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ORIGINAL ARTICLE

Pyrene Based Fluorescent Turn-on Chemosensor for Sequential Detection of Fe^{3+} and Fe^{2+} Ions and its Application in Live Cell Imaging

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Abstract

A simple pyrene-based "turn-on" chemosensor bearing isonizide namely 3-(pyren-1-yl methylene) pentane-2,4-diyldene di(isonicotinohydrazide) (PMPD) was designed and synthesized for detecting $Fe^{3+}$and Fe^{2+} ions in HEPES buffer solution at pH 7.4 (DMSO: H_2O (1:9 *v*/*v*)) medium. The probe shows conducive selectivity for Fe^{2+} and Fe^{3+} ions over the other competitive metal cations. The detection limit was found to be 1.67 μM for Fe^{3+} and 2.02 μM for Fe^{2+}. The recognition mechanism of PMPD towards Fe^{3+} and Fe^{2+} has been examined in detail by absorption, emission, and ESI-MS studies. Moreover, "turn-on" fluorescence behavior of the probe was used to track iron ions in living cells.

Keywords Schiff base · Fluorescence probe · Iron ions · Cell imaging

Introduction

In recent years, devise and synthesis of chemosensors that can detect environmentally and biologically significant metal cations and anions have been of special interest, because of the widespread use of ions in industry, medicine, biology and the environment [1–4]. Many chemosensors have been developed for selective detection of diverse ions due to their advantages of high selectivity and sensitivity even at very low concentration in short time over various analytical methods have been applied including inductively coupled plasma mass spectrometry, atomic absorption spectrometry and electrochemical assays [5–8].

Metal cations, particularly transition metal ions such as Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+}, Pb^{2+} take part major role in the surroundings and human health. Especially, presence of iron ions in human body is important due to its diverse applications in several biological activities such as enzymatic reaction, oxygen-carrying, cellular metabolism and other various bio-syntheses [9, 10]. At cellular level of many biological systems, ferric iron (Fe^{3+}) plays significant role [11]. However, any imbalance in the mobile iron ions pool may results in various diseases. The occurrence of inferior amount of iron ions in the human body could cause diseases such as liver damage, diabetes, cancer and hemochromatosis [12–14], whereas a higher concentration of it induces Fenton reaction producing reactive oxygen species (ROS), causing damage to lipids, nucleic acids and proteins [15–17] which in turn speed up creation of numerous severe diseases like Alzheimer's, Parkinson's, etc. [18–23]. Therefore, it is essential to design and develop new sensors for detecting trace amount of iron ions in biological systems.

In the recent past, a plethora of receptors reported by researcher described the advances of Fe^{3+} sensors, most of them exhibited fluorescence quenching because of the paramagnetic character of Fe^{3+} ions. Recently, few reports regarding "turn on" fluorescent chemosensors are found in the literature. However, these systems have some snags like severe reaction conditions, chemical and physical interference due to similarities of coexisting metal ion or inadequate biocompatibility [24–26]. Hence, extensive attention is given to overcome the above challenges in the design of simple and handy "turn on" fluorescent probes for the detection of Fe^{3+}. Moreover, iron

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present in its Fe^{2+} form together with Fe^{3+} ions in biological systems. Natural catalytic cycles were utilized to stabilize the oxidation states of iron. Hence, it is too worthwhile to design probes that detect iron in its Fe^{2+} form.

To account all, herein we have synthesized a simple pyrene based Schiff base sensor 3-(pyren-1-yl methylene) pentane-2,4-diyldene di(isonicotinohydrazide) (PMPD) in which isonicotinohydrazide moiety is the binding sites and pyrene moiety is the signaling unit for the metal ion detection. The probe is designed to maximize the fluorescence quenching due to photoinduced electron transfer (PET). Upon interaction of the target metal ion with PMPD, inhibition of PET from isonicotinohydrazide moiety to the pyrene fluorophore occur, in turn it is expected turn-on" fluorescence response.

Experimental

Methods and Materials

All the chemicals were purchased from Sigma-Aldrich and commercially available analytical grade solvents were used without further purification. Fresh double distilled water was used throughout the experiments. Melting points were checked in open capillary tubes on a Technico micro heating table and were uncorrected. Analytical data have been obtained by Vario EL III Elemental analyzer. UV-Visible and fluorescence spectra were recorded on Shimadzu 1800 and JASCO FP-8200 spectrophotometer respectively using a 1 cm square quartz cell in DMSO–H_2O (1:9 *v*/v) HEPES buffer solution (pH 7.4). ^{1}H-NMR spectra were measured in DMSO-d_6 or $CDCl_3$ on a Bruker AMX-500 spectrometer at 400 MHz. The chemical shifts (δ) recorded in ppm with reference to tetramethylsilane (TMS). Electrospray ionization mass spectra (ESI-mass) were collected on an advanced Q-TOF micro™ mass spectrometer. Cell imaging was done on the (Eclipse Ti-U, Nikon, USA) fluorescence microscope.

Synthesis of Probe (PMPD)

The probe was synthesized by two steps. First 3-(pyren-1-ylmethylene)pentane-2,4-dione (1) was prepared according to reported procedure [27]. Using the compound 1, following procedure was used to synthesize the probe PMPD.

To a hot ethanol solution (20 mL) of compound 1 (0.314 g, 1 mmol), isonicotinic acid hydrazide (0.274 g, 2 mmol) was added and stirred for 3 h. After completion of the reaction, the precipitate formed was filtered, washed with ethanol to afford a pure pale yellow color compound. Yield: 75%, 0.43 g; M.pt.: 230–240 °C. Anal. Calc. for $C_{34}H_{26}N_6O_2$: C, 74.17; H, 4.76; N, 15.26%. Found: C, 73.62; H, 4.52; N, 14.78%. UV-vis (DMSO, λ_{max}, nm): 290, 380, 401. ^{1}H NMR (DMSO-*d6*, ppm): 12.26 (s, 2H) 9.54 (s, 1H) 9.12–8.41 (d, Ar H) 8.15–8.17 (t, 1H) 1.23 (s, H) ^{13}C-NMR (DMSO-*d6*, ppm): 164.68, 162.12, 148.50, 140.77, 132.58, 131.24, 130.68, 129.08, 127.73, 127.18, 126.12, 125.59, 122.56, 121.52, 117.16, 113.65. (Figure S1 and S2) MS (ESI, *m/z*) 551.2 $[M–H]^+$ (Fig. S4).

Synthesis of PMPD-Fe Complex

To a hot solution of methanol and chloroform mixture (20 mL, 1:1 *v*/v), 0.276 g (0.50 mmol) of the probe and 0.241 g (0.50 mmol) of $FeCl_3·2H_2O$ were added. After stirred for 1.0 h at 65 °C, the orange color complex formed was filtered, washed with cold methanol, dried and subjected to spectroscopic analysis. The complex is air-stable and soluble in DMSO and DMF.

Yield: 80%, 0.68 g; M.pt.: 246–248 °C; Anal. Calc. for $C_{35}H_{29}N_6O_2Cl_2Fe$: C, 60.71; H, 4.22; N, 12.14%. Found: C, 60.24; H, 3.78; N, 11.65%. UV-vis (DMSO, λmax, nm): 290, 384, 405. ^{1}H NMR (DMSO-*d6*, ppm): 9.58 (s, 1H) 9.14–8.41 (d, Ar H) 8.15–8.17 (t, 1H) 1.23 (s, 6H) (Fig. S3) MS (ESI, *m/z*) 656.4 $[M–H]^+$ (Fig. S5).

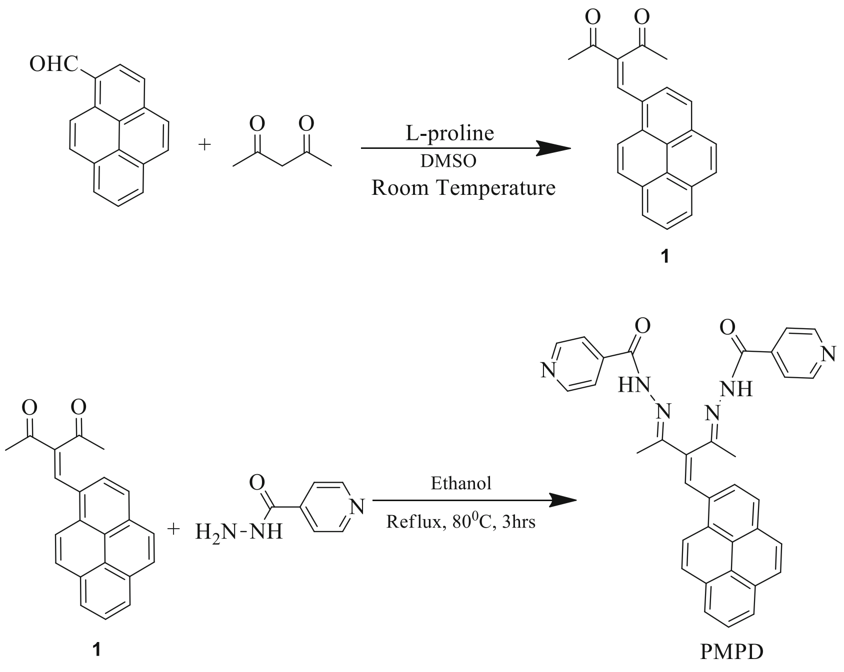
Results and Discussion

The receptor PMPD has been synthesized in a two-step reaction sequence as shown in Scheme 1. The receptor was further characterized by elemental analysis, ^{1}H-NMR and ESI-mass spectral studies.

UV-Visible Spectral Study

The cation recognition property of the probe PMPD was primarily studied by absorption spectroscopy. The absorption spectra of probe (PMPD) with various biologically important metal ions like Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+}, Mg^{2+}, Pb^{2+}, Ag^{+}, Mn^{2+}, Fe^{3+} and Al^{3+} with HEPES buffer in DMSO: H_2O (1:9 *v*/v) at pH 7.4 medium were recorded. As shown in Fig. 1, there is no appreciable changes in absorptions were observed throughout the addition of metal ions with PMPD except iron ions. When iron ions (Fe^{3+} and Fe^{2+}) were added to PMPD, peaks at 290, 380 and 405 nm slightly shifted to red shift with increase in their absorption intensity. During the addition of iron ions, color of the receptor solution changes from pale yellow to greenish yellow. The color change is visible to the naked eye under UV lamp. This observation indicates that iron ions specifically interact with probe than the other metal ions. Besides, in order to determine the extent and strength of binding of Fe^{3+} ions with receptor, UV-Vis titrations (Fig. S6) were done. It was carried out by gradual addition of a standard solution of Fe^{3+} ions (0–100 μM) to the solution of PMPD. The results show that the absorption band intensity was gradually increased at 380 nm (Figure S6)

Scheme 1 Synthetic scheme for the receptor (PMPD)



accompanied with red shift and reaches maximum at 100 μM. The association constant (Ka) for PMPD with Fe^{3+} ions was determined from UV-Vis titration experiment data by non-linear fitting method and it was found to be 2.87×10^6 M^{-1}. A similar tendency for the addition of Fe^{2+} ion with PMPD was observed in the UV-Vis absorption measurements (Fig. S7).

Fluorescence Spectral Study

To find the binding ability of PMPD towards Fe^{3+} and Fe^{2+} ions, emission study was carried out with HEPES buffer at pH 7.4 (DMSO: water (1:9)) medium. The probe PMPD shows very week emission at 480 nm ($\lambda_{ex} = 380$ nm). After confirming the fluorescence property of PMPD, its sensing capability was analyzed. On addition of various metal cations (Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+}, Mn^{2+}, Ag^+, Zn^{2+}, Cd^{2+}, K^+, Pb^{2+} and Al^{3+}) to the PMPD, there was no significant fluorescence enhancement was noticed at 480 nm except in the case of Fe^{3+} ions. On addition of Fe^{3+} ions to PMPD a "turn-on" fluorescence emission response was observed due to inhibition of photoinduced electron transfer (PET) mechanism caused by the electron transfer from the electron donor nitrogen atom to the π-conjugated pyrene fluorophore (Fig. 2).

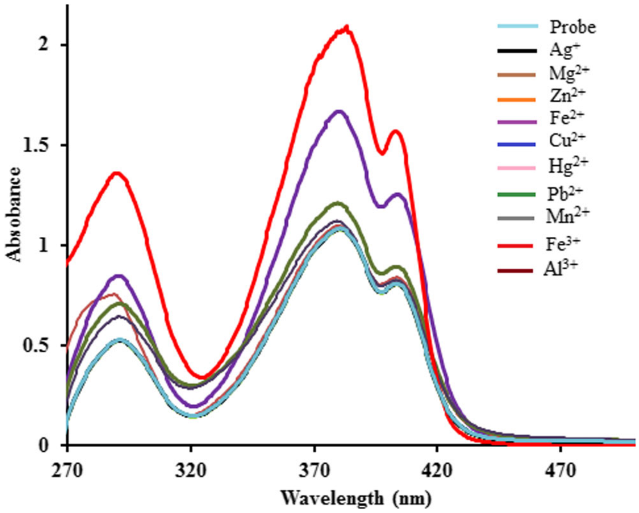


Fig. 1 UV-Vis spectra of probe PMPD (10 μM) in DMSO/H2O (1:9 (*v*/v), 50 mM HEPES, pH = 7.4) with 100 μM of metal ions (Ag^+, Mg^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Mn^{2+}, Fe^{3+} and Al^{3+})

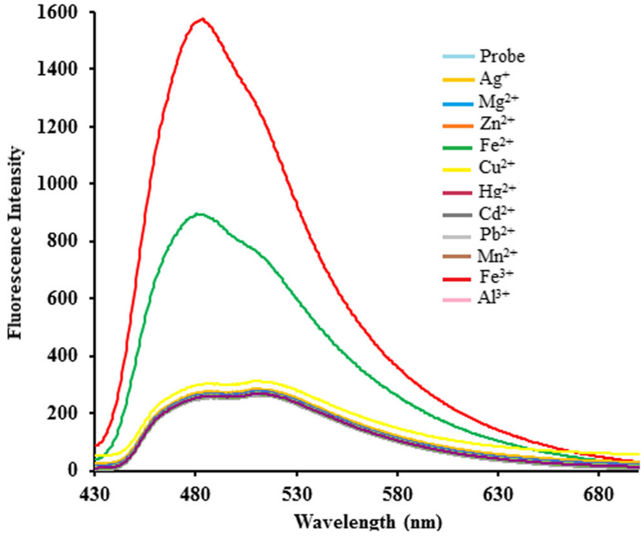


Fig. 2 Fluorescence response of probe PMPD (10 μM) in DMSO/H_2O (1:9 (v/v), HEPES = 50 mM, pH = 7.4) with 100 μM of metal ions (Ag^+, Mg^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, Mn^{2+}, Fe^{3+} and Al^{3+}) (λ_{ex} = 380 nm)

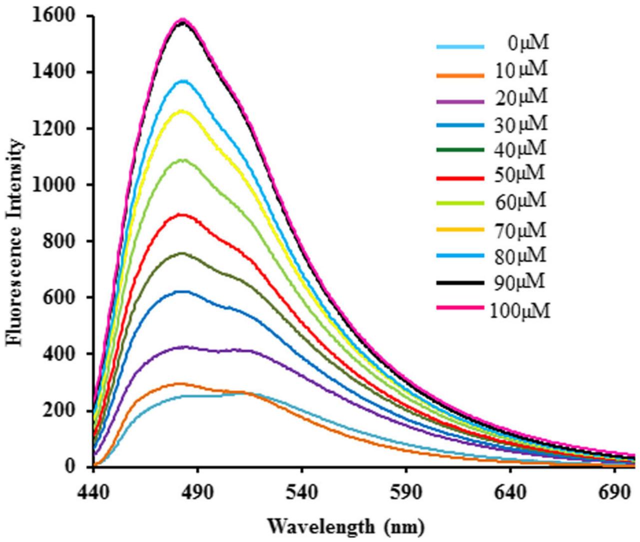


Fig. 3 Fluorescence response of the probe PMPD (10 μM) in DMSO/H_2O (1:9 (v/v), HEPES = 50 mM, pH = 7.4) with 0–100 μM of Fe^{3+}

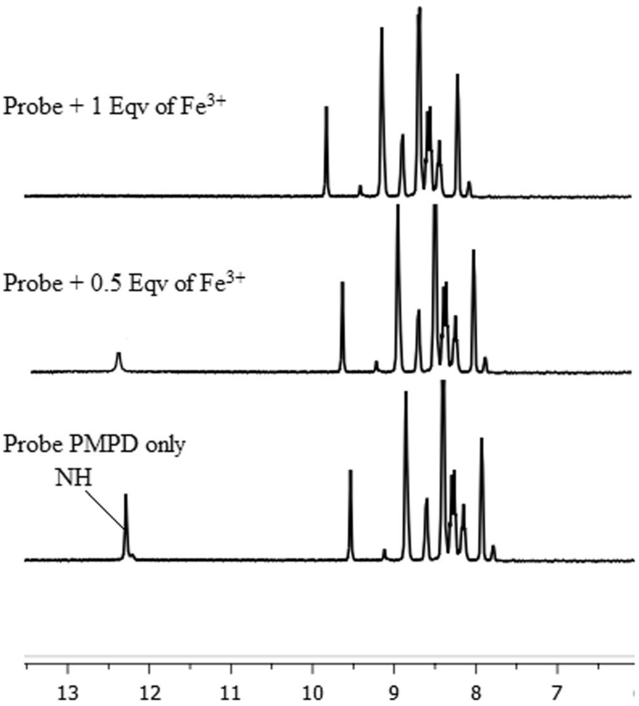


Fig. 5 ^1H NMR titration of probe PMPD with Fe^{3+} ions

To examine the binding affinity of PMPD towards Fe^{3+} ions further, the fluorescence titration experiment was carried out for PMPD for increasing quantity of Fe^{3+} ions (Fig. 3). The fluorescence enhancement of PMPD was steadily increased with the rising amount of Fe^{3+} ions (0-100 μM). The plot of fluorescence intensity at 480 nm vs Fe^{3+} concentration (0–100 μM) shows a linear relationship indicates the receptor PMPD is more appropriate for quantitative recognition of Fe^{3+} ions. The limit of detection (LOD) of Fe^{3+} was measured to be 1.67 μM according to the 3σ method (Fig. S8), which is comparable to those of recently reported fluorescence probes for Fe^{3+} ions detection (Table S1). Moreover, the emission intensity changes upon incremental addition of Fe^{2+} ions (0-100 μM) to PMPD were also examined. The results show that a similar tendency in enhancement was noticed (Fig. S9) and the detection limit was found to be 2.02 μM (Fig. S10). The association constant (K_b) was measured by Benesi-Hildebrand equation from fluorescence titration experiment (Fig. S11 and S12) and the values were calculated to be 3.36 and 3.12 μM^{-1} for Fe^{3+} and Fe^{2+} respectively. The high binding constant and low detection limit values show that PMPD could be a valuable receptor for the detection of iron ions.

To evaluate the activity of newly designed probe, selectivity and interference are two important parameters. Especially for probes having biomedical applications, a selective response towards target over various competing species is basically needed. Therefore, the selectivity study of probe to Fe^{3+} over various metal ions (K^+, Mg^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+}, Ag^+, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Pb^2 and Al^{3+}) was conducted. Moreover,

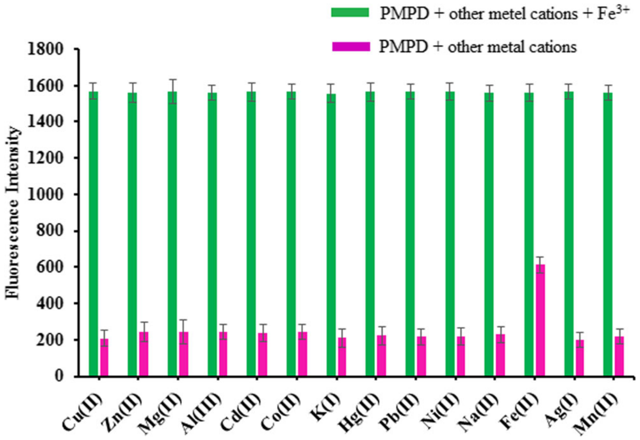


Fig. 4 Interference studies of probe PMPD + other cations, PMPD + other cations with Fe^{3+}in DMSO/H2O (1:9 (v/v), 50 mM HEPES, pH = 7.4) (λex = 380 nm)

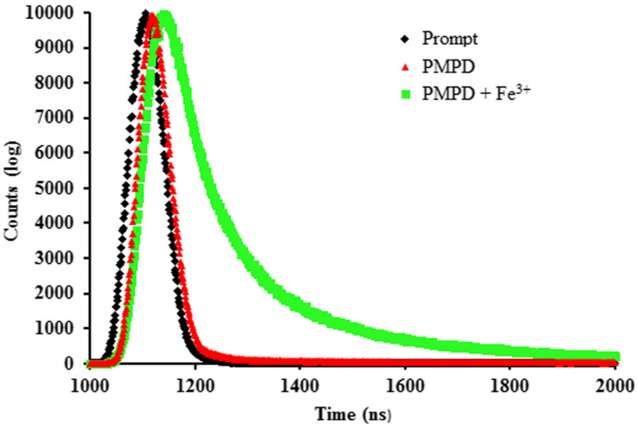
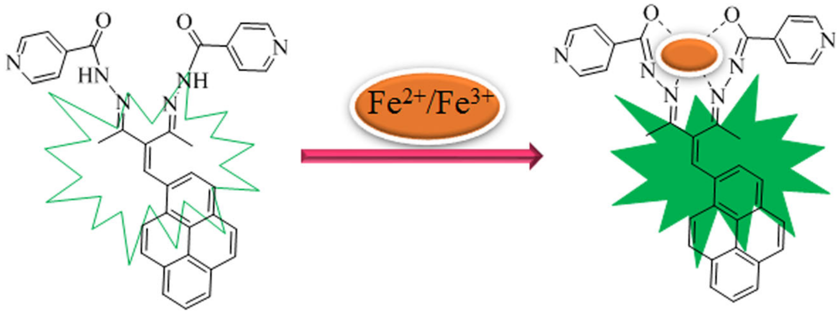


Fig. 6 Fluorescence lifetime decay profiles of probe PMPD and PMPD with Fe^{3+} ion

Scheme 2 Possible mechanism for Fe^{3+}/Fe^{2+} ions detection of PMPD



interference from all the above metal ions for Fe^{3+} detection by probe was also investigated. As shown in Fig. 4 and S13, the probe has shown excellent selectivity and interference for Fe^{3+} and Fe^{2+} detection among above mentioned metal ions. After establishing the binding of PMPD with Fe^{3+} and Fe^{2+} ions, the Job's plot was drawn from fluorescence titration data to determine binding stoichiometry (Fig. S14 and S15). It can be seen that maximum value is found at the mole fraction of 0.50 and 0.51 indicate 1:1 binding stoichiometry. In addition, appearance of molecular ion peak at m/z, 656.4 in the mass spectrum of PMPD-Fe^{3+} ensemble confirms the formation of PMPD-Fe^{3+} complex with 1:1 stoichiometry. The formation of PMPD+Fe^{3+} complex was further proved from ^1H NMR studies (Fig. 5). The probe shows peaks at 12.2 ppm due to amide NH proton. When half equivalent Fe^{3+} ions were added to the probe, intensity of the NH peak decreases. After addition of one equivalent Fe^{3+} ions to the probe, the NH peak completely disappears shows the coordination of Fe^{3+} with the probe via oxygen due to enolisation followed by deprotonation.

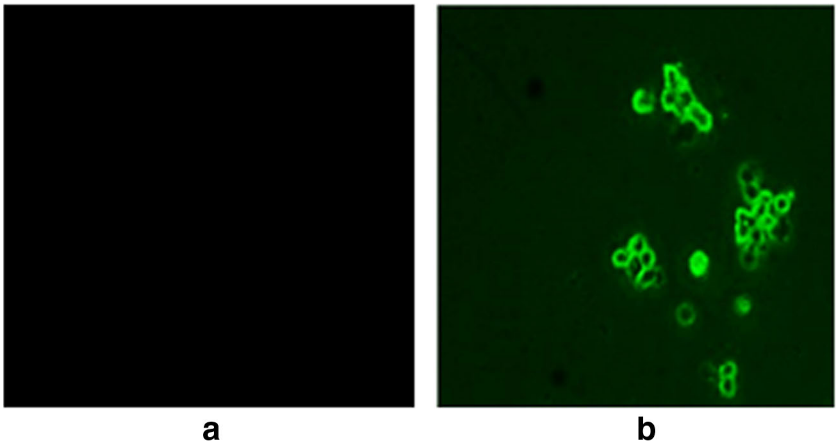
To examine the performance of the probe under physiological conditions, emission spectra of PMPD and PMPD-Fe^{3+}/Fe^{2+} ensembles were recorded under different pH conditions (2–14). As shown in Fig. S16 and S17, the PMPD could response the detection of iron metal ions in 4–9 pH range which is usually suitable for any biological applications. To evaluate the time-dependent measurement of PMPD towards Fe^{3+} ions, the fluorescence life time measurement of PMPD and PMPD-Fe^{3+} ensemble have been done using time-correlated single photon counting (TCSPC) (Fig. 6) and the average life time was calculated using standard methods [28]. The average life time of the PMPD is 1.47 ns, which was significantly increased to 1.99 ns upon the addition of Fe^{3+} ions.

Based on the above results, a possible mechanism for Fe^{3+}/Fe^{2+} detection of probe PMPD was proposed as in Scheme 2.

Application in Bio Imaging

Before cell imaging study, cytotoxicity of PMPD was investigated on living NCCS cells by MTT assays. The results indicate that, up to 50 μM and 24 h, PMPD have not imposed any noticeable cytotoxicity on the cells, suggests 10 μM PMPD possesses nil toxicity for NCCS cells under the experimental conditions (Fig. S18). Therefore, cell imaging studies were done using the non-toxic probe PMPD by fluorescence microscopy. Owing to the weak fluorescence nature, the probe does not exhibit any substantial fluorescence when NCCS cells have been incubated with 10 μM of probe (Fig. 7), while the cells have been treated with 20 μM solution of Fe^{3+} ions, cell fluorescence turned "on" which is acquired with a fluorescence microscope. The observation reveals that the probe PMPD is cell-permeable and quantitatively applicable for detecting Fe^{3+} ions through the formation of intracellular PMPD-iron complex in living cells.

Fig. 7 Fluorescence images of (NCCS) cells treated with (**a**) PMPD (10 μM), (**b**) PMPD (10 μM) + Fe^{3+} (15 μM)



Conclusions

In conclusion, we rationally designed a new fluorescent probe PMPD for detection of Fe^{3+} and Fe^{2+} ions via turn-on fluorescence response. The working mechanism underlying the detection is the chelation between Fe^{3+}/Fe^{2+} and PMPD yields the PMPD-Fe^{3+}/Fe^{2+} complex, accompanied by fluorescence "ON" due to the PET off effect. The detection limit for Fe^{3+} and Fe^{2+} ions was found to be 1.67 and 2.02 μM, respectively. Moreover, with its property of fluorescence switch, PMPD was successfully applied to image Fe^{3+} in living NCCS cells. These results show that the probe PMPD could be useful as a favorable candidate for real-time detection of Fe^{3+} and Fe^{2+} ions.

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