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A turn-on fluorescent probe based on hydroxylamine oxidation for detecting ferric ion selectively in living cells†

Rui Wang,[a] Fabiao Yu,[b] Ping Liu[a] and Lingxin Chen*[a]

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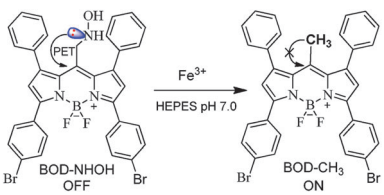
We have described a turn on fluorescent probe BOD-NHOH based on hydroxylamine oxidation for detecting intracellular ferric ions. The probe comprises a signal transducer of BODIPY dye and a Fe^{3+}-response modulator of hydroxylamine. It is readily employed for assessing intracellular ferric ion levels, and confocal imaging is achieved successfully.

The highly sensitive and selective detection of the ferric ion (Fe^{3+}) is of great significance for the study of its physiological functions in organisms.[1,2] Iron is the most abundant transition metal in cellular systems and is of outstanding importance due to its biological functions, including essential roles in oxygen uptake, oxygen metabolism, electron transfer,[3] and transcriptional regulation.[4] Ferric ion deficiency and overload can induce various diseases, such as Parkinson's disease, Alzheimer's disease and cancer.[5] Therefore, much effort has been focused on the investigation of the biological functions and deleterious effects of Fe^{3+} *in vivo*. Recently, a number of sensitive and selective methods have been reported for conducting such research.[6] In comparison with other technologies, fluorescence method provides greater sensitivity, less invasiveness, and more convenience.[7] There has been an explosive increase in the number of fluorescent metal ion probes, which are usually developed by combining a fluorophore with a known metal ion chelator.[8] However, Fe^{3+} is well-known as a fluorescence quencher due to its paramagnetic nature. As a result, many of the available Fe^{3+} probes are based on fluorescence quenching mechanisms.[9] Only a few "turn-on" probes that work with a selective response to Fe^{3+} have been reported.[10] It is generally recognized that one probe with a fluorescence turn-on signal for specific events is more efficient.[7,11] Compared with "turn-off" probes, the greatest advantage of "turn-on" probes is the ease of measuring low-concentration contrast relative to a "dark" background, which reduces the likelihood of false positive signals and increases sensitivity, as demonstrated by numerous studies.[12] Therefore, we have invested much time and effort into dicscovering a new chelator-independent method of detecting Fe^{3+} ions with a turned on chemical switch.

It is noteworthy that hydroxylamine can be easily oxidized by Fe^{3+} while other metal ions have almost no interference.[13] As far as we know, this reaction has never been used to develop a selective fluorescent probe for Fe^{3+}. We anticipated that the lone pair electrons in the ammonia group could quench the fluorescence of an appropriate fluorophore through a fast photoinduced electron transfer (PET) process. With this in mind, we designed and synthesized a turn-on fluorescence probe of 8-hydroxyethanam-ine-4,4-difluoro-1,7-diphenyl-3,5-di(1-bromo-phen-yl)-4-bora-3a,4a-diaza-*s*-indacene (BOD-NHOH) for the detection of Fe^{3+} in living cells, as shown in Scheme 1.

As an overall strategy, the probe is composed of two moieties. One is a fluorophore, for which a BODIPY (4,4-difluoro-4-3a,4a-diza-*s*-indacene) platform is selected as the signal transducer, because the dyes have high molar absorption coefficients and fluorescence quantum yields, sharp absorption and fluorescence peaks in the visible region.[14] The other moiety is the Fe^{3+}-response switch, for which hydroxylamine is chosen as the modulator.[11] After being equipped with hydroxylamine, the fluorescence of the BODIPY platform is quenched by PET between the modulator and the transducer.[15] However, excitingly, the hydroxylamine oxidation can eliminate the PET, triggering an increase of the fluorescence emission, thereby allowing the formation of a "turn-on" fluorescent probe (Scheme 1). As expected, the probe exhibits a selective fluorescence response to Fe^{3+}, instead of other physiological relevant metal ions. We next described the synthesis, spectroscopy, and application of the Fe^{3+}-selective fluorescent probe.

We examined the effect of pH on the fluorescence. The fluorescence of the probe began to quench at pH above 5.8. On the other hand, we explained the phenomenon of the fluorescence



Scheme 1 Structure of BOD-NHOH and proposed mechanism of fluorescence enhancement.

[a] *Key Laboratory of Coastal Zone Environmental Processes, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, P. R. China. E-mail: lxchen@yic.ac.cn; Fax: (+86) 535-2109130; Tel: (+86) 535-2109130*
[b] *School of Chemical Engineering, Dalian University of Technology, Dalian 116024, P. R. China. E-mail: yufabiao@163.com*

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decrease under alkaline conditions in terms of deprotonation of the ammonia group which increased the electron-donating ability of the lone pair electrons in the ammonia group and raised the PET. The pK_a value of protonated hydroxylamine is 5.97.[16] These spectral properties suggest that PET is regulated in the molecule successfully.[12] As shown in Fig. S1†, the pH of the medium had hardly any effect on the oxidized probe (BOD-CH_3) over the pH range from 6.8 to 7.8. Thus, the probe was expected to work well under physiological conditions.[17] Simultaneously, considering the pK_a of the probe and the hydrolysis property of Fe^{3+}, pH 7.0 was chosen as part of the optimal test conditions,[18] *i.e.* pH 7.0, 40 mM HEPES.

The fluorescence and absorption spectra of the probe (1.0 μM) were examined in the HEPES aqueous buffer (pH 7.0, 40 mM). Under these simulated physiological conditions, BOD-NHOH exhibits an absorption maximum at λ_{max} = 580 nm (ε = 1.7 × 10^6 M^{-1} cm^{-1}). There is no significant change in the absorption spectrum upon changing the state from "off" to "on" (Fig. 1a). The fluorescence titration of the probe in the presence of different concentrations of Fe^{3+} was then performed. As shown in Fig. 1b, the fluorescence intensity (λ_{em} = 615 nm) increases significantly upon addition of Fe^{3+}. When increasing the concentration of Fe^{3+} up to 50 μM, there is an impressive fluorescence enhancement from the background level of the probe (from Φ = 0.01 to Φ = 0.35). These spectral properties indicate that the PET process is successfully regulated in the molecule.[19] We tested the ability of the probe to quantify Fe^{3+} in water solution. Fig. 1b inset demonstrates that there is a good linear dependence of fluorescence intensity on Fe^{3+} concentration (0–50 μM), and the regression equation was $F_{615\,nm}$ = 2674.9 [Fe^{3+}] (μM) + 9457.7, with r = 0.997. Upon increasing the Fe^{3+} concentration to 60 μM, the fluorescent emission intensity reaches the saturation threshold (Fig. S2†).

BOD-NHOH exhibits a selective turn-on fluorescence response to Fe^{3+} in water. Responses of 1.0 μM BOD-NHOH to the presence of various biologically relevant metal ions are shown in Fig. 2. The fluorescence profiles of BOD-NHOH are unchanged in the presence of Zn^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Fe^{2+} (all in 0.3 mM), Na^+ and K^+ (both in 120 mM), Ca^{2+} and Mg^{2+} (both in 0.5 mM). Of all the tested metal ions, only the addition of Cu^{2+} (in 0.2 mM) and Ag^+ (in 0.3 mM) gave a limited increase in fluorescence intensity. But lower Cu^{2+} and

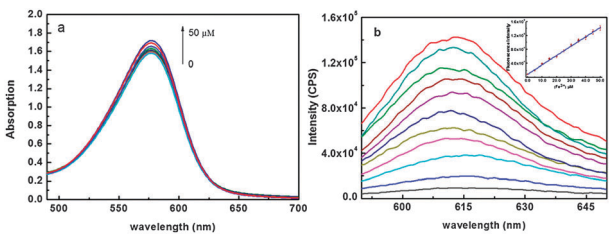

Fig. 1 (a) Absorption spectra of probe (1.0 μM) treated with various concentrations of Fe^{3+} in 40 mM, pH 7.0 HEPES aqueous buffer. (b) Fluorescence responses of 1.0 μM probe to Fe^{3+} concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 μM. Spectra were acquired in 40 mM, pH 7.0 HEPES buffer with excitation at 585 nm and emission ranging from 590 to 650 nm. Inset: Relationship between the fluorescence intensity at 615 nm of probe and [Fe^{3+}].

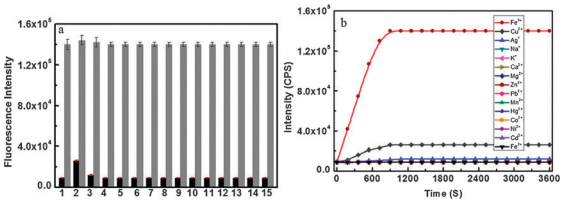

Fig. 2 (a) Fluorescence responses (λ_{ex} = 580 nm, λ_{em} = 610 nm) of 1.0 μM probe to diverse metal ions in 40 mM HEPES (pH 7.0): 1, Fe^{3+} (50.0 μM); 2, Cu^{2+} (0.2 mM); 3, Ag^+ (0.3 mM); 4, Na^+ (120 mM); 5, Ca^{2+} (0.5 mM); 6, Mg^{2+} (0.5 mM); 7, K^+ (120 mM); 8, Zn^{2+}; 9, Pb^{2+}; 10, Mn^{2+}; 11, Hg^{2+}; 12, Co^{2+}; 13, Ni^{2+}; 14, Cd^{2+} (above ions were all 0.3 mM); 15, Fe^{2+} (0.2 mM). Bars present fluorescence responses to various metal ions. In each group, the black bar represents the fluorescence intensity after addition of analytes, and the gray bar represents that after the subsequent addition of 50.0 μM Fe^{3+}. (b) Response of BOD-NHOH to Fe^{3+} and various other cations in (a) over 1 h.

Ag^+ levels (50 μM) will minimize this interference. The results indicate that the present probe shows excellent selectivity for Fe^{3+} over other abundant cellular cations. To check whether the chemoselective hydroxylamine switch might turn on upon incubation with other cations over time, the probe's response to various cations was measured for 1 h. As Fig. 2b demonstrates, BOD-NHOH selectively responded to Fe^{3+} by a turn-on fluorescence switch and avoided a host of other ions.

We next established the ability of BOD-NHOH to track Fe^{3+} levels in living cells by using a MCF-7 cell model exposed to Fe^{3+}. The MCF-7 cells were incubated with 0.01, 0.1, 1, 10, and 100 μM Fe^{3+} solutions for 30 min in RPMI 1640 Medium at 37 °C, and then washed with physiological saline to remove excess Fe^{3+} ions. The treated cells were incubated with BOD-NHOH (10.0 μM) in RPMI 1640 Medium for 15 min. After being washed with physiological saline, the cells were imaged by a confocal fluorescence microscope. As a control, the cells not treated with Fe^{3+} were also imaged. The control experiments showed faint fluorescence (Fig. 3a), but those treated with various concentrations of Fe^{3+} displayed different fluorescence intensities. The confocal fluorescence images grew brighter as the concentration of Fe^{3+} increased from 0.1 to 100 μM (Fig. 3b–f). We selected the whole region in the visual field (Fig. 3b–f) as the

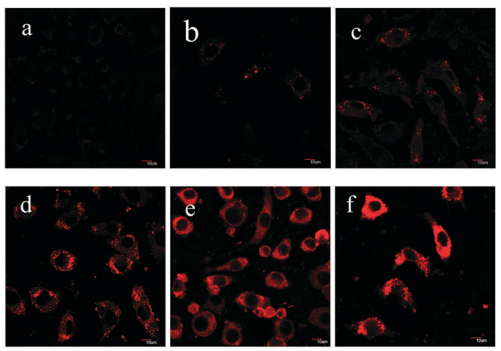

Fig. 3 Confocal fluorescence images of living MCF-7 cells incubated with various concentrations of Fe^{3+}. MCF-7 cells loaded with 10.0 μM BOD-NHOH and Fe^{3+} for 15 min of (a) Control, (b) 0.01 μM, (c) 0.1 μM, (d) 1 μM, (e) 10 μM and (f) 100 μM. Scale bar is 10 μm.

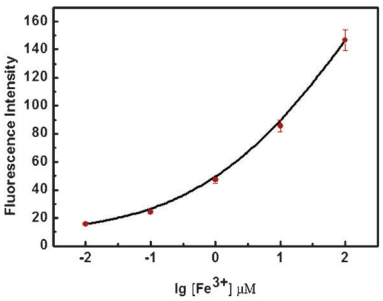

Fig. 4 The relationship between average fluorescence intensity and added various Fe^{3+} concentrations in Fig. 3b–f correspondingly. The whole regions in the visual field (Fig. 3b–f) were selected as the region of interest (ROI), and the average fluorescence intensity was determined *via* confocal laser-scanning microscopy.

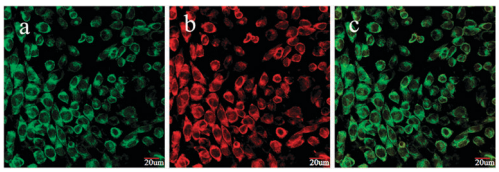

Fig. 5 Confocal fluorescence images of MCF-7 cells incubated with BOD-NHOH (10.0 μM) and rhodamine 6G (1.0 μM) for 15 min. Cells loaded with Fe^{3+} (10 μM) for 30 min, then treated with BOD-NHOH (10.0 μM) and rhodamine 6G (1.0 μM) for 15 min. (a) Green channel with rhodamine 6G. (b) Red channel with BOD-NHOH. (c) Overlay of images showing fluorescence from rhodamine 6G (b) and BOD-NHOH (c). Scale bar is 20 μm.

region of interest (ROI), and the average fluorescence intensity was determined *via* confocal laser-scanning microscopy with various Fe^{3+} concentrations (Fig. 4). The results suggest that BOD-NHOH has good membrane permeability, and these data also establish that BOD-NHOH can respond to intracellular Fe^{3+} level changes within living cells.

We now applied BOD-NHOH to probe the subcellular locations of Fe^{3+} in the MCF-7 cells using confocal fluorescence microscopy. Under the same conditions used in Fig. 3e, the cells were co-stained with BOD-NHOH (10.0 μM) and rhodamine 6G (1.0 μM) for 15 min. Fig. 5 further revealed the location of the probe in the cytoplasm of the living MCF-7 cells.

In summary, we have developed a new fluorescent probe that exhibits high sensitivity and selectivity for monitoring Fe^{3+} both in aqueous solution and living cells. The probe exhibits a turn-on fluorescent response upon detecting Fe^{3+} compared to other biologically relevant metal ions. Confocal microscopy images indicate that BOD-NHOH can be used for detecting changes in Fe^{3+} levels within living cells. To the best of our knowledge, this is the first example of a fluorescence probe that can be used to successfully detect Fe^{3+} based on hydroxylamine oxidation both in aqueous solution and living cells. We anticipate that the new probe will lead to many new opportunities for studying the biological effect of Fe^{3+} in living cells.

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