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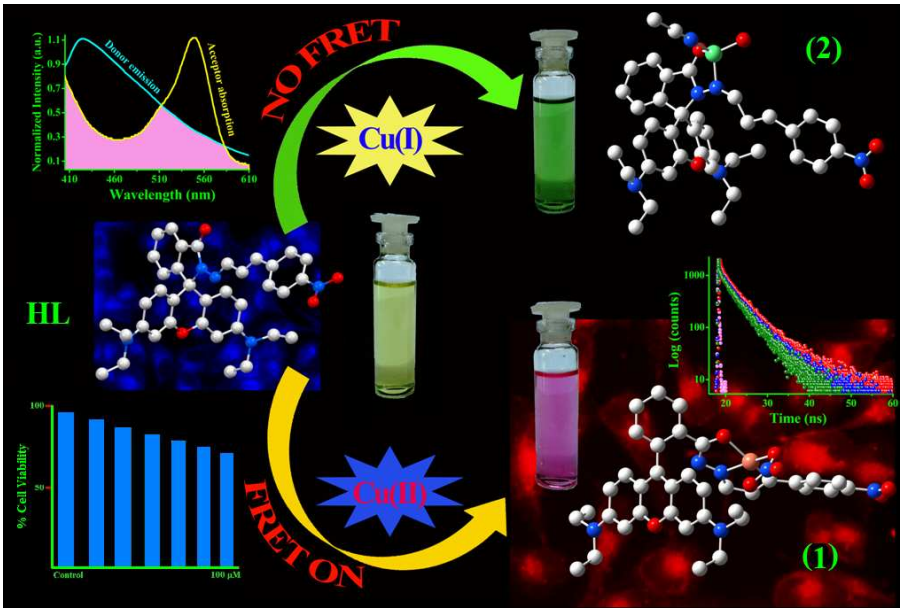
Graphical abstract

Reactions of a newly designed and structurally characterized rhodamine-cinnamaldehyde hybride (**HL**) with copper(I/II) ions in HEPES buffer (1 mM, pH 7.4; acetonitrile/water: 1/5, v/v) at 25 °C help to explore a Cu(II) ions selective chemosensor through FRET process which depends on +2 oxidation state of copper ion exclusively. This non-cytotoxic probe is applicable in cell staining.



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ARTICLE TYPE

Effect of metal oxidation state on FRET: A Cu(I) silent but selectively Cu(II) responsive fluorescent reporter and its bioimaging applications

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Copper(II) and copper(I) complexes of a newly designed and crystallographically characterized Schiff base (**HL**) derived from rhodaminehydrazide and cinnamaldehyde were isolated in pure form formulated as $[Cu(L)(NO_3)]$ (L-Cu) (**1**) and $Cu(HL)(CH_3CN)(H_2O)]ClO_4$ (HL-Cu) (**2**), and characterized by physicochemical and spectroscopic tools. Interestingly, complex **1** but not **2** offered red fluorescence in solution state, and eventually **HL** behaves as a Cu(II) ions selective FRET based fluorosensor in HEPES buffer (1 mM, acetonitrile/water: 1/5, v/v) at 25 °C at biological pH with almost no interference of other competitive ions. The dependency of FRET process on the +2 oxidation state of copper has been nicely supported by exhaustive experimental studies comprised of electronic, fluorimetric and NMR titration, and theoretical calculations. The sensing ability of **HL** has been evaluated by the LOD value towards Cu(II) ions (83.7 nM) and short responsive time (5-10 s). Even the discrimination of copper(I) and copper(II) has also been done using only by UV-Vis spectroscopic study. The efficacy of this bio-friendly probe has been determined by employing **HL** to detect the intercellular distribution of Cu(II) ions in Hela cells developing image under fluorescence microscope.

Introduction

Recently, the development of novel chemosensors for biologically active metal ions has got a huge interest because of their extensive applications in life sciences, medicine, chemistry and biotechnology.[1] Generally, design of a molecular sensor for selective detection of any particular ion is based on the host-guest interaction endorsed by metal-ligand coordination, H-bonding, electrostatic force, and van der Waals and hydrophobic interaction[2] in support of several signaling mechanisms, like chelation enhanced fluorescence (CHEF), intramolecular charge transfer (ICT), excimer/exciplex formation, and Förster/fluorescence resonance energy transfer (FRET) etc.

Among them FRET, a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor part to an acceptor part without emission of a photon, become a imperative physical event with expansive interest to know the molecular level interfaces in living systems and prospective applications in thin film and optoelectronic device development due to its sensitivity to distance and short response time.[3-4] While the efficacy is principally determined by the extent of the spectral overlap between the donor emission and acceptor absorption, the efficiency of energy transfer is influenced by the distance between the donor and the acceptor, and largely the relative orientation of transition dipoles of both the donor and acceptor. Consequently, here, the generated electronic environment of the donor fluorophore ligating with a selective metal ion of preferred oxidation state has been considered to account the transfer of energy for occurring FRET.

Copper being the third most abundant soft transition metal ion and the significant role played by copper ions in the active sites of a large number of metalloproteins in biological systems have stimulated efforts to develop the copper ion selective sensors.[5] Particularly, Cu(II)/Cu(I) redox systems take part in different catalytic processes in the human body like supplying of energy for biochemical reactions, assistance of the formation of cross-links in collagen and elastin, sustaining and repairing of connective tissues related to heart and arteries, controlling of oxidative stress and disorders associated with neurodegenerative diseases including Alzheimer's, Parkinson's, Menkes, Wilson's, and prion diseases.[6-9] Therefore, it is now a challenging task to the researchers not only to explore the copper ion selective fluorosensor but also the bio-friendly chemosensor for a particular oxidation state of copper ion for better understanding of the biological processes. Generally it is expected that the binding of the paramagnetic Cu^{2+} ion to the probe causes a quenching of the fluorescence emission but the binding of diamagnetic Cu^{+} ion do not.[10] However, herein we report a FRET based Cu^{2+} ion selective chemosensor where the enhancement of the fluorescence by binding of Cu^{2+} ions but not Cu^{+} ions was observed. To the best of our knowledge, this type of Cu^{2+} ion selective chemosensor by controlling the transfer of energy for occurring FRET with the help of the oxidation state of the copper ion is

still unexplored.

In this present study, we synthesized and structurally characterised a new Schiff base (**HL**) derived from the reaction of *4-nitrocinnamaldehyde* and *rhodamine-b-hydrazide*. Herein, we explored this organic moiety (**HL**) as a FRET based Cu(II) ions selective chemosensor following the "turn-on" red fluorescence signalling. Interestingly, **HL** exhibited this FRET based fluorescence enhancement upon chelation with Cu(II) ion selectively but no FRET based fluorescence in spite of chelation with Cu(I) ion indicated by a visual color change of the solution of **HL**. The faint yellow colored solution of **HL** was turned into faint green colored due to the ligation of **HL** with Cu(I) ion. The presence of an excess of the other metal ions, *viz.* alkali [Na(I), K(I)], alkaline earth [Mg(II), Ca(II)], transition metal and other metal ions [Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Al(III), Zn(II), Cd(II), Hg(II)] and Pb(II) ions do not hamper this behaviour of **HL** observed in presence of Cu(II) and Cu(I) ions due to the selective formation of **L-Cu (1)** and **HL-Cu (2)** complexes. Moreover, presence of Cu(I) does not affect the selective detection of Cu(II) ions.

Experimental

Preparation of HL

4-nitrocinamaldehyde (532 mg, 3.0 mmol) dissolved in ethanol was added to the ethanolic solution *rhodamine-B hydrazide*[4a] (1.37g, 3.0 mmol) at stirring condition. The resulting mixture was refluxed for 5 h. It was then evaporated to a small volume and cooled, from which yellow colored precipitate was filtered. The pure recrystallized product including the single crystals suitable for X-ray crystallographic study were isolated from acetonitrile/DMF (3:1) mixed solvents on slow evaporation.

$C_{37}H_{37}N_5O_4$: M.P.: 207 °C. Anal. Found: C, 71.99; H, 5.91; N, 11.53; Calc.: C, 72.17; H, 6.06; N, 11.37. HR-MS: $[M + H]^+$, m/z, 616.2907 (100 %) (calcd.: m/z, 616.2926), where M = molecular weight of **HL** (**Fig. S1** ESI†). IR (KBr, cm^{-1}): $\nu_{NH,}$ 3441, $\nu_{C=O}$, 1693; ν_{NO2}, 1423 (**Fig. S2** ESI†). ^{1}H NMR (400 MHz, $CDCl_3$): 8.22-8.20 (d, 1H, CH=N); 8.08-8.05 (d, 2H); 7.93-7.91 (d, 1H); 7.41-7.36 (m, 4H); 7.01-6.99 (d, 1H); 6.94-6.87 (m, 1H); 6.58-6.54(d, 1H); 3.29-3.23 (m, 8H, $4CH_2$); 1.11-1.07 (t, 12H, $4CH_3$) (**Fig. S3** ESI†). ^{13}C NMR (400 MHz, $CDCl_3$): 164.88, 152.87, 149.37, 147.47, 142.43, 136.85, 134.01, 130.73, 128.88, 128.75, 128.14, 127.83, 123.97, 123.67, 123.31, 108.01, 65.85 (spirolactam carbon), 44.02, 11.85. Yield: 82%.

Synthesis of copper(II) complex, $[Cu(L)(NO_3)]$ (L-Cu) (1)

To a solution of **HL** in acetonitrile (615.0 mg, 1.0 mmol) solid copper(II) nitrate trihydrate (242 mg, 1.0 mmol) was added at a time and then the reaction mixture was stirred for 2.0 h at room temperature to ensure the completion. Solvent was removed using a rotary evaporator, while a blood red precipitate was obtained by washing thoroughly with acetonitrile, and then dried in vacuo.

$C_{37}H_{36}CuN_6O_7$: Anal. Found: C, 59.69; H, 4.69; N, 11.67; Calc.: C, 60.03; H, 4.90; N, 11.35. ESI-MS in methanol: $[M + H]^+$, m/z, 740.1713 (obsd. with 40 % abundance) (calcd.: m/z, 740.2049) where M = $[Cu(L)(NO_3)]$ (**Fig. S4** ESI†). Magnetic moment (μ, B.M.): 1.78.

Synthesis of copper(I) complex, $[Cu(HL)(CH_3CN)(H_2O)]ClO_4$ (HL-Cu) (2)

To a solution of **HL** in acetonitrile (615.0 mg, 1.0 mmol) solid *tetrakis(acetonitrile)-copper(I) perchlorate* ($[Cu(AN)_4]ClO_4$; 327.2 mg, 1.0 mmol) was added at a time and then the reaction mixture was stirred for 2.0 h in nitrogen atmosphere at room temperature. A yellowish green precipitate was obtained after evaporation off the solvent by a rotary evaporator. It was then filtered, thoroughly washed with acetonitrile, and then dried in vacuo.

$[C_{39}H_{42}CuN_6O_5]ClO_4$: Anal. Found: C, 63.11; H, 5.52; N, 11.62; Calc.: C, 63.44; H, 5.73; N, 11.38. ESI-MS in methanol: $[M]^+$, m/z, 737.1938 (obsd. with 45 % abundance) (calcd.: m/z, 737.2613) where M = $[Cu^I(HL)(OH_2)(CH_3CN)]$ (**Fig. S5**) and IR spectra of this produced complex (**2**) confirms the formulation as $[Cu^I(HL)(OH_2)(CH_3CN)]ClO_4$ (**Fig. S6** ESI†). ^{1}H NMR (400 MHz, $CDCl_3$): 8.24-8.22 (d, 1H, CH=N); 8.09-8.06 (d, 2H); 7.94-7.92 (d, 1H); 7.43-7.38 (m, 4H); 7.02-7.00 (d, 1H); 6.94-6.87 (m, 1H); 6.58-6.54(d, 1H); 3.36-3.30 (m, 8H, $4CH_2$); 1.18-1.11 (t, 12H, $4CH_3$). ^{13}C NMR (400 MHz, $CDCl_3$): 164.89, 152.98, 149.07, 147.67, 142.64, 136.75, 133.92, 128.68, 127.88, 127.79, 123.96, 123.20, 117.21 (-CN), 108.17, 65.94 (spirolactam carbon), 44.18, 12.17, 1.73 ($-CH_3$ of acetonitrile). Yield: 70%.

X-ray data collection and structural determination

X-ray data were collected on a Bruker's Apex-II CCD diffractometer using Mo Kα (λ=0.71069). The data were corrected for Lorentz and polarization effects and empirical absorption corrections were applied using SADABS from Bruker. A total of 8503 reflections were measured out of which 3602 were independent and 1282 were observed [I>2 σ(I)]. The structure was solved by direct methods using SIR-92[11] and refined by full-matrix least squares refinement methods based on F^2, using SHELX-97.[12] All non-hydrogen atoms were refined anisotropically. All calculations were performed using Wingx package.[13] Important crystal and refinement parameters are given in **Table S1**.

Preparation of cell and *in vitro* cellular imaging with HL

Human cervical cancer (HeLa) cells were used throughout the study. HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 $\mu g.ml^{-1}$ of penicillin, 100$\mu g.ml^{-1}$ of streptomycin and 2 mM Glutamax at 37 °C in a humidified incubator at 5% CO_2. The adherent cultures were grown as monolayer and passaged once in 4-5 days by trypsinizing with 0.25% Trypsin-EDTA. HeLa cells (4 x 10^4 cells/mm^2), plated on cover slips, were incubated with **HL** (10, 5 and 2 μM, 1% DMSO) for 30 min. After washing with 50 mM phosphate buffer, pH 7.4 containing 150 mM NaCl (PBS), required volumes of aluminium nitrate stock solution in DMSO were added such that final $[Al(NO_3)_3]$ adjusted to 2.0 μM, 5.0 μM and 10.0 μM (DMSO will be 1%) and incubated for 30 min. The cells were fixed with 4% paraformaldehyde for 10 min at

room temperature (RT). After washing with PBS, mounted in 90% glycerol solution containing Mowiol, an anti-fade reagent, and sealed. Images were acquired using Apotome.2 fluorescence microscope (Carl Zeiss, Germany) using an oil immersion lens at 63 X magnification. The images were analyzed using the AxioVision Rel 4.8.2 (Carl Zeiss, Germany) software.[14]

Cell cytotoxicity assay

Cytotoxicity of **HL** was carried out using MTT assay which was based on the cleavage of the tetrazolium ring of MTT by mitochondrial dehydrogenases in the viable cells to form formazan as dark blue membrane impermeable species that can be quantified at 540 nm in DMSO solution giving a measure of the number of viable cells.[15] $15x10^3$ Human cervical cancer cell (HeLa) plated in 96-well culture plates were treated with different concentrations of the ligand and incubated for 6 h followed by addition of 25 µl of 4 mg ml^{-1} of MTT to each well and incubated for an additional 3 h. The culture medium was discarded and a 200 µl volume of DMSO was added to dissolve the formazan crystals. The absorbance at 540 nm was determined using an ELISA microplate reader (BioRad, Hercules, CA, USA). The cytotoxicity of the complexes was measured as the percentage ratio of the absorbance of the treated cells over the untreated controls.

Theoretical calculation

To clarify the understanding of the configurations and the mechanism of process of enhancement of fluorescence, DFT calculations of the ground state character of the probe **HL** and [**L-Cu(II)(NO_3)**] complex were performed using **Gaussian-09** software over a **Red Hat Linux IBM cluster**. Molecular level interactions have also been studied using density functional theory (DFT) with the **B3LYP/6-31G (d,p)** functional model and basis set.

Results and discussion

Synthesis and characterization

The organic moiety (**HL**) was synthesized by condensing an ethanolic solution of *4-nitrocinnamaldehyde* and *rhodamine-B hydrazide* in equimolar ratio (**Scheme 1**). The data obtained from the physico-chemical and spectroscopic tools (ESI†) , and the detailed structural analysis using single crystal X-ray crystallography are in good agreement with the formulation of **HL** as shown in **Scheme 1. HL** is soluble in common polar organic solvents and sparingly soluble in water. The ESI mass spectrum of the compound in methanol shows a peak at m/z 616.2907 with 100% abundance assignable to $[M + H]^+$ (calculated value at m/z, 616.2926) where M = molecular weight of **HL** (**Fig. S1** ESI†)**.** The peaks obtained in ^1H and ^{13}CNMR spectra of **HL** have been assigned and these are in accordance with structural formula of the **HL** in the solution state (**Fig. S3** ESI†). An ORTEP view and the packing arrangement of the probe **HL** with the atom numbering scheme is illustrated in **Fig. 1** and **Fig. S7** (ESI†). The crystallographic data and the bond parameters (selected bond distances and angles) are listed in **Tables S1** and **S2**, respectively.

To establish the fact of the copper(II) and copper(I) complex formation (**Scheme S1** ESI†), the **L-Cu** (**1**) and **HL-Cu** (**2**) in solid state were isolated from the reaction of copper(II) nitrate and *tetrakis(acetonitrile)-copper(I) perchlorate* with **HL** respectively in 1:1 mole ratio in the acetonitrile medium. The complexes are soluble in methanol, DMSO and acetonitrile etc. The peaks obtained in ^{13}C spectra and ^1H NMR of copper(I) complex have been assigned and it is in accordance with structural formula of the Cu(I) complex as $[Cu^I(HL)(CH_3CN)(H_2O)]ClO_4$ (**Fig. S8** and **S9** ESI†).

To know the dependency of the complex formation on only oxidation state of copper ion, cupric chloride and cuprous thiocyanate instead of cupric nitrate and *tetrakis(acetonitrile)-copper(I) perchlorate* respectively were allowed to react with **HL** in acetonitrile to obtain corresponding copper(II) and copper(I) complexes. In this study, ESI mass and IR spectra[16] of these produced complexes confirms the formulation as [Cu^{II}**(L)(Cl)**] (**3**) and [Cu^{I}**(HL)(H_2O)(CH_3CN)]SCN** (**4**) (**Fig. S10 (a,b)** ESI†); as the ESI mass spectrum of **3** shows a peak at m/z, 713.2568 with 90% abundance, assignable to $[M + H]^+$ (where M = [Cu^{II}**(L)(Cl)**]; calculated value at m/z, 713.1860) and that of **4** exhibits at m/z, 737.1938 with 45% abundance, assignable to $[M']^+$ (M′ = [Cu^{I}**(HL)(H_2O)(CH_3CN)**]$^+$; calculated value at m/z, 737.2613) (**Fig. S11** and **S12** ESI†). These findings excellently reinforce the fact of the flexidentate behaviour towards copper(II/I) ions as **HL** behaves as tridentate dibasic ligand for Cu(II) and as bidentate neutral ligand for Cu(I).

To understand the electronic configurations of **HL** and (**L-Cu**) (**1**) DFT calculations were performed (**Fig. S13** and **S14** ESI†). The narrowing of the energy gap between the HOMO and LUMO of [**Cu(L)(NO_3)**] compared to **HL** demonstrated the facile conversion, extra stability and have higher conjugation after spirolactam ring opening of the complex [**Cu(L)(NO_3)**]. A greater electronic charge density could be pointed out in the HOMO over the *p-nitrocinamalidene-hydrazido* unit of (**L-Cu**) (**1**) compared to that of **HL**. This feature is due to the strong -R effect of the nitro group of the *p-nitrocinamalidenehydrazido* unit of **HL** (**Fig. S13** ESI†). In **Fig. S14** (ESI†) the optimized structures give the clear idea about spirolactam ring distances.

Spectral characteristics

Absorption study

The electronic spectrum of **HL** (10 µM) recorded in HEPES buffer (1 mM, pH 7.4; acetonitrile/water: 1/5, v/v) exhibited absorption bands at higher energy below 400 nm corresponding to $\pi\rightarrow\pi^*$ (320 nm, $\varepsilon = 5.1x10^4$) and $n\rightarrow\pi^*$ (362 nm, $\varepsilon = 5.8x10^4$) transitions. On stepwise addition of copper(II) ions (0-20 µM) a new absorption peak at *ca.* 553 nm gradually developed due to the formation of a copper(II) complex with a visual colour change from yellow to red in view of opening of the spirolactam ring of **L** in complex **(L-Cu)** (**1**) (**Fig. 2**). While on gradual addition of copper(I) ions, the peak at 320 nm as well as 362 nm gradually increases up to the addition of 20.0 µM of copper(I) ions with a visual colour change from yellow to faint green (**Fig. 3**). But interestingly, the peak at around 550 nm was neither observed nor increased upon gradual addition

of copper(I) ions indicating closed form of the spirolactam ring in the complex (**HL-Cu**) (**2**).

Emission study

The fluorescence emission spectra of **HL** (10 μM) at 415 nm (λ_{ex} = 365 nm) (**Fig. 4**) was attributable for the donor cinnamaldehyde moiety, appeared with a quantum yield[17] of Φ = 0.34. But with increase of copper(II) ions (20 μM), the emission intensity at around 415 nm (donor position) gradually decreased (quantum yield of Φ = 0.11) with concomitant increase of a new peak at around 582 nm (Φ = 0.88) through an isoemissive point at 543 nm. On the other hand this type of spirolactam ring opening followed by FRET (Fröster Resonance Energy Transfer) was not occurred in case of the addition of different copper(I) salts (**Fig. S15** ESI†). Here, a slight quenching of the fluorescence intensity was observed at 415 nm position due to the chelation of diamagnetic copper(I) ion with *p-nitrocinamalidenehydrazido* unit of **HL** but no new peak at around 582 nm was formed. This result ascribed the closed spirolactam ring system of **HL** in the **HL-Cu** (**2**) complex (**Scheme 2**) and as a result no FRET process was operated. The observed results clearly indicate the dependency of FRET process on the oxidation state of copper ion. Fluorescence quantum yields (Φ) of the fluorescent species (at λex = 550 nm) were estimated by integrating the area under the fluorescence curves with the reported method[4b]

In this case the resulting intense fluorescence emission is due to the spirolactam ring-opening form of **L** in **(L-Cu) (1)** system. The *p-nitrocinamalidenehydrazido* unit chelated with copper(II) ion absorbs the radiation of 365 nm and emits at 415 nm which energy is transferred in non-radiative fashion to the xanthene moiety (acceptor) in turn leading to FRET (**Scheme 2**) due to the spirolactam ring opening.[18] This process is associated with a switch on UV-vis spectral response at 553 nm, which has a significant spectral overlap with the emission spectrum of the donor part of probe **HL** (**Fig. 5**).

Job's plot analyses (**Fig. 4** inset**, Fig. S16**) showed that both the complexes (**1** and **2**) formed in solution state in a 1:1 stoichiometric ratio. To evaluate the affinity of the probe towards copper(II) ions, the binding constant (K, 1.17 x 10^4 M^{-1}) was determined from the emission intensity data (**Fig. S17** ESI†) using the modified Benesi-Hildebrand equation corresponding to 1:1 stoichiometry.[19,20]

$$1/(F_x-F_0)=1/(F_{max}-F_0)+(1/K[C])(1/(F_{max}-F_0)$$

where F_0, F_x, and F_∞ are the emission intensities of organic moiety considered in the absence of Cu(II) ions, at an intermediate Cu(II) ions concentration, and at a concentration of complete interaction, respectively, and where [C] is the concentration of Cu(II) ions.

In addition to this dependency of occurring of FRET on the oxidation state of copper ions, the selectivity of **HL** towards Cu(II) ions has also been verified by recording the fluorescence due to Cu(II) ions even in the presence of 50 equivalent concentration of alkali and alkaline earth metal ions [Na(I), K(I), Mg(II), Ca(II)], and 50 equivalent concentration of several transition and other metal ions [Hg(II), Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Al(III), Zn(II), Cd(II) and Pb(II)] (**Fig. S18** and **S19** ESI†). This study reasonably divulges that HL has almost no interference for the detection of Cu(II) ions with an excellent specificity to Cu(II) ions over other cations.

To be acquainted with the role of pH on the fluorescence of **HL**, the fluorescence intensities were measured at various pH values adjusting the pH using HEPES buffer in presence and absence of Cu(II) ions. In the absence of Cu(II) ions, the weak fluorescence intensity of **HL** is almost independent over the pH range 6.0 to 10.0 **(Fig. S20** ESI†), and in presence of Cu(II) ions the enhanced fluorescence intensity compared to that in absence of Cu(II) ions is also independent on the variation of pH over the range of pH 5.5 to 8.5.

The fluorescence average lifetime measurement (at λ_{em} = 415 nm, donor position) of organic moiety (**HL**) in presence and absence of copper(II) ions in HEPES buffer (1 mM, pH 7.4; acetonitrile/water: 1/5, v/v) medium indicated the gradual decrease in life time with increase of copper(II) ions concentration (**Fig. 6**). The average lifetimes were calculated to be 4.64 ns for only **HL**, 4.56 ns for the mixture of **HL:** Cu(II) (1:0.5) and 4.32 ns **HL**: Cu(II) (1:1). This data clearly demonstrated the FRET process. The radiative rate constant k_r and total non-radiative rate constant k_{nr} of the organic moiety, **HL** and Cu(II) complex calculated from the equations: $\tau^{-1} = k_r + k_{nr}$ and $k_r = \Phi_f/\tau$,[21], were tabulated in **Table S3**. The data advocate the fluorescent enhancement due to the increase of the ratio of k_{nr}/k_r from 1.96 for **HL** to 8.24 for (**L-Cu**) (**1**) which is in great agreement of FRET process.

NMR spectral study

To establish the above fact of the formation of the complexes [(**L-Cu**) (**1**) and (**HL-Cu**) (**2**)] and bonding pathway, we tried to obtain the ^{1}HNMR and ^{13}CNMR spectral data of **HL, 1** and **2** in $CDCl_3$. In this study we could not able to do this experiment with **1** because of the paramagnetic nature of copper(II) ion since the recorded magnetic susceptibility data (1.78 B.M) of **1** reveal spin-only value (1.75 B.M.) for the copper(II) complex. The peaks observed in the ^{1}HNMR and ^{13}CNMR spectra of **HL** are almost identical with those in the spectra of **2** with a little change in some characteristic signals (**Figs. S8** and **S9** ESI†), which strongly indicates the coordination of HL with Cu(I) metal ion. The almost unchanged signal due to the spirolactam carbon strongly emphasizes the closed spirolactam ring, not opened even after coordination of copper(I) with **HL** as the signals at δ = 65.85 ppm in **HL** and δ = 65.94 ppm in **2** assignable for this carbon were observed (**Figs. S8** ESI†). Two additinal peaks at δ = 117.21 ppm for C≡N and δ = 1.73 ppm for CH_3 observed in ^{13}CNMR spectrum of **2** are fairly assignable for the two carbons of the coordinated acetonitrile. It is also noted that all the peaks due to the hydrogens in ^{1}HNMR spectrum of **HL** are present in that of **2** with some slight changes for some protons and the findings are in good agreement with the coordination of metal ion (**Figs. S9** ESI†).

Redox-study

To elucidate the understanding of the oxidation state of copper in the complexes **1** and **2**, the metal centred redox properties of

the complexes were examined by cyclic voltammetry using a Pt-wire working electrode in dry acetonitrile and in presence of [*n*-Bu_4N]ClO_4 as the supporting electrolyte (**Fig. S21** and **S22** ESI†). Both the complexes displayed a one electron equivalent quasi-reversible voltammogram corresponding to Cu^{II}/Cu^{I} couple having $i_{pc}/i_{pa} \approx 1$ with the difference in the potential values only depending upon the oxidation state of the copper ion. The $E_{1/2}$ value of -203.5 mV (ΔE = 117 mV) for this couple in **1** in cathodic side while that value of +338.5 mV (ΔE = 187 mV) in **2** in anodic side were obtained.

Analytical figure of merit

To approve the efficacy of this probe towards the detection of copper(II) ions, the detection limit (LOD = 83.70 nM) was calculated from the calibration curve based on the fluorescence enhancement at 582 nm (**Fig. S23**) focusing on the lower concentration region of Cu(II) ions using the equation $3\sigma/S$, where the slope of the curve is S and σ_{zero} is the standard deviation of seven replicate measurements of the zero level[4].

Cell Imaging

To examine the utility of the probe in biological systems, it was applied to human cervical cancer HeLa cell. Here, Cu(II) ions and **HL** were allowed to uptake by the cells of interest and the images of the cells were recorded by fluorescence microscopy at λ_{em} = 415 nm (blue filter) and at λ_{em} = 582 nm (red filter); (λ_{ex} = 365 nm) (**Fig. 7**). This cell imaging study of gradual change of colour from blue (of the probe itself) to red (due to FRET) nicely demonstrates the occurrence of FRET in complex **1,** which is also feasible in biological system. In addition, the *in vitro* study showed that the probe, **HL** has not shown significant cytotoxic effect to the cells upto 8.0 h (IC_{50}> 100μM) **(Fig. S24** ESI†). These results indicate that the probe has a huge potentiality for both *in vitro* and *in vivo* application as Cu(II) sensor as well as imaging in different ways as same manner for live cell imaging can be followed instead of fixed cells.

Conclusion

In conclusion, copper(II) and copper(I) complexes of a newly designed and crystalographically characterized rhodamine hydrazide-cinnamaldehyde conjugate Schiff base (**HL**) have been synthesized duly physico-chemical and thorough spectroscopic characterisation. **HL** acts as a Cu(II) ions selective chemosensor through FRET processes in HEPES buffer (1 mM, pH 7.4; acetonitrile/water: 1/5, v/v) over other competitive ions. The dependency of this FRET process is highly specific on the +2 oxidation state of copper exclusively, though this probe is prone to react with Cu(I) ion to form complex with an inability in opening of the spirolactam ring. The processes have nicely been established by the electronic, fluorimetric and NMR titration along with electrochemical study and computational study. This probe is also useful to detect the Cu(II) ions in Hela cells as **HL** has no cytotoxicity. It is also noteworthy that this Cu(II) ions selective probe is easy to synthesis and comparable with the previously reported FRET-based fluorescence probes in terms of the detection limit so far.[22]

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†Electronic Supplementary Information (ESI) available: [Materials and physical measurements, Schemes, characterization data, tables, figures, and some spectra], See DOI: 10.1039/b000000x/
‡CCDC 998374 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* http://www.ccdc.cam.ac.uk/data_request/cif.

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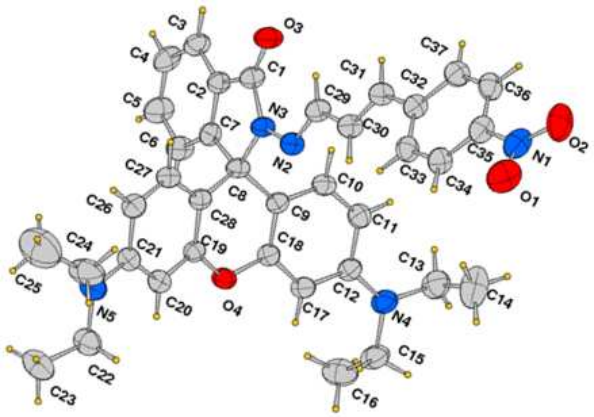


Fig. 1 Molecular views of **HL** with atom numbering scheme (35% ellipsoid probability)

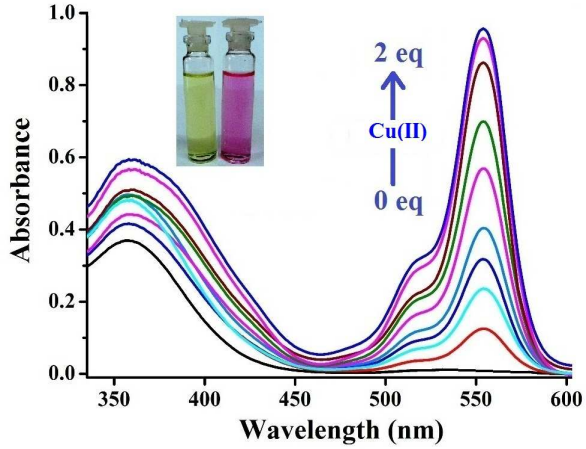


Fig. 2 UV-Vis titration spectra of **HL** with Cu(II) ions in HEPES buffer (1 mM, pH 7.4; acetonitrile/water: 1/5, v/v) at 25 °C. Inset shows the visual color change of **HL** and (**1**)

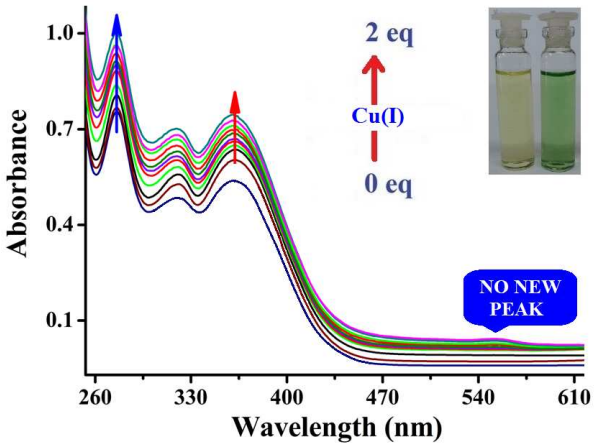


Fig. 3 UV-Vis titration spectra of **HL** with Cu(I) ions in HEPES buffer (1 mM, pH 7.4; acetonitrile/water: 1/5, v/v) at 25 °C Inset shows the visual color change of **HL** and (**2**)

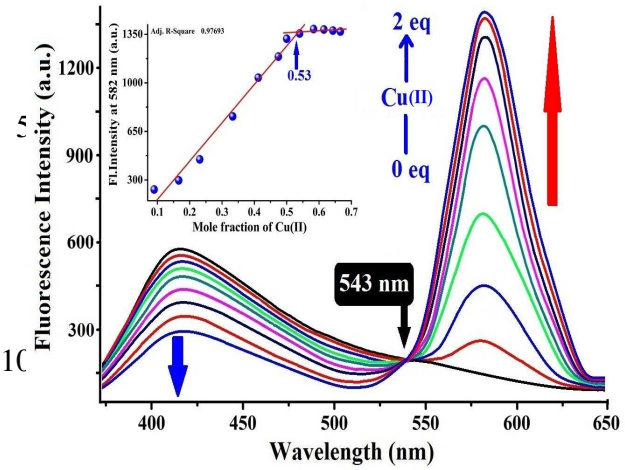


Fig. 4 Fluorescence titration of **HL** with incremental addition of Cu(II) ions in HEPES buffer (1 mM, pH 7.4; acetonitrile/water: 1/5, v/v) at 25 °C

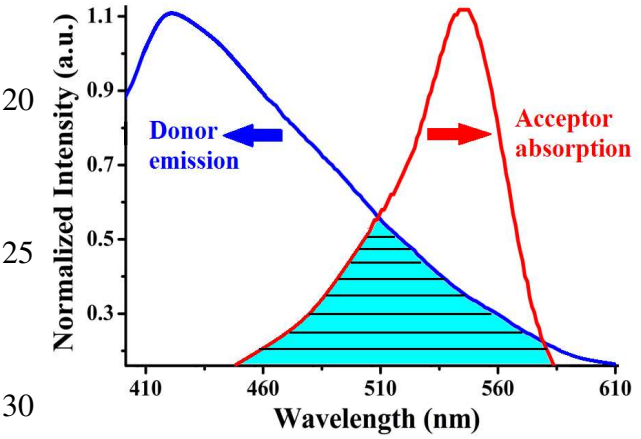


Fig. 5 Overlap (shown with cyan shaded area) between donor emission and acceptor absorption spectra of **HL**

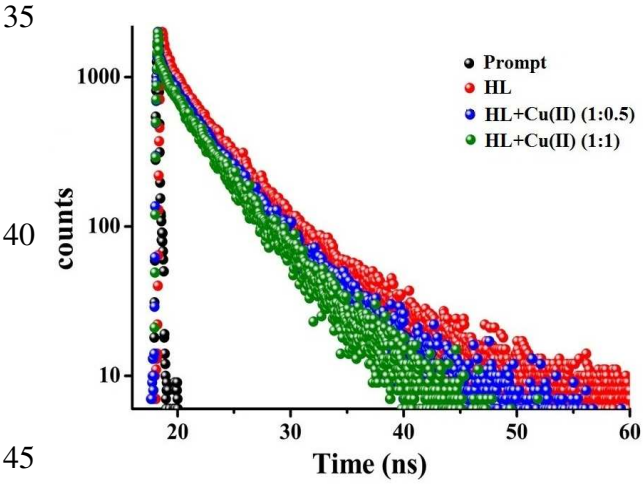


Fig. 6 Fluorescence life time decay profiles of **HL** at 415 nm (donor emission position) with increasing Cu(II) ions

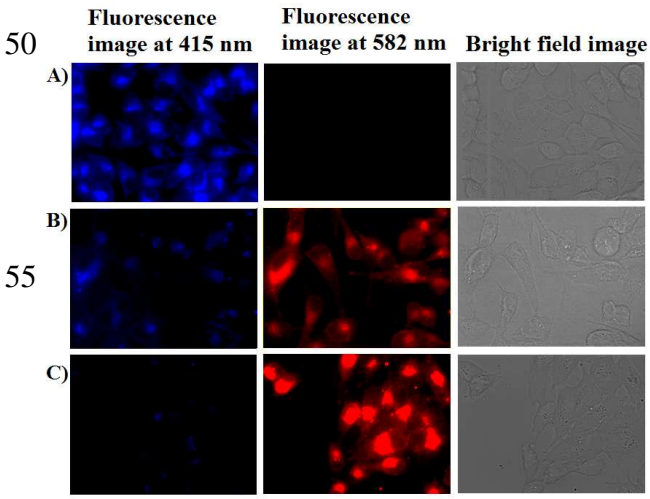
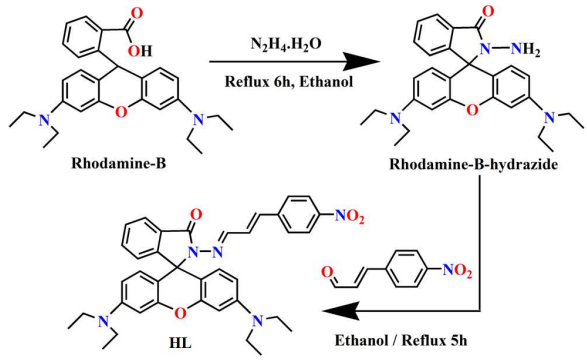
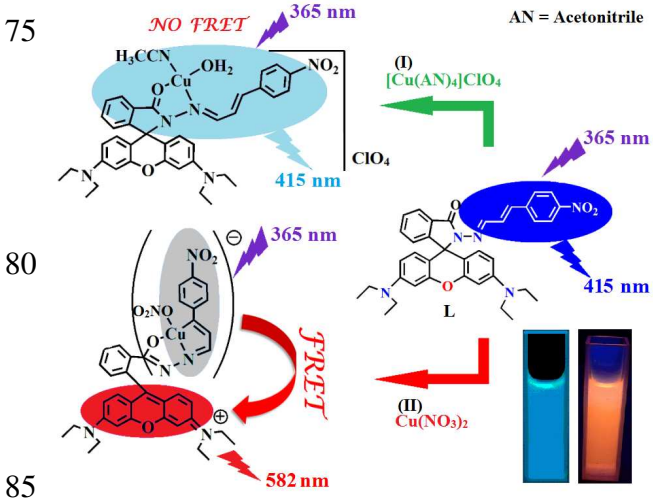


Fig. 7 Fluorescence image of HeLa cells preloaded with **HL** (10 μM) and were incubated with (**A**) 0 μM Cu(II); (**B**) 5 μM Cu(II); (**C**) 10 μM Cu(II) solution [λ_{ex} = 365 nm]



Scheme 1 Schematic representation of synthesis of the probe **HL**



Scheme 2 Plausible mechanistic pathway of **HL** for sensing of Cu(I) and Cu(II)