Fluorescent Probes

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The Development of Fluorescent Probes for Visualizing Intracellular Hydrogen Polysulfides

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Dedicated to Professor Amos B. Smith III on the occasion of his 70th birthday

Abstract: *Endogenous hydrogen polysulfides (H_2S_n; $n>1$) have been recognized as important regulators in sulfur-related redox biology. H_2S_n can activate tumor suppressors, ion channels, and transcription factors with higher potency than H_2S. Although H_2S_n are drawing increasing attention, their exact mechanisms of action are still poorly understood. A major hurdle in this field is the lack of reliable and convenient methods for H_2S_n detection. Herein we report a H_2S_n-mediated benzodithiolone formation under mild conditions. This method takes advantage of the unique dual reactivity of H_2S_n as both a nucleophile and an electrophile. Based on this reaction, three fluorescent probes (**PSP-1**, **PSP-2**, and **PSP-3**) were synthesized and evaluated. Among the probes prepared, **PSP-3** showed a desirable off/on fluorescence response to H_2S_n and high specificity. The probe was successfully applied in visualizing intracellular H_2S_n.*

Reactive sulfur species (RSS) are a group of sulfur-containing molecules that play regulatory roles in biological systems. Important RSS include biothiols and S-modified protein cysteine adducts. RSS also include hydrogen sulfide (H_2S) and sulfane sulfur moieties, such as cysteine persulfides (R-S-SH) and polysulfides (R-S-S_n-S-R′; $n>0$). Understanding the mechanisms of action of RSS derivatives has become a very active research area in modern chemical biology, particularly from the methodological point of view.[1] Among various RSS, hydrogen polysulfides (H_2S_n; $n>1$) have attracted particular attention, mainly because of their involvement in H_2S-related redox biology.[2] H_2S_n can be generated from endogenous H_2S upon reaction with reactive oxygen species (ROS).[3] H_2S can also react with other sulfane sulfurs, such as S_8, to form H_2S_n.[2d,3c,4] Moreover, H_2S_n may have their own biosynthetic pathways. For example, although CSE-mediated cysteine metabolism (CSE = cystathionine γ-lyase) can produce cysteine persulfide,[5] it may also catalyze direct generation of H_2S_n from cystine. A series of recent reports have suggested that H_2S_n derivatives might be a new group of signaling molecules.[2,3c,4] It was found that H_2S_n can activate tumor suppressors, ion channels, and transcription factors with higher potency than H_2S.[4] Some physiological activities that were originally attributed to being mediated by H_2S may actually be mediated by H_2S_n. One such example is S-sulfhydration.[1a,5,6] This posttranslational modification was previously thought to be the result of H_2S activity. However, recent results have demonstrated that H_2S_n are more effective than H_2S in S-sulfhydration.[2,3b–c]

Although H_2S_n molecules have now been recognized as potent physiological mediators, a number of issues remain to be clarified, such as the production and degradation pathways of H_2S_n, their regulatory mechanisms, and potential physiological stimuli that induce those regulatory mechanisms. To address these issues, it is critical to develop effective methods for H_2S_n detection. The traditional method is to measure UV/Vis absorption bands at $\lambda = 290$–300 nm and 370 nm, that is, at the characteristic absorption bands of H_2S_n.[2a] However, this method has low sensitivity and is not applicable for biological detections. Fluorescence assays could be useful because of their high sensitivity and spatiotemporal resolution capability. Although fluorescent probes for H_2S have been extensively studied,[7] the probes for H_2S_n are still underdeveloped because the chemistry of H_2S_n is relatively unknown.

Our laboratory has initiated a program to study new chemistry/reactions of H_2S_n and to develop reaction-based fluorescent probes for H_2S_n. In 2014, we reported the first H_2S_n-specific probes (**DSP**; Scheme 1), which employed a 2-fluoro-5-nitrobenzoic ester template to trap H_2S_n and promote an intramolecular cyclization to release a fluorophore.[8] DSP probes showed satisfactory sensitivity and selectivity for H_2S_n. Several other groups have adopted the same template to develop H_2S_n probes with interesting properties.[9] However, a drawback of this probe type is that 2-fluoro-5-nitrobenzoic esters can also react with biothiols. Although such reactions do not turn on fluorescence, the consumption of the probes is a problem. In theory, the reaction product, that is, thioether **1**, can further react with H_2S_n to switch on fluorescence (Scheme 1). We found that the reaction was somewhat slow and low yielding when the H_2S_n concentration was low (at μM levels). Therefore, a high loading of **DSP**-type probes may be needed for biological detection. Recently, we reported another H_2S_n-specific aziridine-opening reaction and developed a probe (**AP**) based on this reaction.[10] **AP** showed

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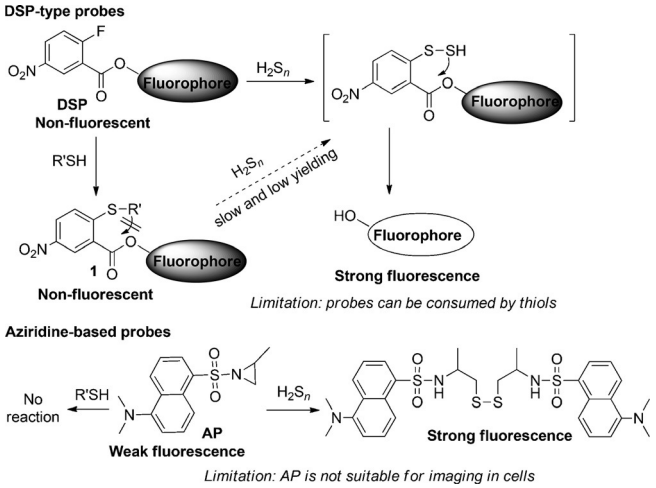
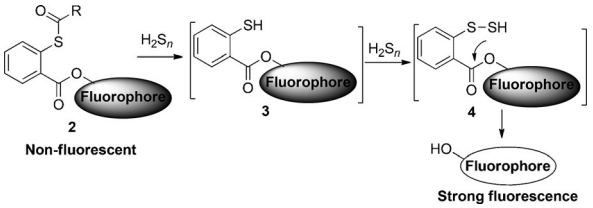


Scheme 1. Previous designs of H_2S_n-specific fluorescent probes.

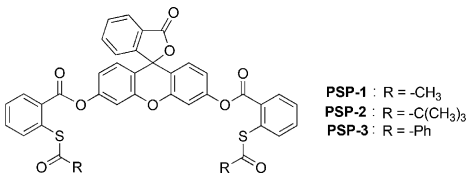


Scheme 2. The design of fluorescent probes exploiting the dual reactivity of H_2S_n.

excellent selectivity for H_2S_n and it did not react with biothiols or H_2S under physiological concentrations. However, due to limited options for aziridine-based fluorophores, the sensitivity of **AP** for H_2S_n was not optimal and attempts to use **AP** for imaging H_2S_n in cells were not successful. Obviously further improvement of the probes is needed. Herein, we report the design, synthesis, and evaluation of a series of new fluorescent probes (**PSP1–3**) that showed high sensitivity and selectivity for H_2S_n and can be used for endogenous H_2S_n imaging.

The key to the development of highly efficient fluorescent probes is to identify a highly specific H_2S_n recognition unit. Ideally, such a recognition unit should only react with H_2S_n and not react with other sulfur species, especially biothiols. From a chemistry perspective, H_2S_n derivatives are quite different from thiols or H_2S. The estimated pK_a values of H_2S_n are in the range of 3 to 5.[11] For comparison, the pK_a values of H_2S and biothiols are in the range of 7 to 9.2. At physiological pH, H_2S_n derivatives are expected to be weak acids and stronger and more reactive nucleophiles than biothiols and H_2S as a result of the alpha effect. Additionally, H_2S_n belong to the sulfane sulfur family. A character of sulfane sulfurs is that they can function as electrophiles and react with certain nucleophiles.[12] Overall, H_2S_n derivatives have a unique dual reactivity and can act as both nucleophiles and electrophiles. Taking advantage of this property, we envisioned that template **2** (Scheme 2) might be specific for H_2S_n derivatives. In **2**, a thioester was employed to trap the nucleophilicity of H_2S_n. One concern with this structure is that the thioester group could also react with biothiols, seeing as reactions between thioesters and thiols are known, for example, in native chemical ligation.[13] However, those reactions are mostly for synthetic purposes with high concentrations of reactants and special solvent conditions. The relatively low concentrations of biothiols in biological systems and the mild, neutral, and aqueous environment may make the reactions slow and low yielding. In addition, we expected that the manipulation of R groups in **2** (in terms of both steric and electronic effects) could significantly alter its reaction rates toward thiols and H_2S_n. If H_2S_n could selectively react with an appropriate thioester group, the product, that is, **3**, should further react with H_2S_n (now serving as an electrophile) to form **4**. The following spontaneous cyclization should release the fluorophore. Overall, this process would be specific for H_2S_n.

Based on this idea, we synthesized three probes (**PSP-1**, **PSP-2**, and **PSP-3**; Scheme 3). Three different R groups (Me, *t*Bu, and Ph) were used to explore the effects of acyl groups on the reactivity of thioesters toward biothiols. We first tested their fluorescence properties and responses to H_2S_n in PBS (phosphate-buffered saline). Freshly prepared Na_2S_2 solutions were used as the equivalents of H_2S_n. All three probes showed almost no fluorescence emission with very low fluorescence quantum yields (Φ; $\Phi_{\mathbf{PSP\text{-}1}} = \Phi_{\mathbf{PSP\text{-}2}} = 0.02$, $\Phi_{\mathbf{PSP\text{-}3}} = 0.01$) because of the protection of the two hydroxy groups of fluorescein. Upon the treatment of the probes with Na_2S_2 (5 equiv) for 30 minutes, the fluorescence intensities of the probes were significantly enhanced (Figure 1 A). **PSP-1** and **PSP-3** exhibited greater increases in fluorescence intensity than **PSP-2**. Presumably the bulky *tert*-butyl group of **PSP-2** decreased the reactivity toward to H_2S_n. We also measured the time-dependent changes in the fluorescence



Scheme 3. The structures of **PSP** probes.

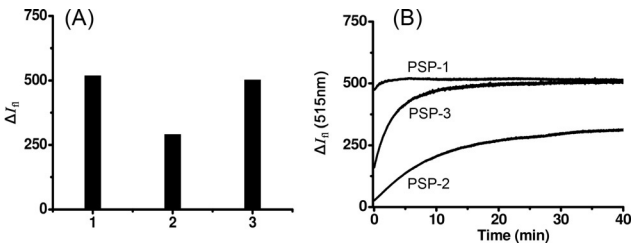
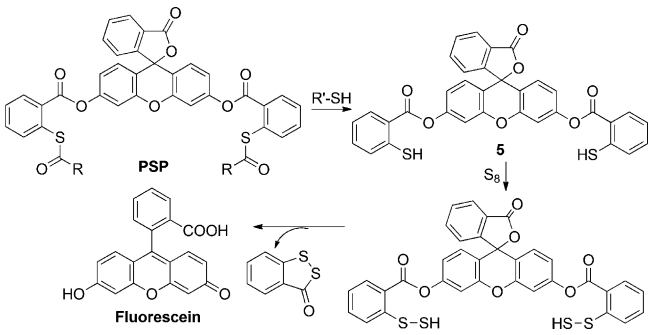


Figure 1. A) Fluorescence intensity increases (ΔI_{fl}) of **PSP** (10 μM) upon treatment with Na_2S_2 (50 μM): 1) **PSP-1**; 2) **PSP-2**; 3) **PSP-3**. B) Time-dependent fluorescence intensity changes of **PSP** (10 μM) in the presence of Na_2S_2 (50 μM).

intensities of the probes in the presence of Na_2S_2 (Figure 1B). The maximum emission intensities of **PSP-1** and **PSP-3** were reached within 2 minutes and 10 minutes respectively, indicating that fluorescence turn-on was fast. The fluorescence turn-on of **PSP-2** was slower (circa 20 min), again suggesting that steric effects played a role in its reactivity. For the purpose of reproducibility, a reaction time of 30 minutes was employed in all of the following experiments.

Next we studied the stability of the probes in the presence of biothiols. As shown in Scheme 4, if the probes undergo



Scheme 4. Possible reactions of **PSP** probes with biothiols and S_8. R′-SH = Cys or GSH.

thioester exchange with biothiols, the resultant product **5** should be sensitive to sulfane sulfurs, such as elemental sulfur (S_8), and the resultant product should be strongly fluorescent. Therefore, measuring the fluorescence of the probes in a mixture of thiols and S_8 should be suitable for evaluating the stability of the probes to thiols.

We then tested the fluorescence responses of the probes (10 μM) to Cys (1 mM) and glutathione (GSH; 5 mM) in the absence or presence of S_8 (50 μM). As shown in Figure 2, Cys, GSH, or S_8 alone did not induce any response. The mixtures of biothiols (GSH or Cys) and S_8 produced strong fluorescence for **PSP-1**. The same mixtures led to almost no increase in fluorescence intensity for **PSP-2** and **PSP-3**. As a positive control, the thioester exchange product **5** was strongly fluorescent in the presence of S_8 under the same conditions. These results confirmed our hypothesis that the acyl group of the thioester could affect its reactivity towards thiols. **PSP-1** appeared to be labile to biothiols while **PSP-2** and **PSP-3** were stable. Therefore, the use of **PSP-2** and **PSP-3** should not be affected by cellular biothiols.

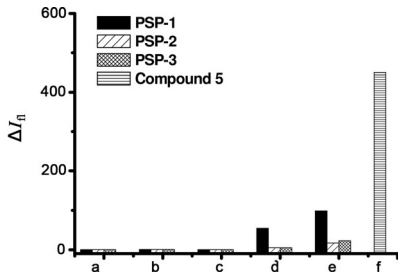


Figure 2. Increases in the fluorescence intensities (ΔI_{fl}) of each probe (10 μM) in the presence of biothiols and S_8: a) Probe + GSH (5 mM); b) Probe + Cys (1 mM); c) Probe + S_8 (50 μM); d) Probe + GSH (5 mM) + S_8 (50 μM); e) Probe + Cys (1 mM) + S_8 (50 μM); f) Compound **5** (10 mM) + S_8 (50 μM).

Having demonstrated the excellent reactivity and stability of **PSP-3**, this probe was selected for further studies. We next validated its selectivity for H_2S_n over other common RSS reagents including homocysteine (Hcy), glutathione disulfide (GSSG), H_2S, SO_3^{2-}, $S_2O_3^{2-}$, SO_4^{2-}, and CH_3SSSCH_3. As shown in Figure 3A, no fluorescence increase was observed

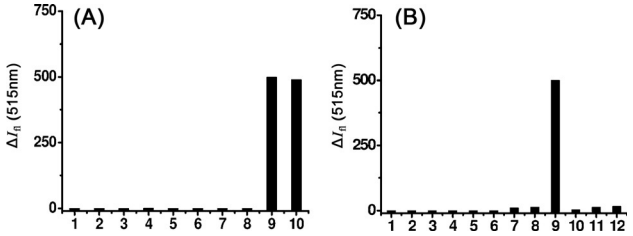


Figure 3. A) Fluorescence intensity (I_{fl}) of **PSP-3** (10 μM) at $\lambda = 515$ nm in the presence of various RSS: 1) probe alone; 2) Hcy (100 μM); 3) GSSG (100 μM); 4) Na_2S (200 μM); 5) $Na_2S_2O_3$ (100 μM); 6) Na_2SO_3 (100 μM); 7) Na_2SO_4 (100 μM); 8) CH_3SSSCH_3 (100 μM); 9) Na_2S_2 (50 μM); 10) Na_2S_4 (50 μM). B) Fluorescence intensity of **PSP-3** (10 μM) in the presence of ROS (with or without H_2S). 1) probe alone; 2) H_2O_2 (200 μM); 3) ClO^- (50 μM); 4) O_2^- (50 μM); 5) $^{\bullet}OH$ (50 μM); 6) $^1O_2$ (50 μM); 7) H_2O_2 (200 μM) + Na_2S (50 μM); 8) H_2O_2 (200 μM) + Na_2S (100 μM); 9) ClO^- (50 μM) + Na_2S (100 μM); 10) O_2^- (50 μM) + Na_2S (100 μM); 11) $^{\bullet}OH$ (50 μM) + Na_2S (100 μM); 12) $^1O_2$ (50 μM) + Na_2S (100 μM).

for these RSS (columns 2–8). Only Na_2S_2 and Na_2S_4 triggered significant increases in fluorescent intensity (columns 9 and 10). Additionally, it is known that H_2S_n could be generated from the reactions between H_2S and ROS. Therefore, we wondered if the probe could sense in situ H_2S_n formation from H_2S and ROS. As shown in Figure 3B, the responses of **PSP-3** to a series of ROS including hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), superoxide (O_2^-), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) were first tested. These ROS did not induce any fluorescence enhancements (columns 2–6). When H_2S (in the form of Na_2S) was added to these ROS, a strong fluorescence signal was observed from ClO^- (column 9), but not from other ROS. These results correspond to previous reports that H_2S_n can be efficiently derived from H_2S and ClO^-, but other ROS are not effective for H_2S_n generation under these concentrations.[3,8–10] The responses of **PSP-3** in the presence of other representative amino acids were also evaluated and no responses were observed (see Figure S1 in the Supporting Information).

To further evaluate the sensitivity of **PSP-3** for H_2S_n, a series of varied concentrations of Na_2S_2 (0–50 μM) were tested. The fluorescence intensities were linearly related to the concentrations of Na_2S_2 employed in the range of 0–20 μM (Figure S2). The detection limit was calculated to be 3 nM. This is the most sensitive probe reported to date for H_2S_n. To clarify the mechanism of fluorescence turn-on, we studied the reactions between the probes and Na_2S_2. All of the reactions gave fluorescein and benzodithiolone as the isolated products in good yields (80–90%; Figure S3). As such, we believe that

fluorescence turn-on is initiated by H_2S_n-mediated thioester cleavage. The resulting thiolate further reacts with H_2S_n and promotes cyclization to form benzodithiolone and release the fluorophore.

Next we wondered if **PSP-3** could be used for imaging H_2S_n in cells. We first validated the capability of **PSP-3** in the visualization of exogenous H_2S_n in cells. As shown in Figure 4,

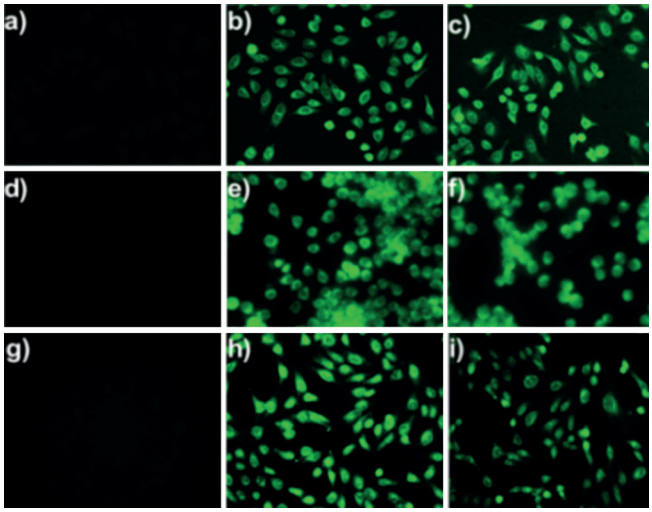


Figure 4. Fluorescence images of exogenous H_2S_n in HeLa (a–c), RAW264.7 (d–f), and Vero (g–i) cells. Cells were incubated with **PSP-3** (5 μM) for 25 min, then washed and subjected to different treatments. a, d, g) Controls (no added Na_2S_2 or Na_2S_4); b, e, h) cells treated with Na_2S_2 (30 μM); c, f, i) cells treated with Na_2S_4 (30 μM).

HeLa, RAW264.7, and Vero cells were respectively incubated with **PSP-3** (5 μM) for 25 minutes. Any extracellular probe was washed off, and no significant fluorescence was observed within the cells (Figure 4 a, d, g). However, when cells were treated with Na_2S_2 or Na_2S_4 (30 μM) for 20 minutes, strong fluorescence was observed. These results demonstrated that **PSP-3** has good cell permeability and could be used to monitor H_2S_n in cells. Additionally, a cell viability assay demonstrated that **PSP-3** has no cytotoxicity to cells (Figure S4).

H_2S_n derivatives are short-lived species and can readily decompose in buffers. With this in mind, in situ generation of H_2S_n from H_2S and ClO^- provides a more reliable and sustainable system for H_2S_n production. Herein **PSP-3** was used to monitor the in situ generation of H_2S_n in cells. As shown in Figure S5, neither ClO^- nor H_2S gave noticeable fluorescence responses whereas the mixture of H_2S/ClO^- led to a strong fluorescence enhancement, which was even brighter than those obtained with exogenous H_2S_n. These results confirmed that in situ generation of H_2S_n by H_2S and ClO^- is a more effective system than simply using Na_2S_2 (or Na_2S_4) to maintain H_2S_n levels in cells.

Having demonstrated the capability of **PSP-3** in detecting exogenous H_2S_n in cells, we then sought to use **PSP-3** to monitor endogenous H_2S_n formation. Our recent studies found that CSE overexpression causes significant elevation of persulfide and polysulfide levels in cells.[5] Therefore, cells which overexpress CSE provide a suitable system for endogenous H_2S_n production. As shown in Figure 5 a–d, normal and CSE-overexpressed[5] COS-7 cells were separately treated with **PSP-3** for 30 minutes. As expected, significant fluorescent enhancement was detected for CSE-overexpressed cells as compared to normal cells. In fact, an appreciable amount of H_2S_n was directly generated from a recombinant CSE in a cell-free reaction mixture containing cystine as a substrate (Figure S6). These results demonstrate that **PSP-3** is suitable for detecting endogenous H_2S_n. Additionally, Kimura et al. recently described a new H_2S biosynthetic pathway from D-Cys in mammalian cells, which occurs predominantly in the kidney and the cerebellum.[14] This process seems to be regulated by 3-mercaptopyruvate sulfurtransferase (3MST) and D-amino acid oxidase (DAO). It was suggested that H_2S produced from D-Cys might be stored as bound sulfane sulfur and the levels of sulfane sulfur can be significantly enhanced by D-Cys. In theory, H_2S_n could be produced from the reactions between H_2S and sulfane sulfurs. Therefore, D-Cys stimulation in Vero cells may be a useful system for endogenous H_2S_n generation and this system was used to further validate the probe's sensitivity for endogenous H_2S_n. As shown in Figure 5 e–h, the Vero cells were incubated with **PSP-3** (5 μM) for 25 minutes at 37 °C. The control (Figure 5 e) showed almost no fluorescence. When cells were treated with D-Cys (30 μM), a modest but obvious fluorescence signal was observed (Figure 5 f). When the concentration of D-Cys was increased to 1 mM, we observed strong fluorescence (Figure 5 g). Moreover, when the cells were first treated with sodium benzoate (a DAO inhibitor), the stimulation by D-Cys (1 mM) led to significantly weakened fluorescence. Taken together, these results indicate that D-Cys and DAO may be responsible for cellular H_2S_n production.

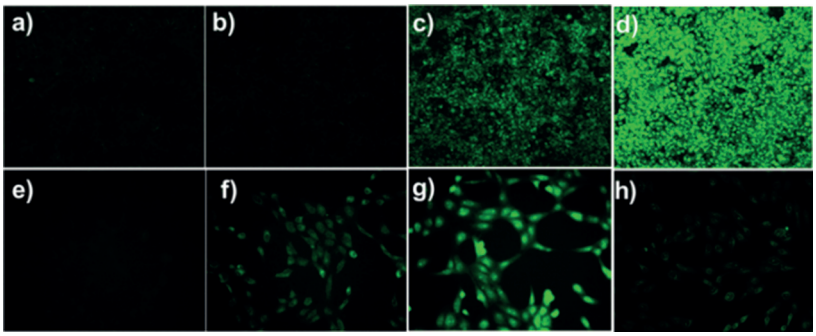


Figure 5. Fluorescence images of endogenous H_2S_n in COS-7 (a–d) and Vero (e–h) cells. a) Normal cells not treated with **PSP-3**; b) Normal cells treated with **PSP-3**; c) CSE-overexpressed cells treated with **PSP-3**; d) normal cells treated with Na_2S_2 (20 μM) and **PSP-3**. e–g) Cells were incubated with **PSP-3** (5 μM) for 25 min, washed, and subjected to different treatments. e) Cells were incubated with FBS-free media (FBS = fetal bovine serum) for 30 min; f) cells were incubated with D-Cys (30 μM) for 30 min; g) cells were incubated with D-Cys (1 mM) for 30 min; h) cells were pretreated with sodium benzoate (250 μM) for 30 min, then washed and incubated with **PSP-3** (5 μM) for 25 min, and then washed and incubated with D-Cys (1 mM) for 30 min.

In summary, we report here the development of three new fluorescent probes (**PSP-1**, **PSP-2**, **PSP-3**) which exploit the unique dual reactivity of H_2S_n as both a nucleophile and an electrophile. Among the probes prepared, **PSP-3** showed an off/on fluorescence response to H_2S_n as well as high specificity. **PSP-3** was also successfully applied in the visualization of both exogenous and endogenous H_2S_n in cells. This novel probe overcomes the major limitations of previously reported H_2S_n probes and it is expected to serve as a useful tool in understanding the physiological functions of H_2S_n.

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