FRET-based sensor for imaging chromium(III) in living cells†

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On the basis of fluorescent resonance energy transfer from 1,8-naphthalimide to rhodamine, a fluorophore dyad (FD8) containing rhodamine and a naphthalimide moiety was synthesized as a Cr^{3+}-selective fluorescent probe for monitoring Cr^{3+} in living cells with ratiometric fluorescent methods.

Förster (fluorescence) resonance energy transfer (FRET) is unique in generating fluorescence signals sensitive to molecular conformation, association and separation in the 1–10 nm range.[1] FRET imaging that affords simultaneous recording of two emission intensities at different wavelengths in the presence and absence of analytes has provided a facile method for visualizing complex biological processes at the molecular level.[2] Most of FRET imaging is focused on recognizing biomacromolecules,[2,3] and only few FRET-based probes have been used to monitor low-weight molecular species[4] (especially metal cations[5]) in living samples although some metal cations play important role in biological systems.

Chromium(III), an essential trace element in human nutrition, has great impacts on the metabolism of carbohydrates, fats, proteins and nucleic acids by activating certain enzymes and stabilizing proteins and nucleic acids.[6] Insufficient dietary intake of Cr^{3+} leads to increases in risk factors associated with diabetes and cardiovascular disease, including elevated levels of circulating insulin, glucose, triglycerides and total cholesterol, and impaired immune function.[7] Exposure to high levels of Cr^{3+} can negatively affect cellular structures, although its toxicity observed *in vivo* is much lower compared to that of Cr^{4+}.[8] To date, however, few methods have been developed to monitor Cr^{3+} transformation in the intracellular compartment or its distribution in living systems.

Because of its paramagnetic property and the lack of a selective multi-chelating ligand for Cr^{3+}, fluorescent turn-on reagents suitable for monitoring intracellular Cr^{3+} remain underdeveloped, although two examples of a selective fluorescent chemosensor of Cr^{3+} in organic solvents have recently been reported.[9] We are interested in developing new Cr^{3+}-selective fluorescent probes for monitoring the transformation and distribution of Cr^{3+} in living samples. To this end, **FD8** (Scheme 1) was designed and synthesized as a novel cell membrane-permeable, Cr^{3+}-selective sensor for FRET imaging in living cells.

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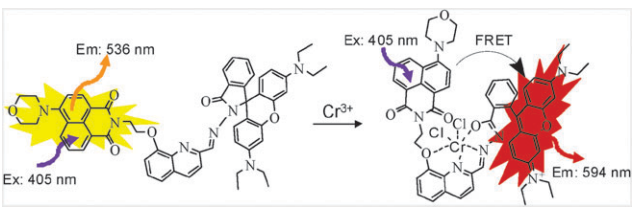
On the basis of the spirolactam (nonfluorescent) to ring-open amide (fluorescent) equilibrium of rhodamine, rhodamine-based dyes are excellent OFF/ON-type fluorescence probes for *in vitro* detection[10] and *in vivo* bioimaging.[11] In the present study, our design strategy for FRET detection of Cr^{3+} is based on modulating the FRET process in a fluorophore dyad comprising a 1,8-naphthalimide donor[12] and a rhodamine acceptor linked by a flexible multi-chelating site (Scheme 1). In the absence of Cr^{3+}, the rhodamine moiety adopts a closed, non-fluorescent spirolactam form, corresponding to weak spectral overlap between 1,8-naphthalimide emission and rhodamine absorption. As a result, FRET is suppressed, and only the yellow emission of the donor is observed upon excitation of the 1,8-naphthalimide chromophore. Binding to Cr^{3+} induces opening of the fluorescent rhodamine moiety, corresponding to intense absorption in the 1,8-naphthalimide emission region. Spectral overlap is enhanced, and excitation of the 1,8-naphthalimide chromophore results in intense strong red emission of rhodamine owing to FRET.

FD8 was synthesized in 40% yield from the reaction of rhodamine hydrazide[11c] with 8-hydroxylquinoline-2-aldehyde in refluxing ethanol, followed by the dehydration reaction with 2-hydroxyethyl-4-(6-morpholin-4-yl-1*H*,3*H*-benzo[*de*])-isoquinoline in anhydrous THF for 24 h (ESI†).

The photophysical properties of **FD8** in ethanol–water (2 : 1, v/v) solution were determined. **FD8** displayed an absorption band centered at ~380 nm and yellow fluorescence centered at 544 nm, which is attributed to an internal charge transfer (ICT) process for the 1,8-naphthalimide chromophore.[12] Notably, addition of Cr^{3+} to the **FD8** solution caused an obvious enhancement of the absorption band centred at 568 nm (Fig. 1(a)), corresponding to a change in color from weak yellow to red–orange as shown in Fig. 2(a). Moreover, the change in the solution color was observed within one minute upon addition of 10 eq. Cr^{3+} to the solution of **FD8** (20 μM) (Fig. S4, ESI†). These facts indicated that **FD8** was induced to form the open-ring structure from the spirolactam form by Cr^{3+}. The transformation of **FD8** was also supported by a 15-fold enhancement of fluorescent emission at 590 nm excited by 515 nm light (Fig. S2, ESI†).

Importantly, When **FD8** was excited by 405 nm, the intensity of the fluorescent peak at 544 nm gradually decreased and that of



Scheme 1 Proposed mechanism of Cr(III)-selective sensor **FD8**.

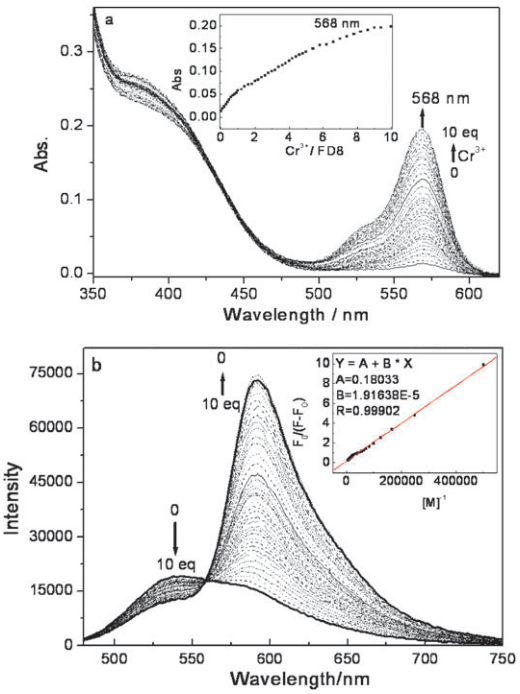


Fig. 1 Absorption (a) and fluorescence (b) spectra of **FD8** (20 μM) in ethanol–water (2 : 1, v/v) solution upon addition of Cr^{3+}. Inset: (a) Curve of absorbance at 568 nm of **FD8** *vs.* different amounts of Cr^{3+}, (b) Curve of fluorescence intensity ratio ($F_0/(F - F_0)$) at 592 nm of **FD8** *vs.* $1/[Cr^{3+}]$ (right) (λ_{ex} = 405 nm). Time intervals between two adjacent measures were 5 min.

a new fluorescent band centered at 592 nm gradually increased, with a well-defined isoemission point at 558 nm (Fig. 1(b)). As a result, an obvious change in fluorescent color from yellow to red was observed (Fig. 2(b)). This observation is consistent with increased FRET from 1,8-naphthalimide (donor) to the open, colored form of rhodamine (acceptor) as shown in Scheme 1. In the presence of Cr^{3+}, the ratio of emission intensities for rhodamine and to 1,8-naphthalimide at 592 and 544 nm (F_{592}/F_{544}) varied from 0.77 to 5.63, corresponding to a 7.6-fold enhancement. Non-linear fitting of the fluorescence titration curve (Fig. 1(b), inset) exhibited a 1 : 1 stoichiometry for Cr^{3+} and **FD8**, with an association constant of $K_a = 9.4 \times 10^3\ M^{-1}$.[11]

This binding mode is supported by the presence of a peak at m/z 1043.1 (calc. 1043.0) corresponding to $[\mathbf{FD8} + CrCl_2]^+$ in the high-resolution mass spectrum of a mixture of **FD8** and 200 μM $CrCl_3$ (Fig. S4, ESI†). Thus, in accordance with the 1 : 1 stoichiometry and the coordination number of six for Cr^{3+}, the carbonyl O, imino N and quinoline N and O atoms are the most likely binding sites of **FD8** for Cr^{3+} (Scheme 1).

Fluorescence measurements of **FD8** with various metal ions revealed excellent selectivity for Cr^{3+}. As shown in Fig. 3,

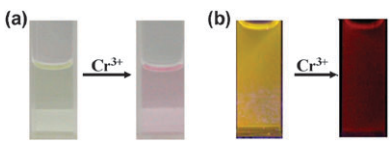


Fig. 2 Change in color (a) and fluorescence (b) of **FD8** (20 μM) upon addition of Cr^{3+} (200 μM).

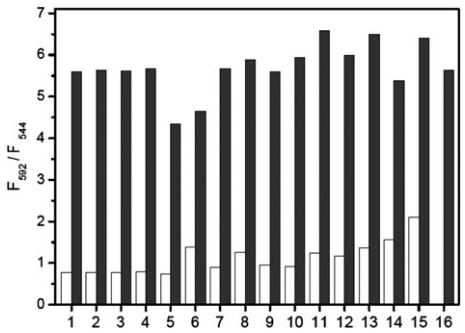


Fig. 3 The ratiometric fluorescence responses (F_{592}/F_{544}) of **FD8** (20 μM) with various metal ions (*x*-axis markers) in ethanol–water (2:1, v/v) solution. Excitation was performed at 405 nm. White bars represent the addition of an excess of the appropriate metal ion (2 mM for Na^+, K^+, Ca^{2+}, Mg^{2+}, 200 μM for all other cations) to a 20 μM solution of **FD8**. Black bars represent the subsequent addition of 200 μM Cr^{3+} to the solution. 1, Na^+; 2, K^+; 3, Mg^{2+}; 4, Ca^{2+}; 5, Mn^{2+}; 6, Ag^+; 7, Co^{2+}; 8, Ni^{2+}; 9, Cu^{2+}; 10, Zn^{2+}; 11, Cd^{2+}; 12, Hg^{2+}; 13, Fe^{2+}; 14, Pb^{2+}; 15, Al^{3+}; 16, Cr^{3+}.

alkali and alkaline-earth metal cations such as Na^+, K^+, Mg^{2+}, Ca^{2+} gave no interference at a 100-fold excess concentration, and transition-metal and heavy-metal ions such as Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Hg^{2+}, Ag^+, Pb^{2+} gave a weak response. Competition experiments for Cr^{3+} mixed with metal ions (200 μM) exhibited no obvious change in F_{592}/F_{544} ratio, although Mn^{2+}, Ag^+ and Al^{3+} induced slight fluorescent quenching or increasing.

A practical bioimaging application of **FD8** for Cr^{3+} in biological samples was developed by confocal fluorescence microscopy on an Olympus IX81 laser scanning microscope. Under selectively excitation of the rhodamine moiety of **FD8** with longer-wavelength light at 515 nm, staining HeLa cells with a 5 μM solution of **FD8** for 30 min at 37 °C led to very weak intracellular fluorescence (Fig. 4(a)). The cells were then supplemented with 50 μM $CrCl_3$ in the growth medium for 30 min at 37 °C and loaded with **FD8** under the same conditions; a significant increase in the fluorescence from the intracellular area was observed (Fig. 4(b)). Bright-field measurements confirmed that the cells after treatment with Cr^{3+} and **FD8** were viable throughout the imaging experiments (Fig. 4(d)). As depicted in

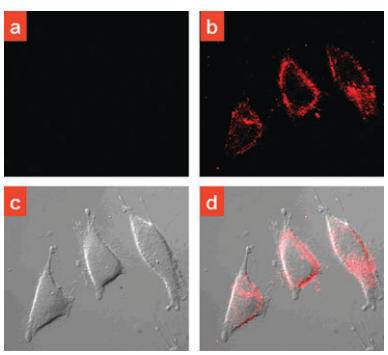


Fig. 4 Confocal fluorescence images in HeLa cells. (a) Cells incubated with **FD8** in PBS buffer for 30 min, (b) Cells incubated with **FD8** for 30 min, washed three times, and then further incubated with 50 μM Cr^{3+} for 30 min (λ_{ex} = 515 nm). (c) Brightfield image of cells shown in panel (b). The overlay image of (b) and (c) is shown in (d).

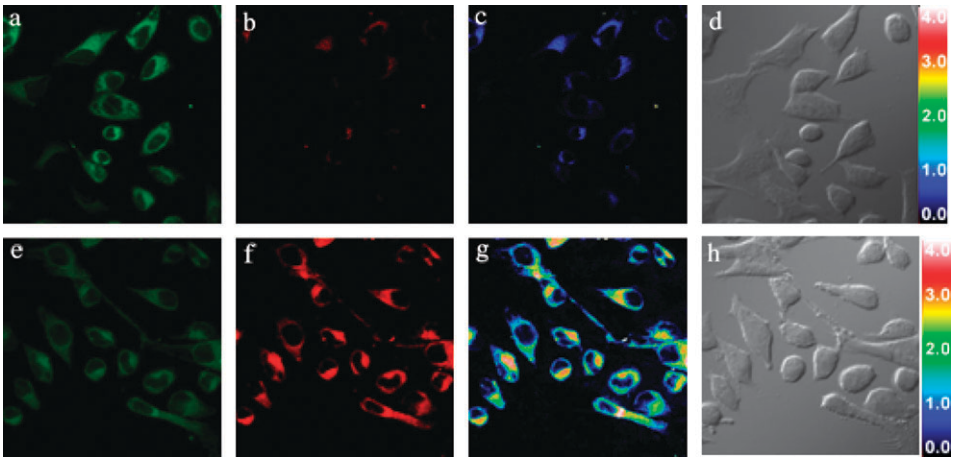


Fig. 5 FRET images in HeLa cells. (Top, a–d) Cells incubated with 5 μM **FD8** for 30 min. (Bottom, e–f) Cells incubated with both 50 μM Cr^{3+} and 5 μM **FD8** for 30 min. Emission was collected by the green channel at 530 ± 20 nm (a and e) and the red channel at 610 ± 40 nm (b and f). (c and g) FRET images with ratio function with red and green channel; (d and h) Bright field imaging (λ_{ex} = 405 nm).

Fig. 4(c), the overlay of fluorescence and bright-field images reveals that the fluorescence signals are localized in the perinuclear area of the cytosol, indicating a subcellular distribution of Cr^{3+} and good cell-membrane permeability of **FD8**.

Furthermore, under excitation of the 1,8-naphthalimide moiety of **FD8** with short-wavelength light at 405 nm, FRET images were easily obtained with the Olympus microscopy software when fluorescence emission were collected at the green (530 ± 20 nm) and red channel (610 ± 40 nm). As shown in Fig. 5. HeLa cells incubated with 5 μM **FD8** for 30 min at 37 °C showed a ratio of the emission at 610 ± 40 and 530 ± 20 nm of <1.0 (Fig. 5(c)). Upon addition of 50 μM $CrCl_3$ to **FD8**-loaded HeLa cells, however, the fluorescence intensity of green channel decreased and that of red channel was enhanced, so the ratio was increased to $\sim$3.0 (Fig. 5(g)). The results suggest that **FD8** could be used for monitoring intracellular Cr^{3+} with FRET methods. To the best of our knowledge, this is the first example of a fluorescent probe for monitoring Cr^{3+} in living cells.

In conclusion, we have demonstrated that **FD8** is a FRET-based probe for Cr^{3+} that exhibits excellent selectivity over other metal ions. Moreover, confocal fluorescence microscopy confirmed that **FD8** can be used for monitoring intracellular Cr^{3+} levels in living cells with general fluorescence and FRET methods. We anticipate that this probe will be of great benefit to biomedical researchers for studying the bioactivity of Cr^{3+} in biological systems.

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