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Synthesis of a highly selective bis-rhodamine chemosensor for naked-eye detection of Cu^{2+} ions and its application in bio-imaging

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ABSTRACT

A bis-rhodamine based fluorescent chemosensor for naked-eye detection of Cu^{2+} with enhanced sensitivity as compared to mono-rhodamine derivative has been synthesized, and its selectivity for Cu^{2+} in the presence of other competitive metal ions (Li^{+}, Na^{+}, K^{+}, Cs^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+}), and application in bio-imaging are demonstrated.

1. Introduction

Development of new, selective, and target ion specific chemosensors that monitor toxic heavy metal ions in biological tissues is an important research area. Copper is one of the important elements in humans, and is present at low levels in a variety of cells and tissues with the highest concentration in the liver [1]. Excess concentration of Cu^{2+} in the neuronal cytoplasm can cause Alzheimer's [2,3] or Parkinson's disease [4]. Cu^{2+} is utilized in several physiological responses and copper containing proteins are useful as redox catalysts in biological processes such as electron transfer or oxidation of various organic substrates [5,6]. Chronic copper overload or exposure to excess copper by accidents and environmental contamination can lead to oxidative damage [7]. Therefore, selective and rapid detection of Cu^{2+} in physiological samples is of toxicological and environmental concern [8–12]. Consequently, synthesis of selective colorimetric probes for naked-eye Cu^{2+} detection assumes importance [13–17]. Significant effort has been focused towards the generation of selective fluorescent probes for transition metal ions [18,19]. Several ON-OFF fluorescent probes for Cu^{2+} have also been reported [20–25] to monitor Cu^{2+} which is known as a fluorescence quencher because of its paramagnetic nature [26].

The rhodamine fluorochrome has attracted considerable interest from chemists because of its excellent photophysical properties [27]. Rhodamine derivatives with closed spirolactam ring are nonfluorescent and colorless, but under strongly acidic conditions, spirolactam ring opening results in intense fluorescence emission and development of pink color [28]. Rhodamine based chemosensors reported in the literature for detection of Cu^{2+} contain at least one potential coordination site for the metal ion binding [29–32]. But, some of them suffer from cross sensitivity towards other metal ions, longer response times, tricky procedures for synthesis and poor sensitivity [25,33–35]. A bis-rhodamine derivative with an appropriate ligand on spirolactam ring capable of binding a single metal ion is expected to display enhanced absorbance as well as fluorescence properties required for trace metal analysis. Herein, we report a simple and efficient procedure for the synthesis of a new bis-rhodamine based chemosensor (**3** in Scheme 1) in which rhodamine itself provides the coordination site. With two Schiff base units, **3** can chelate through its carbonyl O, and imino N atoms with a single metal ion. The spirolactam moiety of the rhodamine group acts as a signal switch, which is expected to turn "ON" when the cation binds.

2. Experimental section

2.1. General

Dry acetonitrile and double distilled water were used throughout the experiment. All the materials for synthesis were

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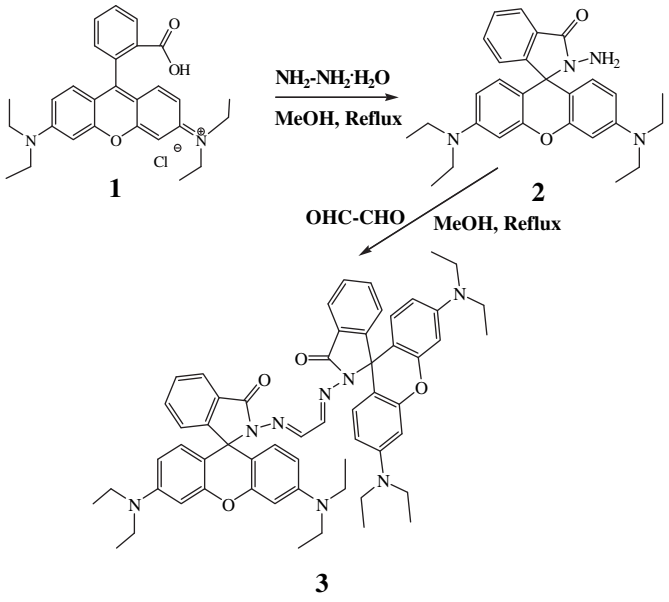

Scheme 1. Synthesis of bis-rhodamine chemosensor 3.

purchased from commercial suppliers and used without further purification. The solutions of metal ions were prepared from the corresponding chloride salts.

Absorption spectra were recorded on a CARY BIO 50 UV–VIS spectrophotometer. Fluorescence measurements were performed on a Perkin Elmer LC 45 Luminescence spectrometer. All pH measurements were made with a Systronics μpH System Model 361. NMR spectra were recorded using a JOEL –ECP500 MHz spectrometer operated at 500 MHz. ESI MS spectra were obtained on a HP 1100 LC-MS Analyzer without using the LC part. Fluorescence imaging experiments were performed using Olympus CK 40 Fluorescence Microscope. All measurements were carried out at room temperature (~298 K).

*2.1.1. Synthesis of rhodamine hydrazide (**2**)*

Rrhodamine hydrazide was synthesized following the reported procedure [36]. To rhodamine B hydrochloride (0.96 g, 2 mmol) dissolved in 15 mL methanol, excess amount of hydrazine hydrate (1 mL, 6.98 mmol) was added and the reaction mixture was refluxed till the pink color disappeared (~3–4 h). After that, the reaction mixture was cooled to room temperature, poured into distilled water and extracted with ethyl acetate (6 × 25 mL). The combined extract was washed with brine, dried with anhydrous sodium sulfate, filtered, and then concentrated under reduced pressure to yield 0.62 g (68%) of rhodamine hydrazide.

^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.16 (t, J = 7.5 Hz,12H, $NCH_2\underline{CH_3}$), 3.32 (q, J = 6.8 Hz, 8H, $N\underline{CH_2}CH_3$), 3.63 (bs, 2H, NH_2), 6.28 (dd, J_1 = 2.3 Hz, J_2 = 2.3 Hz, 2H,Xanthene-H,), 6.43(d, J = 2.3 Hz, 2H, Xanthene-H), 6.45 (d, J = 9.2 Hz, 2H, Xanthene-H,), 7.10 (m, 1H, Ar-H), 7.44 (t, J = 3.5 Hz, 2H, Ar-H), 7.93 (m, 1H, Ar-H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 12.7, 44.5, 66.0, 98.0, 104.5, 108.1,123.1, 123.9, 128.1, 128.2, 130.1, 132.6, 148.9, 151.6,153.9, 166.3.

*2.1.2. Synthesis of bis-rhodamine dye (**3**)*

Rhodamine hydrazide (**2**, 0.46 g, 1 mmol) was dissolved in 20 mL methanol, and glyoxal (0.57 μL, 0.5 mmol) was added dropwise. Addition of glyoxal gave red colour immediately. The mixture was refluxed in an oil-bath for ~3 h and then cooled to room temperature. The yellow colour precipitate obtained was filtered and washed 3 times with 10 mL cold methanol. After drying under reduced pressure, the reaction afforded 0.350 g (75%) **3** as yellow solid.

^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.15 (t, J = 7.0 Hz, 24H, $NCH_2\underline{CH_3}$), 3.32 (q, J = 6.8 Hz, 16H, $N\underline{CH_2}CH_3$), 6.15 (d, J = 7.5 Hz, 4H, Xanthene-H), 6.40 (m, 8H, Xanthene-H), 7.01 (d, J = 7.6 Hz, 2H, imine -H), 7.35 (m, 4H, Ar-H), 7.86 (d, J = 7.0 Hz, 2H, Ar-H), 7.93 (s, 2H, Ar-H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 12.7, 12.8, 44.4, 66.2, 98.0, 98.9, 105.5, 107.9, 108.1,123.4, 123.6, 127.0, 128.1, 133.9, 146.9, 149.0, 152.7, 153.0, 165.9.

ESI MS: calcd for $C_{58}H_{62}N_8O_4$ m/z (M^+) 934.5, found $(M+H)^+$ 935.9.

2.2. Preparation of solutions for absorption and fluorescence measurements

A stock solution of **3** was prepared by dissolving the required amount of **3** (9.35 mg, 1.0 mmol) in 1:1 acetonitrile/water and making up to the mark in a 10 mL volumetric flask. Further dilutions were made to prepare 100 μM solution by diluting appropriately the stock solution. To 1.0 mL of this solution in a 10 mL volumetric flasks was added 9.0 mL 1:1 acetonitrile/water containing different concentrations of metal ions, so as to get an overall dye concentration of 10 μM for the experiments. The contents of the volumetric flasks were shaken for 2 min. and incubated at room temperature for 1 h. Absorption and fluorescence measurements were made using a 3.0 mL cuvette.

3. Results and discussion

High sensitivity and selectivity for the analyte, and suitability of the sensor for bio-imaging applications are crucial in designing modern chemosensors. The bis-rhodamine sensor **3** is colourless in organic media indicating the predominant existance of spirolactam form of **3**. The characteristic peak at ~66 ppm in the ^{13}C NMR spectrum of **3** and disappearance of the peak at ~3.7 ppm in ^{1}H NMR spectrum of **2** revealed the formation of **3** (supporting data Figs. 3 and 4). The vicinal coupling constant (7.0 Hz) observed from the two enamine protons indicates the cis-configuration of enamine moiety. Further evidence to confirm the formation of **3** was obtained from mass analysis (supporting data, Fig. 5). The bis-rhodamine derivative displayed high selectivity and good sensitivity to Cu^{2+} even in the presence of other commonly coexistent heavy metal ions. Also, it was stable under a broad range of pH conditions, and suitable for bio-imaging applications.

For rapid detection, an ideal chemosensor should act instantaneously in response to the metal ion. Interestingly, when micromolar quantities of Cu^{2+} were added to a 20 μM solution of **3** in 1:1 acetonitrile/water an intense purple color developed within a few seconds, which can be ascribed to the Cu^{2+}-induced opening of the spirolactam ring [28]. It is imperative to mention here that even ten fold higher concentrations of other metal ions of interest failed to show any significant interference as shown in Fig. 1. These results

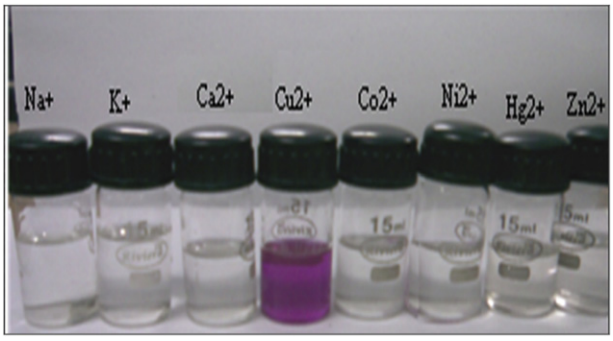

Fig. 1. Effect of addition of various metal ions (50 μM) to 20 μM solution of **3** in 1:1 acetonitrile/water.

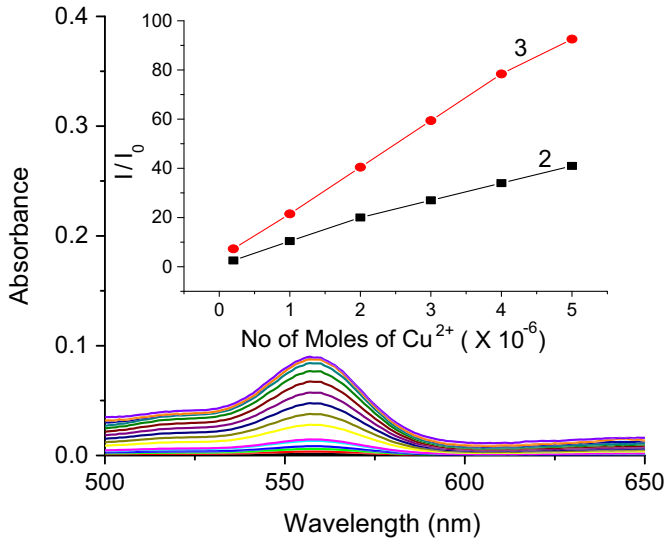

Fig. 2. Absorption spectra of 2 (10 μM) in 1:1 acetonitrile/water and in the presence of various concentrations of Cu^{2+} (0.1 μM–20 μM). Inset: Comparison of absorption intensities of 2 and 3 in the presence of serial concentrations of Cu^{2+}.

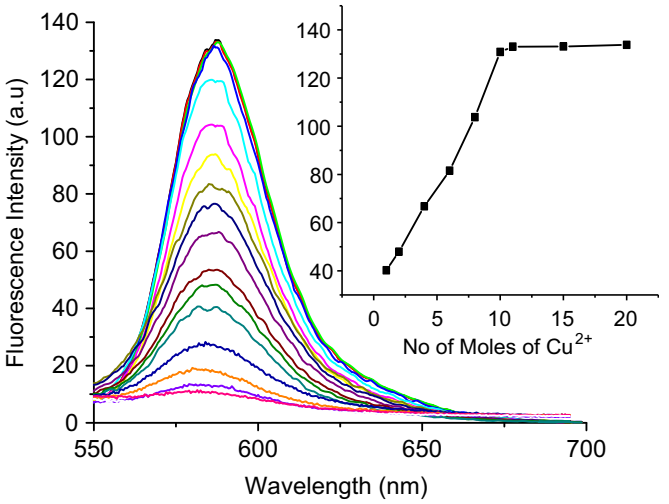

Fig. 4. Fluorescence spectra of 3 (10 μM) in 1:1 acetonitrile/water and in the presence of various concentrations of Cu^{2+} (0.1 μM–20 μM). Inset: Plot of fluorescence emission intensities at 586 nm vs. number of moles of Cu^{2+} added.

suggest that **3** could serve as a potential naked-eye chemosensor selective for Cu^{2+}.

The sensitivities of rhodamine derivatives **2** [37] and **3** to Cu^{2+} ions in aqueous medium are presented in Figs. 2 and 3. At pH 7.2, both the chemosensors **2** and **3** (10 μM) exhibited negligible level of absorbance above 500 nm, however, the absorption increased proportionately upon addition of micromolar quantities of Cu^{2+} ions as evidenced from the new absorption bands at ~556 nm (Fig. 2). It is clear from Fig. 2 (inset) that compound **3** is about one fold more sensitive than compound **2**. Similarly, an incremental increase in the fluorescence emission intensity of **3** was also observed at ~585 nm upon addition of Cu^{2+} ions (Fig. 4). Absorbance as well as fluorescence emission intensities increased as the concentration of Cu^{2+} ions increased and reached the maximum at 10 μM Cu^{2+} ions (see inset to Figs. 3 and 4), suggesting the formation of a 1:1 complex. The stoichiometry of the complex formed was further verified by Job's plot (Fig. 5).

In order to establish the mode of complex formation, ^{13}C-NMR spectra of **3** were recorded using 50 mmoles of **3** and with increasing amounts of Cu^{2+} ions (supporting data, Fig. 8). The drastic reduction in the intensities of signals at 170 ppm and 68 ppm in the presence of 10 mmoles of Cu^{2+} ions clearly ascertained the formation of the complex due to the coordination of Cu^{2+} with carbonyl oxygen resulting in spirolactam ring opening. The ^{13}C spectrum of **3** in the presence of 50 mmoles of Cu^{2+} ions was comparable to the ^{13}C-NMR spectrum of the 1:1 complex prepared separately (supporting data, Figs. 8 and 9). Moreover, the $g_{\parallel}$ (~2.245) and $g_{\perp}$ (~2.073) values deduced from the anisotropic ESR spectrum of the isolated complex suggested a distorted tetragonal geometry for the complex (supporting data, Fig. 10). This proposition was further corroborated by molecular simulation studies (supporting data, Fig. 11) using Gaussian 03 structure programme.

Selectivity is an important parameter to evaluate the performance of a chemosensor. To evaluate the selectivity of **3** we observed the variations in the absorbance and fluorescence

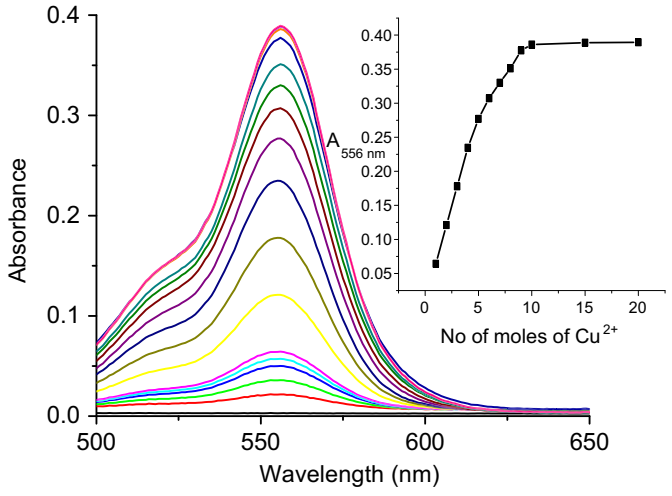

Fig. 3. Absorption spectra of 3 (10 μM) in 1:1 acetonitrile/water and in the presence of various concentrations of Cu^{2+} (0.1 μM–20 μM). Inset: Plot of absorption intensities (A_{556nm}) vs. number of moles of Cu^{2+} added.

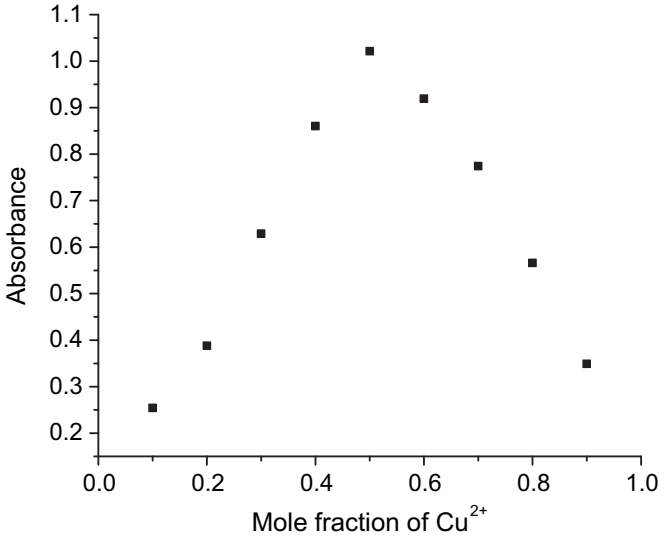

Fig. 5. Job's plot obtained from sensor 3 and Cu^{2+} indicates 1:1 binding mode.

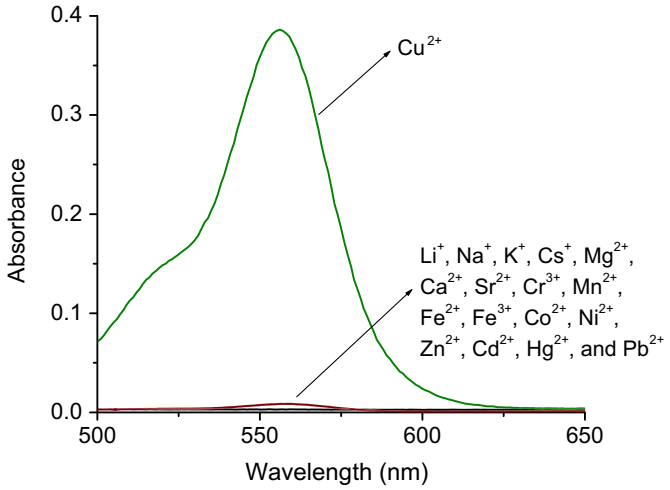

Fig. 6. Change in absorption intensity of 3 (10 μM) in 1:1 acetonitrile/water upon addition of various metal ions (50 μM) Li^{+}, Na^{+}, K^{+}, Cs^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+} and Cu^{2+}.

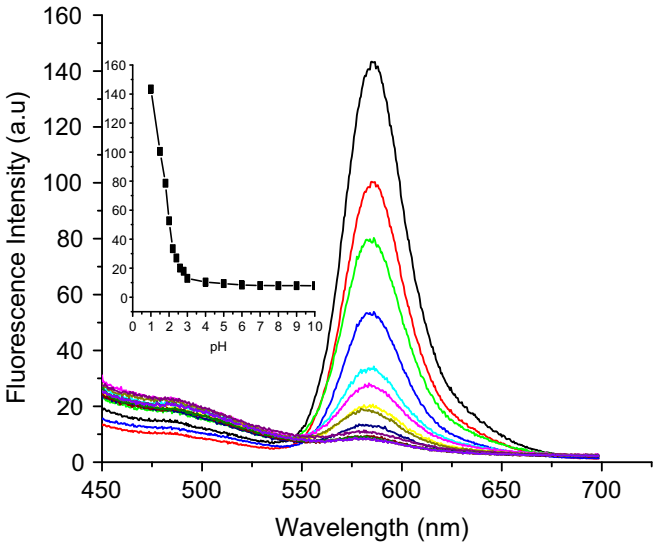

Fig. 8. Effect of pH in the fluorescence emission of 3 in 1:1 acetonitrile/water.

spectra of **3** caused by Cu^{2+} and other possible contaminants such as Li^{+}, Na^{+}, K^{+}, Cs^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+}. As shown in Figs. 6 and 7, the miscellaneous competitive cations did not contribute significantly to absorption or fluorescence behaviour of **3**. Moreover, even in the presence of miscellaneous competitive cations, Cu^{2+} induced absorption and fluorescence changes were similar to those presented in Figs. 3 and 4. In addition, the enhancement in absorbance and fluorescence intensities resulting from the addition of the Cu^{2+} was not influenced by the subsequent addition of miscellaneous cations. Taken together our data suggested a remarkably high selectivity of **3** for Cu^{2+} over other competing cations. It is likely that the addition of Cu^{2+} induces the carbonyl oxygen atom of spirolactam to co-ordinate with Cu^{2+} resulting in the opening of spirolactam ring that leads to the development of pink colour and enhancement in fluorescence intensity [28,37].

The stability of rhodamine derivative **3** over a broad range of pH is an indication of its suitability for applications in biological samples. Significant enhancements in fluorescence emission intensity (Fig. 8) and development of pink colour were observed only below pH 2.8, indicating the opening of spirolactam ring of **3** under strongly acidic conditions. No enhancement in fluorescence intensity is observed above pH 3.0. These results confirm the stability and suitability of compound **3** for biological applications as the pH of biofluids is ~7.4.

To test the applicability of **3** for a possible application in bioimaging, we chose *Escherichia coli*, known Cu^{2+} uptaking bacteria, [38] for the study. *E. coli* cells exposed to Cu^{2+} ions (5 μM) followed by staining with bis-rhodamine fluorochrome **3** (5 μM) were examined under fluorescence microscope. No fluorescence was observed from *E. coli* cells alone, *E. coli* cells exposed only to **3**, and *E. coli* cells exposed only to Cu^{2+}. However, red fluorescence was observed from *E. coli* cells exposed to Cu^{2+} and treated with **3** as

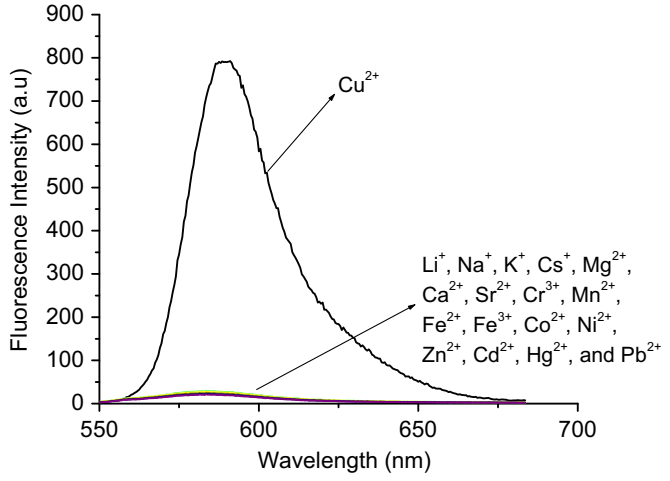

Fig. 7. Change in fluorescence intensity of 3 (10 μM) in 1:1 acetonitrile/water upon addition of various metal ions (50 μM) Li^{+}, Na^{+}, K^{+}, Cs^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+} and Cu^{2+}.

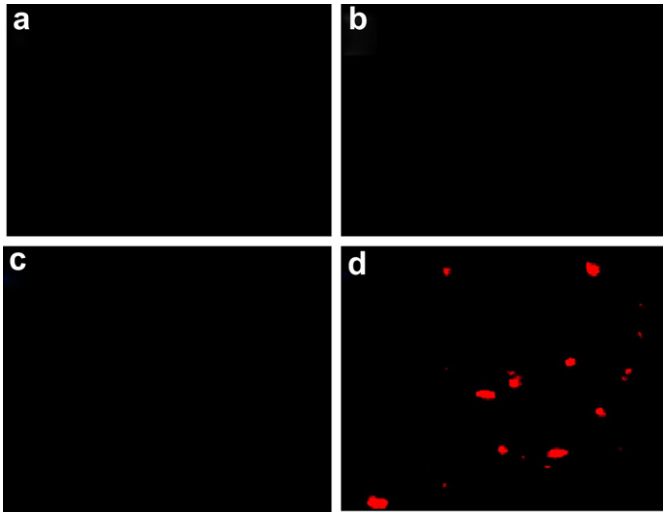

Fig. 9. Fluorescence microscopic images of (a) untreated *E. coli* cells, (b) *E. coli* cells incubated with Cu^{2+} (5 μM), (c) *E. coli* cells incubated only with 3, and (d) *E. coli* cells incubated with Cu^{2+} (5 μM) and stained with 3 (5 μM).

shown in Fig. 9, suggesting an application for the bis-rhodamine derivative.

4. Conclusion

In conclusion, we have synthesized a new bis-rhodamine based chemosensor for naked-eye detection of Cu^{2+} in aqueous medium. The enhanced sensitivity of the bis-rhodamine chemosensor as compared to the mono-rhodamine derivative allows the detection of Cu^{2+} at sub-micro molar concentrations. We have demonstrated the selectivity of the chemosensor for Cu^{2+} over other competing metal ions. We have also demonstrated the suitability of the fluorochrome for bio-imaging of live *E. coli* cells.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.dyepig.2011.04.016.

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