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A novel "off-on" rhodamine-based colorimetric and fluorescent chemosensor based on hydrolysis driven by aqueous medium for the detection of Fe^{3+}

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ABSTRACT

A novel "off-on" colorimetric and fluorescent chemosensor N^2,N^6-bis(2-(rhodamine B amido)ethyl)pyridine-2,6-dicarboxamide (RhBEP) has been designed and synthesized, which can selectively detect the presence of Fe^{3+} with about 75-fold enhancement in fluorescence emission intensity at 585 nm over various environmentally relevant metal cations such as Hg^{2+}, Cu^{2+}, Cr^{3+}, Li^{+}, Na^{+}, K^{+}, Ag^{+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, Al^{3+}, Bi^{3+} and Au^{3+} in PBS reaction media. The remarkable color change from UV-Vis titration experiments indicates that RhBEP can be used as a colorimetric chemosensor for Fe^{3+}. The ultrasensitive detection limit for Fe^{3+} in the fluorescence measurement is down to 2.0×10^{-8} mol·L^{-1}. The recognition mechanism of RhBEP to Fe^{3+} was analyzed by Job's plot and mass spectrometry analysis. In addition, the data from fluorescent cell imaging experiments confirmed that the chemosensor RhBEP has a promising application for the detection of Fe^{3+} in biological systems.

Keywords: Chemosensor; Rhodamine B; Fe^{3+}; Fluorescence enhancement; Living cell imaging

1. Introduction

Fe^{3+} plays an important role in living organisms since it is an important component of hemoglobin, myoglobin and various other enzymes, which can promote the metabolism of vitamin B [1]. In cellular biochemical reaction, Fe^{3+} is an indispensable ion for most organisms due to its role in biological signal transmission and material transportation [2-4]. As one of the most abundant transition metal ions in the human body, both the lack and the excess of Fe^{3+} can cause various dysfunctions of organisms. For instance, the lack of Fe^{3+} can affect the synthesis of hemoglobin and myoglobin and reduce the activities of cytochrome C and ribonucleotide reductase, etc [5-7]. Because these enzymes are closely linked to tissue respiration, bio-oxidation and neurotransmitter's decomposition and synthesis, the lack of Fe^{3+} can cause the decreases of immunity, intelligence and anti-infective capacity, as well as can affect the body's ability in regulating temperature, thereby can lead to neurological disorders [8, 9]. However, excessive intake of Fe^{3+} can cause an imbalance of iron ion in blood, which can lead to oxidative damage to cells and reduced blood circulation to the heart [10-12]. Therefore, it is important to find a fast and sensitive method to identify the distribution of Fe^{3+} in living organisms.

Although some methods have been developed for the detection of Fe^{3+} in the past few decades, these methods have some disadvantages, including low specificity, complicated sample preparation and operation, requirement of skilled personnel, and costly instruments [13-19]. Thus, they are not ideal for the detection of Fe^{3+} in living organisms. In recent years, fluorescence method has been rapidly developed and turned into one of the most extensively used techniques in the field [20-27]. It has various advantages, such as high sensitivity and selectivity, rapid analysis, and simplicity in instrument operation. It does not cause damage to cell while can generate

visually observed signals. Actually, Fluorescence quenching due to the paramagnetic properties of Fe^{3+} when binding of fluorescent chemosensors to Fe^{3+} may result in the difficulty in the detection of Fe^{3+} [28-32]. Compared with the quenchable chemosensors, the enhanced fluorescent chemosensors ("turn-on" or "off-on") have stronger signal intensity, cannot be easily interfered by background interference, and can be used for the detection *in vivo* [33-36]. Thus, it is of great significance to develop an "off-on" fluorescent chemosensors with high sensitivity, selectivity and rapid response for Fe^{3+}.

As we know from the hard-soft acids and bases theory, Fe^{3+} is a hard acid and O and N are hard bases, which can be relatively easily combined. In this paper, we reported the design and synthesis of an "off-on" rhodamine-based chemosensor N^2,N^6-bis(2-(rhodamine B amido)ethyl)pyridine-2,6-dicarboxamide (RhBEP), which demonstrates an ultrasensitive, selective colorimetric and fluorescent response to Fe^{3+} and can realize the visual recognition of Fe^{3+} in biological systems.

2. Experimental details

2.1 Synthesis of RhBEP

The synthetic route of the chemosensor RhBEP was illustrated in Fig. 1. The compound RhBE was synthesized using rhodamine B as a raw material according to the previously described method [37, 38]. Chemosensor RhBEP was synthesized as the follow: pyridine-2,6-dicarbonyl dichloride (0.20 g, 1 mmol) was mixed with 0.55 mL (4 mmol) of triethylamine and 10 mL of CH_2Cl_2 in a 100-mL flask and then stirred at 0°C for 30 min. After that, 20 mL of a solution containing 1.0 g of RhBE (2.1 mmol) was added dropwise at 0°C. The mixture was then stirred for 12 h at room temperature. After the reaction was completed, the reaction solution was dried and washed three times with 100 mL of cold 95% EtOH. Finally, 0.78 g of white powder

was obtained with a yield of 71%. ESI-MS: m/z 1100.31 (Fig. S1). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 9.65 (s, 2H), 8.18 (d, *J* = 7.7 Hz, 2H), 8.04 (d, *J* = 7.2 Hz, 2H), 7.93–7.84 (m, 1H), 7.57-7.39 (m, 4H), 7.07 (d, *J* = 7.1 Hz, 2H), 6.52 (d, *J* = 8.7 Hz, 4H), 6.38 (s, 4H), 6.25 (s, 4H), 3.58 (s, 4H), 3.38 (s, 4H), 3.29 (d, *J* = 7.0 Hz, 16H), 1.15 (t, *J* = 6.9 Hz, 24H) (Fig. S2). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 169.55, 163.91, 153.90, 153.30, 148.67, 138.13, 132.49, 130.62, 128.65, 128.21, 123.84, 123.68, 123.22, 108.16, 105.23, 97.90, 65.54, 44.30, 40.25, 39.82, 12.58 (Fig. S3).

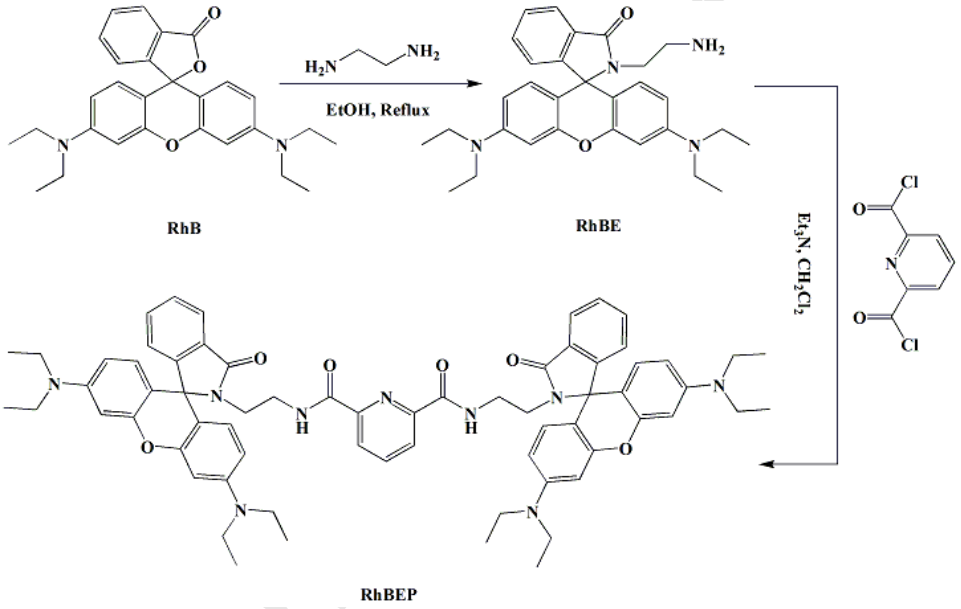

Fig. 1 Synthesis of the chemosensor RhBEP

2.2 Fluorescence measurements

To express concisely, CH_3CN: PBS solution (3:7, *v/v*, 50 mM PBS, pH = 7.4) was defined as the PBS reaction media. Nine-hundred and fifty microliters of PBS reaction media and 10 μL of 1.0×10^{-3} M RhBEP in DMSO stock solution were mixed in a 1-mL centrifuge tube. After that, different concentrations of metal perchlorate salt solution were added and final volume of the solution was adjusted to 1 mL with PBS reaction media. After the reaction was allowed to incubate at room temperature for 20 min, a series of tests including fluorescence selectivity, UV

titration and fluorescence titration tests were performed to investigate the effect of pH and fluorescence imaging on living cells.

2.3 Cell culture and fluorescence imaging

HepG2 cells were cultured in DMEM medium containing 10% bovine serum, 100 units/mL penicillin and 100 mg/L streptomycin in an incubator saturated with 5% CO_2 and 95% air at 37 °C for 24 h. Cells in control group were incubated with RhBEP (10 μM) for 0.5 h prior to the observation under a confocal fluorescence microscope. Prior to fluorescence imaging, cells in experimental group were subjected to exogenous test with different concentrations of Fe^{3+}. The first group was implemented as follow: cells were incubated with RhBEP (10 μM) for 0.5 h. The second group was implemented as follow: cells were incubated with 100 μM $Fe(ClO_4)_3$ for 3h and then RhBEP (10 μM) was added for 0.5 h. The third group was implemented as follow: cells were incubated with 20 μM $Fe(ClO_4)_3$ for 3h and then RhBEP (10 μM) was added for 0.5 h. Prior to fluorescence imaging test, RhBEP and $Fe(ClO_4)_3$ that did not enter into the cells were washed with PBS buffer so that they do not interfere with cell imaging.

3. Results and discussion

3.1 Selectivity towards cations

Due to its significance in the evaluation, the selectivity of chemosensor RhBEP towards various metal cations was examined by fluorescence spectroscopy. First, the selectivity of RhBEP to metal ions in pure acetonitrile was investigated, and the results are shown in Fig. 2 (black bars). After different common metal ions, each at a concentration of 50 μM were added, only Fe^{3+}, Hg^{2+}, Cu^{2+} and Cr^{3+} led to the enhancement of fluorescent signal, among which the enhancement caused by Fe^{3+} was 10 times higher that RhBEP signal, while that caused by Hg^{2+}, Cu^{2+} and Cr^{3+} was

about 5-8 times. This indicates that the chemosensor RhBEP has poor specificity towards metal ions in in pure acetonitrile medium. After that, the selectivity of the chemosensor RhBEP was examined in PBS reaction media. The results were shown in Fig. 2 (red bars). Fe^{3+} caused fluorescence enhancement by 75 times and other metal ions only led to slight enhancement. These data indicate that higher selectivity for Fe^{3+} of the chemosensor RhBEP could be achieved in PBS reaction media. In the addition, the competition experiments have also been carried out. As shown in Fig.S4, the addition of 5 equiv of other competitive metal ions to RhBEP solution scarcely showed fluorescence enhancement for Hg^{2+}, Cu^{2+}, Cr^{3+}, Li^{+}, Na^{+}, K^{+}, Ag^{+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, Al^{3+}, Bi^{3+} and Au^{3+} except for Fe^{3+} (black bar). However, the fluorescence intensity of the mixtures increased after the subsequent addition of the Fe^{3+} (red bar), which suggests that the fluorescent selectivity of RhBEP for Fe^{3+} over other coexisting metal ions is remarkably high (Fig. S4).

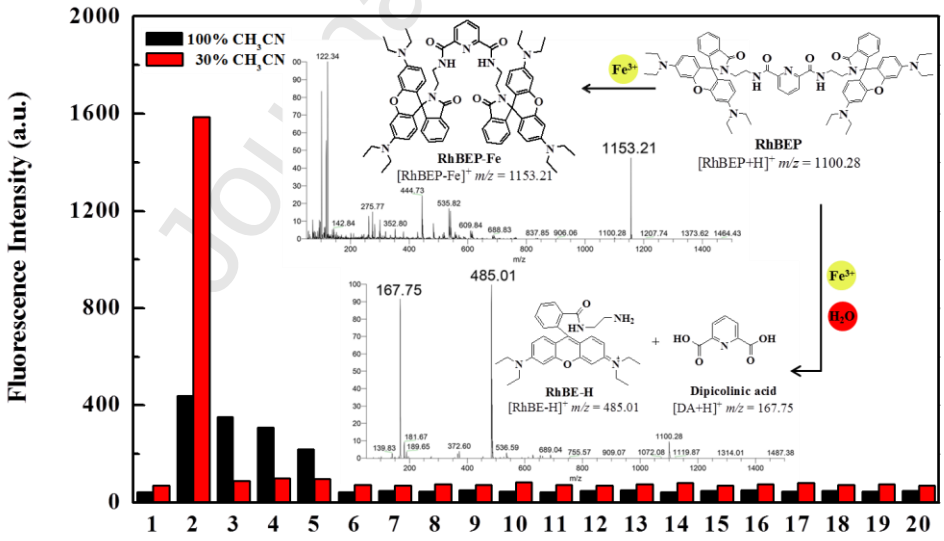

Fig. 2 Fluorescence intensity of RhBEP (10 μM) in 100% CH_3CN (black bars) and 30% CH_3CN (0.1 M PBS, pH 7.4, red bars) at 585 nm, in the absence and presence of different metal ions (50

μM). Excitation wavelength was 520 nm. From 1 to 20: none, Fe^{3+}, Hg^{2+}, Cu^{2+}, Cr^{3+}, Li^{+}, Na^{+}, K^{+}, Ag^{+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, Al^{3+}, Bi^{3+}, and Au^{3+}. Inset: The proposed Fe^{3+} recognition mechanism of the RhBEP, and ESI-MS spectra of RhBEP upon addition of Fe^{3+} in the absence and presence of H_2O

3.2 Fe^{3+}-recognition mechanism of the chemodosimter RhBEP

First, we conducted a Job's plot experiment of the reaction between RhBEP and Fe^{3+} in different media. The results showed that the binding ratio between RhBEP and Fe^{3+} was 1:1 irrespective of the types of media (Fig. S5). The mass spectrum showed a peak at m/z = 1153.21 associated with a 1:1 ratio of RhBEP and Fe^{3+} in pure acetonitrile medium, further indicating that the RhBEP-Fe complex was formed. When PBS reaction media was used, the peak of RhBEP-Fe disappeared, but two new peaks were observed at m/z = 485.01 and 167.75, which correspond to RhBE-H and dipicolinic acid, respectively (Fig. 2, Inset). To further confirm the structure of the new product, the product was separated by column chromatography (silica gel, CH_2Cl_2: MeOH = 15:1), and its structure was characterized by ^1H NMR (Fig. S6). The result showed that the product had the structure of RhBE, which further confirmed that the complex RhBEP-Fe hydrolyzed completely in PBS reaction media. Based on the results, a recognition mechanism was proposed as shown in Fig. 2. In the mechanism, after Fe^{3+} is added, the fluorescence intensity of RhBEP varies with the types of solvents or media. In pure acetonitrile medium, the RhBEP-Fe complex is formed and causes the quenching of fluorescence. By contrast, in the PBS reaction media, the RhBEP exists as RhBE-H with an opened spiro ring that leads to high fluorescence signal.

3.3 Optimization of experimental conditions

The selectivity experiment demonstrated that the presence of water could affect the fluorescence intensity of RhBEP when bound to Fe^{3+}. Therefore, the fluorescence

responses of RhBEP in different ratios of CH_3CN: PBS solution (50 mM, pH = 7.4) were investigated. As shown in Fig. 3a, when the proportion of water increased from 10% to 70%, the fluorescence signal significantly increased (after Fe^{3+} was added). This indicates that the increase of the proportion of water may improve the amount of hydrolysis of the complex RhBEP-Fe^{3+}. When the water content was increased to higher than 70%, the fluorescence signal decreased, indicating that the decreased solubility of RhBEP leads to the quenching of fluorescence [39, 40]. Therefore, CH_3CN: PBS solution (3:7, *v/v*, 50 mM PBS, pH = 7.4) was adopted as the reaction medium.

The effect of pH on the fluorescent signal was also investigated. As illustrated in Fig. 3b, the fluorescence intensity of RhBEP remained unchanged between a pH range of 5 to 12. At pH < 5, the fluorescence intensity increased with the increase of pH since H^+ can open the spiro ring of rhodamine B, thereby led to higher emission of fluorescence. When Fe^{3+} was added to RhBEP solution with different pH, RhBEP emitted strong fluorescent signal within the pH range of 2 to 8, which indicated that the complex between RhBEP and Fe^{3+} underwent hydrolysis at this pH range. At pH > 8, the fluorescence intensity of RhBEP was drastically reduced, which suggested that Fe^{3+} was hydrolyzed into its hydroxide derivative that could not form complex with RhBEP [41]. Therefore, it can be concluded that the optimal pH range for the detection of Fe^{3+} using RhBEP is 5 - 8. The physiological pH (e.g. in the human body) is between 7.35 and 7.45, which lies within the optimal pH range of the Fe^{3+}-specific chemosenor RhBEP. Thus, pH 7.4 was used in subsequent study, in which Fe^{3+} in living cells was detected.

In addition, the effect of reaction time was also investigated. As shown in Fig. 3c, the fluorescence intensity of RhBEP without the addition of Fe^{3+} maintained stable.

After Fe^{3+} was added, the fluorescence intensity of RhBEP rapidly increased within the first 5 min, and the rate of increase was declined from 6 to 15 min. The intensity remained stable after 20 min. Therefore, 20 min was selected as the optimum reaction time.

3.4 UV and fluorescence titration tests

Under the optimal conditions, UV-Vis and fluorescence titration tests were carried out. The UV-Vis titration spectra (Fig. 4a) showed that in the absence of Fe^{3+}, the solution was colorless and had a very weak absorption peak at 560 nm, which indicates that the spiro structure of the chemosensor RhBEP is in a closed state. When

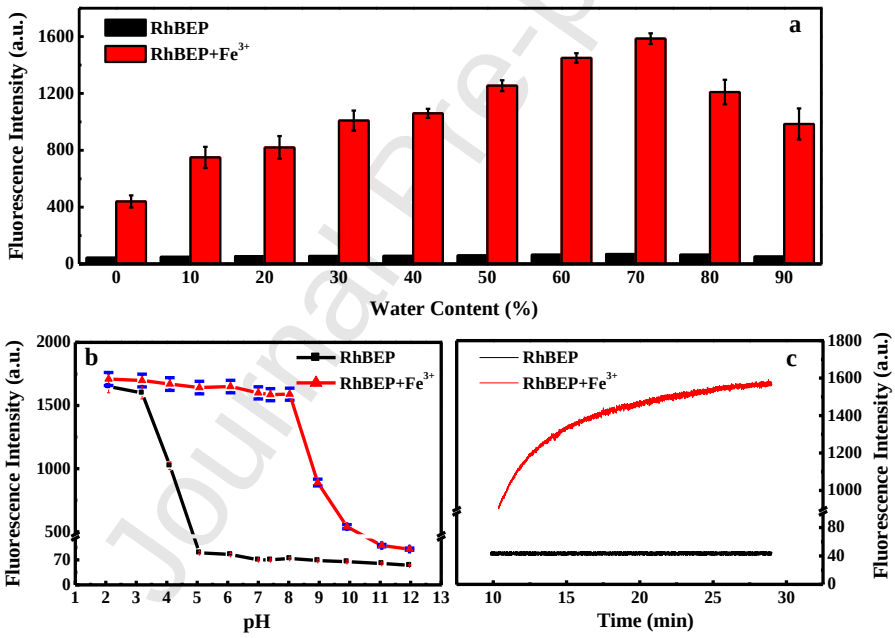

Fig. 3 Fluorescence intensity of RhBEP (10 μM) in the absence and presence of Fe^{3+} (50 μM) at 585 nm. (a) effect of water content. The reaction was performed at room temperature for 20 min with different water content. (b) effects of pH. The reaction was performed at room temperature for 20 min in 0.1 M PBS buffer solution with different pH values adjusted by HCl or NaOH. (c) effect of time. The reaction was conducted at room temperature for different times in 0.1 M PBS buffer solution (pH 7.4).

Fe^{3+} was gradually added, the intensity of the absorption peak at 560 nm was gradually increased, and the solution color turned pink. This indicates that the spiro ring is in open state. This remarkable color change implies that RhBEP can be used as a colorimetric chemosensor to detect Fe^{3+}, and the outcomes can be observed by the naked eye. Fluorescence titration test was also carried out (Fig. 4b). In the absence of Fe^{3+}, no fluorescent emission at 585 nm was observed. When Fe^{3+} was gradually added, the fluorescent emission at 585 nm gradually became intensified, which is in agreement with the UV-Vis titration results. The results also showed that the fluorescence intensity of RhBEP had a linear relationship with Fe^{3+} concentration in the concentration range of 0 to 50 μM ($F = 29.92 [Fe^{3+}] + 11.19$, $r = 0.9987$). The detection limit was found to be 20 nM from ten blank solution measurements, based on the definition by IUPAC ($DL = 3S/\rho$, S: standard deviation of the measurement of the blank; ρ: the slope of linear equation) (Fig. 4c), which was lower than that obtained by previously reported fluorescent probes (Table S1).

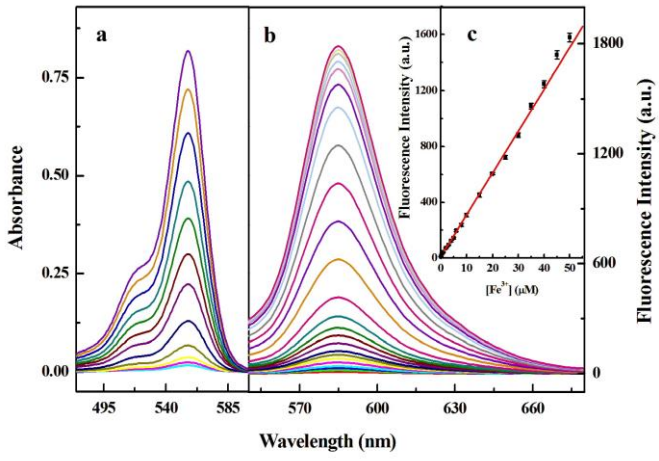

Fig. 4 (a) UV-Vis titration and (b) Fluorescence titration of RhBEP (10 μM) upon addition of Fe^{3+} (0-100 μM). (c) The curve of fluorescence intensity versus Fe^{3+} concentration, fitted with a linear regression equation over Fe^{3+} concentrations of 0-50 μM

3.5 Fluorescent imaging of living cell

The designed chemosensor can play an important role in biological applications if it can be applied to selective detection of target analytes in biological systems [42]. To verify the assumption, cytotoxicity assay and fluorescence imaging were carried out using HepG2 cells. As shown in Fig. S7, at least 85% of HepG2 cells remained viable within 12 h after being treated with RhBEP at a concentration of up to 50 μM. This indicates that RhBEP has low cytotoxicity, thus good biocompatibility. As depicted in Fig.5a, the fluorescent red channel of cells in the control group was not observed under the microscope although after being treated with RhBEP. The results from exogenous test (Fig. 5b-5c) showed that bright red fluorescence was observed in the cells, and the signal intensities varied with the concentration of Fe^{3+}. Meanwhile, nuclear staining with DAPI indicates that the cells are viable throughout the imaging experiments. These results indicate that RhBEP is able to penetrate cell membranes and causes fluorescence signal observed in the cells.

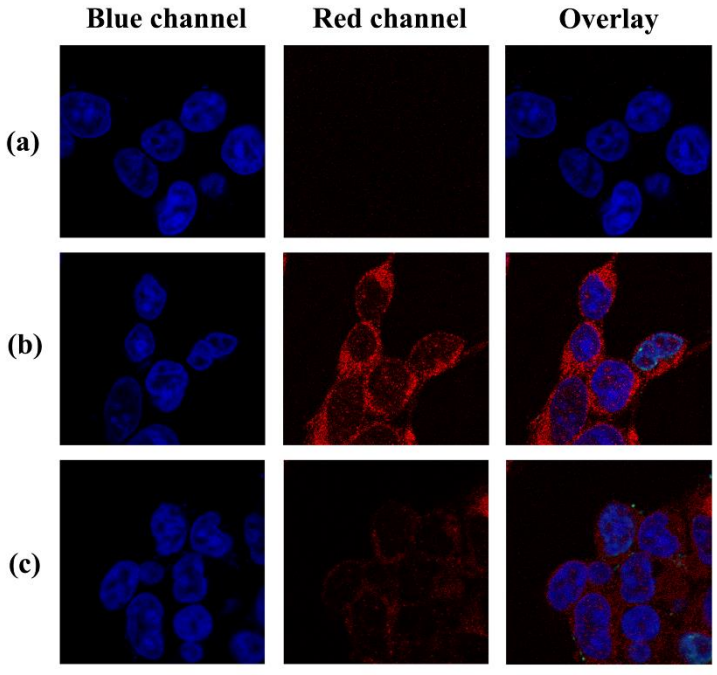

Fig. 5 Fluorescence imaging of Fe^{3+} in HepG2 cells. The cells are treated with DAPI (0.1 mM) nuclear counter-stain (control). (a) cells incubated with RhBEP (10 μM) for 0.5 h; (b) cells incubated with $Fe(ClO_4)_3$ (100 μM) for 3 h, and then RhBEP (10 μM) for 0.5 h. (c) cells incubated

with $Fe(ClO_4)_3$ (20 μM) for 3 h, and then RhBEP (10 μM) for 0.5 h. Blue channel: λ_{ex}= 405 nm, λ_{em}= 425-475 nm; red channel: λ_{ex}=532 nm, λ_{em}= 560-660 nm.

4. Conclusions

In this paper, a novel "off-on" rhodamine-based colorimetric and fluorescent chemosensor RhBEP was designed and synthesized, which showed colorimetric and fluorescent selectivity to Fe^{3+} over other competing ions and "off-on" UV-vis and fluorescence response with high sensitivity due to the hydrolysis of the chemosensor-target complex in PBS reaction media. The recognition mechanism of RhBEP was confirmed by Job's plot and mass spectrometry. In addition, the exogenous tests were applied to investigate the ability of RhBEP in detecting Fe^{3+} in human hepatocarcinoma cells. This study offers an effective analytical method for real-time detection of Fe^{3+} in biological systems.

Acknowledgements

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Author contribution statement

Xiaochun Wang: Conceptualization, Methodology, Validation, Investigation, Writing-Original Draft, Funding acquisition.

Tiechun Li: Resources, Supervision, Project administration.

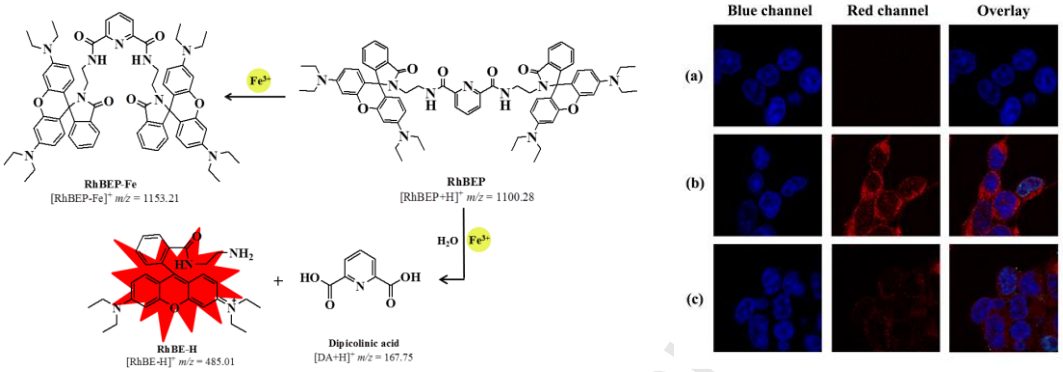
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical Abstract

A novel "off-on" colorimetric and fluorescent chemosensor RhBEP was designed and synthesized, which exhibits not only high sensitivity and selectivity for Fe^{3+} over other coexistent metal ions, but also cell permeability and suitability for detecting Fe^{3+} in the living cells by confocal microscopy.



Highlight

A novel “off-on” chemosensor N^2,N^6-bis(2-(rhodamine B amido)ethyl)pyridine-2,6-dicarboxamide (RhBEP) was developed

Chemosensor RhBEP exhibits high sensitivity and selectivity for Fe^{3+} over other coexistent metal ions

Chemosensor RhBEP is cell permeable and suitable for detecting Fe^{3+} in the living cells by confocal microscopy