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NIR- and FRET-Based Sensing of Cu^{2+} and S^{2-} in Physiological Conditions and in Live Cells

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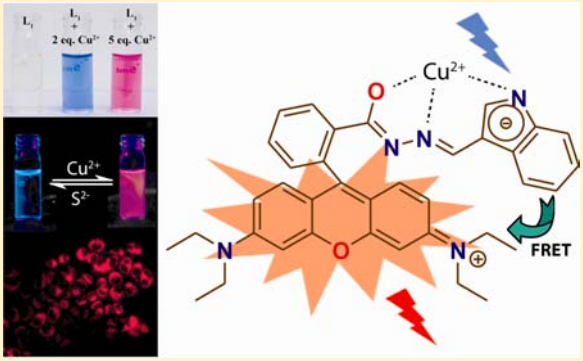
Supporting Information

ABSTRACT: We have synthesized a new indole functionalized rhodamine derivative $\mathbf{L_1}$ which specifically binds to Cu^{2+} in the presence of large excess of other competing ions with visually observable changes in their electronic and fluorescence spectral behavior. These spectral changes are significant enough in the NIR and visible region of the spectrum and thus enable naked eye detection. The receptor, $\mathbf{L_1}$, could be employed as a resonance energy transfer (RET) based sensor for detection of Cu^{2+} based on the process involving the donor indole and the acceptor Cu^{2+} bound xanthene fragment. Studies reveal that $\mathbf{L_1}$**−Cu** complex is selectively and fully reversible in presence of sulfide anions. Further, fluorescence microscopic studies confirmed that the reagent $\mathbf{L_1}$ could also be used as an imaging probe for detection of uptake of these ions in HeLa cells.



INTRODUCTION

The general basis of designing a molecular sensor for selective recognition of different species depends upon host−guest interaction promoted by hydrogen bonding, electrostatic force, metal−ligand coordination, and hydrophobic and van der Waals interaction.[1] In recent years, the development of novel colorimetric and fluorescent sensors of biologically active metal ions has been extensively investigated because of their potential applications in life sciences, medicine, chemistry, and biotechnology.[2] Copper is one of the most abundant essential trace elements found in the human body and has a fundamental role to sustain important physiological processes.[3] Copper-containing enzymes play a significant role in different catalytic processes starting from providing energy for biochemical reactions to assisting the formation of cross-links in collagen and elastin, and thereby maintaining and repairing connective tissues related to heart and arteries.[4] On the basis of research findings, it has been suggested that copper deficiency can increase the risk of developing coronary heart disease.[5] However, copper in excessive amounts could lead to detrimental effects by causing oxidative stress and disorders associated with neurodegenerative diseases including Alzheimer's, Parkinson's, Menkes, Wilson's, and prion diseases.[6,7] Owing to the significant physiological relevance and associated biomedical implications, there is considerable interest in developing selective and sensitive copper sensors. However, the challenge is in the development of sensors which are biocompatible and functional in the complex biological milieu.

Development of selective and efficient signaling units for detection of various chemically and biologically important anions has also attained significant interest.[8] Being one of the biologically and environmentally important anions, sulfide is largely used in industrial processes, for instances, conversion into sulfur, preparation of sulfuric acid and dyes, cosmetic manufacturing, production of wood pulp, etc.[9] Apart from industrial processes sulfide anions can also be generated due to microbial reduction of sulfate by anaerobic bacteria or formed from the sulfur-containing amino acids in meat proteins.[9] Consequently, there are enough risks for the sulfide ions to be exposed to drinking water. Sulfide can damage mucous membranes and can cause unconsciousness and respiratory problems.[9,10] The protonated forms HS^- or H_2S are even more toxic than the sulfide itself. At a low concentration, H_2S can produce dizziness, while at a higher concentration it can result in loss of consciousness, permanent damage of brain tissues, or even death through suffocation.[11] Therefore, development of a quick and sensitive method for immediate sulfide detection in aqueous media and in biological systems is of high interest.

In the recent literature, a large number of colorimetric and fluorescent chemosensors have been reported for selective sensing of copper and sulfide ions with absorbance and emission in the visible region.[12−22] Although chemosensors with spectroscopic responses (absorbance/emission) in the visible region have important roles in various research areas, recently molecular probes with near-infrared (NIR, 700−1000 nm) optical responses are particularly gaining special interest.[23] As for NIR radiation the possibility of scattering is minimized, so unlike UV−vis radiation, NIR radiation provides high sample penetration. Additionally, the autofluorescence generated from the chromophores and macromolecules present in the analytic

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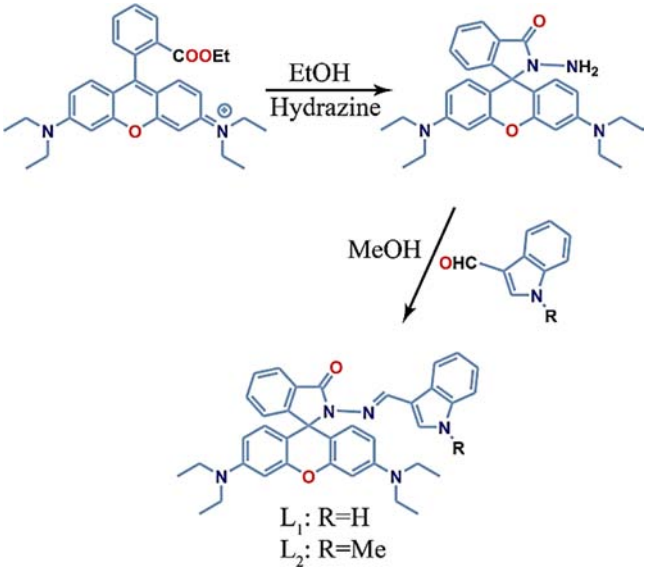
samples are less likely to interfere. This phenomenon enables the assessment of molecular and physiological events in several layers deep inside the analyte samples and tissues.[24] There have been few reports on UV−vis−NIR sensing of cation and anions.[25−29]

Together with visible and NIR absorbance signals, a fluorescence response at a visible region would be an added advantage to the sensing of cations and anions over the methods involving just one kind of optical response. Moreover, the fluorescence chemosensors are known for their efficiency with regard to sensitivity, specificity, and fast response real-time monitoring.[30] Commonly, one of the three different photoinduced processes are involved for the signaling event of luminescence based chemosensors, namely, PET (photoinduced electron transfer),[31] PCT (photoinduced charge transfer),[32] and RET (resonance energy transfer).[33] RET is a nonradiative energy transfer process in which the excitation energy of the donor is transferred to the nearby acceptor via long-range dipole−dipole interaction and/or short-range multipolar interaction. RET-based probe molecules are particularly favorable for biological applications rather than single dye-based probes, as the RET-based process is not dependent on the concentration of a single emissive probe, and one can quantitively measure the analyte concentration by using the ratio of intensities of the well separated fluorescence peaks with rational intensities at two different wavelengths for free probe and analyte bound probe.[34]

Encouraged by the biological importance of Cu^{2+} and S^{2-} ions, we envisioned combining the advantage of the characteristics of a NIR optical response with the sensitivity of a RET based fluorophoric response for the construction of a chemosensor probe (Scheme 1), that may be potentially useful

Scheme 1. Synthetic Scheme for the Ligands L_1 and L_2



for the detection of Cu^{2+} as well as S^{2-} ions at physiological pH. In our continuous effort to design a sensor for various analytes,[35] here we discuss the metal and anion sensing capabilities of an indole functionalized rhodamine based fluorophoric ligand L_1 and the absorption and fluorescence behavior of L_1 upon metal complexation. We have also demonstrated by fluorescence microscopic studies that ligand L_1 could readily detect the presence of intracellular Cu^{2+} as well as S^{2-} ions in live HeLa cells via a characteristic fluorescence switch ON/OFF mechanism. We have reasonably chosen rhodamine and indole derivatives as the two fluorophores for designing the receptor L_1; the binding of Cu^{2+} with L_1 triggers the opening of the spyrolactum ring of the rhodamine derivative, whose absorption spectra shows a significant spectral overlap with the emission spectra of the indole moiety and offers the chance of a RET process. In addition to the FRET signal, an increased absorbance in the NIR region of the UV−vis spectra is also noticed. The "switch ON" behavior of the receptor L_1 is observed selectively in the presence of Cu^{2+} ions. The formation of the L_1−Cu complex is reversible, but the reversibility of the L_1−Cu system to regenerate L_1 is noticeable only in the presence of S^{2-} anions. Details about the synthesis of the receptor L_1 and the control compound L_2 are discussed below, and their characterization data are presented in the Supporting Information.

EXPERIMENTAL SECTION

General Information and Materials. All of the materials for synthesis were purchased from commercial suppliers and used without further purification. The absorption spectra were recorded on a Perkin-Elmer Lamda-25 UV−vis spectrophotometer using 10 mm path length quartz cuvettes in the range 300−800 nm wavelengths, while the fluorescence measurements were carried on a Horiba Fluoromax-4 spectrofluorometer using 10 mm path length quartz cuvettes with a slit width of 5 nm at 298 K. The mass spectra of L_1 and the L_1−Cu complex were obtained using Waters Q-ToF Premier mass spectrometer, and the mass spectrum of L_2 was obtained using Agilent Technologies 6520 Accurate mass spectrometer. NMR spectra were recorded on a Varian FT-400 MHz instrument. The chemical shifts were recorded in parts per million (ppm) on the scale. The following abbreviations are used to describe spin multiplicities in ^{1}H NMR spectra: s = singlet; d = doublet; t = triplet; m = multiplet. Elemental analyses were performed with a Perkin-Elmer 2400 elemental analyzer.

Synthesis of L_1. Rhodamine B hydrazide was prepared following a literature method.[36] Rhodamine B hydrazide (456 mg, 1 mmol) and indole-3-carboxaldehyde (145 mg, 1 mmol) were dissolved in 20 mL of methanol. To this was added approximately 2 drops of acetic acid, and the resulting solution was refluxed for 10 h. An off-white precipitate was found. The reaction mixture was allowed to attain room temperature, and then the precipitate was collected through filtration. The residue was washed thoroughly with methanol to isolate L_1 in pure form with 83% yield (the yield was calculated based on the starting reagents). ^{1}H NMR [400 MHz, $CDCl_3$, $SiMe_4$, J (Hz), δ (ppm)]: 9.26 (1H, s), 8.51 (1H, s), 7.96 (1H, d, J = 8.4), 7.90 (1H, d, J = 7.6), 7.49 (2H, t, J = 3.6), 7.05−7.20 (7H, m), 6.54 (1H, d, J = 8.8), 6.46 (1H, d, J = 2.4), 6.24 (1H, d, J = 2.4), 6.22 (1H, d, J = 2.4), 3.28 (8H, q, J = 7.2), 1.11 (12H, t, J = 7.2). ^{13}C NMR [100 MHz, $CDCl_3$, $SiMe_4$, δ (ppm)]: 164.20, 153.85, 151.09, 148.90, 148.01, 136.78, 132.87, 131.39, 128.79, 128.50, 124.85, 124.16, 123.11, 122.91, 122.75, 120.88, 113.62, 111.31, 108.04, 106.94, 102.02, 98.01, 66.80, 44.47, 12.77. ESI-MS (positive mode, m/z) Calcd for $C_{37}H_{37}N_5O_2$: 583.29. Found: 584.30 ($M + H^+$). Anal. Calcd. for $C_{37}H_{37}N_5O_2$ (583.29): C 76.13, H 6.39, N 12.00, O 5.48. Found: C 76.06, H 6.34, N 12.08, O 5.45;

Synthesis of L_2. 1-Methyl indole-3-carboxaldehyde was prepared by the reported procedure.[37] Rhodamine B hydrazide (456 mg, 1 mmol) and 1-methyl indole-3-carboxaldehyde (159 mg, 1 mmol) were dissolved in 20 mL of methanol. To this was added approximately 2 drops of acetic acid, and the resulting solution was refluxed for 10 h. A white precipitate was found. The reaction mixture was allowed to attain room temperature, and then the precipitate was collected through filtration. The residue was washed thoroughly with methanol to isolate L_2 in pure form with 70% yield (the yield was calculated based on the starting reagents). ^{1}H NMR [400 MHz, $CDCl_3$, $SiMe_4$, J (Hz), δ (ppm)]: 10.00 (1H, s), 8.12 (1H, d, J = 7.2), 7.92 (1H, d, J = 2.8), 7.44 (2H, t, J = 2.8), 7.08−7.21 (7H, m), 6.47 (1H, d, J = 9.2), 6.40 (1H, d, J = 2.0), 6.25 (2H, q, J = 2.4, J = 4.0), 4.76 (3H, s),

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3.32 (8H, q, J = 7.2), 1.16 (12H, t, J = 7.2). ^{13}C NMR [100 MHz, $CDCl_3$, $SiMe_4$, δ (ppm)]: 185.44, 168.99, 168.68, 157.34, 154.03, 153.51, 149.01, 137.30, 132.64, 131.17, 129.84, 129.98, 128.75, 128.21, 125.54, 124.74, 123.99, 122.95, 122.84, 121.74, 121.40, 121.11, 119.20, 114.96, 112.28, 111.82, 108.38, 108.31, 105.59, 98.08, 97.94, 65.46, 44.48, 41.87, 12.76. ESI-MS (positive mode, m/z) Calcd for $C_{38}H_{39}N_5O_2$: 597.31. Found: 598.3188 (M + H$^+$). Anal. Calcd for $C_{38}H_{39}N_5O_2$ (597.31): C 76.35, H 6.58, N 11.72, O 5.35. Found: C 76.38, H 6.51, N 11.76, O 5.29;

UV−Vis and Fluorescence Spectral Studies. Stock solutions of various ions (1×10^{-3} mol L^{-1}) were prepared in deionized water. A stock solution of $\mathbf{L_1}$ (1×10^{-3} mol L^{-1}) was prepared in DMSO. The solution of $\mathbf{L_1}$ was then diluted to 1×10^{-5} mol L^{-1} with CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). In titration experiments, each time a 1×10^{-3} L solution of $\mathbf{L_1}$ (1×10^{-5} mol L^{-1}) was filled in a quartz optical cell of 1 cm optical path length, and the ion stock solutions were added into the quartz optical cell gradually by using a micropipet. Spectral data were recorded at 1 min after the addition of the ions. In selectivity experiments, the test samples were prepared by placing appropriate amounts of the anions/cations stock into 2 mL of solution of $\mathbf{L_1}$ (2×10^{-5} mol L^{-1}). For fluorescence measurements, excitation was provided at 340 and 495 nm, and emission was collected from 390/500 to 650/700 nm.

Evaluation of the Apparent Binding Constant for the Formation of $\mathbf{L_1}$·Cu^{2+}. Receptor $\mathbf{L_1}$ with an effective concentration of 10.0×10^{-6} M in an acetonitrile/aqueous HEPES buffer (1 mM; 1:4, v/v; pH 7.3) was used for the emission titration studies with a Cu^{2+} solution. A stock solution of $Cu(NO_3)_2$, having a concentration of 0.2×10^{-3} M, in an acetonitrile/aqueous HEPES buffer (1:4, v/v; pH 7.3) solution was used. The effective Cu^{2+} concentration was varied between 0 and 10×10^{-5} M for this titration. The solution pH was adjusted to 7.3 using an aqueous HEPES buffer solution having an effective concentration of 1 mM.

Calculations for the Apparent Binding Constants Using Spectrophotometric Titration Data. The apparent binding constant for the formation of the respective complexes were evaluated using the Benesi−Hildebrand (B−H) plot (eq 1).[35h,38]

$$1/(A - A_0) = 1/\{K(A_{max} - A_0)C\} + 1/(A_{max} - A_0) \quad (1)$$

A_0 is the absorbance of $\mathbf{L_1}$ at absorbance maximum (λ = 702 nm), A is the observed absorbance at that particular wavelength in the presence of a certain concentration of the metal ion (C), A_{max} is the maximum absorbance value that was obtained at λ = 702 nm during titration with varying metal ion concentration, K is the apparent binding constant (M^{-1}) and was determined from the slope of the linear plot, and C is the concentration of the Cu^{2+} ion added during titration studies.

Finding the Detection Limit. The detection limit was calculated on the basis of the UV−vis titration. The fluorescence emission spectrum of $\mathbf{L_1}$ was measured 10 times, and the standard deviation of blank measurement was achieved. To gain the slope, the ratio of the UV−vis absorbance at 702 nm was plotted as a concentration of Cu^{2+}.

So the detection limit was calculated with the following equation

$$\text{detection limit} = 3\sigma/k \quad (2)$$

where σ is the standard deviation of blank measurement, and k is the slope between the ratio of UV−vis absorbance versus [Cu^{2+}].

Evaluation of Different Parameters for FRET Process. The Förster distance R_0 was calculated using the expression shown in eq 3

$$R_0 = 0.211[(J)Q(n^{-4})(\kappa^2)] \quad (3)$$

where n is the refractive index of the medium in between donor and acceptor and was taken approximately to be equal to 1.4. κ^2 is the dipole orientation factor. Depending upon the relative orientation of donor and acceptor, the value is in the range 0−4, and it is often assumed to be $^2/_3$. Q is the fluorescence quantum yield of the donor in the absence of acceptor. J is the spectral overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor and is shown in the following eq 4

$$J = \int f_D(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda \quad (4)$$

where $f_D(\lambda)$ is the normalized emission of the donor and $\varepsilon(\lambda)$ is the molar absorption coefficient (M^{-1} cm^{-1}) of the donor. Energy transfer efficiency (Φ_{ET}) was evaluated using the expression shown in eq 5

$$\Phi_{ET} = 1 - (F'_D/F_D) \quad (5)$$

where F'_D and F_D denote the donor fluorescence intensity with and without an acceptor, respectively.

Energy transfer rate constant (K_{ET}) was calculated using eq 6

$$\Phi_{ET} = K_{ET}/(1/\tau_D + K_{ET}) \quad (6)$$

where τ_D denotes the fluorescence lifetime of the donor fragment in the absence of acceptor.

Cytotoxic Effect on HeLa Cells. The cytotoxic effect of compound $\mathbf{L_1}$ and $\mathbf{L_1}$**−Cu** complex was determined by an MTT assay following the manufacturer instruction (Sigma-Aldrich, MO). HeLa cells were initially propagated in a 25 cm^2 tissue culture flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 μg/mL), and streptomycin (100 μg/mL) in a CO_2 incubator. For cytotoxicity assay, cells were seeded into 96-well plates (approximately 10^4 cells per well), and various concentrations of compound $\mathbf{L_1}$ and $\mathbf{L_1}$**−Cu** complex (15, 25, 50, 75, and 100 μM) made in DMEM were added to the cells and incubated for 24 h. Solvent control samples (cells treated with DMSO alone) and cells treated with $Cu(ClO_4)_2$ alone were also included in parallel sets. Following incubation, the growth media was removed, and fresh DMEM containing MTT solution was added. The plate was incubated for 3−4 h at 37 °C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured in a microtiter plate reader (Infinite M200, TECAN, Switzerland) at 550 nm. The assay was performed in six sets for each concentration of compound $\mathbf{L_1}$ and $\mathbf{L_1}$**−Cu** complex. Data analysis and calculation of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation). For statistical analysis, a one way analysis of variance (ANOVA) was performed using Sigma plot.

Cell Culture and Imaging Studies. HeLa cells were procured from National Center for Cell Sciences (NCCS), Pune, India. The cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 μg/mL), and streptomycin (100 μg/mL). Cells were maintained under a humidified atmosphere of 5% CO_2 and at 37 °C incubator as mentioned before. For cell imaging studies, cells were seeded into a 6 well plate and incubated at 37 °C in a CO_2 incubator for 3 days. After 3 days cells were washed three times with phosphate buffered saline (pH 7.4) and incubated with 10 μM $\mathbf{L_1}$ in DMEM at 37 °C for 1 h in a CO_2 incubator and observed under epifluorescence microscope (Nikon eclipse Ti). The cells were again washed thrice with PBS (pH 7.4) to remove the free $\mathbf{L_1}$, and then incubated in phosphate buffered saline with 20 μM $Cu(ClO_4)_2$ for 1 h. Again, images were taken using epifluorescence microscope. The cells were then treated with 30 μM of Na_2S solution, after incubation for 1 h; the cells were washed with PBS three times to remove free compound and ions before analysis. Then, fluorescence microscopic images were acquired.

RESULT AND DISCUSSION

UV−Vis Spectroscopic Studies of $\mathbf{L_1}$ in Presence of Cu^{2+}. UV−vis spectra recorded for $\mathbf{L_1}$ in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4, v/v) indicated an absorption maximum at 324 nm, which may possibly be attributed to intramolecular $\pi-\pi^*$ charge transfer (CT) transition. According to previous studies certain transition-metal ions bind selectively with suitable derivatives of rhodamine,[39] wherein metal−ligand binding induces opening of the spirolactam ring and generation of the xanthene form. This structural change is manifested in the electronic and fluorescence spectral patterns. Thus, the selectivity of $\mathbf{L_1}$ was checked with perchlorate or nitrate salts of Na$^+$, K$^+$, Ca^{2+}, Mg^{2+}, Cr^{3+}, Hg^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Ag$^+$ in CH_3CN/aqueous

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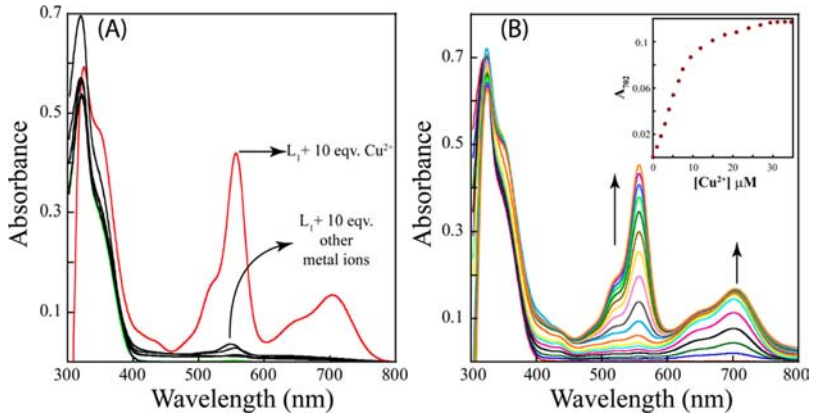
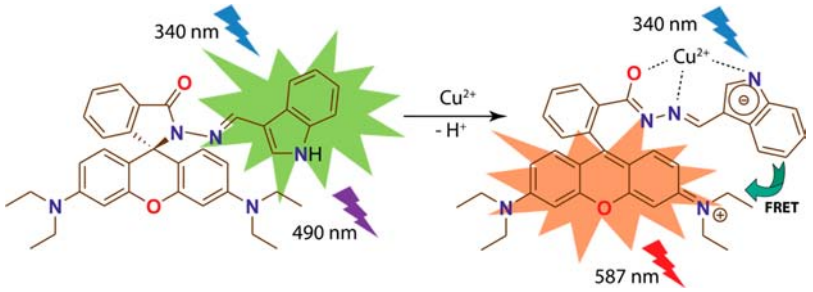


Figure 1. (A) UV−vis absorption spectra of receptor $\mathbf{L_1}$ (10 μM) observed upon addition of 10 equivalent metal ions (perchlorate or nitrate salts of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cr^{3+}, Hg^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Ag^+) in a CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). (B) UV−vis titration spectra of $\mathbf{L_1}$ (10 μM) upon incremental addition of $Cu(ClO_4)_2$ in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). Inset: Changes in the absorbance at 702 nm with incremental addition of Cu^{2+}.

Scheme 2. Cu^{2+}-Induced FRET OFF→ON of the Receptor $\mathbf{L_1}$



HEPES buffer (1 mM, pH 7.3; 1:4 v/v). A significant change in UV−vis spectral pattern was observed only in presence of Cu^{2+}, among all the other metal ions used (Figure 1A). During sequential titration (0 equiv to 3 equiv of Cu^{2+}), a NIR absorption band appeared around 702 nm along with a hump in the visible region near 557 nm (Figure 1B), and the solution turned from colorless to blue (Figure S8). With further increase in the concentration of Cu^{2+} the color of the solution changed from blue to pink with the emergence of a sharp peak near 557 nm of the electronic spectra. It is significant to mention that the detection limit of $\mathbf{L_1}$ for Cu^{2+} ions was found to be 3.6 ppb, which is much lower than the U.S. EPA maximum allowable limit for Cu^{2+} ions (1.3 ppm) in drinking water.

In the recent literature, deprotonation induced NIR sensing by indole moiety was reported by P. Ghosh and co-workers.[38b] In the present study, visual color change of the solution from colorless to blue is a consequence of the ratiometric deprotonation of the NH proton of indole moiety in presence of the metal cation. Such a huge shift of absorption spectra could be attributed to the high conjugation and planarity of the indole moiety of $\mathbf{L_1}$ with the binding site of the receptor (Scheme 2) which favors maximum distribution of the negative charge of the deprotonated receptor in the presence of the guest metal ion. Regardless of the deprotonation of indole moiety, the formation of the peak near 557 nm is mostly due to the breakage of the spirolactum ring structure of $\mathbf{L_1}$ and subsequent formation of the xanthene form. The spectral changes due to formation of the $\mathbf{L_1}$**−Cu** complex and the deprotonation of the indole unit occur simultaneously with addition of Cu^{2+}. However, in the present case the individual steps cannot be analyzed in solution. Therefore, the apparent binding constant for the formation of $\mathbf{L_1}$**−Cu** complex is calculated on the basis of change in absorbance at 702 nm by considering a 1:1 binding stoichiometry. The apparent binding constant (K) determined by the B−H method was found to be 1.71×10^4 M^{-1}.

To verify the significance of the deprotonation of indole −NH in generating the NIR peak, a new control compound $\mathbf{L_2}$ was synthesized where the indole −NH was replaced by $-CH_3$. As expected, compound $\mathbf{L_2}$ failed to show a NIR peak in presence of Cu^{2+}. The only change of the absorption spectra of $\mathbf{L_2}$ in presence of Cu^{2+} ions was the formation of a sharp peak near 550 nm, predominantly due to the rupture of the spirolactum ring of rhodamine into the xanthene form (Figure S9).

Fluorescence Spectroscopic Studies of $\mathbf{L_1}$ in Presence of Cu^{2+}. The selective binding of $\mathbf{L_1}$ with Cu^{2+} among all other metal ions was also studied using the emission spectroscopy of the solution of $\mathbf{L_1}$ (10.0×10^{-6} M) in the absence and presence of an excess (10 equiv) of each of the metal ions in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v) (Figure 2A). As the receptor $\mathbf{L_1}$ bears two different fluorophore units, we consider it to be appropriate to study the metal binding event of $\mathbf{L_1}$ at two different excitation wavelengths corresponding to the excitation wavelength of the xanthene unit (495 nm) and the indole unit (340 nm), respectively.

As evident from Figure 2B, excitation of the initial solution of receptor $\mathbf{L_1}$ at 495 nm wavelength did not show any significant emission over the range from 500 to 700 nm. This supports the facts that in absence of metal ions the receptor remains in the spirolactum form, and the nonexistence of the highly conjugated xanthene form results in the suppression of emission in the above-mentioned region. Addition of Cu^{2+} to this receptor solution induces a significant switch ON fluorescence response

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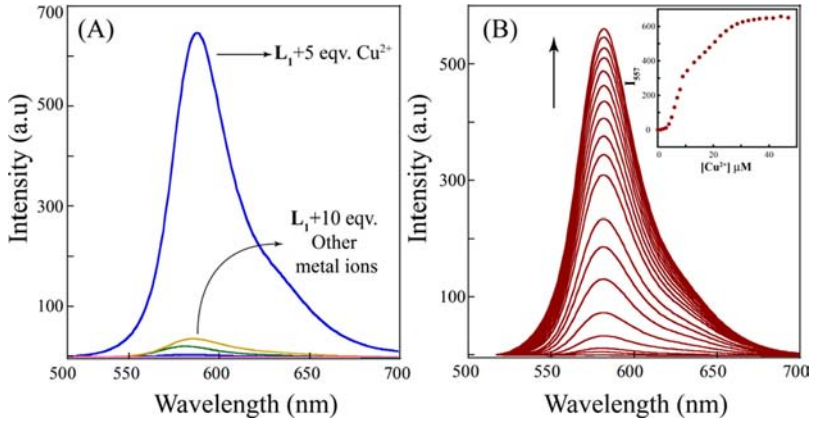

Figure 2. (A) Changes of the fluorescence emission of receptor $\mathbf{L_1}$ (10 μM) observed upon addition of metal ions (perchlorate or nitrate salts of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cr^{3+}, Hg^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Ag^+) (10 equiv) in a CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). (B) Fluorescence titration spectra of $\mathbf{L_1}$ (10 μM) upon incremental addition of 5 equiv of $Cu(ClO_4)_2$ in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v) λ_{ex} = 495 nm. Inset: Changes in the fluorescence intensity at 557 nm with incremental addition of Cu^{2+}.

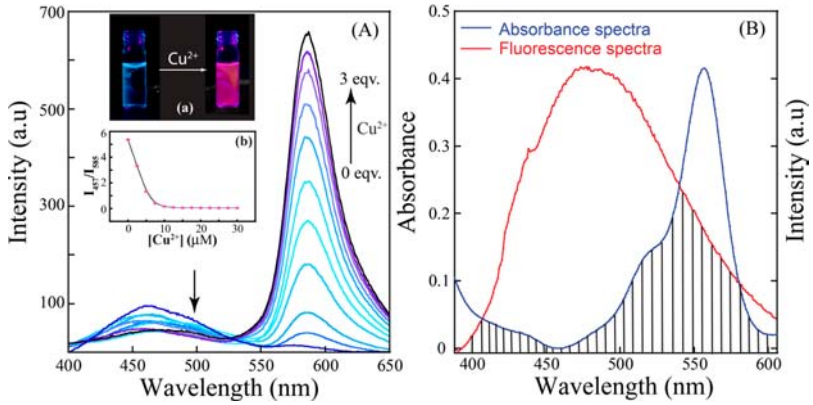

Figure 3. Fluorescence spectra (λ_{ex} = 340 nm) of (A) $\mathbf{L_1}$ (10 μM) with varying [Cu^{2+}] (0–3 equiv), and (B) the overlap (shown with vertical stripes) between emission and absorption spectra of the donor and acceptor, respectively. All studies were carried out in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v) medium. Inset: (a) Visual change of the color of the $\mathbf{L_1}$ solution in presence of Cu^{2+} under UV light. (b) The changes of fluorescent intensity ratios at 457 and 585 nm (I_{457}/I_{585}) with increasing concentration of Cu^{2+}.

near 580 nm, with a visual display of reddish-pink fluorescence. Switch ON responses for the absorption spectral band at 557 nm and the emission band at ~580 nm on binding to Cu^{2+} suggest opening of the spirolactam ring in $\mathbf{L_1}$ on metal ion coordination. It can also be observed from Figure 2A that the metal–ligand binding induced ring-opening of $\mathbf{L_1}$ and the generation of xanthene moiety is very much selective toward Cu^{2+} ions and does not reveal any noticeable spectral change for other tested metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cr^{3+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Ag^+). To gain an insight into the properties of $\mathbf{L_1}$ as a receptor for Cu^{2+}, a titration of the receptor was performed with increasing concentration of Cu^{2+}. As described in Figure 2B the fluorescence intensity of a 10 μM solution of $\mathbf{L_1}$ was enhanced with incremental addition of Cu^{2+} ions, which also confirmed that receptor $\mathbf{L_1}$ exhibited a high sensitivity toward Cu^{2+}, with near about 600-fold increase of its fluorescence intensity upon addition of only 5.0 equiv of Cu^{2+} ions.

The binding of Cu^{2+} ion induces opening of the spirolactam ring in $\mathbf{L_1}$ with an associated switch on UV–vis spectral response in the range 500–580 nm, which has a significant spectral overlap with the emission spectrum of the indole fragment, and this fact unlocks a plausible route for nonradiative transfer of excitation energy between donor indole to acceptor xanthene moiety and initiates an intramolecular FRET process. In the free ligand $\mathbf{L_1}$ the FRET pathway is totally suppressed, and only an emission maximum near 480 nm is observed when excited at 340 nm. Binding of the receptor to Cu^{2+} induces the FRET process to produce an intense rhodamine-based red emission; i.e., energy transfer from indole to xanthene is due to the ring-opening resulting in increase of overlap integral between indole and xanthene moiety.[34g] Thus, when titrated with increasing concentration of Cu^{2+} the emission band with a λ_{max} near 457 nm starts to decrease along with a concomitant generation of a new fluorescence band at 585 nm. This change in fluorescence was also observed visually, and the color changed to reddish-pink as shown in the inset of Figure 3A.

The singlet–singlet excitation energy-transfer efficiency (Φ_{ET}) between donor and acceptor was evaluated from steady state fluorescence data. The value for Φ_{ET} was found to be 51.5% while the Förster critical distance (R_0) was calculated as 35.8 Å.

The complex formed between $\mathbf{L_1}$ and Cu^{2+} is found to be 1:1 in stoichiometry, which is established with the help of Job's plot (Figure S10) from fluorescence spectrometry. Further confirmation of 1:1 stoichiometry was obtained from the ESI-MS studies. The molecular-ion peak is observed at m/z 708.21 in the mass spectrum which is related to the mass of [$\mathbf{L_1}$ + Cu^{2+} + NO_3] (Figure S11).

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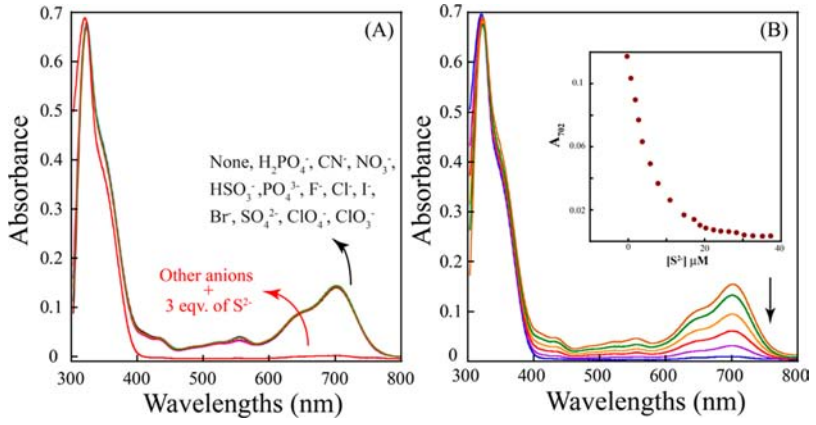

Figure 4. (A) Changes in the absorption spectra of $\mathbf{L_1}$**−Cu** complex in presence of different anions. (B) UV−vis titration spectra of $\mathbf{L_1}$ (10 μM) with 2 equiv of Cu^{2+} upon addition of sodium sulfide (30 μM) in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). Inset: Changes in the absorbance at 702 nm with incremental addition of S^{2-}.

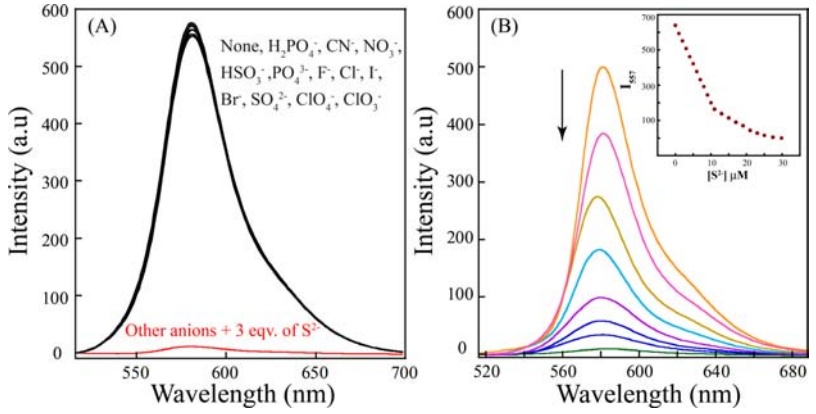

Figure 5. (A) Changes in the fluorescence spectra of $\mathbf{L_1}$**−Cu** complex in presence of different anions (λ_{ex} = 495 nm). (B) Fluorescence titration spectra (λ_{ex} = 495 nm) of $\mathbf{L_1}$ (10 μM) with 2 equiv of Cu^{2+} upon addition of sodium sulfide (30 μM) in aqueous CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). Inset: Changes in the fluorescence intensity at 557 nm with incremental addition of S^{2-}.

The role of the C═O amide bond on binding Cu^{2+} cations was examined using FTIR techniques. The FTIR spectra of $\mathbf{L_1}$ revealed that the peak at 1702 cm^{-1}, the characteristic stretching frequency for the C═O amide bond of the rhodamine unit, shifted to 1630 cm^{-1} in presence of 2 equiv of the Cu^{2+} ion (Figure S12). Such shift in the stretching frequency of C═O amide bond of the rhodamine unit on binding to a metal ion has been reported earlier.[40] Thus, FTIR studies suggest that the shift in the stretching frequency of C═O bond was due to its participation in the binding event of the metal cation.

UV−Vis Spectroscopic Studies of $\mathbf{L_1}$−Cu Complex in Presence of S^{2-}. Hence from the above-mentioned studies we can conclude that $\mathbf{L_1}$ selectively binds with Cu^{2+} to form $\mathbf{L_1}$**−Cu** complex with considerable change in its spectral properties. We have further studied the influence of different anions on the rupture of this metal−ligand complex and their effect on the reversibility of this complex to regenerate $\mathbf{L_1}$. The optical properties of the $\mathbf{L_1}$**−Cu** complex were studied in presence of different anions such as F^-, Cl^-, Br^-, I^-, CN^-, $H_2PO_4^-$, NO_3^-, ClO_3^-, ClO_4^-, SO_4^{2-}, HSO_3^-, PO_4^{3-}, and S^{2-}. It is worth mentioning that the regeneration of compound $\mathbf{L_1}$ is observed only by adding S^{2-} to the solution containing $\mathbf{L_1}$**−Cu**, whereas other anions failed to produce any discernible spectral change (Figure 4A). For further understanding, a solution of $\mathbf{L_1}$ in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v) containing 2 equiv of Cu^{2+} is titrated in presence of sulfide anions. The UV−vis spectral pattern of the titration experiment (Figure 4B) was similar but in reverse direction to the titration curve obtained with Cu^{2+} (Figure 1B). This fact is evidence that ligand $\mathbf{L_1}$ is regained from complex in presence of S^{2-}.

Fluorescence Spectroscopic Studies of $\mathbf{L_1}$−Cu Complex in Presence of S^{2-}. Apart from the results obtained from UV−vis studies, the fluorescence spectroscopy also shows that the emission of the $\mathbf{L_1}$**−Cu** complex returns to its native $\mathbf{L_1}$ state, selectively in presence of sulfide anions (Figure 5A). To further understand the fluorescence "ON−OFF" switching property of the sensor, we have performed fluorescence titration experiment. The fluorescence intensity of the compound $\mathbf{L_1}$ is enhanced to a moderate level in presence of 2 equiv of Cu^{2+} ions; the resulting $\mathbf{L_1}$**−Cu** complex is then titrated by the addition of various amounts of sulfide ions. Figure 5B shows that the intensity of the fluorescence emission decreases with increasing concentration of sulfide anion, and on addition of near about 3 equiv of S^{2-} anion both the intensity and overall pattern of emission spectrum closely match those of compound $\mathbf{L_1}$ (Figure 2B), so the fluorescence intensity along with the maximum emission peak are totally regenerated. As the fluorescence spectrum was recorded within 15 s after sulfide anion addition, and the intensity does not change with time, so the monitoring system is virtually real-time and stable. The results of the spectroscopic studies indicated that the sensor $\mathbf{L_1}$ was recycled during the detection of sulfide anions.

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From our Cu^{2+} binding studies it was evident that the binding induced breakage of the spirolactum ring of $\mathbf{L_1}$ initiates the FRET pathway for efficient transfer of energy from indole moiety to the xanthene unit in the $\mathbf{L_1}$-**Cu** complex. It is obvious that if the regeneration of $\mathbf{L_1}$ from $\mathbf{L_1}$-**Cu** complex is possible in presence of sulfide anion then the removal of Cu^{2+} must affect the FRET process. Thus, continuous addition of sulfide ions to the $\mathbf{L_1}$-**Cu** complex generates a spectrum (Figure 6) which is

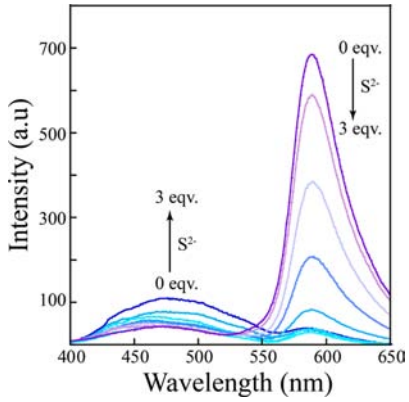

Figure 6. Fluorescence titration spectra (λ_{ex} = 340 nm) of L_1 (10 μM) with 2 equiv of Cu^{2+} upon continuous addition of sodium sulfide (up to 30 μM) in CH_3CN/aqueous HEPES buffer solution (1 mM, pH 7.3; 1:4 v/v).

equivalent to the spectra of $\mathbf{L_1}$ (when excited at 340 nm). The above-mentioned fact provides strong evidence of the dissociation of $\mathbf{L_1}$-**Cu** complex in presence of S^{2-} anions to restore the native structure of $\mathbf{L_1}$.

In order to verify the reason of the fluorescence "OFF–ON" property, the mass spectrum of the $\mathbf{L_1}$**–Cu** system was also studied in presence of S^{2-}. The mass spectrum of the $\mathbf{L_1}$**–Cu** system shows a molecular-ion peak at m/z 708.21, corresponding to $[\mathbf{L_1} + Cu + NO_3]$, while subsequent addition of S^{2-} ions to the above solution gives a molecular ion peak at m/z 584.30 (Figure S13) which confirmed the identity of free $\mathbf{L_1}$ and substantiated the mechanism of the sensing of sulfide anions. The sensing efficiency of $\mathbf{L_1}$ and $\mathbf{L_1}$**–Cu** complex was further tested in presence of other cations and anions, which may interfere in estimation of copper and sulfide (Figure 7). The receptor $\mathbf{L_1}$ and the $\mathbf{L_1}$**–Cu** complex both performed well in presence of other ions and sensed the respective analytes from a competitive environment.

Biological Studies of $\mathbf{L_1}$ in Presence of Cu^{2+} and S^{2-}. Owing to its favorable binding properties with copper(II) and intense emission in visible region, it was conceived that compound $\mathbf{L_1}$ could be exploited for fluorescence imaging of live cells, particularly for sensitive detection of intracellular Cu^{2+}. However, to pursue this goal, it was pertinent to assess the cytotoxic effect of compound $\mathbf{L_1}$ on live cells. Varying concentrations of compound $\mathbf{L_1}$ and $\mathbf{L_1}$**–Cu** complex were thus chosen, and their cytotoxic effect on HeLa cells was ascertained following an exposure period of 24 h. The well-established MTT assay, which is based on mitochondrial dehydrogenase activity of viable cells, was adopted. It is quite evident from Figure 8 that compound $\mathbf{L_1}$ failed to exert any effect on the

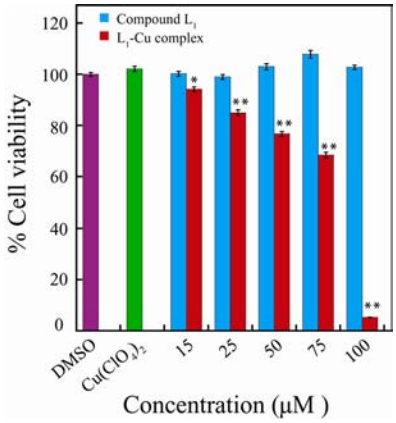

Figure 8. MTT assay to determine the cytotoxic effect of compound $\mathbf{L_1}$ and $\mathbf{L_1}$**–Cu** complex on HeLa cells. Statistically significant values derived by ANOVA are indicated by asterisk marks. * indicates P value <0.05, and ** indicates P value <0.001.

viability of HeLa cells, irrespective of the chosen concentrations of the compound. However, exposure of HeLa cells to the lowest concentration of $\mathbf{L_1}$**–Cu** complex (15 μM) resulted in a decline in cell viability. In presence of higher concentrations of $\mathbf{L_1}$**–Cu** complex, the effect was more prominent and indicated a dose-dependent cytotoxic effect on HeLa cells. Previous literature reports also suggest cytotoxic and antiproliferative effects

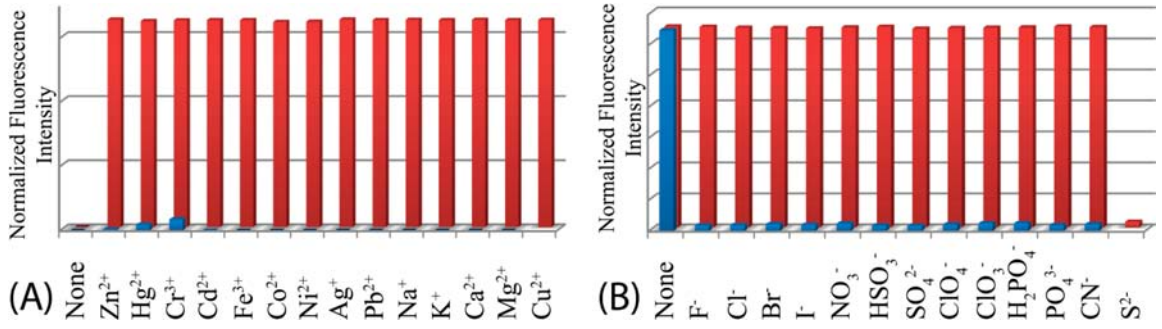

Figure 7. (A) Normalized fluorescence responses of $\mathbf{L_1}$ (10 μM) to various cations in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). The blue bars represent the emission intensities of $\mathbf{L_1}$ in the presence of cations of interest (50 μM). The red bars represent the change of the emission that occurs upon the subsequent addition of Cu^{2+} to the above solution. (B) Normalized fluorescence responses of $\mathbf{L_1}$**–Cu** to various anions in CH_3CN/aqueous HEPES buffer (1 mM, pH 7.3; 1:4 v/v). The red bars represent the emission intensities of $\mathbf{L_1}$**–Cu** in the presence of anions of interest (50 μM). The blue bars represent the change of the emission that occurs upon the subsequent addition of S^{2-} to the above solution. The intensities were recorded at 585 nm.

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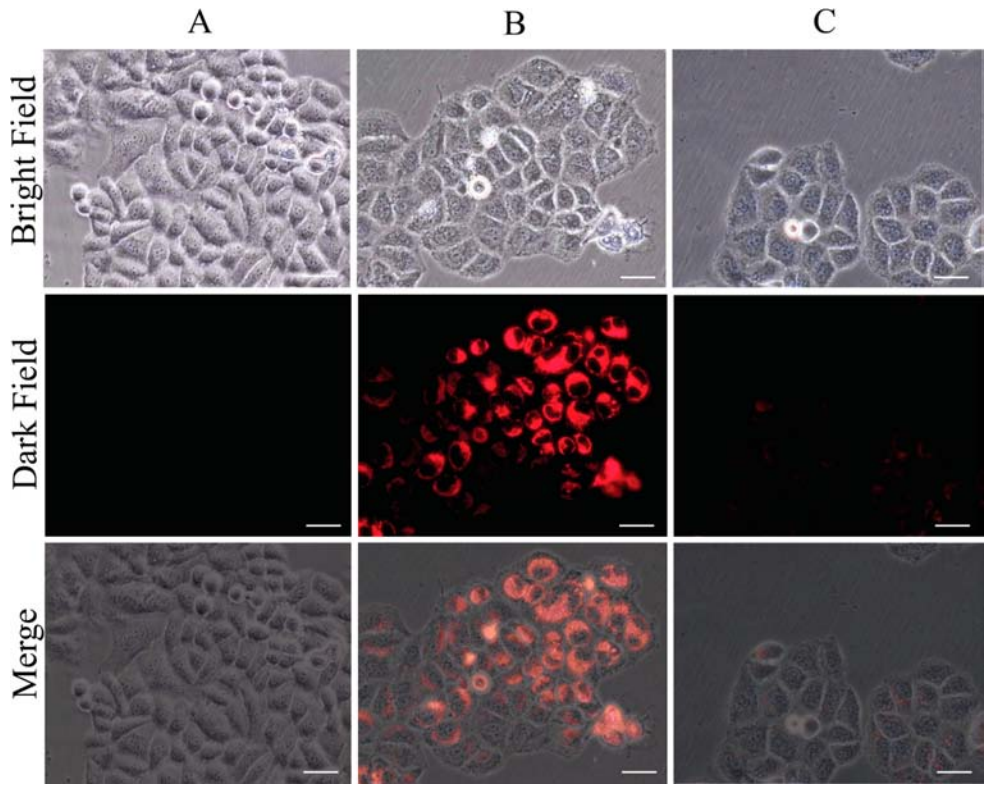

Figure 9. Fluorescence microscopic images of HeLa cells (A) after treating with 10 μM $\mathbf{L_1}$ (under green light), (B) after adding 20 μM of Cu^{2+} (under green light) to the $\mathbf{L_1}$ treated cells, and (C) after adding 30 μM S^{2-} (under green light) to the ($\mathbf{L_1}$ + Cu^{2+}) treated cells. Scale bar for the images is 50 μm.

of copper complex on cancer cells.[41] The viability of HeLa cells was not influenced by the solvent (DMSO) or the copper salt (Figure 8), substantiating that the observed cytotoxic effect could be attributed to $\mathbf{L_1}$**−Cu** complex.

The results obtained in the *in vitro* cytotoxic assay suggested that, in order to pursue fluorescence imaging studies of $\mathbf{L_1}$**−Cu** complex in live cells, it would be prudent to choose a working concentration below 15 μM for compound $\mathbf{L_1}$. Hence, to assess the effectiveness of compound $\mathbf{L_1}$ as a probe for intracellular detection of Cu^{2+} by fluorescence microscopy, HeLa cells were treated with 10 μM $\mathbf{L_1}$ solution for 1 h followed by incubation with 20 μM $Cu(ClO_4)_2$ to promote formation of $\mathbf{L_1}$**−Cu** complex. On the basis of the established 1:1 stoichiometry of binding between $\mathbf{L_1}$ and Cu^{2+} (Figure S10 and S11), it can be reasonably assumed that the concentration of $\mathbf{L_1}$**−Cu** complex formed in HeLa cells would be much lower than the concentration (15 μM) at which a marginal cytotoxic effect of the complex was observed (Figure 8).

Fluorescence microscopic studies revealed a lack of fluorescence for HeLa cells treated with compound $\mathbf{L_1}$ alone (Figure 9, panel A). Upon incubation with $Cu(ClO_4)_2$, a striking switch-ON fluorescence was observed inside HeLa cells, which indicated the formation of $\mathbf{L_1}$**−Cu** complex, as observed earlier in solution studies. Further, an intense red fluorescence was conspicuous in the perinuclear region of HeLa cells (Figure 9, panel B). Interestingly, sulfide sensing inside HeLa cells by $\mathbf{L_1}$**−Cu** complex could also be pursued as evident from the remarkable switch-OFF of the red fluorescence emission inside cells following incubation with Na_2S solution (Figure 9, panel C). Essentially, the fluorescence microscopic analysis strongly suggested that compound $\mathbf{L_1}$ could readily cross the membrane barrier, permeate into HeLa cells, and rapidly sense intracellular Cu^{2+} and S^{2-}. It is significant to mention here that brightfield images of treated cells did not reveal any gross morphological perturbations, which suggested that HeLa cells were viable. This finding is encouraging for future *in vivo* biomedical applications of the sensor.

■ CONCLUSION

In summary, we have developed an NIR sensitive receptor $\mathbf{L_1}$ which selectively binds with Cu^{2+} ions and propels a switch ON response in optical and fluorescence spectra in the visible region. Apart from the NIR and visible changes the FRET-based fluorescence response makes it a dual probe for naked eye detection through change in color and fluorescence. The detection limit for Cu^{2+} was found to be much lower than the permissible Cu^{2+} concentration in drinking water as per standard norms. The complex formed between $\mathbf{L_1}$ and Cu^{2+} is dissociable only in presence of sulfide anion, which makes the $\mathbf{L_1}$**−Cu** complex an efficient sensor for sulfide anions. From the extensive spectroscopic studies it is clear that the receptor $\mathbf{L_1}$ and $\mathbf{L_1}$**−Cu** complex could be used as a ratiometric sensor for the detection of Cu^{2+} and S^{2-}, respectively, based on NIR response and RET process involving the donor indole and the acceptor Cu^{2+} bound xanthene moiety of $\mathbf{L_1}$. The receptor $\mathbf{L_1}$ shows intense change in its fluorescence emission when bound to Cu^{2+} in physiological conditions. Hence, the effectiveness of compound $\mathbf{L_1}$ as a probe for intracellular detection of Cu^{2+} by fluorescence microscopy was also studied. Moreover, the fluorescence microscopic analysis strongly suggested that compound $\mathbf{L_1}$ could readily cross the membrane barrier, permeate into HeLa cells, and rapidly sense intracellular Cu^{2+} and S^{2-}.

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■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR, ^{13}C NMR, mass spectra, and IR spectra as characterization data of L_1 and **L_1–Cu** complex. Benesi–Hildebrand plot, Job's plot, UV–vis spectra, and photograph of the **L_1** solution in presence and absence of Cu^{2+} ions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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