Microchim Acta (2015) 182:1535–1541
DOI 10.1007/s00604-015-1482-z

ORIGINAL PAPER

Highly sensitive DNA-based fluorometric mercury(II) bioassay based on graphene oxide and exonuclease III-assisted signal amplification

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Received: 24 December 2014 / Accepted: 14 March 2015 / Published online: 7 April 2015

Abstract The article describes a fluorometric and sensitive assay for mercury(II) ions (Hg^{2+}). It is based on the following scheme and experimental steps: (1) Hg^{2+} triggers the self-hybridization of thymine-rich ss-DNA labeled with a fluorescence tag to form a ds-DNA; (2) in the absence of Hg^{2+}, labeled ss-DNA will be adsorbed on the surface of graphene oxide (GO) and its fluorescence is quenched; (3) the ds-DNA formed in the presence of Hg^{2+} is cleaved by the catalytic action of exonuclease III; (4) the cleaved labeled DNA fragments do not adsorb on the surface of GO, this resulting in an increase in fluorescence intensity. The induction of the process by Hg^{2+} leads to a strong amplification of fluorescence, while the fluorescence of uncleaved labeled ss-DNA is quenched because it is adsorbed on the surface of GO in the absence of Hg^{2+}. This assay displays a detection limit of 0.1 nM (which is below the 10 nM upper limit in drinking water according to the US EPA and can be performed with 8 min.

Keywords Graphene oxide · Mercury ions · Exonuclease III · Fluorescence bioassay · Signal amplification

Electronic supplementary material The online version of this article (doi:10.1007/s00604-015-1482-z) contains supplementary material, which is available to authorized users.

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Introduction

Contamination of the environment with heavy metal ions has been recognized as an important worldwide issue for decades [1, 2]. Hg^{2+}, as one of the most ubiquitous heavy metals pollutants, is found in the atmosphere, soil, surface waters and even food [3]. According to the U.S. Environmental Protection agency (EPA), an annual total global mercury emission from natural sources as well as human activities is over 7500 tons [4]. Mercury ions can cause serious and permanent damage to the nervous system, kidney, liver, brain and other organs [5]. Because of the high toxicity of Hg^{2+}, the EPA and the international World Health Organization (WHO) have regulated the upper limit of Hg^{2+} levels in drinking water as 10 nM and 30 nM, respectively [6]. Therefore, there is a need to develop efficient, facile, sensitive and selective analytical methods for determination of Hg2+.

Conventional analytical methods, such as cold-vapor atomic fluorescence spectrometry (CVAFS) [7], cold-vapor atomic absorption spectroscopy (CV-AAS) [8], inductively coupled plasma atomic emission spectrometry (ICPAES) [9], inductively coupled plasma mass spectrometry (ICP-MS) [10], have been used in the detection of Hg^{2+}. These methods are very sensitive and selective. Nevertheless, they require complicated and expensive instruments operated by professional operators, limiting their wide application. Recently, Ono et al. reported that the Hg^{2+} can be specifically bind to two DNA thymine bases (T) and form stable T-T mismatches in DNA duplexes [11]. The T-Hg^{2+}-T pair mediated by mercury ions is more stable than the natural adenine-thymine (A-T) base pair. Moreover, this T-T mismatch shows high specificity for Hg^{2+} against other metal ions [12]. Based on these findings, various types of Hg^{2+} detection assays have been developed in recent years [13–15]. Among these methods, fluorescence bioassays using labeled oligonucleotides (OND), due to its simple

operation, fast response and high sensitivity, have attracted particular attention in various fields [16].

Graphene, a single-atom-thick and two-dimensional carbon nanomaterial with extraordinary electronic, mechanical and optical properties, has attracted great research interests in various fields [5, 6]. Graphene oxide (GO), an oxide form of graphene, displaying good water-solubility and flexibility for modification, has been widely applied in biological and biomedical areas [17]. In addition, GO also has some important characteristics such as highly efficient fluorescence quencher; high affinity to single-stranded DNA (ss-DNA) than double-stranded DNA (ds-DNA) or well folded ss-DNA; as well as high affinity to long ss-DNA than short ss-DNA [18]. More recently, by making use of these properties of GO, researchers have developed many fluorescent assays for Hg^{2+} detection [5, 6]. However, most of these methods didn't adopt the strategy of signal amplification, leading to their detection limit above the toxic level of Hg^{2+} in drinking water set by the EPA (10 nM) [19].

Signal amplification strategy based on target recycling, in which a target molecule circularly interacts with different nucleic acid-based signaling probes, is a very useful approach to improving detection sensitivity of bioassays [20, 21]. Nucleases such as Rnase H, nicking endonuclease, DNase I, duplex-specific nuclease, endonuclease IV, exonuclease III (Exo III) are often employed as the cleavage enzymes to promote the target recycling [22]. Different from the sequence-specific nicking endonuclease, Exo III, as exonuclease-assisted target recycling amplification, does not require any specific recognition sequence [23]. Exo III catalyzes the stepwise removal of mononucleotides from the 3-hydroxyl ends of dsDNA with blunt or recessed 3-terminus, while it has limited activity on ssDNA or 3-protruding termini of dsDNA [22]. In view of these features, Exo III has been widely used in the highly sensitive detection assays for DNA, RNA, protein and metal ions [22–25]. Based on these general concepts and researches, we present a highly sensitive and selective fluorescent bioassay for the detection of Hg^{2+} by combination of GO with Exo III-assisted signal amplification. Moreover, the fabricated sensing platform was successfully applied to the determination of Hg^{2+} in lake water samples.

Experimental

Apparatus

Fluorescence measurements were carried out on a Hitachi F-4600 spectrophotometer (Hitachi Co. Ltd, Japan) equipped with a xenon lamp excitation source at room temperature. The excitation was set at 495 nm and the emission spectra were collected from 510 to 600 nm. The fluorescence intensity at 518 nm was used to choose the optimal experimental conditions and evaluate the performance of the proposed sensing system. The excitation and emission slit widths were both set at 5 nm.

Materials and reagents

The Exo III was purchased from Takara Biotechnology Co. Ltd (Dalian, China, www.takara.com.cn) and DNA oligonucleotides were synthesized and purified using HPLC by Sangon Biotechnology Co. Ltd (shanghai, China, www.sangon.com). The sequence of the fluorescent dye-labeled ssDNA is 5'-CATTCTTTCTTCCCCT TGTT TGTTT-FAM-3'. GO was synthesized according to our previous report [17]. Graphite powder was oxidized by a mixture of H_2SO_4 and $KMnO_4$. Water was added to end the reaction. Adequate H_2O_2 (30 %) was added until the color of the mixture turned to bright yellow. The mixture was washed with dilute HCl solution to remove metal ions, and then washed with water until the solution reached a neutral pH. The resulting solid was dried in air. Finally, the synthesized product was further purified by dialysis to remove the remaining metal species, and dispersed in water under sonication to get the homogeneous GO suspension (0.5 mg mL^{-1}). Tris-hydroxymethyl aminomethane (Tris), the metal salts and all the other reagents employed were of analytical grade and used without further purification. Ultrapure water obtained from a Millipore water purification system (18.2 MΩ cm resistivity, Milli-Q Direct 8) was used in all runs.

Preparation of working solution

The GO stock solution was stable in a few days, but tended to form cluster if it was placed for a long time. Therefore, the GO stock solution should be sonicated for 1 h prior to each use. 1 μM FAM-labeled ssDNA probe stock solution was prepared with Tris buffer (20 mM, 100 mM NaCl, pH 8.2). The standard stock solution of Hg^{2+} was prepared by dissolving mercuric acetate with 0.1 % acetic acid. Aliquots of various concentrations of Hg^{2+} solutions were obtained by serial dilution of the stock solution with ultrapure water. The buffer for enzyme digestion was Tris buffer (10 mM, 10 mM KCl, 10 mM $MgCl_2$, pH 8.0, buffer 1). The NEBuffer was always used as the assisted solution of the Exo III. But the NEBuffer contained the dithiothreitol which forms a precipitate with Hg^{2+}. In this work, the activity of Exo III also has no obvious effect without adding the NEBuffer. Fluorescence measurements were performed in the buffer 2 which consisted of Tris buffer (20 mM, 100 mM NaCl, 2 mM $MgCl_2$, pH 7.4). All the solutions were stored at 4 °C.

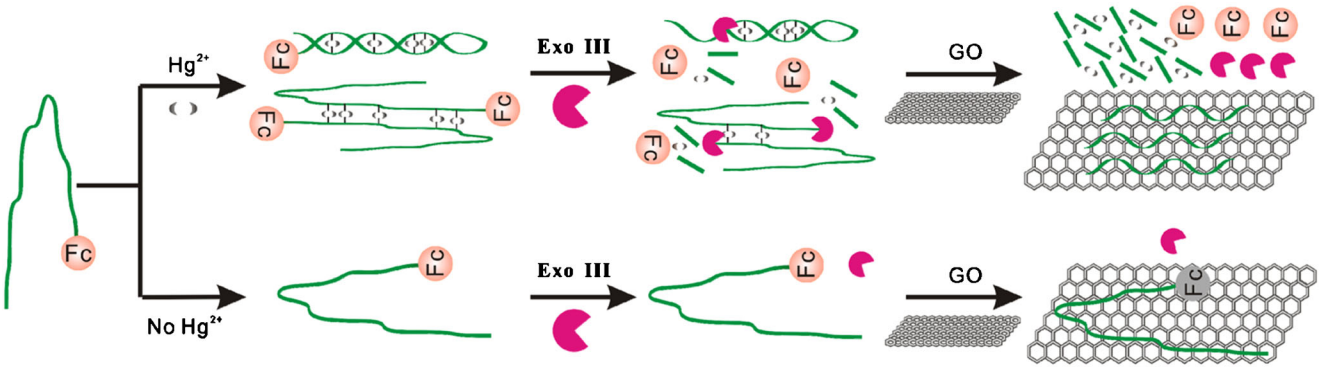

Fig. 1 Schematic illustration of fluorometric Hg^{2+} detection by combining graphene oxide with Exo III-assisted signal amplification

Fluorescence detection of Hg^{2+}

Thirty nanometer FAM-labeled T-rich ss-DNA probe and a desired concentration of Hg^{2+} were first mixed and kept at room temperature for 2 min, followed by adding 1 unit of Exo III and buffer 1. Subsequently, the whole 200 μL solution was homogeneously mixed and incubated at 37 °C for 5 min. Finally, the reaction solution was added with GO solution and diluted with buffer 2 to 1 mL. After homogeneous mixing, the fluorescence of the mixture was measured at room temperature. In control experiments, the measurement process was all the same with the above except the addition of Hg^{2+}. Unless otherwise noted, each fluorescence measurement was repeated three times, and the standard deviation was plotted as the error bar.

To evaluate effects of potential experimental conditions on Hg^{2+} detection, other metal ions, including Fe^{3+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+} at a concentration of 500 nM, were added respectively (in the presence and absence of 50 nM Hg^{2+}) and the measurement was performed respectively under the same conditions . To investigate the specificity of the assay, the fluorescence response of 50 nM Hg^{2+} was respectively compared with that of other potentially interfering metal ions (in the presence of 50 nM Hg^{2+}) at high concentrations (500 nM). To evaluate the selectivity of the assay, the fluorescence intensity of 50 nM Hg^{2+} was respectively compared with that of other potentially interfering metal ions (in the presence of 50 nM Hg^{2+}) at high concentrations (500 nM).

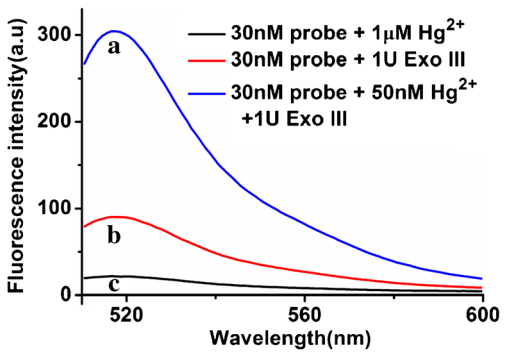

Fig. 2 The fluorescent spectra of the bioassay system for the detection of Hg^{2+} under different conditions (GO concentration is 50 μg mL^{-1}). Excitation wavelength is 495 nm and emission wavelength is 518 nm

Real sample analysis

To investigate the practical application of the sensing platform, the water sample from Huangjiahu Lake in Wuhan was filtered through a 0.22 μm membrane. The lake water was spiked with a stock solution of Hg^{2+}. Next, the high concentration Tris buffer was added to the mixture. The fluorescence measurement of Hg^{2+} was then performed in the same manner.

Results and discussion

Bioassay strategy

The inspiration of our sensing strategy for the detection of Hg^{2+} is based on the following factors: (1) Exo III digests dsDNA with blunt or recessed 3-terminus, but it has limited activity to ssDNA or dsDNA with protruding 3-terminus [20, 22], (2) GO absorbs the FAM-labeled ssDNA probe and quench the fluorescence of probe, while the affinity between very short ssDNA and GO is negligible [26]. (3) Hg^{2+}

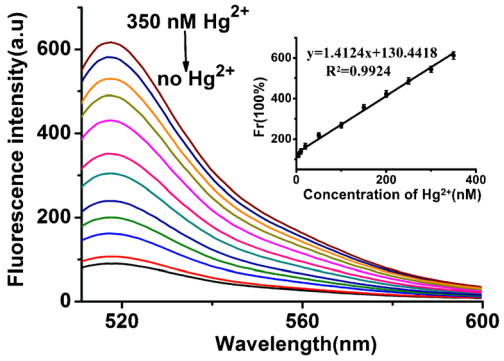

Fig. 3 Fluorescence emission spectra of this assay at different Hg^{2+} concentrations: 0, 0.1, 5, 10, 20, 50, 80, 150, 200, 250, 300, and 350 nM. Inset: the calibration curve and the linear equation from 3 to 350 nM Hg^{2+}

Table 1 Merits of nanomaterial-based methods for determination of Hg(II)

Materials[a]	Method[b]	Time	Linear range	LOD	Real sample	Advantages	Disadvantages	Ref.
CDs/GO	F	34 h	5–200 nM	2.6 nM	Citrus leaf	High selectivity	Time-consuming probe preparation	[28]
Au NPs	A	5 min	NA	3 μM	NA	Simple and economical	Poor sensitivity	[34]
Exo III/ CNTs/ AgNCs	F	160 min	0.1–200 nM	33 pM	River water	High sensitivity and selectivity	Complicated system and high cost	[1]
DNAzyme /HRP	A	155 min	10–100 pM	9.7 pM	Tape water	High sensitivity	Relatively long analysis time and complicated system	[32]
SWCNTs CNTs	E	55 min	0.1–1.0 pM	16.3 fM	NA	High sensitivity and selectivity	Time-consuming sensor preparation	[31]
exo III	E	70 min	NA	0.2 nM	NA	High sensitivity and selectivity	Time-consuming sensor preparation	[20]
QDs/ S NP	F	78 h	10–400 nM	6.6 nM	NA	High selectivity	Time-consuming probe preparation	[29]
PI/PC/Ag NCs	F	8 h 35 min	0.05–1.0 and 0.5–10 μM	3.0 nM/ 9.0 nM	Lake water	Relatively economical	Time-consuming probe preparation	[3]
SWCNTs	F	20 min	0.05–8.0 mM	14.5 nM	NA	High selectivity and simple	Relatively poor sensitivity	[4]
GO	F	140 min	0–1.0 nM	0.3 nM	Drinking water	High sensitivity and selectivity	Time-consuming	[5]
HRP/Au NPs	E	45 min	0.1–1000 nM	52 pM	River water	High sensitivity and selectivity	Time-consuming sensor preparation	[33]
Exo III/ GO	F	8 min	3–350 nM	0.1 nM	Lake water	Fast and high sensitivity	Relatively costly	this work

[a] *Ag NCs* silver nanoclusters, *Au NPs* gold nanoparticles *SNP* silver nanoparticle, *QDs* quantum dots, *SWCNTs* single-wall carbon nanotubes, *GO* graphene oxide, *HRP* horseradish peroxidase, *PI* polyinosinic acid, *PC* polycytidylic acid

[b] *F* fluorescence, *E* electrochemistry, *A* absorbance

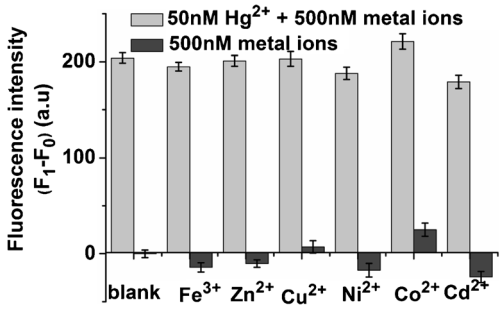

Fig. 4 The fluorescence intensity (F_1–F_0) of various 500 nM metal ions in the presence and absence of 50 nM Hg^{2+}

mediates the T-rich ssDNA to self-form the well folded dsDNA [12]. (4) the well folded dsDNA with T-Hg^{2+}-T base pairs can be digested by Exo III with the same efficiency as normal dsDNA [27]. The schematic description of the sensing strategy is illustrated in Fig. 1. In the absence of Hg^{2+}, the FAM-labeled ssDNA probe is presented as a random coil structure and resistant to Exo III digestion. So the probe is adsorbed on the surface of GO and the fluorescence is quenched. On the contrary, in the presence of Hg^{2+}, the fluorescence dye-labeled probe forms into a hairpin structure or a mismatch dsDNA between different probes via the Hg^{2+}-mediated coordination of T-Hg^{2+}-T base pairs. The hairpin structure is consisted of a 4-nt loop (CCCC) and a 10-bp stem with a recessed 3-terminus. The Exo III catalyzes the stepwise removal of mononucleotides from the 3-hydroxyl ends of the hairpin structure and mismatch dsDNA, resulting in the release of the target Hg^{2+} and FAM-labeled mononucleotide. The FAM-labeled mononucleotide does not adsorb on the surface of GO so that the fluorescence intensity of the solution has obvious increase with the progress of the triggered activity of Exo III. Moreover, the released Hg^{2+} then mediates the new probe, forming the well folded dsDNA and initiating a new cycle of digestion. Through such a catalytic cycle, small amounts of Hg^{2+} can exponentially trigger the cleavage of FAM-labeled DNA probe by being recycled hundreds of times. So this Exo III-assisted signal amplification technique can offer an ultrahigh sensitivity for the detection of extremely low concentrations of Hg^{2+}.

Assay feasibility

In order to verify the feasibility of the proposed sensing system for Hg^{2+} assay, the fluorescence signals of Hg^{2+} measurements in the presence of Exo III as well as a series of control experiments were depicted in Fig. 2. As shown in Fig. 2c, the very weak fluorescence showed that the great majority of the FAM-labeled probe was adsorbed on the surface of GO just in the presence of Hg^{2+}. Upon addition of Exo III, the fluorescence intensity was dramatically increased (shown in Fig. 2a), revealing that Exo III triggered the enzyme digestion reaction and formed the FAM-labeled mononucleotides which were not adsorbed on the surface of GO. Nevertheless, a non-negligible signal was observed just in the presence of Exo III (shown in Fig. 2b), revealing that Exo III non-specifically digests the probe and thus increase the background signal of the sensing platform. These results obviously indicate that the Exo III-assisted cleavage cycle triggered by Hg^{2+} has been successfully achieved. The quantitative assay of Hg^{2+} was realized by using the relative fluorescence change: $Fr=[(F_1-F_0)/F_0]\times 100$ %. F_1 and F_0 are the fluorescence intensities at 517 nm in the presence and absence of Hg^{2+}, respectively.

Optimization of assay conditions

The concentration of Exo III and GO, enzymatic reaction temperature and time were investigated systematically in order to obtain the optimal experimental conditions and attain a high signal-to-noise ratio for the detection of Hg^{2+} by fixing the 30 nM probe (The details can be found in Electronic Supplementary Material and Figure S1). 1 U mL^{-1} Exo III, 50 μg mL^{-1} GO, 37 °C enzymatic reaction temperature, 5 min enzymatic reaction time were chosen as the optimum condition.

Sensitivity of the assay towards Hg^{2+} detection

Under the optimal experiment conditions, the sensitivity of the bioassay was evaluated. In the presence of various Hg^{2+} concentrations, the fluorescence emission spectra of the bioassay were shown in Fig. 3. Clearly, the fluorescence signal was dynamically enhanced with increased Hg^{2+} concentrations from 0 to 350 nM. However, when the Hg^{2+} ions concentration was higher than 0.5 μM, the fluorescence signal reached a

Table 2 Recovery of Hg^{2+} added to real lake water samples

Sample	Hg^{2+} added (nM)	Hg^{2+} found (nM)	Recovery (%)	RSD (%)
1	0	0	–	–
2	5.0	4.7 [a]	94.0	3.9
3	20.0	21.2 [a]	106.0	4.7
4	100.0	93.6 [a]	93.6	4.9

[a] mean values of three assays

plateau and decreased with further increase of the Hg^{2+} concentrations (The results were not shown). This may be ascribed to the fact that the very high Hg^{2+} ions concentrations diminish the activity of Exo III. The inset depicted the relationship between Fr and the Hg^{2+} concentrations. As shown in the inset of Fig. 3, the value of Fr was proportional to the Hg^{2+} concentrations in the range of 3 nM to 350 nM. The linear regression equation was Fr=1.4124C+ 130.4188 with a correlation coefficient of 0.9924, and the detection limit was determined to be 0.1 nM (three times the standard deviation of the blank solution. C is the concentration of Hg^{2+}). The detection limit of this assay is far below the maximum allowable level of Hg^{2+} in drinking water (10 nM) set by the U.S. EPA. The sensitivity of this assay is also higher than that reported in most of the previous reported methods (as shown in Table 1). These results indicate that the designed bioassay might possess a great application for the detection of Hg^{2+} in environmental fields.

Selectivity and specificity

In order to evaluate the selectivity and specificity of the bioassay system for possessing great application, various metal ions were added to test the change of the fluorescence intensity. Firstly, we investigated whether these metal ions may markedly change the fluorescence intensity of the bioassay. A variety of environmentally relevant metal ions such as Fe^{3+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+} were respectively added in the absence of Hg^{2+}. Compared with the blank solution, the fluorescence intensity just had slight change after adding these metal ions (shown in Fig. 4). This indicates that these potentially interfering metal ions do not mediate the same formation of dsDNA as Hg^{2+} does. In this work, we added 50 μM potentially interfering metal ions in the presence of 50 nM Hg^{2+}. It was found that there was no strongly significant effects upon addition of Fe^{3+}, Zn^{2+} and Cu^{2+} ions (the decrease of fluorescence intensity was all less than 20 %), but addition of Cd^{2+} and Ni^{+} ions led to a large decrease in the fluorescence intensity (The results were not shown). Therefore, 500 nM of these potentially interfering metal ions were respectively added in the presence of 50 nM Hg^{2+} in the work. As demonstrated in Fig. 4, the bioassay can detect Hg^{2+} in the presence of other potentially interfering metal ions at high concentrations. All the above observations show that this bioassay for Hg^{2+} detection has high selectivity and specificity against the interferences of other metal ions, which are mainly attributed to the specific T-Hg^{2+}-T base pairing [30]. Nevertheless, in view of the strong interference of high concentrations of metal ions in real samples (for instance, Cd^{2+} and Ni^{+} ions), the potential solution to reduce the interference of high concentrations of these metal ions in real samples may be that citric acid is added to the analyzed samples, resulting in the chelating ligand between citric acid and heavy metal ions, as described in the previously reported work [16].

Real samples assay

In order to evaluate the performance of this bioassay for the detection of Hg^{2+} in natural samples, the lake water samples spiked with three different concentrations of Hg^{2+} were applied. According to the linear regression equation in the range from 3 to 350 nM Hg^{2+}, the 'Found' Hg^{2+} concentrations in the lake water samples were obtained. All the measurements were performed three times, and the results were summarized in Table 2. We observed that the recoveries were in the range of 94.6–106.0 %, and the average RSD was less than 5.0 %. The results clearly indicate that the bioassay could realize the quantification analysis of Hg^{2+} in real samples.

Conclusions

In summary, a fluorescence bioassay based on GO and Exo III-assisted signal amplification has been developed for the highly sensitive and selective detection of Hg^{2+} in the present work. Mediated by Hg^{2+}, the FAM-labeled ssDNA forms the well-folded dsDNA and is digested by the Exo III, resulting in the release of FAM-labeled mononucleotide and Hg^{2+} ions. The FAM-labeled mononucleotide is not adsorbed on the surface of GO, leading to the increase of the fluorescence intensity. Furthermore, the released Hg^{2+} mediates a new cycle of digestion. In such a way, a small amount of Hg^{2+} can induce the large number of enzyme digestion reactions and form a significantly amplified fluorescence signal. This bioassay possesses high sensitivity and selectivity, and has fast response (8 min) in comparison to the published work (as shown in table 1). The practical applications in lake water also indicate that this bioassay exhibits great perspective for the detection of Hg^{2+} in the environmental samples.

Acknowledgments The authors acknowledge the support of Natural Science Foundation of China (Nos. 21275040 and 21475034), and the Natural Science Foundation of Hubei Province (No. 2013CFA061).

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