A new rhodamine based ‘turn-on’ Cu\(^{2+}\) ion selective chemosensor in aqueous system applicable in bioimaging

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Abstract. A new rhodamine-based Schiff base (L) has been synthesized and characterized by physicochemical and spectroscopic tools. This organic molecule selectively reacts with Cu\(^{2+}\) ions with a remarkably significant optical change, which supports the development of a chemosensor for Cu\(^{2+}\) ions as low as nanomolar level in aqueous medium. On the basis of the experimental work, the ‘turn-on’ colorimetric/fluorimetric spectroscopic change is due to Cu\(^{2+}\) ion-assisted hydrolysis followed by spirolactam ring opening of the probe (L) in 20 mM HEPES buffer [pH 7.4; water/acetonitrile (9:1 v/v)]. The competitive ions do not affect the selectivity and specificity of the probe (L) in the detection of Cu\(^{2+}\) ions. The cell imaging study using fluorescence microscope showed that this non-cytotoxic probe is useful to detect the distribution of Cu\(^{2+}\) ions in AGS cells lines.

Keywords. Hydrolysis; Cu\(^{2+}\) ions; rhodamine-B; cell imaging.

1. Introduction

A chemosensor is such a compound that exhibits a very distinguishable and a significant change in electrical, electronic, magnetic, or optical signal when it binds to a specific guest counterpart like cations, anions or molecules, etc.\(^{1}\) As the third most abundant transition metal ion, Cu\(^{2+}\) (after Fe\(^{2+}\) and Zn\(^{2+}\)) plays a pivotal role in a variety of fundamental physiological processes in organisms ranging from bacteria to mammals involving cellular energy generation, oxygen transport and activation, and signal transduction.\(^{2-4}\) In many metalloenzymes, including superoxide dismutase, cytochrome c oxidase, and tyrosinase copper ion plays a crucial role as a catalytic cofactor.\(^{5}\) Due to its widespread use, Cu\(^{2+}\) ion is also a significant metal pollutant in the environment.\(^{6}\) Though Cu\(^{2+}\) plays important role in many biochemical processes, alteration in the cellular homeostasis of copper ions was reported to be connected with some serious neurodegenerative diseases like Alzheimer’s disease,\(^{7}\) Indian childhood cirrhosis (ICC),\(^{8}\) Indian prion disease,\(^{9}\) and Menkes and Wilson diseases.\(^{10,11}\) A high amount of copper for a short period of time can also lead to gastrointestinal disturbance, liver or kidney damage.\(^{12}\) Free copper ions in a live cell catalyze the formation of reactive oxygen species (ROS) that can damage lipids, nucleic acids, and proteins. As a result, the maximum permissible level of copper in drinking water has been set at 1.3 ppm (\(\sim 20 \mu M\)) by the U.S. Environmental Protection Agency (EPA).\(^{13}\)

Though, numerous methods for the detection of trace level of copper in the samples are available which include electrochemistry,\(^{14}\) atomic absorption spectrometry,\(^{15}\) inductively coupled plasma mass spectroscopy (ICPMS),\(^{16}\) inductively coupled plasma atomic emission spectrometry (ICP-AES)\(^{17}\) and voltammetry.\(^{18}\) But these techniques are usually complicated, time-consuming and costly too.

Of the many different kinds of optical sensors, fluorescent chemosensors have several advantages over the other methods due to their intrinsic sensitivity, upfront application to fiber optical-based detection, and real-time monitoring with fast response time.\(^{19,20}\) Due to its intrinsic paramagnetic properties, Cu\(^{2+}\) ion has the propensity to quench the fluorescence of fluorescent
metal chelators conferring a non-fluorescent state.⁴¹,⁴² In addition, most of the copper-selective sensors suffer from the interfering effect of cations such as Ni²⁺, Ag⁺, Hg²⁺ ions, etc.⁴³-⁴⁶ Therefore, the development of a highly sensitive, selective turn-on fluorogenic probe working under a physiological condition with a fast response is highly desirable.

Rhodamine derivatives are non-fluorescent and colorless, whereas ring-opening of the corresponding spirolactam gives rise to strong fluorescence emission and a pink color. Several photosensing processes viz., photo-induced electron transfer (PET),²⁷ photo-induced charge transfer (PCT),²⁸ fluorescence resonance energy transfer (FRET),²⁹ intermolecular charge transfer (ICT),³⁰ chelation enhanced fluorescence (CHEF),³¹ etc., are well known. Some hydroxynaphthalidine derivatives and calixarene functionalised nanoparticles have been reported as Cu²⁺ ions sensor.³²,³³ Rhodamine and its derivatives have been widely used to design fluorescent chemosensors due to their good photostability, high extinction coefficient, high fluorescence quantum yield and broad fluorescence in the visible region of electromagnetic spectrum. Several rhodamine-based chemosensors for recognition of various metal ions are reported.³⁴-³⁹ With these considerations, herein, we report synthesis of a salicylalddehyde appended rhodamine hydrazone derivative (L) for selective determination of Cu²⁺ ions at physiological condition without interfering. Experimental findings suggest Cu²⁺ ions assisted spirolactam ring opening of (L) and the subsequent hydrolysis is responsible for fluorescence enhancement leading to off-on sensing.

2. Experimental

2.1 Materials and general methods

2.1a Materials: High-purity HEPES buffer and rhodamine B dye were purchased from Himedia Laboratories, India. 3,5-Di-tert-butyl-2-hydroxybenzaldehyde was purchased from Sigma Aldrich and different inorganic salts were purchased from Emplura, Merck. Analytical grade different solvents were purchased from commercial sources and they were used in this work without further purification (if not mentioned). Here, Milli-Q 18 Ω water was used throughout the experiments. UV-Vis and IR spectral data were collected using a Shimadzu (model UV-1800) spectrophotometer and a Prestige-21 SHIMADZU FTIR spectrometer, respectively. A Bruker Avance DPX 500 MHz spectrometer ¹H NMR spectra were recorded in DMSO-d₆, pH value of solution was adjusted using either 50 mM HCl or NaOH solution and the pH values were measured by using a Systronics digital pH meter (model 335). A Thermochem Exactive plus mass spectrometer was used to get the electrospray ionization mass spectra. Steady state fluorescence emission and excitation spectra were obtained with the help of a Hitachi-7000 spectrofluorimeter.

2.1b General methods: The fluorescence properties of the probe L were measured in 10% CH₃CN 20 mM HEPES buffer medium (pH 7.4) at 25 °C. To investigate the effect of different pH on the emission intensity of L, pH study was performed in 20 mM HEPES buffer solution by maintaining the pH using 50 mM HCl or 50 mM NaOH solution. Cell imaging study was carried out at biological pH ∼ 7.4 using 20 mM HEPES buffer solution. To check the selectivity of this probe L towards different metal ions, stock solutions (∼ 10⁻² M) for L was prepared using different salts; such as, the chloride salts of Ni²⁺, Co²⁺, Cu²⁺, Cr³⁺, Hg²⁺, Ca²⁺, Mg²⁺ and Fe³⁺ ions; nitrate salts of Na⁺, K⁺, Cr³⁺ and Ag⁺ ions; acetate salts of Mn²⁺ and Zn²⁺ ions; and ferrous sulphate in 10% CH₃CN 20 mM HEPES buffer (pH 7.4) solvent. During this selectivity measurement, the concentration of these metal ions was taken fifty times greater than that of the probe (L). For fluorimetric titration study, solution of copper(II) chloride dihydrate in 10% CH₃CN 20 mM HEPES buffer (pH 7.4) was used. During this study, probe concentration was 20.0 μM and gradually Cu²⁺ solution were added by varying Cu²⁺ concentration from 0 to 60.0 μM and the corresponding spectra were recorded.

The path length of the cells used for absorption and emission studies was 1 cm. For UV-Visible and fluorescence titrations, a stock solution of L was prepared in 10% CH₃CN 20 mM HEPES buffer (pH 7.4) at room temperature. Working solutions of L and Cu²⁺ ions were prepared from their respective stock solutions. Fluorescence measurements were performed using a 5 nm × 5 nm slit setting for both excitation and emission spectra. All the fluorescence and absorbance spectra were taken after 20 min of mixing the Cu²⁺ ions and L. A series of solutions containing L and CuCl₂·2H₂O were prepared such that the total concentration of L (20 mM) remained constant in all the sets. The organic molecule (L) shows a very weak emission at 572 nm in 10% CH₃CN 20 mM HEPES buffer (pH 7.4) at 25 °C when excited at 550 nm.

2.2 Synthesis of the probe (L)

The probe L was synthesised by a two step reaction (Scheme 1). At first, the rhodamine B-hydrazide was prepared following a literature method.⁴⁰ In brief, 85% hydrazine hydrate (4 mL) was added to a solution of rhodamine B (1.0 g, 2.1 mmol) in ethanol (40 mL). The solution was refluxed for 6 h. Then, the reaction mixture was evaporated under reduced pressure to give an orange coloured oil, which was then recrystallized from methanol-water to afford rhodamine B-hydrazide as a light-orange crystal (77%).

In the second step, 3,5-Di-tert-butyl-2-hydroxybenzaldehyde (234.33 mg, 1.0 mmol) dissolved in ethanol was added to the ethanolic solution of rhodamine-B hydrazide (456.25
2.4 Cell cytotoxicity assay

To verify the usefulness of this probe in the biological system, the cytotoxicity of L was checked through MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay experiment. To the respective probe (5, 10, 25, 50, and 100 μM) solutions, 10 μL of MTT solution (10 mg/mL PBS) was added to each well of a 96-well culture plate and incubated continuously at 37°C for 8 h. All the mediums were replaced with 100 μL of acidic isopropanol in the wells. The intracellular formazan crystals (blue-violet) formed were solubilized with 0.04 M acidic isopropanol and the absorbance of the solution was measured at 550 nm with a microplate reader. Absorbance values were recorded as the mean ± S.D. of three independent experiments.

3. Results and Discussion

3.1 Synthesis and characterization

The rhodamine B-hydrazide derivative probe (L) was prepared from the reaction 3,5-Di-tert-butyl-2-hydroxybenzaldehyde (TBHB) and rhodamine B-hydrazide in ethanol (Scheme 1). The crude white precipitate obtained from the reaction mixture was purified through crystallisation from the solution of L in acetonitrile/methanol (3:1) mixed solvents on slow evaporation over a few days. The crystallised product was characterized by physicochemical and spectroscopic tools (Figures S1–S3, in Supplementary Information).

3.2 Absorption and fluorescence spectroscopic studies of L

The UV-Visible spectra of L recorded in 10% CH₃CN 20 mM HEPES buffer (pH 7.4) at 25°C shows an absorption maximum at 314 nm, which may be attributed to the intramolecular π-π* charge transfer transition.

On the stepwise addition of Cu²⁺ ions (0–60 μM) to the solution of L in 10% CH₃CN 20 mM HEPES buffer (pH 7.4), the absorption intensity at 314 nm increased gradually and a new peak at 560 nm was generated by ring opening with a visual color change from colorless to pink (Figure 1). In the presence of an excess of the biologically relevant transition metal ions (Cr³⁺, Mn²⁺, Fe³⁺, Co²⁺, Ni²⁺, Zn²⁺, Cd²⁺), alkali metal ions (Na⁺, K⁺), alkaline earth metal ions (Ca²⁺, Mg²⁺, Al³⁺) and other heavy metal ions (Hg²⁺, Pb²⁺), no new band was produced as they were unable to go through the ring opening step.

The emission spectrum of L excited at 550 nm shows a fluorescence maximum at 572 nm in 10% CH₃CN.
L showed an almost 68-fold increase in its fluorescence intensity with the addition of only 3.0 equivalents of Cu$^{2+}$ ions. This consequential strong fluorescence emission is due to the ring-opening of the spirolactam system of rhodamine-B (Scheme 2). In addition, this visual and fluorescence color change due to Cu$^{2+}$ ions were not perturbed by the presence of an excess of the biologically relevance transition (Cr$^{3+}$, Mn$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Cd$^{2+}$), alkali, alkaline earth metal ions (Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, Al$^{3+}$) and other heavy metal ions (Hg$^{2+}$, Pb$^{2+}$) (Figure S4 in SI) and also these ions did not offer such visual and fluorescent color change property through ring opening. It reveals that the probe L has an excellent selectivity and specificity towards Cu$^{2+}$ ions over the other cations.

3.3 pH study

The effect of pH on the emission characteristics of L has been examined. Different sets of an equimolar mixture of L and Cu$^{2+}$ ions were adjusted to different pH (pH 4.0–11.0) and their emission intensities were measured (viz., Figure 3). This study clearly showed the maximum emission intensity of the [Cu$^{2+}$ + L] system at pH 7.4,
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3.4 Mechanism

In the presence of Cu$^{2+}$ ions, L undergoes hydrolysis leading to open ring rhodamine unit, evidenced by the mass spectra and IR spectra (Figures S5 and S6 in Supplementary Information) of the products obtained from the final mixture of the probe and the added 3.0 equivalents of Cu$^{2+}$ ions. As a result of hydrolysis of the probe followed by the ring opening, the increase of the emission intensity was observed with increasing addition of Cu$^{2+}$ ions (Figure 2). It may be due to more and more hydrolysis of L in this condition followed by the formation of copper(II) complex of the fragmented part, irreversibly (Figure S5 on SI). However, no colorimetric change or no fluorescence enhancement of L occurred in the presence of other ions but interestingly, changes in color/fluorescence was observed only in the presence of Cu$^{2+}$ ions in similar condition. Based on the MS spectral analysis, it has also been observed that the selective Cu$^{2+}$ ions-assisted hydrolysis has occurred and it is different from the previous reports.43–45 The emission and absorption spectra of L in the presence of Cu$^{2+}$ ions was measured for a time period of 30 min and it was found that after ~20 min the emission and absorbance reach a saturation level (Figure 4 and Figure S7 in SI). From the above discussions, the plausible mechanistic pathway of selective sensing Cu$^{2+}$ ions by the probe L is presented in Scheme 2.

3.5 Analytical figure of merit

To investigate the selectivity, representative ions such as Na$^{+}$, K$^{+}$, Ca$^{2+}$, Mg$^{2+}$, Cr$^{3+}$, Mn$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Pb$^{2+}$, Ba$^{2+}$, Sr$^{2+}$, Ag$^{+}$ and Au$^{3+}$ ions were added to a solution of L, keeping the other experimental conditions unchanged. Only in the case of addition of Cu$^{2+}$ ions a visible color change (colorless to pink) occurred along with an enhancement of fluorescence intensity. No significant change in the fluorescence spectra of L (20 μM) upon the addition of metal ions is also checked (Figure 5). This selectivity of Cu$^{2+}$ ion over all other ions is due to the selective hydrolysis of L. From the linear response curve (Figure S8 in Supplementary Information), it is revealed that the probe exhibits a linearity with concentration of Cu$^{2+}$ ions up to ~ 46 μM.

The detection limit (LOD) was calculated from the calibration curve based on the fluorescence enhancement
Figure 7. Fluorescence and bright field images of AGS cells after incubation with L (20 μM) followed by addition of Cu^{2+} ions, (1, 1') 0 μM, (2, 2') 10 μM and (3, 3') 20 μM, respectively. All the samples were excited at 550 nm by using a [63X] objective.

3.6 Application

To investigate the utility of this probe (L) in biological systems, it was applied to AGS cell line. Here, L and Cu^{2+} were allowed to be taken up consecutively by the cells and the images of the cells were captured by fluorescence microscopy by excitation at ~ 550 nm (Figure 7). Additionally, in vitro study showed that the probe L is non-cytotoxic towards the cells up to 8.0 h (Figure S9 in SI). These results indicate that the probe has a huge potentiality for both in vitro and in vivo applications as a Cu^{2+} sensor as well as for live cell imaging.

4. Conclusions

A new fluorescence turn-on rhodamine-based Schiff base (L) has been synthesized and characterized by physicochemical and spectroscopic methods. The spectrophotometric color formation (colorless to reddish pink) and fluorimetric emission due to the selective Cu^{2+}
ion assisted hydrolysis, followed by spirolactam ring opening of the L in 10% CH3CN 20 mM HEPES buffer (pH 7.4) at room temperature helps to detect Cu2+ ions as low as 30 nM in physiological condition. Interestingly, the presence of several competitive ions did not affect this color change. This non-toxic probe is also useful to identify the distribution of Cu2+ ions in living cells.

Supporting Information (SI)

Supplementary Information associated with this article is available at www.ias.ac.in/chemsci.

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