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A long-wavelength fluorescent turn-on probe for video detection of biological thiols in living cells

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We have designed and synthesized the compound FR-thiol based on a new far-red platform as a turn-on fluorescent probe for biological thiols. The turn-on probe FR-thiol displayed a high selectivity and sensitivity to thiols. Finally, we have demonstrated that FR-thiol is suitable for video observation of biological thiols in living cells.

Introduction

Small-molecular-weight biological thiols, including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play a critical role in many biological processes. However, abnormal levels of Cys, Hcy, and GSH are implicated in a variety of diseases, such as liver damage, skin lesions, slowed growth, edema, and high sensitivity and selectivity. It is of intense interest for the detection of biological thiols with high sensitivity and selectivity.

So far, several analytical techniques, including high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), electrochemical assay, UV/Vis spectroscopy, Fourier transform infrared (FTIR) spectroscopy, mass spectrometry, and fluorescence spectroscopy, have been devoted to the detection of thiols. Although these methods provide sensitive analysis, they require complicated sample preparation, sophisticated instrumentation, or destruction of tissues or cells. By contrast, fluorescence sensing via microscopy is noninvasive.

Thus, the development of fluorescent probes for biological thiols is of high interest. However, fluorescent thiols probes with both maximal absorption and emission wavelength in the far-red or near-infrared region are relatively few, although they are highly desirable for biological imaging of thiols in living cells. Thus, it is necessary to construct long-wavelength fluorescent probes with favorable properties for monitoring thiols in living cells.

Herein, we rationally designed probe FR-thiol as a novel fluorescence turn-on probe for thiols. The probe exhibits large turn-on signal upon treatment with biological thiols. Importantly, we have demonstrated that the probe could detect thiol in biological samples.

Results and Discussion

Design and synthesis of probe FR-thiol

The protection/deprotection approach has been extended to cage fluorescent dyes to construct the fluorescent probes for the particular analyte. In this work, we designed a fluorescent turn-on probe based on the protection/deprotection approach. As is well known, an ideal fluorescent probe consists of two parts, a recognition moiety with high selectivity for one kind of analyte only (responsible for the selective reaction with the target analyte) and a fluorescent dyes moiety that is highly sensitive to the environment (the analyte of interest and leading to the release of the free fluorescent molecule). As shown in Fig. 1, probe FR-thiol is composed of a new long-wavelength fluorescent dye and a 2,4-dinitrobenzenesulfonate group. The 2,4-dinitrobenzenesulfonate group was chosen for reaction site based on several considerations. Firstly, the 2,4-dinitrobenzenesulfonate group is a specific reaction site for thiol. Secondly, it can act as a protection group for this new dye. We thus anticipated that probe FR-thiol may be essentially non-fluorescent. However, upon reaction with thiol to release the 2,4-dinitrobenzenesulfonate unit, the fluorescence should be recovered.

Probe FR-thiol was readily prepared in three steps (Scheme S1). Reference compound FR-OCH3 was synthesized by saccharidized condensation reaction between benzocyclohexanone derivatives 1 and salicylaldehyde derivatives 2. The starting materials 1 and 2 were commercially available. All new compounds were carefully characterized by 1H NMR, 13C NMR,
MS (ESI), and HRMS (ESI).

**Optical properties**

To further support our design concept of the fluorescent turn-on probe FR-thiol, we then proceeded to investigate their optical properties. The absorption and emission profiles of compounds FR-OCH3 and FR-OH in distinct solvents (CH3CH2OH, PBS containing 10% CH3CH2OH) are shown in Fig. 2. Firstly, we investigated the spectral properties of FR-OCH3 and FR-OH dyes in protic solvent CH3CH2OH. FR-OCH3 and FR-OH dyes have a maximum absorption peak at around 561 nm and 594 nm, respectively. Fluorescence spectra of FR-OH exhibits strong fluorescence with a maximum emission peak at around 624 nm in CH3CH2OH. Reference compound FR-OCH3, which bears no electron-donating group, displays weak fluorescence. To get more insight into the photophysical properties of this FR-OCH3 and FR-OH dyes, we continue to test the spectral properties of these dyes in PBS containing 10% CH3CH2OH. Upon excitation at 550 nm, compound FR-OH displays a strong emission bands at around 624 nm and FR-OCH3 shows a weak emission (Fig. 2).

The molar extinction coefficients of the new FR-OCH3 and FR-OH dyes are 39480 M-1cm-1 and 61920 M-1cm-1, respectively. Importantly, compound FR-OH has a fluorescence quantum yield of 0.57 in CH3CH2OH, which is relatively large for classical Rhodamine dyes. Furthermore, the fluorescence quantum yield of compound FR-OCH3 is only 0.016, much less than that of compound FR-OH. This is attributed to the alkyloxy substituent of FR-OCH3 which reduces the electron-donating ability of the oxygen atom and thus forfids the formation of the zwitterionic resonance form.

Thus, these data suggest that the optical properties of new far-red dye FR-OH can be regulated by alkylation on the hydroxyl group.

To further understand the role of the hydroxyl group playing on the photophysical properties, we then examined the fluorescence profiles of the new FR-OH dye at different pH values. As shown in Figure S1, with the enhancement of pH from 3.0-10.0, the absorption band at around 550 nm undergoes a red-shift to a peak at around 584 nm. As shown in Fig. 3, The emission profiles of compound FR-OH is pH-dependent. Upon excitation at 550 nm, enhancement of pH from 3.0-10.0 induces a fluorescence turn-on signal in the far-red region (Fig. 3). The pKa of compound FR-OH was calculated to be 6.45 based on the Henderson–Hasselbach-type mass action equation. These results implies the protection/depredation approach can be extended to cage fluorescent dyes to construct the fluorescent probes.

**Sensing of the probe to thiol**

With the probe FR-thiol in hand, we firstly evaluated the capability of probe FR-thiol to detect cysteine in PBS. As designed, the free probe is almost non-fluorescent in PBS (Figure 4a). However, addition of cysteine induces a dramatic change in the fluorescence spectra. A significant fluorescence turn-on response at 624 nm (up to 20-fold) was observed. Consistently, a marked red-shift from 544 to 580 nm was noted in the absorption spectra upon treatment of the probe with cysteine (Figure S2). Both the mass spectrometry and NMR analyses confirmed that the fluorescence turn-on is indeed due to the thiol-mediated removal of the 2,4-dinitrobenzenesulfonyl moiety (Figure S3 and Figure S4). The detection limit for FR-thiol was calculated to be 1.87 × 10⁻⁷ M (Figure S5), indicating that the probe is highly sensitive to cysteine.

We also evaluated the capability of FR-thiol to detect other biological thiols (GSH) in PBS buffer. The titrations of the novel probe FR-thiol (5 µM) with GSH were conducted in PBS buffer (pH 7.4, containing 10% EtOH as a cosolvent). Fig 4b shows the fluorescence emission spectra (excitated at 550nm) of compounds FR-thiol treated with different concentrations of GSH. When the GSH concentration was increased, the intensity of the maximum emission peak at 624 nm progressively increased. A maximal fluorescence enhancement (about 11-fold) was obtained when the concentration of GSH reached 5.0 equiv. These data
demonstrate that the probe is capable of monitoring biological thiol in PBS.

Fig. 4 (a) Emission spectra (excitated at 550 nm) of the novel probe FR-thiol (5 µM) in the presence of various amounts of cysteine (0–50 µM) in PBS (pH 7.4, containing 10% EtOH as a cosolvent). Inset: fluorescence intensity changes at 624 nm of the probe (5 µM) with the amount of cysteine; (b) Emission spectra (excitated at 550 nm) of the novel probe FR-thiol (5 µM) in the presence of various amounts of GSH (0–50 µM) in PBS (pH 7.4, containing 10% EtOH as a cosolvent). Inset: fluorescence intensity changes at 624 nm of the probe (5 µM) with the amount of GSH.

Selectivity studies

To verify the specific response of probe FR-thiol toward biological thiols, we then examined the selectivity of the probe FR-thiol toward other disturb species. (such as representative amino acids, glucose, metal ions, reactive oxygen species, reducing agents, nucleosides, and small-molecule thiols) in buffer solution (25 mm phosphate buffer, pH 7.4) and monitored by emission spectroscopy. As exhibited in Fig. 5, introduction of Cys/Hcy/GSH to a solution of probe FR-thiol resulted in a visible increase of fluorescence intensity. However, no noticeable changes were observed upon addition of amino acids (Gly, Arg, Glu, Val, Ser, Leu, and Lys), metal ions (K+, Ca2+, Na+, Mg2+, and Zn2+), a reactive oxygen species (hydrogen peroxide), a reducing agent (nicotinamide adenine dinucleotide (NADH)), or glucose, which indicates that the probe FR-thiol showed high selectivity to thiols over other interference ions examined.


To further examine the effective applications of the probe, the fluorescence responses of FR-thiol to cysteine in the presence of typical competing ions were studied. As shown in Fig. 6, most of competing ions only exhibited minimum interference in the detection of cysteine. The result showed that probe FR-thiol can selectively respond to cysteine even under competition from other related interference ions.


Response time of probe FR-thiol

The time course of probe FR-thiol in the absence or presence of thiold was displayed in Figure. S5. Upon addition of thiold (100 µM) at room temperature, a dramatic enhancement in the fluorescence intensity at 624 nm was observed within 15 min, denoting the rapid reaction of probe FR-thiol with thiold. Probe FR-thiol responds rapidly to thiold (Figure S6), and the pseudo-first-order rate constant was calculated to be $k = -0.2044 \text{ min}^{-1}$ for cysteine and $k = -3525 \text{ min}^{-1}$ for GSH, respectively. (Figure S6),
These data indicated that the probe is highly rapid and may be suitable for studies of thiol in the living systems.

Detection of thiol in living cells

For the preliminary fluorescence imaging applications, the probe **FR-thiol** was incubated with the living HeLa cells pretreated with or without N-ethylmaleimide (as a thiol blocking agent). As shown in Fig. 7, the cells treated without N-ethylmaleimide (as a thiol blocking agent) exhibit strong fluorescence in the red channel. By contrast, when the cells pretreated with N-ethylmaleimide, then incubated with **FR-thiol** showed much weak fluorescence in the red channel was observed (Fig. 7). These data established that the probe **FR-thiol** is cell membrane permeable and can report biological thiols in the living cells. Furthermore, the results of the nuclear staining with Hoechst 33258 (Fig. 7) implied that the cells were alive during the imaging experiments.

**Fig. 7.** (a-d) HeLa cells pre-incubated with N-ethylmaleimide (1 mM) for 30 min. and then co-incubated with **FR-thiol** (5 µM) and Hoechst 33258 (4.5 µM) for 30 min. (a) Brightfield image; (b) Emission from the red channel; (c) emission from the blue channel (nuclear staining); and (d) overlay of the blue and red channels. (e-h) HeLa cells only co-incubated with **FR-thiol** (5 µM) and Hoechst 33258 (4.5 µM) for 30 min. (e) Brightfield image; (f) Emission from the red channel; (g) emission from the blue channel (nuclear staining); and (h) overlay of the blue and red channels.

The **FR-OH** dye may be suitable for time-dependent video observation of biological thiols in living cells in light of their good optical properties. Toward this end, we successfully video image of **FR-thiol** responding to biological thiol in HeLa cells. As shown in Fig. 8, the fluorescence intensity in HeLa cells is time-dependent. Interestingly, this is consistent with the results of the time-dependent fluorescence changes of the probe upon incubation with biological thiols in the buffer (Figure S5). The HeLa cells were still alive after 30 min, indicating that the probe has minimum cytotoxicity (Fig. S7), in accordance with the results of the nuclear staining with Hoechst 33258 in living cells (Fig. 8).

**Fig. 8.** Dynamic fluorescent pictures HeLa cells co-incubated with **FR-thiol**. Images were taken after only co-incubated with **FR-thiol** (5 µM).

Conclusions

In conclusion, a long-wavelength protection/deprotection dye platform was developed. Then, we have designed and synthesized probe **FR-thiol** as a new turn-on fluorescent probe for thiols based on this protection/deprotection dye platform. The turn-on probe **FR-thiol** displayed a high selectivity to thiols, and it is highly sensitive to thiols with a low detection limit. Furthermore, the turn-on probe can be employed to detect thiols in living cells. Finally, we have demonstrated that **FR-thiol** is suitable for time-dependent video detection of biological thiols in living cells.

Notes and references


15. The pKa was calculated according to the Henderson–Hasselbach–mass action equation (log[(Fmax − F)/(F − Fin)] = pKa − pH).
