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journal homepage: [www.elsevier.com/locate/jlumin](http://www.elsevier.com/locate/jlumin)A new rhodamine B based fluorometric chemodosimeter for Cu<sup>2+</sup> ion in aqueous and cellular mediaSuresh Kumar Kempahanumakkagaari <sup>a</sup>, Ramakrishnappa Thippeswamy <sup>a,\*</sup>, Pandurangappa Malingappa <sup>b</sup><sup>a</sup> Center for Nano and Material Sciences, Global Campus, Jain University, Jakkasandra (P) Kankapura (T), Bangalore 560001, Karnataka, India<sup>b</sup> Department of Studies in Chemistry, Central College Campus, Dr Ambedkar Veedi, Bangalore University, Bangalore 560001, India

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## ABSTRACT

A simple, sensitive and selective fluorescent chemo dosimeter rhodamine B phenyl hydrazide (RBPH) for Cu<sup>2+</sup> was proposed. This probe is non fluorescent and colorless but exhibits fluorescent enhancement at 580 nm and displayed color change from colorless to pink for Cu<sup>2+</sup> in the pH range 1–6. Fluorescence microscope experimental results reveals that this chemo sensor is cell permeable and can be used for fluorescence imaging of Cu<sup>2+</sup> ions in living cells. This probe can detect Cu<sup>2+</sup> with good linear relationships from 10 to 100 nM with  $r=0.99971$  then limit of detection was found to be 0.015 nM with  $\pm 0.91\%$  RSD at 10 nM concentrations.

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## 1. Introduction

The designing of new colorimetric and fluorescent chemosensors for environmentally and physiologically important analytes is becoming popular due to their simplicity, selectivity, and sensitivity [1]. Among various environmental and physiologically important analytes copper is most studied element due to its significant impact on environment as well as in physiology of the various organisms [2]. It is found to be highly toxic to some organisms such as many bacteria and viruses [3]. Due to its toxicity to bacteria, higher levels of copper hamper the self-purification capability of the sea or rivers and destroy the biological reprocessing systems in water. Copper is the third most abundant heavy transition essential element next to iron and zinc in the human body. It performs an important role in many fundamental physiological processes in the human body [4]. Alteration in the cellular homeostasis of this element results in some serious neurodegenerative disorders such as Alzheimer's disease and prion diseases, and other diseases such as Menkes, Wilson diseases and familial amyotrophic lateral sclerosis. In particular, exposure to a high level of copper even for a short period of time can cause gastrointestinal disturbance, and long-term exposure causes kidney damage [5]. In recent years this element has been suspected to cause infant liver damage due to an excessive intake

[6]. In order to understand the impact of this element on human health, facile techniques enabling to monitor the concentration of copper ions in biological samples and to visualize its sub cellular distribution in biological processes are of considerable significance. To visualizing the distribution of copper ion in living cells during physiological processes, fluorescence bioimaging found to be the best choice due to its high sensitivity and less cell damage [7–12]. Therefore, fluorescence probes are particularly attractive compared to other instrumental methods [13]. The development of highly sensitive, selective, and cell membrane permeable probes that exhibit a visible “turn-on” fluorescent emission in aqueous media is very much necessary for fluorescence imaging of metal ions in living cells. Till date a number of fluorescence probes has been reported for imaging various intracellular heavy metal ions such as Zn<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>+</sup>, Pb<sup>2+</sup>, Fe<sup>3+</sup>, Hg<sup>2+</sup> and Cu<sup>2+</sup> [14–20].

Xanthene derivatives possess very attractive spectroscopic properties such as large molar extinction coefficient, high fluorescence quantum yield, and visible light excitation as well as long wavelength emission [21–27]. These derivatives are colorless and non fluorescent, in spiro ring closed form whereas ring-opened forms are colored and have strong fluorescence emissions. So, xanthene framework seems to be an ideal model to construct “turn-on” fluorescent chemosensors. Rhodamine B hydrazide the fluorescent chemodosimeter for Cu<sup>2+</sup> was the first xanthene derivative utilized for metal ion detection [28]. This chemodosimeter showed an irreversible fluorescence enhancement toward Cu<sup>2+</sup> in water with high selectivity. Inspired by this successful work, increasing attention has focused on rhodamine spirolactam-based fluorescent

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chemosensors and chemodosimeters for various metal ions and other biologically important species in recent years [29]. A reversible fluorescence “turn-on” monoboronic acid-conjugated rhodamine based probe have proposed for  $\text{Cu}^{2+}$  with high selectivity, and they successfully applied it to image  $\text{Cu}^{2+}$  in living cells and organisms. However, it shows moderate sensitivity which might limit its application in detection of  $\text{Cu}^{2+}$  in aqueous samples with very low concentrations [30]. Then 2-picoyl functionalized coumarin based fluorogenic probe for imaging  $\text{Cu}^{2+}$  in living cells was reported but however, it showed a “turn-off” fluorescence signal with incubation of  $\text{Cu}^{2+}$  [31]. Therefore, the search for highly sensitive, selective, irreversible and “turn-on” fluorescent probes for monitoring  $\text{Cu}^{2+}$  in biological samples and in living cells is still very active. These poses a significant challenge for chemical research because these type of chemodosimeters has several advantages like specific ion-induced reactions, remarkable spectroscopic modulations and they are generally irreversible reactions resulting in a significant chemical transformation involving the breaking and formation of several covalent bonds. Compared with the other methods of sensing, this approach has the advantages of high selectivity as well as a cumulative effect related to the ion concentration. Especially while applying for monitoring heavy metal ions in cellular media the irreversible chemodosimeters shows higher quantum yields compared to reversible chemosensors because metal bound dyes has less quantum yields in cellular environment [32]. One of the chemodosimeter based on rhodamine derivative have been reported for imaging  $\text{Cu}^{2+}$  in living HeLa cells with high sensitivity and fast response time but this probe cannot distinctly recognize  $\text{Cu}^{2+}$  from  $\text{Hg}^{2+}$ , and thereby, is not suitable for monitoring the concentration of copper ions in complicated cellular environment [33]. Recently two fluorescent rhodamine derivatives were reported for  $\text{Cu}^{2+}$  ions but they possess comparatively short emission wavelengths [34–36]. The short emission wavelength probes are disadvantageous for cell imaging because the cells have to irradiate with high energy radiations which lead to the cell toxicity [37,38]. In order to overcome above said properties there is need to derivatise the rhodamine framework with suitable moiety which can distinctly recognize  $\text{Cu}^{2+}$  from  $\text{Hg}^{2+}$  and possess long absorption and emission wave lengths results in less cell toxicity the necessary condition in cell labeling studies. Herein, we are reporting the novel rhodamine based chemodosimeter rhodamine B phenyl hydrazide for  $\text{Cu}^{2+}$  ions which contains reactive phenyl hydrazide group [39]. Due to the presence of reactive phenyl hydrazide group the probe exhibits above said silent features like specific irreversible “turn-on” absorbance/fluorescence changes with  $\text{Cu}^{2+}$  ions in the pH range 1–6 and the absorbance/emission wave lengths (550/580 nm) are longer compared to some of the reported probes [34–36]. The probe also has higher quantum yields compare to the reported rhodamine probes [30–39]. Due to its less cell toxicity, water solubility and cell permeability it has been utilized for the fluorescence imaging of  $\text{Cu}^{2+}$  ions in cellular media and for determination of copper in biological various biological fluids.

## 2. Experimental

### 2.1. Apparatus

All Fluorescence studies were done using Shimadzu Spectrofluorimeter (model RF 5301PC). Absorbance studies were done using a Shimadzu Spectrophotometer (model UV-1800) with 1 cm quartz cuvettes.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded with a Bruker 400 MHz spectrometer. Mass spectral data was obtained using Thermo Finnigan Deca QXP Mass Spectrometer. All pH measurements were carried out using Control Dynamics digital

pH meter (model APX 175). Ceti-EpiFluorescent microscope has been used for fluorescence imaging studies.

### 2.2. Reagents and solutions

Rhodamine B phenyl hydrazide (10 mM) was prepared by dissolving 0.532 g in 1:10 acetonitrile/water (% v/v). Standard  $\text{Cu}^{2+}$  solution was prepared by dissolving  $\text{CuCl}_2$  (Merck, AR grade, Mumbai, India) in distilled water. Robinson buffer solutions of pH 1–12 were prepared by using the solutions of 0.02 M acetic acid, 0.02 M orthophosphoric acid, 0.02 M sodium tetra borate and 0.4 M NaOH. PBS (phosphate buffer saline) was purchased from the Sigma-Aldrich. All solvents used for synthesis and measurements were redistilled before use. All other chemicals were of analytical-reagent grade and were used without further purification. The blood and urine samples were collected from the diagnostic laboratory, Bangalore, Karnataka, India.

### 2.3. Cell culture

The Breast cancer cell line was provided by the national center for cell sciences, Pune, India.

Cells were grown in 5% fetal bovine serum (FBS) in an atmosphere of 5%  $\text{CO}_2$ , 95% air at 37 °C. Cells were plated on 18 mm glass cover slips and allowed to adhere for 24 h. Experiments to assess  $\text{Cu}^{2+}$  uptake were performed in the same media supplemented with 10 mM  $\text{CuCl}_2$  for 2 h.

### 2.4. Fluorescence imaging

The 48 h grown culture was incubated with 10  $\mu\text{M}$  RBPH for 2 h. Excitation of RBPH loaded cells at 561 nm was carried out. Emission was collected in the range 550–670 nm. Before the experiments, cells were washed with distilled water and washed five times with phosphate buffer saline (PBS) in order to remove the unabsorbed probe to overcome the background fluorescence followed by incubated with 100 mM of  $\text{Cu}^{2+}$  ions for 2 h at 25 °C. Cell imaging was then carried out after washing the cells.

### 2.5. Sample preparation

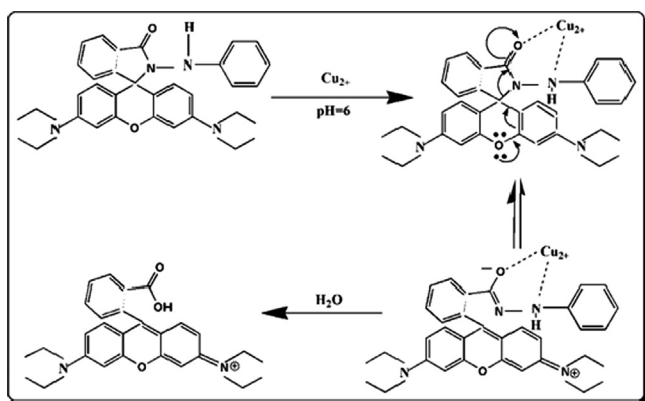
The preserved human blood (1 mL) and urine (1 mL) samples were transferred into each 25 mL beakers. Then samples were ashed in a Muffle furnace at 500 °C for a 4 h in the presence of 1 mL concentrated nitric acid [40]. Then, the content of each beaker were cooled at room temperature, 1.5 mL of concentrated hydrochloric acid was added to each beaker and warmed slightly. The content of each beaker was filtered and neutralized with dilute ammonia in the presence of 2 mL of 0.01% (w/v) tartrate solution, transferred quantitatively into a 10 mL calibrated flask and made up to the mark with distilled water. A suitable aliquot of the above solution was taken and copper content was determined as described under the procedure section.

### 2.6. Recommended procedure

In a set of 10 mL calibrated standard flasks  $\text{Cu}^{2+}$  solutions in the concentrations range 10–100 nM were taken. To this 1 mL of 10 mM RBPH and 1 mL of Robinson buffer solution of pH 6 were added and leave the solutions for 2 min. Then made up to the mark with distilled water and measure the fluorescence intensity of the solutions ( $\lambda_{\text{ex/em}}=561/580$  nm).

### 3. Results and discussion

The RBPH is colorless and non-fluorescent in acetonitrile–water system (1:10 v/v) due to its spiro-closed structure. But upon the addition of  $\text{Cu}^{2+}$  to RBPH in the pH range 1–6 the solution turns pink and becomes highly fluorescent due to the transformation of spiro-closed form to open form [21–27]. The above transformation was achieved by coordination of  $\text{Cu}^{2+}$  to the 'O' atom of the carbonyl group and 'N' atom of the imido group of phenyl hydrazide moiety of the probe leads to the formation of the complex and subsequent hydrolysis of this complex leads to the formation of delocalized xanthenic conformation of the rhodamine moiety [21,22]. The spirolactam ring opening of the RBPH by  $\text{Cu}^{2+}$  was confirmed by various spectroscopic techniques like mass and NMR spectroscopy. The mass spectrum of the RBPH shows  $m/z$   $[\text{M}+\text{H}]^+ = 533$  whereas upon treating RBPH with  $\text{Cu}^{2+}$  and resulting compound shows  $m/z$   $[\text{M}+\text{H}]^+ = 443.47$  corresponds to the rhodamine B compound (Fig. S1 supporting information) which confirms the formation of rhodamine B through rhodamine– $\text{Cu}^{2+}$  complex formation and subsequent hydrolysis. The proton NMR spectra of RBPH contains the  $\text{NHC}_6\text{H}_5$  signal at 5.9 ppm whereas the product obtained in the reaction lacks this peak instead its proton NMR spectra well matches with that of proton NMR spectra (not shown) of authentic rhodamine B (Fig. S2 supporting information). This confirms that rhodamine tautomer of spiro-open structure was obtained in the above reaction. Based on the above spectroscopic results the possible mechanism of spirolactam ring opening of RBPH by  $\text{Cu}^{2+}$  was deduced (Scheme 1). The RBPH has very less absorption in the visible region but upon the addition of  $\text{Cu}^{2+}$  RBPH showed a new maximum absorption band centered at 561 nm. No other metal ions as well as nonmetal ions induce this change with the probe (Fig. 1). In order to know the stoichiometric ratio of RBPH to  $\text{Cu}^{2+}$  the absorbance values were plotted against the  $[\text{Cu}^{2+}]/[\text{Cu}^{2+}] + [\text{RBPH}]$  (Fig. 2). This study has revealed that the stoichiometry between  $\text{Cu}^{2+}$  and RBPH has been found to be 1:1. Similarly the RBPH has very less excitation and emission intensities centered at  $\lambda_{\text{ex/em}} = 561/580$  nm whereas addition of  $\text{Cu}^{2+}$  results in drastic increase in excitation and emission wavelengths and the increase in emission intensity is found to be linear with  $\text{Cu}^{2+}$  concentration (10–100 nM) (Fig. 3). To check whether the fluorescence or colourimetric signaling of RBPH towards  $\text{Cu}^{2+}$  is reversible or irreversible we added 1 M EDTA to the solution containing RBPH and  $\text{Cu}^{2+}$ . The fluorescence intensity as well as the absorbance of the solution was not altered indicating the irreversible interaction of the probe and the  $\text{Cu}^{2+}$  ions. So the interaction of RBPH with  $\text{Cu}^{2+}$  is irreversible i.e., cation catalyzed ring opening induces to the ring opening rather than metal ion coordination.



Scheme 1. Mechanistic pathway of spirolactam ring opening process.

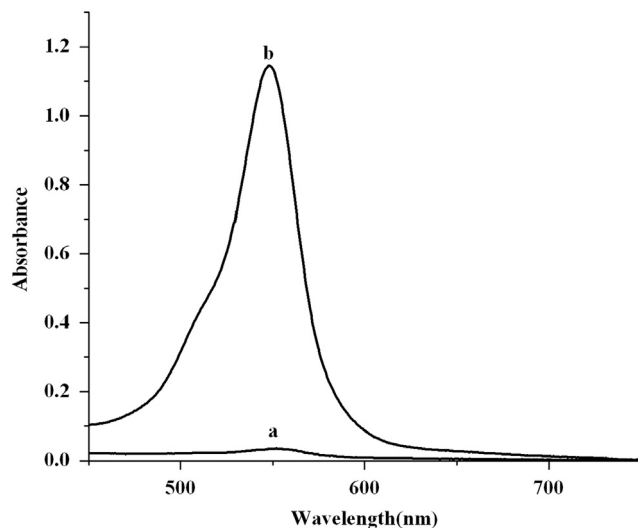


Fig. 1. Absorption spectra of 1 mL of 10 mM RBPH in 1:10 acetonitrile water (%V/V) + 1 mL Robinson buffer solution of pH 6 (a) reagent blank and (b) in the presence of 500 nM of  $\text{Cu}^{2+}$  ions.

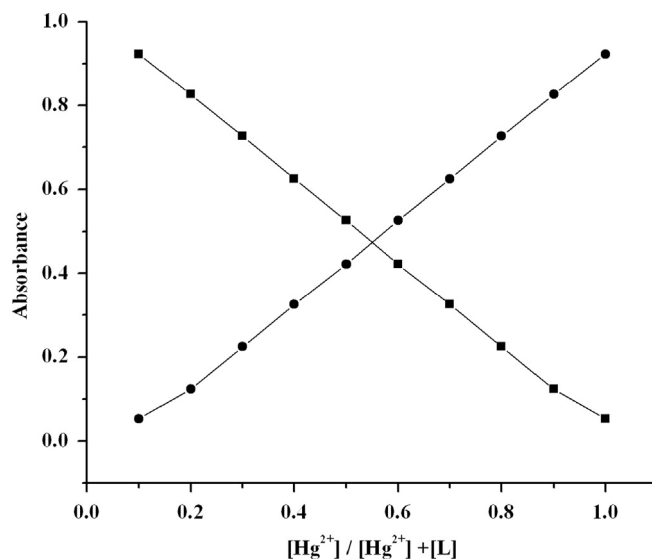


Fig. 2. Jobs plot of  $[\text{Cu}^{2+}]/([\text{Cu}^{2+}] + \text{RBPH})$  (10 mL of 10 mM) in 1:10 acetonitrile water (%v/v),  $\text{Cu}^{2+} = 50$  nM.

#### 3.1. Optimization studies

The various reaction parameters that affecting the fluorescence emission measurements like pH, concentration of the probe, time and temperature has been studied.

##### 3.1.1. Effect of pH

The spectroscopic characters of the probe were studied in the pH range 1–12 in Robinson buffer solution. In 10 mL standard flasks 1 mL of 10 mM RBPH in 1:10 ethanol water (%v/v), 1 mL of Robinson buffer solution of pH 1–12 and 20 nM of  $\text{Cu}^{2+}$  were added and leave the solutions for 2 min. Then measure fluorescence emission intensities at  $\lambda_{\text{ex/em}} = 561/580$  nm. The probe possesses very low fluorescence emission in the pH range 1–12 but upon the addition of  $\text{Cu}^{2+}$  ions the fluorescence emission ( $\lambda_{\text{ex/em}} = 561/580$  nm) drastically increases with increase in pH up to 6 and then it decreases after 7. This is because the probe exists in structure I (Scheme 2) in the pH range 1–6 and in the pH range 8–12 it exhibits structure II (Scheme 2) due to the deprotonation of the imido nitrogen.

The  $\text{Cu}^{2+}$  ions does not forms complex with second structure because it possess tertiary amine where as divalent copper ions has less ligating affinity with them. The order of ligating affinity with of  $\text{Cu}^{2+}$  ions with amines ( $-\text{NH}_2$ ,  $-\text{NHR}$ ,  $-\text{NR}_2$ ) [41]. Then structure II (Scheme 2) holds a rigid double bond of  $\text{C}=\text{N}$ , which inhibits the binding of divalent copper ions with the oxygen atom of carbonyl group. Different from structure II the structure I possess a flexible single bond of  $\text{C}-\text{N}$ , which increases the flexibility of the whole molecule and is favorable for the oxygen atom of carbonyl nitrogen atom of amido group to coordinate with  $\text{Cu}^{2+}$  ions [35]. So the probe exhibits absorbance/fluorescence changes in the pH range 1–6. Again the emission values were found to be maximum at pH 6. So the pH 6 is considered as optimum reaction pH where maximum fluorescence emission can be obtained (Fig. 4).

### 3.1.2. Effect of probe concentration

The optimum concentration of the probe required for maximum fluorescence emission was studied by keeping all other parameters constant. In 10 mL standard flasks different volumes of 10 mM RBPH in 1:10 ethanol water (%v/v), 1 mL of Robinson buffer solution of pH 6 and 20 nM of  $\text{Cu}^{2+}$  solution were added and left the solutions for 2 min. Then measure fluorescence emission intensities at  $\lambda_{\text{ex/em}}=561/580$  nm. The fluorescence emission increases with increase in concentration of the probe and remains constant after 10 mM. So this concentration was achieved by adding 1 mL of 10 mM concentration RBPH solution (Fig. 5).

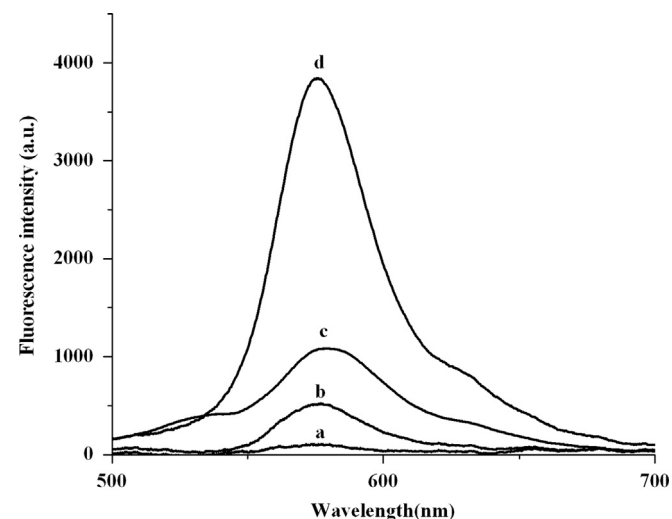
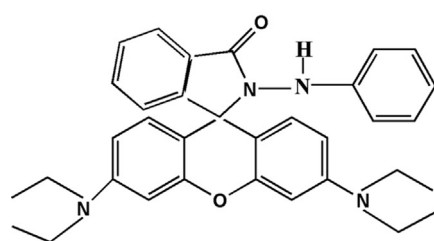
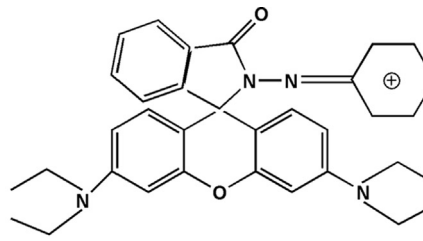


Fig. 3. Fluorescence emission spectra of 1 mL of 10 mM RBPH in 1:10 acetonitrile water (% V/V)+1 mL. Robinson buffer solution of pH 6 (a) reagent blank (b) in the presence of 10 nM  $\text{Cu}^{2+}$  ions (c) in the presence of 60 nM of  $\text{Cu}^{2+}$  ions and (d) in the presence of 200 nM of  $\text{Cu}^{2+}$  ions.



Structure I

pH 1-6



Structure II

pH 8-12

Scheme 2. Different structures of the RBPH in different pH conditions.

### 3.1.3. Rate of reaction

The reaction between RBPH and  $\text{Cu}^{2+}$  ions was found to be instantaneous due to the presence of active phenyl hydrazide group but for time taken for complete reaction was studied by

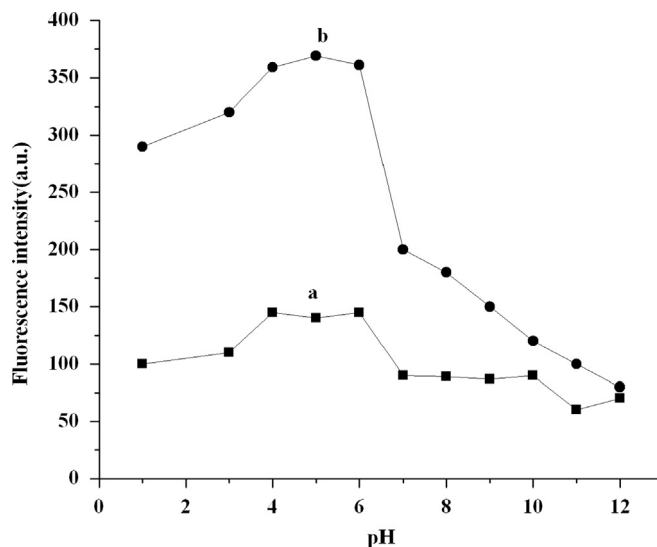


Fig. 4. Effect of pH 1 mL of 10 mM RBPH in 1:10 acetonitrile water (% V/V)+1 mL Robinson buffer solution of pH 6 in the presence of 30 nM of  $\text{Cu}^{2+}$  ions as well as reagent blank.

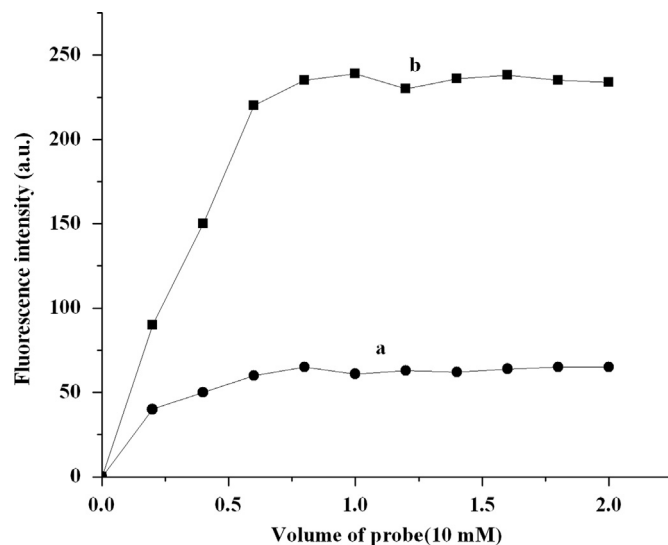


Fig. 5. Effect of probe concentration. 1 mL of 10 mM RBPH in 1:10 acetonitrile water (% V/V)+1 mL. Robinson buffer solution of pH 6 in the presence of 30 nM of  $\text{Cu}^{2+}$  ions as well as reagent blank.

keeping all other reaction parameters constant. In a 10 mL standard flask 1 mL of 10 mM RBPH, 1 mL of Robinson buffer of pH 6 was added. To this 20 nM of  $\text{Hg}^{2+}$  solution was added and the fluorescence emission ( $\lambda_{\text{ex/em}}=561/580$  nm) values were measured and plotted as function of time. As the time increases the fluorescence emission intensity increases and it remains constant after 2 min, but the fluorescence intensity of the probe in buffer solution does not changes after 1 h also (Fig. 6).

### 3.1.4. Interference study

The absorption properties of RBPH in the presence of various competitive anions and metal ions which are commonly present in water samples such as  $\text{Cl}^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{S}^{2-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{ClO}^-$  and also metal ions like  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Hg}^{2+}$  were studied (Fig. 7). It revealed that no other anions as metal ions leads to significant spectral changes in the studied pH range (1–7) and even  $\text{Hg}^{2+}$  kept silence in this pH range. Thus, RBPH can function as a highly selective chemosensor for  $\text{Cu}^{2+}$ . As shown in

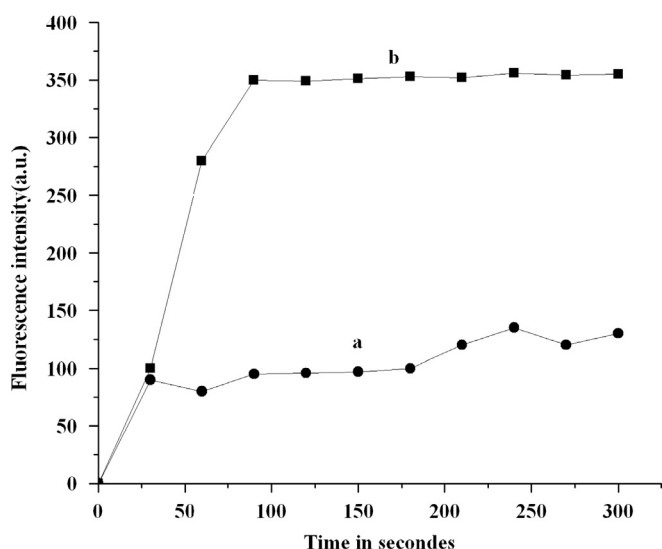


Fig. 6. Effect of time. 1 mL of 10 mM RBPH in 1:10 acetonitrile water (% V/V)+1 mL Robinson buffer solution of pH 6 in the presence of 30 nM of  $\text{Cu}^{2+}$  ions as well as reagent blank.

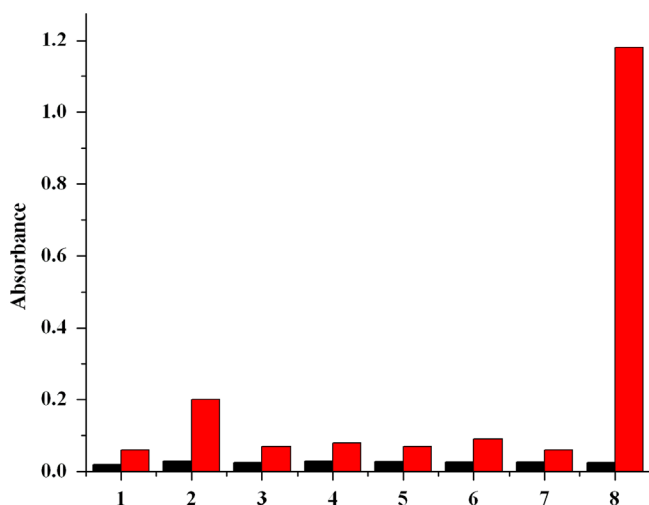


Fig. 7. Absorbance values of 1 mL of 10 mM RBPH in 1:10 acetonitrile water (% V/V)+1 mL Robinson buffer solution of pH 6. (1) Reagent blank+100 nM  $\text{Pb}^{2+}$ , (2) reagent blank+100 nM  $\text{Hg}^{2+}$ , (3) reagent blank+100 nM  $\text{Mg}^{2+}$ , (4) reagent blank+100 nM  $\text{Ni}^{2+}$ , (5) reagent blank+100 nM  $\text{Ca}^{2+}$ , (6) reagent blank+100 nM  $\text{Ni}^{2+}$ , (7) reagent blank+100 nM  $\text{Cd}^{2+}$  and (8) reagent blank+100 nM  $\text{Cu}^{2+}$  ions.

Figs. 8 and 9, the addition of 10 nM of the  $\text{Cu}^{2+}$  resulted in a significant enhancement of the emission intensity at 580 nm in the pH range 1–6. However, the addition of anions as well as cations as mentioned above brings no obvious effect on the fluorescence emission. It is noted that the addition of higher concentration of  $\text{Hg}^{2+}$  (100 ng) produced a enhancement of the emission intensity as well as absorbance values in the pH range 8–12 but not in the pH range 1–7 as that of  $\text{Cu}^{2+}$ . The absorbance/fluorescence changes of the probe with  $\text{Hg}^{2+}$  ions in the pH range 8–12 is due to the transformation of the probe from structure I–II (Scheme 2). The slight absorbance/fluorescence changes of the probe with  $\text{Hg}^{2+}$  ions can be explained based on the effect of “copper triangle” of the periodic table [41].

### 3.1.5. Analytical features of the method

The analytical features of the proposed protocol have been summarized in Table 1.

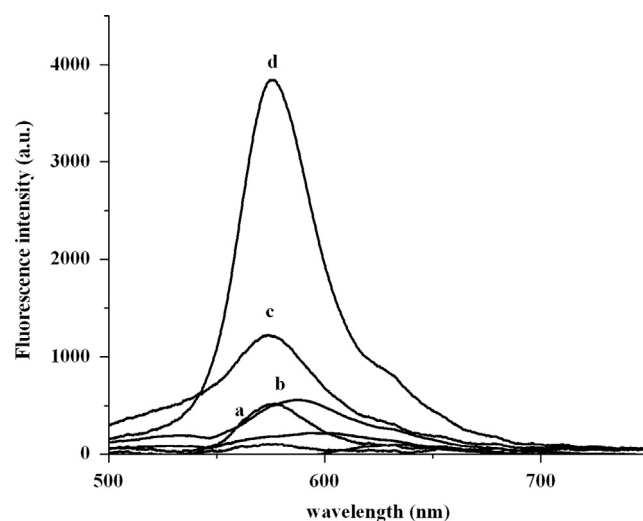


Fig. 8. Fluorescence emission spectra of 1 mL of 10 mM RBPH in 1:10 acetonitrile water (% V/V)+1 mL Robinson buffer solution of pH 6 (a) reagent blank, (b) in the presence of 200 nM of other ions, (c) in the presence of 200 nM of  $\text{Hg}^{2+}$  ions and (d) in the presence of 200 nM of  $\text{Cu}^{2+}$  ions.

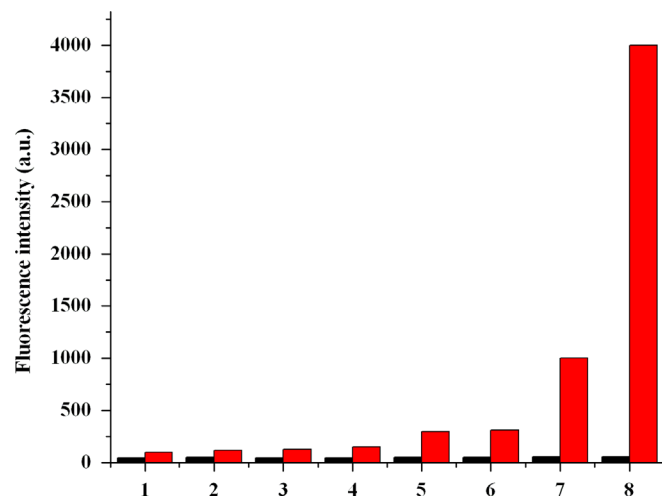


Fig. 9. Fluorescence emission intensities of 1 mL of 10 mM RBPH in 1:10 acetonitrile water (% V/V)+1 mL Robinson buffer solution of pH 6. (1) Reagent blank+100 nM  $\text{Pb}^{2+}$ , (2) reagent blank+100 nM  $\text{Cd}^{2+}$ , (3) reagent blank+100 nM  $\text{Mg}^{2+}$ , (4) reagent blank+100 nM  $\text{Ni}^{2+}$ , (5) reagent blank+100 nM  $\text{Ca}^{2+}$ , (6) reagent blank+100 nM  $\text{Cr}^{6+}$ , (7) reagent blank+100 nM  $\text{Hg}^{2+}$  and (8) reagent blank+100 nM  $\text{Cu}^{2+}$  ions.



### 3.2. Application study

#### 3.2.1. Determination of blood in biological samples

The developed method was applied for the determination of copper (by oxidizing the samples which converts Cu or Cu<sup>+</sup> to Cu<sup>2+</sup>) present in blood serum and urine samples of the healthy and Wilsons disease affected males. The results confirmed that the drastic amounts of copper present in the patients affected with the

disease when compared to the healthy individuals. In order to ascertain the recovery of the method the known amounts of Cu<sup>2+</sup> ions were spiked into the serum samples and analyzed and it was found to be more than 99%. The results are presented in Tables 2 and 3.

**Table 1**

Analytical merits of the method.

Analytical feature	nM mL <sup>-1</sup>
Linear range	10–100
Limit of detection <sup>a</sup>	1.019
Limit of quantification <sup>b</sup>	12.19
Fluorescence quantum yield (Φ <sub>F</sub> )	0.61
RSD (n=10) Hg <sup>2+</sup> = 10 nM	± 0.99971

<sup>a</sup> Calculated based on standard deviation values of 5 reagent blank determinations (3σ).

<sup>b</sup> Calculated based on standard deviation values of 5 reagent blank determinations (10σ).

#### 3.2.2. Fluorescence imaging of Cu<sup>2+</sup> ions

From the fluorescence microscopic studies, we are proposing that RBPH can permeate into cells and it is suited for fluorescence imaging of Cu<sup>2+</sup> ions in living cells because of its favorable amphiphilic nature and fluorescence properties exhibiting in the physiological pH conditions. Cultured MCF-7 cells were incubated with 1 mM RBPH for 30 min at room temperature and subjected to fluorescence measurement but these cells fail to show fluorescence property (Fig. 10B). After the cells were incubated with 100 nM CuCl<sub>2</sub> for another 30 min, a brighter fluorescence was observed (Fig. 10C). A bright field microscopic image (Fig. 10A) shows that the cells were viable throughout the imaging experiments. These results demonstrate that RBPH is cell permeable and capable of sensing Cu (II) in living cells.

**Table 2**

Detection of Cu<sup>2+</sup> in blood serum samples.

Sample no	Cu <sup>2+</sup> (nM mL <sup>-1</sup> )		Cu <sup>2+</sup> (nM mL <sup>-1</sup> )		Added Cu <sup>2+</sup> (nM mL <sup>-1</sup> ) (C)	Total Cu <sup>2+</sup> (nM mL <sup>-1</sup> )		% Recovery	
	(A)		(B)						
	Present method	AAS method	Present method	AAS method		Present method	AAS method	Present method	AAS method
1.	101.12 ± 1.08	102.13 ± 1.02	421.21 ± 2.03	422.09 ± 3.10	15.0	436.09 ± 3.01	436.89 ± 1.01	99.84	99.95
2.	104.21 ± 2.02	105.39 ± 1.01	521.24 ± 3.01	520.32 ± 2.01	10.0	529.13 ± 2.9	530.12 ± 2.01	99.62	99.96
3.	108.99 ± 3.07	109.21 ± 2.94	225.19 ± 2.3	225.15 ± 1.02	30.0	255.90 ± 3.9	255.13 ± 3.1	99.72	99.99
4.	101.22 ± 2.02	101.03 ± 1.91	330.09 ± 1.2	332.01 ± 2.09	10.0	341.10 ± 2.79	341.10 ± 1.3	100.27	99.70
5.	118.29 ± 2.05	118.12 ± 1.20	359.08 ± 2.03	358.99 ± 3.02	15.0	372.81 ± 2.1	373.84 ± 2.1	99.66	99.95
6.	122.13 ± 2.09	121.03 ± 1.20	289.10 ± 3.01	287.23 ± 2.07	25.0	312.11 ± 3.1	312.11 ± 2.8	99.36	99.96

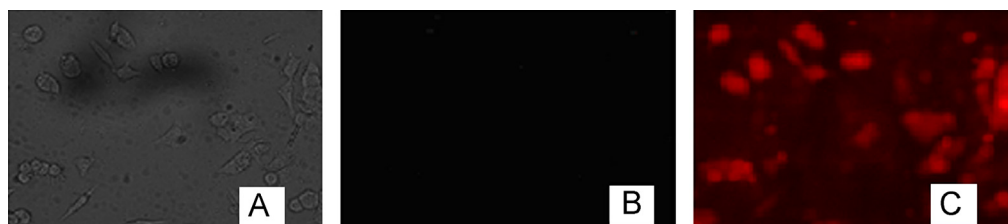
(A) Healthy males blood, (B) Wilsons disease Patients blood and (C) Spiked to Wilsons disease Patients blood.

**Table 3**

Detection of Cu<sup>2+</sup> in urine samples.

Sample no	Cu <sup>2+</sup> (nM mL <sup>-1</sup> )		Cu <sup>2+</sup> (nM mL <sup>-1</sup> )		Added Cu <sup>2+</sup> (nM mL <sup>-1</sup> ) (C)	Total Cu <sup>2+</sup> (nM mL <sup>-1</sup> )		% Recovery	
	(A)		(B)						
	Present method	AAS method	Present method	AAS method		Present method	AAS method	Present method	AAS method
1.	37.02 ± 0.82	36.13 ± 0.02	120.21 ± 2.09	121.02 ± 3.10	25.0	144.99 ± 3.01	146.89 ± 1.71	99.84	100.59
2.	34.21 ± 0.92	34.19 ± 0.57	121.24 ± 3.02	120.30 ± 2.01	30.0	149.15 ± 2.9	150.99 ± 2.91	98.61	100.45
3.	38.09 ± 0.72	38.91 ± 0.94	151.19 ± 2.6	152.12 ± 1.02	15.0	165.19 ± 3.9	166.18 ± 3.6	99.39	99.43
4.	36.22 ± 0.92	35.93 ± 0.91	130.07 ± 1.2	130.09 ± 2.09	20.0	151.10 ± 2.79	149.15 ± 1.3	100.68	99.37
5.	36.89 ± 0.59	32.12 ± 1.20	159.88 ± 2.03	158.95 ± 3.02	10.0	168.89 ± 2.1	169.89 ± 2.5	99.41	99.96
6.	37.05 ± 0.99	121.03 ± 1.20	169.19 ± 3.01	168.99 ± 2.09	25.5	189.12 ± 4.1	192.16 ± 2.1	97.13	98.80

(A) Healthy male's urine, (B) Wilsons disease Patients blood and (C) Spiked to Wilson's disease Patients blood.



**Fig. 10.** Fluorescence imaging results of (A) MCF-7 cells in the bright field (B) MCF-7 cells after incubated with RBPH for 30 min (100 mM) and (C) MCF-7 cells after incubated with RBPH for 30 min (100 mM) after with 100 nM CuCl<sub>2</sub> for 30 min.

#### 4. Conclusions

In conclusion, we are proposing new rhodamine-based chemosensor that can sense  $\text{Cu}^{2+}$  fluorometrically through spiro lactam ring opening strategy. Selective binding of the compound to  $\text{Cu}^{2+}$  and spiro lactam ring opening results in the immediate release of rhodamine B and remarkable fluorescence enhancement at 580 nm emission wavelengths, which proved that RBPH could serve as a sensitive and selective chemosensor of  $\text{Cu}^{2+}$ . The probe has been utilized for detection of copper in various biological fluids and also for fluorescence imaging of  $\text{Cu}^{2+}$  ions in cellular media.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jlum.2013.09.010>.

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