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A Rhodamine-Based Chemosensor that Works in the Biological System

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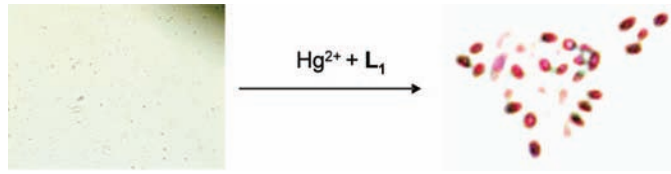
ABSTRACT



A new rhodamine-based reversible chemosensor (L_1) is reported, which could bind Hg^{2+} and Cu^{2+} in aqueous methanol solution with detectable change in color. Cu^{2+} and Hg^{2+} ions responded differently toward the fluorescence output signals on binding to L_1. L_1 could also be used as a selective probe for monitoring Hg^{2+} adsorbed on bacteria using an optical microscope.

Mercury is an unsafe toxin that has posed a great threat to our environment.[1] Oxidation of mercury vapor in atmosphere to water-soluble Hg^{2+} ions and its consequent metabolism by aquatic microbes produces methyl mercury, which bioaccumulates through the food chain.[2] This is expected to have a severe effect on human health and the environment. The best way to detect Hg^{2+} that has gone into the food chain or contaminated the environment is to monitor the extent of mercury present in microorganisms such as bacteria, which survive in waste water or effluents. Recently, Chang and co-workers reported a fluorescent probe for the detection of mercury in fish, where the appearance of the new fluorescence could be detected by confocal laser microscopy.[3] In the past few years, a number of fluorescent chemosensors for the selective detection of Hg^{2+} ions have been reported.[4] However, examples for the detection of mercury in biological systems are extremely rare.[5] Limiting factors for design of such a sensor molecule are low solubility in water, cross-sensitivity toward other metal ions, and spectral/optical sensitivity in physiological conditions. Generally, Hg^{2+} is known to cause fluorescence quenching of the fluorophores via the spin-orbit coupling effect,[6] and this is reflected in the *turn-off* fluorescence response reported in most instances.[7] Very recently, few rhodamine-based fluorescent probes are

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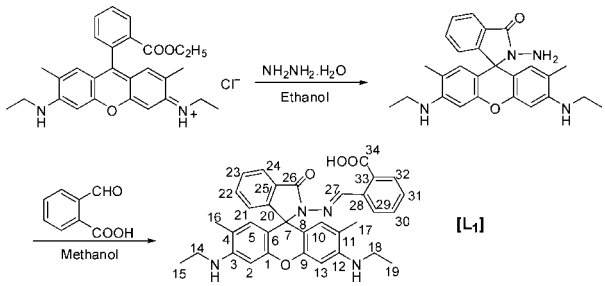
reported which show a selective *turn-on* response to Hg^{2+},[8] although reports on the use of such probe molecules for detection of Hg^{2+} uptake by live microorganisms are rare.[5] Fluorescent probes, which show fluorescence enhancement on binding to the cation of interest, are preferred as sensors as these allow a lower detection limit and high-speed spatial resolution via microscopic imaging.[3,9]

Rhodamine-based dyes are known for their excellent spectroscopic properties with a large molar extinction coefficient (ε) and high fluorescence quantum yield (Φ). Earlier, rhodamine-based spirolactam (fluorescence "*off*" state) was employed as a molecular scaffold to design chemosensors for selective recognition of Cu^{2+} and Pb^{2+}[10,11] as coordination of these metal ions induced spirolactam ring opening (fluorescence "*on*" state) and thereby allowed detection through an enhancement in fluorescence intensity.[12]

Here, we report a new rhodamine-based spirolactam derivative ($\mathbf{L_1}$) as a chemosensor for Hg^{2+} and Cu^{2+}, when binding phenomena could be probed through binding-induced changes in an electronic spectral pattern. Further, binding of these metal ions to $\mathbf{L_1}$ caused color changes, which could also be detected by the naked eye. Interestingly, binding of only Hg^{2+} to $\mathbf{L_1}$ caused significant fluorescence enhancement in an aqueous−methanol mixture. In this mixed solvent media, two different modes of binding for Hg^{2+} and Cu^{2+} to $\mathbf{L_1}$ were observed. Cu^{2+} formed a 1:1 complex ($Cu\mathbf{L_1}$), whereas Hg^{2+} formed a 2:1 complex ($Hg(\mathbf{L_1})_2$). The newly synthesized rhodamine 6G derivative ($\mathbf{L_1}$) (Scheme 1) was prepared in high yield (see Supporting Information).

Scheme 1. Synthetic Route of $\mathbf{L_1}$



The proposed molecular structure and its purity were confirmed by various spectroscopic analyses (see Supporting Information), and this was unequivocally corroborated on the basis of the single-crystal X-ray analysis (Figure 1).[13]

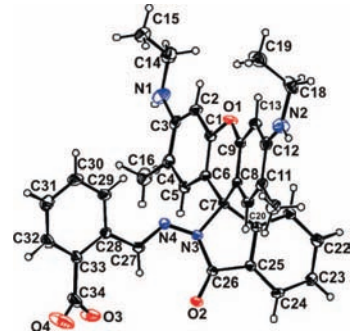


Figure 1. ORTEP diagram of the compound $\mathbf{L_1}$ (40% probability level for the thermal ellipsoids).

This compound ($\mathbf{L_1}$) remained colorless in water−methanol (1:1, v/v) solution at pH 7.0. This indicates that the spirolactam form of $\mathbf{L_1}$ predominantly existed under this condition. The ^{13}C NMR spectrum was recorded for $\mathbf{L_1}$. A characteristic peak for the C_7-atom appeared near 66 ppm and confirmed this proposition.[14] Spectrophotometric titrations for $\mathbf{L_1}$ with varying pH revealed that $\mathbf{L_1}$ retained the spirocyclic form within the pH range of 5.0−13.0 (see Supporting Information). Below pH 5.0, the fluorescence intensity tended to increase with a further decrease in the pH of the solution, which signified the spirolactam ring opening—as the acyclic form of rhodamine derivatives are known to be strongly fluorescent.

Electronic spectra of $\mathbf{L_1}$ (20 μM), recorded in the water/methanol (1:1, v/v) mixed solvent at neutral pH, exhibited a very weak band above 530 nm, which could be attributed to

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(13) Crystal data for the compound: CCDC no. 685033; molecular formula: $C_{34}H_{31}N_4O_4$, $M = 559.63$, crystal size: $0.40 \times 0.36 \times 0.20$ mm^3, Triclinic, space group P-1 with $a = 9.132(3)$ Å, $b = 11.574(4)$ Å, $c = 13.473(4)$ Å, $\alpha = 92.904\ (5)°$, $\beta = 92.889(5)°$, $\gamma = 97.025(5)°$, $V = 1409.2(8)$ Å^3, $Z = 2$, $D_{calcd} = 1.319$ g/cm, $T = 100(2)$ K, $F(000) = 590$, Absorption coefficient $= 0.088$ mm^{-1}, $\lambda = 0.71073$ Å, 11 469 reflections were collected, 6158 observed reflections with ($I \geq 2\sigma(I)$), R(int) = 0.0633. $R1 = 0.0780$, w$R2 = 0.1987$, goodness of fit on $F^2 = 1.057$. The largest difference peak and hole: 0.867 and −0.411 eÅ^{-3}, respectively.

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the presence of a trace (~0.22%) amount of the ring-opened form of $\mathbf{L_1}$. On addition of Hg^{2+} and Cu^{2+}, a new absorption band appeared at 534 and 528 nm, respectively (Figure 2).

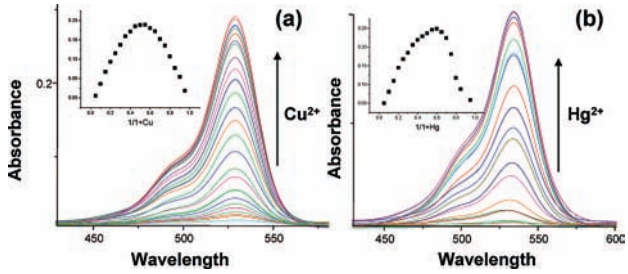

Figure 2. Absorption spectra of $\mathbf{L_1}$ (20 μM) in water−methanol (1:1, v/v) at pH 7.0 (a) upon addition of 0−35 mol equiv of Cu^{2+}. Inset: Job's plot that indicates the 1:1 stoichiometry for complex formation. (b) Upon addition of 0−8 mol equiv of Hg^{2+}. Inset: Job's plot that indicates 2:1 stoichiometry for complex formation. ($[\mathbf{L_1}] + [Cu^{2+}]$ or $[Hg^{2+}] = 100$ μM).

This enhancement in absorbance clearly suggests the formation of the delocalized xanthane moiety of the rhodamine group, associated with a distinct color change from colorless to pink. Other metal ions, such as Co^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Li^{+}, K^{+}, and Na^{+} did not show any significant color and spectral change under identical conditions (see Supporting Information).

An association constant ($K_a^{Hg^{2+}}$) of $8.0 \times 10^5 \pm 0.1$ M^{-2} L^2 was evaluated assuming a 2:1 stoichiometry for $[Hg(\mathbf{L_1})_2]$ (Figures 2 and 3) from the nonlinear fitting of the titration

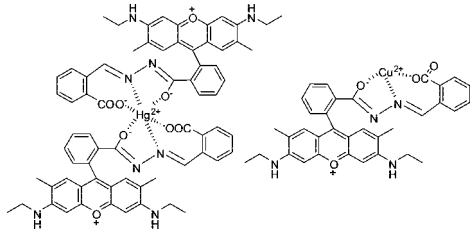

Figure 3. Proposed binding mode of $\mathbf{L_1}$ with Hg^{2+} and Cu^{2+}.

curve. This binding stoichiometry was also confirmed from Job's plots.[15]

Spectrophotometric titrations with Cu^{2+} revealed a 1:1 complex formation ($\mathbf{CuL_1}$) (Figures 2 and 3), and the association constant ($K_a^{Cu^{2+}}$) of $1.68 \times 10^5 \pm 0.012$ M^{-1} L was evaluated. This stoichiometry was also confirmed by ESI−MS mass spectra (see Supporting Information). The peak at $m/z = 623.3$ corresponding to $\mathbf{CuL_1}$ was observed, whereas $\mathbf{L_1}$ without Cu^{2+} exhibited peaks only at $m/z = 561.5$, which corresponds to $[\mathbf{L_1} + H]^+$. The association constant values reported here are an average of at least five independent experimental results in each case. Presumably, the larger covalent radius (1.49×10^{-10} m) for Hg^{2+} as compared to the Cu^{2+} (1.17×10^{-10} m) accounts for the difference in the coordination behavior of $\mathbf{L_1}$.

On addition and gradual increase of $[Hg^{2+}]$ to the nonfluorescent aqueous methanol solution (1:1, v/v) of $\mathbf{L_1}$, a significant enhancement in fluorescence intensity at 554 nm was observed following excitation at 500 nm, and the emission quantum yield (Φ) was found to be 0.82 relative to rhodamine 6G. No such change was observed on addition of Cu^{2+}. The quenching of the fluorescence of the open ring form of $\mathbf{L_1}$ by Cu^{2+} could be explained based on the well-known paramagnetic effect of the d^9 Cu(II) system.[16] Fluorescence intensity of $\mathbf{L_1}$ (10 μM) upon addition of Hg^{2+} (0−8 mol equiv) was found to enhance by 90 fold at 554 nm (Figure 4). Various alkali, alkaline earth metal ions,

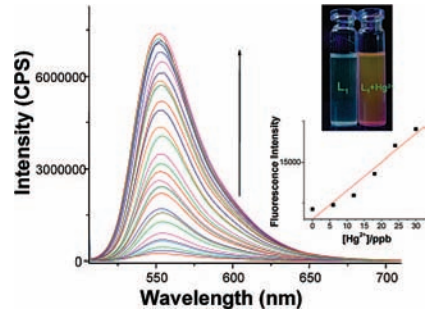

Figure 4. Change in fluorescence spectra of $\mathbf{L_1}$ (10 μM) upon addition of Hg^{2+} (0−8 equiv) in water−methanol (1:1, v/v) at pH 7. λ_{ext} at 500 nm. Inset: Emission intensity of $\mathbf{L_1}$ (2×10^{-5} M) at 554 nm as a function of $[Hg^{2+}]$ in ppb level.

and transition metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Zn^{2+}, and Pb^{2+}) did not show any significant fluorescence enhancement at 554 nm even upon addition of 25 mol equiv of respective metal ions. Relative fluorescence enhancement of $\mathbf{L_1}$ in the absence and presence of various other metal ions and thereby its selectivity for Hg^{2+} is shown in Figure 5.

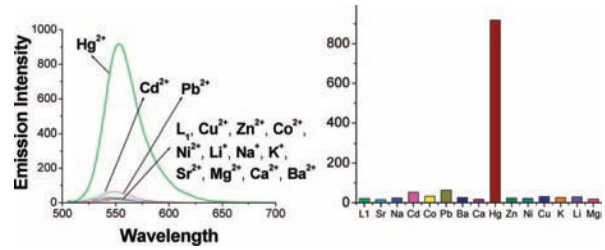

Figure 5. Fluorescence spectra of $\mathbf{L_1}$ (8 μM) in water−methanol (1:1, v/v) at pH 7 with respective metal cations (33 equiv). λ_{ext} at 500 nm.

Competitive recognition of Hg^{2+} in the presence of various other metal ions, in even higher concentration, was also

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studied, which revealed that Hg^{2+} present in 33 mol equiv could be detected even in the presence of 33 mol equiv of other metal ions like Na^+, K^+, Ba^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Zn^{2+}, and Pb^{2+} (see Supporting Information). The lower detection limit for Hg^{2+}, using this chemosensor (**L_1**), was also evaluated. The fluorescence titration profile of **L_1** (20 μM) with Hg^{2+} demonstrates that Hg^{2+} could be detected at the parts per billion level.[17] Please note that the signal-to-noise ratio for this specific concentration was at least three.

Consequently, it was of great interest to investigate the reversibility of the system. I^- is known to have a strong binding affinity for Hg^{2+},[4i,5d] and upon addition of aqueous methanol solution of 5 equiv of KI to a solution mixture of **L_1** (20 μM) and Hg^{2+} (8 μM), color changed from pink to colorless. Simultaneously, about 95% of the fluorescence intensity gets quenched (see Supporting Information) signifying decomplexation of Hg^{2+} by I^- and followed by a spirolactam ring closure reaction (see Supporting Information). Thus, **L_1** can be classified as a reversible chemosensor for Hg^{2+}.

The Hg^{2+} ion is one of the most potent pollutants present in various effluents. There are several microorganisms which exist in soil and various effluents and are known to have a high affinity for adsorption of the Hg^{2+} ion.

Pseudomonas putida, a Gram -*ve* bacteria is one such bacteria.[18,19] To evaluate the application potential of this newly developed chemosensor for Hg^{2+}, we have used this bacterial cell for detection of Hg^{2+} adsorbed when exposed to the Hg^{2+} solution. *Pseudomonas putida* was cultured in the King's B (KB) medium (Peptone 20 g, glycerol 15 g, K_2HPO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, distilled water 1000 mL, pH 7.2). The cells were harvested and vortexed for making the homogeneous suspension in sterile distilled water. These bacterial cells were exposed to Hg^{2+} (10.0 μM) in water–ethanol (7:3, v/v) for 10 min at 25 °C and then successively exposed to **L_1** (20.0 μM) under the same conditions and monitored through a light microscope (AXIO IMAGER, Carl Zeiss; 100×). Microscopic images revealed that, after treatment with Hg^{2+} and **L_1** the color of bacterial cells changed to pink (Figure 6). These results demonstrate that **L_1** could be used for detecting Hg^{2+} adsorbed on the cell wall of the bacteria. Adsorption of the Hg^{2+} ion on the cell wall was also confirmed by SEM images (Figure 7)

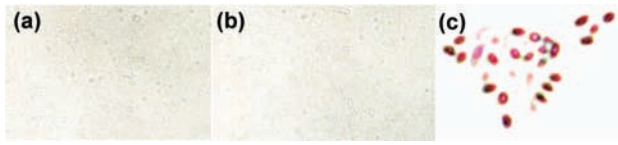

Figure 6. Light microscopy images (100×) of (a) blank cells of *Pseudomonas putida*, (b) bacteria cells exposed to Hg^{2+} solution (10 μM), and (c) cells exposed to aqueous solution of Hg^{2+} (10 μM) and then to a water–ethanol (7/3, v/v) solution of **L_1** (20 μM).

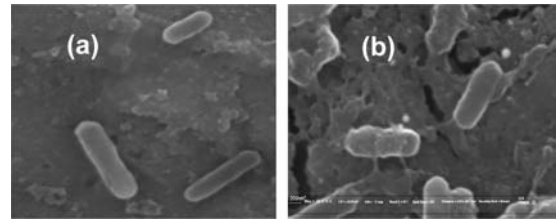

Figure 7. SEM images of *Pseudomonas putida* (a) before and (b) after being exposed to Hg^{2+} and then to **L_1**.

recorded for *Pseudomonas putida*, before and after exposing the live cells to the Hg^{2+} ion and then to **L_1**.

The difference in the relative contrasts in the SEM images of the sample surfaces signifies the nonhomogeneous chemical nature of the sample surface. Relatively brighter cell exterior for *Gram -ve* bacteria indicates the accumulation of higher charges on the extracellular surface.

In conclusion, we have synthesized a new rhodamine-based chemosensor **L_1** that displayed 1:2 complex formation with Hg^{2+} and 1:1 complex formation with Cu^{2+} in aqueous solution. Complex formation processes could be monitored by the spectral changes, as well as through color changes, which could be detected by the naked eye. Binding to Hg^{2+} caused a significant enhancement in the observed fluorescence at 554 nm. This could also be visualized in the dark when irradiated with 365 nm of light. Remarkably, high selectivity for the Hg^{2+} ion in aqueous solution could be demonstrated when it was probed using the fluorescence emission mode. Further, this sensor molecule could detect the Hg^{2+} ion adsorbed on the cell surface of the microorganism such as *Pseudomonas putida* and thus could be useful for determining the amount of Hg^{2+} that could enter in the food chain affecting human beings along with phytoplanktons and zooplanktons.

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Supporting Information Available: Synthetic details, characterization data for the compound **L_1**, and selected spectroscopic data of **L1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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