Recent Developments in Research Reactive Sulfur Species

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Recent advances in probe design to detect reactive sulfur species and in the chemical reactions employed for fluorescence switching

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Reactive sulfur species, including hydrogen sulfide, hydropersulfide, and polysulfide, have many roles in biological systems. For example, hydrogen sulfide is involved in the relaxation of vascular smooth muscles and mediation of neurotransmission, while sulfane sulfur, which exists in cysteine persulfide/polysulfide, and glutathione persulfide/polysulfide, is involved in physiological antioxidation and cytoprotection mechanisms. Fluorescence imaging is well suited for real-time monitoring of reactive sulfur species in living cells, and many fluorescent probes for reactive sulfur species have been reported. In such probes, the choice of detection chemistry is extremely important, not only to achieve effective fluorescence switching and high selectivity, but also because the reactions may be applicable to develop other chemical tools, such as reactive sulfur species donors/scavengers. Here, we present an overview of both widely used and recently developed fluorescent probes for reactive sulfur species, focusing especially on the chemical reactions employed in them for fluorescence switching. We also briefly introduce some applications of fluorescent probes for hydrogen sulfide and sulfane sulfur.

Key Words: fluorescent probe, hydrogen sulfide, sulfane sulfur, HSNO, reactive sulfur species

Reactive sulfur species (RSS), including hydrogen sulfide (H_2S), hydropersulfide (R-S-SH), and polysulfide ($\text{-S-S}_n\text{-S-}$), play important roles in many physiological processes in biological systems. For example, H_2S is involved in relaxation of vascular smooth muscles^(1,2) and mediation of neurotransmission.^(3,4) Also, sulfane sulfur (S^0), which is a sulfur atom with six valence electrons and no charge, existing in the forms of cysteine persulfide or polysulfide and glutathione persulfide or polysulfide, is involved in physiological antioxidation and cytoprotection mechanisms⁽⁵⁾. Thus, there is a need for real-time detection methods to study the biological actions of RSS in living cells or tissues. Fluorescent probes are particularly suitable for this purpose,⁽⁶⁾ and many fluorescent probes for RSS have been reported.

In this review, we provide an overview of fluorescent probes for RSS, focusing on the underlying chemical reactions used to achieve fluorescence switching in response to RSS. These chemical reactions may also be applicable to the development of chemical tools such as RSS donors/scavengers. To illustrate representative mechanisms, we first focus on some well-established fluorescent probes that have been widely used in

biological studies, and then we provide an overview of more recent fluorescent probes that have been reported since 2018.

Well-established Fluorescent Probes for H_2S

Fluorescent probes utilizing the reduction of an azide group with H_2S . H_2S reduces an azide group to amine, and this reaction has often been used for fluorescence switching. Lippert and colleagues reported the first H_2S -detecting fluorescent probes using azide reduction, SF1 and SF2 (Fig. 1A).⁽⁷⁾ Both probes are azide-caged rhodamine analogues forming a closed form and weakly fluorescent before reaction with H_2S . However, in the presence of 100 μM NaHS (an H_2S donor), 10 μM SF1 and SF2 in open form show 7- and 9-fold increases of fluorescence intensity and the *in vitro* detection limit was 5–10 μM . These probes are selective for H_2S over other RSS, reactive oxygen species (ROS) and reactive nitrogen species (NOS) although a slight fluorescence enhancement was observed with other species than H_2S . Many fluorescent probes with azide group as a reaction site have been reported since the development of SF1 and SF2 and we will introduce some examples in the section of “recent developments in fluorescent probes for H_2S ”.

Fluorescent probes utilizing the nucleophilicity of H_2S . Xian's group^(8,9) developed H_2S -detecting fluorescent probes, WSP1–5, which undergo selective disulfide exchange reaction with H_2S to form benzodithiolone (Fig. 1B). To develop probes with high selectivity, they focused on the difference of nucleophilicity between H_2S and other biothiols such as glutathione (GSH) and cysteine. H_2S can undergo nucleophilic reaction with the probes twice, because it is a non-substituted thiol. As shown in Fig. 1B, H_2S firstly reacts with the electrophilic group in the probe to form an intermediate containing a R-S-SH group. The persulfide group triggers intermolecular cyclization, releasing the fluorophore, which exhibits strong fluorescence. Biothiols can also react with the probes at their electrophilic moiety, but the product does not undergo the subsequent intramolecular cyclization.

Fluorescent probe using complexation of H_2S with Cu^{2+} . Our group⁽¹⁰⁾ has developed a fluorescent probe for H_2S , HSip-1, which employs azamacrocyclic copper (II) ion (Cu^{2+}) complex chemistry to detect H_2S (Fig. 1C). HSip-1 is weakly fluorescent

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Fig. 1. Established fluorescent probes for H_2S that have been utilized in biological studies. Chemical structures and reaction mechanisms of (A) SF1-2, (B) WSP series, and (C) HSip-1.

before the reaction with H_2S , but shows a rapid 50-fold fluorescence increase upon addition of $10\ \mu\text{M}$ H_2S . Cu^{2+} is released from the azamacrocyclic ring by reaction with H_2S , forming the CuS complex, and this leads to a fluorescence increase due to loss of the fluorescence quenching effect of Cu^{2+} on the nearby fluorophore. HSip-1 shows high selectivity for H_2S over biothiols such as reduced GSH, cysteine, and homocysteine. We success-

fully applied it for high-throughput screening (HTS) to find selective inhibitors of 3-mercaptopyruvate sulfurtransferase (3MST), an RSS-generating enzyme.⁽¹¹⁾ In addition, Ezeriņa *et al.*⁽¹²⁾ used HSip-1 to measure H_2S generation *in cellulo*, and investigated the mechanism of the antioxidant effect of *N*-acetylcysteine (NAC).

In this section, we have described well-established H_2S -detecting fluorescent probes based on representative detection

A

B

Fig. 2. Established fluorescent probes for sulfane sulfur. Chemical structures and reaction mechanisms of (A) SSP series and (B) SSip-1.

mechanisms. Many fluorescent probes using these mechanisms have been designed for specific purposes, and recent developments will be discussed later. These include organelle-targeting probes, and near-infrared (NIR) fluorescent probes with low phototoxicity and low background fluorescence.

Well-established Fluorescent Probes for Sulfane Sulfur

Fluorescent probes utilizing the binding ability of sulfane sulfur to another sulfur atom. Sulfane sulfur can bind reversibly to another sulfur atom, and this unique feature has been used for the development of fluorescent probes. In this section, we introduce two representative fluorescent probes for sulfane sulfur. In 2013, Xian's group⁽¹³⁾ reported a series of sulfane sulfur-detecting fluorescent probes, the SSP series, by utilizing the ability of sulfane sulfur to form persulfides with other thiols (Fig. 2A). In their probes, sulfane sulfur forms a persulfide group with the fluorescent probe, and then intramolecular nucleophilic attack on the ester group releases the fluorophore, generating strong fluorescence (Fig. 2A). These probes are widely used in biological studies. For example, SSP4 was used to detect H_2S_3 produced by 3MST in COS cells in response to addition of the substrate, 3MP, to the medium.⁽¹⁴⁾ Xian's group also developed the DSP series⁽¹⁵⁾ and the PSP series⁽¹⁶⁾ for selectively detecting hydrogen polysulfides among sulfane sulfur species by utilizing the high nucleophilicity of hydrogen polysulfides. Another fluorescent probe, SSip-1, also utilizes this characteristic reactivity of sulfane sulfur (Fig. 2B).⁽¹⁷⁾ SSip-1 is a reversible probe that can detect the dynamics of intracellular sulfane sulfur,

whereas most previously developed probes are irreversible. SSip-1 is weakly fluorescent, because the 2-thio RB moiety works as a Förster resonance energy transfer (FRET) acceptor, quenching the fluorescence of the fluorescein moiety (the FRET donor). Sulfane sulfur forms a persulfide with the thiol group of SSip-1, and this induces intramolecular spirocyclization, resulting in strong fluorescence. Miyamoto *et al.*⁽¹⁸⁾ used SSip-1 in a study of polysulfides generated by the interaction between H_2S and a nitric oxide (NO) donor in the presence of reducing substances, and concluded that H_2S and NO react to produce H_2S_n , which activates transient receptor potential ankyrin 1 (TRPA1).

Recent Developments in Fluorescent Probes for H_2S

Fluorescent probes for H_2S using azide reduction. An azide group can be easily introduced into molecules as a reaction site for H_2S , and several H_2S -detecting fluorescent probes using azide reduction have recently been reported, in addition to the SF series shown in Fig. 1A. Some of them were designed for organelle-targeted imaging. Moreover, NIR fluorescent probes can be used for the fluorescence imaging of whole bodies with minimal interference from tissue autofluorescence. Zhou *et al.*⁽¹⁹⁾ developed a mitochondria-targeted near-infrared (NIR) probe, Mito- N_3 (Fig. 3A). The probe has no fluorescence before reaction with H_2S owing to photoinduced electron transfer. The azide group in Mito- N_3 reacts with H_2S and the probe releases the fluorophore, resulting in a fluorescence enhancement at 736 nm. The detection limit of the probe was calculated to be as low as 20 nM and this high sensitivity is one of the advantages of this

Fig. 3. Recently reported fluorescent probes for H_2S . (A) Chemical structures of Mito- N_3 , Gol-NH and TCAN. These probes have an azide group as the reaction site for H_2S . (B) Chemical structure and reaction mechanism of N_3 -CR- PO_4 . This fluorescent probe can simultaneously detect H_2S and phosphatase activity. (C, D) Chemical structures of DC-NBD and NCR. These fluorescent probes contain an NBD moiety as the reaction site with H_2S . (E) Chemical structures of the PTZ series.

probe. In addition, this fluorescent probe shows low cytotoxicity and high membrane permeability, and is suitable for use in living cells and tissues. Another organelle-targeting probe was developed by Zhu and colleagues in 2020 (Fig. 3A).⁽²⁰⁾ They reported a Golgi-targeting fluorescent probe, Gol-NH. This probe has an azide group as a reaction site with H_2S , and a phenylsulfonamide moiety as a Golgi-targeting group, which binds to cyclooxygenase-2 (COX-2) localized in the Golgi complex. This probe is expected to be useful for studying the roles of H_2S in the Golgi.

Other recently developed fluorescent probes have plural reaction sites for RSS, biothiols and enzymes, and can detect H_2S and other biomolecules simultaneously. Qi *et al.*⁽²¹⁾ developed TCAN which can detect Cys/Hcy or GSH, and H_2S at the same time (Fig. 3A). In this probe, phenyl azide-functionalized tetrahydroquinoxaline for the detection of H_2S and nitrobenzoxadiazole (NBD)-masked 7-hydroxycoumarin for the detection of H_2S , Cys/Hcy or GSH are linked together. The probe is almost non-fluorescent, but when it reacts with Cys/Hcy or GSH, the ether bond in the NBD moiety is cleaved and TCA, a blue-emitting

dye, is generated. The reaction with Cys/Hcy produces NBD-*S*-Cys/Hcy, and then *S,N*-intramolecular rearrangement occurs to generate NBD-*N*-Cys/Hcy, which emits in the green region. H_2S reacts with both the azide group and the NBD moiety, and then elimination and cyclization reactions occur to give a hydroxycoumarin dye with blue emission and a tetrahydroquinoxaline coumarin with red emission. By using the two reaction sites (azide group and NBD moiety) and making use of successive reactions, the probe can provide three different signal patterns: blue-green for Cys/Hcy, blue for GSH, and blue-red for H_2S .

Ou *et al.*⁽²²⁾ reported a dual-reactive fluorescent probe, N_3 -CR- PO_4 , which can detect phosphatase activity and H_2S at the same time (Fig. 3B); this is useful because H_2S is thought to be a regulator of phosphatase activity. By using a single probe to investigate the correlation of phosphatase activity and H_2S level in biological systems, they could eliminate various potential errors associated with the use of separate probes to measure H_2S and phosphatase activity, such as differences in cellular uptake and spectral interference. A phosphate-conjugated rhodol and a

Fig. 3. (continue)

coumarin with an azide group are connected by a linker in this fluorescent probe. Addition of H_2S increases the fluorescent peak at 445 nm ($\lambda_{\text{ex}} = 360$ nm), while enzymatic reaction with phosphatase increases the fluorescence at 545 nm ($\lambda_{\text{ex}} = 510$ nm). In the presence of both H_2S and phosphatase, the fluorescence peak at 545 nm ($\lambda_{\text{ex}} = 360$ nm) was increased. This probe relies on FRET from the coumarin moiety to the rhodol moiety. In live-cell imaging with this probe, it was found that the H_2S level tightly controls the phosphatase activity, and even a slight change of H_2S level in cells resulted in a sharp decrease of phosphatase activity.

Fluorescent probes for H_2S using an NBD moiety. Gong *et al.*⁽²³⁾ developed a NIR fluorescent probe using NBD ether as the reaction site (DC-NBD) (Fig. 3C). After reaction with H_2S , the fluorophore is released and fluorescence enhancement at 744 nm is seen within 3 min. This probe has a larger Stokes shift and a faster response time than most previously developed NIR probes. Although the NBD moiety can react with biothiols (Cys, Hcy, GSH, etc.) and H_2S , DC-NBD unexpectedly showed high selectivity for H_2S . There may be two reasons for this. One is that H_2S and biothiols have different pK_a values of the thiol group, and H_2S shows stronger nucleophilicity under physiological condi-

tions. The other is that H_2S is sterically smaller than biothiols, and can more easily attack the NBD moiety. However, the precise reason for the difference in the reactivity of the NBD moiety between TCAN and DC-NBD is unclear.

Another fluorescent probe using the NBD moiety was developed by Qiao *et al.*⁽²⁴⁾ for simultaneous detection of Cys/Hcy, GSH and H_2S (Fig. 3D). This fluorescent probe contains two fluorophores, coumarin and resorufin, and two reaction sites. The NBD amine moiety was expected to react with H_2S selectively. Therefore, the NBD amine moiety was used as the reaction site for H_2S , and a disulfide bond connecting the two fluorophores was used as the reaction site for Cys/Hcy, GSH and H_2S .

Fluorescent probes for H_2S using a C=C bond. Wang and colleagues⁽²⁵⁾ reported a new design strategy to detect H_2S (Fig. 3E). They discovered the reductive cleavage of a C=C bond by H_2S under mild conditions and utilized this reaction for probe development. They designed and developed a series of fluorescent probes, PTZ-P1–4 (Fig. 3E), in which the strongly fluorescent dye phenothiazine (PTZ) is connected to a dicyano moiety. The probes show no fluorescence due to strong intramolecular charge transfer (ICT) before the reaction with H_2S . But, upon reaction with H_2S ,

Fig. 4. Recently reported fluorescent probes for sulfane sulfur, hydropersulfide and hydropolysulfide. (A) Chemical structures of fluorescent probes containing a 2-fluoro-5-nitrobenzoate moiety, KB1, PZC-S_n, and NIPY-NF, are shown. The reaction mechanism is also illustrated. (B) Chemical structure and reaction mechanism of ACC-Cl. (C) Chemical structures of fluorescent probes using a thiol group as the reaction site for sulfane sulfur: BD-SH, SSNIP, and Mito-SeH. (D) Chemical structure of HQO-PSP. (E–G) Chemical structures and reaction mechanisms of a fluorescent probe for H₂S_n, FP-CF₃ (E), H1, a photoactivatable fluorescent probe for sulfane sulfur (F), and MCP1, a fluorescent probe for simultaneous differentiation of H₂S, H₂S_n, and biothiols (G).

the C=C bond is cleaved and intramolecular cyclization reaction occurs, switching on the fluorescence. Further development of H₂S fluorescent probes based on this approach is expected.

Recent Developments in Fluorescent Probes for Sulfane Sulfur (Persulfide and Polysulfide)

Fluorescent probes for persulfide and polysulfide using a 2-fluoro-5-nitrobenzoate moiety. Several NIR probes or organelle-targeting probes for sulfane sulfur (persulfide and polysulfide) have recently been developed. Three fluorescent probes having a 2-fluoro-5-nitrobenzoate moiety as a reaction site, like the DSP series⁽¹⁵⁾ for hydrogen persulfides and polysulfides, have been reported by different groups. KB1, developed by Li

and colleagues (Fig. 4A),⁽²⁶⁾ is a NIR fluorescent probe based on dicyanomethylene-benzopyran dye. Another group reported PZC-S_n as a red-emitting fluorescent probe with large Stokes shift for *in vivo* imaging, based on the phenothiazine coumarin scaffold.⁽²⁷⁾ In addition, NIPY-NF was developed by Ren and colleagues⁽²⁸⁾ as a lysosome-targetable fluorescent probe. These probes all employ the same chemical reaction for detecting hydrogen polysulfides (persulfides). First, hydrogen polysulfide reacts with the probe at the 2-fluoro-5-nitrobenzoate moiety, introducing a persulfide group in place of the F atom. This triggers an intramolecular cyclization reaction, resulting in release of the fluorophore. Chen *et al.*⁽²⁹⁾ reported a new strategy to detect hydrogen polysulfides using a coumarin moiety bearing a Cl atom instead of 2-fluoro-5-nitrobenzene (Fig. 4B). They developed a

Fig. 4. (continue)

rationetric fluorescent probe, ACC-Cl, which contains 4-chloro-7-diethylaminocoumarin ester as the reactive site for hydrogen polysulfides. Before reaction with hydrogen polysulfides, the fluorescence of the 4-chloro-7-diethylamino-coumarin ester moiety is observed, but upon reaction with polysulfides, substitution-cyclization reaction occurs to release the fluorescent dye, 7-hydroxy-4-methylcoumarin, resulting in a fluorescence spectral change which makes it possible to detect polysulfides (persulfides) in a ratiometric manner. This probe exemplifies a new strategy to develop ratiometric fluorescent probes for hydrogen polysulfides.

Fluorescent probes for persulfide and polysulfide using a thiol group (-SH) or a selenol group (-SeH). There are several reports of sulfane sulfur-detecting fluorescent probes using a thiol group as the reaction site like the SSP series.⁽¹³⁾ For example, BD-SH having the BODIPY scaffold,⁽³⁰⁾ and SSNIP constructed by linking 2-thiobenzoic acid and 2-[2-(4-hydroxystyryl)-4H-chromen-4-ylidene] malononitrile⁽³¹⁾ have been reported by

different groups (Fig. 4C). Both probes are NIR probes for the detection of total sulfane sulfur *in vivo*.

A NIR fluorescent probe, Mito-SeH, which uses a selenol group as a more electrophilic reaction site than thiol, has been reported by Gao *et al.* (Fig. 4C).⁽³²⁾ In this molecular design, a 2-hydroselenobenzoate group, which serves as a reaction site for sulfane sulfurs, is conjugated to the BODIPY scaffold bearing a mitochondria-targeted unit, the triphenylphosphonium cation. In the reaction of Mito-SeH with sulfane sulfurs, the selenol group is converted to -Se-SH, and then intramolecular cyclization occurs, leading to release of the fluorophore.

The 2-(benzothio) benzoate moiety is expected to react selectively with hydrogen persulfides over other biothiols, minimizing consumption of the probe. In 2015, Chen *et al.*⁽¹⁶⁾ utilize this moiety for the development of a fluorescent probe for hydrogen polysulfides, PSP-3. In addition, HQO-PSP, a red-emitting and mitochondria-targeting fluorescent probe having a phenyl 2-

Fig. 5. Chemical structure and reaction mechanism of TAP-1, a fluorescent probe for HSNO.

(benzoylthio) benzoate moiety like PSP-3 and a heptamethine cyanine scaffold, was reported by Meng (Fig. 4D).⁽³³⁾ This probe shows no fluorescence before reaction with hydrogen polysulfides (persulfides), but the thioester group of the probe is cleaved by hydrogen polysulfides (persulfides), generating an intermediate with a thiol group. Hydrogen polysulfides further react with this thiol group to form a persulfide group, which results in spontaneous intramolecular cyclization, with release of the fluorophore.

Fluorescent probes for persulfide and polysulfide using a trifluoromethyl-substituted acrylate ester group. Guo *et al.*⁽³⁴⁾ developed a series of fluorescent probes for hydrogen polysulfides, the FP series, based on a 3-benzothiazol-7-hydroxycoumarin dye bearing different acrylate groups (-H, -Me, -Ph, -CF₃). The probe with the trifluoromethyl-substituted acrylate group, FP-CF₃, was chosen as the best candidate among the four synthesized probes (Fig. 4E). The acrylate ester group is expected to react with hydrogen polysulfides, followed by intramolecular cyclization and fluorophore release. FP-CF₃ is selective for hydrogen polysulfides over biothiols, including cysteine and homocysteine/glutathione, and works rapidly, with a response time of less than 60 s and a signal-to-background ratio of over 44. Acrylate ester has been used in the development of cysteine fluorescent probes, but here the reaction was utilized for the development of a hydrogen polysulfide fluorescent probe.

Fluorescent probe for persulfide and polysulfide using an aldehyde group. Han *et al.*⁽³⁵⁾ have reported a photoactivatable and mitochondria-targeting fluorescent probe for hydrogen polysulfides, H1, utilizing an aldehyde group as the reaction site (Fig. 4F). H1 consists of the fluorescein scaffold, a mitochondria-targeting group (the triphenylphosphonium cation) and a 2-nitrobenzyl-based photocleavage moiety. The aldehyde group is masked with the benzyl group in this probe, so that H1 cannot react with hydrogen polysulfides in the absence of UV irradiation. After irradiation, the produced aldehyde group is attacked by hydropersulfides/polysulfides, and the resulting intermediate undergoes cyclization followed by cleavage of the ester bond to afford the benzodithiolone product with release of the fluorophore.

Fluorescent probe for simultaneous differentiation of H₂S, H₂S_n and biothiols using a highly reactive double bond as a reaction site. MCP1, a fluorescent probe for detecting H₂S, H₂S_n and biothiols simultaneously, has been reported by Chen (Fig. 4G).⁽³⁶⁾ MCP1 has three reaction sites: the substituted phenoxy group, the activated α , β -unsaturated bond and the cyano group. The substituted phenoxy group reacts with H₂S, and is replaced by a thiol group. Then, intermolecular cyclization via

reaction between the thiol and cyano groups occurs, resulting in a fluorescence enhancement at 508 nm. On the other hand, biothiols react with the activated α , β -unsaturated bond, and the probe shows an emission peak at 469 nm. H₂S_n reacts with the probe at the α , β -unsaturated bond, and intramolecular cyclization occurs via reaction between the produced persulfide group and the phenoxy group, resulting in a fluorescence enhancement at 576 nm. Thus, MCP1 shows distinct emission peaks at 508, 576, and 469 nm in response to H₂S, H₂S_n, and biothiols, respectively.

Fluorescent Probe for HSNO

Chen *et al.*⁽³⁷⁾ have reported a fluorescent probe, TAP-1, to detect HSNO (Fig. 5). TAP-1 has two reaction sites, 2-mercaptobenzoate and *o*-phenylenediamine. The S atom in HSNO reacts with the 2-mercaptobenzoate moiety, and the N atom in HSNO reacts with the *o*-phenylenediamine moiety to afford benzotriazole. Polysulfides or persulfides can react with the 2-mercaptobenzoate moiety in TAP-1, but without the reaction at *o*-phenylenediamine the resulting product is only weakly fluorescent. This makes it possible to detect HSNO selectively in the presence of polysulfides or persulfides.

Conclusion and Perspective

In this review article, we have summarized fluorescent probes available for detecting RSS including H₂S, sulfane sulfurs, biothiols, and HSNO, considering well-established fluorescent probes and very recently reported fluorescent probes separately. The latter category includes NIR fluorescent probes for both H₂S and sulfane sulfur, multi-functional fluorescent probes simultaneously detecting multiple RSS including biothiols, and organelle-targeting fluorescent probes, as well as a probe targeting HSNO. We have focused especially on detection chemistry for RSS, since this provides a basis for the development of new fluorescent probes to examine the physiological functions of RSS, as well as new chemical tools such as RSS donors/scavengers.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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