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Development of FRET-Based Ratiometric Fluorescent Cu^{2+} Chemodosimeters and the Applications for Living Cell Imaging

Lin Yuan, Weiying Lin,* Bin Chen, and Yinan Xie

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, Hunan 410082, People's Republic of China

weiyinglin@hnu.edu.cn

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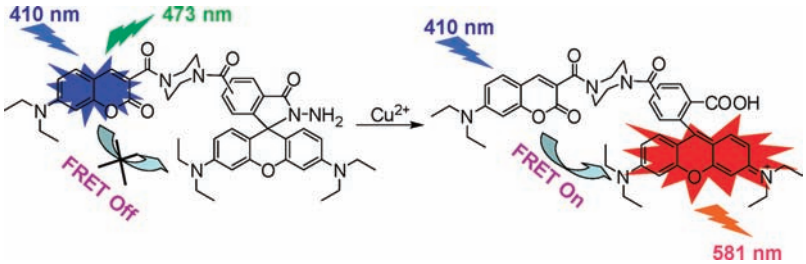
ABSTRACT



Coumarin−rhodamine-based compounds 1a,b were rationally designed and synthesized as novel FRET ratiometric fluorescent chemodosimeters. Ratiometric chemodosimeters 1a,b exhibit several favorable features, including a large variation in the emission ratio, well-resolved emission peaks, high sensitivity, high selectivity, low cytotoxicity, and good cell membrane permeability. Importantly, these excellent attributes enable us to demonstrate ratiometric imaging of Cu^{2+} in living cells by using these novel ratiometric fluorescent chemodosimeters.

As the third most abundant essential trace element after iron and zinc in the human body, copper ions play an important role in various physiological processes.[1−3] The total copper content of the adult human body contains typically is 70−80 mg of copper under normal conditions.[2] However, copper ions in abnormal levels are toxic and can cause oxidative stress and neurological disorders, including Alzheimer's, Parkinson's, Menkes', and Wilson's diseases.[3] Thus, it is of high importance to detect copper ions.

In the past few years, although a wide variety of techniques have been developed to detect copper ions, fluorescence sensing has become the gold standard, due to its high sensitivity, high selectivity, and useful applications in the environment, chemistry, biology, and medicine.[4] Thus, the design and synthesis of fluorescent copper probes has received intense attention,[5] most of which, however, are fluorescence intensity-based probes. Although turn-on probes have high sensitivity due to the lack of background signal, a major limitation of intensity-based probes is that

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variations in the sample environment may influence the fluorescence intensity measurements. In principle, this problem can be alleviated by using ratiometric fluorescent probes. Ratiometric probes allow the measurement of fluorescence intensities at two wavelengths, which should provide a built-in correction for environmental effects.[4d,6] Although some ratiometric fluorescent copper probes have been constructed in recent years,[7] reports on the ratiometric imaging of copper ions in living cells are very scarce. Thus, it is still highly challenging to design small-molecule ratiometric fluorescent probes for Cu^{2+} with favorable photophysical properties suitable for ratiometric imaging of copper ions in living cells.

Förster resonance energy transfer (FRET) is a nonradiative process in which an excited dye donor transfers energy to a dye acceptor through long-range dipole–dipole interactions.[8] FRET is the commonly exploited sensing mechanism for design of ratiometric fluorescent probes.[9] Recently, some FRET-based ratiometric fluorescent probes for Cu^{2+} [7a,b] have been reported, none of which, however, have been applied for ratiometric imaging of Cu^{2+} in living cells.

To design small-molecule FRET ratiometric fluorescent probes for ratiometric detection of Cu^{2+} in living cells, it is required to formulate a FRET platform, which consists of an energy donor, a linker, and an energy acceptor. Then an appropriate interaction site for Cu^{2+} is introduced on the FRET platform to modulate the energy transfer efficiency. In general, the emission spectrum of the donor should have reasonable overlap with the absorption spectrum of the acceptor.[10] Furthermore, for practical applications, some design criteria involving energy donors, acceptors, and linkers should be considered. (1) The absorption spectrum of the donor should be well separated from that of the acceptor to ensure independent excitation at the absorption wavelengths of the donor and acceptor, respectively. (2) The emission spectrum of the donor should be well resolved from that of the acceptor for high accuracy in the measurement of fluorescence intensity ratios. (3) Appropriate linkers should be selected to avoid the static fluorescence quenching. Obviously, these requirements render the selection of suitable energy donors, energy acceptors, and linkers demanding.

Herein, we report compounds **1a**,**b** (Scheme 1) as new ratiometric fluorescent chemodosimeters for Cu^{2+}. The novel ratiometric chemodosimeters **1a**,**b** were rationally designed on the basis of a coumarin–rhodamine scaffold as the FRET platform, which bears a large emission wavelength shift (around 110 nm) between the coumarin emission and rhodamine emission.[9g] Furthermore, there is proper spectral overlap ($J_{DA} = 3.6 \times 10^{14}\ M^{-1}\ cm^{-1}\ nm^4$) between the coumarin emission and rhodamine absorption in compounds **4a**,**b**.[10,9e] A piperazine moiety was chosen as the rigid linker, which may avoid the likely static fluorescence quenching. Furthermore, the piperazine linker renders a suitable distance between the energy donor (coumarin) and acceptor (rhodamine) (Figure S1, Supporting Information). These two factors should ensure a high energy transfer efficiency from the coumarin energy donor to the rhodamine acceptor. A hydrazide functional group was chosen as the potential reaction site for Cu^{2+}.[11] Notably, one unique feature of our design is that the energy donor (coumarin) is distant from the reaction site of Cu^{2+}. Thus, we envisioned that the interactions between the reaction site and Cu^{2+} would not be affected by the coumarin donor, and the sensitivity of the FRET chemodosimeter should be comparable to that of the rhodamine-based fluorescence turn-on type Cu^{2+} chemodosimeters.[11] In the absence of Cu^{2+}, the excited energy of the coumarin donor could not be transferred to the rhodamine acceptor, as the rhodamine acceptor is in the closed form. Thus, the FRET should be off in the free **1a** or **1b**. In other words, upon excitation at the coumarin donor, only the emission of the coumarin dye is observed. However, we envisioned that addition of Cu^{2+} may induce the rhodamine acceptor to be in the ring-opened form via Cu^{2+}-promoted hydrolysis of rhodamine-B hydrazide to rhodamine-B, as reported previously.[11] Thus, the FRET is switched on when the new ratiometric chemodosimeter **1a** or **1b** is incubated with Cu^{2+} and turned into **4a** or **4b**, respectively. Obviously, we should note the emission of the rhodamine acceptor upon excitation at the coumarin donor.

Chemodosimeters **1a**,**b** were synthesized on the basis of the route shown in Scheme 1. Condensation of piperazyl-coumarin **3** with the mixture of 4′- and 5′-carboxyrhodamines **2a,b** by standard coupling chemistry yielded the mixture of FRET dyads **4a**,**b.** We then tried to separate compounds **4a**,**b** by standard column chromatography. Unfortunately, this attempt was proved to be very difficult, although we could separate the mixture of compounds **4a**,**b**

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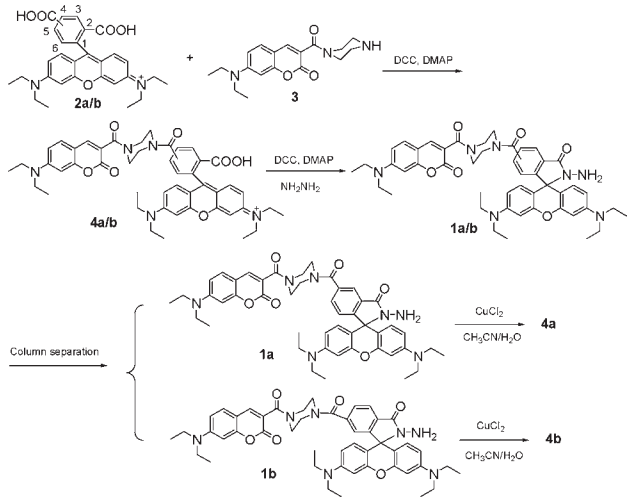
Scheme 1. Design and Synthesis of FRET-Based Ratiometric Fluorescent Cu^{2+} Chemodosimeters **1a**,**b**



by preparative thin-layer chromatography (CH_2Cl_2/CH_3OH 9/1). Therefore, we decided to transform the mixture of compounds **4a**,**b** to compounds **1a**,**b,** which are much less polar and have good solubility in many organic solvents, and the separation of compounds **1a**,**b** by standard column chromatography may be feasible. Indeed, the separation of the mixture of compounds **1a**,**b** could be readily conducted by standard column chromatography to give the individual compound in the pure isomeric form.

The titrations of the novel ratiometric probe **1a** (1 μM) with Cu^{2+} were conducted in HEPES buffer (pH 7.0, containing 20% CH_3CN as a cosolvent). As shown in Figure 1A, upon excitation at 410 nm (the coumarin maximal absorption), the free **1a** displays a single emission band centered at 473 nm, attributed to the emission of coumarin. There is no FRET in the free **1a**, as the rhodamine acceptor is in the ring-closed form. In contrast, addition of Cu^{2+} decreases significantly the fluorescence intensity at around 473 nm and simultaneously a new red-shifted emission band at around 581 nm, ascribed to the emission of the rhodamine acceptor, is formed. Thus, addition of Cu^{2+} elicits a large red shift (108 nm) in emission, which almost completely resolves the two emission peaks. Notably, this is highly favorable for the accuracy in determination of the fluorescence ratios. The observed rate constants of **1a** (1 μM) to Cu^{2+} (50 μM) were determined as 0.135 min^{-1} (Figure S2) under the pseudo-first-order conditions. The changes in the absorption spectra (Figure S3) are in good agreement with the variations in the emission profile. The ratios of fluorescence intensities at 581 and 473 nm (I_{581}/I_{473}) exhibit a drastic change from 0.047 in the absence of Cu^{2+} to 9.8 in the presence of 50 μM Cu^{2+} (Figure 1B), a 208-fold variation in the emission ratios. Importantly, the chemodosimeter shows an excellent linear relationship between the emission ratios and the concentrations of Cu^{2+} from 0.08 μM to 30 μM (Figure S4), suggesting that the chemodosimeter is potentially useful for quantitative determination of Cu^{2+}. Like **1a**, the chemodosimeter **1b** also shows the significant fluorescence (Figures S5 and S6) and absorption changes (Figure S7).

As shown in Scheme 1, treatment of the chemodosimeter **1a** or **1b** with Cu^{2+} could afford compound **4a** or **4b**, respectively (see the Supporting Information regarding the synthesis of compounds **4a**,**b** from **1a**,**b** for details). Furthermore, the excitation and emission spectra of compounds **4a**,**b** are consistent with those of **1a**,**b** in the presence of 50 μM Cu^{2+} (Figures S8 and S9). This unambiguously confirms that the ratiometric response is indeed due to the Cu^{2+}-promoted hydrolysis of **1a**,**b** to **4a**,**b**.[11]

As a representative example, the different energy transfer efficiencies in compounds **1a** and **4a** were explained by theoretical calculations (Figure S10). For **1a**, the HOMO–LUMO energy gap of the rhodamine energy acceptor (ΔE = 3.27 eV) is greater than that of the coumarin energy donor (ΔE = 2.50 eV), suppressing the energy transfer from coumarin to rhodamine. However, for **4a**, the energy gap of rhodamine (ΔE = 1.81 eV) is markedly less than that of the coumarin group (ΔE = 2.51 eV), facilitating the intramolecular FRET.[7b]

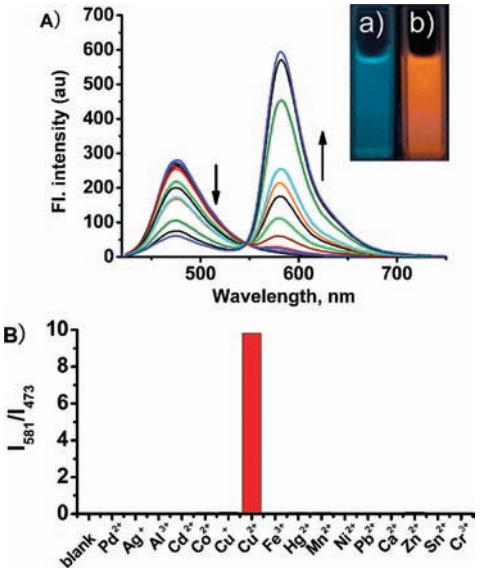


Figure 1. (A) Emission spectra (excitation at 410 nm) of the novel ratiometric chemodosimeter **1a** (1 μM) in the presence of various amounts of Cu^{2+} (0–50 μM) in HEPES buffer (pH 7.0, containing 20% CH_3CN as a cosolvent). The inset shows the visual fluorescence color of chemodosimeter **1a** (1 μM) before (left) and after (right) addition of 50 μM of Cu^{2+} on excitation at 365 nm using a hand-held UV lamp. (B) Fluorescence ratio (I_{581}/I_{473}) of chemodosimeter **1a** (1 μM) in the presence of various analytes (50 μM) in HEPES buffer (pH 7.0, containing 20% CH_3CN as a cosolvent).

The detection limit (S/N = 3) of the ratiometric chemodosimeter **1a** was determined to be 13 nM in HEPES buffer

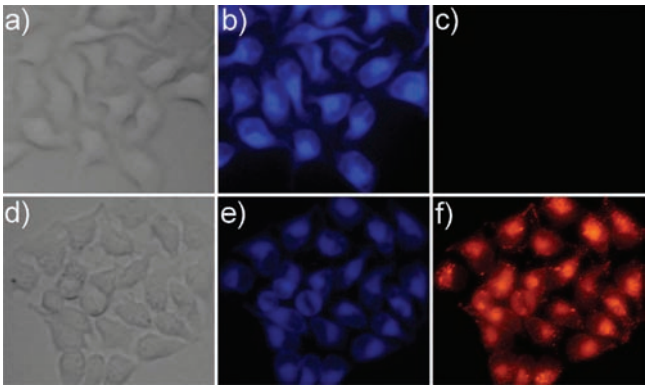


Figure 2. Images of HeLa cells treated with the ratiometric chemodosimeter **1a**: (a) bright field image of HeLa cells incubated with chemodosimeter **1a** (5 μM); (b) fluorescence image of (a) from blue channel; (c) fluorescence image of (a) from red channel; (d) bright field image of HeLa cells incubated with chemodosimeter **1a** (5 μM) for 30 min, and then further incubation with Cu^{2+} (100 μM) for 30 min at 37 °C; (e) fluorescence image of (d) from blue channel; (f) fluorescence image of (d) from red channel.

(pH 7.0, containing 20% CH_3CN as a cosolvent), which is comparable to that of the rhodamine-based fluorescence turn-on type Cu^{2+} chemodosimeters,[11] confirming the validity of our FRET design strategy. Additionally, the ratiometric chemodosimeters **1a**,**b** both exhibit a high selectivity for Cu^{2+} over other metal ions represented by Ag^{+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Al^{3+}, Ca^{2+}, Sn^{2+}, and Zn^{2+} (Figure 1B and Figure S11). The high selectivity is further supported by the observation that other species have only negligible interference with the fluorescence response (Figures S12 and S13). Thus, the chemodosimeters **1a**,**b** can both selectively sense Cu^{2+} in a ratiometric fashion.

As a representative example, the ratiometric chemodosimeter **1a** was applied for ratiometric fluorescence imaging in living cells. HeLa cells were incubated with only chemodosimeter **1a** (5 μM) for 30 min at 37 °C, and the cells show intense fluorescence in the blue channel (Figure 2b) but almost no fluorescence in the red channel (Figure 2c). However, treatment of $CuCl_2$ with **1a**-loaded cells elicit a partial fluorescence decrease in the blue channel (Figure 2e) and strong fluorescence in the red channel (Figure 2f), consistent with Cu^{2+}-induced ratiometric fluorescence response. The ratiometric fluorescence images are shown in Figure S14. Thus, the results indicate that chemodosimeter **1a** is cell membrane permeable and could be employed for ratiometric fluorescence imaging of Cu^{2+} fluctuations in the living cells. In addition, the MTT assays (Figure S15) suggest that the ratiometric chemodosimeter has low cytotoxicity to the cells.[12]

In conclusion, we have judiciously designed and synthesized compounds **1a**,**b** as novel fluorescent chemodosimeters suitable for ratiometric fluorescent imaging of Cu^{2+} in living cells on the basis of the Cu^{2+}-induced FRET off-on. Remarkably, the new ratiometric fluorescent chemodosimeters exhibit a very large variation in the fluorescence ratio (I_{581}/I_{473}) and two well-resolved emission peaks. The other prominent features of **1a**,**b** include high sensitivity, high specificity, low cytotoxicity, and good cell membrane permeability. Significantly, we have demonstrated ratiometric imaging of Cu^{2+} in living cells by using **1a**. The FRET technology on the basis of the coumarin–rhodamine platform has a great potential for development of a wide variety of ratiometric fluorescent chemodosimeters due to its excellent photophysical properties.

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Supporting Information Available. Text, tables, and figures giving experimental procedures, characterization data, and some spectra. This material is available free of charge via the Internet at http://pubs.acs.org.