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PAPER

A rhodamine appended tripodal receptor as a ratiometric probe for Hg^{2+} ions†

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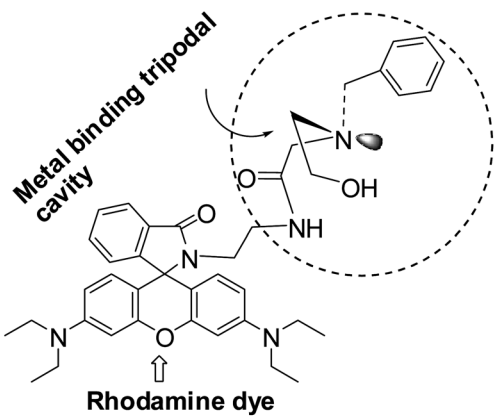
A new rhodamine appended tripodal receptor **1** has been designed and synthesized. The receptor selectively recognizes Hg^{2+} ions in CH_3CN–water (4 : 1, v/v; 10 μM tris HCl buffer, pH 7.0) by displaying a ratiometric change in emission. Additionally, the visual detection is possible by a sharp change in color. The receptor shows *in vitro* detection of Hg^{2+} ions in human cervical cancer (HeLa) cells.

Introduction

Design and synthesis of fluorescent chemosensors for the selective sensing and recognition of the toxic heavy metal ions, such as Hg^{2+}, Pb^{2+} and Cd^{2+} ions *etc*.,[1] is of considerable importance because of the severe immunotoxic, genotoxic, and neurotoxic effects of those metal ions. Among the different heavy metal ions, mercury ions (Hg^{2+}) are considered to be dangerous as they can accumulate in the human body and affect a wide variety of diseases even in a low concentration, such as prenatal brain damage, serious cognitive disorders, and Minamata disease.[2] Therefore, it is important to develop highly sensitive and selective assays for Hg^{2+} ions. In recent years, considerable efforts have been made in the design of fluorescent chemosensors of different architectures for the selective sensing of Hg^{2+} ions.[3–6] The receptors belonging to this category are of three types depending on the nature of their responsive modes (turn-on, turn-off and ratiometric). Among the three modes, ratiometric probes (especially, rhodamine-based) for the detection of Hg^{2+} ions[4] are considerably important and they are relatively less in number than the 'turn-on'[5] based receptors. The ratiometric chemosensors offer advantages over the conventional monitoring of fluorescence intensity at a single wavelength. A dual emission system can minimize the measurement errors because of the factors such as phototransformation, receptor concentrations, and environmental effects.[6]

As the continuation of our work on the sensing of cations[7] and anions[8] of biological significance, we report in this full account a new receptor module **1** in which the tripodal metal binder scaffold (see the encircled part in structure **1**) is connected to the rhodamine dye. In our earlier publication,[7a] we reported a new synthesis and the metal binding characteristic features of a tripodal receptor. To further explore this tripodal centre in wider aspect, we presently focus our attention with the rhodamine labelled new structure **1** to detect the metal ion both by fluorometrically and colorimetrically in semi-aqueous system. It is mentionable that rhodamine B and its derivatives (RhB) are well known for their good photo stability, high extinction coefficient (>75 000 cm^{-1} M^{-1}), and high fluorescence quantum yield.[9] The functioning of this moiety in the chemosensor towards metal ion is related with the switching of the spirocyclic form (which is colorless and non-fluorescent) to the ring-opened amide form which is pink and strongly fluorescent.[4,5] A number of rhodamine labeled receptors of this class are known which show color change and selective "turn-on" response to Hg^{2+} ions.[5] However, in the present case, receptor **1** shows high sensitivity and selectivity towards Hg^{+2} ions by exhibiting both colorimetric and ratiometric fluorescence responses in CH_3CN–water (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0).



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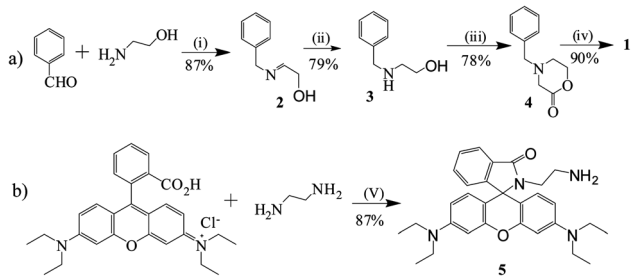
† Electronic supplementary information (ESI) available: Figures showing the change in fluorescence and UV-vis titrations of receptor **1** with various metal ions, Job plot, spectral data (NMR, mass) for **1**. See DOI: 10.1039/c2ob00009a



Scheme 1 (a) (i) Dry MeOH, reflux, 9 h; (ii) $NaBH_4$, dry MeOH, reflux, 3 h; (iii) Ethyl 2-chloroethanoate, K_2CO_3, dry acetone, reflux, 7 h; (iv) **5**, THF, stirring, 4 h; (b) (v), EtOH, reflux, 9 h.

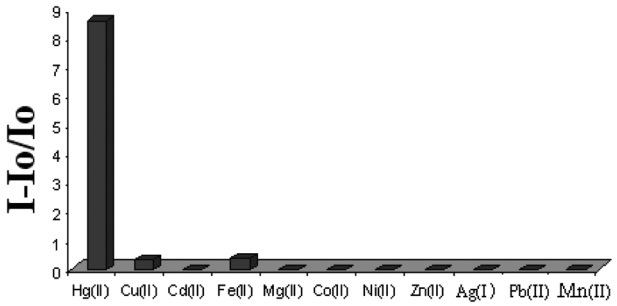


Fig. 1 Change in fluorescence ratio of **1** (c = 4.41 × 10^{-5} M) at 580 nm upon addition of 18 equiv. amounts of cations.

Results and discussion

The receptor **1** was synthesised according to Scheme 1. Initially the intermediate compound 4-benzylmorpholin-2-one **4** was synthesized according to our reported procedure.[7c] The Schiff base **2**, which was obtained from the condensation of benzaldehyde with 2-aminoethanol, was reduced to the amine **3** using $NaBH_4$ in MeOH. The reaction of the amine **3** with ethyl 2-chloroethanoate under refluxing condition gave 4-benzylmorpholin-2-one **4** in appreciable yield. Further reaction of the intermediate compound **4** with the rhodamine labelled amine **5**[10] furnished the desired compound **1** in good yield. The compound **1** was fully characterized by ^{1}H NMR, ^{13}C, FTIR, mass and elemental analyses.

The spectroscopic properties of the receptor **1** towards the metal ions such as Hg^{2+}, Cu^{2+}, Cd^{2+}, Fe^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+} and Ag^{+} (taken as their perchlorate salts) were rated in CH_3CN–H_2O solution ($CH_3CN : H_2O$ = 4 : 1, v/v, 10 μM tris HCl buffer, pH = 7.0). In this context, a higher content of water in aqueous measuring solution is seemed to be desirable. But such an approach is constrained by the limited solubility of **1** in water. As a reasonable compromise, a 4 : 1 ($CH_3CN : H_2O$) aqueous CH_3CN solution was used in the subsequent study using 10 μM tris HCl buffer for maintaining pH 7. Initially, the emission of **1** was monitored upon successive addition of the metal ions to the receptor solution in aq. CH_3CN ($CH_3CN : H_2O$ = 4 : 1, v/v, 10 μM tris HCl buffer, pH = 7.0). On excitation at 510 nm, a nonstructured emission at 536 nm underwent significant quenching upon interaction with all the metal ions studied. However, the change in fluorescence ratio at 536 nm for all the metal ions was found to be almost the same in magnitude and there are no distinguishable attributes (Fig. 3S; ESI†). In contrast, in the case of Hg^{2+}, a new peak at 580 nm appears with remarkable intensity. Fig. 1, in this regard, shows the change in fluorescence ratio $[(I–I_0)/I_0]$ of **1** at 580 nm in the presence of 18 equiv. amounts of the different metal ions. As can be seen from Fig. 1, the receptor is highly sensitive to Hg^{2+} ion. In comparison, other metal ions merely perturbed the emission of **1** at this wavelength (ESI†).

Fig. 2 describes the emission titration spectra obtained from the gradual addition of $Hg(ClO_4)_2$ solution to the solution of **1** (c = 4.41 × 10^{-5} M) in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0). While the emission centered at 536 nm decreases, a new band at 580 nm begins to appear on progression

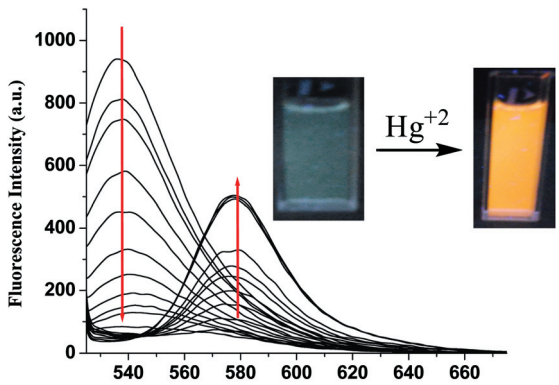


Fig. 2 Fluorescence titration spectra of **1** (c = 4.41 × 10^{-5} M) in CH_3CN–water (4 : 1, v/v; 10 μM tris HCl buffer, pH 7.0) upon addition of Hg^{2+}; Inset: Colour change of the receptor solution under illumination of UV light.

of the titration of **1** with Hg^{2+} and exhibits a ratiometric change. The ratiometric calibration curve is displayed in Fig. 3a.

Importantly, the increase in concentration of buffer in CH_3CN–H_2O solution ($CH_3CN : H_2O$ = 4 : 1, v/v, 35 μM tris HCl buffer, pH = 7.0) did not alter the ratiometric nature of emission spectra as well as the extent of change in intensity while titration was performed with Hg^{2+} ions (Fig. 5S in ESI†). This experimental finding (*i.e.*, ratiometric fluorescence change) distinguishes the Hg^{2+} ion from the other metal ions examined. To the best of our knowledge, such rhodamine-based receptor for ratiometric detection of Hg^{2+} is a new addition to the existing reports[4] in the literature.

Furthermore, the plot of ratio of fluorescence response at the wavelengths 536 and 580 nm is shown in Fig. 3b, which indicates that the response factor is noteworthy for Hg^{2+} ions. This is also found to be true when the ratiometric fluorescent response of **1** was recorded in the presence of 18 equiv. amounts of other metal ions considered in the study. Fig. 4 signifies this feature. It is quite comprehensible from Fig. 4 that the ratiometric behaviour of **1** is unperturbed in the presence of other metal ions and thereby indicated its high selectivity and sensitivity towards Hg^{2+} ions. As can be seen from Fig. 4, Ag^{+} and Pb^{2+} ions which interfere in Hg^{2+}-ratiometric sensors,[11] did not perturb the ratiometric behaviour of **1** towards Hg^{2+} ions.

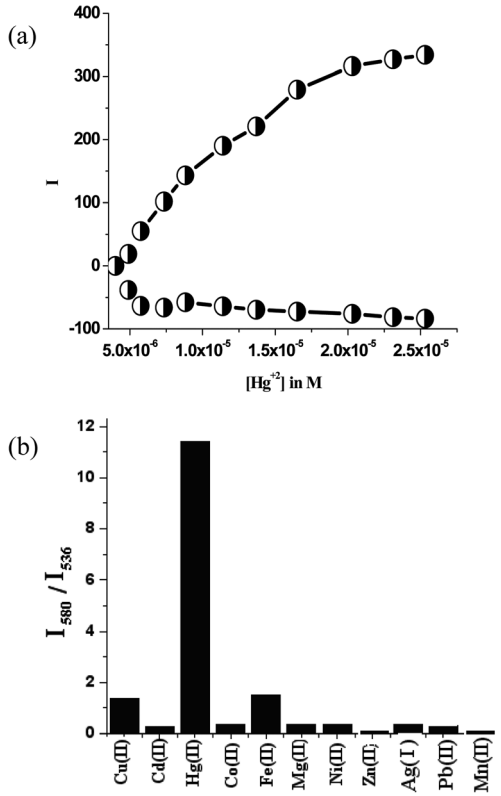


Fig. 3 (a) Ratiometric calibration curve for **1** (c = 4.41 × 10^{-5} M) with Hg^{+2}; (b) Ratiometric fluorescent response of **1** (c = 4.41 × 10^{-5} M) with the addition of 18 equiv. amounts of each metal ion (c = 8.82 × 10^{-4} M) in CH_3CN–water (4 : 1, v/v; 10 μM tris HCl buffer; pH = 7.0).

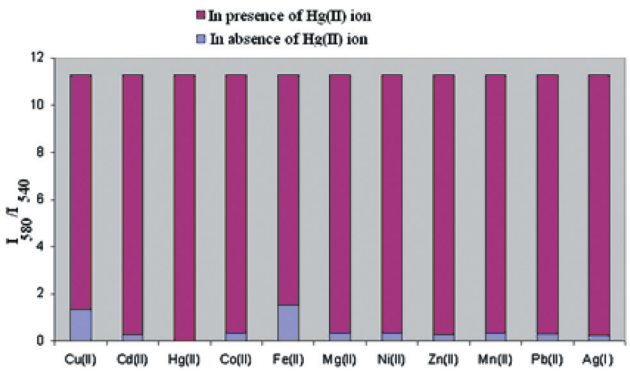


Fig. 4 Ratiometric fluorescent response of sensor **1** (c = 4.41 × 10^{-5} M) to Hg^{2+} (c = 8.82 × 10^{-4} M) over the selected metal ions (c = 8.82 × 10^{-4} M).

In our opinion, the ratiometric response of **1** towards the Hg^{2+} ion is attributed to the involvement of the binding centre in different ways. We believe that initially, the aliphatic tertiary amine nitrogen together with the alcoholic and amide functionalities in the tripodal centre interact with the Hg^{2+} ion (see Form **1A** in Fig. 5) in the same way as described in our earlier report.[7c] Due to this interaction the emission intensity at 536 nm decreases. Then the closely spaced spirocyclic form of the rhodamine part is opened and provides an amide ion to chelate the

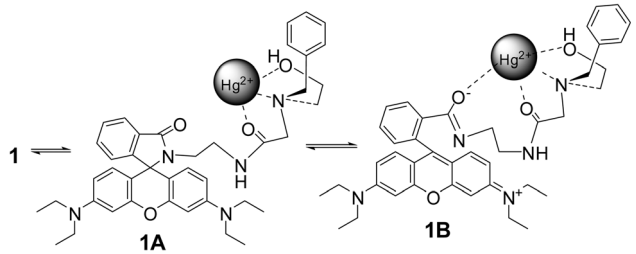


Fig. 5 Suggested modes of interaction of **1** with Hg^{2+} ion in solution.

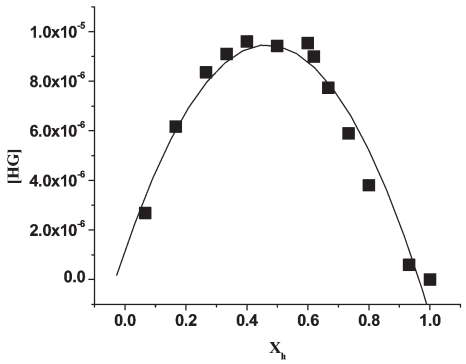


Fig. 6 Fluorescence Job plot for **1** with Hg^{+2} in CH_3CN–water (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0) ([H] = [G] = 4.41 × 10^{-5} M).

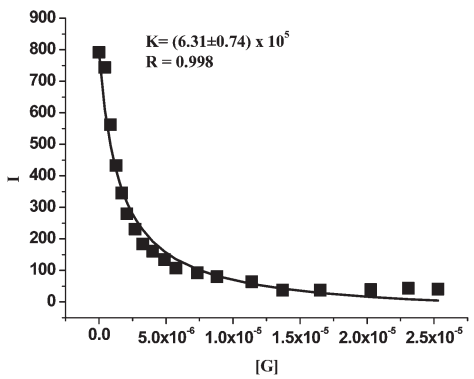


Fig. 7 Nonlinear curve fitting of the fluorescence titration data for **1** (c = 4.41 × 10^{-5} M) with Hg^{2+} in CH_3CN–water (4 : 1, v/v; 10 μM tris HCl buffer; pH = 7.0) upon addition of Hg^{+2}.

Hg^{2+} ion strongly (Form **1B** in Fig. 5) for which a new peak at 580 nm begins to appear with significant growth on progression of the titration. The absence of this peak in the cases of all the metal ions except Hg^{2+} indicates that the ring opening step is not occurring during the course of interaction. In the interaction process, the stoichiometry of the Hg-complex was evaluated to be 1 : 1, as evident from the Job's method of continuous variations (Fig. 6).[12] From the titration data, the binding constant (K_a) for the formation of **1**. Hg^{2+} complex was estimated by a nonlinear curve fitting procedure and it was found to be (6.31 ± 0.74) × 10^5 M^{-1} (Fig. 7). The nonlinear fit was done using eqn (1)[13]

Table 1 Binding constant values (K_a in M^{-1}) for **1** with the metal ions in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0) by fluorescence method

Guests	K_a (M^{-1}) at 536 nm
Hg^{+2}	$(6.31 \pm 0.74) \times 10^5$
Cu^{+2}	$(9.40 \pm 0.25) \times 10^4$
Fe^{+2}	$(3.20 \pm 0.37) \times 10^4$
Mg^{+2}	$(2.43 \pm 0.09) \times 10^4$
Co^{+2}	$(2.04 \pm 0.09) \times 10^4$
Cd^{+2}	$(1.74 \pm 0.13) \times 10^4$
Zn^{+2}	$(3.50 \pm 0.39) \times 10^4$
Ni^{+2}	$(2.70 \pm 0.14) \times 10^4$
Mn^{2+}	$(4.28 \pm 0.82) \times 10^4$
Pb^{2+}	$(1.50 \pm 0.14) \times 10^4$
Ag^{+}	$(1.41 \pm 0.21) \times 10^4$

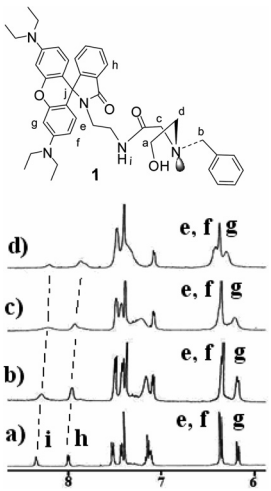


Fig. 8 Partial ^1H NMR of (a) **1** (7.96×10^{-3} M) and with (b) 0.5 equiv. (c) 1 equiv. and (d) 2 equiv. amounts of Hg^{2+} in CD_3Cl containing 4% d_6-DMSO.

$$I = I_0 + (I_{lim} - I_0)/2C_H\{C_H + C_G + 1/K_a - [(C_H + C_G + 1/K_a)^2 - 4C_HC_G]^{1/2}\} \quad (1)$$

where I represents the intensity; I_0 represents the intensity of pure host; C_H and C_G are corresponding concentrations of host and cationic guest; K_a is the binding constant. The binding constant (K_a) and correlation coefficients (R) were obtained from a non-linear least-square analysis of I *vs.* C_H and C_G.

Using eqn (1), the binding constant values for the other metal ions were also determined (Table 1) and they were found to be less than that of Hg^{2+}. This underlines the fact that the strong interaction of Hg^{2+} over the rest of the metal ions is due to the existence of the Form **1B**. The formation of **1B** through ring opening was established from both the ^1H NMR and FTIR studies. In FTIR, the amide carbonyl stretching of the spirolactam part appeared at 1683 cm^{-1} changed to a lower wavenumber (1678 cm^{-1}) in the presence of Hg^{2+}. Also in ^1H NMR, the ring protons (e, f and g; see the labeling in Fig. 8) of the rhodamine part moved to the downfield direction ($\Delta\delta_e = 0.02$, $\Delta\delta_f = 0.07$ and $\Delta\delta_g = 0.05$) in the presence of 2 equiv. amounts of Hg^{2+} ions. Fig. 8, in this regard, shows the change in ^1H NMR of **1** in

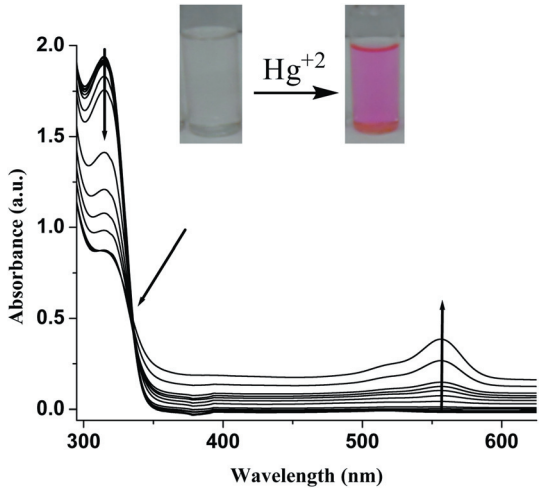


Fig. 9 Absorption titration spectra of **1** ($c = 4.41 \times 10^{-5}$ M) in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0) upon addition of Hg^{+2}.

the absence and presence of different amounts of Hg^{2+}ions. The 'h' proton showed an upfield shift of 0.13 ppm. This upfield movement is attributed to the increase in electron density arising from the opening of the spirolactam ring. Indeed, in ^{13}C NMR the disappearance of the signal at 66.03 ppm for the tertiary carbon of the spirolactam ring of **1** (labeled as 'j'; see Fig. 8) upon addition of 1.2 equiv. amounts of Hg^{2+} (ESI†) convincingly proved the ring opening[14] to give the form **1B** in Fig. 5. Furthermore, the cooperative involvement of the tripodal cavity in complexation is proved from the downfield shift of the signals for 'a', 'b', 'c' and 'd' type protons by 0.13, 0.22, 0.09 and 0.02 ppm, respectively in the presence of 2 equiv. amounts of Hg^{2+} ions. The amide proton (i-type) appeared at 8.34 ppm underwent a small upfield shift by 0.14 ppm. This suggests that the amide oxygen instead of nitrogen participates in the metal coordination.

Interestingly, during the fluorometric titration of **1** with Hg^{2+} ions the colourless solution of the receptor became pink. This pink color is attributed to the opening of the spirolactam ring and generation of the delocalised xanthene moiety.[4,5,15] Under the illumination of UV light a yellowish brown colour of the receptor solution containing Hg^{2+} was noticed (inset of Fig. 2). This was not observed with other metal ions except Cu^{2+} and Fe^{2+} ions. In case of Cu^{2+} and Fe^{2+} ions, the colourless solution of **1** turned into a very faint pink colour. Therefore, the receptor **1** is also capable of reporting the presence of Hg^{2+} ion in solution colorimetrically. But it is interesting to note that the receptor **1** did not show any color change while performing the titration using 8.7×10^{-6} M concentration of Hg^{2+}. Even the ratiometric behavior in emission was not observed (Fig. 6S in ESI†).

The UV-vis spectrum of **1** ($c = 4.41 \times 10^{-5}$ M) in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0) shows a strong absorption at 315 nm which decreases followed by an appearance of a new absorption at 555 nm upon gradual addition of mercury solution and resulted in an isosbestic point at 333 nm (Fig. 9). The growth of the absorption peak at 555 nm is the consequence of the opening of the spiroring in **1**.[11c,15] This was not

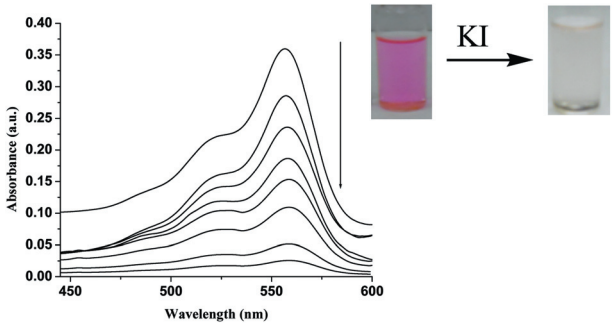


Fig. 10 Change in absorbance of **1**-Hg^{+2} complex in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0) upon addition of KI (c = 1.5 × 10^{-3} M); Inset: colour change upon addition of KI.

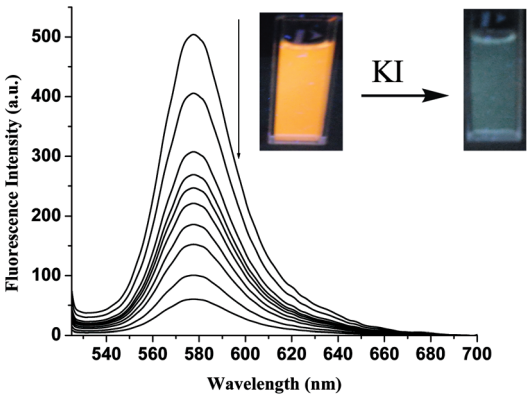


Fig. 11 Change in fluorescence of **1**-Hg^{+2} complex (c = 6.81 × 10^{-5} M) in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0) upon addition of KI (c = 1.5 × 10^{-3} M); Inset: colour change under exposure of UV light upon addition of KI.

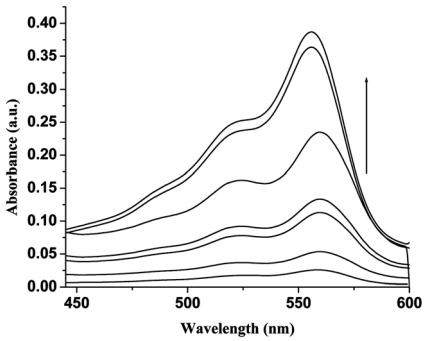


Fig. 12 Change in absorbance from the addition of $Hg(ClO_4)_2$ (c = 1.5 × 10^{-3} M) to the solution of **1**-Hg^{+2} containing KI in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0).

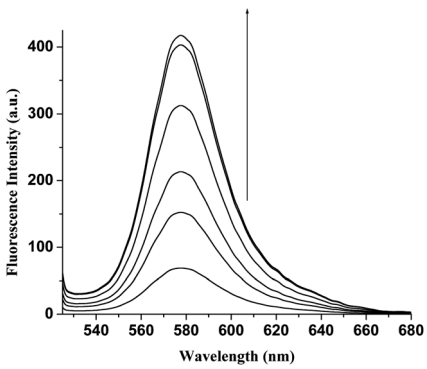


Fig. 13 Change in emission from the addition of $Hg(ClO_4)_2$ (c = 1.5 × 10^{-3} M) to the solution of **1**-Hg^{+2} containing KI in CH_3CN–H_2O (4 : 1, v/v; 10 μM tris HCl buffer; pH 7.0).

observed when the titrations were conducted with other metal ions (ESI†). The stoichiometry of the Hg-complex in the ground state was also found to be 1 : 1 (ESI†).

To check the reversibility in the complexation, a KI-adding experiment was performed. Addition of KI to the solution of complex of **1** with Hg^{2+} brought the reverse change in the absorption spectra (Fig. 10). A similar finding was observed in emission (Fig. 11). Interestingly, while KI diminished the absorbance and emission significantly, further addition of excess Hg^{2+} ions could recover both the absorbance and emission signals. In this regard, Figs. 12 and 13 represent the recovery of both the absorbance and emission respectively. In the event the pink color of the solution is also retrieved.

Similar to many reported rhodamine spirolactam-based fluorescent probes, the fluorescence enhancement response of **1** toward Hg^{2+} is most likely the result of the spiro ring-opening mechanism rather than an ion-catalyzed hydrolysis. The above-mentioned KI experiment could serve as experimental evidence to support this reversible spiro ring-opening mechanism. Like KI, addition of aq. solutions Na_2EDTA and cysteine brought about similar change in both absorbance and emission spectra (Fig. 7S in ESI†) and further confirmed the reversibility in the binding process.

The potential biological application of the receptor was evaluated for *in vitro* detection of Hg^{2+} ions in human cervical cancer (HeLa) cells. The HeLa cells were incubated with 5 μl of receptor **1** (10 μM in CH_3CN–H_2O (4 : 1, v/v)) in DMEM (Dulbecco's modified Eagle's medium) medium (without FBS) for 30 min at 37 °C and washed with phosphate buffered saline (PBS) buffer (pH = 7.4) to remove excess of receptor **1**. DMEM medium (without FBS) was again added to the cells. The cells were then treated with 5 μl of mercury perchlorate (30 μM) and incubated again for 30 min at 37 °C. A control set of cells which was devoid of Hg^{2+} ion was kept. The addition of receptor **1** to the cells did not show any cytotoxicity as evident from the morphology of the cells.

In this regard, Fig. 14a and 14b represent the bright field images of the cells before and after treatment of the cells with **1**, respectively. Cells incubated with receptor **1** without Hg^{2+} (Fig. 14c) and cells incubated with Hg^{2+} without receptor **1** (Fig. 14d) did not show any fluorescence properties. In contrast, cells incubated with the receptor **1** and then with Hg^{2+} ions showed the occurrence of red fluorescence indicating the permeability of the receptors inside the cells and binding of Hg^{2+} with the receptor (Figs. 14e and 14f).

The KI-adding experiments which could serve as experimental evidence to support the reversibility in structural change, were also applied to human cervical cancer (HeLa) cells. Red coloured cells obtained from the incubation of the receptor

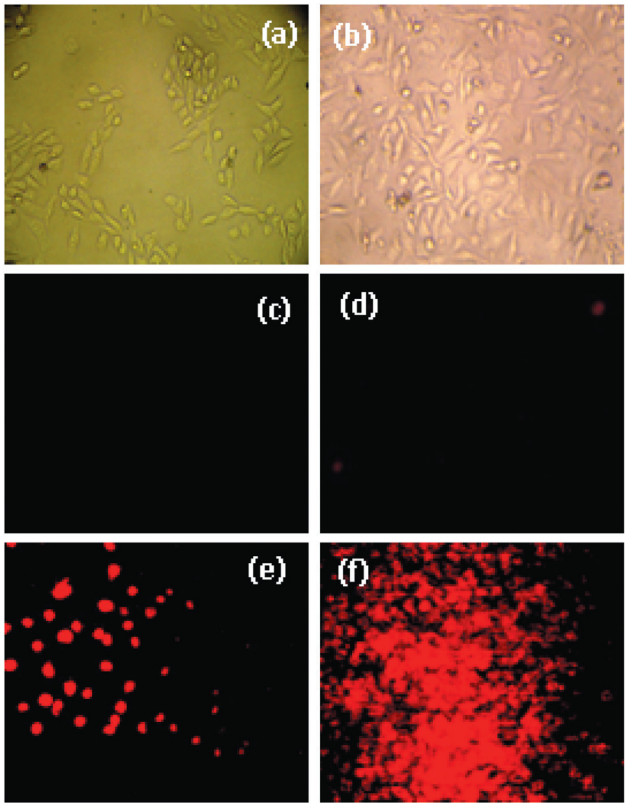


Fig. 14 Fluorescence and bright field images of HeLa cells: (a) Bright field image of normal cells, (b) Bright field image of cells treated with receptor **1** (10 μM) for 1 h at 37 °C, (c) Fluorescence image of cells treated with **1** (10 μM) for 1 h at 37 °C, (d) Fluorescence image of cells treated with $Hg(ClO_4)_2$ (30 μM) for 1 h at 37 °C, (e) Red fluorescence images of cells upon treatment with receptor **1** (10 μM) and then $Hg(ClO_4)_2$ (30 μM) for 1 h at 37 °C, (f) Red fluorescence images of cells upon treatment with **1** (10 μM) and then $Hg(ClO_4)_2$ (30.0 μM) for 2 h at 37 °C; λ_{ex} = 510 nm.

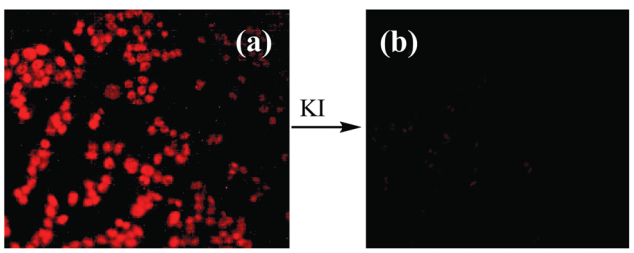


Fig. 15 Fluorescence images of HeLa cells: (a) Red fluorescence images of cells upon treatment with receptor **1** (10 μM) and then Hg $(ClO_4)_2$ (30 μM) after 1 h, (b) Red fluorescence images of cells upon addition of KI (30 μM) to the ensemble of **1** (10 μM) and $Hg(ClO_4)_2$ (30 μM) after 1 h.

followed by treatment with Hg^{2+} became invisible in fluorescence upon addition of KI (30 μM) (Fig. 15).

Conclusions

In conclusion, we have thus synthesized a rhodamine labeled flexible tripodal cavity **1** which selectively recognizes Hg^{2+} ions through a change in color as well as emission in ratiometric fashion. This ratiometric chemosensor for Hg^{2+} ions is a new example among the few reports in the literature.[4] The cooperative action of the binding groups of the tripodal cavity and the amide ion generated from the spirolactam ring opening favors the strong chelation of Hg^{2+} over a series of other studied cations. The selectivity towards Hg^{2+} over the heavy transition metal ion Cu^{2+} (usually interfere with the binding of Hg^{2+}) is noteworthy with respect to ratiometric emission characteristic of **1**. Additionally, the chemosensor is also noted to be efficient in reporting the presence of Hg^{2+} inside the cell. Thus, the experimental findings in this full account together with our previous observations underlines the truth that the 4-substituted morpholin-2-one can be opened up in various ways[7a,c] to meet the molecular diversity very easily for the sensing of different cations.

Experimental

4-Benzylmorpholin-2-one (4)

Ethanolamine (0.51 g, 8.29 mmol) was added to a solution of benzaldehyde (0.8 g, 7.54 mmol) in dry methanol (30 mL) and the reaction mixture was refluxed for 9 h. The solution was cooled and stirred at 0 °C for 2 h to give yellow precipitate. The precipitate was filtered off and washed with methanol several times and finally dried under vacuum to give foam like yellow solid **2** (0.97 g, 87%), mp: 110–112 °C. In the next step, $NaBH_4$ (0.304 g, 8.04 mmol) was added to a stirred solution of Schiff-base **2** (0.8 g, 5.36 mmol) in dry methanol (30 mL) portion wise at 0 °C under nitrogen atmosphere. The reaction mixture was refluxed for 3 h. The solvent was removed under vacuum; water was added and extracted with CH_2Cl_2 (30 mL × 3). The organic layer was separated and dried over Na_2SO_4 and the solvent was removed in a rotary evaporator. Finally, the crude product was purified by column chromatography using 2% methanol in $CHCl_3$ as eluent to give yellowish solid **3** (0.64 g, 79%), mp: 92 °C.

K_2CO_3 (0.5 g, 3.97 mmol) was added to a solution of **3** (0.5 g, 3.31 mmol) in dry acetone (40 mL), followed by addition of ethyl 2-chloroethanoate (0.48 g, 3.97 mmol). The reaction mixture was refluxed for 7 h under nitrogen atmosphere. After completion of the reaction, K_2CO_3 was filtered off and the filtrate was evaporated on a rotary evaporator. Water was added to the residue and the aqueous layer was extracted with CH_2Cl_2 (30 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification of this crude product by column chromatography gave the lactone **4** (0.49 g, 78%) as light yellow solid, mp 122 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 7.36–7.27 (m, 5H), 4.40 (t, 2H, J = 4 Hz), 3.61 (s, 2H), 3.33 (s, 2H), 2.67 (t, 2H, J = 4 Hz) ppm; FT IR (ν in cm^{-1}, KBr) 3474, 3080, 2959, 2810, 1747, 1455.

2-(Benzyl(2-hydroxyethyl)amino)-*N*-(2-(3′,6′-bis(diethylamino)-3-oxospiro[isoindoline-1,9′-xanthene]-2-yl)ethyl)acetamide (1)

Amine **5** (0.211 g, 0.436 mmol), which was obtained according to the reported procedure,[10] was added dropwise to a stirred solution of compound **4** (0.1 g, 0.522 mmol) in THF (20 mL). Stirring was continued for 4 h. After completion of reaction,

monitored by TLC, THF was evaporated off and water was added to the residue. The aqueous layer was extracted with $CHCl_3$ (25 mL × 3) and dried over anhydrous Na_2SO_4. Purification of the crude mass by silica gel column chromatography using 3% CH_3OH in $CHCl_3$ as eluent yielded the product **1** (0.268 g, 90%), mp 142 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.44 (bt, 1H), 8.01 (d, 1H, J = 8 Hz), 7.50 (dd, 2H, J_1 = 8 Hz, J_2 = 4 Hz), 7.43 (d, 2H, J = 8 Hz), 7.31 (t, 1H, J = 8 Hz), 7.16–7.09 (m, 4H), 6.36–6.34 (m, 4H), 6.16 (dd, 2H, J_1 = 8 Hz, J_2 = 4 Hz), 3.77 (t, 2H, J = 4 Hz), 3.67 (s, 2H), 3.38–3.28 (m, 10H), 3.05 (s, 2H), 2.84 (m, 2H), 2.73–2.71 (m, 2H), 1.15 (t, 12H, J = 7.20 Hz), ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.4, 170.1, 153.9, 153.2, 148.9, 137.9, 133.0,130.2, 129.3, 128.9, 128.4, 128.3, 127.2, 123.9, 123.0, 108.2, 104.2, 97.6, 66.0, 59.7, 59.4, 58.8, 58.5, 44.3, 40.5, 39.2, 12.5; Mass (LCMS) 676.2 $(M + 1)^+$, Anal. Calcd for $C_{41}H_{49}N_5O_4$: C, 72.86; H, 7.31; N, 10.36. Found: C, 72.84; H, 7.29; N, 10.39.

General procedure for fluorescence and UV-vis titrations

Stock solutions of the receptor were prepared in 4 : 1(v/v) $CH_3CN : H_2O$ containing 10 mM Tris/HCl buffer (pH = 7.0) in the concentration range ~10^{-5} M. 2.5 ml of the receptor solution was taken in the cuvette. Stock solutions of guests in the concentration range ~10^{-4} M, were prepared in the same solvents and were individually added in different amounts to the receptor solution. Upon addition of metal ions, the change in emission of the receptor was noted. The same stock solutions for receptor and guests were used to perform the UV-vis titration experiment. The metal solution was successively added in different amounts to the receptor solution (2.5 mL) in the cuvette and the absorption spectra were recorded. Both fluorescence and UV-vis titration experiments were carried out at 25 °C.

Job plots

The stoichiometry was determined by the continuous variation method (Job plot).[12] In this method, solutions of host and guests of equal concentrations were prepared in the solvents used in the experiment. Then host and guest solutions were mixed in different proportions maintaining a total volume of 3 mL of the mixture. All the prepared solutions were kept for 1 h with occasional shaking at room temperature. Then emission and absorbance of the solutions of different compositions were recorded. The concentration of the complex, *i.e.* [HG], was calculated using the equation $[HG] = \Delta I/I_0 \times [H]$ or $[HG] = \Delta A/A_0 \times [H]$ where $\Delta I/I_0$ and $\Delta A/A_0$ indicate the relative emission and absorbance intensities respectively. [H] corresponds the concentration of pure host. The mole fraction of the host (X_H) was plotted against concentration of the complex [HG]. In the plot, the mole fraction of the host at which the concentration of the host–guest complex concentration [HG] is at a maximum, gives the stoichiometry of the complex.

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