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A selective turn-on fluorescent sensor for Fe^{III} and application to bioimaging

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Abstract—A novel compound FD1 was demonstrated as a turn-on fluorescent sensor for imaging of iron(III) ion in biological samples. Based on the spirolactam (nonfluorescence) to ring-open amide (fluorescence) equilibrium, FD1 exhibited high selectivity and sensitivity for Fe^{3+} over other metal ions. Moreover, fluorescent microscopy experiments further established that FD1 could be used for sensing Fe^{3+} within living cells.

The design and synthesis for selective and sensitive fluorescent sensors for metal ions has tremendously gained in importance,[1] because metal ions involved in a variety of fundamental physiological processes in organisms. As an important physiologically relevant metal ion, Fe^{III} ion plays an indispensable role in many biochemical processes at the cellular level,[2] and both its deficiency and excess can induce a variety of diseases[3] with iron trafficing, storage, and balance being tightly regulated in an organism.[4] As a consequence, intense research efforts have been directed to develop sensitive and selective sensors for Fe^{3+}.[5–7] However, for the paramagnetic nature of iron(III) ion, the fluorescent indication for Fe^{3+} is mostly signaled by fluorescence quenching.[6] Especially, the lack of suitable turn-on fluorescent iron indicators[7] is even more obvious when judged in terms of application in bioimaging, although significant progress has been made in fluorescent molecular sensors for intracellular imaging main group I and II metal ions[8,9] and transition metal ions (such as Zn^{2+},[10] Cu^{+},[11] Pb^{2+},[12] and Hg^{2+}[13]).

Compared with the single-photon excited fluorescent bioimaging technique, two-photon laser scanning microscopy imaging (TPLSM) has the advantage of high transmission at low incident intensity, reduced photodamage, improved depth penetration, and reduced background cellular autofluorescence, and has recently attracted a great deal of interest since the pioneering work of the Webb group on TPLSM.[14] To image the distribution of ions in cellular processes, suitable two-photon chemosensors with turn-on fluorescence should be developed. However, the cases of two-photon excited fluorescence (TPEF) sensor for ions are still limited to proton, fluoride anion, main group I and II metal ions, and cysteine/homocysteine.[15] Herein, we are interested in developing a TPEF probe which could be utilized for sensing Fe^{3+} in living cells. A major challenge to achieve this goal is developing a fluorescent probe that exhibits an increasing visible fluorescent emission upon the addition of Fe^{3+} over physiologically relevant metal ions. Based on the well-known spirolactam (nonfluorescence) to ring-open amide (fluorescence) equilibrium of rhodamine,[16] we described a significant emission-increasing probe FD1 (Scheme 1) for Fe^{3+}, and demonstrated its utility in bioimaging.

FD1 was synthesized by treatment of rhodamine B hydrazide[17] with acetone in a yield of ca. 40%.[18] Single

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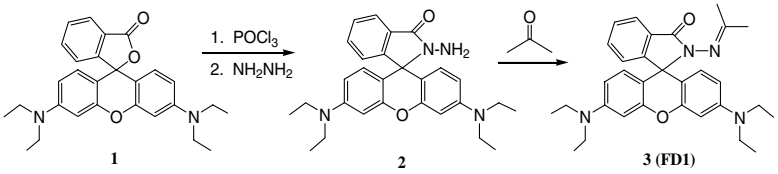

Scheme 1. Synthetic route of FD1.

crystal of FD1 was obtained by slow evaporation of the acetone solution. The single-crystal X-ray diffraction study showed that the molecular structure of FD1 is of spirocyclic form in the crystal (Fig. 1). The characteristic peak of 66.2 ppm (9-carbon) in the ^{13}C NMR spectrum of FD1 also supported this consideration.

Absorption spectra of FD1 were performed in a CH_3CN solution diluted 20 times with HEPES buffer (20 mM, pH 7). $FeCl_3$ was used as Fe(III) source. A weak absorption peak at 561 nm was observed in FD1 solution which appeared to be almost colorless. The adsorption peak intensity increased dramatically with each addition of Fe^{3+} (Fig. 2) and the color of solution changed to red-orange, which can be attributed to the delocalized xanthene moiety of the ring-open amide form of FD1. The significant color change in FD1 solution upon addition of Fe^{3+} indicated that FD1 was a sensitive 'naked-eye' indicator for Fe^{3+}. Binding assays using the method of continuous variations (Job's plot) were consistent with a 1:1 stoichiometry of the FD1–Fe^{3+} complex.

The fluorescence enhancement effects of various amounts of Fe^{3+} on FD1 were investigated under excitation at $\lambda_{ex} = 510$ nm (Fig. 3). No obvious fluorescent emission was observed in FD1 solution with the absence of Fe^{3+}. When Fe^{3+} was introduced into a 10 μM FD1 solution, an obvious fluorescence peak was observed and also enhanced upon further addition of Fe^{3+}, whereas other metal ions displayed much weaker response. The fluorescence intensity at 583 nm increased by a factor of 112 when Fe^{3+} concentration increased

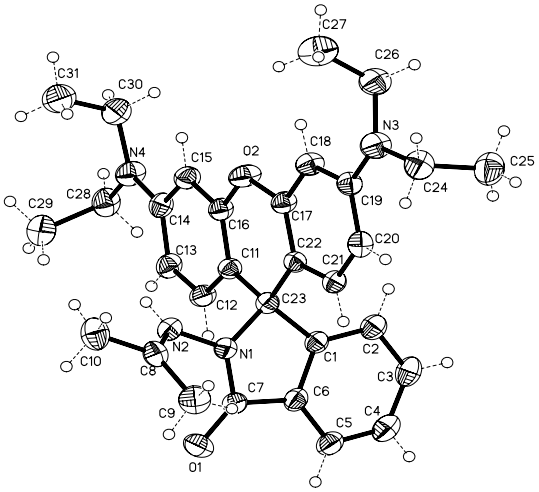

Figure 1. The ORTEP drawing of FD1 (30% ellipsoid). H atoms were omitted for clarity.[19]

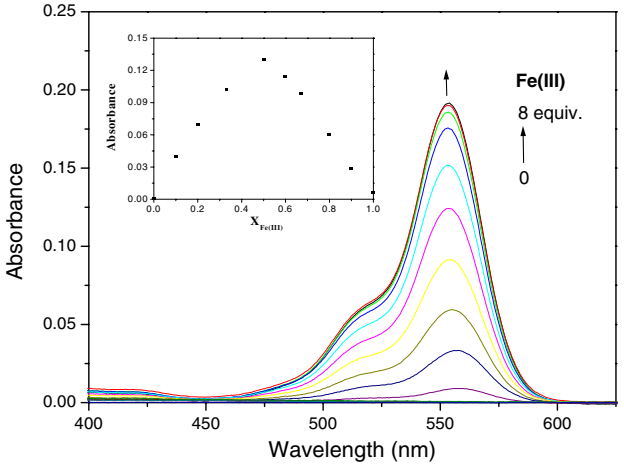

Figure 2. UV–vis absorption spectra of FD1 (10 μM) upon the addition of Fe^{3+} (0–75 μM) in the CH_3CN solution diluted 20 times with HEPES buffer (20 mM pH 7) at 25 °C. Each spectrum is obtained after Fe^{3+} addition for 5 min. Inset: Job' plot of FD1 and Fe^{3+}. The total concentration of FD1 and Fe^{3+} was kept at a fixed 10 μM. The data are consistent with 1:1 Fe^{3+}–FD1 complex.

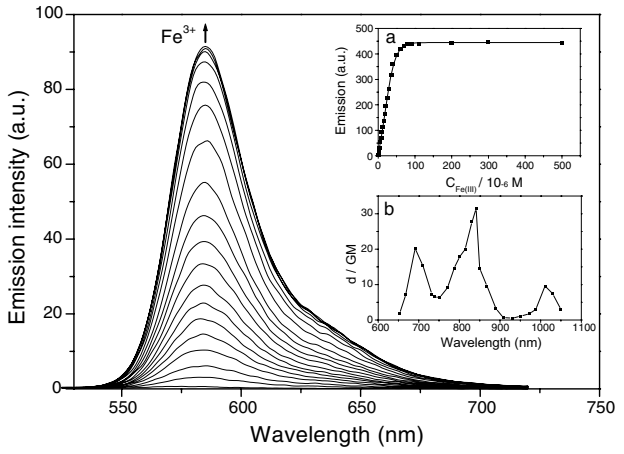

Figure 3. Fluorescence responses of FD1 (10 μM) upon the addition of Fe^{3+} at 25 °C. Each spectrum was obtained in the CH_3CN solution diluted 20 times with HEPES buffer (20 mM, pH 7) after Fe^{3+} addition for 5 min. Inset: (a) the plot of fluorescent emission intensity at 583 nm as a function of Fe^{3+} concentration ($\lambda_{ex} = 510$ nm) and (b) two-photon absorption spectrum of FD1 upon the addition of 80 μM Fe^{3+}.

from 0 to 80 μM. The stability constant of the complex was calculated by the linear Benesi–Hildebrand expression:[20]

$$\frac{1}{\Delta I} = \frac{1}{[\mathrm{FD1}]\Delta\phi} + \frac{1}{K_{\mathrm{ass}}[\mathrm{FD1}]\Delta\phi}\frac{1}{[\mathrm{Fe}^{3+}]}$$

where ΔI is the change in the fluorescence intensity at 583 nm; K_{ass} is the stability constant; $\Delta\phi$ ($\Delta\phi = \phi_{complex} - \phi_{FD1}$) is the difference of fluorescence quantum yields between the complex and FD1; and [FD1] and [Fe^{3+}] are the concentrations of FD1 and Fe^{3+}, respectively. On the basis of the plot of $1/\Delta I$ and $1/[Fe^{3+}]$, the stability constant was calculated to be $2.3 \times 10^4\ M^{-1}$.

Furthermore, the two-photon related photophysical properties of FD1 were investigated in the absence and presence of Fe^{3+}, and the two-photon absorption cross-sections were determined by the method of two-photon induced fluorescence using Rhodamine B as a standard with known two-photon absorption (TPA) cross-section (δ) (Supplementary data).[21] A similar response to the TPEF titration of Fe^{3+} with those of FD1 in single-photon related photophysical properties was observed. No obvious TPEF signal was recorded under the excitation of fs laser from 650 to 1050 nm, indicating that FD1 was not TPEF-active. However, the TPEF intensity of FD1 ($\lambda_{ex} = 800$ nm) increased gradually upon addition of Fe^{3+} (Supplementary data). As shown in the inset of Figure 3, FD1 exhibited two-photon active properties with TPA cross-section of 31 GM in the presence of 80 μM Fe^{3+}. This result indicated that FD1 could be used as TPEF sensor for Fe^{3+}.

The selective coordination studies of FD1 were then extended to related heavy, transition, and main group metal ions by fluorescence spectroscopy. The representative chromogenic behavior of FD1 toward metal ions in aqueous solution is shown in Figure 4. Upon the addition of 80 μM metal ions, very mild fluorescence enhancement factor (FEF) in FD1 solution was observed for Cu^{2+} (18-fold), while Na^+, K^+, Cu^+, Ag^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Fe^{2+} showed very weak response (Fig. 4).

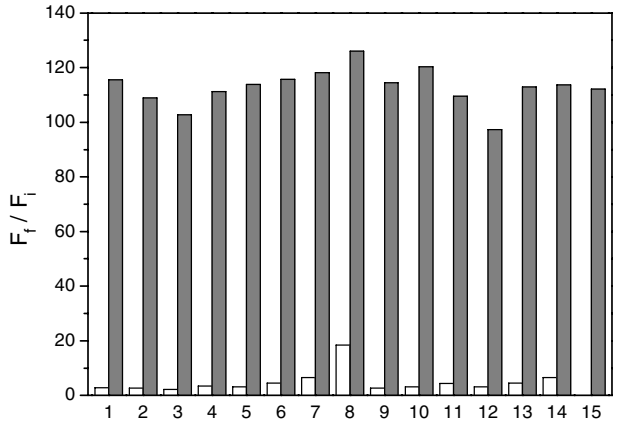

Figure 4. Fluorescence responses of FD1 (10 μM) to various metal ions (80 μM, *x*-axis markers) in 20 mM HEPES, pH 7. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). White bars represent the addition of an excess of the appropriate metal ion (1 mM for Na^+, K^+, Mg^{2+}, and Ca^{2+}, 80 μM for all other cations) to a 10 μM solution of FD1. Black bars represent the addition of Fe^{3+} (80 μM) to the solution. ($\lambda_{ex} = 510$ nm). 1, Na^+; 2, K^+; 3, Cu^+; 4, Ag^+; 5, Ca^{2+}; 6, Cd^{2+}; 7, Co^{2+}; 8, Cu^{2+}; 9, Cr^{2+}; 10, Zn^{2+}; 11, Mg^{2+}; 12, Mn^{2+}; 13, Ni^{2+}; 14, Pb^{2+}; 15, Fe^{3+}.

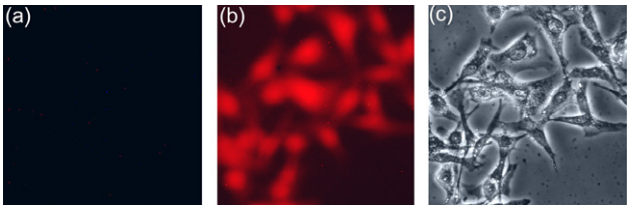

Figure 5. Fluorescence and phase contrast images of live PIEC cells: (a) fluorescence image of PIEC cells at 25 °C; (b) fluorescence image of PIEC cells incubated with 5 μM FD1 for 15 min at 25 °C; (c) brightfield image of live PIEC cells shown in panel b to confirm viability.

However, only the addition of Fe^{3+} resulted in a prominent enhancement (112-fold) of fluorescence emission at 583 nm, which indicated the high selectivity of FD1 to Fe^{3+}. The competition experiment was also carried out by adding Fe^{3+} to FD1 solution in the presence of other metal ions, as shown in Figure 4. The results indicated that the recognition of Fe^{3+} by FD1 was not significantly interfered by these commonly coexistent ions.

To develop practical applicability of FD1 toward Fe^{3+}, we next assessed the ability of FD1 to operate within living pig iliac artery endothelium cells (PIEC) by fluorescence microscopy (Fig. 5). PIEC cells incubated with or without 5 μM FD1 for 15 min at 25 °C gave no intracellular background fluorescence (Fig. 5a). Moreover, no intracellular fluorescence was detected for PIEC cells supplementing cells with 50 μM $FeCl_3$ in the growth medium for 5 h at 37 °C. However, as shown in Figure 5b, bright intracellular fluorescence was observed for supplementing cells with 50 μM $FeCl_3$ in the growth medium for 5 h at 37 °C and then staining with FD1 under the same loading conditions. It can be seen from the brightfield transmission measurement after Fe^{3+} and FD1 incubation (Fig. 5c) that the PIEC cells were viable throughout the imaging experiments. These facts implied that FD1 was membrane-permeable and could sense intracellular Fe^{3+} in living cells.

In summary, we demonstrated a unique fluorescent chemosensor FD1 for Fe^{3+} and its bioimaging applications. FD1 exhibited a turn-on fluorescent response for detecting Fe^{3+} with excellent selectivity over other metal ions. Moreover, fluorescent microscopy experiments indicated that FD1 could be used as fluorescent probe for sensing Fe^{3+} in living cells. We anticipate that the turn-on fluorescent properties of this probe presage many opportunities for studying the biological effect of Fe^{3+} in future application.

Acknowledgements

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Supplementary data

Synthetic and experimental details (PDF) are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.03.112.

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18. *Data for FD1*: mp 210–213 °C; ^{1}H NMR (400 Hz, $CDCl_3$): δ 7.89–7.92 (m, 1H), 7.42–7.47 (m, 2H), 7.09–7.13 (m, 1H), 6.53 (d, $J = 8.8$ Hz, 2H), 6.38 (d, $J = 2.4$ Hz, 2H), 6.26–6.29 (dd, $J = 8.8$ Hz, $J = 2.8$ Hz, 2H), 3.30–3.35 (m, 8H), 1.96 (s, 3H), 1.81 (s, 3H), 1.16 (t, $J = 7.2$ Hz, 12H); ^{13}C NMR (100 Hz, $CDCl_3$): δ 12.6, 21.4, 25.5, 44.3, 66.2, 97.9, 106.5, 107.8, 122.8, 123.8, 128.0, 128.5, 130.8, 132.1, 148.6, 151.8, 153.6, 160.6, 174.2. MS (EI): m/z 496.5 (M^+). Anal. Calcd for $C_{31}H_{36}N_4O_2$: C, 74.97; H, 7.31; N, 11.28. Found: C, 74.84; H, 7.17; N, 11.32.
19. *Crystal data for* $C_{31}H_{36}N_4O_2$: Fw = 496.65, Monoclinic ($P2(1)/c$), $a = 12.199(2)$ Å, $b = 12.303(2)$ Å, $c = 18.536(3)$ Å, $\alpha = 90°$, $\beta = 107.501(3)°$, $\gamma = 90°$, $V = 2653.3(8)$ Å^3, $\rho_{calcd} = 1.246$ g cm^{-3}, $Z = 4$, $\mu = 0.079$ mm^{-1}, R_1 [$I > 2\sigma(I)$] = 0.0613, wR_2 [$I > 2\sigma(I)$] = 0.1401, R_1 (all data) = 0.1500, wR_2 (all data) = 0.1788, GOF = 1.046. CCDC deposition number: CCDC 605536.
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