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Rhodamine based "Turn-On" Fluorescent Probe for Pb(II) and their live cell imaging

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A novel "*turn-on*" fluorescent chemosensor RDP-1 based on rhodamine tri methoxy benzaldehyde conjugate was synthesized, which showed high selectivity and sensitivity towards recognition of Pb^{2+} in aqueous media over other metal ions and also color changes from colorless to pink, allowing colorimetric detection of Pb^{2+} by the naked eye. The sensitivity of probe RDP-1 towards Pb^{2+} was demonstrated in living cells, and can be used for selective imaging of Pb^{2+} in living cells.

Introduction

Selective and sensitive detection of toxic heavy metal ions has been of great interest because these metal ions have caused adverse health effect and environmental problems.[1] Lead(II) is a very important hazardous metal ion because of its widespread application in our daily lives.[2] Nonetheless, it should be noted that it is the second most abundant toxic metal ion responsible for large number of adverse human disorders like memory loss, anaemia, mental retardation, cardiac and neurologic diseases, even when present in low concentrations.[3] Although lead(II) finds wide applications in a number of commonly used materials such as gasoline, batteries, pigments etc., lead poisoning still remains one of the major unresolved problems.[4-6] Based on its toxicity, the permissible limit set by the US Environmental Protection Agency (EPA) and Bureau of Indian Standards is 0.05 mg L^{-1} and 0.1 mg L^{-1} respectively.[7] The determination of lead by analytical methods such as flame or plasma techniques can lead to low precision and sensitivity due to the effects of high back-ground and chemical interferences.[8] Thus, fluorimetric methods are an alternative approach that can provide better selectivity, sensitivity with low cost. Fluorescence based sensors for metal ions gained interest in recent years due to their aids in environmental monitoring by their ability to detect metal ions in very low concentration and also as imaging agents.[9]

Due to operational simplicity, high sensitivity, and low cost, fluorescent detection has become the most promising strategy for detection of analytes.[10] There are many effective fluorescent sensors that have been successfully developed for alkali and alkaline earth metal and heavy metal cations.[9a] Although some sensors are available there is a need for good sensor because its applicability of sensing in water and many reported fluorescent chemosensors generally undergo fluorescence quenching upon binding with these metal ions *via* spin-orbit coupling[9a,111], energy or electron transfer.[12] As fluorescence is the most efficient approach to detect low concentrations of analytes, there are many efforts devoted for the development of chemical sensors for Pb^{2+}. Even though many different kinds of sensors for Pb^{2+} have been reported, most of them suffer from limitations such as poor water solubility, low sensitivity, and low selectivity.[13] In general, the "*turn-on*" response for detecting metal ions is highly preferable in practical applications because the "*turn-off*" response can experience interference by other external factors.[14] Thus, the synthesis of new fluorescent chemosensors that selectively and sensitively detect heavy metal ions in aqueous solutions by "*turn-on*" response is highly challenging. Fluorescence

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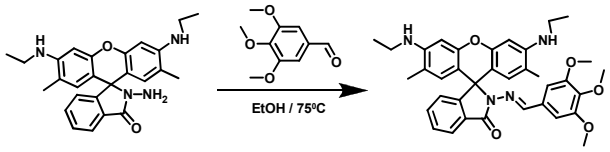
detection of Pb^{2+} ion with chromogenic reagents offers a promising approach for simple and rapid tracking in biological, toxicological, and environmental samples.[15–17] However, it was found that these systems exhibit low aqueous solubility, which involved extractive procedures and thus became more tedious and time consuming, which in turn limit their practical applicability.[18-20] Rhodamine dyes exist in two forms one is spiro form and another one ring opened form, the spiro form is colorless and non-fluorescent whereas ring opened form is pink colored and fluorescent, which makes rhodamines feasible to develop "*turn-on*" chemosensors.[21] To best of our knowledge, there are very few reports on Pb^{2+} sensing based on rhodamine dyes through spirocyclic ring-opening mechanism.[21-24] In this work we have developed a new system for detecting Pb^{2+} fluorogenically as well as colorimetrically. In the present study we report the probe RDP-1 as a highly selective and sensitive colorimetric fluorescent sensor for Pb^{2+}.

Result and Discussion:

Addition of rhodamine 6G hydrazide to 3,4,5-trimethoxy benzaldehyde in ethanol under refluxing conditions yields the formation of the sensing probe RDP-1 (Scheme 1) with 80% yield. The probe RDP-1 was characterised by ^{1}H, ^{13}C-NMR, ESI-MS and elemental analysis. (Fig. S1 to S3 in ESI†)



The probe did not show any observable absorption in the visible region evidencing that the probe RDP-1 was persisting as the lactam form in the solution. Upon addition of Pb^{2+}, an absorption peak emerged at 530 nm and also colour changes immediately from colourless to pink (**Fig. 1**) and the peak centred at 530 nm emerged in the UV-vis spectra only with the introduction of Pb^{2+} ions. The peak at 530 nm is a characteristic peak for the amide form of the rhodamine dye. This absorption change in visible region makes feasible the colorimetric detection of Pb^{2+} by colour changes from colourless to pink. To check the selectivity, the Probe RDP-1 is investigated in the presence of other metal ions Na^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Fe^{2+}, Cr^{3+}, Ni^{2+}, Al^{3+}, Ag^{+} and Co^{2+} by UV-vis spectroscopy (**Fig. 2**). The absence of colour change for other metal ions also revealed that the probe RDP-1 selectively senses Pb^{2+}. The change in the absorbance wavelength makes feasible to distinguish Pb^{2+} from other metal ions.

Fig. 1 Photograph showing colorimetric change during addition of Pb^{2+} to the probe RDP-1.

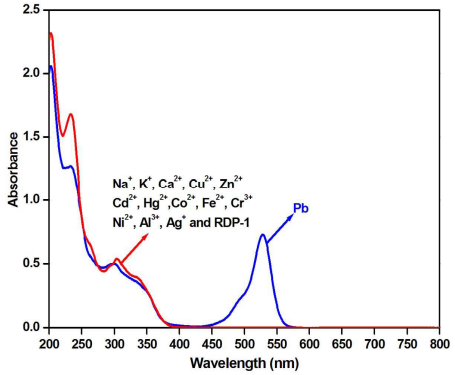


Fig. 2 UV–vis absorption spectra of probe RDP-1 (10 μM) upon addition of various metal ions in HEPES buffer (1×10^{-5} M). Inset shows the changes in the absorption intensity at 530 nm upon addition of Pb^{2+} (0-10 μM).

The incremental addition of Pb^{2+} ion leads to a gradual enhancement in the absorption intensity at 530 nm. Hence the probe RDP-1 can be utilised as a colorimetric sensor for Pb^{2+}.

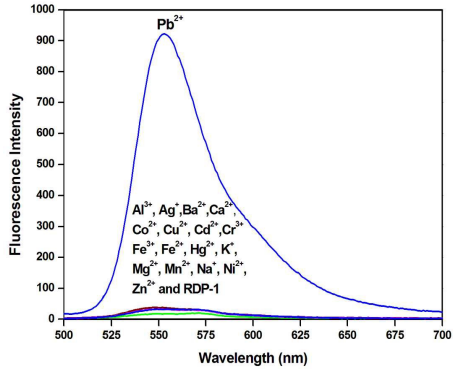


Fig. 3 Fluorescence spectra of probe RDP-1 (10 μM) upon addition of various metal ions in HEPES buffer (1×10^{-5} M), λex = 500 nm.

The fluorescent properties of the probe RDP-1 in the presence of various cations were studied in HEPES buffer solution. The probe RDP-1 did not displayed any appreciable emission upon excitation at 500 nm. Upon addition of Pb^{2+} ions culminated in an intense emission band enhancement at 552 nm (**Fig. 3**). The fluorescence titration of RDP-1 with 0-1.5 equivalent of Pb^{2+} ions resulted in

gradual enhancement in the fluorescence intensity at 552 nm(**Fig. 4**) to 100 folds, whereas addition of other metal ions such as Na^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{2+}, Cr^{3+}, Ni^{2+}, Al^{3+}, Ag^{+} and Co^{2+} did not result in any observable fluorescence enhancement (**Fig. 5**).

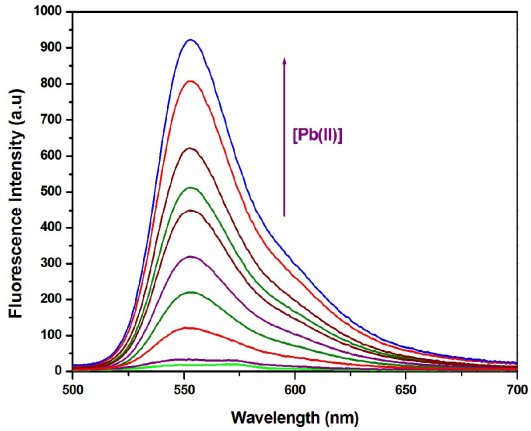


Fig. 4 Concentration-dependent fluorescence enhancement of RDP-1 (10 μM) on the addition of various amounts of Pb^{2+} (0- 10 μM) in HEPES buffer solution (pH 7.54), λex = 500 nm.

The increase in fluorescence intensity was attributed to conversion of rhodamine spiro form to amide form induced by Pb^{2+} ion; formation of RDP-1+ Pb^{2+} complex.[25] From the absorption and fluorometric results it is revealed that the probe RDP-1 is very selective and sensitive towards Pb^{2+} over other metal ions. To confirm the stoichiometry of the binding of probe RDP-1 with Pb^{2+} Job's plots analysis was carried out. The plot of the fluorescence intensity variation at 550 nm against mole fraction of the Pb^{2+} clearly showed the maxima with a mole fraction at 0.5 indicating 1 : 1 stoichiometry and further supported by the peak at 815.27 (RDP-1 + Pb^{2+}+H^{+}) ESIMS analysis(Fig. S4 and S5).

Similarly Job's plot were derived from fluorescence analysis also conforms to the 1:1 binding of RDP-1 with Pb^{2+} ions. The linear plot was obtained when plotting fluorescence intensity vs. concentration of Pb^{2+} ions indicating that the probe RDP-1 can be used to determine Pb^{2+} ion concentration. The titration profile shows that a steady increase in fluorescence intensity with increasing concentration of Pb^{2+}, after addition of 1 equivalent it reaches the saturation level. The detection limit is found to be 15 nM.[25] The association constant between RDP-1 and Pb^{2+} was 3.432×10^{2}, determined[26] from fluorimetric titration data.

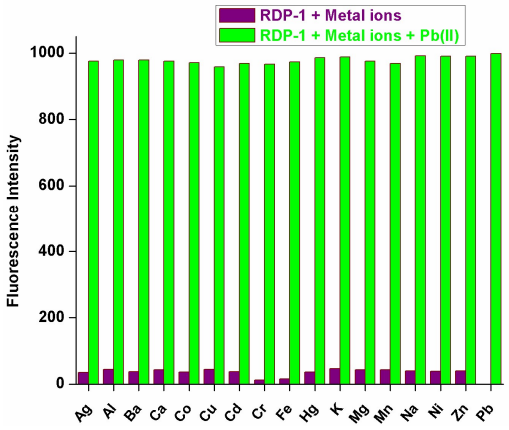


Fig. 5 Fluorescence response of 10 μM RDP-1 to various mixture of Pb^{2+} and other metal ions (Total concentration: 10 μM (5 μM of Pb(II) + 5 μM of other metal ions) . The violet bars represent the addition of the corresponding metal ion to RDP-1. The green bars represent the change of the emission that occurs upon the subsequent addition of Pb^{2+} to the above solution. Excitation at 500 nm, (slit width = 5 nm).

In order to check the applicability of the probe for Pb^{2+} sensing in different pH, pH metric titrations were carried out. The probe RDP-1 showed enhanced fluorescence in the high acidic pH but it is stable in the rest pH range whereas RDP-1+ Pb^{2+} did not showed any fluorescence in highly basic range, due to the formation of $Pb(OH)_2$. This clearly indicates the applicability of the probe in sensing Pb^{2+} ion in physiological pH range. The selectivity of the Probe RDP-1 towards Pb^{2+} is explained in terms of preferred interaction and size offered for coordination by the ligands. The probe RDP-1 perfectly accommodates Pb^{2+} ions whereas other metal ions doesn't bind effectively because of soft-soft interaction of the probe RDP-1towards Pb^{2+} ions. It is evidenced by the higher association constant of the RDP-1with Pb^{2+} ions. Therefore coordination of the Pb^{2+} ions leads to ring opening of the rhodamine dye that leads to the colorimetric and fluorescence change (Scheme-2).

The response time of the probe RDP-1 towards Pb^{2+}sensing was calculated by fluorescence spectroscopy in different time intervals. It is observed that the RDP-1 shows both colorimetric and fluorescence change immediately after addition of Pb^{2+}ions. Hence the probe RDP-1 responds towards Pb^{2+} in faster manner. (Fig.S9)

We next evaluated the potential utility of probe RDP-1 for fluorescence imaging of Pb^{2+} in living cell. The cytotoxicity of RDP-1 to HeLa cells was evaluated by standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The results clearly indicate that the RDP-1 was non-toxic to the HeLa cells under the experimental conditions (figure-S11). The HeLa cells

were incubated with probe RDP-1 (10.0 μM) in HEPES buffer at 37°C for 30 min and imaged through confocal fluorescence microscopy with 500 nm excitation. Then it was treated with $PbCl_2$ (10 μM) for 30 min, the intracellular uptake of Pb^{2+} ions complexes with probe RDP-1 yielded fluorescent ensemble. Therefore, the "*off-on*" fluorescence imaging of probe RDP-1 was accomplished in HeLa cells by the intracellular conversion of rhodamine spiro form to amide form modulated by Pb^{2+} ion (**Fig. 6**). The significant red fluorescence from the intracellular region proves that the probe RDP-1 is applicable for imaging Pb^{2+} in living cells. The bioimaging in HeLa cells confirms the fluorescence enhancement with excellent cell permeability. It shows that RDP-1 is biocompatible in nature and can be used for detecting Pb^{2+} ions in cells rapidly.

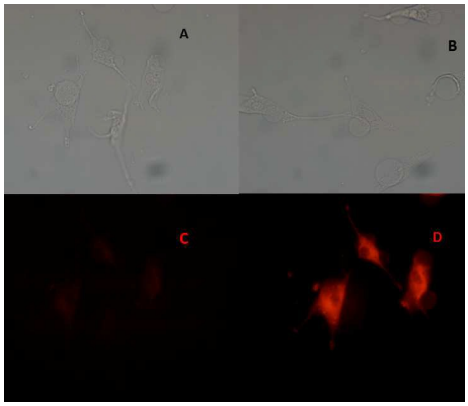


Fig. 6 (a) and (b) Bright-field image of RDP-1 and RDP-1+$PbCl_2$ treated HeLa cells respectively. (c) and (d) fluorescence image of HeLa cells incubated with RDP-1 subsequently treated with (10 μM) Pb^{2+} λex = 500 nm.

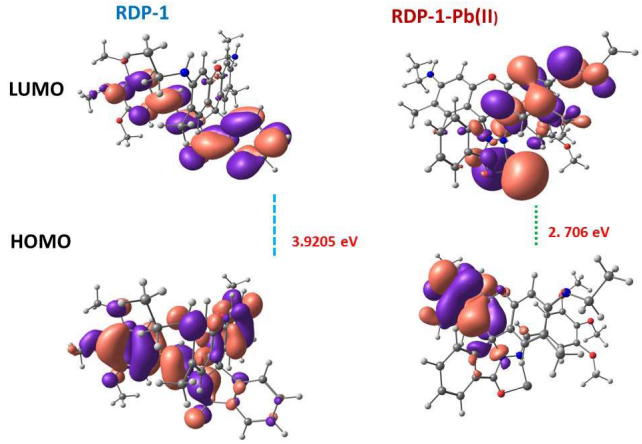
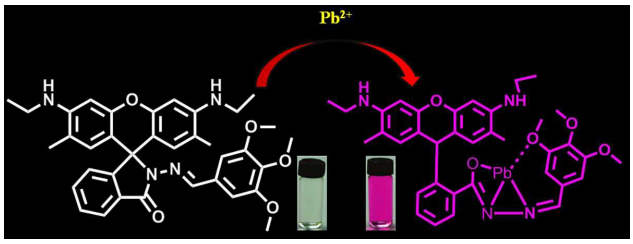


Fig.7 Frontier molecular orbitals of RDP-1 and RDP-1+ Pb^{2+} obtained from the DFT calculations using Gaussian 09 program.



Scheme-2: plausible mechanism for Pb^{2+} sensing by RDP-1.

To get an insight into the electronic structure and the photophysical properties of RDP-1 and RDP-1+Pb^{2+} adduct, density functional theory (DFT) calculations were carried out with the Gaussian 09 program[27] with the B3LYP/6-311G basis sets. From the optimized geometries the TDDFT calculations were carried out using aforementioned sets. Frontier molecular orbitals derived from the optimized geometries, the HOMO and LUMO of RDP-1 is localized over the whole xanthenyl ring and methoxy unit, respectively, whereas in RDP-1+ Pb^{2+} the xanthenyl unit has HOMO character and the methoxy unit with reasonable contribution from Pb^{2+} has LUMO character (**Fig. 7**). This clearly indicates that the clear operation of internal charge transfer that is the cause for observed UV-absorption and fluorescence change.

Conclusion

We have designed and synthesized a rhodamine based derivative RDP-1 as highly sensitive and selective chemosensor for Pb^{2+} ion. The probe RDP-1 facilitates the detection of Pb^{2+} even at nanomolar concentration (15 nM). DFT calculations reveals that the observed photophysical change due to the change in internal charge transfer followed by spiro ring opening. Further the sensor RDP-1 has the feasibility of detecting Pb^{2+} in HeLa cells under physiological conditions.

Experimental section

Synthesis of RDP-1:

Rhodamine 6G hydrazide (1.09 mmol) and 3,4,5-trimethoxybenzaldehyde (1.09 mmol) were refluxed in 10 ml ethanol for about 6 hours in the presence of catalytic amount of acetic acid. The reaction mixture upon cooling to room temperature, had thrown out a colourless solid. The precipitated solid was then purified by column chromatography using dichloromethane - ethyl acetate (9:1) to yield colourless solid. Probe: ^{1}HNMR (300 MHz, $CDCl_3$): 8.445 (s,1H), 8.034-8.019(1H,d), 7.517-7.490(2H,dd,J=12Hz), 7.119-7.094(1H, m), 6.801-6.774(2H, d, J= 8.1Hz), 6.421(s,2H), 6.348(s,2H), 3.93(s,3H), 3.840(s,3H), 3.747(s,3H),3.502(s,2H, Br), 3.237-3.214(m,4H), 1.874(s,6H), 1.361-1.310(t,6H, J= 9Hz). 13C-

NMR(75 MHz): 164.6, 152.9,151.5, 151.4, 147.4, 145.9, 139.4,133.3, 130.8, 129.3,128.2, 127.5, 123.75, 123.26, 117.8, 106.4, 104.3,96.5, 66.0, 60.7, 56.0, 38.2, 16.5, 14.6 Elemental analysis: calculated: C, 71.27; H, 6.31; N, 9.23; observed: C, 71.19; H, 6.28; N, 9.13%; MS (ESI): 606.57, calculated: 606.28.

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