Rhodamine-azobenzene based single molecular probe for multiple ions sensing: Cu$^{2+}$, Al$^{3+}$, Cr$^{3+}$ and its imaging in human lymphocyte cells

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ABSTRACT

A photoinduced electron transfer (PET) and chelation-enhanced fluorescence (CHEF) regulated rhodamine-azobenzene chemosensor (L) was synthesized for chemoselective detection of Al$^{3+}$, Cr$^{3+}$, and Cu$^{2+}$ by UV–Visible absorption study whereas Al$^{3+}$ and Cr$^{3+}$ by fluorimetric study in EtOH-H$_2$O solvent. L showed a clear fluorescence emission enhancement of 21 and 16 fold upon addition of Al$^{3+}$ and Cr$^{3+}$ due to the 1:1 host-guest complexation, respectively. This is first report on rhodamine-azobenzene based Cr$^{3+}$ chemosensor. The complex formation, restricted imine isomerization, inhibition of PET (photo-induced electron transfer) process with the concomitant opening of the spirolactam ring induced a turn-on fluorescence response. The higher binding constants 6.7 × 10$^3$ M$^{-1}$ and 3.8 × 10$^3$ M$^{-1}$ for Al$^{3+}$ and Cr$^{3+}$, respectively and lower detection limits 1 × 10$^{-6}$ M and 2 × 10$^{-6}$ M for Al$^{3+}$ and Cr$^{3+}$, respectively in a buffered solution with high reversible nature describes the potential of L as an effective tool for detecting Al$^{3+}$ and Cr$^{3+}$ in a biological system with higher intracellular resolution. Finally, L was used to map the intracellular concentration of Al$^{3+}$ and Cr$^{3+}$ in human lymphocyte cells (HLCs) at physiological pH very effectively. Altogether, our findings will pave the way for designing new chemosensors for multiple analytes and those chemosensors will be effective for cell imaging study.

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1. Introduction

Environmental pollution caused by the high concentration of heavy metals in sewage remains a global challenging problem. Heavy metal ions like Cu$^{2+}$, Al$^{3+}$, and Cr$^{3+}$ provide essential indispensable nutrients for life when taken in regulated amount, but their uncontrolled or over-exposure generates acute biological and physiological disorder [1–3]. Cu$^{2+}$, a cofactor of various enzymes like cytochrome co-oxidase, tyrosinase, and superoxide dismutase, plays a significant role in a physiological system such as iron regulation [4–6]. It turns as a toxic element when taken in excess of recommended amounts of 1.3 ppm (~20 μM) in drinking water and generated oxidative stress neurodegenerative diseases such as Alzheimer’s disease, Wilson’s disease, Menke’s disease, gastrointestinal lipid metabolism disorders [7]. The toxic carcinogenic Cr$^{6+}$ is converted to Cr$^{3+}$ by bacterial reduction and bind nonspecifically with DNA to damage the intracellular activity like DNA replication and transcription [8]. Although Cr$^{3+}$ is physiologically less harmful compared to Cr$^{4+}$ and Cr$^{6+}$, its deficiency increases the risk of diabetes, cardiovascular disease, and cancer [9]. Extensive use of other metal ions such as aluminum in modern life increases the risk of aluminum toxicity which brings calcium metabolism disorder, interferes the concentration of iron in the blood causing Osteomalacia, microcytic hypochromic anemia. Unregulated intake of aluminum cause encephalopathy, myopathy, dementia, Guamanian amyotrophic lateral sclerosis, Parkinson’s disease, and Alzheimer’s disease [10]. Therefore, a new challenging field is emerged for developing artificial chemosensor capable of recognizing the environmentally and physiologically important analytes in a rapid, inexpensive and sensitive way [11]. Due to high selectivity, sensitivity and easy operational use over other methods, colorimetric and fluorometric probes become the first choice for practical applications [12]. Meanwhile, the idea of designing a new cost-effective molecular probe which binds more than one analyte using a single detection method or an array of detection methods gain the importance over one-to-one normal sensors [13–15]. To date, in comparison to diamagnetic Al$^{3+}$, there are very few ‘Switch-On’ sensors reported for paramagnetic Cu$^{2+}$ (d$^9$) and Cr$^{3+}$ (d$^3$) because of their fluorescence quenching property [16]. Higher photostability and longer absorption-

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emission wavelength along with the ability to trigger a dramatic change in colour by the opening of the non-fluorescent closed shell to zwitter-ionic fluorescent spirolactam form make Rhodamine derivatives very reliable platform for constructing Off-On fluorescence sensor [17,18].

In this work, we have designed and synthesized a new (E)-2-hydroxy-3-methoxy-4-(4-nitrophenyl)diazenyl)benzaldehyde appended rhodamine hydrazone derivative (L) in a mixed solvent as a single chemosensor for sensing multiple analytes such as Cu^{2+}, Al^{3+}, and Cr^{3+}. The outline of the synthesis is depicted in Scheme 1. The sensor L exhibits the ‘Turn-On’ property via the chelation-enhanced fluorescence (CHEF) process [19] and inhibited PET which was supported by the different photophysical process. We have constructed INHIBIT molecular Logic gate using the sensing properties. The experimental findings are well-correlated with theoretical results using density functional theory calculations. In addition, L was successfully applied in the imaging of Al^{3+} and Cr^{3+} in human lymphocyte cells (HLCs).

2. Experimental section

2.1. Materials and instruments

3-Ethoxysalicylaldehyde (99%), p-nitroaniline (97%), Rhodamine B (99.0%) from Sigma-Aldrich, high-purity HEPES (99.0%), Na_{2}EDTA (98.0%), perchlorates and nitrate salts of the different metal cations (Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Cr^{3+}, Al^{3+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, Pd^{2+}, Mg^{2+}, Pb^{2+} Na^{+}, Hg^{2+}) and all solvents were purchased from Merck (India) Ltd. and were used as received without further purifications. All spectroscopic data were recorded using HPLC grade solvent. UV–Vis absorption study was performed with Evolution-201 spectrometer and XENO Flash (PTI) fluorescence spectrophotometer with quartz cuvette (path length = 1 cm) was utilized to record the emission spectra with the excitation wavelength 520 nm for both ligand and complex at room temperature. ¹H NMR spectra and ¹³C NMR spectra were recorded on Brucker 400 MHz instruments using TMS as an internal standard. Chemical shifts (δ) were reported in ppm units and ¹H–¹H coupling constants in Hz. IR spectra (KBr pellet, 400–4000 cm⁻¹) were recorded on a Perkin-Elmer infrared spectrophotometer (Model: 883). Elemental analysis for carbon, hydrogen, and nitrogen was performed with Perkin Elmer 2400 CHN analyzer. Electrospray ionization MS (ESI-MS) on Qtof Micro YA263 mass spectrometer. Fluorescence quantum yield was calculated using fluorescein an optically matching standard (Φr = 0.79). HORIBA Jobin Yvon Fluorocube-01-NL fluorescence lifetime spectrometer was used to record the time Correlated Single Photon Counting (TCSPEC) data. Gaussian software was used to have DFT data. Cell imaging was carried out with Fluorescence microscope (LEICA DFC295, Germany).

2.2. Isolation and culture of human lymphocyte cell (HLCs)

5 mL of blood samples were collected from healthy young volunteers according to the method of Hudson and Hay [20].About 4 mL of blood was layered onto the same amount of histopaque 1077 (Sigma-Aldrich Co. LLC, US) and centrifuged at 2000 rpm for 30 min at room temperature. To a centrifuge tube, the upper lymphocytes monolayer was transferred and washed three times in phosphate buffer solution (pH 7.4). The human lymphocyte cells (HLCs) were cultured by resuspending in RPMI medium supplemented with 10% FBS and incubated for 24 h at 37 °C in a 95% humidified and 5% CO₂ atmosphere in a CO₂ incubator and used for the experimentation.

2.3. Cell cytotoxicity study

In order to measure the cytotoxicity of the ligand, yellow colored tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was executed in human lymphocyte cells (HLCs) according to standard procedure [21]. In brief, overnight culture of HLCs were seeded in 96-well plates and incubated with a series of concentrations of L (5, 10, 20, 50 and 100 μM) at 37 °C in a 5% CO₂ atmosphere for 24 h. After the removal of the culture medium, cells were washed by PBS (pH 7.4). 10 μl of MTT solution (1 mg mL⁻¹) in PBS was added in each well and cell were again incubated for 3 h at 37 °C. After the incubation, 0.1% DMSO was added to each well. The absorbance of the intracellular formazan crystal (blue-violet) was measured at the wavelength of 540 nm by ELISA ANALYSER (Bio-Rad, Model 680). The cell viability was quantified as the optical density ratio of the ligand treated group to HLC-control. Values are mean (M) ± standard error of mean (SEM) of three independent experiments.

%cell viability = (OD of the sample)/(OD of control) × 100

The cell cytotoxicity was calculated using the following formula:

%cell cytotoxicity = 100% − %cell viability

2.4. Computational method

DFT study is an important tool to obtain better insights into the geometry, electronic structure, and optical properties of these systems. Ground state electronic structure calculations in gas phase of both the complexes have been carried out using DFT [22] method associated with the conductor-like polarizable continuum model (CPCM) [23–25]. Becke’s hybrid function [26] with the Lee-Yang-Parr (LYP) correlation function [27] was used through the study. The geometry of the ligand and complex was fully optimized without any symmetry constraints. On the basis of the optimized ground state geometry, the absorption spectral properties in ethanol (EtOH) media were calculated by time-dependent density functional theory (TDDFT) [28–30] approach associated with the conductor-like polarizable continuum model (CPCM). We computed the lowest 40 singlet – singlet transition and results of the TD calculations were qualitatively very similar. The TDDFT approach had been demonstrated to be reliable for calculating the spectral properties of many transition metal complexes [31–33]. Due to the presence of electronic correlation in the TDDFT (B_{3}LYP) method, it yields more accurate electronic excitation energies. Hence
2.5. Fluorescence lifetime measurements

Fluorescence lifetimes were measured by the method of Time-Correlated Single-Photon Counting (TCSPC) using a HORIBA JobinYvon Fluorocube-OI-NL fluorescence lifetime spectrometer. The sample was excited using a laser diode at 450 nm and the signals were collected at the magic angle of 54.7° to eliminate any considerable contribution from fluorescence anisotropy decay [36]. The typical time resolution of our experimental setup is ~100 ps. The decays were deconvoluted using DAS-6 decay analysis software. The acceptability of the fits was judged by χ² criteria and visual inspection of the residuals of the fitted function to the data. Mean (average) fluorescence lifetimes were calculated using the following equation [37,38],

\[
\tau = \frac{\sum_{i} \alpha_i \tau_i}{\sum_{i} \alpha_i},
\]

where \(\alpha_i\) is the pre-exponential factor corresponding to the \(i\)th decay time constant, \(\tau_i\).

2.6. Synthesis of rhodamine B hydrazide

Rhodamine B hydrazide was synthesized according to the previously reported method [39].

2.7. Synthesis of 3-ethoxy-2-hydroxy-4-((4-nitrophenyl) diazenyl) benzaldehyde

The diazo compound was synthesized according to the literature procedure [40]. A clear green solution of p-nitroaniline (0.414 g, 3 mmol) in HCl (30%, 6 mL) was prepared in between 0 and 5 °C by keeping the beaker on ice-salt bath with constant stirring. Colour of the solution changed from green to pale yellow during dropwise addition of cold aqueous NaNO₂ (0.207 g, 3.0 mmol). This diazotized solution was added to the alkaline solution of 3-ethoxy salicylaldehyde (0.5 g, 3 mmol) in water (20 mL) containing NaOH (0.12 g, 3 mmol) and (0.17 g, 1.63 mmol) at 5°C. After overnight stirring of the resulting solution at room temperature, a dark brown cake was produced, and it was filtered using Whatman-41. The dark-brown residue was resuspended after repeating washing by 10% NaCl. The solution made slightly acidic (pH 4–5) by adding 2–3 drops of conc. HCl. Thereafter the yellow colour dye was dried by keeping in a vacuum desiccator and purity was confirmed by chromatography. The yield of the product was 85%. 1H NMR: [Fig. S1, ESI] [400 MHz, DMSO-d₆, δ (ppm)] δ 10.32 (1H, s, Ar=CHO), 8.35–8.33 (d, 2H, J = 8 Hz, Ar=H) 7.98–7.95 (d, 2H, J = 12 Hz, Ar=H) 7.87 (s, 1H, Ar=H) 7.62 (s, 1H, Ar=H) 4.18–4.13 (8H, q, J = 12 Hz, OCH₂Ar) 1.38–1.35 (3H, t, J = 8 Hz, OCH₂CH₃). Melting point (M.P.) 184°C.

2.8. Synthesis of chemosensor L

An easy and convenient one-step condensation method was applied to the synthesis of L from Rhodamine B hydrazide and aldehyde precursor (Scheme 1). Rhodamine B hydrazide (0.460 g, 1.008 mmol) and 2-ethoxy-6-methyl-4-((4-nitrophenyl)diazenyl)phenol (0.319 g, 1.008 mmol) were dissolved in dry EtOH (20 mL) by stirring at room temp. The resulting solution was refluxed for 6-h with constant monitoring by thin layer chromatography (TLC). At the end of the reaction, the volume of the reaction mixture is reduced to 1/3 of its original volume so that a deep red colored solid appeared. The deep red colored solid was separated by simple filtration and washed repeatedly by cold EtOH-Ether (1:1 ratio) after cooling the reaction at room temperature. Yield ~85%. M.P. 220°C.

1H NMR [Fig. S2, ESI] [400 MHz, DMSO-d₆, δ (ppm)] 6.1092 (1H, s, –OH), 9.16 (1H, s, –CH=N) 8.39–8.37 (2H, d, J = 8 Hz) 8.00–7.98 (2H, d, J = 8 Hz), 7.94–7.92 (1H, d, J = 8 Hz), 7.76 (1H, s), 7.63–7.56 (2H, m), 7.43 (1H, s), 7.11–7.10 (1H, d, J = 4 Hz), 6.46–6.44 (4H, m), 6.37–6.34 (2H, dd, J = 12 & 8 Hz), 4.11–4.10 (2H, q, J = 8 Hz), 2.50–2.48 (8H, q, J = 4 Hz, OCH₂), 1.35–1.31 (3H, t, J = 8 Hz, OCH₂CH₃), 1.07–1.03 (12H, t, J = 8 Hz, N(CH₂)₂CH₃), 13C NMR [Fig. S3, ESI] [125 MHz CDCl₃, δ (ppm)]: 129.00, 159.34, 149.22, 148.45, 144.72, 145.22, 148.45, 148.57, 151.95, 153.11, 157.27, 157.87, 158.28, 162.07. 13C NMR [Fig. S7, ESI] [125 MHz CDCl₃, δ (ppm)]: 128.11, 128.41, 133.88, 134.65, 144.73, 145.31, 146.08, 149.07,152.04,153.14,159.70,164.32. Elemental analysis (calcld. %) for C₄₃H₄₃N₇O₆: C, 68.48; H, 5.78; N, 13.03; O, 12.73; found (%): C, 68.48; H, 5.78; N, 13.03; O, 12.70. ESI-MS: [Fig. S4, ESI] m/z calculated for C₄₃H₄₃N₇O₆ [M + H⁺]: 754.33, found 754.66.

2.9. Synthesis of L-Al³⁺ and L-Cr³⁺ Complex

Al(NO₃)₃.9H₂O (0.19 g, 0.506 mmol) and CrCl₃ (0.080 g, 0.506 mmol) were added to the two separate R.B. each containing (0.381 g, 0.506 mmol) of L in acetonitrile. The solution was filtered after overnight stirring at room temperature. The deep maroon colored solid filtrate appeared in both case after several times washing with MeOH kept in vacuum for drying. 1H NMR [Fig. S5, ESI] [400 MHz, DMSO-d₆, δ (ppm)] δ 9.44 (1H, s, –CH=N) 8.41–8.39 (2H, d, J = 8 Hz), 8.00–7.93 (3H, M), 7.78 (1H, s), 7.62–7.60 (2H, m), 7.44 (1H, s), 7.12–7.10 (1H, d, J = 8 Hz), 6.46–6.44 (4H, m), 6.37–6.32 (2H, m), 4.11–4.09 (2H, q, J = 8 Hz), 2.51–2.49 (8H, q, J = 4 Hz, OCH₂), 1.36–1.33 (3H, t, J = 8 Hz, OCH₂CH₃), 1.08–1.04 (12H, t, J = 8 Hz, N(CH₂)₂CH₃), 13C NMR [Fig. S5, ESI] [125 MHz CDCl₃, δ (ppm)]: 128.09, 128.69, 131.46, 145.22, 145.25, 14.17, 148.41, 149.06, 151.93, 153.11, 164.40. ESI-MS: [Fig.S8, S9, ESI] m/z calculated for C₄₃H₄₃N₇O₆ [M + Al³⁺]: 814.1625, found 814.1625 and for C₄₃H₄₃N₇O₆ [M + Cr³⁺]: 804.51, found 804.51.

2.10. Association constant

The association constant value (Kₐ) was determined from absorption data using the following Benesi–Hildebrand (B-H) equations [41].

\[
\frac{1}{(A - A_0)} = \frac{K(A_{\text{max}} - A_0)C}{A_{\text{max}} - A_0} + \frac{1}{A_{\text{max}} - A_0}
\]

where \(A_0\) is the absorbance maxima of sensor L, \(A\) is the observed absorbance at that particular wavelength at different concentration of the metal ion \(C\), \(A_{\text{max}}\) is the maximum absorbance value at \(C = \text{max}\) nm and 581 nm for Al³⁺ and Cr³⁺, respectively during titration with varying \(C\). K is the association constant and was determined from the ratio of slope and intercept of the linear plot, and \(C\) is the concentration of the M³⁺ ion added during titration studies.

The binding constant is determined from fluorescence intensity data using Benesi–Hildebrand equation:

\[
\frac{1}{(I - I_0)} = \frac{(l_1 - l_0) + (l_1 - l_0)K[M^{n+}]}{m}
\]

where \([M^{n+}]\) is the metal ion concentration, \(I_0\) and \(I\) indicate emission intensities in the absence of, at intermediate and at infinite concentrations of metal ions, respectively. An aqueous stock solution of \(M^{n+}\) in
H₂O with an exact concentration of 1 × 10⁻³ mol L⁻¹, in an aqueous HEPES buffer (pH 7.2) and an effective concentration of 2 × 10⁻⁵ mol L⁻¹ ligand solution was prepared in the EtOH-H₂O medium at pH 7.2 for this purpose.

A linear relationship between 1/(A-A₀) vs. 1/[M⁰⁺] plot indicates 1:1 binding stoichiometry and the binding constant calculated to be 6.7435 × 10⁴ M⁻¹, 3.8 × 10⁵ M⁻¹ respectively for Al³⁺ and Cr³⁺ and 5.685 × 10⁵ M⁻¹ for Cu²⁺ (Fig. S10, S11, S12 ESI).

2.11. Procedure for metal ion sensing

A stock solution of the sensor L was prepared with concentration 1 × 10⁻³ in Ethanol-aqueous HEPES buffer (5 µM, pH 7.2, 9:1, v/v) for both spectroscopic studies. Metal solution of different concentrations (C = 1 × 10⁻², C = 1 × 10⁻²) were prepared from Ethanol-de-ionized water. Absorbance and fluorometric selectivity were examined by taking 20 µL of the stock solution in 2 mL ethanol in a quartz cuvette (path length, 1 cm) maintaining the final conc.10 × 10⁻⁶ M along with 50 µL (C = 1 × 10⁻²) metal solution. Titration experiment was carried out by the gradual addition of metal ion (C = 1 × 10⁻²) to sensor solution.

2.12. Fluorescence quantum yield

Fluorescein an optically matching standard (Φ₟ = 0.79) was used in spectroscopic grade EtOH to determine the Fluorescence Quantum Yield by the following Equation [42].

\[ \Phi_ϕ = \Phi_ϕ' \left( \frac{A_s/F_s}{A_r/F_r} \right) (\eta_r)^2 \]

where As and Ar are the absorbance of the sample and reference solutions, respectively, at the same excitation wavelength, ϕ₟ and ϕᵣ are the corresponding relative integrated fluorescence intensities, and η is the refractive index of the solvents. The Quantum Yield of ligand Φ = 2.94%, changes to 11 fold Φ = 32.6% for Al³⁺ and 3.61 fold Φ = 10.6% for Cr³⁺.

2.13. Binding ratio measurements (Job’s plot)

Job’s plot measurement was made from UV–Vis titration. Receptor solution was taken in 10 vials at regular intervals starting from 20 µL to 2 µL whereas metal solution was injected into it in reverse order at the same regular interval. The total solution volume of each vial is fixed to 2 mL. Absorption data was recorded out at room temperature after shaking the vials for 2 min [43].

3. Results and discussion

3.1. Selectivity studies

Selectivity of L is ascertained spectrometrically by taking 25 equivalent of several competitive metal ions (Ba²⁺, Ca²⁺, Cd²⁺, Co²⁺, Cu²⁺, Cr³⁺, Al³⁺, Mn²⁺, Fe³⁺, Zn²⁺, Ni²⁺, Pd²⁺, Mg²⁺, Pb²⁺, Na⁺, Hg²⁺) in a solution containing 20 µL L in 2 mL EtOH-H₂O (9:1,v/v, 5 µM HEPES pH 7.2) solvent. The result exhibited that Al³⁺, Cr³⁺ and Cu²⁺ exhibit sharp change in absorbance whereas Al³⁺ and Cr³⁺ show distinct change in PL intensity with respect to other metal ions. Al³⁺, Cr³⁺, and Cu²⁺ altered the absorbance spectral line with the appearance of three bands at λₘₐₓ = 551, 554, and 557 nm, respectively under the identical condition. Under this optimal condition (i.e.; pH = 7.2) L changes its fluorescence spectral line in presence of Cr³⁺ and Al³⁺ at 581 nm & 582 nm, respectively. The superior dual fluorometric selectivity of L was experienced in the naked eye after 366 nm radiation of UV light where the colour of the L changed immediately from colourless to deep pink after the addition of Al³⁺ and Cr³⁺. Such interesting findings motivate our groups to explore the L as a single probe for dual metal sensing by fluorometric method. Though in ambient light, L shows colormetric distinction with Ba²⁺-Cr³⁺-Co²⁺ (dark yellow), Fe³⁺ and Pd²⁺ along with the Al³⁺, Cr³⁺, and Cu²⁺ metal ions (Fig. 1), but in UV–Visible absorption study, our sensor shows a prominent change in absorbance only with three metal ions (Cu²⁺, Al³⁺, and Cr³⁺) whereas Cr³⁺ and Al³⁺ are distinguished from other metal ions by PL study. Oxophilic, diamagnetic, hard acid Al³⁺ with its high ionic potential and low ionic radius provides strong binding energy with the hard base than other paramagnetic divalent and trivalent metal ions, and hence make them as a strong guest for L [43,44]. Again particularly inter-system crossing in excited singlet state by transferring electron from paramagnetic Cu²⁺ (d⁹) to the Rhodamine-B fluorophore

(a)

(b)

Fig. 1. Visual display of L plus metal ions: (a) under normal illumination (b) and demonstrating fluorescence under 365 nm illumination.
promotes the intersystem absorption phenomena making it a natural fluorescence quencher [45,46].

3.2. Spectroscopic recognition

3.2.1. UV–Visible absorption studies

The extraordinary dual sensitivity by this newly designed rhodamine-based probe was investigated by UV–Vis and fluorescence method, using different metal solutions in EtOH-H2O (9:1) at optimized pH 7.2. The colour of L changes upon addition of Ba2+, Ca2+, Cd2+, Cr3+, Cu2+, Fe3+, Hg2+, Mg2+, Mn2+, Ni2+, Pb2+, Pd2+, Zn2+ and Al3+ in aforementioned ligand solution taken in a quartz tube containing 2 mL EtOH-H2O (9:1, v/v, pH = 7.2) using 5 μM HEPES buffer at 25 °C. Interestingly, as depicted in Fig. 2, UV–Visible spectral results revealed that among the all coexisting metal ions only Cu2+, Al3+, and Cr3+ alters the spectral line at λmax = 551, 554 and 557 nm, respectively. To have a better quantitative appraisal of the reversible interaction of the sensor L, absorption spectral variation was carried out between sensor L with Mn2+ and resulting chelated complex with a more powerful hexadentate chelating agent Na2EDTA. Gradual incremental addition of Al3+, Cr3+, and Cu2+, to the fixed solution of sensor L (2 mL) in EtOH-H2O (9:1, v/v) at pH = 7.2 a progressive increase of the absorption peak for the guest metals were observed along with deepening of pink colour. After addition of 46 μM Al3+, 36 μM Cr3+ and 50 μM Cu2+, no further increment was observed at λmax = 554, 557 and 551 nm, respectively, for Al3+, Cr3+ and Cu2+.

This indicates the chelating saturation of sensor L for guest metal ions due to the transformation of Rhodamine B from close spirrolactam ring to stable metal-induced open conjugated xanthene form (Fig. 3(a–c)) [47]. However, complete bleaching of pink colour, as well as the disappearance of aforementioned peaks (Fig. 3(d–f)) by the incremental addition of Na2EDTA, clearly indicates reversible binding nature of sensor ligand towards the guest cation. Association constant was calculated from absorbance vs. [M]1/2 curve by Benesi-Hildebrand linear curve fitting methods and was found to be kass = 6.7435 × 106 M−1, 3.8 × 106 M−1, and 5.685 × 105 M−1, respectively, for Al3+, Cr3+, and Cu2+. (Fig. S10, S11, S12 ESI). The calculated LOD value 1.07 × 10−6 M, 2.02 × 10−6 M, and 1.6 × 10−6 M for Al3+, Cr3+ and Cu2+ based on UV–Visible absorption data by 3σ method [48] also satisfies the limit of drinking water for both Al3+ and Cr3+ (Fig. S13, S14, S15 ESI).
As our purpose is to establish the designed sensor as an effective fluorescent probe so we are not interested to explore the fluorometric sensing property of Cu$^{2+}$ as it does not obliterate the fluorescent spectral nature of the sensor L. The selectivity tuning of L towards Al$^{3+}$ and Cr$^{3+}$ was explored by the fluorometric method.

3.3. Fluorescence study

A better understanding of emissive behavior of sensor L is explored by choosing an appropriate solvent. The emissive behavior of L-Al$^{3+}$ and L-Cr$^{3+}$ in different solvents (EtOH, MeOH, CH$_3$CN, DMF, and DMSO) were tested with the findings that in both cases EtOH was the suitable solvent for the study of emissive property (Fig. 4). The selectivity and sensitivity trait of L towards Al$^{3+}$ and Cr$^{3+}$ with respect to other alkali and alkaline earth metal ions except for a very weak absorption for Cu$^{2+}$ was detected at $\lambda_{\text{max}} = 574$ nm and fascinatingly, enough under the same experimental condition, Al$^{3+}$ and Cr$^{3+}$ generate a remarkable fluorescence enhancement of 21 fold and 16 fold at $\lambda_{\text{max}} = 582$ nm and 581 nm, respectively (Fig. 5) as a result of metal-induced CHEF process and inhibition of PET process, when 25 eq. of different metal ions were added to the sensor solution.

The onset of Photo-induced electron transfer (PET) process by $\pi$-N bond isomerization along with a donation of an electron from HOMO of Schiff base N and $\pi$-OH of hydroxyl aldehyde to fluorophore LUMO is mainly responsible for ‘Turn-Off’ emissive behavior of the sensor [49]. Again the inhibition of isomerization and appropriate complexation in presence of specific guest and functional group attached to the fluorophore makes the PET process invalid and initiates the chelation-enhanced fluorescence process (CHEF) by transferring HOMO electron of the fluorophore to its LUMO arresting the previous electron transfer [50] (Scheme 2). This strong ‘Turn-On’ selectivity of sensor L also observed when sensor solution treated separately with each of 25 eq. Al$^{3+}$ and Cr$^{3+}$ under long-range UV lamp of (366 nm). The almost immediate transformation of colourless sensor solution to pink colour supports the ‘Turn-On’ property of the sensor.

To elucidate the reversible binding mode of sensor L fluorescence titration was performed with Al$^{3+}$ and Cr$^{3+}$. Fluorescence intensity increases maximally up to $\lambda_{\text{max}} = 582$ nm and 581 nm with the gradual addition of 46 $\mu$M Al$^{3+}$ and 36 $\mu$M of Cr$^{3+}$ (Fig. 6(a–b)) after which increment ceases down with a quantum yield ($\Phi = 11.08$) for Al$^{3+}$ and ($\Phi = 3.61$) for Cr$^{3+}$, respectively. The fluorescence intensity decreases gradually almost by the similar manner with the incremental addition of Na$_2$EDTA (Fig. 6(c–d)) due to the removal of metal ions from the L-M$^{3+}$ complex by the formation of strong chelate with EDTA. The lower quantum yield and lower emission intensity of Cr$^{3+}$ in compared to Al$^{3+}$ is attributed to the higher oxophilic nature higher CFSE value and hard acid character of Al$^{3+}$, which encourages the higher CHEF effect of the sensor for Al$^{3+}$.

3.4. Interference studies

For effective use of the probe, achieving high selectivity as a host for Al$^{3+}$ and Cr$^{3+}$ in presence of other potentially challenging metal cation is a very important index. In order to check the cross sensitivity, we do perform the selectivity of our probe for a wide range of cations along...
with Al\textsuperscript{3+} and Cr\textsuperscript{3+} in the EtOH-H\textsubscript{2}O medium. Clearly, it is revealed from the graph that the emission intensity of the probe remains almost unchanged when 5 equivalents of other metal ions added to the buffer solution of the probe as that of Al\textsuperscript{3+} and Cr\textsuperscript{3+} (Fig. 7). Surprisingly in both case after the addition of 10 equivalents of Cu\textsuperscript{2+} and Fe\textsuperscript{3+}, there was a rapid quenching of fluorescent intensity. Fe\textsuperscript{3+} quenches the intensity because of the stability of its half-filled (d\textsuperscript{5}) shell [51] and the onset of non-radiative decay caused by the electron transfer from redox active (d\textsuperscript{9}) Cu\textsuperscript{2+} to the excited fluorophore of rhodamine moiety is one of the probable reasons for fluorescence quenching.

Therefore, the dual metal experiment clearly evidenced that our probe can be used as a dual target recognizing agent in a single detection method.

3.5. Reversibility

The practical usability of \( L \) depends mainly on its alternating enhancing and reviving mechanism. This reversibility is investigated to monitor the important changes in the biological system. Hexadentate chelating agent Na\textsubscript{2}EDTA bleach the ‘signal-on’ absorption band of both Al\textsuperscript{3+} and

![Scheme 2. Schematic presentation showing the possible PET-CHEF mechanism for Al\textsuperscript{3+} and Cr\textsuperscript{3+} sensing.](image)

---

**Fig. 6.** Emission spectral change of the ligand \( L \) (10 \( \mu \)M) upon incremental addition of (a) Al\textsuperscript{3+} and (b) Cr\textsuperscript{3+} in EtOH-H\textsubscript{2}O [9:1, v/v, 5 \( \mu \)M HEPES pH 7.2] at 25 °C. Emission spectral change of the (c) \( L-\text{Al}^{3+} \) and (d) \( L-\text{Cr}^{3+} \) complex upon incremental addition of Na\textsubscript{2}EDTA in EtOH-H\textsubscript{2}O [9:1, v/v, 5 \( \mu \)M HEPES pH 7.2] at 25 °C.
Cr$^{3+}$ through the process of demetallation from L-Mn$^{2+}$ complex by regenerating the spirolactam ring. Our reversibility experiment well substantiates this theory. Addition of Na$_2$EDTA to the L-Mn$^{2+}$ complex solution completely arrests the fluorescent by returning it to the 520 nm indicating the generation of metal-free ligand. Turned-on property of L is regained upon addition of either Al$^{3+}$ or Cr$^{3+}$. This result is recreated after sequential several additions of metals and Na$_2$EDTA, which clearly indicates that the receptor reversibly recognized Al$^{3+}$ and Cr$^{3+}$ (Fig. 8).

3.6. pH study

The L can only be used effectively in a complex biological system if it can withstand in physiological pH without hydrolysis by the interfering proton. Although we have used HEPES buffer solution to arrest the hydrolysis of guest metal ion throughout the sensing process so as to eliminate any type of chance that may be caused by the participation of proton in incremental fluorescent luminescence, the pH study has been performed over a long-range of pH (2−12) in EtOH-H$_2$O (9:1, v/v) medium both in the presence and absence of metal ions [52]. At lower pH, the protonation induced spirolactam ring opening affects the gradual increment of fluorescence intensity of probe L irrespective of the inclusion of metal ions. However, in presence of metal ions, the probe L shows excellent fluorescent sensing ability which remains almost unaltered throughout the physiological range of pH. On another site, there is no observable emission for the probe L in absence of analytes within this physiological range as it remains in its spirolactam form in this pH (Fig. 9) [53]. This very conclusive study clearly negates any type of interference of the proton in physiological pH and indicates that the luminescence in the physiological pH is only due to L-Mn$^{2+}$

![Fig. 7. Relative emission intensity of L after addition of 5 equivalents of competing for metal ions as that of Al$^{3+}$/Cr$^{3+}$ to the solution of L in EtOH-H$_2$O [9:1, v/v, 5 μM HEPES pH 7.2] at 25 °C.](image)

![Fig. 8. Changes of emission intensity at 582 nm (λ$_{ex}$ = 520 nm, 10 μM) in EtOH-H$_2$O (9:1, v/v at pH = 7.2 HEPES buffer) upon repetitive addition of (a) Al$^{3+}$ (1.0 equiv.) and Na$_2$EDTA solution. (b) changes of emission intensity at 581 nm (λ$_{ex}$ = 520 nm, 10 μM) in EtOH-H$_2$O 9:1 (v/v at pH = 7.2 HEPES buffer) upon repetitive addition of Cr$^{3+}$ (1.0 equiv.) and Na$_2$EDTA solution.](image)

![Fig. 9. Fluorescence response of (a) L-Al$^{3+}$ (brown line) at λ$_{max}$ = 582 nm, (b) L-Cr$^{3+}$ (pink line) at λ$_{max}$ = 581 nm, and (c) L (red line) as a function of pH in EtOH-H$_2$O 9:1 (v/v at pH = 7.2 HEPES buffer) at 25 °C at the excitation wavelength of 520 nm. The pH was adjusted using 1 M aqueous solutions of HCl.](image)
binding spirolactam ring opening. So clearly this novel sensor is a biocompatible one for detection of Al\(^{3+}\) and Cr\(^{3+}\).

### 3.7. Plausible mechanism

The conspicuous ‘Turn-On’ behaviour of \(L\) towards Al\(^{3+}\) and Cr\(^{3+}\) is a combination of the PET-CHEF process. The Turn Off fluorescent property which is possibly responsible for a lower quantum yield of \(L\) is due to PET process where lone pair from N center of spirolactam moves to the azobenzene moiety making the spirolactam tautomer colourless [54]. On the other hand, colourless non-fluorescent to the pink fluorescent make-over of \(L\) is an obvious result of the binding-induced spirolactam ring opening process [55]. The coordinating site offered by the host to the guest through keto group and the hydroxyl group is confirmed from the shifting of keto carbonyl IR peak of spirolactam ring at 1710 cm\(^{-1}\) to 1646 cm\(^{-1}\) and 1647 cm\(^{-1}\) for L-Al\(^{3+}\) and L-Cr\(^{3+}\), respectively (Fig. S16, S17, and S18, ESI). The lower wave no. of \(\delta\) supports the complexation of metal ion (Al\(^{3+}\), and Cr\(^{3+}\)) with the ligand [56,57]. Disappearing of \(^1\)H NMR peak at (6) 66.03 ppm (Fig. S6, S7 ESI) for sp\(^2\) carbon and also disappearing of \(^1\)H NMR peak at (6) 10.92 ppm (Fig. S5, ESI) for –OH proton along with shifting of –CH=N peak at (6) 9.44 ppm supports the formation of L-Al\(^{3+}\) and L-Cr\(^{3+}\) complexation [58]. Again, the L-M\(^{n+}\) complexation as well as the better complexing ability of L-Al\(^{3+}\) is also be confirmed from the higher \(\tau_{av}\) value of L-M\(^{n+}\) than bare L and higher \(\tau_{av}\) value of L-Al\(^{3+}\) than L-Cr\(^{3+}\) complex (Table-5). Furthermore, the 1:1 binding pattern of the L-M\(^{n+}\) complex was confirmed from UV–Vis absorption by the Job’s plot for L and M\(^{n+}\) (Fig. S19, S20, S21 ESI). A thorough inspection of the Job’s plot exposed that absorption reaches its maxima near about 0.5-mol fraction of Cr\(^{3+}\) and Al\(^{3+}\) indicating ideal binding mode for L-Al\(^{3+}\) and L-Cr\(^{3+}\) complex, respectively. The superiority of our sensor \(L\) has been established by comparing the detection limit with many other reported probes [Fig. S22 ESI].

### 3.8. Logic gate interpretation

The quick and subtle response of our probes towards the dual metal inspired us to check its reversibility by constructing a double input and single output based noncommutative type logic gate [59,60]. The INHIBIT logic gate is constructed in the maximum emission of Al\(^{3+}\) and Cr\(^{3+}\) at \(\lambda_{em} = 582\ nm\) and 581 nm with the basic theory of Boolean algebra where 1 represents ‘YES’/ON-state and 0 pointing ‘NO’/OFF-state. In the absence of both metal and EDTA, the output is zero indicating off state. In variation 2 when the input is only metal without EDTA maximum emission intensity is observed for Al\(^{3+}\) and Cr\(^{3+}\) at \(\lambda_{em} = 582\ nm\) and 581 nm, respectively, pointing 1 (on) state, in the third row where EDTA is the only chemical entity to input, output is zero and in the fourth row where both the metals and EDTA is used as input, output again becomes zero. Therefore, input 1 performs the yes operation and the input 2 satisfies the criteria of NOT gate [61]. Considering all the results among the 16th TRUTH Table our truth table matches with the truth table of INHIBIT logic gate (Fig. 10).

### 3.9. Geometry optimization and electronic structure

The optimized geometries of the Al\(^{3+}\) complex and its Cr\(^{3+}\) complex are shown in Fig. 11. The ground state geometry optimization for the complexes 1 and 2 was performed in the gas phase. Main optimized geometrical parameters of the complex 1 are listed in Table 1 and for complex 2 in Table 2. In case of complex 1, the Al\(^{3+}\) center is penta-coordinated with one di-deprotonated tridentate chelate H\(_2\)L ligand (with NO\(_2\) donor sites) and one water molecule and one nitrate ion. The geometry of the penta-coordinated metal center is measured by the Addison parameter (\(\tau\)), which is 0.006 for 1 in this case [\(\tau = (\alpha - \beta)/2\) for 1].

![Fig. 10. (a) Truth table (b) circuit diagram for Inhibit Logic Gate.](image)

![Fig. 11. DFT/B3LYP optimized geometry of complexes [Al(L)(H\(_2\)O)(NO\(_3\))\(^2\)]\(^+\) (1) and as well as [Cr(L)(H\(_2\)O)]\(^3+\) (2).](image)
Table 1
Selected optimized geometrical parameters for [Al(H2O)[NO3]]+ (1) in the ground state calculated at B3LYP levels.

<table>
<thead>
<tr>
<th>Bonds</th>
<th>Values (Å)</th>
<th>Bond angles</th>
<th>Values (°)</th>
</tr>
</thead>
<tbody>
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<td>Al1-N1</td>
<td>1.956</td>
<td>N1-Al1-O1</td>
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Table 2
Selected bond distance and bond angles of [Cr(L)(H2O)]2+ (2).

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<th>Values (Å)</th>
<th>Bond angles</th>
<th>Values (°)</th>
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<td>O1w-Cr1-O2w</td>
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</table>

The complex 1 shows a sharp absorption band at 544 nm in EtOH solution at room temperature. This experimental value was supported by the TD-DFT calculation. This absorption band is assigned to the S0 → S2 transition which is in good agreement with experimental results of 547 nm. The absorption energies along with their oscillator strengths, the main configurations and their assignments calculated using TD-DFT method using the ground state geometry for complex 1 is discussed here and the related data are given in Table 3.

Table 3
Selected parameters for the vertical excitation (UV–Vis absorptions) of complex 1 in terms of molecular orbital contribution of the transition; electronic excitation energies (eV) and oscillator strengths (f) in ethanol.

<table>
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<tr>
<th>Electronic transition</th>
<th>Composition</th>
<th>Excitation energy</th>
<th>Oscillator strength (f)</th>
<th>CI</th>
<th>λexp (nm)</th>
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<td>S0 → S26</td>
<td>HOMO → LUMO + 1</td>
<td>2.5414 eV (547)</td>
<td>0.0472</td>
<td>0.66278</td>
<td>554</td>
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<tr>
<td>HOMO → LUMO + 1</td>
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<td>HOMO → LUMO + 1</td>
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</tr>
<tr>
<td>+ 1</td>
<td></td>
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<td>554</td>
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</tr>
</tbody>
</table>

3.10. Time-correlated single photon counting (TCSPC) study

The enhancement of fluorescence of L upon binding with Al3+ and Cr3+ has been supported by the results obtained from fluorescence decay measurements, using the TCSPC technique (Fig. 15). The decay behavior of the bare probe and its metal complex is best fitted to bi-exponential functions. Bare L showed two components having lifetimes 0.13 and 2.34 ns respectively. The populations of the same were 0.94 and 0.06% respectively. The average lifetime of L was calculated to be 0.54 ns. Upon addition of Al3+, two components were obtained having lifetimes 0.38 and 1.89 ns respectively, having populations of 0.78 and 0.22% respectively (Table 5). The average lifetime was calculated to be 1.31 ns. A similar experiment performed with comparable Cr3+ showed a bi-exponential decay trace with time constants τ1 = 0.36 (0.77%) and τ2 = 1.72 (0.23%). The average lifetime was calculated to be 1.19 ns. Upon binding of Al3+ and Cr3+ to the (L) formation of a tight binding complex occurs along with the opening of the spirolactam ring to convert the free (hanging) Rhodamine B part of the complex, thereby freezing of non-radiative pathways generated by rigid structure of the complex than the comparatively more flexible structure of the bare ligand [62]. As a result the average lifetime increased.

3.11. Cell imaging study

The overnight sub-confluent culture of human lymphocyte cells (HLC) was washed with phosphate buffer saline (PBS; pH 7.4) and incubated with DMEM containing L with the final concentration at 5 μM for...
3 h at 37 °C in CO2 incubator. After incubation, fluorescence images of HLCs were captured under a fluorescence microscope (LEICA DFC295, Germany) in 40× magnification. Similarly, a fluorescence image of HLCs (pre-incubated with 5 μM) was taken after addition of Al3+ and Cr3+ salt solution at the concentration of 10 μM for 1 h separately with an excitation wavelength 582 and 581 nm [63].

Fig. 12. Frontier molecular orbitals of optimized complexes.

Fig. 13. Frontier molecular orbitals involved in the UV–Vis absorption of complex 1.
3.11.1. In vitro cell cytotoxicity

To reproduce the capability of the fluorescence-based probe for intracellular imaging of Al\(^{3+}\) and Cr\(^{3+}\), firstly, it was of prime importance to verify the cytotoxic effect of the ligand on normal human cells. To establish the biocompatibility of the ligand in the cellular environment, the performed cell viability study by standard MTT assay in human lymphocyte cells (HLCs) revealed that 93.6 ± 0.54% cell viability was observed at the concentration of 5 \(\mu\)M in the presence of \(L\) compared to HLC-control cells. The viability of the cells has been observed in a dose-dependent manner up to the concentration of 100 \(\mu\)M. Ligand showed 45.3 ± 0.48% cytotoxicity at 100 \(\mu\)M concentration after 24 h incubation, whereas only 6.2 ± 0.51% cytotoxicity at 5 \(\mu\)M concentration at the same incubation time. It has been assumed that ligand did not influence the viability of HLCs at least 24 h of its treatment at concentration of 5 \(\mu\)M (Fig. 16).

3.12. Fluorescence cell imaging study

No intracellular fluorescence was observed in a fluorescence cell imaging study, after the incubation with the ligand at a concentration of 5 \(\mu\)M at 37 °C for 1 h. However, intensive intracellular green switch-on dose-dependent manner up to the concentration of 100 \(\mu\)M. Ligand showed 45.3 ± 0.48% cytotoxicity at 100 \(\mu\)M concentration after 24 h incubation, whereas only 6.2 ± 0.51% cytotoxicity at 5 \(\mu\)M concentration at the same incubation time. It has been assumed that ligand did not influence the viability of HLCs at least 24 h of its treatment at concentration of 5 \(\mu\)M (Fig. 16).

3.12. Fluorescence cell imaging study

No intracellular fluorescence was observed in a fluorescence cell imaging study, after the incubation with the ligand at a concentration of 5 \(\mu\)M at 37 °C for 1 h. However, intensive intracellular green switch-on
fluorescence was observed after the incubation with exogenous Al³⁺ ion (10 μM) and Cr³⁺ (10 μM) solution and the fluorescence intensity of L-Al³⁺ is quite higher than the L-Cr³⁺ salt solution (Fig. 17). The fluorescence image of the L with Al³⁺ and Cr³⁺ salt offers the confirmatory evidence that the L easily infiltrates the cell membrane and binds with intracellular Al³⁺ and Cr³⁺ forming the L-Al³⁺ and L-Cr³⁺ complex respectively. The present study proposes that L could be utilized as an efficacious signature of the selective sensor for bio-imaging Al³⁺ ions at the specified doses and incubation time without showing any cytotoxic effect.

4. Conclusion

In summary, we have demonstrated an inexpensive, solvatrchromic-based highly efficient, fast turn-on probe, L. The L in its stable metal-induced conjugated chelated form expresses its strong off-on selectivity response towards Al³⁺, Cr³⁺, and Cu²⁺ detected by UV-Visible absorption whereas Al³⁺ and Cr³⁺ detected by fluorimetric study. Larger association constants 6.7435 × 10^3 M effaceable signature of the selective sensor for bio-imaging Al³⁺ ions respectively. The present study proposes that L could be utilized as an ineffaceable signature of the selective sensor for bio-imaging Al³⁺ ions at the specified doses and incubation time without showing any cytotoxic effect.

Conflicts of interest

There is no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.04.056.

References
