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ORIGINAL PAPER

A Fluorescent Chemodosimeter for Hg^{2+} Based on a Spirolactam Ring-Opening Strategy and its Application Towards Mercury Determination in Aqueous and Cellular Media

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Abstract A novel fluorescent chemosensor rhodamine B phenyl hydrazide (RBPH) for Hg^{2+} was designed and synthesized. This probe is highly sensitive, selective, and irreversible for Hg^{2+} and exhibits fluorescent response at 580 nm. RBPH also displayed detectable color change from colorless to pink upon treatment with Hg^{2+}. This property has been utilized as naked eye detection for Hg^{2+} in various industrial samples. Fluorescence microscopic experiments demonstrated that this chemosensor is cell permeable and can be used for fluorescence imaging of Hg^{2+} in cellular media. This probe can detect Hg^{2+} with good linear relationships from 1 to 100 nM with r=0.99983 and the limit of detection were found to be 0.019 nM with±0.91 % RSD at 10 nM concentrations.

Keywords Chemodosimeter · Hg^{2+} ions · Naked eye sensor · Fluorescence imaging

Introduction

The design and synthesis of selective and sensitive chemosensors capable of monitoring heavy metal ions has attracted considerable attention due to their wide use by humans in their day to day life and their subsequent impact on the environment and nature [1, 2]. The mercury is considered as highly dangerous, toxic and easily converted into most toxic form like methyl mercury by bacteria and it is widely distributed in the environment due to the various human activities and subsequently bio accumulates through the food chain. The USEPA set 2 ppb of Hg^{2+} in drinking water as threshold limit value [3]. Therefore, the effective and selective detection of mercury is of great significance for biochemistry, environmental science and medicine [4, 5]. An ideal chemosensor should thus display a very low detection limit in water and in various environmental samples and retain its selectivity toward mercury. Scientists have been using many instrumentation techniques like AAS, AES, XFS, ICP-AES, ICP-MS etc. and these instruments are expensive and requires sample preparation. To overcome these difficulties peoples now a day's using fluorescence-based methods, because these methods offers a promising approach for mercury screening due to their simplicity, selectivity, high sensitivity, adaptability and online imaging capabilities[6–8]. To date, several kinds of small molecular chemosensors, chemodosimeters, materials and bio molecules have been reported as fluorescence sensors for mercury detection at lower detection limits [9–12].

Owing to the outstanding spectroscopic properties and binding-promoted fluorescence-enhancing process of rhodamine based dyes, many of these derivatives are reported in recent years [13]. These derivatives possess special structures such as the non fluorescent circular spiro-lactams or thiospirolactones, which block the fluorescent signal being, realized [14–17]. Upon the reaction with Hg^{2+}, the non fluorescent spirocyclic forms of the chemosensors were decomposed irreversibly to the fluorescent carboxylic species, resulting in the highly sensitive detections of Hg^{2+}. Rhodamine-based chemosensors have been widely used for

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detecting intracellular analytes [18, 19]. These reported chemosensors have demonstrated reasonable selectivity and sufficient turn-on ratios for Hg^{2+} detections over other M^{n+} ions [20–22]. However, there are only a few successful examples of fluorescent probes for detecting Hg^{2+} ions in biological samples for distinguishing between the safe and toxic levels of inorganic mercury [23–30]. Taking into account the pronounced limitation for heavy metal detection in the natural biological samples (the low quantum efficiency of metal-bound dyes in biological systems), it is still an outstanding challenge to create new synthetic molecules that meet prerequisite of appropriate selectivity, and optical sensitivity in natural settings [31]. Recently reported rhodamine derivatives for Hg^{2+} ions are inheritance with one or more limitations. Some of them have comparatively short emission wave lengths, which are not favorable for cell imaging studies [32]. Few of them require organic solvent as a cosolvent since they are not soluble in aqueous conditions [33, 34]. These probes have less sensitivity as well as cross sensitivities towards other metal ions [35]. Recently Vijay luxami et.al reported naphthalimide rhodamine dyad for Hg^{2+} ions. It shows reasonable selectivity and long linear range (50 nm to 2 μM) but the synthetic procedure is tedious involving multiple steps [36].

In order to over come above said limitations in the present manuscript, we are reporting the synthesis and applications of RBPH, a bright and specific fluorescent sensor for bioimaging of Hg^{2+} ions in cells and its dramatic color change with Hg^{2+} can be utilized in the naked eye detection of mercury in various industrial effluents. The RBPH is advantageous over other reported rhodamine dyes especially in fluorescence imaging studies because the fluorescence emission species in our study is the free rhodamine B species which has higher quantum yields ($Ø_F$=0.61) compared to the metal bound dyes which has very less quantum yields in the cellular medium($Ø_F$=0.001 to 0.48) [22, 37] .

Experimental

Apparatus

Fluorescence measurements were recorded using Shimadzu Spectrofluorimeter (model RF 5301PC). Absorbance measurements were made using a Shimadzu Spectrophotometer (model UV-1800) with 1 cm quartz cuvettes. ^{1}H and ^{13}C NMR measurements were performed with a Bruker 400 MHz spectrometer. Mass spectral data was obtained using Thermo Finnigin Deca QXP Mass Spectrometer. Elemental analysis was carried out using Elementar (model Vario super user). All pH measurements were carried out using Systronics digital pH meter (model 802). Ceti - Epifluorescent microscope has been used for fluorescence imaging studies.

Reagents and Solutions

Rhodamine B was purchased from Sigma-Aldrich. Phenyl Hydrazine hydrate and Ethanol (HPLC grade) were purchased from SD Fine Chem. Ltd. 10 mM of rhodamine B phenyl hydrazide was prepared by dissolving it in 1:10 acetonitrile/water (% v/v). Standard Hg^{2+} solution was prepared by dissolving $HgBr_2$ (Merck, AR grade, Mumbai, INDIA.) in distilled water. Robinson buffer solutions of pH 7–12 were prepared by using the solutions of 0.02 M acetic acid, 0.02 M orthophosphoric acid, 0.02 M sodium tetraborate and 0.4 M NaOH. PBS (phosphate buffer saline) was purchased from the Sigma-Aldrich. All solvents used for synthesis and measurements were redistilled before use. All other chemicals were of analytical-reagent grade and were used without further purification. The industrial effluents were collected from the Department of Pollution Control Board, Bangalore, Karnataka, India. MCF-7 cell lines were procured from National Centre for Cell Sciences, Pune, India.

Cell Cultures and Cell Labeling

The obtained cell line was cultured on a cover slip with RPMI 1640 with 5 % fetal bovine serum at 37 °C in a humidified atmosphere with 5 % CO_2. Prior to the experiments, the MCF-7 cells were incubated with 100 mM RBPH for 30 min at room temperature to allow the probe to permeate into the cells. The cells were then washed with PBS (phosphate buffer saline) in order to remove excess probe to avoid background fluorescence and then cells were incubated with 100 nM $Hg(NO_3)_2$ for another 30 min. Following incubation, the cells were imaged with λex/λem=561/580 nm.

Recommended Procedure

In a 10 mL calibrated standard flasks with different concentrations of Hg^{2+} solutions in the range 1–100 nM were taken. To this 1 mL of 10 mM RBPH followed by 1 mL of Robinson buffer solution having pH 7 were added and leave the solutions for 2 min. Then made up to the mark with distilled water and measure the fluorescence emission intensity of the solutions (λ ex/em=530/580 nm).

Results and Discussion

Synthesis and Spectral Characterization of RBPH

The rhodamine B (0.60 g, 1.2 mM) solution was prepared in a 250 mL round bottom flask by dissolving in 20 mL ethanol followed by phenyl hydrazine (5 mL, 50 mM) was added drop wise. The reaction mixture was refluxed for 4 h. Finally solvent was removed under reduced pressure. The obtained residue

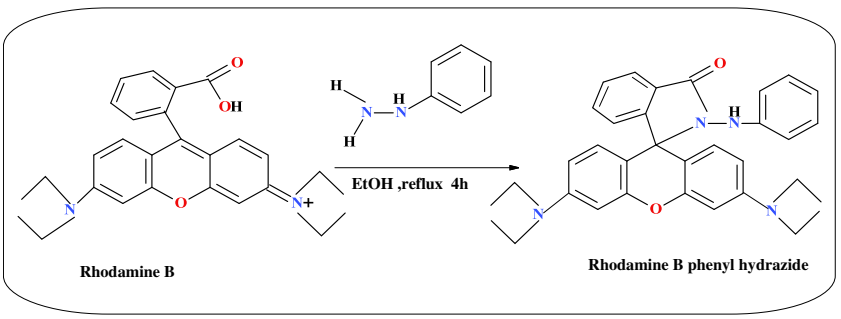
was dissolved in dichloromethane (50 mL) followed by washed with saturated $NaHCO_3$ solution. The organic phase was separated. The remaining mixture was again washed with water (50 mL×3) and dried over anhydrous Na_2SO_4. This process was repeated until to separate all the organic and water phase clearly from the mixture. Then it is subject to filtration. After filtration, the solid was washed with acetonitrile (5 mL×2), dried under vacuum (Scheme 1). RBPH was obtained as yellow powder in 88 % yield; ESIMS: m/z $[M + H]^+$=533.7; Calcd: 533.69. (Refer supplementary data, Fig. S1), ^1H NMR (400 MHz, $CDCl_3$, ppm): δ 1.2 (t, 12H, *J*=7.2 HZ, NCH_2CH_3), 3.3 (m, 8H, *J*=7.2 HZ, NCH_2CH_3), 5.8 (s,1H, NHC_6H_5), 6.5 (s, 6H, xanthene-H),6.4 (d, 2H, *J*=8.0 HZ, Ar-H), 6.6 (t, 1H, *J*=7.2 HZ, Ar-H), 6.9(t, 2H, Ar-H), 7.1 (d, 1H,*J*=4.0 HZ, Ar-H), 7.6 (m, 2H, Ar-H), 7.9 (d, 1H, *J*=4.0 HZ, Ar-H) (Refer supplementary data, Fig. S2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 166, 152, 150, 148.2, 148.1, 133. 130, 128,129.7, 128.9, 124.5, 123.4, 118.1, 113.7, 108.1, 97.3, 66.6, 38.7, 14.7 (Refer supplementary data, Fig. S3). Anal. Calcd. for $C_{34}H_{36}N_4O_2$: C, 81.05 %; H, 5.8 %; N, 9.00 %. Found: C, 81.09 %; H, 5.76 %; N, 9.06 %.

Absorption and Emission Properties of RBPH

The spectral properties of the sensor have been investigated in acetonitrile – water system (1:10 v/v) in Robinson buffer solution. The RBPH is colorless and has negligible absorption band centered at 561 nm. Similarly it has week emission band centered at 630 nm when excited at 580 nm (Refer supplementary data, Fig. S4). This is due to its spiro closed structure similar to that of the other rhodamine derivatives [38, 39]. But upon the addition of Hg^{2+} the color less solution turns pink and intensity of the absorption band will be centered at 561 nm, which linearly increases with Hg^{2+} ions concentration. Similarly fluorescence emission band arises at 580 (λ ex/em=530/580 nm). In the presence of Hg^{2+} ions the spirolactam ring opening occurs resulting in the blue shift of the emission band from 630 to 580 nm (Figs. 1 and 2). The enhancement in absorbance at 561 nm followed by blue shift and increment in fluorescence emission intensity at 580 clearly suggested the spirolactam ring opening process of RBPH by Hg^{2+} which lead to the formation of spiro open structured delocalized xanthene conformation of the rhodamine moiety. In order to know the stoichiometric ratio of RBPH to Hg^{2+} the Jobs continuous variation method was adopted [40]. The absorbance values were plotted against the [RBPH] / [RBPH] + $[Hg^{2+}]$ (Fig. S5). This study has revealed that the stoichiometry between Hg^{2+} and RBPH is 1:1. The association constant (K_a) of RBPH with Hg^{2+} cannot be obtained due to the irreversible interaction [41]. Furthermore the absorbance values obtained by titrating the different concentrations of Hg^{2+} (0–100 μM) with 10 mM RBPH reveals that the absorbance linearly increases with Hg^{2+} concentration up to 100 μM with R=0.9957 (Fig. 1). The fluorescence emission values (λ ex/em=530/580 nm) obtained by titration experiments (using 2 mM RBPH and Hg^{2+} (0–100 nM) revels that the fluorescence emission intensity linearly increases up to 100 nM of Hg^{2+} with R=0.9986 (Fig. 2). The fluorescence quantum yield ($Ø_F$) of RBPH with 10 nM Hg^{2+} at 580 nM was found to be 0.61 which is greater than that of the reported fluorescent rhodamine derivatives for Hg^{2+} [22, 37, 42]. Rhodamine 6G in ethanol ($Ø_F$)=0.69 is used as standard method for quantum yield calculations [43]. To check whether the fluorescence signaling of RBPH towards Hg^{2+} is reversible or irreversible we added 1 M sulfide solution to the solution containing RBPH and Hg^{2+}. The fluorescence intensity as well as the absorbance of the solution was not altered indicates the irreversible interaction of the probe and the Hg^{2+} ions. So the interaction of RBPH with Hg^{2+} is irreversible i.e., cation catalyzed ring opening rather than metal ion coordination. There is no effect of counter ion present in Hg^{2+} salts on fluorescent emission intensity as well as the absorbance. This was confirmed by examining the absorbance and fluorescent emission intensity values of different cupric salts like $HgCl_2$, Hg $(NO_3)_2$ and $HgBr_2$ with RBPH in Robinson buffer medium of pH 7.

Evidence for Spirolactam Ring Opening Process

The transformation of RBPH to Rhodamine B through spirolactam ring opening process by Hg^{2+} was confirmed by various spectroscopic studies. The mass spectrum of the RBPH



Scheme 1 Synthesis of RBPH from the rhodamine B

shows m/z $[M + H]^+$=533.7, but after treating RBPH with Hg^{2+} the resulting compound shows m/z $[M + H]^+$=443.47 corresponds to the rhodamine B compound (Refer supplementary data, Fig. S1 and S6) which confirms that the formation of rhodamine B through rhodamine - Hg^{2+} complex and subsequent hydrolysis. The proton NMR spectra of RBPH contains the NHC_6H_5 signal at 5.9 ppm where as the product obtained in the reaction lacks this peak instead its proton NMR spectra well matches with that of proton NMR spectra of authentic rhodamine B (not shown) (Refer supplementary data, Fig. S2 and Fig. S7). This confirms that rhodamine tautomer of spiro open structure was obtained in the above reaction. Based on the above spectroscopic results the possible mechanism of spirolactam ring opening of RBPH by Hg^{2+} was deduced (Scheme 2). The phenyl hydrazide group recognizes Hg^{2+} results in the complex formation and subsequent hydrolysis of the complex results in the release of rhodamine B.

Optimization of Reaction Parameters

The various reaction parameters like pH, reaction kinetics and probe concentration that affects the spectral properties of the probe with respect to fluorescence assay has been optimized.

Effect of pH

The spectral properties of the RBPH has been studied in the acetonitrile water system (1:10) in the presence of Robinson buffer solution. In a 10 mL standard flask 1 ml of 10 mM RBPH, 10 nM of Hg^{2+} and 1 mL of Robinson buffer solution of pH 1–12 was added and fluorescence emission intensity was measured (λ ex/em=530/580 nm) and plotted as a function of pH. The RBPH shows negligible fluorescent emission intensities in the pH range 1–12, but upon the addition of Hg^{2+} (10 nM) the emission intensity increases with increase in pH and becomes constant after 7. No other metal/non metal ions produce considerable emission intensity in this pH range except Cu^{2+} (10 nM) which shows similar effect in the pH range 1–4 as that of Hg^{2+}. So interference of Cu^{2+} ions can overcome by maintaining the pH of the solution above 7 and its interference was explained based on the effect of "copper triangle" of the periodic table [44].

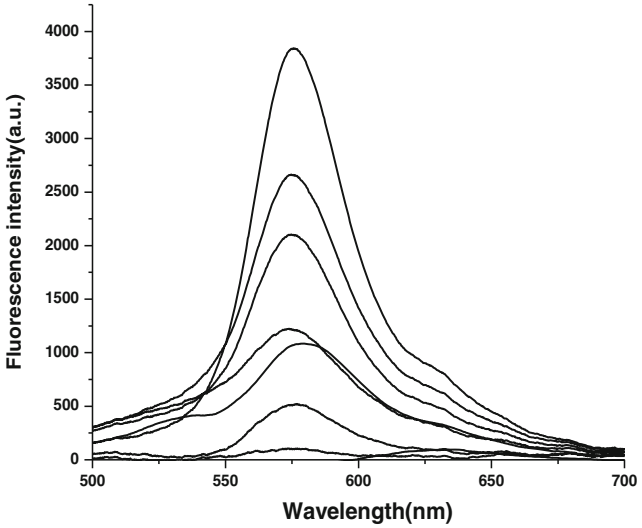


Fig. 2 Fluorescence emission spectra of 1 mL of 10 mM RBPH 1:10 acetonitrile/water (% v/v)+1 mL of Robinson buffer solution of pH 7+0–50 nM Hg^{2+} solution (λex/em=530/580 nm) Inset showing calibration plot of fluorescence emission against $[Hg^{2+}]$

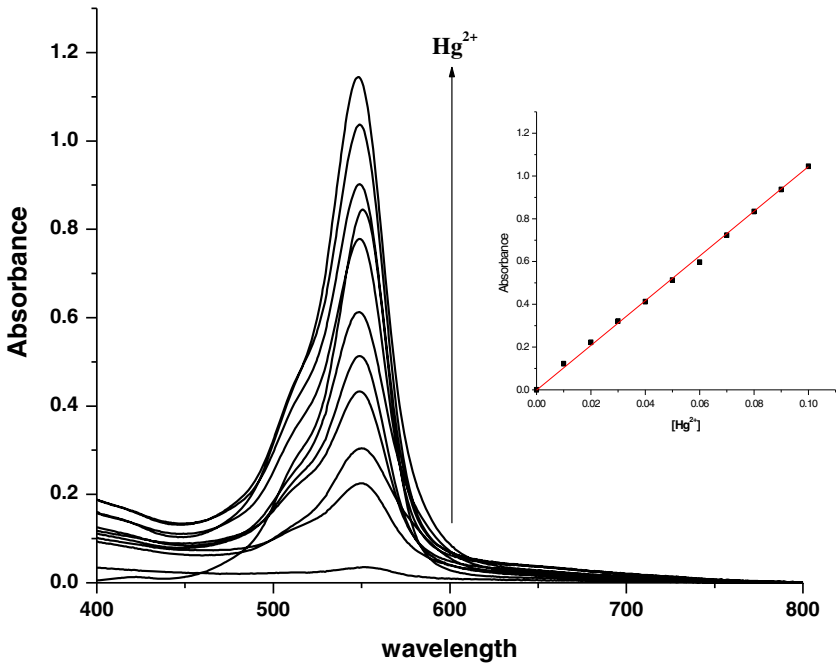
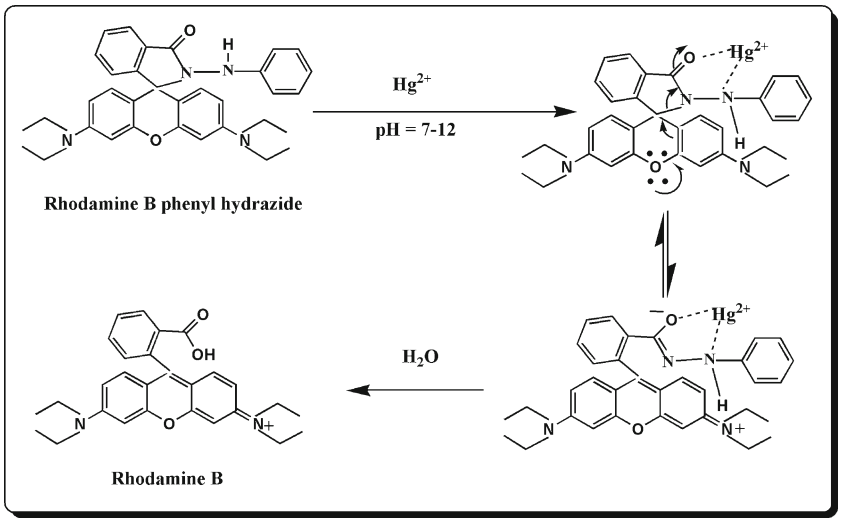


Fig. 1 Absorption spectra of 1 mL of 10 mM RBPH1:10 acetonitrile/water (% v/v)+1 mL of Robinson buffer solution of pH 7+0–100 μM Hg^{2+} solution (In set showing the calibration plot of absorbance against $[Hg^{2+}]$

Scheme 2 Mechanistic pathway of spirolactam ring opening process



Reaction Kinetics

The reaction kinetics of RBPH with Hg^{2+} for fluorescence measurement was studied by keeping all other parameters constant. In 10 mL standard flasks 1 ml of 10 mM RBPH, 1 mL of pH 7 Robinson buffer solution followed by 10 nM of Hg^{2+} were added and fluorescence emission intensity was measured (λ ex/em=530/580 nm) and it was plotted as a function of time. The back ground fluorescence of the reagent blank remains same even after 1 h but fluorescence emission intensity of the sample increases with increase in time and it becomes constant after 1.5 min. So 1.5 min time has been considered as optimum for completion of the reaction. The quick response time of the probe towards Hg^{2+} through spirolactam ring opening process compared to reported probes may be due to the presence of active phenyl group which readily hydrolysis to give spiro ring opened rhodamine moiety. A similar probe with sulfur atoms requires elevated temperatures and lethargic ring open kinetics. The ring opening kinetics in the case of rhodamine B hydrazide with Hg^{2+} was also found to be very slow [45, 46].

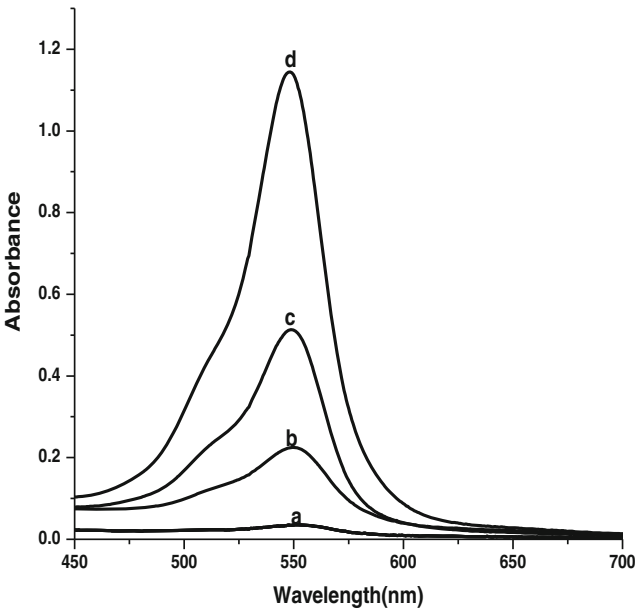


Fig. 3 Fluorescence emission spectra of in the presence of 1 mL Robinson buffer solution of pH 7+1 mL of 10 mM RBPH in 1:10 acetonitrile water (% v/v) (**a**) Reagent blank (**b**) In the presence of 1,000 nM of other ions (**c**) In the presence of 100 nM of Cu^{2+} ions (**d**) In the presence of 10 nM of Hg^{2+} ions (λex/em=530/580 nm)

Effect of Probe Concentration

The optimum concentration of the probe required to obtain maximum emission intensity was studied by keeping all other parameters constant. In 10 mL standard flask different volumes of 10 mM RBPH in1:10 acetonitrile water (% v/v) (% v/v), 1 mL of pH 7 Robinson buffer solution followed by 10 nM of Hg^{2+} was added, the resulting solution was made up to the

Table 1 Analytical merits of the method

Analytical feature	nM/mL
Linear range	0.1–50
Limit of detection[a]	0.019
Limit of quantification[b]	0.19
Fluorescence quantum yield ($Ø_F$)	0.61
RSD (n=10) Hg^{2+}=10 nM	± 0.991

[a] Calculated based on standard deviation values of 5 reagent blank determinations (3 σ)

[b] Calculated based on standard deviation values of 5 reagent blank determinations (10 σ)

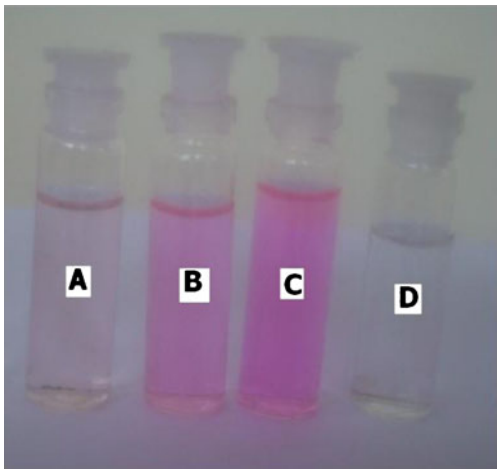


Fig. 4 Naked detection of Hg^{2+} in industrial samples, each sample tube contains 1 mL of 10 mM RBPH in1:10 acetonitrile water (% v/v)+1 mL of Robinson buffer solution of pH 7 and 3 mL of pretreated effluent (**a**) Chrome plating industry effluent (**b**) Lead-acid battery effluent (**c**) Chloro-alkali industry effluent (**d**) Textile dyeing industry effluent

mark with distilled water and kept aside for 2 min. Then fluorescence emission intensity was measured (λex/em=530/580 nm) and plotted as a function of concentration of the probe. Similarly reagent blank was also prepared by following the above procedure and its fluorescence emission intensity was measured (λex/em=561/580 nm). In the case of reagent blank, emission intensity found to be almost same with respect to the probe concentration. But in the case of sample it increases with increase in probe concentration up to 1 mL and then remains constant. Finally we keep this concentration as optimum for further measurements.

Interference Study

The Interference property of most commonly available metal and non metal ions in water samples such as Ca^{2+},K^{+},Mg^{2+}, Pb^{2+},Ni^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Cl^{-}, NO_2^{-}, NO_3^{-}, S^{2-}, SO_4^{2-}, and ClO^{-} with respect to absorption property of RBPH has been studied. It revealed that no other anions and cations leads to significant absorbance changes have been observed pH range (7–12) (Refer supplementary data Fig. S8). But as a special case 10 nM of Cu^{2+} produces pink color with the probe in acidic pH (1–5) but not in the studied pH range (7–12). Thus, RBPH can function as a highly selective chemo sensor for Hg^{2+} in this particular pH range. Similarly the probe has negligible emission intensity but addition of 10 nM of the Hg^{2+} resulted a significant enhancement of the emission intensity at 580 nm. However, the addition of anions as well as cations as mentioned above brings no obvious effect on the fluorescence emission except Cu^{2+} ions (Fig. 3 and Fig. S9 Refer supplementary data). But the addition of higher concentration of Cu^{2+} (100 ng) produced a slight enhancement of the emission intensity in the pH range 1–4 but not in the pH range 7–12 as that of Hg^{2+}.

Analytical Merits of the Method

The various analytical features of the method in relation to the Hg^{2+} determination in aqueous as well as in intracellular medium has been listed in the Table 1.

Naked Eye Detection of Hg^{2+}

The probe is colorless in presence of Robinson buffer solution of pH 7 but after the addition of Hg^{2+} ions, the solution turns pink colored within a minute. No other metal/non metal ion induces this change with probe including Cu^{2+} ions at this pH range studied. The color change patterns of the RBPH with various concentrations of Hg^{2+} ions and other metal ions are shown in the Fig. S10. This dramatic color change of the RBPH with Hg^{2+} ions can be utilized as a naked eye sensor [45, 46]. This property can be utilized for naked eye sensing of mercury in various aqueous environmental samples especially in industries for testing effluents after pretreatment, before dumping into the environment [47–49]. The present method can be utilized as naked eye determination of Hg^{2+} in pretreated industrial effluents like chloro-alkali industry, lead-acid battery, textile dyeing industry and chrome plating industry. To the 3 mL of effluent, 1 mL of 10 mM RBPH in1:10 acetonitrile water (% v/v) was added. The resulting solution has been adjusted pH 7 using Robinson buffer solution and leave for 2 min for color development. The concentration of the Hg^{2+} ions in the test samples can be determined by comparing the color and fluorescence emission intensity with standard mercury solution. The naked eye de-

Fig. 5 Fluorescence imaging results of (**a**) MCF-7 cells in the bright field (**b** and **c**) MCF-7 cells after incubated with RBPH for 30 min (100 mM) and (**c**) after with 100 nM Hg $(NO_3)_2$ for 30 min (λ ex/em=530/580 nm)

tection of Hg^{2+} ions in various industrial effluents were demonstrated in the Fig. 4. The absorption as well as fluorescence emission intensities of above said industrial effluents are given in Fig. S11 and S12 (Refer supplementary data).

Fluorescence Imaging of Hg^{2+}

The selective fluorescence signaling of the probe towards Hg^{2+} in presence of other biologically important metal ions in the pH range (7–9). We thought of utilizing this desirable property of intra cellular fluorescence imaging of Hg^{2+} ions. From the fluorescence microscopic studies we came to know that RBPH can permeate into cells and it is suited for fluorescence imaging of Hg^{2+} in living cells because of its favorable amphiphilic and fluorescence properties. Cultured MCF-7 cells were incubated with 10 mM RBPH for 30 min at room temperature and fluorescence emission was measured at 580 nm with excitation wave length set at 561 nm. But the fluorescence was not detected in the cells (Fig. 5b). After the cells were incubated with 100 nM Hg $(NO_3)_2$ for another 30 min, a brighter fluorescence was observed (Fig. 5c). A bright field microscopic image (Fig. 5a) shows that the cells were viable throughout the imaging experiments. These results demonstrate that RBPH is cell permeable and capable of sensing Hg^{2+} in living cells.

Conclusions

In conclusion, we have synthesized a new rhodamine-based chemosensor that can sense Hg^{2+} through visible color change and this property has been utilized as naked eye detection in various industrial samples. Selective binding of the compound to Hg^{2+} and spirolactam ring opening results in the release rhodamine B results in immediate and remarkable fluorescence enhancement at 580 nm emission wavelengths, which proved that RBPH could serve as a sensitive and selective fluorescent chemosensor of Hg^{2+}. Furthermore, we have demonstrated that RBPH is applicable for Hg^{2+} imaging in cellular media. The RBPH posse's higher quantum yields compared to other reported rhodamine fluorescent probes for Hg^{2+} ions.

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