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Fluorometric determination of lead(II) and mercury(II) based on their interaction with a complex formed between graphene oxide and a DNAzyme

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Abstract

The authors have designed a DNAzyme where graphene oxide (GO) interacts with the ssDNA stem loop region. The DNAzyme strand and substrate strand are hybridized and bind to the surface of GO which act as a signal reporter, while GO act as a strong quencher. The presence of Pb(II) ion disturbs the GO-DNAzyme complex and causes internal cleavage of the DNAzyme complex. On addition of Thioflavin T (ThT) as a quadruplex inducer, fluorescence intensity (best measured at excitation/emission peaks of 425/490 nm) is strongly enhanced. Subsequent addition of Hg(II) to ThT/G-quadruplex complex decreases fluorescence because the G-quadruplex is unwinding to form a T-Hg(II)-T dsDNA system. Therefore, the change in fluorescence intensity of ThT is directly correlated to the concentration of Pb(II) and Hg(II). As a result, the assay is highly selective and sensitive. The limits of detection are 96 pM for Pb(II) and 356 pM for Hg(II). Moreover, the method was applied to the detection of the two ions in spiked real samples and gave satisfactory results.

Introduction

Lead and mercury ions are also the most toxic heavy metal ions. Even at low concentration of Lead and mercury, it is a threat to the human health and environment due to its ability of accumulation in ecological system. Lead poisoning induces a wide variety of serious human diseases, including harm hypertension, nervous system, kidney damage and reproductive problems [1–3]. Exposure to mercury may cause severe damages like kidney failure, brain damage, various cognitive and motion disorders [4, 5]. Much effort has been made to apply the common analytical techniques for the detection of Pb(II) and Hg(II) ions including atomic adsorption spectroscopy (AAS), atomic plasma emission spectroscopy, inductively coupled plasma mass spectrometry (ICP-MS) and electrochemical methods [6–8]. These methods require expensive sophisticated instruments, well trained manual operation, difficult to prepare sample and time consuming [9]. During which, fluorescent methods have attracted great attention because of their distinctive advantages such as simplicity, highly sensitive and selective.

DNAzyme are single strand DNA with catalytic activity. DNAzyme are attractive molecules due to high catalytic activity, trouble-free synthesis and more stable than other enzymes. A number of DNAzymes that are highly selective divalent metal ions such as Pb^{2+}, Cu^{2+}, UO_2^{2+}, Zn^{2+} and Mn^{2+} as cofactors have been isolated through in vitro selection [10–14]. Furthermore, the modification of fluorescent dye on DNAzyme leads to complicated synthetic route, high cost and attached fluorophore disturb the catalytic activity of DNAzyme. Therefore, label-free DNAzyme have great interest for potentially increasing sensitive detection of metal ions.

Graphene oxide (GO) has attracted a growing attention in the biological field because of excellent properties such as electronic, mechanical, surface functionality, aqueous dispersibility and enhances fluorescence quenching ability. In addition, GO binds single-strand DNA (ssDNA) through

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hydrophobic, hydrogen bonding and π-stacking interactions between nucleobases and GO [15–19]. On the other hand double strand DNA does not adsorbed onto the GO surface because the efficient shielding of nucleobases within in the negatively charged phosphate backbone of dsDNA [20–22]. Based on these properties GO with DNA based biosensor has been considered as a promising high sensitive detection of metal ions when compared with other type of sensors. G-quadruplex is non-canonical four standard DNA structure formed from guanine rich sequences of DNA or RNA. Generally, G-quadruplex based sensor exhibits high sensitive detection of metal ions. In addition, the fluorogenic dye ThT can induce the quadruplex formation with metal ions and enhances the fluorescence intensity which is superior to other quadruplex binding dyes [23, 24]. G-quadruplex structures greatly enhance the fluorescence compared to other single and double strand DNA structures.

In this study, we explore a rapid and inexpensive fluorescence detection of Pb(II) and Hg(II) ions on GO-DNAzyme complex. The hybridized DNAzyme-substrate strand is adsorbed on the surface of GO, with the absence of Pb(II) ion, which causes no fluorescence signal. When the Pb(II) ion introduced into the DNAzyme, it cleavages and release fragments in GO surface. In addition of ThT as an efficient inducer, the free of GT rich fragments to fold into G-quadruplex structure then produces the fluorescence light up having enhancement. Subsequently the addition of Hg(II) ion into the above GO/DNAzyme-Pb(II)/ThT/G-quadruplex system, the G-quadruplex structure can be released to mediate T-Hg(II) -T dsDNA and the fluorescence signal is decreased. We herein report, a new approach for the introduction of this assay using single mechanisms which can help to detection of Pb(II) and Hg(II) ions. To the best of our knowledge, so far there has been no report in the literature for such type system. Finally, this method is successfully applied to detect Pb(II) and Hg(II) ions in environmental samples.

Experimental

DNA sequences

The GR-5 DNAzyme and substrate strand DNA were received from Allied Scientific Products (Kolkata, India) (http://www.alliedscientific.net) and the sequences are shown as follows: GR-5 DNAzyme: (5′- CCC AAA ACC CTC TGA AGT AGC GCC GCC GTA TAG TAC CCT - 3), Substrate DNA: (5′- TCA GGG TTT TGG GTT TTT TGG GTT TTG GGA GAG TArA TAT CAG GGA CAC TT-3′.

Materials

Instruments

FT-IR spectrum was measured using a Nicolet 400 Fourier transform infrared spectrometer (Madison, WI).The X-ray diffraction (XRD) pattern was carried out with a PAN analytical X'pert pro X-ray diffractometer with Cu target (λ = 1.5406 Å).All fluorescence spectra of the sample were carried out on a HORIBA JOBIN YVON Fluoromax-4 Spectrofluorometer with a xenon lamp excitation source. The excitation and emission slit width wavelength were set in 10 nm in wave length ranging from 440 nm to 600 nm and sample is excited by 425 nm. Atomic force microscopy (AFM) image was performed at ambient temperature on an Agilent Pico LE atomic force microscope. Laser Raman micro spectrometer (Renishaw, UK) with a sample wavelength 514 nm was used to characterize the structural information of GO. Circular Dichroism (CD) measurements were carried out on a JASCO J-815 spectrometer (JASCO International Co. Ltd., Japan). The spectra were measured in the wavelength range 200–500 nm using quartz cuvette with 1.0 mm path length.

Fluorescence detection of Pb(II) and Hg(II)

The preparation of GR-5 DNAzyme: 5 μL of 2 μM substrate DNA and 10 μL of 2 μM GR-5 DNAzyme were hybridized in a buffer (50 mM of HEPES (50 mM NaCl, 5 mM $MgCl_2$, pH = 7.4) at 37 °C for 30 min. To detect Pb(II), 10 μL of DNAzyme, 800 μL of buffer (50 mM of HEPES (50 mM NaCl, 5 mM $MgCl_2$, pH = 7.4) and 80 μL GO (60 $μg.mL^{-1}$) were sequentially added into plastic centrifuge tube. After 30 min of reaction, the solution was transferred into cuvette.

Subsequently, the freshly prepared 10 μL of Pb(II) stock solution was added in different concentrations (0 to 2 nM) and incubated for 40 min. Then 80 μL of 10 μM of ThT was added and reacted for 30 min at 37 °C to form GO-DNAzyme/Pb(II)/ ThT/G-quadruplex. The fluorescence intensity was measured by excitation at 425 nm and emission was observed at 490 nm. After the detection of Hg(II), the final concentration of GO (80 μL), 10 μL of DNAzyme and 10 μL of 2 nM Pb(II) ion was mixed and diluted with the buffer to a final volume of 800 μL and incubated for 30 min. Then 80 μL of ThT was added to the same solution and incubated for 30 min. Next, different concentrations (0 to 3 nM) of 10 μL of Hg(II) ion were added to the solution. The fluorescence spectra were measured at the excitation and emission wavelength of 425 nm and 490 nm respectively.

Fig. 1 Fluorescence spectral changes for (**a**) GO- DNAzyme (GO (60 μg.mL^{-1}), S(r) (60 nM), GR-5 (60 nM); (**b**) GO-DNAzyme- Pb^{2+} (GO (60 μg.mL^{-1}), S(r) (60 nM), GR-5 (60 nM), Pb^{2+} (0.1 nM); (**c**) GO-DNAzyme-Pb^{2+}-ThT; (**d**) GO-DNAzyme-Pb^{2+}- ThT- Hg^{2+} (GO (60 μg.mL^{-1}), S(r) (60 nM), GR-5 (60 nM), Pb^{2+} (0.1 nM), Hg^{2+} (0.2 nM)

Results and discussion

Mechanism

The depicted mechanism of GO-DNAzyme complex based fluorescent assay for detection of Pb(II) and Hg(II) ions is shown in Scheme.1. The substrate strand was designed by many GT rich sequences which are hybridized DNAzyme strand and formation of DNAzyme-substrate complex having single strand stem loop region. A GR-5 DNAzyme is readily bind to the surface of GO via the π-stacking interaction and hydrogen bonding between the GO and DNA bases [15–19]. GO is act as an excellent super quencher which was employed as an independent quencher to suppress background. The optimum amount of GO assured that strong binding between the ssDNA loop region of DNAzyme-substrate strand and
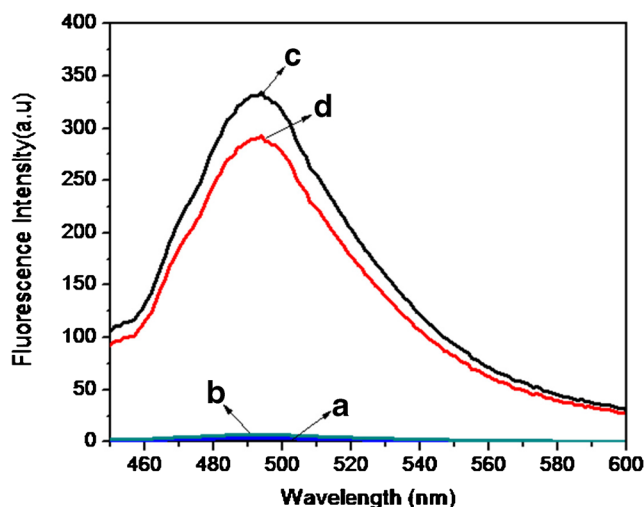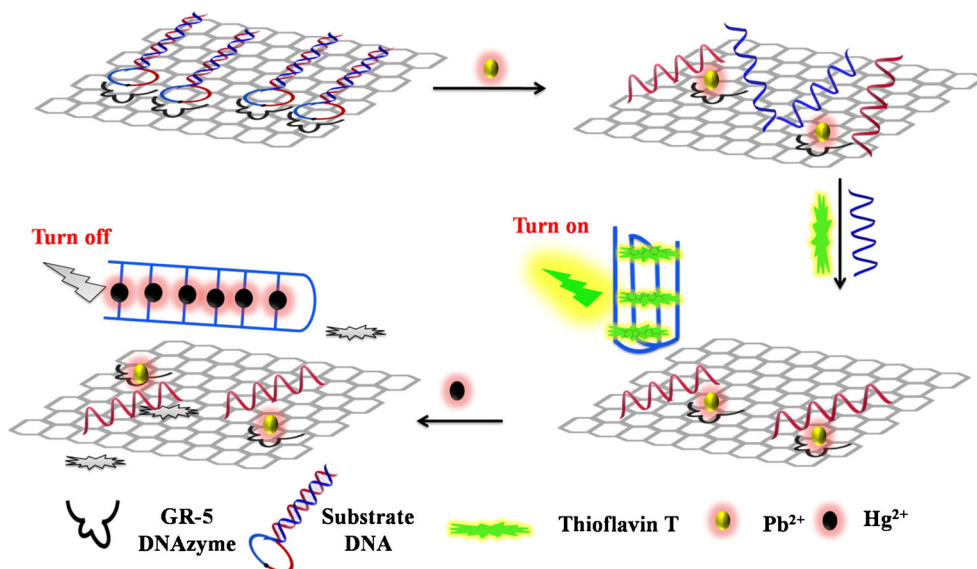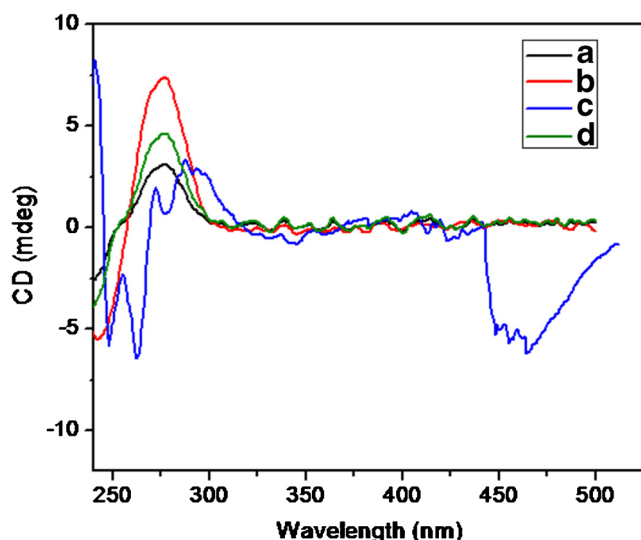
graphene surface. Then, Pb(II) ion was introduced into GO-DNAzyme complex; the substrate strand cleaved at the rA sites and disturbs the GO–DNAzyme complex, and thus released the shorter GT rich sequences to form a G-quadruplex structure, which strongly binding with ThT to form the ThT-G-quadruplex resulting in significant enhancement of fluorescence signal.

It is noting that the GO-DNAzyme/Pb(II)/ThT system is act another important application for the detection of Hg(II). Interestingly, in addition Hg(II) ion in the system the fluorescence intensity dramatically decreased. Based on the interaction of Hg(II) ion to form mediated T-Hg(II)-T complex

Scheme 1 A Schematic illustration of the fluorescent detection of Pb (II) and Hg (II)

Fig. 2 **a** GR-5 DNAzyme (10 μM); **b** GR-5 DNAzyme (10 μM) & Substrate strand (10 μM); **c** GR-5 DNAzyme (10 μM), substrate strand (10 μM), Pb^{2+} (10 μM) &ThT (6 μM); (d) GR-5 DNAzyme (10 μM), substrate strand (10 μM), Pb^{2+} (10 μM), ThT (6 μM) & Hg^{2+}(10 μM)

through folding of them into hairpin duplex structure and released the G-quadruplex [24]. As a result this system acts as fluorescence off assay for Hg(II) ion. To verify the possibility of designed GO-DNAzyme structure based fluorescent detection of Pb(II) and Hg(II) ion the fluorescence intensities were depicted in Fig.1. As shown in Fig. 1(a & b), the introduction of Pb(II) ion react with GO-DNAzyme structure resulting in low fluorescence signal. Upon the addition of ThT to form the G-quadruplex structure, an increase in fluorescence signal was observed in Fig.1(c). Further, in presence of required concentration of Hg(II) ion in same system a decrease in fluorescence signal is shown in Fig. 1(d).

Characterization

CD spectra analysis

The CD spectrum was valuable tool and it is applied for the study of oligo-nucleotides structure. Fig. 2 shows a plot of CD
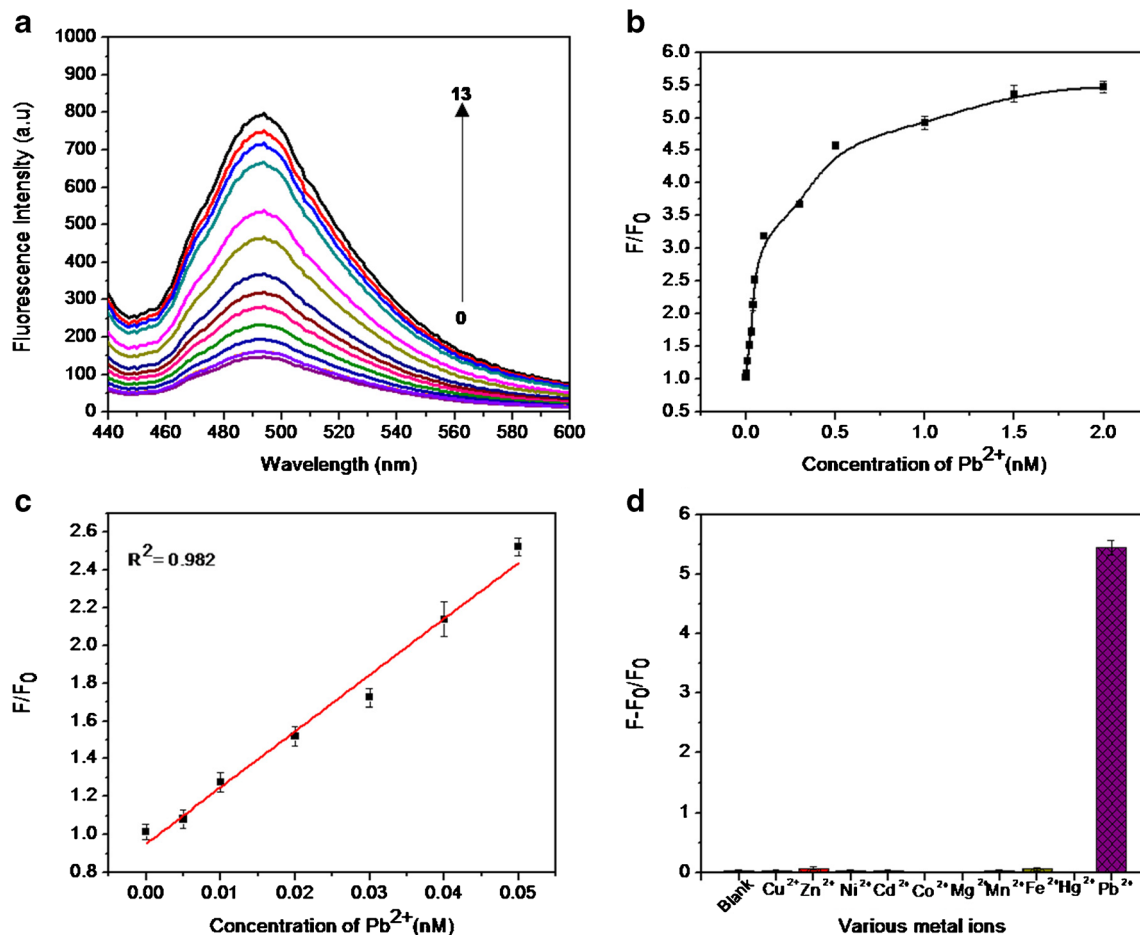
Fig. 3 **a** Fluorescence spectra of Pb^{2+} GO (60 μg.mL^{-1}), S(r) (60 nM), GR-5 (60 nM) and ThT (12 μM) at different concentrations (0, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.3, 0.5, 1, 1.5 and 2 nM) in 50 mM HEPES (pH = 7.4) at 37 °C; **b** Fluorescence intensity against the concentrations of Pb^{2+}; **c** The linear relationship between the fluorescence intensity and concentrations of Pb^{2+} (0–0.05 nM); (d) Selectivity of Pb^{2+} (5 nM) and different metal ions (10 nM). The excitation and emission wavelengths are 425 and 490 nm, respectively. Error bars represent the standard deviation in three individual experiments
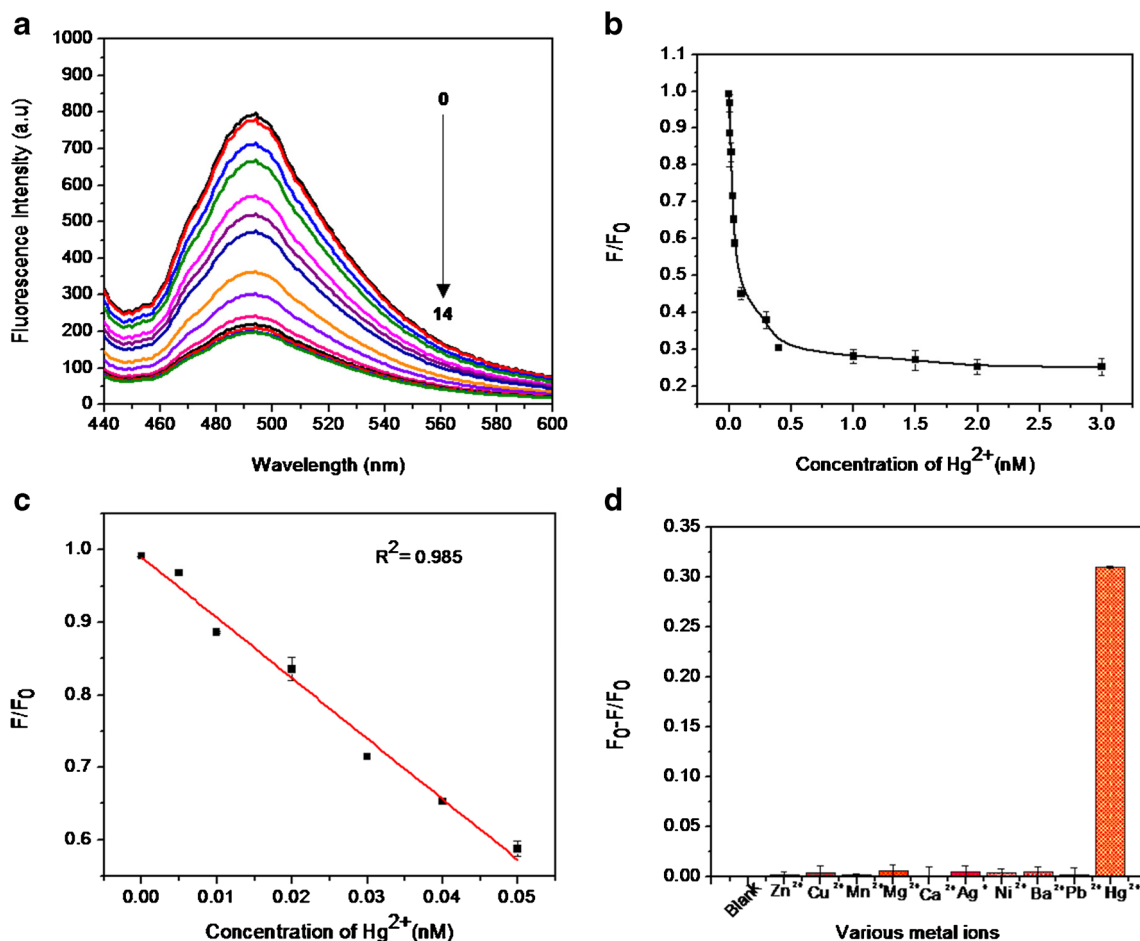
Fig. 4 **a** Fluorescence spectra of Hg^{2+} (GO (60 $\mu g.mL^{-1}$), S(r) (60 nM), GR-5 (60 nM), ThT (12 μM) and Pb^{2+} (2 nM)) at different concentrations (0, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.3, 0.5, 1, 1.5, 2 and 3 nM) in 50 mM HEPES (pH = 7.4) at 37 °C; **b** Fluorescence intensity against the concentrations of Hg^{2+}; **c** The linear relationship between the fluorescence intensity and concentrations of Hg^{2+} (0–0.05 nM); (d) Selectivity of Hg^{2+} (5 nM) and different metal ions (10 nM). The excitation and emission wavelengths are 425 and 490 nm, respectively. Error bars represent the standard deviation in three individual experiments

spectra of substrate strand has a positive peak at 278 nm and negative peak at 245 nm, which is the characteristic peak of single strand DNA. By comparing curve (a), the CD spectra of curve (b) is dramatically increased the peak intensity in positive peak at 278 nm and negative peak 245 nm, which indicate that substrate strand and enzyme strand were mixed and formed duplex DNA structure. Upon the addition of Pb(II)

ion and ThT, it is observed that formation of new peak showed antiparallel quadruplex structure has a positive peak at around 294 nm, and a negative peak at around 262 nm [23, 24] (curve (c)), which revealed that in presence of Pb(II) ion cleaved the substrate strand and released fragments are bind to the ThT. The negative peak at around 450 nm indicate that interactions between the ThT and G-quadruplex. Then, addition of Hg(II)

Table 1 Comparison of fluorescence based detection methods for Pb(II)

Method	Sensing Probe	Detection limit	Real water samples	Reference
Fluorescence	GO/AGRO100	400 pM	Not reported	[26]
Fluorescence	Gudruplex/GO/AO	3 nM	Lake and Tap water	[27]
Fluorescence	PTCDI/aptamer	0.483 nM	Water samples	[28]
Fluorescence	GR-5 DNAzyme	3 nM	River water	[29]
Fluorescence	Single labeled G-qudruplex/GO	400 pM	Tap water	[30]
Fluorescence	GO/GR-5 DNAzyme/FAM	300 pM	River water	[31]
Fluorescence	GO/GR-5 DNAzyme/ThT	96 pM	Tap and Lake water	This work

Table 2 Comparison of fluorescence based detection methods for Hg(II)

Method	Sensing Probe	Detection limit	Real water samples	Reference
Fluorescence	Single strand DNA/ThT	5 nM	Tap and River water	[24]
Fluorescence	GO-based DNA duplex poly (T)	500 pM	River water	[32]
Fluorescence	GO/aptamer	920 pM	Not reported	[33]
Fluorescence	CA-CdTe QDs/AuNps/aptamer	0.025 nM	Tap and lake water	[34]
Fluorescence	T-Hg^{2+}-T mis-matched DNA	9.5 nM	Lake water	[35]
Fluorescence	ssDNA/SYBERGreen	3 nM	Lake and Drinking water	[36]
Fluorescence	GO/GR-5 DNAzyme/ThT	356 pM	Tap and lake water	This work

ion in same system the antiparallel quadruplex is disappeared (curve (d)).

Optimization of method

The following parameters were optimized: (a) sample pH value; (b) amount of GO; (c) concentration of GR-5 DNAzyme; (d) concentration of ThT; (e) reaction time. The Signal-to-background ratio (SBR) was used to evaluate the analytical performances. Respective data and Figures are given in the Electronic Supporting Material. The following experimental conditions were found to give best results: (a) A sample pH value of 7.5 (Fig. S2, ESM); (b) an amount of GO of 60 μg (Fig. S3, ESM); (c) a concentration of GR-5 DNAzyme of 60 nM (Fig. S4, ESM); (d) a concentration of ThT of 12 μM (Fig. S5, ESM); (e) reaction time of 40 min (Fig. S6, ESM);

Sensitivity of Pb(II) and Hg(II) ions

In order to evaluate the quantitative determination of Pb(II) ion, the fluorescence spectra of the system depends on the different concentrations of Pb(II) ion under optimization conditions. The hybridized DNAzyme-substrate complex is adsorbed on the surface of GO, with the absence of Pb(II) ion, which causes no fluorescence signal. When the Pb(II) ion introduced into the DNAzyme, it cleavages and release fragments in GO surface. Upon the addition of quadruplex dye ThT is interacted with cleaved substrate strand, result

shows the fluorescence signal (Fig. 3a).The remarkable fluorescence intensity is increased with increasing the concentrations of Pb(II) ion at 0 to 2 nM. The linear correlation coefficient ($R^2 = 0.982$, Fig. 3c) between the fluorescence intensity and concentration of Pb(II) ion in the range from 0 to 0.05 nM. The detection limit of Pb(II) ion is 96 pM calculated to the 3σ/slop rule. The significant detection of Pb(II) ion in different concentration level based on GO-DNAzyme/Pb(II)/ThT act as another fluorescent assay of to detect the Hg(II) ion. At first, 2 nM of Pb(II) ion was added in to the GO-DNAzyme/ThT system and incubated for 40 min, the cleaved substrate strand is completely converted into G-quadruplex structure and shows strong fluorescence enhancement. With the addition of Hg(II) ion, the concentration increases, a gradual decrease in the fluorescence intensity is monitored and reached maximum concentration at 3 nM (Fig. 4a). A good linear relationship ($R^2 = 0.985$, Fig. 4c) is observed in the range from 0 to 0.05 nM and the limit of detection is 356 pM calculated to the 3σ/slop rule.

Selectivity of Pb(II) and Hg(II) ions

The selectivity of the GO-DNAzyme/ ThT assay was studied in the presence of other metal ions such as Ca(II), Ba(II), Cu(II), Zn(II), Ni(II), Cd(II), Co(II), Mg(II), Mn(II) and Fe(II). It can be seen from Fig. S8a the coexistence of interference metal ions, the response of this assay to Pb(II) is almost unaffected. However, after addition of Hg(II) ion to the

Table 3 Results of Pb(II) and Hg(II) in real water samples

Sample	Spiked (nM)	Detected ±SD		Recovery (%)	
		Pb(II)	Hg(II)	Pb(II)	Hg(II)
Tap water	0.1	0.101[a] ± 0.55[b]	0.096[a] ± 0.55[b]	101	96.5
	0.2	0.203[a] ± 0.41[b]	0.019[a] ± 0.66[b]	101.5	95
	0.3	0.297[a] ± 1.5[b]	0.303[a] ± 2.4[b]	99.3	101
Lake water	0.1	0.100[a] ± 0.63[b]	0.101[a] ± 0.76[b]	100.2	101
	0.2	0.197[a] ± 0.75[b]	0.195[a] ± 0.10[b]	98.5	97.5
	0.3	0.301[a] ± 1.18[b]	0.302[a] ± 0.28[b]	100.3	100.6

[a] Mean values of three determinations [b] Standard deviation

system the fluorescence intensity drastically decreased. So further to evaluate the selectivity of this strategy toward Pb(II) was studied by adding Pb(II), Ca(II), Ba(II), Cu(II), Zn(II), Ni(II), Cd(II), Co(II), Mg(II), Mn(II), Fe(II) and Hg(II) ions separately into the system. Figure 3d shows that only Pb(II) induces the fluorescence enhancement among all other metal ions. This fluorescent assay exhibits a high selectivity toward Pb(II). The conceivable reason is as follows: The deprotonated species of the hydrated Pb(II) effectively interact with 2′-OH on the rA site to perform the nucleophile attack reaction for the pKa values of 7.2–7.8 [25]. The important features of Pb(II) are to induce an effective RNA cleavage and the DNAzyme affords a scaffold to make use of Pb(II) for this sensing system successfully.

To selectivity of Hg(II) ion is evaluated by GO-DNAzyme/ Pb(II)/ThT system and comparing its fluorescence responses of Hg(II) ion and other metal ions (Zn(II), Cu(II), Mn(II), Mg(II), Ca(II), Ag(I), Ni(II), Ba(II) and Pb(II)). The fluorescence intensity is not decreased in addition of other metal ions except Hg(II) ion as shown in Fig. 4d & S8b. These results indicate that the excellent selectivity of these system for Hg(II) ions and did not interference of other metal ions. It demonstrated that the assay has good selectivity for Hg(II) ions. Comparison of several methods used for the detection of Pb(II) and Hg(II) ions are listed in Tables 1 & 2.

Analysis of Pb(II) and Hg(II) ions in real water samples

The practical applicability of this strategy was evaluated by the detection of Pb(II) and Hg(II) in real water samples. The water samples (tap and lake water) were collected from near SRM University campus Tamil nadu, India. First, the water samples were filtered to remove insoluble substance. Then different amounts (0.1 nM, 0.2 nM & 0.3 nM) of Pb(II) and Hg(II) ions spiked in real water samples and diluted with the buffer. The application of our method and the evaluation of the concentrations of Pb(II) and Hg(II) are demonstrated. The observed results are summarized in Table 3.The average recoveries are found to vary from 95 to 101%, which results in indicating that this method is applicable for the Pb(II) and Hg(II) ions analysis in real water samples.

Conclusion

In summary, we have reported on fluorescence biosensing platform for the detection of Pb(II) and Hg(II) ions based on self-assembled GO-DNAzyme complex. The remarkable fluorescent intensity of ThT fluorescent probe was binding with G-quadruplex. This method exhibits an ultrasensitive detection with a detection limit of 96 pM of Pb(II) and 356 pM of Hg(II) respectively, within the range of 0 to 0.05 nM.

Therefore, this sensing strategy holds a great potential for detection of Pb(II) and Hg(II) ions in real water samples with satisfying results. The limitation of the study is that the selectivity needs to be improved. Thus, the new method is inexpensive, highly sensitive and label-free.

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