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Visible light excitable ON fluorescence and naked eye detection of Cu^{2+} *via* hydrolysis of rhodamine–thiophene conjugate: human breast cancer cell (MCF7) imaging studies†

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Thiophene appended rhodaminehydrazone derivative, **RDHDTCA** allows selective colorimetric and fluorescence recognition of Cu^{2+} as low as 2.4×10^{-8} M. **RDHDTCA** is capable to detect intracellular Cu^{2+} in human breast cancer cells, MCF7 under fluorescence microscope.

Introduction

Rhodamine derivatives possess excellent photo-physical properties[1] and transform from spirocyclic form to open-ring form in the presence of analytes capable to activate the carbonyl group.[2] Consequently the fluorescence properties also change significantly. Fluorescence sensors offer several advantages in terms of sensitivity, selectivity, simplicity, response time and spatial resolution. Several photosensing processes *viz.* photo-induced electron transfer (PET),[3] photo-induced charge transfer (PCT),[4] fluorescence resonance energy transfer (FRET),[5] inter molecular charge transfer (ICT),[6] chelation enhanced fluorescence (CHEF),[7] perturbation of optical transitions and polarizabilities, excimer/exciplex formation,[8a,b] modification of redox potentials in ground or excited states, and photo-regulation of binding properties are well known. Copper, the third most abundant soft transition metal ion in the human body, is an essential element for various biochemical and physiological processes. Due to its redox sensitivities, it acts as a vital cofactor for varieties of enzymes such as tyrosinase, cytochrome oxidase, and Cu/Zn superoxide dismutase *etc.*[9] However, at higher concentration, Cu^{2+} is a significant environmental pollutant and exerts toxicity to all living organisms.[10] Excess accumulation of copper in the human body causes gastrointestinal disturbance, neurodegenerative diseases, and liver/kidney damage. Thus, development of a Cu^{2+} selective, water soluble fluorescence probe functioning at physiological pH is highly desirable. Although, a significant number of rhodamine-based fluorescent probes for different metal ions *viz.* zinc,[11] iron,[12] chromium[13] and mercury[14] have been developed, relatively few have been reported for copper.[15] Herein, we report a thiophene appended rhodaminehydrazone derivative, **RDHDTCA** for selective determination of Cu^{2+} at physiological pH. Experimental data suggest Cu^{2+} assisted spirolactam ring opening of **RDHDTCA** and subsequent hydrolysis are responsible for fluorescence enhancement.

Results and discussion

Single crystals of **RDHDTCA** are grown from its ethanol solution and characterized by X-ray crystallography (Fig. 1). The most significant geometric data, which are in the expected range for rhodaminehydrazone derivatives,[16] are shown in Tables S1 and S2 (see ESI†). The crystal structure clearly shows the formation of a spirolactam-ring, where two planes of the spiro compound are almost in mutually vertical position. The dihedral angle between the spirolactam-ring and the xanthene-ring planes is about 88.7°. Weak intermolecular C–H···N (3.5 Å) and C–H···O (3.3 and 3.4 Å, bifurcated) hydrogen bonds along with other weaker interactions are responsible for the crystal packing. The absence of significant interactions involving the aldehyde residue explains the disorder found for the corresponding branch.

The effect of pH on the emission characteristics of **RDHDTCA** has been examined. Different sets of an equimolar mixture of **RDHDTCA** and Cu^{2+} are adjusted to different pH

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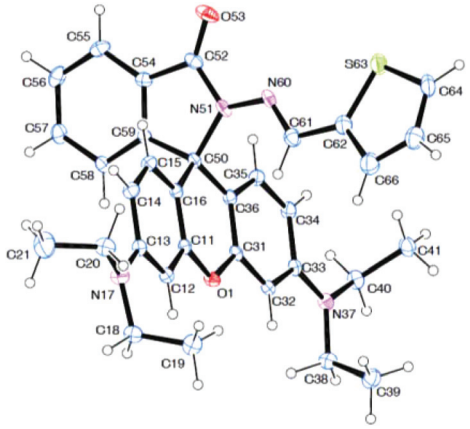


Fig. 1 View of the molecular structure of **RDHDTCA** showing only one of the three sets of positions found for the disordered aldehyde residue. Displacement atomic ellipsoids are drawn at 50% probability level.

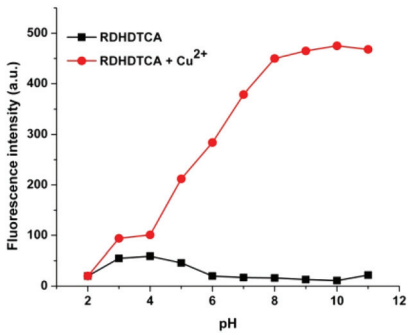


Fig. 2 Effect of pH on the emission intensity of free **RDHDTCA** (20 μM) and [Cu^{2+}–**RDHDTCA**] system (10 : 1, mole ratio, λ_{ex} = 500 nm, λ_{em} = 581 nm).

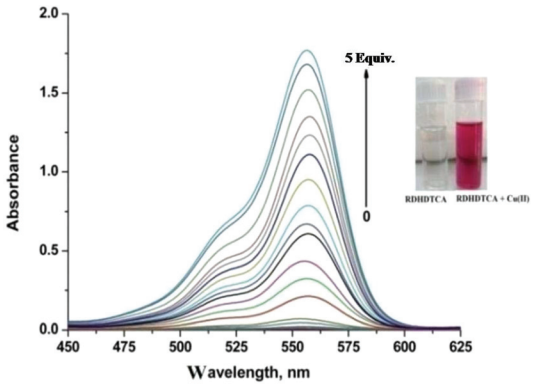


Fig. 3 Changes in the absorbance spectra of **RDHDTCA** (20 μM, CH_3CN–water = 9 : 1, v/v) upon gradual addition of Cu^{2+} (0, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 , 100 μM). Inset: color changes of **RDHDTCA** in the presence of Cu^{2+}. All spectra are recorded after 40 minutes of mixing of Cu^{2+} and **RDHDTCA**.

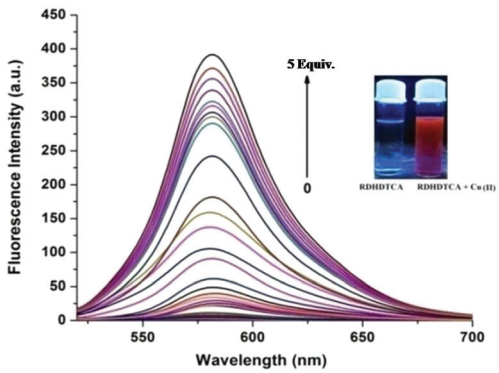


Fig. 4 Changes in the fluorescence spectra of **RDHDTCA** (20 μM, CH_3CN–water = 9 : 1, v/v) upon gradual addition of Cu^{2+} (0, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 μM) (λ_{ex} = 500 nm). Inset: color changes of **RDHDTCA** in the presence of Cu^{2+}. All spectra are recorded after 40 minutes of mixing of Cu^{2+} and **RDHDTCA**.

(pH 2.0–11.0) and their emission intensities are measured. Fig. 2 indicates that over pH 7.4, the emission intensity of the [Cu^{2+}–**RDHDTCA**] system becomes maximum allowing determination of Cu^{2+} at physiological pH.

Fig. 3 shows the changes in the absorbance spectra of **RDHDTCA** upon gradual addition of Cu^{2+}. The absorbance of free **RDHDTCA** gradually enhances with increasing concentration of Cu^{2+} while the colorless **RDHDTCA** solution turns to red-pink indicating the ring-opening of rhodamine unit of **RDHDTCA**.

Changes in the emission spectra of **RDHDTCA** upon gradual addition of Cu^{2+} are presented in Fig. 4. Addition of Cu^{2+} to **RDHDTCA** results a 581 nm emission band along with generation of pink-red color, attributed to Cu^{2+} induced spirolactam ring opening of **RDHDTCA**. The sigmoidal nature of fluorescence enhancement may be rationalized in the light of Cu^{2+} assisted hydrolysis of **RDHDTCA** (*vide infra*). With increasing Cu^{2+} concentration, more and more **RDHDTCA** being chelated followed by their hydrolysis, so the emission intensity increases. Moreover, at higher [Cu^{2+}] region, a small addition of Cu^{2+} causes much higher fluorescence enhancement. This is because, Cu^{2+} released to the medium after hydrolysis, may be available for chelation to other **RDHDTCA** molecules, so a lower amount of external Cu^{2+} is necessary for the same extent of fluorescence enhancement (Fig. S1, ESI†).

The Job's plot (Fig. S2, ESI†) indicates a binding stoichiometry of **RDHDTCA** to Cu^{2+} as 1 : 1 (mole ratio). The association constant (K_a) of **RDHDTCA** for Cu^{2+} is determined by fluorescence (Fig. S3, ESI†) and UV-Vis (Fig. S4, ESI†) techniques following the Benesi–Hildebrand method.[17,18] The values are 4.297×10^4 M^{-1} and 3.3×10^4 M^{-1} respectively. Quantum yield of free **RDHDTCA** (1.19×10^{-2}) increases 8.54 fold in the presence of Cu^{2+} (details in ESI†). In the presence of water, the [**RDHDTCA**–Cu^{2+}] system further undergoes rapid hydrolysis leading to open ring rhodamine unit, evidenced by the mass spectra of the final stable product (Fig. S5, ESI†).

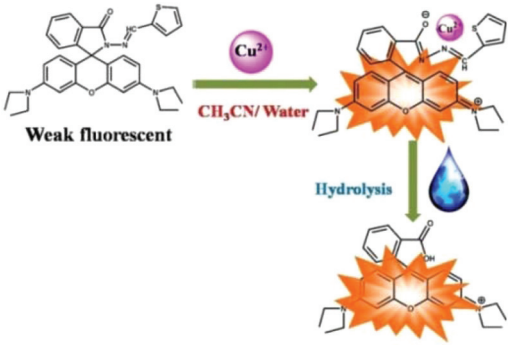


Fig. 5 Proposed Cu^{2+} sensing mechanism.

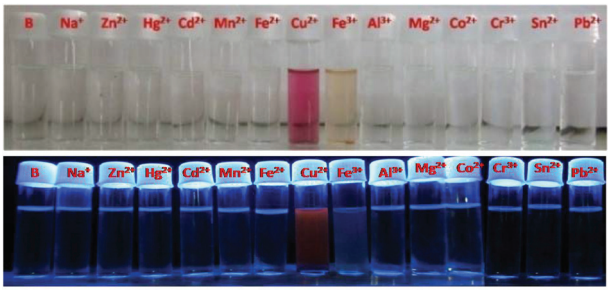


Fig. 6 Color of **RDHDTCA** (20 μM) in the presence of different cations: naked eye (top); under UV lamp (bottom) in CH_3CN–water (9 : 1, v/v).

From the above discussions, the plausible binding interaction of **RDHDTCA** for Cu^{2+} is presented in Fig. 5. The limit of detection (LOD) for Cu^{2+} is 2.4×10^{-8} M (Fig. S6, ESI†).

Selectivity and interferences

Fig. 6 shows the naked eye and UV illuminated color of **RDHDTCA** in the presence of different cations. Common cations *viz.* Na^+, Mg^{2+}, Hg^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Cr^{3+}, Sn^{2+}, Pb^{2+}, Cd^2, Al^{3+} and Zn^{2+} do not show any color change of free **RDHDTCA**. Only Fe^{3+}/Fe^{2+} show slight coloration, however it can be masked by F^- ion without disturbing the [**RDHDTCA**–Cu^{2+}] system. Again, except for iron, other cations, present in excess together with Cu^{2+} (50 equivalent) in a ternary mixture, do not show any significant change to the emission intensities of the [**RDHDTCA**–Cu^{2+}] system. Insignificant effect of iron is nullified using F^- ion as masking agent. Thus, **RDHDTCA** can selectively sense Cu^{2+} through fluorescence enhancement (Fig. S7 and S8, ESI†).

Cell imaging

RDHDTCA is capable to detect intracellular Cu^{2+} in human breast cancer cells, MCF7 under fluorescence microscope. Cells treated only with **RDHDTCA** (in the absence of Cu^{2+}) are used as control. Fig. 7 indicates that **RDHDTCA** is able to permeate the cell membrane and stain Cu^{2+} without any harm (cells remain alive even after several hours of exposure to 20 μM **RDHDTCA**).

Fig. 7 Fluorescence microscope images of MCF7 cells: (A) and (B) are images of cells after 2 h incubation with Cu^{2+} (50 μM) and **RDHDTCA** (20 μM) respectively, while (C) shows the cells after 2 h incubation with 50 μM Cu^{2+} followed by the addition of 20 μM **RDHDTCA** solution.

Conclusion

Thiophene appended rhodaminehydrazone derivative functions as an excellent colorimetric and fluorescent probe for trace level determination of Cu^{2+} at physiological pH with LOD, 2.4×10^{-8} M. Initial chelation of **RDHDTCA** to Cu^{2+} followed by hydrolysis is attributed to the fluorescence enhancement. **RDHDTCA** is capable to detect intracellular Cu^{2+} in human breast cancer cells, MCF7 under fluorescence microscope.

Experimental

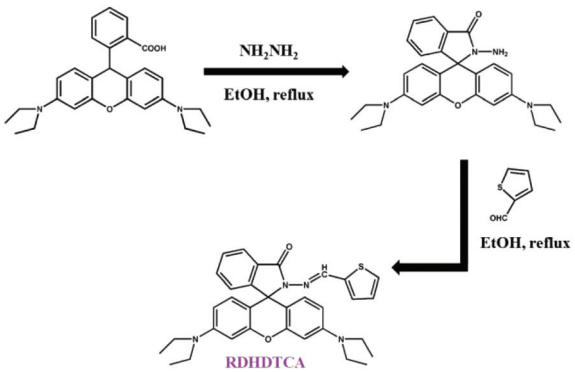
General procedure

High-purity rhodamine B, thiophene-2-carboxaldehyde, hydrazine hydrate (Sigma Aldrich, India) and $Cu(NO_3)_3{\cdot}3H_2O$ (Merck, India) are used. Spectroscopic grade solvents are used. Metal salts used are either in nitrate or chloride forms. Other analytical reagent grade chemicals are used without further purification except when specified. Mili-Q Milipore® 18.2 MΩ cm^{-1} water is used in all the experiments. A Shimadzu Multi Spec 1501 spectrophotometer is used for recording UV-Vis spectra. FTIR spectra are recorded on a PerkinElmer FTIR (model RX1) spectrophotometer. Mass spectra are carried out using a QTOF Micro YA 263 mass spectrometer in ES positive mode. ^1H NMR spectra are recorded using Bruker Advance DPX 300 (300 MHz) in $CDCl_3$. The steady-state fluorescence emission and excitation spectra are recorded with a Perkin Elmer LS 55 precisely, spectrofluorimeter. Systronics digital pH meter (model 335) is used to measure the solution pH. HCl or NaOH (50 μM) are used for pH adjustment.

Synthesis of rhodaminehydrazone unit

N-(Rhodamine-B) lactam-hydrazine is prepared by refluxing rhodamine B and hydrazine hydrate for 3 hour following the literature method.[19]

Synthesis of RDHDTCA. *N*-(Rhodamine-B) lactam-hydrazine (0.20 g, 0.4386 mmol) is dissolved in 10 mL ethanol, and added drop-wise to the ethanol solution of thiophene-2-carboxaldehyde (49 g, 0.4375 mmol in 10 mL) under stirring condition. The reaction mixture is refluxed for 6 hours (Scheme 1), filtered to remove suspended material and the



Scheme 1 Synthesis of **RDHDTCA**.

filtrate is kept undisturbed. After 3 days, pink color crystals suitable for single crystal X-ray diffraction analysis are collected. ^{1}H NMR (300 MHz, $CDCl_3$) (Fig. S9, ESI†): 1.13 (12H, m, J = 6.9 Hz), 3.28 (8H, m), 6.23 (2H, d, J = 8.3 Hz), 6.45 (2H, m), 6.89 (2H, m), 7.07 (1H, d, J = 2.8), 7.12 (1H, d, J = 6.7), 7.22 (1H, d, J = 4.5), 7.26 (1H, s), 7.48 (2H, s), 7.96 (1H, d, J = 2), 8.91 (1H, s); elemental analysis data as calculated for $C_{33}H_{34}N_4O_2S$ (%): C, 71.97; H, 6.22 and N, 10.17; S, 11.64; Found (%): 72.02, 6.31, 10.14 and 11.53. QTOF-MS ES$^+$ (Fig. S10, ESI†): $[M + H]^+$ = 551.92; FTIR/cm^{-1} (Fig. S11, ESI†): ν(C═O) 1721, ν(C═N) 1614.

Synthesis of [RDHDTCA–Cu^{2+}] complex. In an ethanol solution of **RDHDTCA** (200 mg, 0.36 mmol), 2 mL solution of $Cu(NO_3)_2{\cdot}3H_2O$ (0.006 g, 0.024 mmol) in ethanol is added drop-wise and stirred for 5 minutes. The solution is kept for several days to finally obtain a deep pink compound. QTOF-MS ES$^+$ (Fig. S5, ESI†): $[M + H]^+$ = 443.13; FTIR/cm^{-1} (Fig. S11, ESI†), ν(C═O), 1585.

Single crystal X-ray structure analysis

Single crystal X-ray diffraction data for **RDHDTCA** are collected at 100(2) K, on a Bruker SMART 1000 CCD diffractometer using graphite-monochromated Mo-Kα radiation (λ = 0.71073 Å). Some significant crystal parameters and refinement data are summarized in Table S3 (see ESI†). Data are processed and corrected for Lorentz and polarization effects. Multi-scan absorption corrections are performed using the SADABS routine.[20,21] The structure is solved by standard direct methods[22] and refined by full matrix least squares on F^2.[23] All non-hydrogen atoms are anisotropically refined. Hydrogen atoms are included in the structure factor calculation in geometrically idealized positions, with thermal parameters depending of the parent atom, by using a riding model. Since some disorder is found in the whole aldehyde residue (Fig. S12, ESI†), the corresponding atoms are modeled in three alternative positions with occupation sites of 36.7, 44.6 (atoms labeled B) and 18.7 (atoms labeled C). Hydrogen bonds along with other weaker interactions are responsible for the crystal packing (Fig. S13, ESI†). The absence of significant interactions involving the aldehyde residue explains the disorder found for the corresponding branch.

In vitro cell imaging

Human breast cancer cell line MCF7 is grown in DMEM (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, USA), 2 mM glutamine, 100 U mL^{-1} penicillin–streptomycin solution (Gibco, Invitrogen, USA) in the presence of 5% CO_2 at 37 °C. For *in vitro* imaging studies, the cells are seeded in 6 well culture plate with a seeding density of 10^5 cells per well. After reaching 60%–70% confluence, the previous media is replaced with serum free media, supplemented with Cu^{2+} and **RDHDTCA** at 50 μM and 20 μM, and incubated for 2 h to facilitate metal ion or **RDHDTCA** uptake by the cells. The cells are then observed under an inverted microscope (Dewinter, Italy) at different magnifications to examine any adverse effect on cellular morphology. **RDHDTCA** treated cells are then incubated with Cu^{2+} for 15–30 min and observed under an inverted fluorescence microscope at different magnifications with blue filter. Images are taken through an attached ccd camera with the help of Bio-Wizard 4.2 software. Control experiment is done using medium devoid of Cu^{2+} salt.

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