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Turn on fluorescent chemosensor containing rhodamine B fluorophore for selective sensing and *in vivo* fluorescent imaging of Fe^{3+} ions in HeLa cell line and zebrafish

Natarajan Vijay[a], Shu Pao Wu[b], Sivan Velmathi[a,*]

[a] Organic and Polymer Synthesis Laboratory, Department of Chemistry, National Institute of Technology, Tiruchirappalli-620 015, India
[b] Department of Applied Chemistry, National Chiao Tung University, Hsinchu, Taiwan

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ABSTRACT

Rhodamine dyes are utilized effectively as a chemosensor to monitor biologically important metal ions and effectively applied for fluorescent imaging studies in living systems. Herein, rhodamine-B armed fluorescent chemosensor (RhBNC) was designed and synthesized by condensation reaction between rhodamine B hydrazide and naphthyl chromone with ONO binding site in good yield. Upon sensing study towards metal ions, the probe showed selective turn-on fluorescent change for Fe^{3+} over other cations even in the presence of competing trivalent metal ions as well. The fluorescence switch is "on" when Fe^{3+} ions binds to RhBNC with orange fluorescence. The probe can detect lower concentration of Fe^{3+} ions with detection limit of 0.16 μM. Due to its lower detection limit; it can be applied to monitor Fe^{3+} ion presence in a living system by fluorescent imaging technique. The probe was employed effectively to ascertain the presence of Fe^{3+} and to image Fe^{3+} ions in HeLa cell line and zebrafish.

1. Introduction

Selective and sensitive detection of heavy metal ions in aqueous solution attracts enormous importance in monitoring and studying the physiological functions of these ions in living organisms [1,2]. Conventional spectroscopic methods failed due to their complicated experimental procedure and requirement of sophisticated instrument for the detection. So, there is a requirement of smart and simple detection method to monitor the amount of trace level metal ions in a living system under its physiological condition. Recently, monitoring the biologically important heavy metals by optical methods grabbed much attention due to their relatively lower cost compared to instrumental methods [3]. Nowadays, Small molecule fluorescent probes are utilized effectively for monitoring the particular analyte in a living system. Those possesses high sensitivity and selectivity with simple detection procedure. Hence, this decade several studies focused on the construction of selective and sensitive chemosensors using spectral methods includes both spectrophotometric and spectrofluorometric methods for the detection [4]. In case of chemosensor, the sensing study is based on quenching/growing of emission/absorption peaks of the receptor induced by the analyte provides the unique changes in detection. Fluorescent signalling supplies high sensitivity and easy handling as well as being cheaper to use. Utilizing the fluorescent probe for heavy metal ion detection is a reliable technique compared to sophisticated instrumental technique. Basically, colorimeric or fluorometric receptors were designed with chromophore / fluorophore with binding unit and employed as a receptor for sensing of targeted analytes [5,6]. In this decade, rhodamine dyes were applied effectively as a heavy metal ion sensor due to their very good photophysical properties, higher fluorescence quantum yield and tuneable selectivity nature [7,8]. Rhodamine based chemosensor showed turn on fluorescence change for metal ions when it binds with metal ions. This is because of the spirolactam ring opening/closing mechanism of the receptor with addition of metal ions [9]. Rhodamine dye is a good fluorophore with emission maximum of 580 nm. Rhodamine derived receptors were applied widely for bioimaging of particular analyte in a living cell, which gives promising results due to its photophysical properties and the minimal background noise from the living system [10,11]. Iron is found to be a predominant microelement, play a number of biologically and pathologically important roles to maintain a normal physiological function in a system. It is a crucial element for oxygen sensing in living system where metabolism is been linked with oxygen metabolism [12]. It is also important to maintain redox balance of labile iron species which can generate iron catalyzed reactive oxygen species (ROS)

* Corresponding author.
E-mail address: velmathis@nitt.edu (S. Velmathi).

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[13–15]. It is important to prevent the ROS generation for survival. Both inadequacy and excess of iron concentration in our body can cause the cellular homeostasis and can cause various biological diseases [16–20]. We are living in world where we are surrounded with iron and its derivatives, either direct or indirect manner. We are consuming iron in our daily life even through food and water. Thus it is important to monitor it's amount in living system by simple method. Utilizing the organic receptor as a selective probe to trace out Fe^{3+} ions in a living system is an easy and reliable way. To discover the presence of Fe^{3+} ions, number of chemosensors were reported in recent days [21–24]. Some rhodamine derived fluorescent probes were also reported for sensing of Fe^{3+} by fluorescent change and colorimetric change [10,25–27]. But, most of the probes showed either limited selectivity over other trivalent ions, or poor solubility and higher detection limit. Particularly, most of the reported Fe^{3+} probes failed in selectivity over existing trivalent ions. Moreover, paramagnetic fluorescence quenching of Fe^{3+} ions becomes major obstacle in designing fluorescent probe for Fe^{3+} ions sensing [28,29]. So the requirement of selective fluorescent probe for Fe^{3+} is still in need. Herein, we have designed and developed rhodamine B derived fluorescent probe for selective detection of Fe^{3+}. The probe was constructed with ONO (*via* Oxygen-Nitrogen-Oxygen) binding unit with rhodamine B hydrazide and naphthyl chromone. The probe shows excellent selectivity for Fe^{3+} over other metal ions and trivalent ions as well. It exhibits turn on fluorescence for Fe^{3+} with 20 fold fluorescence enhancement. It can even detect Fe^{3+} in lower concentration as well with the detection limit of 0.16 μM. Thus, it can be applied for the detection of Fe^{3+} in a living system. Hence, we have performed fluorescent imaging of Fe^{3+} ions in HeLa cell line and zebrafish and the probe shows red emission for Fe^{3+} in both zebra fish and HeLa cell line.

2. Experimental Section

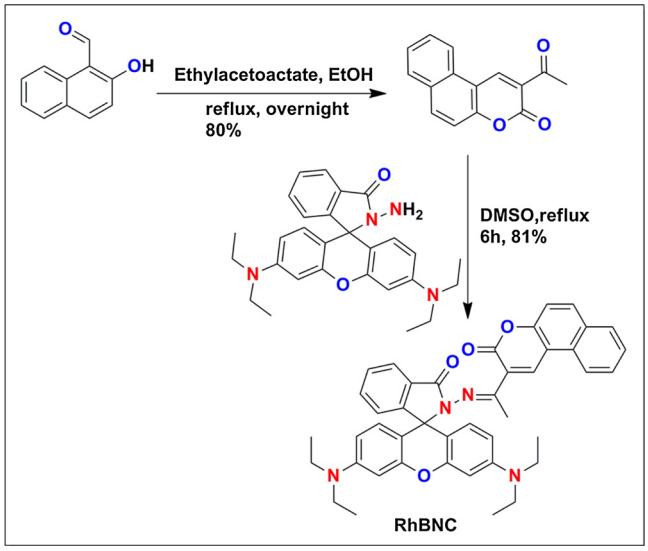
2.1. Instrumental techniques and methods

All the reagents, chemicals and solvents were obtained from commercially available sources (Sigma and Merck) and used without additional purification throughout the studies. Double distilled water is utilized for doing all the experiments. The product formation was confirmed by the characterization techniques like FT-IR, ^{1}H & ^{13}C NMR, ESI-Mass analysis and the complete photophysical studies were carried out using UV/Vis and Fluorescence spectrophotometer. Shimadzu UV-2600 spectrophotometer was used to get the absorption spectra using quartz cell having 1 cm path length. RF-5301PC spectrofluorophotometer was utilized to get the emission spectra with scanning rate of 500 nm/min. Bruker AV-500 spectrometer (Bruker, Switzerland) was used to obtain ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra using $CDCl_3$ as a solvent. Nicolet IS 5 FTIR spectrometer was used to record the IR spectra by KBr pressed disc method. The stock solutions of metal ions that applied for the sensing studies were prepared by dissolving the manganese acetate and other metal chloride salts of corresponding metal salts such as (Cr^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Al^{3+}) in double distilled water to afford 1.5 mM solutions and the stock solutions of anions were prepared by dissolving tetra-*n*-butylammonium salts of corresponding anions (CN^{-}, F^{-}, Cl^{-}, Br^{-}, I^{-}, AcO^{-}, H_2PO4-, HSO_4^{-}, $NO_3^{-}$and ^{-}OH) in double-distilled water to get 1.5 mM solutions. 1.5 mM solution of the probe RhBNC stock solution was prepared in DMSO and kept in normal day light condition for several months; no characteristic change was observed.

2.2. Synthesis of naphthylchromone

2-Hydroxy-1-naphthaldehyde 172.00 mg (1 mmol) was taken in 10 mL absolute ethanol with ethylacetoactate 150 μL (1.2 mmol) in a round bottom flask. The reaction mixture was refluxed overnight in an oil bath. The reaction completion was monitored through TLC and the reaction mixture was cooled to room temperature and filtered using funnel and washed with ethanol to get the pure product as a yellow solid (Scheme 1). Yield: 80% (202 mg). Melting point: 195 ^{0}C. FT-IR (ν/cm^{-1}): ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): 9.29 (s, 1 H), 8.3 (d, 1H, J:17 Hz), 8.1 (d, 1H, J: 18 Hz), 7.9 (d, 1H, J:16 Hz), 7.7 (t, 1H, J: 15 Hz), 7,6 (t, 1H, J: 15 Hz), 7.4 (d, 1H, J: 18 Hz), 2.788 (s, 3 H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 195.63,

159.47, 156.19, 143.34, 136.34, 130.23, 129.87, 129.25, 126.68, 122.45, 121.72, 116.52, 112.80 and 30.72.



Scheme 1. Synthesis route of probe (RhBNC).

2.3. Synthesis of probe (RhBNC)

Rhodamine-B-hydrazide can be synthesized by reaction between rhodamine B and hydrazine hydrate by following the previously reported procedure [30,31]. Rhodamine-B hydrazide (0.5 mmol, 228 mg) and naphthylchromone (0.5 mmol, 119 mg) were taken in RB flask and dissolved in 0.5 mL of DMSO. The reaction mixture was heated to 120 °C for 6 h in an oil bath. The reaction completion was monitored thorough TLC and the reaction mixture was poured to ice-cold water. The resulting precipitate was filtered, washed with cold water and dried over vacuum to get desired product as greenish yellow solid (Scheme 1). Yield: 81% (275 mg). Melting point: 208 °C. FT-IR (ν/cm^{-1}): 3449 cm^{-1}, 3060 cm^{-1}, 1735 cm^{-1}, 1612 cm^{-1}, 1513 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): 8.67 (s, 1 H), 8.1 (d, 1H, J: 16 Hz), 8.0 (d, 1H, J: 8 Hz), 7.7 (q, 2 H), 7.6 (t, 1H, J: 15 Hz), 7.5 (d, 2H, 6), 7.5 (t, 1H, J: 14 Hz), 7.2 (d, 1H, J: 10 Hz), 7.2 (d, 1H, J: 17 Hz), 6.6 (d, 2H, J: 18 Hz), 6.443 (s, 2 H), 6.3 (d, 2H, J:17 Hz) 3.3 (d, 8H, J: 13 Hz), 2.347 (s, 3 H) and 1.1 (t, 12H, J: 17 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 153.9, 150.8, 148.8, 139.5, 134.1, 132.5, 131.3, 130.1, 129.4, 128.9, 128.5, 128.2, 126.0, 124.1, 124.0, 123.2, 121.6, 116.3, 113.0, 107.9, 106.8, 97.6, 44.3, 19.9, 12.6. ESI Mass: 676.3, m + 1 677.3 (ESI: Fig. 1–9).

2.4. Physical constants determination

Stoichiometric ratio (R:M) of RhBNC with Fe^{3+} ions were calculated by Job's plot method. To find out the stoichiometry; emission at 580 nm was plotted against the mole ratio of the ([Fe^{3+}] / [Fe^{3+}] + RhBNC) (Job's plot). Benesi-Hildebrand equation was used to calculate the binding constant (Ka) and limits of detection (LODs) of the receptor RhBNC using the fluorescence incremental titration data. For that, fluorescence incremental titration experiment was performed with gradual increment of Fe^{3+} ion concentration (0–2eq.).

2.5. Cytotoxicity assay

Cell viability experiment for RhBNC in HeLa cell lines was measured by using the MTT (methyl thiazolyl tetrazolium) assay experiment. For this experiment the desired cell line were cultured in multi well cell-culture plate and different concentrations (5, 10, 15, 20, 25 μM) of receptor were treated with the cells. After that, the cells were allowed for incubation for 24 h at 37 °C under 5% CO_2. Then, to each of the well 10 μL MTT (5 mg mL^{-1}) was added and allowed for incubation again for 4 h with same condition. After 4 h incubation the media were removed and 200 μL DMSO and 25 μL Sorensen's glycine buffer (0.1 M glycine and 0.1 M NaCl) was added to that. Then the absorbance was measured by single time Multiskan GO microplate reader (at 570 nm). The cell viability was calculated using the given equation:

Cell viability (%) = Mean absorbance (Treated cell) / Mean absorbance (Control cell).

2.6. Cell culture for HeLa cell line

For bioimaging studies the HeLa cell line was obtained from the Food Industry Research and Development Institute (Taiwan). The desired HeLa cell lines were cultured in DMEM which is having 10% fetal bovine serum (FBS) in addition and is kept at 37 °C under 5% CO_2 atmosphere. After the culturing, the cells were treated with 20 μM probe (RhBNC) in 2 mL of DMSO and incubated for 30 min at 37 °C. Then the media was removed and the cells were washed with 1 M PBS twice to remove excess probe. Then, 20 μM Fe^{3+} (in DMSO) was added to the cell and incubated, allowed for 30 min and the media were removed. Finally, the cell lines were plated on 18 mm glass coverslips and allowed to adhere for 24 h before imaging. Fluorescence imaging was performed with Leica TCS SP5 X AOBS Confocal Microscope (Germany) and a 63-oil-immersion lens has been utilized as an objective lens. The excitation wavelength was fixed at 540 nm and emission was recorded at 580 nm ± 10 nm. Institutional Animal Care and Use Committee (IACUC) of NCTU accepted National Institute of Health, Taiwan guidelines was followed for imaging of zebrafish and maintained the condition according to the guideline. The fish was maintained at ambient temperature with good condition. The both gender fishes (Male and female) are maintained in same container at 28 °C with 10 h/14 h cycle (dark/light respectively) for mating purpose, and then the egg fertilization was induced by sun light. Egg fertilization was good in this condition; most off the eggs were fertilized. The zebrafish culturing was carried out in 6 well cell culturing plate with addition of 5 mL fish medium along with 1-phenyl-2-thiourea (PTU) in each well at 30 °C for 1day. Followed by this, zebrafish were anaesthetized using 50 mg/L tricaine and treated with 20 μM probe at 28 °C for 30 min. Then, the remaining probe which is present in the outside of the fish was removed by washing it with 1 M PBS and then the fish was treated with 20 μM of Fe^{3+} ions in DMSO and kept for incubation at 28 °C for 30 min. Then again the PBS wash has been carried out to remove the excess Fe^{3+} present in the fish. Then finally, zebrafish imaging was performed with Leica TCS SP5 X AOBS Confocal Fluorescence Microscope.

3. Results and discussions

3.1. Synthesis and characterization

The real application of the fluorescent probe can be achieved when it shows exclusive sensitivity towards particular analyte with distinct change. In order to achieve that, herein the fluorescent probe RhBNC was designed for the detection of Fe^{3+} ions. The probe was constructed by utilizing Rhodamine B as a fluorophore, with naphthyl chromone as binding unit for Fe^{3+}. In case of rhodamine based probe with ONO as binding unit will selectively detect Fe^{3+}. In that motive, the probe was designed and synthesized in very good yield (Scheme 1). The product obtained as a yellow solid with melting point 208 °C. Synthesized probe was well characterized and the product formation was confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR and ESI-Mass spectroscopic methods (Fig. ESI 1–9).

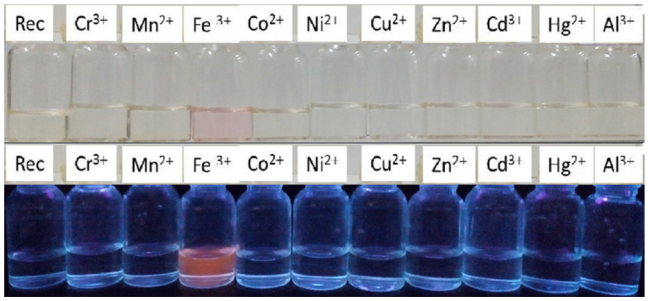


Fig. 1. Color change experiment for RhBNC (50 μM receptor in THF upon addition of 2 eq. of cations in H_2O; a) Day light image; b) Fluorescence image.

3.2. Visual sensing studies

Things, that seeing visually will make more effect and will be remembered for long time. Visual color change experiment is a key fact in sensing field. The visual color change of probe (RhBNC) with cations (Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Al^{3+}) were observed by naked eye experiment. The experiments were carried out in different solvents such as ACN, DMSO, ethanol, MeOH and THF to find out the selectivity of the probe over biologically existing cations. The probe was taken in glass vial having 3 mL THF solvent with a final concentration of 50 μM. To that solution 2 eq. of metal ions were added which was dissolved in double distilled water. The images were taken in normal day light and under UV light (Fig. 1). The probe (RhBNC) shows excellent selectivity towards Fe^{3+} over other cations in THF solvent medium with a colour change from colourless to appearance of pale pink colour.

3.3. Photophysical studies

In continuation of colorimetric studies, the sensing action was monitored through photophysical experiments using UV–vis and fluorescence spectrophotometer. To investigate the efficiency of the probe as a metal ion sensor the photophysical changes of probe with various metal ions were verified in THF solvent medium. The absorption spectrum of the probe exhibit bands in the range of 350–400 nm corresponding to π-π* and n- π* transition in chromone and rhodamine moiety. Noticeable change was not observed after adding 2 eq. metal ions, in absorption spectra because not much colour change in naked eye experiment as well. For Fe^{3+} very small change was found in absorption spectra in the range of 350–400 nm (Fig. 2(a)). The fluorescent change of the probe towards various metal ions was checked in THF solvent medium using fluorescent spectrometer. In a fluorescence spectrometer the probe was excited at 520 nm and the emission was collected at 540 nm. The probe alone doesn't exhibit any fluorescence in 550–600 nm range, after addition of metal ions, Fe^{3+} ions alone causes 20 fold fluorescence enhancement with λ_{max}at 580 nm (Fig. 2(b)). Only the addition of Fe^{3+} causes turn on fluorescence change with distinct colour change. This is due to the ring opening of the spirolactam present in rhodamine dye triggered by the addition of Fe^{3+} ions [32,33].

3.4. Incremental addition and selectivity

Fluorescence titration experiment was performed with Fe^{3+} to find out its binding ability with probe. Initially, 50 μM of probe was taken in

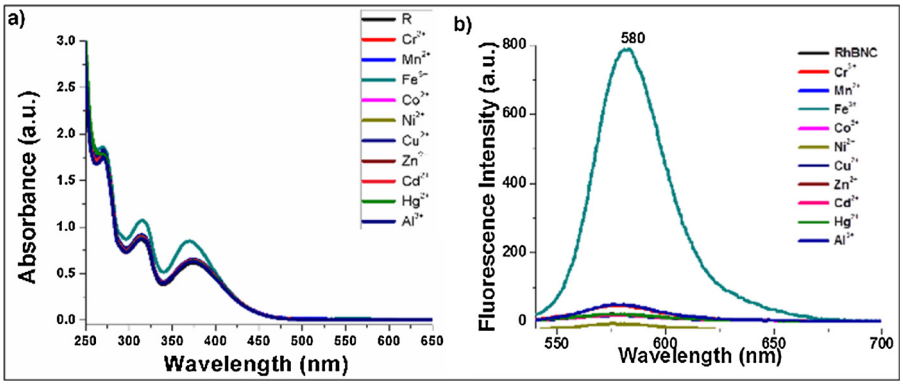


Fig. 2. a) UV–vis spectrum of RhBNC (50 μM) with different cations (100 μM) in THF; b) Fluorescence spectrum of RhBNC (50 μM) with different cations (100 μM) in THF.

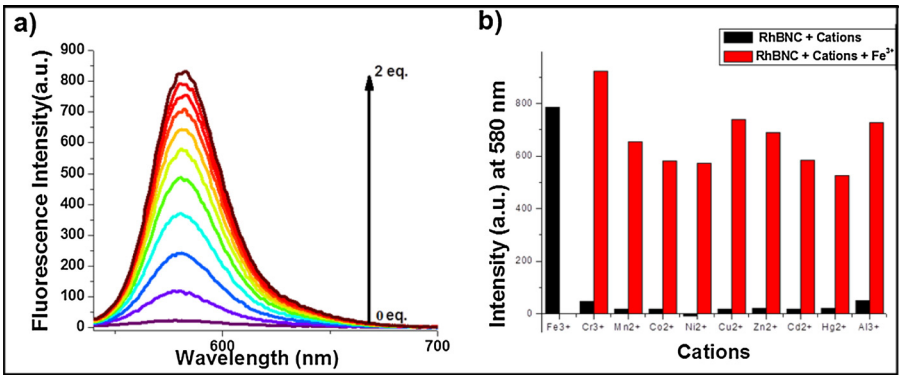


Fig. 3. a) Fluorescence spectrum of RhBNC (50 μM) with Fe^{3+} (0–2 eq.) in THF; b) Fluorescence intensity of RhBNC (50 μM) with Fe^{3+} (2 eq.) at 580 nm in the presence of other cations (2 eq.) in THF (Black bar: Intensity of RhBNC with cations; Red bar: Intensity of RhBNC with cations and Fe^{3+}).

THF and Fe^{3+} was added gradually (0–2 eq.). The fluorescence intensity at 580 nm increases gradually with increasing the Fe^{3+} concentration (Fig. 3(a)). The complete application of the probe will be fulfilled, if it works even in the presence of the other metal ions. Because, in real time application the probe should work inside the biological medium with the presence of other competing metal ions. In order to ensure the selectivity of the probe (RhBNC) towards Fe^{3+} with the presence of other metal ion such as Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Al^{3+} competition experiment was performed with the presence of above mentioned metal ions. Initially 50 μM of probe taken in THF solvent medium and 2 eq. of various metal ions were added to that and the fluorescence spectrum was recorded with similar excitation (520 nm) and the emission spectrum was collected from 540 nm. Then the intensity at 580 nm was plotted (Fig. 3(b)) (Black bar). To that 2 eq. of Fe^{3+} was added and the fluorescence spectrum was taken and the intensity at 580 nm was monitored and plotted again (red bar). The probe shows 20 fold fluorescence enhancement for Fe^{3+} with the presence of other metal ions as well. The probe shows excellent selectivity for Fe^{3+} with 'turn on' fluorescence even in the presence of higher concentration of competitive metal ions.

3.5. Stoichiometry, binding constant and detection limit calculation

To understand the clear sensing mechanism and to carry out further applications, stoichiometry between probe and Fe^{3+} need to be verified. It is calculated using mole ratio plot methods and Job's plot method. The fluorescence change at 580 nm was plotted against the concentration of Fe^{3+} ions added to the probe. The result reveals that the binding ratio is 1:1 between probe and Fe^{3+} (Fig. ESI: 13). Furthermore, the binding constant and limit of detection of the probe towards Fe^{3+} was calculated using Benesi-Hildebrand equation from the fluorescence titration experiment. During fluorescence titration, the intensity at 580 nm gradually increases while increasing the concentration of Fe^{3+} ions. From the above titration, we have plotted the graph according to Benesi-Hildebrand equation. Binding constant of the probe with Fe^{3+} was calculated according to the equation (1).

$$1/(I\text{-}I_0) = 1/Ka\ (I_{max} - I_0)\ [Fe^{3+}]\ +\ 1/\ (I_{max}\text{-}I_0) \quad (1)$$

I and I_0 were fluorescence intensity of RhBNC at 580 nm in presence and absence of Fe^{3+} ions respectively and I_{max} is the saturated fluorescence intensity with the presence of high concentration of Fe^{3+} ions and $[Fe^{3+}]$ is concentrations of iron added to the probe. The calculated binding constant (Ka) is 7450. The U.S. Environmental Protection Agency (EPA) permitted amount of Fe^{3+} ions in drinking water was 0.3 mg L^{-1}(equivalent to 5.4 μM). To detect iron even at lower concentration, we have calculated the limit of detection of the probe towards Fe^{3+} ions detection using fluorescent titration experiment. We have followed the equation that based on definition provided by IUPAC.

The IUPAC provided equation was,

Limit of Detection = 3*Standard deviation of blank / slope between intensity and concentration

The detection limit of the probe for the detection of Fe^{3+} ions was very low, 0.16 μM. It is very much lower than EPA permitted level of Fe^{3+} ions concentration in drinking water. So, the probe can be applied for detection of Fe^{3+} ions in practical applications.

3.6. Proposed sensing mechanism

The significant colour change and fluorescence change for Fe^{3+} with the probe need to be analyzed and the clear sensing mechanism should be proposed. The results reveal that, the probe followed general

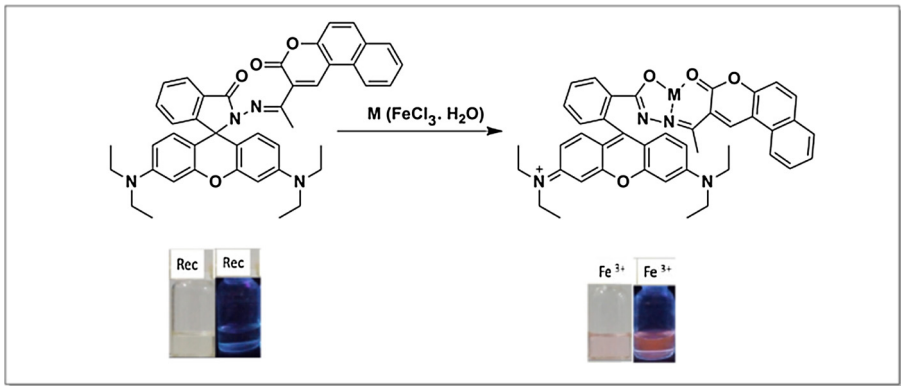


Fig. 4. Proposed sensing mechanism of the probe for Fe^{3+} ions sensing event.

rhodamine-B mechanism for the sensing event. The metal ion induces spirolactam ring opening and results with selective colour change and fluorescence change in the range 550–600 nm (Fig. 4). Rhodamine-B dye with ONO binding mostly utilized as Fe^{3+} sensor with distinct colour change and fluorescence change [25,34,35]. The probe worked as we expected, it makes ONO binding with Fe^{3+} ions and gives selective change over the other cations. The binding was confirmed with stoichiometry ratio between probe and Fe^{3+} by Jobs plot method. It showed 1:1 binding mode with Fe^{3+}. To confirm further, RhBNC-Fe^{3+} complex was synthesized and formation of the product was confirmed by ESI mass analysis (Fig. ESI: 9 & 10). Calculated mass of the probe (RhBNC) was 676.3 and mass peak obtained at 677.3 (m + 1) and calculated mass for RhBNC-Fe^{3+} complex is 855.2 and obtained mass peak at 856.2 (m + 1), confirms the 1:1 stoichiometry between probe RhBNC and Fe^{3+}.

3.7. Cell imaging and zebrafish imaging

Once, the primary photophysical studies were completed with the probe, it is employed in real time application studies. The predominant application of this study is to monitor the presence of Fe^{3+} ions in living system. In prior to carry out the bioimaging studies, the cell viability experiments were performed with the probe in order to find out the toxicity of the probe towards living cells. MTT assay experiment was performed with HeLa cell line with various concentration of RhBNC (< 25 μM). In MTT assay experiment the probe shows more than 80% cell viability towards HeLa cell line (Fig. ESI: 12). So the probe can be applied for biological studies as it shows less cell death. Fluorescence imaging of HeLa cell line towards Fe^{3+} was performed using Leica fluorescence microscope. The probe RhBNC (20 μM) treated with HeLa cell line and incubated for 30 min and imaged using the microscope. It doesn't show any emission in cell line before it treated with Fe^{3+} ions (Fig. 5 channel A). After that cell line was treated with 20 μM Fe^{3+} ions dissolved in DMSO and incubated for 30 min. After the incubation the cell was imaged using the microscope. It gives bright red emission when excited at 560 ± 10 nm.

To account for the cell permeability and the fluorescence in the cytoplasm, initially fluorescent image was captured only with nuclear staining dye. It showed blue emission in the nucleus. After the treatment of probe with Fe^{3+} it showed red emission in cytoplasm only (Fig. 5 channel B). So the probe possesses very good cell permeability with less cell death, further it applied for *in vivo* fluorescent imaging of Fe^{3+}. This experiment was performed with zebrafish. Three days old zebrafish was treated with probe (20 μM) for 30 min and fluorescent image was captured using the microscope; no characteristic emission was observed (Fig. 6, panel A). Then the fish was allowed to treat with Fe^{3+} for half an hour and the emission image was captured with microscope. It exhibited bright red emission in the fish stomach as like HeLa cell line (Fig. 6, panel B).

4. Conclusion

In summary, rhodamine-B derived fluorescent probe was designed and synthesized for selective detection of Fe^{3+} ions in aqueous medium. The receptor shows turn on fluorescent change selectively for Fe^{3+} ions over other cations. It can detect the Fe^{3+} in lower concentration as well with the detection limit of 0.16 μM. The receptor shows less cell death in cell viability test towards HeLa cell line, hence it is applied successfully for monitoring the intracellular Fe^{3+} ions in living cells. This experiment demonstrated by fluorescent imaging of Fe^{3+} in HeLa cell line using fluorescence microscope. Finally, *in vivo* fluorescent imaging studies were performed for Fe^{3+} using zebrafish. The probe can be applied to monitor the Fe^{3+} in a living system with selectively over other trivalent ions and other metal ions.

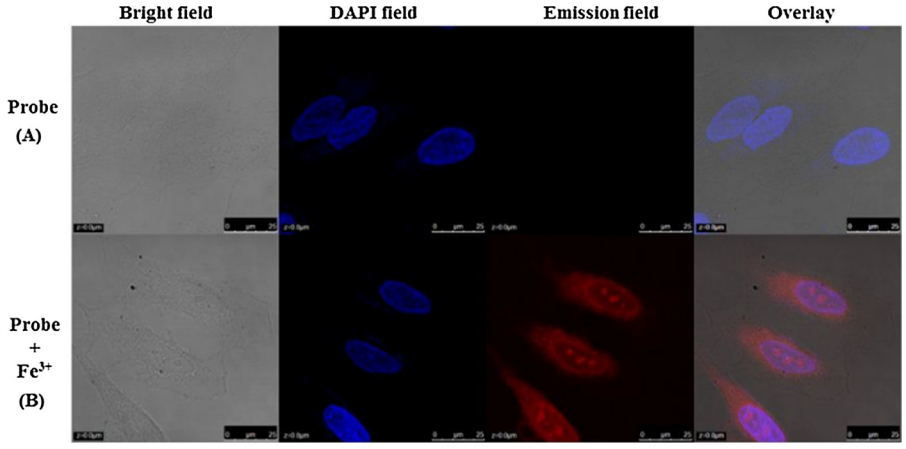


Fig. 5. Fluorescence images of HeLa cell line. (First) Bright field; (Second) DAPI (4′,6-diamidino-2-phenylindole, nuclear stain); (Third) Fluorescence; (Fourth) merged image. (A) The cells were treated with Probe (20 μM) for 30 min. (B) Cells were incubated with probe (RhBNC) (20 μM) for 30 min and then treated with Fe^{3+} (20 μM).

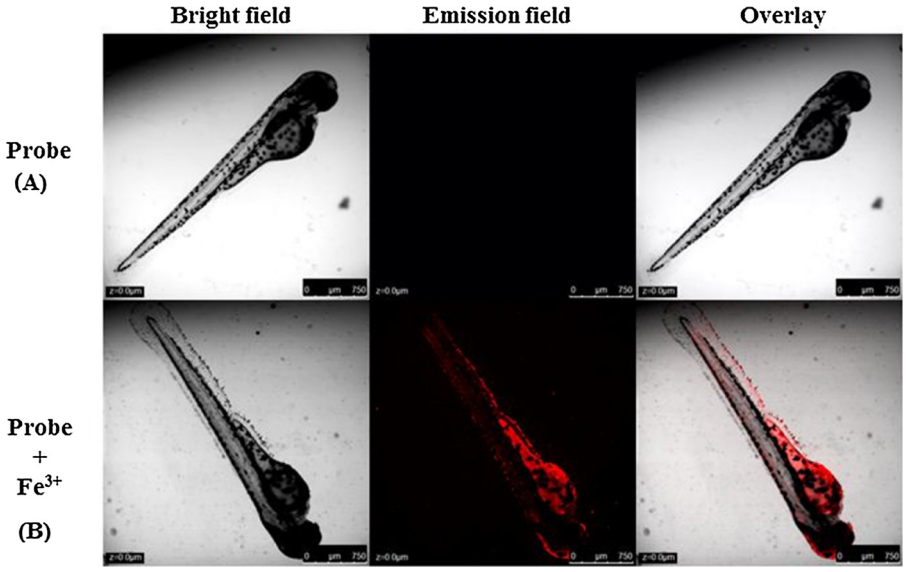


Fig. 6. Confocal Fluorescence images of zebrafish embryos (3 days old). (A) The fish was treated with probe (20 μM) for 30 min. (B) The zebrafish were treated with probe (20 μM) and then treated with Fe^{3+} (20 μM) for 30 min.

Declaration of Competing Interest

There is no conflict of interest to declare in this submission.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jphotochem.2019.112060.

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