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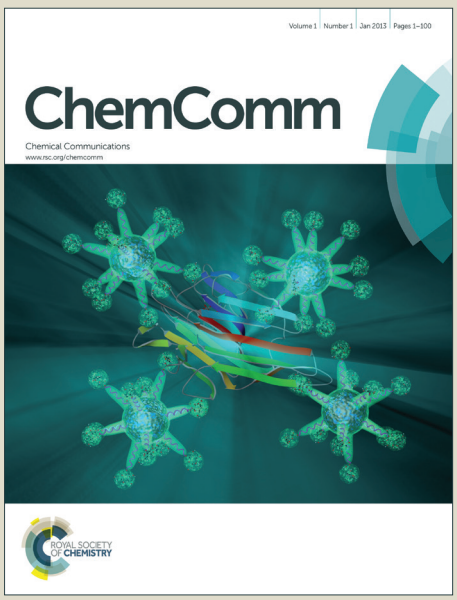

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Thiol-Thiosulfonate Reaction Providing Novel Strategy for Turn-on Thiol Sensing

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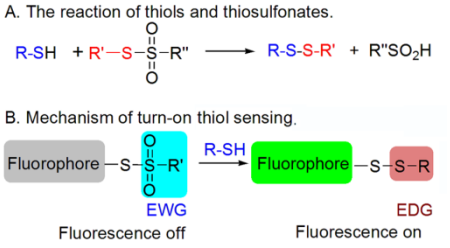
The first thiol-specific turn-on probe, BODIPY-TS, utilizing the thiosulfonate scaffold as a thiol recognition unit was reported. BODIPY-TS displays low toxicity, and has high sensitivity, fast response and quantitative reaction towards thiols. The structural novelty of BODIPY-TS would guide the development of novel thiol probes.

Small molecule thiols, such as cysteine (Cys) and glutathione (GSH), and protein thiols, play vital roles in regulating multiple redox-mediated signaling pathways. Alternations of the cellular thiol homeostasis have been implicated into a number of diseases, including cancer, neurodegenerative disorders and cardiovascular diseases.[1-3] Among various methods in detecting thiols, fluorescence-based assays are powerful and popular due to their high sensitivity, operational simplicity and good biocompatibility. Thus, numerous thiol-responsive small molecule fluorescent probes have been rationally developed in the past years.[4-15]

Scheme 1 Thiol-thiosulfonate reaction.



Manipulation of the electronic characters of the substituents attached to a fluorophore from an electron donating group (EDG) to an electron withdrawing group (EWG) or vice versa usually changes the absorbance/fluorescence profiles through the alteration of the intramolecular charge transfer (ICT) or photo-induced electron transfer (PET) process. The specific reaction of thiols and thiosulfonates (Scheme 1A) has long been known,[16, 17] and led to the development of several thiol-reactive agents. For example, S-methyl methanethiosulfonate (MMTS) is a classic thiol-targeted molecule widely used to block protein thiols.[18, 19] However, this reaction has never been applied to design optical probes. In the reaction, replacement of the sulfinate moiety by the sulfide also inverts the group from an EWG to EDG. Thus, we speculated that attachment of the thiosulfonate unit to certain fluorophores might generate novel turn-on probes for thiols (Scheme 1B).

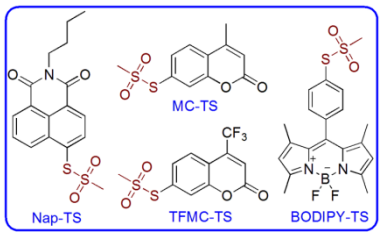


Fig. 1 Chemical structures of potential probes.

As part of our continuing interest in discovering and developing novel small molecule regulators of cellular redox systems,[20-24] we reported, for the first time, the strategy for thiol probes by employing the specific reaction between thiols and thiosulfonates. The thiosulfonate scaffold was introduced into different fluorophores to construct the potential probes (Fig. 1). The results demonstrate that BODIPY-TS was exclusively switched on by thiols with ~13-fold increase of the fluorescence intensity. Analysis of the reaction between Cys and BODIPY-TS revealed that nearly quantitative conversion of the probe to the corresponding disulfide product achieved. The response of BODIPY-TS toward thiols is fast (~5 min) and the detection limit could be down to a few nanomolar. Submicromolar of BODIPY-TS efficiently images the thiols in live cells within a few minutes.

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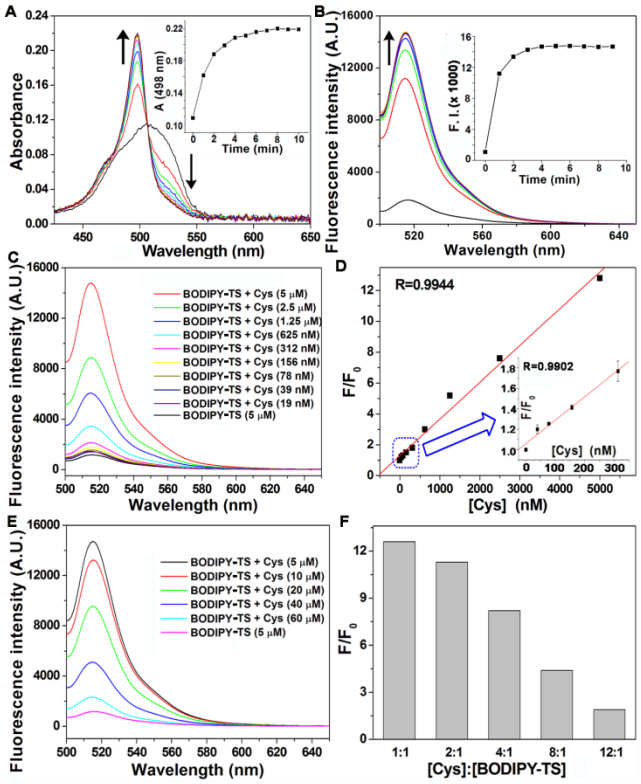


Fig. 2 Response of BODIPY-TS to Cys. Time-dependent absorbance (A) and fluorescence (B) spectra of BODIPY-TS towards Cys. The spectra were scanned every 1 min for 10 min after mixing the probe (5 μM) with Cys (5 μM) in PBS. The insets show the dynamic changes of absorbance at 498 nm and emission at 515 nm. (C) Dose-dependent emission spectra of BODIPY-TS toward Cys. BODIPY-TS (5 μM) was incubated with varying concentrations of Cys in PBS. The emission spectra were recorded after 5 min. (D) Plotting the fold of fluorescence increase (F/F_0) at 515 nm versus the concentrations of Cys from (C). The inset shows the detection limit of BODIPY-TS could be down to 39 nM of Cys. (E) Dose-dependent quench of fluorescence by excessive Cys. BODIPY-TS (5 μM) was incubated with increasing concentrations of Cys (5-60 μM) for 5 min, and the emission spectra were recorded. (F) Plotting the F/F_0 at 515 nm versus the increasing ration of Cys from (E). For all fluorescence spectra, λ_{ex}=490 nm.

The thiosulfonate unit was constructed via coupling sodium methanesulfinate with the corresponding thiols in acetonitrile as illustrated in Scheme S1.[20, 22, 25-28] The original spectra (^{1}H NMR, ^{13}C NMR and MS) of the final probes were included in the Supporting Information (Fig. S3-14). We initially examined the absorbance and fluorescence spectra of the thiosulfonate probes in responding to Cys. Upon addition of Cys (1 equiv.), the absorbance spectra of all compounds were changed distinctly (Fig. S1). The maximum absorbance of Nap-TS, MC-TS and TFMC-TS was all red-shifted, while that of BODIPY-TS was blue-shifted. The fluorescence spectra revealed that Nap-TS and MC-TS gave the on-off signal, while no significant alteration of the emission spectra for TFMC-TS. In contrast, BODIPY-TS showed >10-fold increment of the intensity (Fig. S2). Accordingly, BODIPY-TS was selected for studying its response to Cys in detail.

BODYPY-TS has maximum absorbance at ~510 nm. After addition of Cys, its maximum absorbance is blue-shifted to ~495 nm (Fig. 2A). The response of BODIPY-TS to Cys is fast, and the reaction completes within 5 min. The inset shows the time-dependent changes of absorbance at 498 nm. Usually, BODIPY dyes display narrow Gaussian-shaped absorption bands.[29, 30] We noticed that BODIPY-TS exhibits a broad and structureless band. This is probably due to the electron-withdrawing capacity of the sulfonyl group. After addition of Cys, the absorbance was restored to a sharp and narrow band. The fluorescence spectra were shown in Fig 2B. BODYPY-TS gives a weak emission centered at 515 nm when excited at 490 nm (ϕ=0.051, absolute quantum yield). The fluorescence intensity increased ~13-fold after addition of Cys (ϕ =0.356, absolute quantum yield) (Fig. 1B). The inset shows the dynamic changes of the emission, and the fluorescence intensity reaches a plateau after 5 min, consistent with the observation from the absorbance spectra. The fluorescence intensity of the probe at varying Cys concentrations (0-1 equiv. of BODYPY-TS) was also determined (Fig. 2C). The response of BODIPY-TS towards Cys is linear within the range of 0-1 equiv. of Cys (R^2=0.9944, Fig. 2D). The experimental detection limit of Cys could be down to 39 nM, which displays a ~1.2-fold increase of the intensity with good reproducibility (R^2=0.9902, the inset in Fig. 2D). Increasing the Cys concentration to >1 equiv. of BODYPY-TS causes gradual decreases of the fluorescence (Fig. 2 E & F). This is probably due to the decomposition of the disulfide product of the reaction (vide infra) by the excessive Cys.

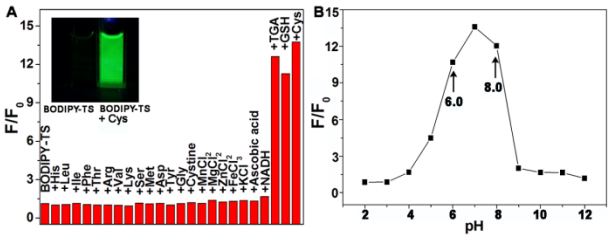
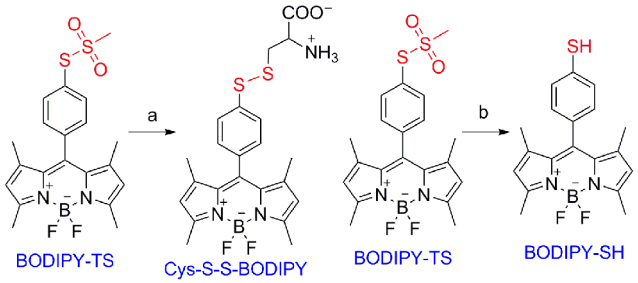


Fig. 3 (A) Selective recognition of thiols by BODIPY-TS. The fluorescence change at 515 nm (λ_{ex}=490 nm) was determined after mixing the probe (5 μM) with various analytes for 5 min in PBS. The concentrations of TGA, GSH and Cys are 5 μM, while others are 50 μM. The photo of the inset was taken under UV illumination (365 nm). (B). Influence of pH on the response of BODIPY-TS to Cys. The fluorescence change at 515 nm (λ_{ex}=490 nm) was determined after mixing the probe (5 μM) with Cys (5 μM) for 5 min in buffers with the pH ranging from 2.0 to 12.0.

We then evaluated the selectivity of BODIPY-TS towards thiols. As shown in Fig. 3A, amino acids without a sulfhydryl group give no response to the probe. NADH, ascorbate and inorganic salts are also negative to the probe. However, the fluorescence could be

switched on with >10-fold increment of the intensity by thioglycolic acid (TGA), GSH or Cys, indicating the exclusive recognition of the probe by thiols. The photo in Fig. 3A was acquired under UV illumination (365 nm) after mixing BODIPY-TS (5 μM) with the control buffer (left) or Cys (5 μM, right) for 5 min at room temperature. The pH-dependent response of BODIPY-TS was shown in Fig. 3B. The emission intensity increases as the pH rises, and reaches a maximal value at ~7.0. This may be attributed to that the higher pH facilitates the ionization of Cys to thiolate, and hence promotes the reaction. As the pH further increases from 8.0 to 12.0, the emission intensity decreases sharply, which could be due to the instability of the disulfide product under alkaline circumstance. BODIPY-TS exhibits stable response to Cys at the pH range of 6.0 to 8.0, suggesting that it is suitable for applying under physiological environments.

Scheme 2 Reaction of BODIPY-TS with Cys.



Reaction conditions: a) Cys (0.93 eq), , EtOH/H2O (1:1), r. t. 5 min, 85%; b) Cys (5 eq), EtOH/H2O (1:1), r. t. 30 min, 78%.

As BODIPY-TS has been demonstrated exclusive response to thiols, we then employed Cys as a model thiol to study the reaction details. Slight excess of BODIPY-TS (0.07 mmol) was mixed with Cys (0.065 mmol) in ethanol/H_2O. The reaction runs smoothly at room temperature, and completes within 5 min monitored by TLC. The only product was identified as the mixed disulfide compound (Cys-S-S-BODIPY, Scheme 2) with 85% isolated yield, indicating a clear reaction of BODIPY-TS with Cys. We also run a reaction of BODIPY-TS with excess Cys (5 eq.), and the product was identified as BODIPY-SH in 78% yield (Scheme 2). The original spectra (NMR and MS) of Cys-S-S-BODIPY and BODIPY-SH were included in the Supporting Information (Fig. S15-19). It was reported that attachment of 2, 4-dinitrobenzene moiety to the BODIPY scaffold quenches the later fluorescence via a PET process.[31, 32] In analogy to this, we reasoned that the weak fluorescence of BODIPY-TS is likely due to the PET process as the benzene ring in the molecule is more electron-deficient compared to that in Cys-S-S-BODIPY. Conversion of BODIPY-TS to Cys-S-S-BODIPY increases the electron density of the benzene ring and blocks the PET process, thus enhancing the emission intensity.

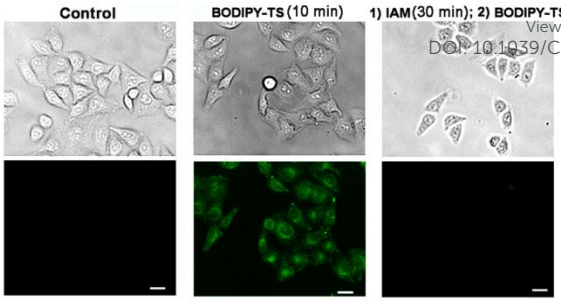


Fig. 4 Imaging thiols in live HepG2 cells. The cells were treated with vehicle or IAM followed by incubation with BODIPY-TS (0.5 μM) and the bright-field (top panel) and fluorescence (bottom panel) images were acquired. Scale bars: 20 μm.

With these favorable properties of BODIPY-TS in hands, we then performed experiments to demonstrate the practical applications of the probe in thiol sensing in live cells (Fig. 4). The control cells without the probe treatment show non-detectable fluorescence (left panel). As expected, the live HepG2 cells stained with submicromolar of BODIPY-TS for 10 min give bright green fluorescence (middle panel). Pretreatment of the cells with a thiol-specific alkylating agent iodoactamide (IAM) completely suppresses the fluorescence (right panel), supporting the specific recognition of cellular thiols by the probe.

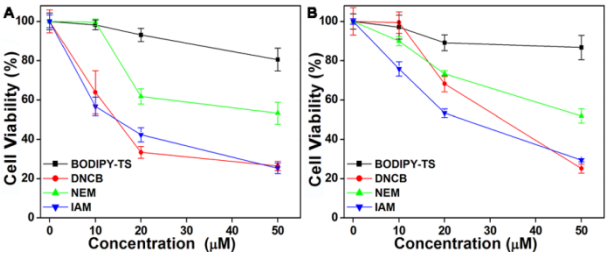


Fig. 5 Cytotoxicity of BODIBY-TS and the general thiol-reactive agents to HeLa (A) and HepG2 (B) cells. The cells were treated with different compounds at indicated concentrations for 24 h, and the cell viability was determined by the MTT assay. Data were expressed as the mean±SD.

Most of the thiol probes contain a thiol-reactive motif that selectively and irreversibly reacts with the sulfhydryl group,[6-10] thus inevitably depleting cellular thiols. As thiols are vital for cell function, these probes might elicit potential toxicity. In contrast, BODIPY-TS reacts with thiols to form mixed disulfide compounds, which are physiologically reversible for regeneration of the thiol. We thus hypothesized that BODIPY-TS might have lower toxicity. The MTT assay was adopted to determine the cytotoxicity of BODIPY-TS and some commercially thiol-reactive agents, *i. e.*, N-ethylmaleimide (NEM), 2, 4-dinitro-1-choloro-benzene (DNCB) and IAM. Compared to NEM, DNCB and IAM, BODIPY-TS exhibits much less cytotoxicity (Fig. 5). As the core structure of NEM, DNCB or IAM

is commonly employed in a majority of thiol probes,[6-10] the low cytotoxicity of BODIPY-TS provides another advantage for biological thiol sensing.

In summary, the thiosulfonate scaffold was employed to create a turn-on thiol probe BODIPY-TS for the first time. The probe has low toxicity, and features high selectivity, low detection limit, fast response and quantitative reaction to thiols. The structural novelty of BODIPY-TS would guide the development of novel thiol sensors and protein thiol labeling agents.

ACKNOWLEDGEMENTS

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TOC

Thiosulfonate scaffold was applied to design selective and turn-on thiol probes for the first time.