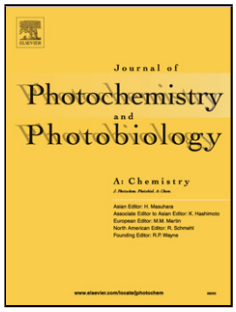

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A novel turn-on colorimetric and fluorescent sensor for Fe^{3+} and its application in living cells

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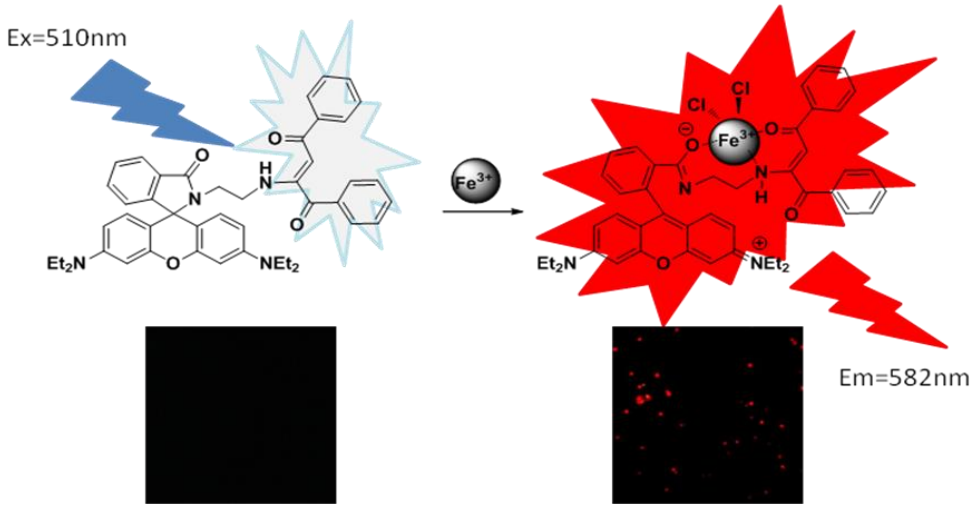
Graphical Abstract



Highlights

- A novel rhodamine-based colorimetric and fluorescent sensor **1** was synthesized.
- Sensor **1** displayed a distinct color change from colorless to pink and a significant fluorescence enhancement after binding of Fe^{3+}.
- Fluorescent microscopy experiments established that **1** could be used for sensing Fe^{3+} in living cells.

Abstract

A novel turn-on colorimetric and fluorescent sensor **1** based on rhodamine B was developed as a colorimetric and fluorescent chemosensor for Fe^{3+}. Upon addition of Fe^{3+} in aqueous ethanol, the **1** displayed a distinct color change from colorless to pink and a significant fluorescence enhancement, which can be directly detected by the naked eye. The stoichiometry of **1** to Fe^{3+} complex was found to be 1:1 and the detection limit was determined as low as 7.68×10^{-7}M. The results suggest that the sensor **1** may provide a conveniently method for visual detection of Fe^{3+} with high sensitivity. Furthermore the sensor has been utilized for fluorescence imaging of Fe^{3+} in living cells.

Key words

Rhodamine B, colorimetric, fluorescent sensor, Fe^{3+}, bioimaging

1. Introdution

The development of fluorescent chemosensors for a variety of metal ions have received increasing attention because of their fundamental role in medical, environmental and biological applications [1,2]. Among the metal ions, Iron performs a crucial role in a wide range of biochemical processes [3,4], several biological functions depend directly or indirectly on the proper concentration and oxidation states of iron to maintain the homoeostatic mechanism of biosystem. Deficiency or overdose of iron can lead to serious diseases, including Alzheimer's, Huntington's and Parkinson's disease [5-7]. Therefore, design of fluorescent chemosensors for detecting Fe^{3+} is of great importance. However, Fe^{3+} is a well-known fluorescence quencher due to its paramagnetic nature, which makes it difficult to develop a sensitive turn-on fluorescent sensor. Only limited number of turn-on fluorescent chemosensors is available in literature [8-19]. Moreover, among these fluorescent sensors, only a few sensors have been applied in bioimaging [15-19]. Therefore, design of turn-on fluorescent sensor for Fe^{3+} imaging in living cells remains a challenging work.

Rhodamine-based fluorogenic and chromogenic probes have received increasing interest in recent years by virtue of their properties of long-wavelength emission, high fluorescence quantum yield and large molar extinction coefficient, and many fluorescent probes based on metal induced spiroring opening have been developed [20–33], because they can perform not only great fluorescence intensity enhancement toward some specific cations, but also a strong colour development against the

colourless blank during the sensing event. In continuing of our study on developing molecular sensors for Fe^{3+} detection [34, 35], herein, we introduce a novel and simple method to prepare rhodamine B-based chemosensor by three steps reaction. The sensor **1** showed "off–on" type chromogenic behavior toward Fe^{3+} over other metal ions. Moreover, fluorescent microscopy experiments indicated that **1**could be used as fluorescent probe for sensing Fe^{3+} in living cells.

2. Experimental

2.1. Reagents

All the reagents were purchased from commercial suppliers and used without further purification. The salts used in stock solutions of metal ions were $CoCl_2 \cdot 6H_2O$, $ZnCl_2$, $MnCl_2 \cdot 4H_2O$, KCl, NaCl, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, $CdCl_2 \cdot 2H_2O$, $HgCl_2$, $MgCl_2 \cdot 6H_2O$, $CrCl_3 \cdot 6H_2O$, $Pb(NO_3)_2$, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $Al(NO_3)_3 \cdot 9H_2O$.

2.2. Apparatus

NMR spectra were measured on a Varian Mercury 300 spectrometer operating at 300 MHz for ^1H and 75 MHz for ^{13}C relative to tetramethylsilane as internal standard. MS spectra were obtained on a Finnigan Trace MS spectrometer. IR spectra were recorded on a Perkin-Elmer PE-983 infrared spectrometer as KBr pellets with absorption reported in cm^{-1}. Absorption spectra were determined on UV-2501 PC spectrophotometer. Fluorescence spectra measurements were performed on a FluoroMax-P spectrofluorimeter equipped with a xenon discharge lamp, 1 cm quartz cells at room temperature (about 298K).

*2.3. Synthesis of rhodamine derivate (**1**)*

The synthetic route of the compound **1** is shown in Scheme 1. 2-(methylthio)-1,4-diphenylbut-2-ene-1,4-dione (**2**) [36] and rhodamine B ethylenediamine (**3**) [37] was prepared according to literatures. To a 100 mL flask, rhodamine B ethylenediamine **3** (0.48g, 1.0 mmol) was dissolved in 50 mL ethanol. After the addition of compound **2** (0.28g, 1.0 mmol), the stirred mixture was heated to reflux for 24h. The residue was recrystallized from ethanol to afford pure compound **1** (0.51g, 71%) as a yellow solid. M.p.: 124-125^{o}C. IR (KBr, cm^{-1}): 3449, 2969, 1681, 1624, 1514, 1384, 1221, 1113, 700; ^{1}H NMR ($CDCl_3$, 300MHz): δ10.6(s,1H),7.94-7.97(m,2H), 7.76- 7.79(m,1H), 7.50-7.63 (m,2H), 7.32-7.47(m,8H),7.03(m,1H), 6.41-6.44(m,3H), 6.22-6.34(m,2H), 5.63(s,1H), 3.22-3.34(m,9H) ,3.01-3.04 (m,2H), 1.62(s,2H), 1.15(t, 12H); ^{13}C NMR (75 MHz, $CDCl_3$):191.2, 189.5, 168.3, 160.6, 153.7, 152.9, 148.6, 139.4, 134.5, 134.4, 132.4, 131.1, 130.5, 130.1, 128.7, 128.4, 128.1, 127.0, 123.6, 122.8, 108.1, 104.8, 97.6, 90.9, 64.7, 53.3, 44.2, 42.7, 40.9, 12.5; ESI-MS: m/z 719.46 $[M+H]^+$. Anal.calcd for $C_{46}H_{46}N_4O_4$: C76.85 H 6.45 N7.79; found C76.63 H 6.25 N7.64.

(Scheme 1)

2.4. Analytical procedure

The stock solutions of **1**(1.0×10^{-5} M) were prepared by dissolving **1** in EtOH/ water (9:1, v/v) containing HEPES buffer (10 mM, pH =7.2). The cationic stocks were all in H_2O with a concentration of 3.0×10^{-3} M for UV–vis absorption and fluorescence spectra analysis. For metal ion absorption and fluorescence titration

experiments, each time 3 mL solution of **1** filled in a quartz cell of 1 cm optical path length, and we increased concentrations of metal ions by stepwise addition of different equivalents using a micro-syringe. After each addition of Fe^{3+} ion, the solution was stirred for 3 min. The volume of cationic stock solution added was less than 100 μL with the purpose of keeping the total volume of testing solution without obvious change. For all measurements of fluorescence spectra of **1**, the excitation was at 510 nm.

2.5. Cell culture

Cell line Hela was purchased from ATCC (American Type Culture Collection, Rockville, MD, USA), and maintained in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibico, USA) in a humidified incubator with 5% CO_2 at 37°C. Hela cells plated on 18mm glass coverslips in 6-well plates and allowed to adhere for 24h.

2.6. Fluoreszence imagine

1×10^{-2}M $FeCl_3$ were prepared in PBS (phosphate-buffered saline, PH =7.2), and sensor **1** was dissolved in DMSO. The cells plated on glass cover lips cultured in DEME were treated with final concentration 1×10^{-5}M $FeCl_3$ solution and incubated at 37°C for 30 min. The treated cells were washed with PBS 3 times to remove remaining metal ions. 2ml DMEM was added to the cell culture, which was then treated with final concentration 1×10^{-5} M solution of sensor **1**, and the sample were incubated at 37°C for 30 min. the culture medium with sensor **1** was removed, and washed 3 times with PBS before observation. Fluorescence imaging was performed

under an Olympus SZX16 microscope with an Olympus DP72 digital camera with cell Sens version1.6 software, and processed with Adobe Photoshop CS4 software.

3. Results and discussion

*3.1. Synthesis and structural characteristics of **1***

The compound **1** was obtained by the reaction of rhodamine B ethylenediamine (**3)** with 2-(methylthio)-1,4-diphenylbut-2-ene-1,4-dione (**2**) in EtOH under reflux. The yield of **1** was 71%. The structures of **1** were identified by using ^{1}H NMR, ^{13}C NMR, ESI-MS (Figures S1-S3, in the ESI).

3.2. *Spectral characteristics*

To examine the binding properties of **1** with metal ions, the absorption spectra of **1**(10μM) in EtOH/water (9:1, v/v) containing HEPES buffer (10 mM, pH =7.2) was first explored in the presence of 10 equivalent of different metal ions (K^{+}, Na^{+}, Cr^{3+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Fe^{3+}, Cu^{2+} , Pb^{2+} , Fe^{2+} , and Al^{3+}), and the results were depicted in Figure 1. The sensor **1** exhibited c abroad band at 319 nm (log ε =5.04) and no absorption was observed in the visible range, suggesting that the closed spirolactone form of Rhodamine B presented. Upon binding of metal ions it was found that coordination of Fe^{3+} to **1** resulted in the enhancement of its absorbance band at 319 nm (log ε =5.28) and a new absorption band was appeared at 552 nm (log ε = 5.10). At the same time, the solution changed from colorless to pink (Inset Figure 1). The new absorption band at 552 nm was ascribed to the ring opened rhodamine moiety, which indicated that the interaction of **1** with Fe^{3+} can triggered the formation of the ring-opened form of **1** from the spirolactam form. Other metal ions did not

cause this change under the same conditions. Accordingly, **1** can serve as a "naked-eye" Fe^{3+} indicator in aqueous media.

(Figure 1)

The fluorescence spectra of **1** with respective metal cations are shown in Figure 2. The sensor **1** without Fe^{3+} showed no apparent fluorescence emission at 582nm. In fact, **1** also did not give any observable response for many metal ions such as K^{+}, Na^{+}, Cr^{3+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Fe^{2+}, and Al^{3+}. However, addition of Fe^{3+} created a remarkable fluorescence enhancement at 582nm. In agreement to the fluorescent spectra, the emission color change from colorless to yellow can be clearly observed under UV light in presence of Fe^{3+}, while other ions did not induce any significant emission color change. The specific response of **1** towards Fe^{3+} was assumed to be based on the opening function of the spirolactam ring. Meanwhile, the reaction of Fe^{3+} with a chelating agent induces rigidity in the resulting molecule and tends to produce a large chelation enhancement of the fluorescence (CHEF) which induces the large enhancement of fluorescence. These results demonstrated that **1** can be considered as a new "off-on" chemosensor for detecting Fe^{3+}.

To get an insight into the sensing properties of **1** to Fe^{3+}, fluorescence titration experiments were then performed (Figure 3). The fluorescence intensity of **1** (10 μM) at 582 nm was gradually increased with the addition of increasing amount of Fe^{3+} (10 μM-100 μM). The enhanced intensity of 1-Fe^{3+} displayed a good linear regression

relationship (R_2 = 0.9916) (Inset Figure 3).

(Figure 2)

The fluorescence spectra of **1** upon titration with Fe^{3+} were then performed (Figure 3). The fluorescence intensity of **1** (10 μM) at 588 nm was gradually increased with the addition of increasing amount of Fe^{3+} (10 μM-100 μM). The enhanced intensity of **1**-Fe displayed a good linear regression relationship (R_2 = 0.994) (Figure 3, inset). The fluorescence quantum yield (Φ_u) of **1** in the present of 100 μM Fe^{3+} was calculated to be 0.61 with respect to rhodamine B in ethanol solution (Φ_s=0.89) [38].

(Figure 3)

To investigate the binding stoichiometry between **1** and the Fe^{3+} ion, the Job's plot experiment[39]was carried out by keeping the total concentration of **1** and Fe^{3+}ions at 10 μM and changing the molar ration of Fe^{3+} ([Fe^{3+}]/[**1**+ Fe^{3+}]) from 0 to 1. As shown in Figure 4, the result shows that a maximum at a molar fraction of 0.5, indicating the formation of 1:1complex of **1** and Fe^{3+}. The 1:1 complexation is also supported from ESI-MS spectra analyses (Fig. S4), which show a prominent peak at 844.16 (calculated value, 844.22) due to $[1+ Fe^{3+}+2Cl^-)]^+$.

(Figure 4)

The association constant (*K*a) of **1**–Fe^{3+} complexes was determined by the Benesi–Hildebrand Eq. (1) [40]:

$$\frac{1}{F - F_o} = \frac{1}{\{K_a \times (F_{max} - F_o) \times [Fe^{3+}]\}} + \frac{1}{F_{max} - F_o} \qquad (1)$$

Where *F* is the fluorescence intensity at 588 nm at any given Fe^{3+}concentration, F_0 is the fluorescence intensity at 588 nm in the absence of Fe^{3+}, and F_{max} is the maxima fluorescence intensity at 588 nm in the presence of Fe^{3+} in solution. The association constant *K*a was evaluated graphically by plotting 1/ ($F-F_0$) against 1/[Fe^{3+}] (Figure 5). Data were linearly fitted according to Eq. (1) and the *K*a value was obtained from the slope and intercept of the line. The *K*a value of 1- Fe^{3+} complexe was 5.43×10^3 M^{-1}. The detection limit, based on the definition by IUPAC (C_{DL} =3Sb/m) [41], was found to be 7.68×10^{-7} M from 10 blank solutions, which is allowed for the detection of micromolar concentration range of Fe^{3+}. All of these suggested that the receptor can be used as a selective fluorescent sensor for Fe^{3+} in environmental analysis and analytical chemistry.

(Figure 5)

To check the practical applicability of **1** as a selective fluorescent sensor for Fe^{3+}, competition experiment, the systems of other metal ions and Fe^{3+} coexisted was examined in aqueous ethanol. As shown in Figure 6, we found that all the coexistent metal ions had no obvious interference with the detection of Fe^{3+}.

(Figure 6)

To investigate the binding mechanism, IR spectra experiments of **1** and **1**-Fe^{3+} complexs were measured (Figure 7). The IR spectra were primarily characterized by

band in the double-bond region. Two bands, 1681cm^{-1} and 1624 cm^{-1}, were associated with the amide carbonyl and ketone carbonyl absorption in chemosensor **1**, respectively. Upon addition of Fe^{3+}, the carbonyl groups IR peak at 1681cm^{-1} and 1624 cm^{-1} disappeared and a new IR peak appeared at 1631 cm^{-1}. This suggests that the ketone and amide carbonyl O of **1** is actually involved in the coordination with Fe^{3+}.

(Figure 7)

According to the above experiments, a proposed binding mechanism of Fe^{3+} with **1** was shown in Scheme 2. As it would be expected, the Fe^{3+} binds with **1**, and opens the spirolactam ring that results in the fluorescence enhancement and development of pink color.

(Scheme 2)

To access the feasibility of Fe^{3+} detection in living cells, the fluorescence imaging was recorded using confocal fluorescence microscopy. Figure 8 shows the results of confocal fluorescence imaging of Fe^{3+}in live HeLa cells. As expected, HeLa cells incubated with **1** gave no intracellular background fluorescence (Figure 8a). By contrast, when **1** deposited HeLa cells were incubated with 10μM Fe^{3+} for 30 min, intense red fluorescence was observed (Figure 8c). It is well known that the Fe^{3+} could be taken up into the living cells by diffusion through porins, and transport by transferrin and lactoferrin[42]. So, we conclude the remarkable fluorescence

enhancement was because of the complexation of **1** with Fe^{3+} in the living cells. An overlay of fluorescence and bright-field images (Figure 8d) shows that the fluorescence signals is localized in the intracellular area, indicating a subcellular distribution of Fe^{3+} and good cell-membrane permeability of chemosensor **1**.

(Figure 8)

4. Conclusion

In summary, we demonstrated a novel fluorescent chemosensor **1** for Fe^{3+} and its bioimaging applications. **1** exhibited a turn-on fluorescent response for detecting Fe^{3+} with excellent selectivity over other metal ions. Moreover, fluorescent microscopy experiments indicated that **1**could be used as fluorescent probe for sensing Fe^{3+} in living cells.

Acknowledgments

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Captions:

Figure 1. Absorption spectral changes of compound 1 (10 μM) in EtOH/water (9:1, v/v) containing HEPES buffer (10mM, pH =7.2) upon additions of various metal ions (100 μM). Inset: the color changes of **1** before and after addition of Fe^{3+}.

Figure 2. fluorescence spectral changes of compound **1** (10μM) in EtOH/water (9:1, v/v) containing HEPES buffer (10mM, pH =7.2) upon additions of various metal ions (50 μM). λ_{ex} = 510 nm. Inset: color of **1** and **1**+Fe^{3+} system under UV lamp.

Figure 3. Emision spectra of **1** (10μM) in EtOH/water (9:1, v/v) containing HEPES buffer (10 mM, pH =7.2) upon the addition of Fe^{3+} (0–10eq) at 25ºC.

Figure 4. Job' plot of **1** and Fe^{3+}. The total concentration of **1** and Fe^{3+} was kept at a fixed 10μM.

Figure 5. Benesi–Hildebrand plot of sensor **1** with Fe^{3+}.

Figure 6. Competitive experiments in the **1**+Fe^{3+} system with interfering metal ions. [**1**] = 10 μM, [Fe^{3+}] = 100 μM, and [M^{n+}] = 100 μM. λ_{ex} = 510 nm.

Figure 7. Infrared spectra (KBr) of free **1** and **1**-Fe^{3+} complex at room temperature.

Figure 8. Fluorescence images of Fe^{3+} in HeLa cells. (a) Fluorescence images of HeLa cells treated with **1**; (b) bright-field image of cells shown in panel; (c) fluorescence images after presence of Fe^{3+}; (d) overlay image of (b) and (c)

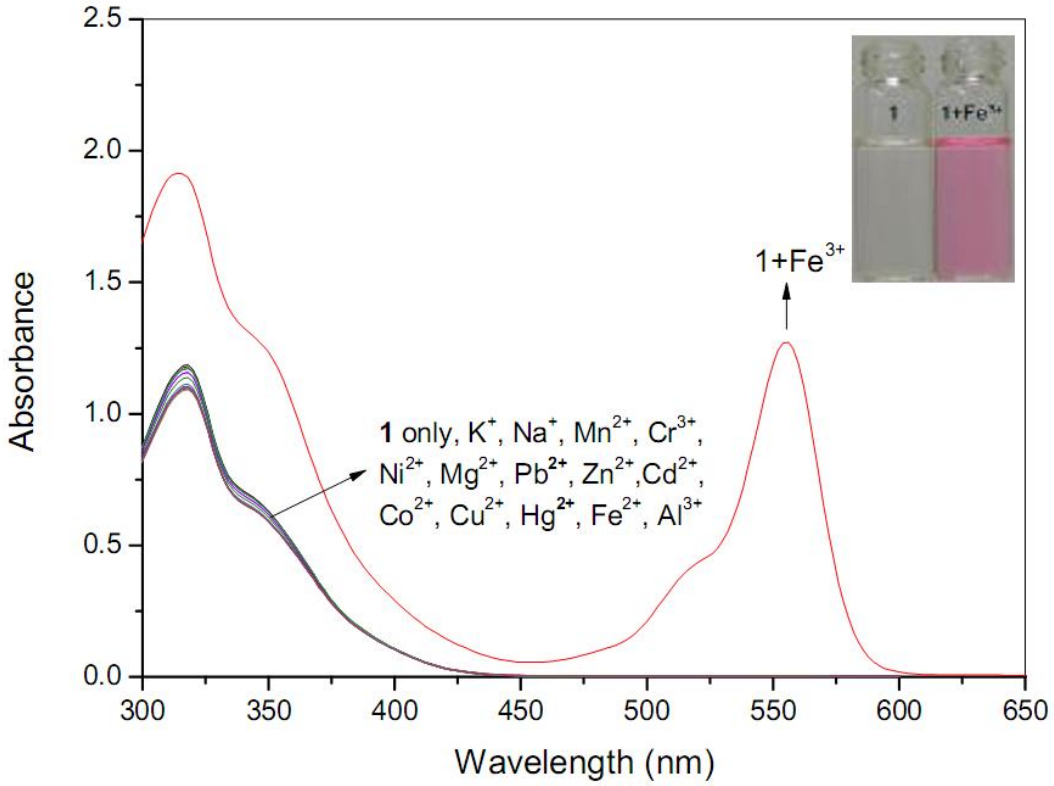


Figure 1

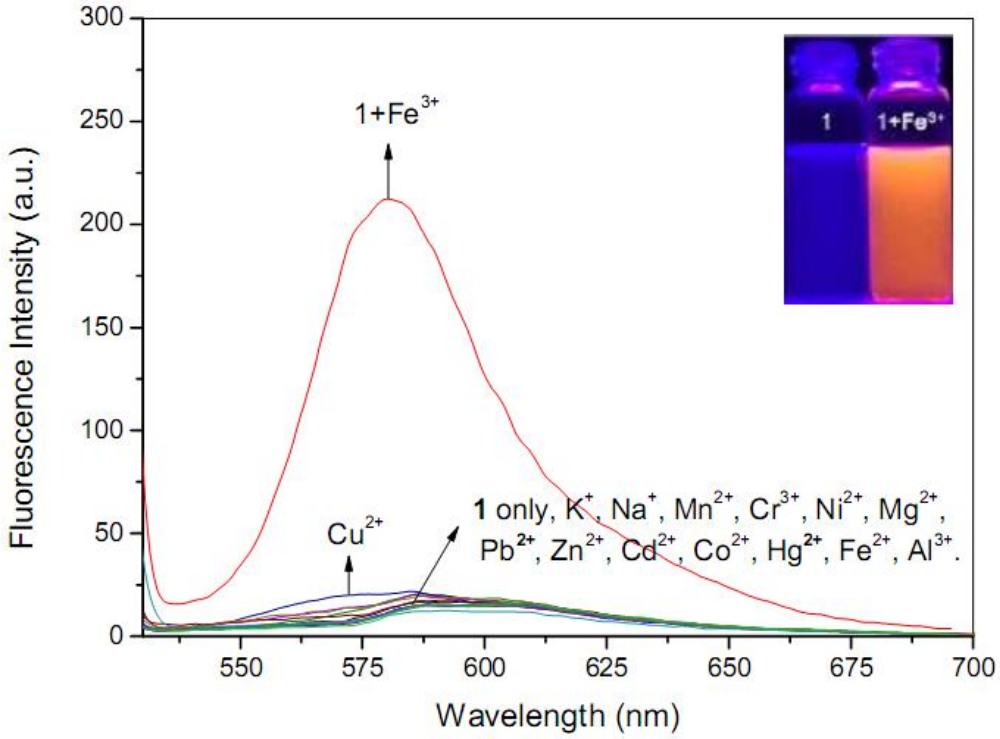


Figure 2

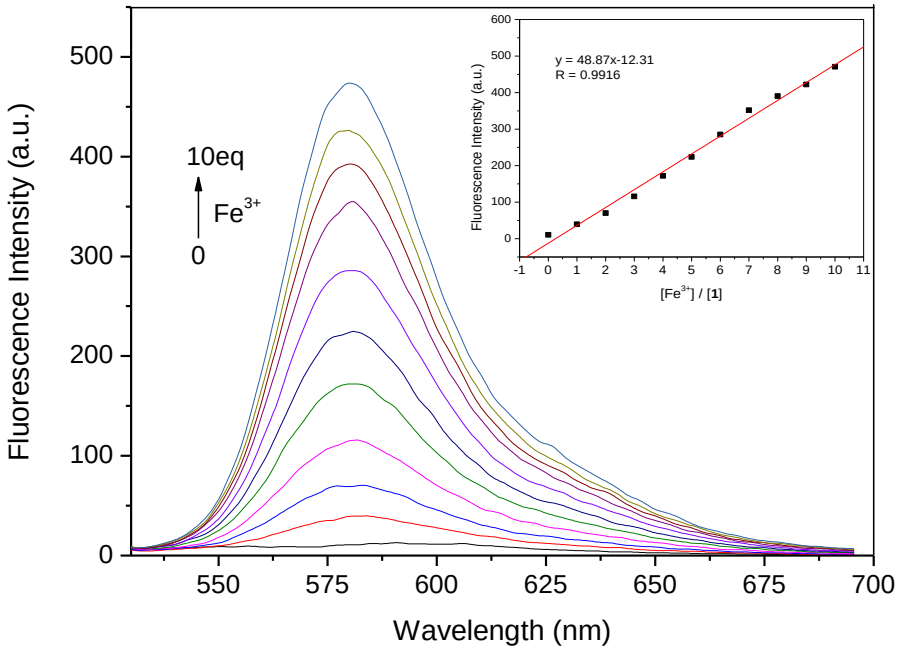


Figure 3

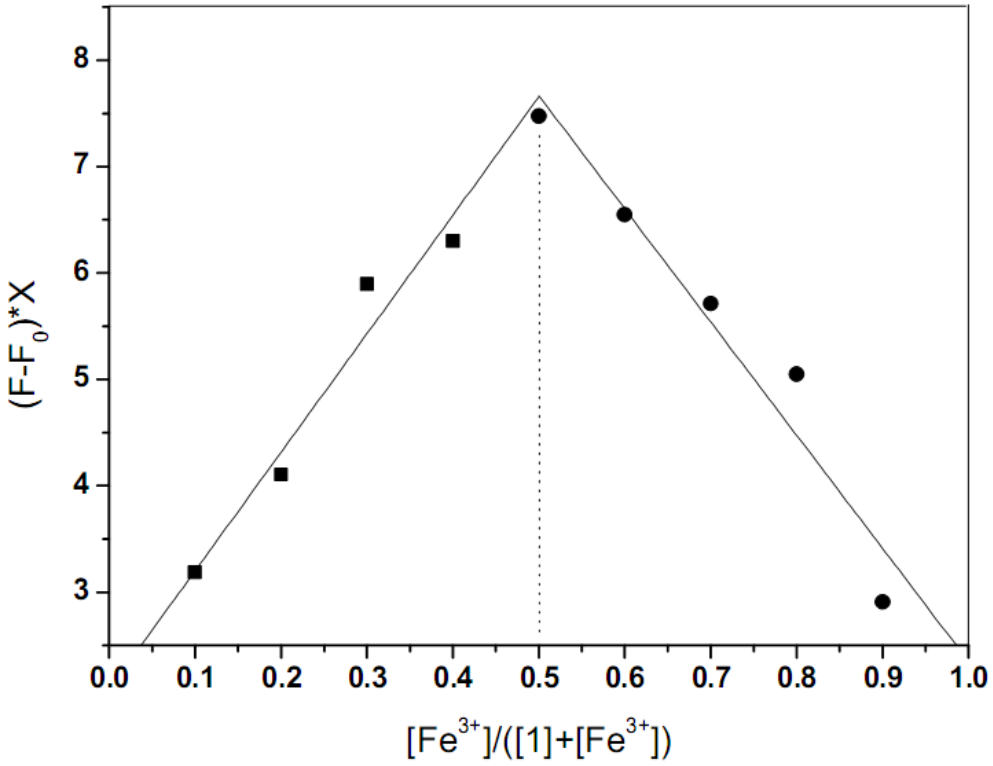


Figure 4

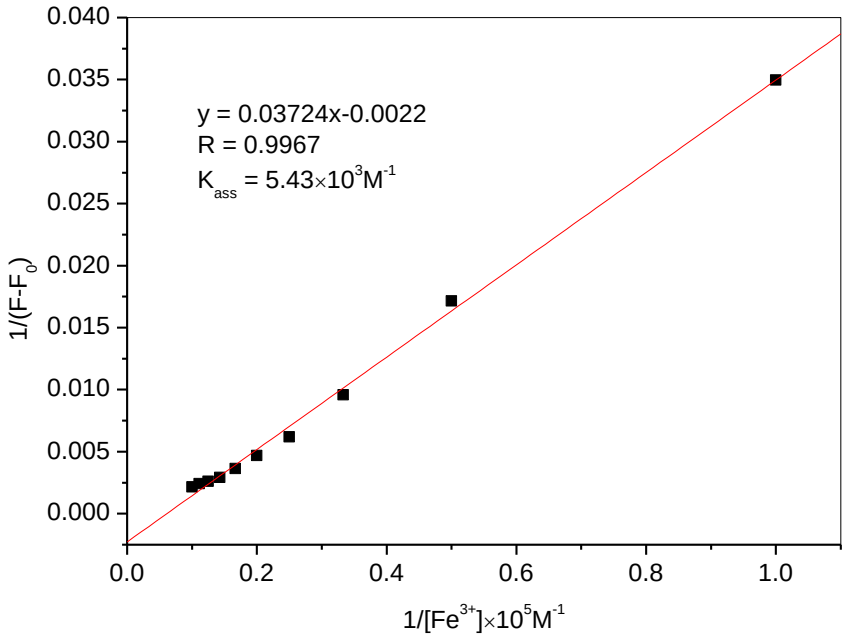


Figure 5

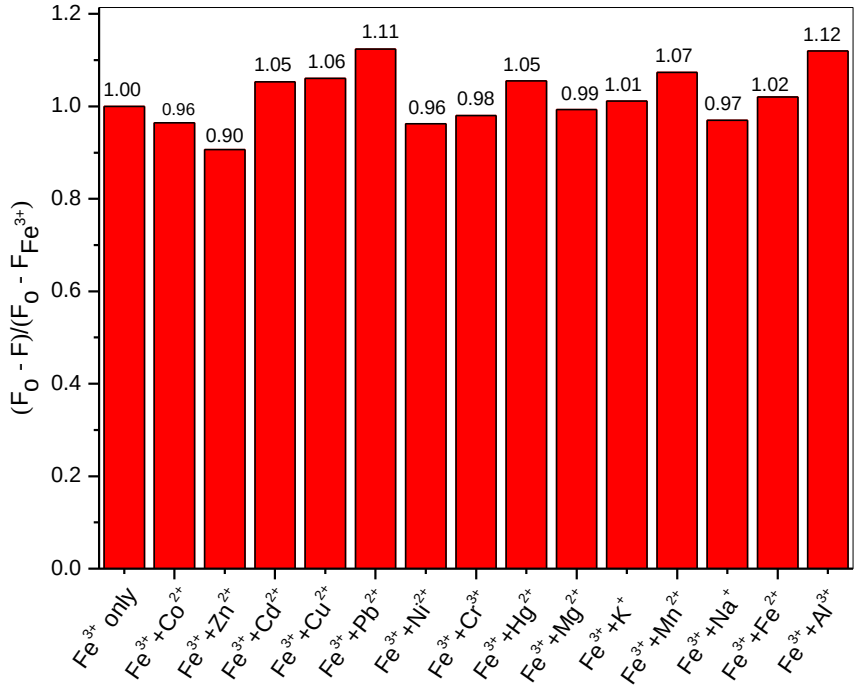


Figure 6

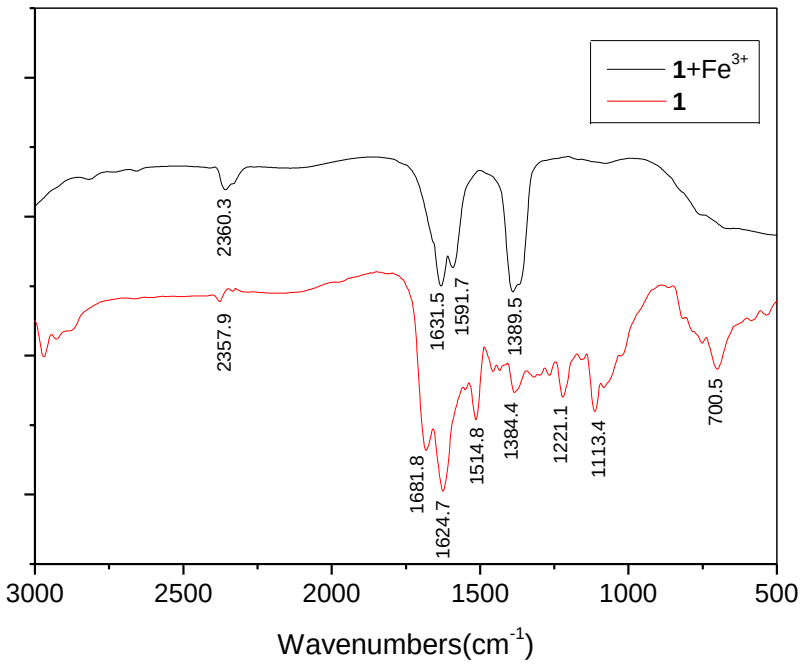


Figure 7

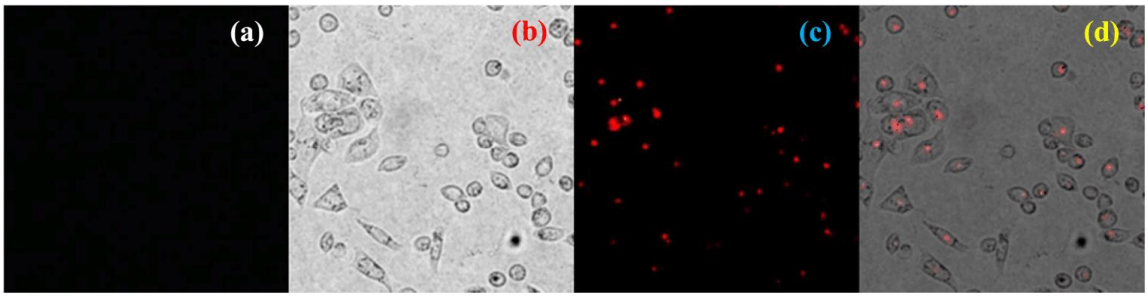
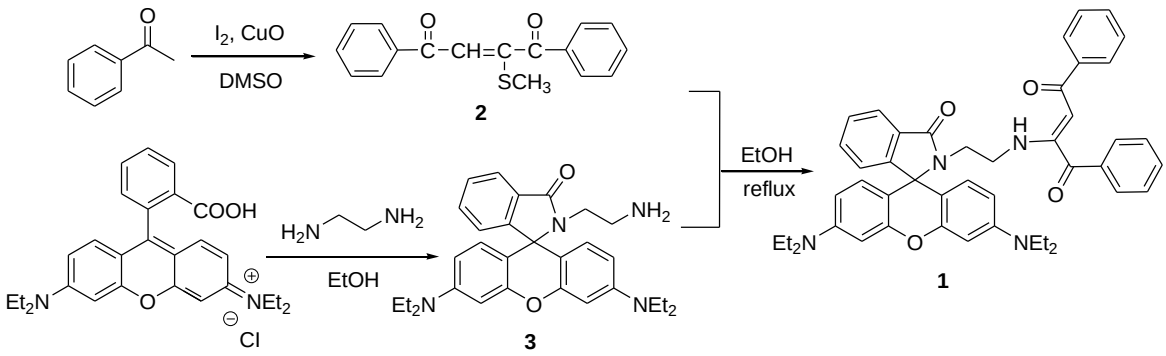


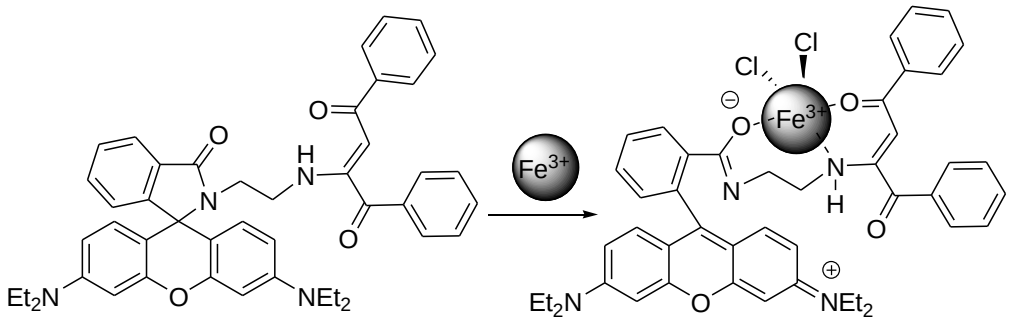
Figure 8

Scheme 1. Synthesis of rhodamine derivate **1**

Scheme 2. Proposed binding mechanism for **1** with Fe^{3+}.



Scheme 1



$[M+H]^+ = 719.78$ $[M+Fe^{3+}+2Cl^-]^+ = 844.16$

Scheme 2.