence intensity of SG was logarithmically related to the target Hg^{2+} concentration across the range from 0.1 nM to 50 nM (Fig. 2d). The linear regression equation can be expressed as: IF=285.3215+23.5405log [Hg^{2+}] (M) (r = 0.9974). We can find that as low as 0.1 nM Hg^{2+} can produce obvious fluorescence enhancement. In the Hg^{2+} response range of 0.1 to 50 nM, 0.091 nM is the experimentally estimated detection limit based on 3s/Slope (where the s is the standard deviation of blank sample, and Slope is the slope of the linear equation), which excels or is comparable to most of previously reported optical Hg^{2+} detection sensor. The performance of some Hg^{2+} sensors using different detection methods is summarized in Table 2. Most of the selected sensors are based on the T-Hg^{2+}-T chemistry and the other are the recent published new sensors based on all kinds of nanomaterials, which demonstrates that our sensor has very high sensitivity.

3.5. Selectivity

To further evaluate the selectivity of the EFA and fluorescence detection platform, the sensor was interrogated with a spectrum of environmentally relevant metal ions, including K^+, Na^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Ag^+, Ni^{2+}, and Ba^{2+}ions (each 1 μM). As shown in Fig. 3, 50 nM Hg^{2+} could produce a significant fluorescence enhancement, whereas all other metal ions at a concentration of 1 μM only yield similar fluorescence intensity as blank sample. The concentration of other ions was much higher than that of Hg^{2+} solution but produced negligible response, indicating that the EFA and label-free fluorescence platform was highly selective for Hg^{2+} over the other metal ions and may have potential application for analysis of complex

samples.

3.6. Practical application

The analytical reliability and application potential of the proposed method was evaluated by recovery experiments with tap water containing two different concentrations of Hg^{2+} (10 nM and 50 nM). After discharging tap water for 20 min and boiled for 5 min to remove chlorine, the tap water was collected. The water samples were spiked with Hg^{2+} at different concentration levels (10 nM and 50 nM) and then analyzed with the method proposed with three replicates. Recovery from 93% to 108% was obtained (Table S1 in Supporting Information), which confirms that the suspected interfering materials in the tap water did not influence the Hg^{2+} ion detection with the described method. Further, the spiked concentration of Hg^{2+} was analyzed by standard ICP-AES. The results show that there is no obvious difference between the ICP-AES and the proposed method (Table S1 in Supporting Information), indicating that the proposed method has good accuracy.

4. Conclusions

In summary, we proposed a sensitive Hg^{2+} sensor with logarithmically linear range of 0.1 nM-50 nM and low detection limit 0.091 nM by employing superquencher GO, T-Hg^{2+}-T mediated strand displacement and DNA intercalator SG. The method shows several combined advantages. First, this is a label-free approach, and SG could differentiate stem-loop hairpin DNA and dsDNA through preferential intercalation into dsDNA and different adsorption capacity of them to GO. Second, the method is

enzyme-free, which is cost-effective and simple. Third, it is an isothermal amplified method, which does not need high temperature operation. Fourth, under optimized conditions, 0.091 nM Hg^{2+} could be detected.

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Table 1 Sequences of oligonucleotide

DNA	5'-3'
H1	GCT TTT CAA GTC TGA TAA GCT ACC ATG TGT AGA TAG CTT ATC AGA CT
H2	TAA GCT ATC TAC ACA TGG TAG CTT ATC AGA CTC CAT GTG TAG A
Assistant DNAT	TAG CTT ATC AGA CTT GAA TTG C

Table 2 Comparison of methods for the determination of Hg^{2+}

Sensing method	Transducer used	Linear range	Detection limit	reference number
Hg(2+)-triggered toehold binding and exonuclease III-assisted signal amplification	Strip biosensor	1 pM-100 nM	1 pM	[16]
DNA/Nanoparticle Conjugates	Colorimetric	-	1 uM	[38]
DNA-Functionalized Gold Nanoparticles	Colorimetric	0-2 uM	100 nM	[39]
T-Hg^{2+}-T	Fluorescence	0-66.43 nM	1.33 nM	[40]
via single ion-induced three-way junction of DNA	electrochemical	0.005-100 nM	5 pM	[12]
T-Hg^{2+}-T	Fluorescence	0.1-10 nM	0.5 nM	[41]
Cationic porphyrin	Fluorescence	0.1 nM-1 uM	0.1 nM	[42]
Ag nanoparticles	Absorbance	0.2-3 nM	0.2 nM	[43]

GO-based fluorescent sensor by using hybridization chain reactions	Fluorescence	0-1.0 nM,	0.3 nM	[29]
Gold nanostar dimer	SERS spectroscopy	0.002-1 ng/mL	0.8 pg/mL	[44]
Gold nanoparticles	Colorimetric	50-500 nM	30 nM	[45]
Ag nanoparticles	Colorimetric	25-500 nM	17 nM	[46]
double-stranded DNA modified Gold nanoparticles	RRS	2.5-60 nM	0.4 nM	[47]
Conjugated-oligoelectrolyte-substituted POSS and gold nanocluster	FRET	-	0.1 nM	[48]
Gold nanoparticles	Colorimetric	0.1 mM-1 nM	0.6 nM	[49]
Magnetic beads	Electrochemilumine scence	5-250 nM	5 nM	[50]
Quantum dots and gold nanoparticles	TGFRET	1-10 nM	0.49 nM	[51]
label-free and isothermal enzyme-free amplified fluorescence platform	Fluorescence	0.1-50 nM	0.091 nM	Our method

SERS: Surface-enhanced Raman scattering spectroscopy
RRS: Resonance Rayleigh scattering
FRET: Fluorescence resonance energy transfer
TGFRET: Time-gated fluorescence resonance energy transfer

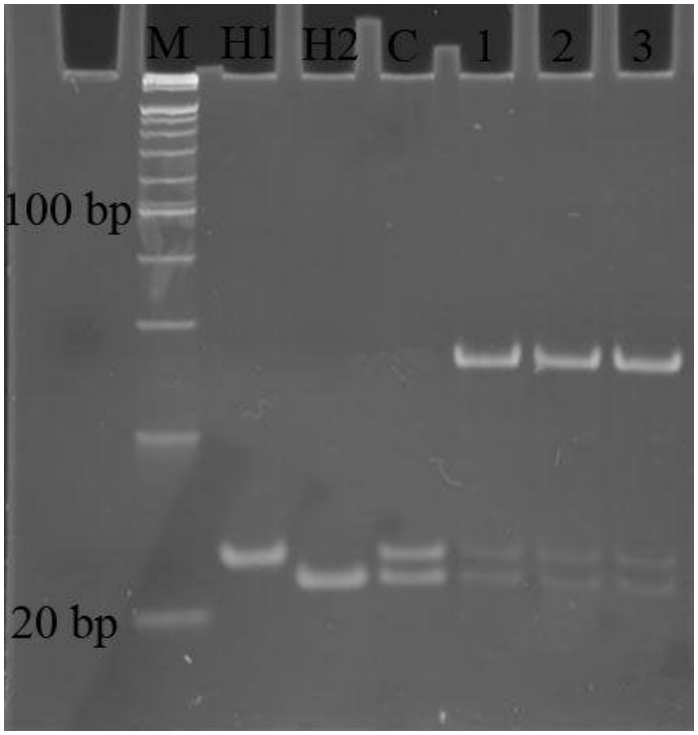
Figure Captions:

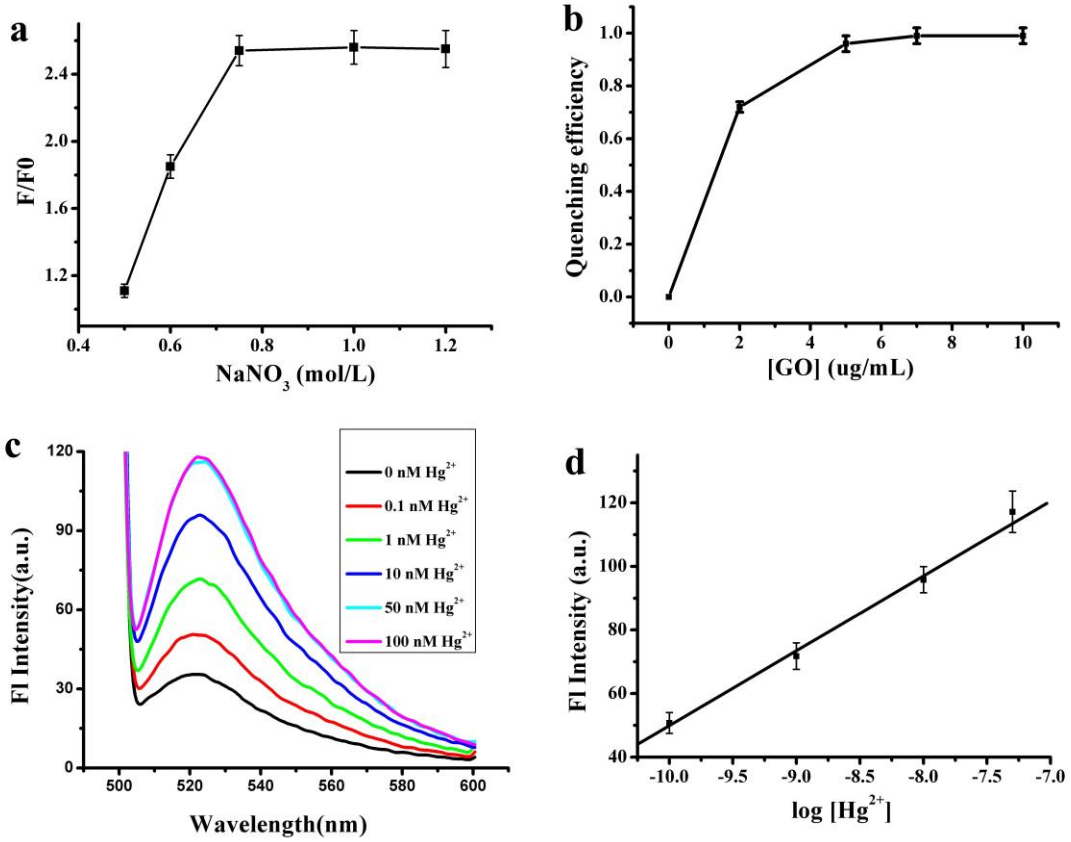
Scheme 1. Schematic illustration of the EFA and GO-based label-free fluorescence platform for the detection of Hg^{2+}

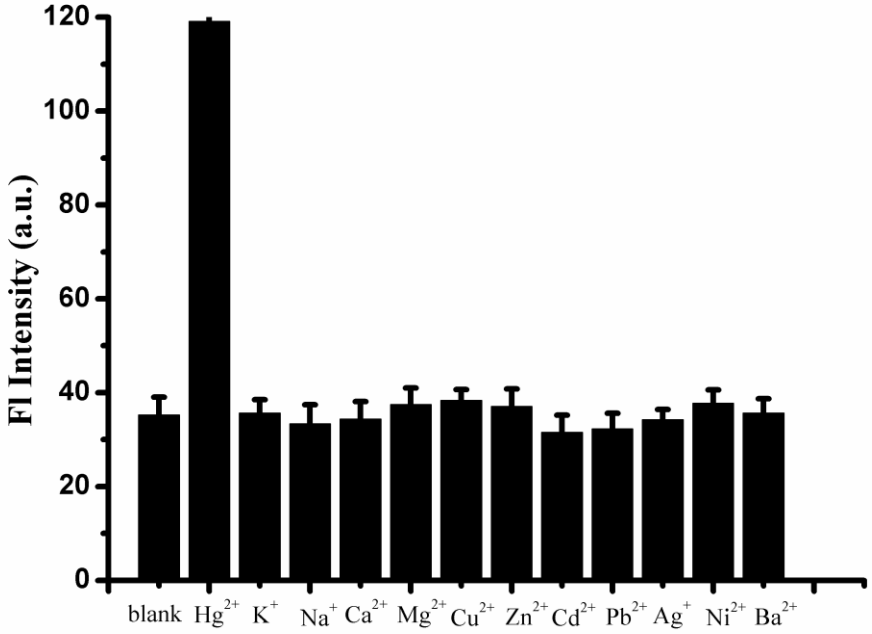
Fig. 1. Polyacrylamide gel electrophoresis of the EFA products initiated by Hg^{2+}. M: DNA ladder, H1: hairpin probe H1 (1 μM), H2: hairpin probe H2 (1 μM), C: negative control without Hg^{2+}(1 μM H1+ 1 μM H2+ 200 nM T_2), 1-3: three paralleled amplification experiments using 100 nM mol/L of Hg^{2+} as target.

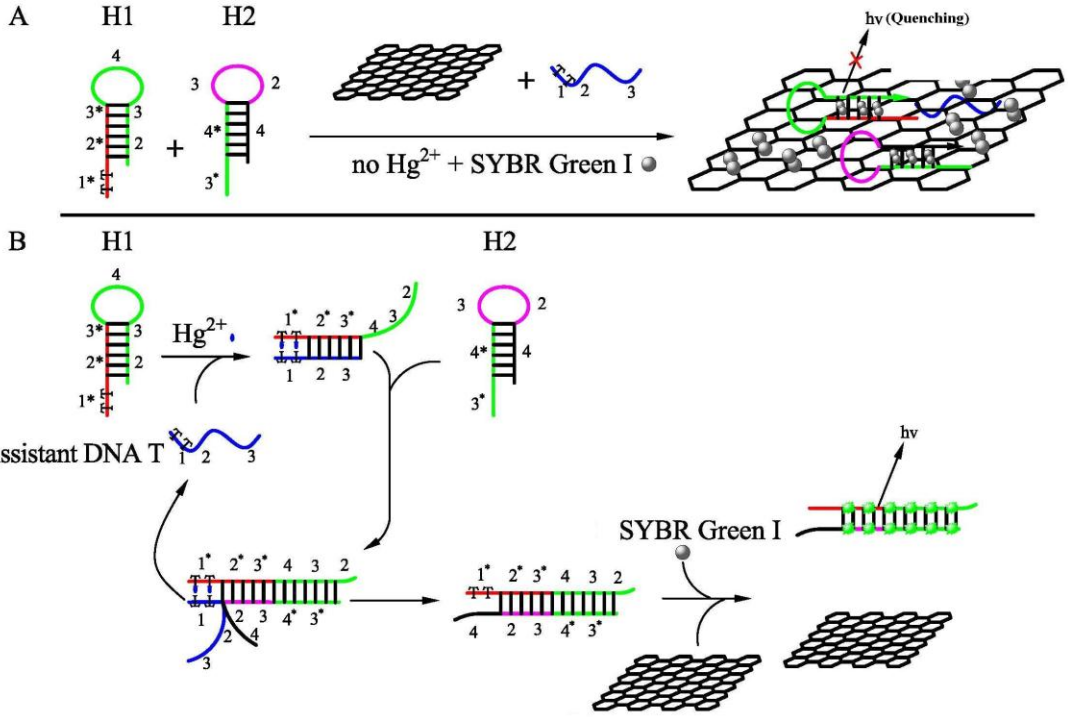
Fig. 2. Optimizations of experimental parameters. (a) Evaluation of the effect of salt concentration (NaNO3) on the F/F0 (F represents the fluorescence intensity upon addition of 10 nM Hg^{2+}, F0 represents the fluorescence intensity without Hg^{2+}). (b) Concentration optimizations of GO. (c) Fluorescence spectroscopy of EFA products from different concentrations of Hg^{2+}. (d) Fluorescence intensity of EFA/GO/SG vs. lg[Hg^{2+}].

Fig. 3. The difference in fluorescence intensity between the blank and solutions containing different ions. [H1]= [H2] =50 nM, [T]= 10 nM, [SG]= 1×, [Hg^{2+}]=50 nM ，[K^+]=[Na^+]=[Ca^{2+}]=[Mg^{2+}]=[Cu^{2+}]=[Zn^{2+}]=[Cd^{2+}]=[Pb^{2+}]=[Ag^+]=[Ni^{2+}]=1 μM. The error bars represent the standard deviation of three independent measurements.

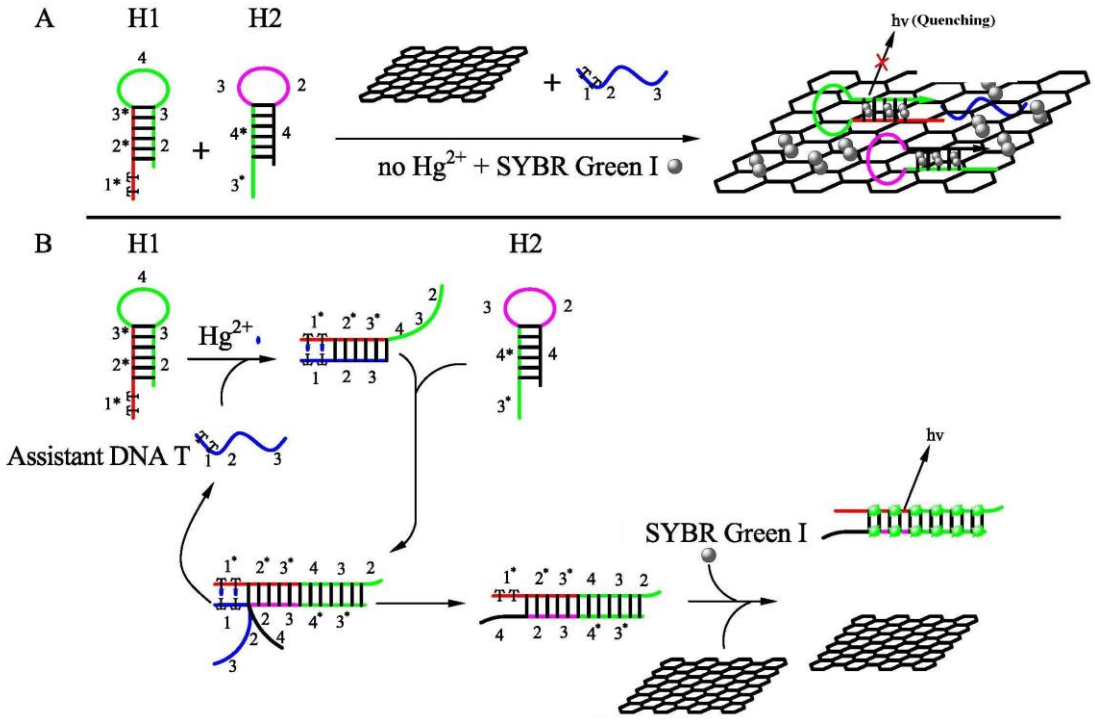





Fl Intensity (a.u.)
120
100
80
60
40
20
0
blank
Hg2+
K+
Na+
Ca2+
Mg2+
Cu2+
Zn2+
Cd2+
Pb2+
Ag+
Ni2+
Ba2+


A
H1
H2
hv (Quenching)
no Hg2+ + SYBR Green I
B
H1
H2
Hg2+
Assistant DNA T
hv
SYBR Green I

Graphical abstract



Highlights:

1. A novel and convenient biosensor for Mercury (II) detection was developed based on an isothermal enzyme-free amplification (EFA) and label-free fluorescence platform.

2. The enhanced signals were obtained by the recycling of Hg^{2+} and an assistant DNA.

3. The method has good sensitivity and high selectivity and can be applied for practical determination.