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Highly selective, sensitive and quantitative detection of Hg^{2+} in aqueous medium under broad pH range†

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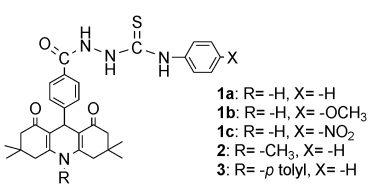
Amidothiourea linked acridinedione derivatives selectively detect Hg^{2+} in unbuffered aqueous solution under broad pH range with both single- and two-photon excitation. The observed linear fluorescence intensity change allows the quantitative detection of Hg^{2+} in the concentration range of 22 nM–0.33 μM with the lower detection limit of 2 nM.

The development of selective and sensitive detection of toxic heavy metal ions has attracted considerable attention because of the wide use of these metal ions and their subsequent impact on the environment.[1] Among these, Hg^{2+} is considered as highly dangerous, because both elemental and ionic mercury can be converted into methyl mercury by bacteria in the environment, which subsequently bioaccumulates through the food chain.[2] The Environmental Protection Agency (EPA) standard for the maximum allowable level of inorganic Hg^{2+} in drinking water is 2 ppb.[3] Several optical methods using organic fluorophores,[4] nanoparticles,[5] semiconductors,[6] DNAzymes,[7] and oligonucleotide[8] have been developed for simple and rapid detection of Hg^{2+}. Among the optical sensors, the fluorescence based approach is a promising tool for a simple and sensitive detection of Hg^{2+} and also for rapid tracking in biological, toxicological and other environments.[9] To date, a number of functional fluoroionophores exhibiting optical sensing ability have been developed for the monitoring of Hg^{2+} ions.[10] Another approach, which makes use of a chemodosimeter[11] through a specific chemical reaction between dosimeter molecule and target species to form a coloured or a fluorescent product, has drawn much attention in recent years. Because of the strong thiophilic affinity of Hg^{2+}, fluorescence changes associated with mercury-promoted desulfurization reactions, including hydrolysis,[11a,b] cyclizations,[11c,d] ring opening of rhodamine spiro systems[11e–h] and eliminations,[11i] have been used in the design of chemodosimeters for Hg^{2+}. Even though a few fluorescent Hg^{2+} probes with good water solubility, high selectivity and sensitivity are reported,[11j,12] detailed investigations on the optical changes and how individual sensors provide optical feedback are lacking. As far as quantitative practical Hg^{2+} detection is concerned, a linear fluorescence response, uniform fluorescence output at broad pH range, compatibility with aqueous medium, higher selectivity, sensitivity, fast response and easy synthetic procedures of probes are most important. The Hg^{2+} probes reported so far have achieved only a few of the above mentioned criteria and do not satisfy all the desired features.

Recently, two-photon microscopy (TPM) has become a vital tool in biology.[13] Compared to traditional fluorescence microscopy, TPM offers intrinsic 3D resolution combined with reduced photo-toxicity, increased specimen penetration, and negligible background fluorescence. However, two-photon (TP) probes for Hg^{2+} are rare.[14] In this paper, we report amidothiourea linked acridinedione (ADD) dosimeters that exhibit all the important features of Hg^{2+} detection, including two-photon excited fluorescence.

Amidothiourea linked ADD dyes (Scheme 1) were synthesized and characterized by the known procedure.[15] These dyes show the absorption and emission maxima in water in the region of 390 and 440 nm, respectively, which are assigned to the intramolecular charge transfer (ICT) from the ring nitrogen atom to the ring carbonyl centre within the ADD moiety.

Addition of Hg^{2+} to an aqueous solution of **1a** did not lead to any significant change in the longer wavelength absorption maximum (Fig. S1a†), which indicates the absence of any ground-state interaction between the Hg^{2+} reaction centre and ADD chromophore. The corresponding fluorescence spectrum (Fig. S1b†) shows a 92-fold fluorescence quenching, without any spectral shift. The fluorescence intensity decrease of **1a** was found to limit with the addition of one equivalent of Hg^{2+} that reveals a 1 : 1 stoichiometry reaction. Unlike other



Scheme 1 Structure of Hg^{2+} probes.

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† Electronic supplementary information (ESI) available: Experimental procedures and characterisation of compounds, emission spectra with the addition of Hg^{2+}. See DOI: 10.1039/c1cc12018b
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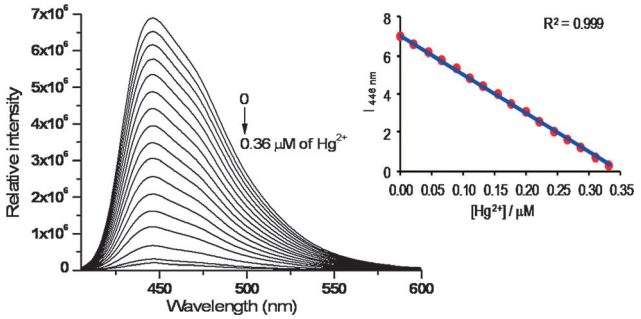


Fig. 1 Fluorescence quenching of **1a** (0.34 μM) upon addition of Hg^{2+} in water–MeOH (99 : 1, v/v), λ_{exc} = 385 nm. Inset shows the changes in the fluorescence intensity at 446 nm upon addition of Hg^{2+} (0–0.33 μM)

reported dosimetric molecules, probe **1a** showed a fluorescence response towards Hg^{2+} within 30 s. No further change in the fluorescence intensity was seen even at prolonged period (up to 20 min), which allows a rapid real time detection of Hg^{2+}. For the practical quantitative detection, fluorescence spectral changes should vary linearly with the concentration of Hg^{2+}.To test this, we carried out fluorescence titration studies of **1a** (0.34 μM) with Hg^{2+} (Fig. 1). A linear response of the fluorescence intensity as a function of [Hg^{2+}] was observed from 22 nM to 0.33 μM (R^2 = 0.999) (inset of Fig. 1). The observed linearity in the nanomolar concentration range allows the quantitative detection of Hg^{2+} in aqueous medium. The detection limit (DL) can be calculated with the equation,[16] DL = $3S_0/m$, where "m" is the calibration sensitivity of the fluorescence intensity change ($\Delta F = F_0 - F$) *vs.* [Hg^{2+}], and "S_0" is the standard deviation of the blank signal (F_0) obtained without Hg^{2+}. From this, the lower detection limit was found to be 2 nM, which is close to the EPA standard.

Changes in the fluorescence intensity of **1a** caused by other metal ions, including Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^+ were also measured as shown in Fig. 2a. Among the metal ions investigated, Cu^{2+} and Ag^+ show 4.9- and 3.4-fold fluorescence quenching, respectively, whereas other metal ions did not show any significant change in the emission intensity even at higher concentration, when compared to Hg^{2+}. Fluorescence quenching of **1a** with Hg^{2+} in the presence of each of the above metal ions (20 μM each) is observed to be similar to that of **1a** with Hg^{2+} alone (*i.e.*, 92-fold quenching), except for Cu^{2+} and Ag^+, which show 95.0 and 94.2 fold fluorescence quenching, respectively. For the rapid monitoring of aqueous Hg^{2+} in

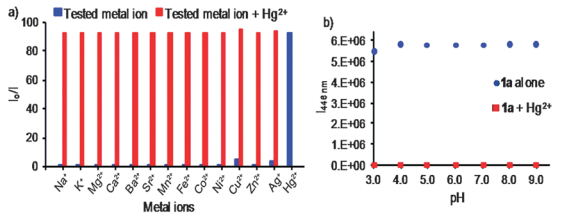
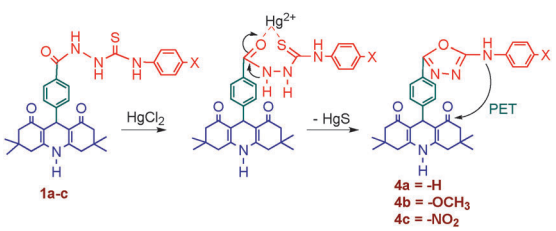


Fig. 2 (a) Fluorescence intensity ratio (I_0/I) of **1a** (3.38 μM) in water–MeOH (99 : 1) upon addition of different metal ions (5 times higher concentration than that of Hg^{2+}); (b) Fluorescence intensity changes of **1a** (3.38 μM) (●) and **1a** (3.38 μM) + Hg^{2+} (3.60 μM) (■) at various pH.

environmental or biological samples, the fluorescence intensity should be resistant to changes in pH that may occur in unbuffered natural systems, or in the analysis of samples from mildly acidic and/or basic environments. The changes in the fluorescence intensity were monitored at solution pH values ranging 3–9, within which most biological samples can be tested. The emission intensity at 446 nm was greatly reduced in strongly acidic medium (pH < 3) due to the protonation of the ADD carbonyl oxygen.[17] On the other hand, in highly basic medium (pH > 11), fluorescence intensity at 446 nm is decreased with the formation of a new emission peak at 515 nm, due to the deprotonation of ring amino hydrogen.[17] No substantial change in the fluorescence intensity of **1a** was observed in the pH range of 4 to 9 (Fig. 2b). Addition of Hg^{2+} to this solution caused a similar (*ca.* 90-fold) fluorescence quenching. The uniform activity over such a wide range of pH makes this molecule suitable for the analysis of environmental samples that would occur well within this extended range of pH, or for use in unbuffered medium.

The high thiophilicity of Hg^{2+} promoted desulfurization leading to the formation of dosimetric product, 1,3,4-oxadiazole (Scheme 2).[18] Due to the removal of thiocarbonyl group, electron density around the aniline moiety is increased, which promotes an efficient intramolecular through-space photo-induced electron transfer (PET) from the aniline moiety to the relatively electron deficient excited state ADD fluorophore. A similar kind of PET process from methoxybenzene substituted at the 9th position of ADD is already reported.[19] To confirm the involvement of a PET process, we have synthesized two more ADD derivatives with strong electron releasing and withdrawing groups at the PET donating aniline moiety. Substitution of electron releasing OCH_3 group (**1b**) leads to a 118-fold fluorescence quenching with the addition of Hg^{2+} (Fig. S2†) due to the enhanced PET in the dosimetric product **4b**, whereas, compound **1c** having electron withdrawing NO_2 group shows only 1.6-fold fluorescence quenching (Fig. S3†) with the addition of Hg^{2+}, due to the diminished PET process in the product **4c**.

Involvement of a PET process was further confirmed from two more ADD derivatives, **2** and **3** which have methyl and tolyl group, respectively at the PET acceptor moiety. Addition of Hg^{2+} to **2** and **3** shows 16- and 20-fold fluorescence quenching (Fig. S4 and S5†), respectively, which is very low as compared to that of **1a** with Hg^{2+}. The presence of the electron releasing methyl group at the PET acceptor (ADD moiety) decreases the extent of PET in the dosimetric product and results in the decreased fluorescence quenching of **2** with the addition of Hg^{2+}, as compared to that of **1a** with Hg^{2+}.



Scheme 2 Hg^{2+} promoted desulfurization and the formation of 1,3,4-oxadiazole derivative.

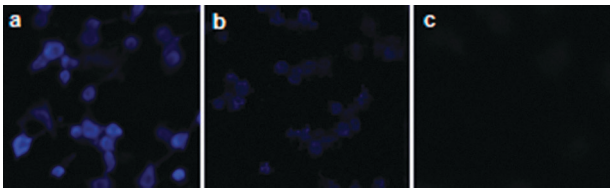


Fig. 3 TPM images of H9C2(2-) cells labelled with **3** (8 μM) upon addition of (a) 0 μM, (b) 2 μM and (c) 10 μM of Hg^{2+}. The TPEF was collected at 420–520 nm upon excitation at 760 nm with fs pulse.

The observed electronic effects and lower fluorescence quantum yield and shorter lifetime of **4a** and **4b** (Table S1†) clearly confirm the operation of PET process in these dosimetric products.

To explore the utility of two-photon excited fluorescence, we have tested the ability of these molecules to detect Hg^{2+} by two-photon excitation. The linear dependence of output fluorescence intensity (I_{out}) on the square of input laser power (mw^2) (Fig. S6†) confirms that this is a two-photon excitation mechanism. Among all the ADD derivatives, compound **3** shows a relatively higher TPA cross section of 14.6 GM, due to the increased conjugation from tolyl group. The two-photon fluorescence excitation spectra (Fig. S7†) of **3** was determined by the two-photon excited fluorescence (TPEF) method, which shows a 10 nm blue shift when compared to one-photon spectra, as observed for many other fluorophores.[20] Compound **3** exhibits a significant TPEF centred at 450 nm. Addition of Hg^{2+} to **3** leads to a gradual fluorescence intensity decrease (Fig. S8†) as observed in single photon excitation. Addition of Hg^{2+} did not show any significant variation in the TP absorption cross section; hence the observed quenching in the intensity is only due to the decreased quantum yield of **3** in the presence of Hg^{2+}. To demonstrate the potential applications of **3** for TPM imaging in living cells, H9C2(2-) cells were cultured and stained with **3** for 15 min, washed with PBS buffer (pH 7.4) to remove the remaining **3**, the treated cells were then incubated with Hg^{2+} in culture medium for 15 min. While the cells treated with only **3** shows bright fluorescence (Fig. 3a), the cells treated with both **3** and Hg^{2+} did not show any fluorescence (Fig. 3c). The fluorescence images clearly indicate that the probe **3** can detect a minimum of <2 μM of Hg^{2+} in live H9C2(2-) cells.

We have developed a selective and sensitive Hg^{2+} probe for the quantitative detection in aqueous medium. The ability of this probe to function in unbuffered aqueous solution with linear and fast fluorescence response will be useful for rapid and quantitative detection of Hg^{2+} in environmental samples. The operation of PET process in the fluorescence signalling action was confirmed by varying the electron releasing and withdrawing groups at the PET donor and acceptor moieties. Even with the lower TPA cross section, we could detect Hg^{2+} by TPM imaging.

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