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Construction of a FRET-based ratiometric fluorescent thiol probe†

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We have rationally constructed a novel FRET-based ratiometric thiol probe suitable for ratiometric imaging in living cells based on the native chemical ligation reaction.

Small-molecular-weight biological thiols, for instance cysteine (Cys), play a critical role in many biological processes. However, abnormal levels of Cys are implicated in a variety of diseases, such as liver damage, skin lesions, and slowed growth.[1] Thus, it is of intense interest to develop sensitive and selective fluorescent methods[2] for the detection of biological thiols. Some fluorescent probes for thiols have been developed.[3] However, a vast majority of them respond to thiols with optical signal changes only in the fluorescent intensity. The intensity-based probes can be affected by the variations in the sample environment. By contrast, ratiometric fluorescent probes allow the measurement of emission intensities at two wavelengths, which should provide a built-in correction for environmental effects.

Förster resonance energy transfer (FRET) is a signaling mechanism may be exploited for ratiometric fluorescent probe development. Recently, two research groups constructed FRET-based thiol probes by linking a fluorescent dye with a dye quencher.[3c,4] However, their FRET-based probes only exhibited a fluorescence turn-on response to thiols. Thus, notably, their FRET-based probes are not ratiometric. In this communication, we describe the construction of the first FRET-based ratiometric thiol probe based on the native chemical ligation reaction.

The native chemical ligation (NCL) reaction has been widely used in peptide synthesis.[5] Native chemical ligation of peptide segments involves reaction between a peptide-α-thioester and an N-terminal cysteine-peptide. The prominent features of the NCL reaction include: (1) the reaction is highly chemoselective for thiols and devoid of any interference from other biologically relevant species; (2) the reaction is *in vivo* compatible and can proceed inside living cells. It is important to note that the NCL reaction has not been previously exploited to design small-molecule fluorescent thiol probes.

In this work, we have judiciously designed a NCL-based ratiometric fluorescent thiol probe (**NRFTP**, Fig. 1, Fig. S1 (ESI†)) for ratiometric imaging of thiols in living cells. The probe is composed of a rhodamine dye, a thioester group, a piperazyl moiety, and a Bodipy dye. The selection of Bodipy and rhodamine as the fluorophores is based on the consideration that the emission of Bodipy has a strong overlap with the absorption of rhodamine. Thus, they are suitable as a FRET dyad in which Bodipy and rhodamine act as the energy donor and acceptor, respectively.[6] The piperazyl moiety was chosen as the rigid linker to facilitate the energy transfer between the Bodipy donor and the rhodamine acceptor. The distance from the boron atom in the Bodipy dye to the oxygen atom in the xanthene ring of the rhodamine moiety was calculated to be 22.88 Å (Fig. S2, ESI†). Thus, we expected that the excited energy of the Bodipy donor could be efficiently transferred to the rhodamine acceptor in the free **NRFTP**. To employ the NCL reaction for the thiol probe design, a thioester group is required as the potential reaction site for thiols. Notably, to promote the NCL reaction, we judiciously selected a thiophenylester moiety as the reaction site of the probe, as it is a better leaving group than a thioalkylester group in physiological conditions.[5,7] According to the NCL reaction, we reasoned that when the probe is treated with Cysteine (Cys), the nucleophilic thiol group of Cys could attack the electrophilic carbon of the thioester group leading to the cleavage of the FRET dyad. Since the distance is infinite after the thiol-induced cleavage of the FRET dyad, the FRET should be switched off in the presence of thiols. Thus, the thiol-induced cleavage of the probe may afford the Bodipy moiety **8** and rhodamine moiety **10**. For the formation of the rhodamine moiety, according to the NCL reaction, a native amide bond will form between the rhodamine dye and Cys after the expected *trans*-thioesterification and intramolecular rearrangement reactions. At the physiological pH, the resulting N atom of the amide group may further attack the electrophilic 9-position carbon of the conjugated xanthene ring to give the non-fluorescent deconjugated spirolactam compound **10** based on the well-known rhodamine chemistry.

A versatile new convergent strategy was designed to synthesize **NRFTP** in 12 steps. The Bodipy donor building block **2** and the linker building block **6** were prepared as shown in Schemes

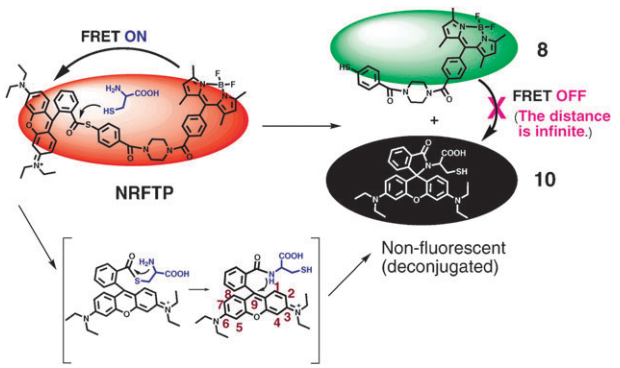

Fig. 1 Design of ratiometric fluorescent thiol probe **NRFTP** based on the NCL reaction.

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† Electronic supplementary information (ESI) available: Experimental procedures, characterization data for the probe, and some spectra of the probe. See DOI: 10.1039/c0cc03806g

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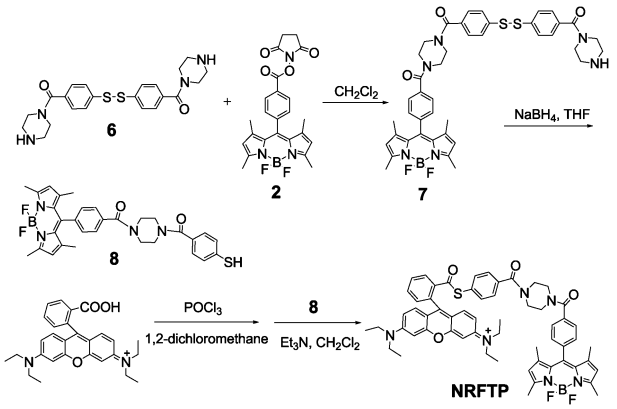

Scheme 1 Synthesis of ratiometric thiol probe **NRFTP**.

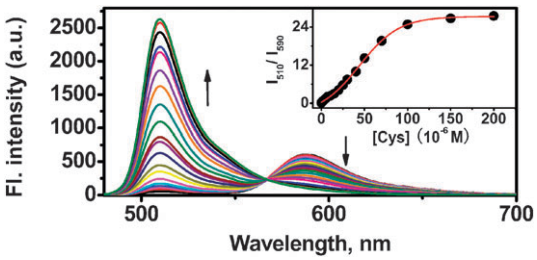

Fig. 2 Fluorescence spectra (with excitation at 470 nm) of **NRFTP** (1.3 μM) upon addition of increasing concentrations (0–200 μM) of Cys in 25 mM potassium phosphate buffer (pH 7.4, containing 45% CH_3CN as a co-solvent). The inset shows the changes of fluorescent intensity ratios at 510 and 590 nm (I_{510}/I_{590}) to increasing concentration of Cys.

S1 and S2 (ESI†), respectively. Reaction of amine **6** with succinimidyl ester **2** in reflux afforded amide **7** (Scheme 1). The disulfide bond of compound **7** was reduced by $NaBH_4$ at room temperature to give the key intermediate Bodipy sulfhydryl **8**. Rhodamine B was converted into the acid chloride derivative, which was then coupled with Bodipy sulfhydryl **8** to provide **NRFTP**. The reference thioester **9** was prepared by a similar procedure (Scheme S3, ESI†).

The absorption spectrum of **NRFTP** exhibited the characteristic absorption of the Bodipy donor with a peak at 500 nm and the typical absorption of the rhodamine acceptor with a peak at around 562 nm (Fig. S3, ESI†), indicating that there are little or no electronic interactions between the Bodipy donor and the rhodamine acceptor in the ground state. When **NRFTP** was excited at the Bodipy donor absorption (Fig. S4, ESI†), the featured emission of the Bodipy donor at around 510 nm was nearly completely quenched when compared with that of the reference Bodipy donor **8**. Concurrently, the emission of the rhodamine acceptor at around 590 nm displayed a marked fluorescence enhancement relative to that of the reference rhodamine acceptor **9** at the same concentration. This suggests that the excitation energy of the Bodipy donor is efficiently transferred to the rhodamine acceptor. The intramolecular energy transfer efficiency from the Bodipy donor to the rhodamine acceptor in **NRFTP** was calculated to be 98.4% (see the ESI†). The efficient intramolecular energy transfer was collaborated by the excellent correspondence between the absorption and excitation spectra of the probe (Fig. S5, ESI†).

The free probe only showed the emission of rhodamine at 590 nm with excitation at 470 nm. However, when **NRFTP** was titrated with increasing concentrations of Cys (Fig. 2), the intensity of the rhodamine emission at 590 nm gradually decreased with the concomitant growth of a new emission peak of Bodipy at 510 nm, indicating that the FRET is off. A well-defined isoemission point was noted at 567 nm. The changes in the absorption spectra (Fig. S6, ESI†) are in good agreement with the variation in the emission profile. As shown in the inset of Fig. 2, the fluorescent intensity ratios at 510 and 590 nm (I_{510}/I_{590}) exhibited a drastic change from 0.09 in the absence of Cys to 24.82 in the presence of Cys (100 μM), a 275-fold enhancement in the emission ratios. It should be noted that such a dramatic change of signal ratios at two wavelengths is highly desirable for ratiometric probes, as the sensitivity as well as the dynamic range of ratiometric probes are controlled by the ratios. The emission ratios have an excellent linear relationship with the concentrations of Cys from 1.0×10^{-7} to 1000×10^{-7} M (Fig. S7, ESI†), suggesting that the probe is potentially useful for quantitative determination of thiol concentrations in a large dynamic range. The detection limit of the probe was determined to be 8.2×10^{-8} M (S/N = 3). A similar ratiometric fluorescence response was noted when the probe was titrated with other small-molecular-weight biological thiols, homocysteine or glutathione (Fig. S8, ESI†).

Furthermore, when excited at 550 nm, the fluorescent intensity of the probe at 590 nm was gradually decreased with the addition of elevated concentrations of Cys (Fig. S9, ESI†), consistent with the decreasing of rhodamine absorption at 562 nm (Fig. S6, ESI†). These data suggest that the conjugated xanthene structure in the rhodamine acceptor was destroyed, as anticipated. To further confirm the design hypothesis, the reaction products **8** and **10** of **NRFTP** with Cys were isolated and subjected to the standard NMR and mass spectroscopy characterization (Fig. S12–S15, ESI†), demonstrating that the ratiometric fluorescence response of the probe to Cys is indeed due to the NCL reaction.

The free **NRFTP** is highly stable in the assay conditions (Fig. S16, ESI†), and the fluorescence intensity is almost unchanged with excitation at 470 nm for 30 min (Fig. S17, ESI†). The time course of the fluorescent intensity ratio (I_{510}/I_{590}) in the absence or presence of Cys was displayed in Fig. S18 (ESI†). Upon introduction of Cys, a dramatic enhancement in the emission intensity ratios was noted, and the ratios essentially reached the maximum in 20 minutes. Thus, an assay time of 20 min was selected in the evaluation of the selectivity and sensitivity of the probe to Cys. The free probe is stable over a wide pH range of 2.7–10.0, and the probe can be employed to detect thiols under the physiological pH value (Fig. S19, ESI†).

To examine the selectivity, **NRFTP** was treated with various biologically relevant species including amino acids (Cys, Phe, Ala, Gly, Glu, Arg, Lys, Tys, Leu, Ser, Val), glucose, metal ions (K^+, Ca^{2+}, Zn^{2+}, Fe^{3+}), a reactive oxygen species (H_2O_2), and a reducing agent [nicotinamide adenine dinucleotide (NADH)]. The probe is highly specific for thiols over other biologically relevant species (Fig. 3 and Fig. S20 (ESI†)), in good agreement with the chemoselective nature of the NCL

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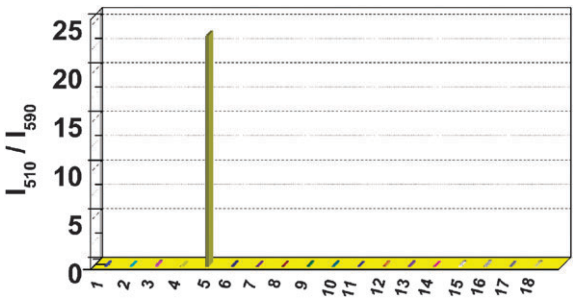

Fig. 3 Ratiometric response (I_{510}/I_{590}) of **NRFTP** (1.3 μM) to 100 μM of various species: (1) KCl; (2) $CaCl_2$; (3) $ZnCl_2$; (4) $FeCl_3$; (5) Cys; (6) glucose; (7) NaDH; (8) H_2O_2; (9) Phe; (10) Ala; (11) Gly; (12) Glu; (13) Arg; (14) Lys; (15) Tys; (16) Leu; (17) Ser; (18) Val. λ_{ex} = 470 nm.

reaction. In addition, the visual response of the probe to various species indicates that the probe can be used conveniently for thiol detection by simple visual inspection (Fig. S21, ESI†).

Probe **NRFTP** was then applied for thiol detection in real biological samples. Aliquots of reduced serum sample were added to a solution of **NRFTP** (1.3 μM) (see the ESI†). As exhibited in Fig. S22 (ESI†), the increase in the amount of newborn-calf serum elicited a linear enhancement in the emission ratios (I_{510}/I_{590}), suggesting that the probe is capable of ratiometric sensing of thiols in the plasma sample. For quantitative measurement, a standard addition method with Cys as the standard was used to estimate the unknown concentration of total thiols in a human urine sample from a healthy volunteer. The total content of thiols in the urine sample was analyzed to be 40 ± 3 μM, which is well within the reported thiol concentration range for the urine samples from the healthy individuals.[8]

The probe has relatively low toxicity to the HeLa cells (Fig. S23, ESI†). The suitable amphipathicity and low toxicity of probe **NRFTP** may render it applicable for ratiometric imaging in living cells. To examine this possibility, the living Hela cells were treated with **NRFTP**. The dual-channel fluorescence images recorded at 515 ± 10 nm and 610 nm ± 10 nm with excitation at 488 nm were shown in Fig. 4. Incubation of living HeLa cells with **NRFTP** (2 μM) for 30 min provided a strong green fluorescence in the Bodipy emission window (Fig. 4b) and almost no red fluorescence in the rhodamine emission window (Fig. 4c). By contrast, in a control experiment, the Hela cells were pre-treated with *N*-ethylmaleimide (1 mM, as a thiol-reactive reagent) for 30 min, and further incubated with the probe (2 μM) for 30 min, giving intense red fluorescence (Fig. 4g) but essentially no green fluorescence (Fig. 4f), in good agreement with the emission profile of the free probe. The ratiometric imaging data are shown in Fig. S24 (ESI†). Thus, these results establish that **NRFTP** is cell membrane permeable and able to display FRET-based ratiometric fluorescence response to intracellular thiols.

In summary, the FRET-based ratiometric thiol probe **NRFTP** was constructed based on the NCL reaction. The favorable features of the probe include high stability and functioning well at physiological pH, high selectivity, high

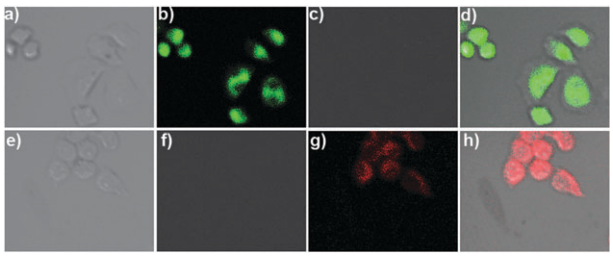

Fig. 4 Confocal fluorescence images of living HeLa cells (excitation at 488 nm). (a) Bright-field image of HeLa cells incubated with **NRFTP** (2 μM) for 30 min; (b) fluorescence image of (a) with emission at 515 ± 10 nm; (c) fluorescence image of (a) with emission at 610 ± 10 nm; (d) overlay of the images of (a), (b), and (c). (e) Bright-field image of HeLa cells pre-treated with 1 mM *N*-ethylmaleimide for 30 min, and then incubated with the probe (2 μM) for 30 min; (f) fluorescence image of (e) with emission at 515 ± 10 nm; (g) fluorescence image of (e) with emission at 610 ± 10 nm; (h) overlay of the images of (e), (f), and (g).

sensitivity, a large ratio signal variation, and a large linear dynamic range. The probe has been applied for ratiometric detection of thiols in biological fluids. Importantly, the FRET-based ratiometric imaging of thiols in living cells has been demonstrated by employing **NRFTP**. Thus, we expect that **NRFTP** will be a useful molecular tool for diverse biological applications including the determination of thiol levels in biological fluids, fluorescence labelling of proteins, and the assaying of enzymes with a thiol as a product for enzyme–inhibitor screening. Furthermore, the NCL-based thiol probe design concept should be widely applicable for construction of ratiometric thiol probes.

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