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A Single Fluorescent Probe to Visualize Hydrogen Sulfide and Hydrogen Polysulfides with Different Fluorescence Signals

Dr. Wei Chen^[a], Armando Pacheco^[a], Yoko Takano^[b], Jacob J. Day^[a], Prof. Dr. Kenjiro Hanaoka^[b], and Prof. Dr. Ming Xian^[a]

^[a]Department of Chemistry, Washington State University, Pullman, WA 99164 (USA)

^[b]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033(Japan)

Abstract

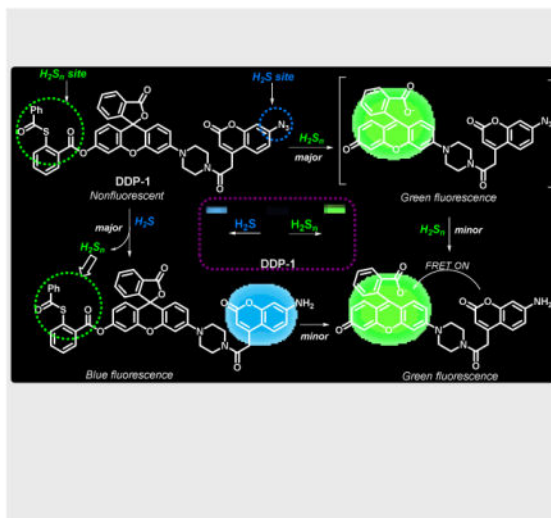
Hydrogen sulfide (H_2S) and hydrogen polysulfides (H_2S_n , $n > 1$) are endogenous regulators of many physiological processes. In order to better understand the symbiotic relationship and cellular cross-talk between H_2S and H_2S_n , it is highly desirable to develop single fluorescent probes which enable dual-channel discrimination between H_2S and H_2S_n . Herein we report the rational design, synthesis, and evaluation of the first dual-detection fluorescent probe **DDP-1** that can visualize H_2S and H_2S_n with different fluorescence signals. The probe showed high selectivity and sensitivity to H_2S and H_2S_n in aqueous media and in cells.

Dual-channel Sensing

The first single fluorescent probe **DDP-1** that can visualize H_2S and H_2S_n with different emission channels was discovered. H_2S can be identified by the simultaneous appearance of two fluorescence signals in blue and green channels, whereas H_2S_n can be characterized by the single emission in green channel

Correspondence to: Ming Xian.

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Keywords

hydrogen polysulfides; sulfide; fluorescence; cyclization; fluorescent probes

Due to their vital physiological functions, reactive sulfur species (RSS) form an important and ever-increasing research field.^[1] Among RSS, H₂S is perhaps most attractive as it has been characterized as a crucial gaseous transmitter.^[2] While the research on H₂S is still actively ongoing, a new hot topic about RSS has recently emerged which focuses on the chemical biology of hydrogen polysulfides (H₂S_n, n > 1).^[3] Endogenous H₂S_n may be generated from H₂S upon reacting with reactive oxygen species (ROS) like ClO⁻.^[4] Cystathionine γ -lyase (CSE) and cystathionine- β -synthase (CBS), the enzymes responsible for H₂S biosynthesis, were found to also produce persulfides (RSSH), which could further be converted to H₂S_n.^[5] Very recently 3-mercaptopyruvate sulfurtransferase (3MST) was identified to be an important enzyme in brain for H₂S_n generation.^[6] The significance of H₂S_n in redox biology has only been recognized recently. Evidences suggest that H₂S_n might be the actual signaling molecules that activate ion channels, transcription factors, and tumor suppressors with higher potency than H₂S.^[3,7] One example is protein S-sulfhydration,^[5,8] which was previously thought to be resulted from H₂S. Recent studies found that H₂S_n were much more effective in causing S-sulfhydration than H₂S.^[3,7]

Given the importance of H₂S and H₂S_n in redox biology, convenient and accurate detection methods for these species are invaluable research tools. In the past five years, the detection of H₂S has received wide attention and a large number of fluorescent probes for H₂S have been developed.^[9] It should be noted that in all of those studies the selectivity of the probes for H₂S versus H₂S_n was not considered, mainly because the significance of H₂S_n in biological samples was not recognized. On the other hand, the detection of H₂S_n is much less studied, due to very limited understanding of the chemical reactivity of H₂S_n.^[10] Very recently our laboratory and several others have developed a few fluorescent probes for

H_2S_n .^[11] In these works the selectivity of probes for H_2S_n versus H_2S was verified and H_2S did not trigger any fluorescent signals for these probes.

With the increasing knowledge available for sensing H_2S and H_2S_n , it is now possible to develop fluorescent probes for dual-channel differentiation of H_2S and H_2S_n . Such probes will be very useful for understanding the mutual relationship and cellular cross-talk between H_2S and H_2S_n . While simply mixing two specific probes might be able to detect both analytes with distinct fluorescence signals, this strategy suffers from limitations such as 1) potential interference between two probes, 2) larger invasive effects, and 3) possible different localization and metabolisms of the probes.^[12] Therefore, it is highly desirable (but also challenging) to develop a single fluorescent probe which enable visualization of H_2S and H_2S_n using different emission channels. Such dual-detection probes have not been reported so far. Herein, we report the rational design, synthesis, and evaluation of a single fluorescent probe that can differentiate H_2S and H_2S_n in aqueous media and in cells.

As shown in Scheme 1, we expected the dual-detection probe would contain two separated and quenched fluorophores A and B. Ideally each pseudo-fluorophore should only react with one species (H_2S or H_2S_n) to cause the corresponding fluorescence ‘turn-on’. As such, H_2S and H_2S_n can be determined by different fluorescence emission wavelengths. Even if H_2S and H_2S_n are present together, the ratio of the two emission intensity might be able to determine the ratio of the two sulfur species.

To achieve this design, the key is to construct two specific reactive sites on the probe for H_2S and H_2S_n . In our studies of H_2S_n probes, we found phenyl 2-(benzoylthio)benzoate-based probes exhibited high sensitivity and selectivity for H_2S_n .^[11b] Importantly, H_2S did not show any response to this type of probes. Therefore, phenyl 2-(benzoylthio)benzoate should be a suitable choice as H_2S_n reactive site. On the other hand, the selection of the H_2S reaction site was more difficult as previously reported H_2S probes did not verify their selectivity for H_2S_n . So far three types of reactions have been used in the development of H_2S probes: a) H_2S -mediated reductions (mostly using azides), b) H_2S -mediated nucleophilic reactions, and c) metal-sulfide formations.^[9] As H_2S_n are expected to be stronger nucleophiles than H_2S , nucleophilic reactions or metal-sulfide formations are likely to occur with H_2S_n , leading to poor selectivity. H_2S -mediated azide reduction might be useful. H_2S_n are oxidation products of H_2S and might have weaker reducing ability (at least for some azides). Therefore, azide-based fluorophores might be able to differentiate H_2S from H_2S_n .

With this consideration in mind, we first compared the fluorescence responses of several azide-based fluorophores to H_2S and H_2S_n . Na_2S and Na_2S_2 were used in buffers as H_2S and H_2S_n equivalents, respectively. One example using 4-methyl-7-azidocoumarin (C7-Az) is shown in Figure 1 and Figure S1. As expected, the treatment of C7-Az with H_2S_n led to much decreased fluorescence signals as compared to H_2S treatment. These results indicated this azide moiety has higher reactivity to H_2S than H_2S_n , and could be used in dual-detection.

Next we proposed a dual-detection probe **DDP-1** (Scheme 2). In this design, four factors were considered: (1) coumarin and rhodol were selected as two fluorophores due to their excellent solubility, high quantum yields, and well-separated maximum emission wavelengths (~445 nm for coumarin and ~542 nm for rhodol). As such, dual-color imaging of H₂S and H₂S_n from different emission channels was possible. (2) A rigid piperazine linker was used to bridge the two fluorophores and provided an advantage for Förster resonance energy transfer (FRET) in the coumarin-rhodol scaffold, which preventing the π - π stacking between dyes.^[13] (3) Azidation of coumarin and phenyl 2-(benzoylthio)benzoate-protected rhodol should effectively quench the fluorescence of the probe via the intramolecular spirocyclization and the intramolecular charge transfer (ICT) effect, respectively. The probe should bear very low background fluorescence that is favorable for high sensitivity. (4) The azide and phenyl 2-(benzoylthio)benzoate moieties provide selective reaction sites for H₂S and H₂S_n.

The fluorescence turn-on mechanism of **DDP-1** is also proposed in Scheme 2. When the probe is treated with H₂S_n, phenyl 2-(benzoylthio)benzoate should be preferably reacted to release the fluorescence of rhodol (to form **1**). Even if the azide group of **1** is partially reduced by H₂S_n to form a small amount of **3**, it should not affect the fluorescence emission channel due to FRET between the two fluorophores (Figure S2). Overall the reaction with H₂S_n should only produce green fluorescence of rhodol. In contrast, the reaction between **DDP-1** and H₂S is more complicated. Previous results have demonstrated that H₂S cannot turn on phenyl 2-(benzoylthio)benzoate-based fluorophore.^[11b] Therefore we expected H₂S would preferably react with the azide moiety to produce **2** and release blue fluorescence of coumarin. It should be noted that recent studies demonstrated that the reaction of H₂S with azides led to the formation of H₂S_n.^[14] Therefore, **3** would also be formed in this process, which should exhibit green fluorescence of rhodol because of FRET. However, less than 0.5 equivalent of H₂S_n are generated from the reaction of 1 equivalent of H₂S and azide. Moreover, the reaction with phenyl 2-(benzoylthio)benzoate consumes at least 2 equivalent of H₂S_n. Therefore, only a small amount of **3** would be produced during this process. Overall the reaction between **DDP-1** and H₂S should produce the emission signals of both coumarin (major) and rhodol (minor). Taken together, it is anticipated that probe **DDP-1** could detect H₂S and H₂S_n from distinct emission channels.

The proposed probe was then prepared and characterized (see Supporting Information). We first tested the probe's fluorescence response to H₂S and H₂S_n in PBS buffers under the excitation wavelength of coumarin ($\lambda_{\text{ex}} = 360$ nm). As shown in Figure S3, **DDP-1** alone showed almost no fluorescence. Upon reacting with Na₂S or Na₂S₂, **DDP-1** gave appreciable fluorescence enhancements in 1 hour. As expected, different fluorescence emission behaviors were observed for Na₂S and Na₂S₂. Na₂S led to two distinct emissions at 452 nm and 542 nm (blue-green fluorescence). While Na₂S₂ induced only one strong emission at 542 nm (green fluorescence). Therefore, H₂S could be easily identified by the simultaneous appearance of the two well-separated emissions, whereas H₂S_n could be characterized by the single emission at 542 nm. In addition, these distinguished fluorescence color changes might be favorable for the simple detection of H₂S and H₂S_n by the naked eyes.

Next we studied the sensitivity of **DDP-1** for H_2S and H_2S_n using varied concentrations of Na_2S or Na_2S_2 (0~150 μM). As shown in Figure 2, the increase in fluorescence intensity ($\lambda_{\text{ex}} = 360 \text{ nm}$) with the gradual increase of Na_2S or Na_2S_2 concentrations was observed. A good linear relation was obtained (Figure S4). For H_2S , the fluorescence intensity at 452 nm increased linearly with Na_2S concentration from 0 to 20 μM . The fluorescence intensity at 542 nm increased linearly with Na_2S in the concentration range of 0~40 μM . The detection limits ($S/N=3$) were 100 nM and 150 nM for H_2S , corresponding emission at 452 and 542 nm. The fluorescence intensity at 542 nm increased linearly with Na_2S_2 concentrations from 0 to 20 μM . The detection limit was calculated to be 24 nM. These results indicated that **DDP-1** displayed much higher sensitivity to H_2S_n than H_2S . The effects of pH in these reactions were also studied. **DDP-1** was found to work effectively at neutral to basic pH (7–10) (Figure S5).

We then wondered if the probe **DDP-1** could give meaningful responses when H_2S and H_2S_n co-exist. To this end, we tested fluorescence changes of varying $\text{Na}_2\text{S}_2/\text{Na}_2\text{S}$ mixture solutions, while the total sulfur concentration was fixed in samples (200 μM). As H_2S is a much more stable species than H_2S_n and the concentration of H_2S is likely to be higher than H_2S_n in biological systems, we varied $[\text{H}_2\text{S}_2]/[\text{H}_2\text{S}]$ ratios from 0 to 1. The fluorescence signals of these solutions were measured by the probe. As shown in Figure 3, following the increases of $[\text{H}_2\text{S}_2]/[\text{H}_2\text{S}]$ ratios, the emission at 452 nm decreased with a concurrent increase at 542 nm. The $F_{542 \text{ nm}}/F_{452 \text{ nm}}$ ratios increased linearly with $[\text{H}_2\text{S}_2]/[\text{H}_2\text{S}]$ ratios in the range of 0~0.176 (Figure S6). These results indicated the probe could be used for the ratiometric detection of relative H_2S_n and H_2S concentrations when they coexist.

To verify the specificity of **DDP-1** for H_2S and H_2S_n , its responses to a series of biologically relevant RSS (GSH, Cys, Hcy, GSSG, SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, $\text{CH}_3\text{SSSCH}_3$, and S_8) were tested. As shown in Figure 4, these RSS did not cause any fluorescence increase. Only Na_2S_2 , Na_2S_3 , and Na_2S_4 triggered significant fluorescence increases. We also examined the responses of **DDP-1** to common ROS, such as H_2O_2 , ClO^- , superoxide (O_2^-), hydroxyl radical ($\bullet\text{OH}$), and singlet oxygen ($^1\text{O}_2$). No fluorescence increase was detected for these species. Moreover, as H_2S_n could be efficiently generated from H_2S and ClO^- , the probe was used to analyze *in-situ* generation of H_2S_n . When Na_2S (100 μM) and ClO^- (50 μM) co-existed, a very strong fluorescence emission at 542 nm was observed. We also tested the specificity of **DDP-1** under the excitation wavelength of rhodol ($\lambda_{\text{ex}} = 515 \text{ nm}$). H_2S_n induced significant fluorescence increase while H_2S gave only small fluorescent enhancement (Figure S7). Almost no fluorescence changes were detected for other RSS or ROS. The responses of **DDP-1** to representative amino acids and ascorbic acid were also tested, they did not induce any response. These results demonstrated the excellent specificity of **DDP-1** for H_2S and H_2S_n .

Finally we evaluated the ability of **DDP-1** to detect H_2S and H_2S_n in cells. As shown in Figure 5, HeLa cells were first incubated with **DDP-1** (20 μM) for 30 min. Then extracellular probe was washed off. Only very weak fluorescence was observed in blue and green channels. When cells were treated with Na_2S (100 μM), an apparent fluorescence enhancement was detected in blue and green channel. When cells were treated with Na_2S_2 or *in-situ* generated H_2S_n , the fluorescence signals in blue channel decreased with a

coincident increase in green channel. These results proved that **DDP-1** is cell membrane permeable and could be used for the detection of H₂S and H₂S_n from distinct emission channels in cells. Additionally, the cell viability assay implied that **DDP-1** has low cytotoxicity and good biocompatibility (Figure S8).

In summary, we report in this study the rational design, synthesis, and evaluation of the first single fluorescent probe **DDP-1** that can clearly differentiate H₂S and H₂S_n with different fluorescence signals. This also represents a significant improvement in the development of H₂S probes as previously reported H₂S probes can hardly discriminate H₂S_n. This novel probe is expected to serve as a useful tool in understanding the redox signaling of H₂S and H₂S_n.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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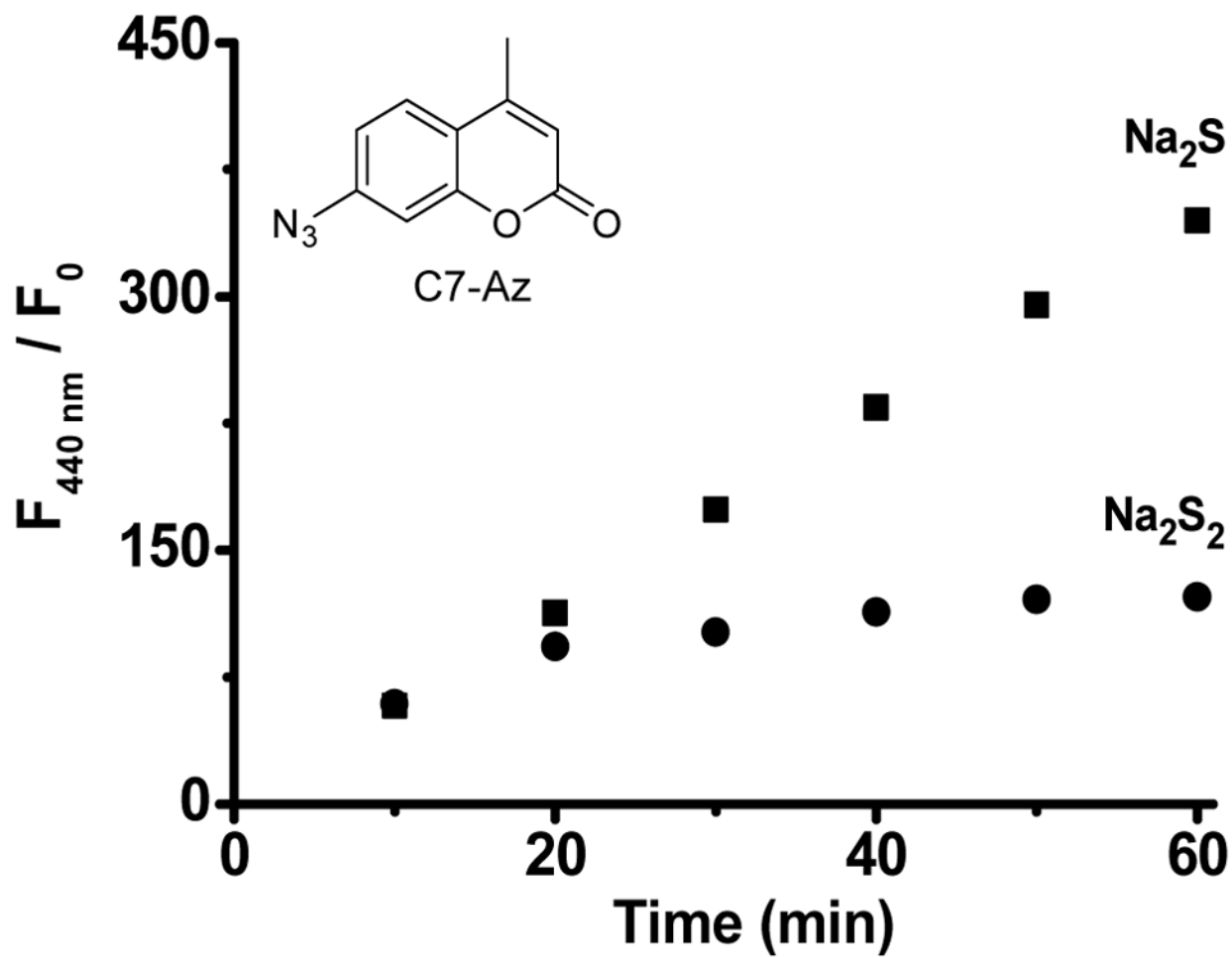


Figure 1. Time-dependent fluorescence intensity changes of C7-Az (10 μM) with 50 μM Na₂S (■) or 50 μM Na₂S₂ (●).

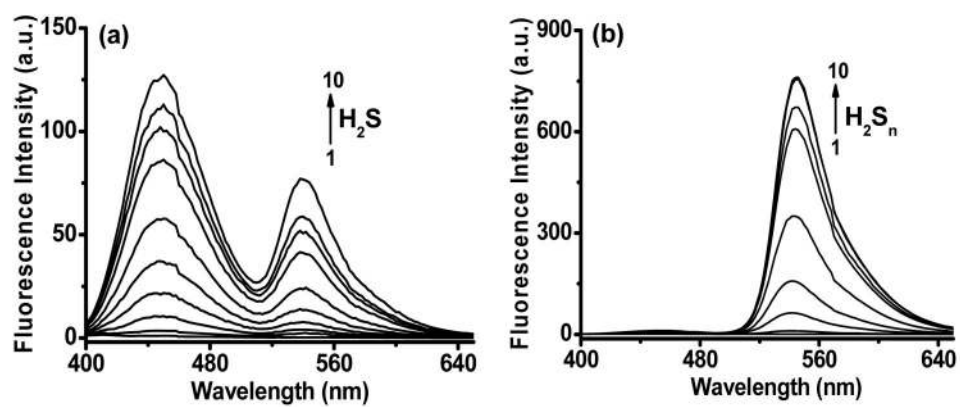


Figure 2. Fluorescence spectra of **DDP-1** (10 μM) under various concentrations of (a) H_2S (0, 1, 5, 10, 20, 40, 75, 100, 120, 150 μM for curves 1–10, respectively); (b) H_2S_n (0, 1, 5, 10, 20, 45, 70, 100, 120, 150 μM for curves 1–10, respectively).

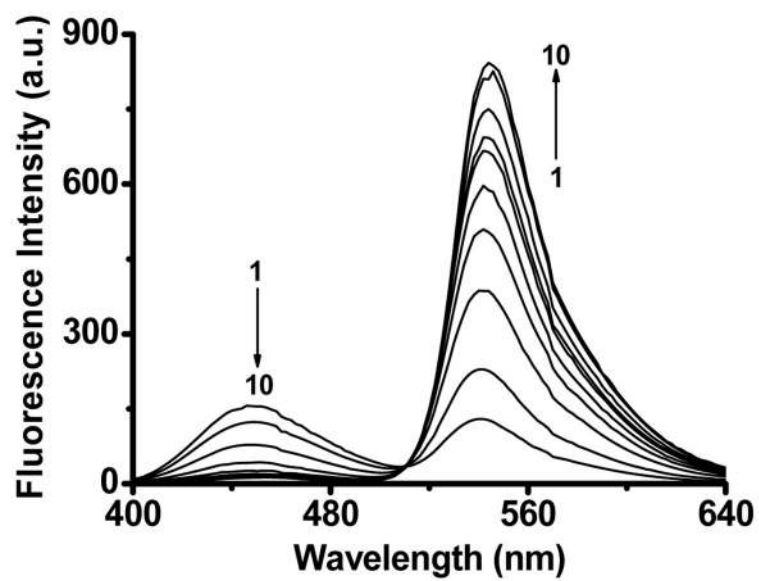


Figure 3. Fluorescence spectra ($\lambda_{\text{ex}} = 360 \text{ nm}$) of **DDP-1** ($10 \mu\text{M}$) with varying $\text{Na}_2\text{S}_2/\text{Na}_2\text{S}$ mixture solutions ($\text{Na}_2\text{S}_2/\text{Na}_2\text{S}$ ratios were 0, 0.01, 0.026, 0.053, 0.081, 0.111, 0.176, 0.333, 0.667, 1 for curves 1–10, respectively).

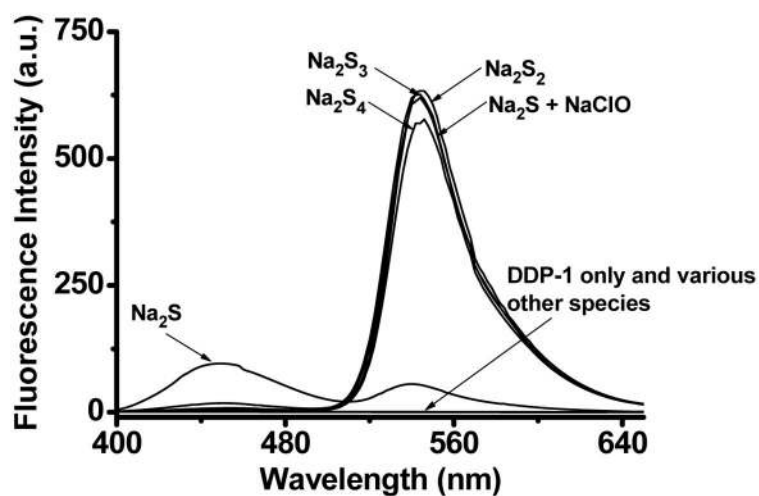


Figure 4.

Fluorescence spectra ($\lambda_{\text{ex}} = 360 \text{ nm}$) of **DDP-1** ($10 \mu\text{M}$) in the presence of various reactive species. (1) Probe only; (2) 5 mM GSH; (3) 1 mM Cys; (4) 100 μM Hcy; (5) 100 μM GSSG; (6) 100 μM $\text{Na}_2\text{S}_2\text{O}_3$; (7) 100 μM Na_2SO_3 ; (8) 50 μM $\text{CH}_3\text{SSSCH}_3$; (9) 50 μM S_8 ; (10) 250 μM H_2O_2 ; (11) 50 μM ClO^- ; (12) 50 μM O_2^- ; (13) 50 μM $\bullet\text{OH}$; (14) 50 μM $^1\text{O}_2$; (15) 100 μM Alanine; (16) 100 μM Serine; (17) 100 μM Arginine; (18) 100 μM Isoleucine; (19) 100 μM Lysine; (20) 100 μM Ascorbic acid; (21) 100 μM Na_2S ; (22) 50 μM Na_2S_2 ; (23) 50 μM Na_2S_3 ; (24) 50 μM Na_2S_4 ; (25) 100 μM Na_2S + 50 μM ClO^- .

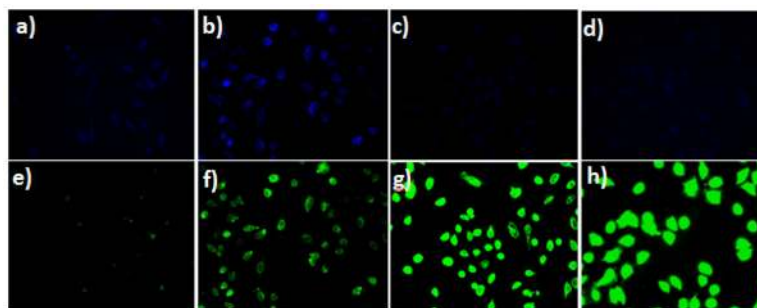
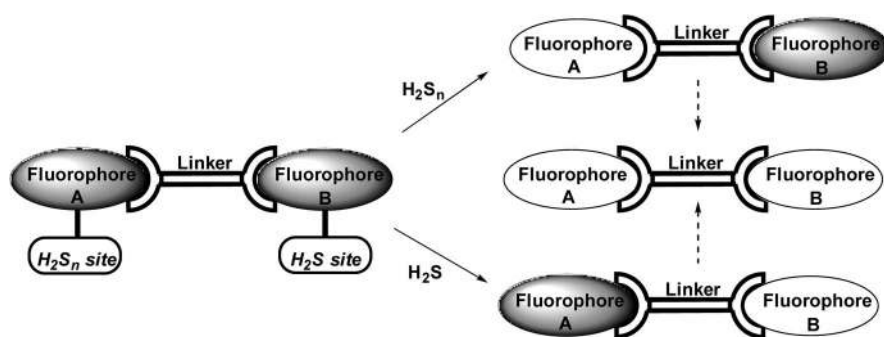
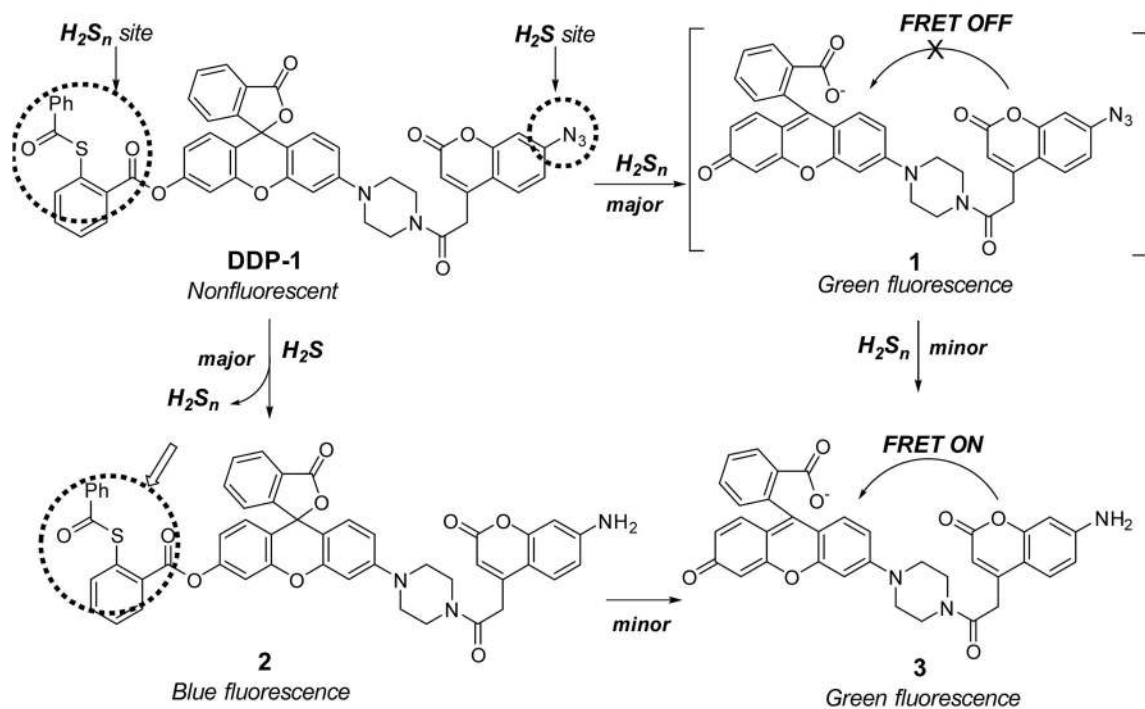


Figure 5. HeLa cells were incubated with **DDP-1** (20 μM) for 30 min, then washed, and subjected to different treatments. a, e) Controls (no added Na_2S , NaClO , or Na_2S_2); b, f) Na_2S (100 μM); c, g) the mixture of Na_2S (100 μM) and NaClO (50 μM); d, h) Na_2S_2 (50 μM). a–d) Fluorescence image of HeLa cells at the blue channel; e–h) fluorescence image of the corresponding image (a–d) from green channel.

**Scheme 1.**

The design of dual detection probes for H_2S and H_2S_n .



Scheme 2.
Possible reactions of **DDP-1** with H_2S and H_2S_n .