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Fluorescent probes for hydrogen sulfide detection and bioimaging

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In comparison with other biological detection technologies, fluorescence bioimaging technology has become a powerful supporting tool for intracellular detection, and can provide attractive facilities for investigating physiological and pathological processes of interest with high spatial and temporal resolution, less invasiveness, and a rapid response. Due to the versatile roles of hydrogen sulfide (H_2S) in cellular signal transduction and intracellular redox status regulation, fluorescent probes for the detection of this third signalling gasotransmitter have rapidly increased in number in recent years. These probes can offer powerful means to investigate the physiological actions of H_2S in its native environments without disturbing its endogenous distribution. In this feature article, we address the synthesis and design strategies for the development of fluorescent probes for H_2S based on the reaction type between H_2S and the probes. Moreover, we also highlight fluorescent probes for other reactive sulfur species, such as sulfane sulfurs and SO_2 derivatives.

Introduction

Fluorescence bioimaging technology can be distinguished readily amongst biological detection technologies by its many advantages, such as good sensitivity, excellent selectivity, rapid response, and non-invasive detection. In biological systems, features of physiologically active species include low concentration, high reactivity and short lifetimes. Therefore, it still remains a huge challenge to determine the intracellular concentration of these species accurately. In order to meet these urgent needs, reaction-based fluorescent probes have emerged.[1,2]

In general, the design and synthetic strategies of these probes depend on the chemical properties of the physiologically active species. The reaction-based fluorescent probes are mainly composed of two moieties: the fluorescence signal transducer and the fluorescence modulator (Fig. 1). The fluorescence signal transducer moiety transduces molecular recognition into a fluorescence signal that can be detected. It is essential to choose a suitable fluorophore platform as the signal transducer. High quantum yield, photostability and bio-compatibility are critical for bioimaging, such that the minimum dosage of the probe can avoid disturbing the natural

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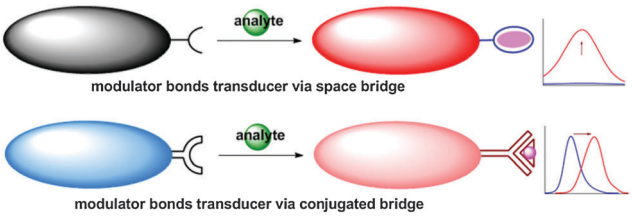
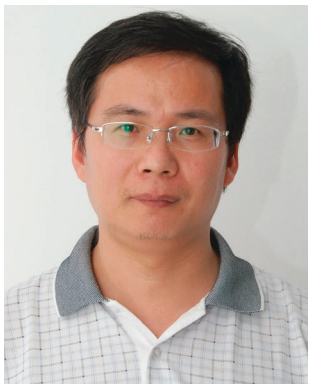


Fig. 1 Schematic illustration of the design of fluorescent probes.

distribution of the physiologically active species. The fluorescence modulator moiety manipulates the molecular recognition process. A desirable fluorescence modulator can only be triggered by a single reaction switch. The selected reaction is also screened to ensure it possesses reasonable reaction kinetics under physiological conditions. After integrating the modulator into the transducer *via* a conjugated or a space bridge, a reaction-based fluorescent probe is generated (Fig. 1). All the features of the fluorescence response can be employed as outputs based on their signal changes, such as absorption, emission spectra, and fluorescence lifetime. It is generally recognized that turn-on fluorescent probes are more efficient compared with turn-off probes. Turn-on signals provide ease of measuring low concentrations against a dark background, which can reduce false positive signals and increase sensitivity. Appropriate fluorescent probes also possess near-infrared absorption and emission spectra (including two-photon and multi-photon), because the light in this region shows maximized tissue penetration while minimizing the absorbance of heme in hemoglobin and myoglobin, water, and lipids. Moreover, ratiometric probes benefit from measuring the ratio of the emission intensity at two different wavelengths, since the interference caused by factors such as uneven loading and



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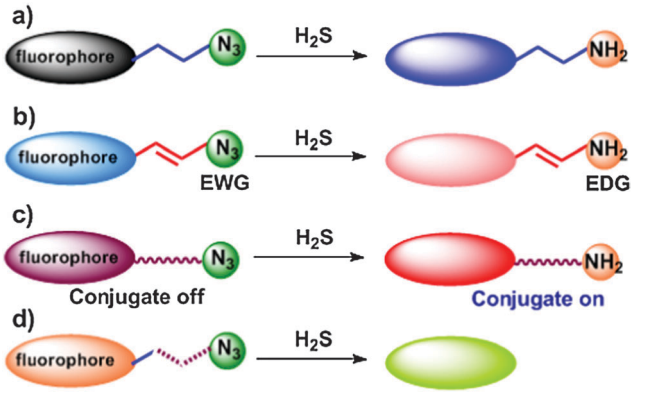

Fig. 2 Summary of strategies for fluorescent probes based on reducing azides to amines.

the inhomogeneous distribution of fluorescent probes in cells can be cancelled out. Finally, the probes should have low cytotoxicity and appropriate water solubility.

Intracellular reactive sulfur species (RSS) is a general term for sulfur-containing biomolecules. These molecules play critical roles in physiological and pathological processes. Glutathione (GSH), the most abundant intracellular nonprotein thiol, can control intracellular redox activity, intracellular signal transduction, and gene regulation. Cysteine (Cys) is implicated in slow growth in children, liver damage, skin lesions, and loss of muscle and fat. Homocysteine (Hcy) is a risk factor for Alzheimer's disease and cobalamin (vitamin B12) deficiency.[3] H_2S has been identified as the third gasotransmitter following nitric oxide (NO) and carbon monoxide (CO). At physiological levels, H_2S regulates the intracellular redox status and fundamental signalling processes, including regulation of vascular tone, myocardial contractility, neurotransmission, and insulin secretion. Abnormal levels of H_2S in cells can induce many diseases, such as Alzheimer's disease, liver cirrhosis, gastric mucosal injury and arterial and pulmonary hypertension.[4] In recent years, the development of fluorescent probes for H_2S has rapidly increased, benefiting from the chemical reactions of H_2S.

The fluorescent probes for GSH, Cys and Hcy have been well reviewed.[5,6] Hitherto, there have been few reviews on the progress of fluorescent probes for H_2S.[7] Now, we overview the synthesis and design strategies for the development of fluorescent probes based on the reaction type between H_2S and the probes. We classify these probes according to their reaction type with H_2S: (a) H_2S reductive reactions: reducing azides to give amines, reducing nitro/azanol to give amines, and reducing selenoxide to give selenide; (b) H_2S nucleophilic reactions: Michael addition reactions, dual nucleophilic reactions, double bond addition reactions, and thiolysis reactions; (c) copper sulfide precipitation reaction. Moreover, we also introduce fluorescent probes for other reactive sulfur species, such as sulfane sulfurs and SO_2 derivatives. The detection of sulfane sulfurs is mainly based on the nucleophilic addition reaction, and the detection of SO_2 derivatives is based on their nucleophilic and reductive properties.

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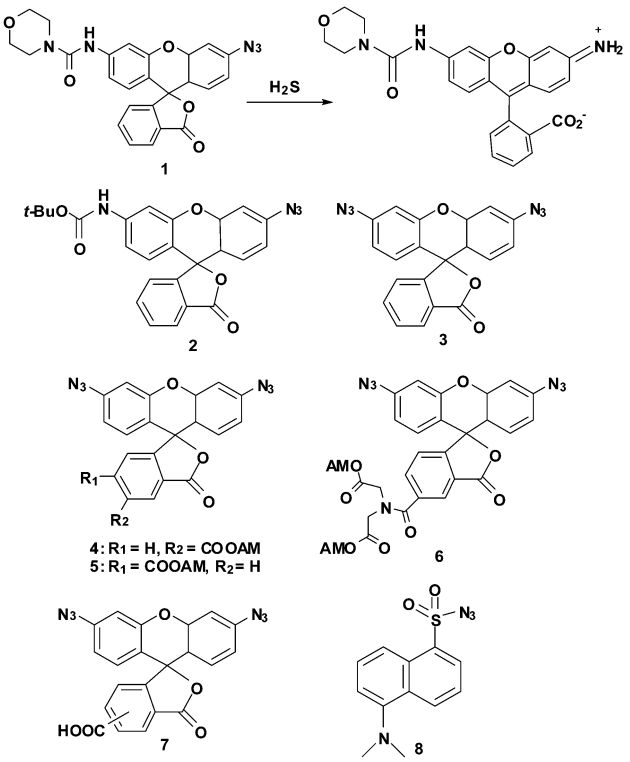
Fluorescent probes based on reducing azides to amines

Azides and other oxidized nitrogen species can be reduced to amines by H_2S faster than by GSH and other thiols, signifying a promising method for H_2S detection. Upon reduction by H_2S, the electron-withdrawing azido group will be converted into an electron-donating amino group. Therefore, exploiting the electron-donating ability of different substituent groups will result in versatile fluorescent probes (Fig. 2). The fluorescent probes employing the photoinduced electron transfer (PET) mechanism are typically constructed by connecting an electron donor/acceptor recognition group to a fluorophore *via* a space bridge. The design principles of such probes are clear, and the resulting processes will quench or increase the fluorescence of these probes (Fig. 2a). The fluorescent probes that adopt an internal charge transfer (ICT) mechanism typically contain a strong push–pull electronic system, wherein the electron donating group (EDG) and the electron withdrawing group (EWG) are conjugated to the fluorophore. Depending on the ICT mechanism, ratiometric probes can be readily available (Fig. 2b). The changes in the π-conjugated systems triggered by chemical reactions are often followed by a notable alteration in spectroscopic properties, which is advantageous to obtain turn-on/ratiometric fluorescent probes (Fig. 2c). Moreover, design strategies that take advantage of protecting group chemistry will result in excellent probes (Fig. 2d). The approaches mentioned above have since been widely adopted for H_2S detection (Fig. 2).

Chang *et al.* have exploited the selective H_2S-mediated reduction of azides to develop a series of fluorescent probes for intracellular H_2S detection (**1–6**).[8,9] The fluorophores of these probes were based on Rhodamine 110. The detection mechanism was illustrated in Fig. 2c. After caging Rhodamine 110 by azides at the 3 or 6 positions, the probes **1–6** adopted a closed lactone conformation, and exhibited no absorption features. When azides were reduced to amines by H_2S, the spiro-rings of **1–6** opened, and the π-conjugated structure was recovered. Therefore, these H_2S probes gave a turn-on response. Probes **1** and **2** could detect H_2S in live HEK293T cells using confocal microscopy, and it took about 1 h to saturate the fluorescence response. Under test conditions, Probe **1** gave a fluorescence quantum yield, Φ, of 0.60. For Probe **2**, Φ = 0.51. The detection limit of **1** was 10 μM. However, the concentration of H_2S changed acutely in cells, and it was difficult for **1** and **2** to capture H_2S opportunely in cells. Later, the same group optimized these design strategies and improved the sensitivity and cellular retention in their new probes. They reported bis-azido probes (**3–6**) for increasing H_2S sensitivity, and they incorporated an acetoxymethyl ester-protected carboxy group into the new probes to increase cellular trappability (**4–6**). It is worth noting that probe **6** could, directly and in real-time, detect endogenous H_2S which was produced in live human umbilical vein endothelial cells upon stimulation with vascular endothelial growth factor (VEGF). The detection limit of **6** was 500 nM. They also revealed that endogenous H_2S production was related to NADPH oxidase-derived hydrogen peroxide (H_2O_2). This experimental result would establish a link for H_2S/H_2O_2 crosstalk. Under test conditions, probes **3**, **4**, **5** and **6** displayed fluorescence quantum yields of 0.92, 0.18, 0.18 and 0.17, respectively. Based on Rhodamine 110 as the fluorophore, Sun *et al.* also developed a fluorescent probe **7** to trap intracellular H_2S in HeLa cells.[10] Probe **7** displayed a 120-fold fluorescence enhancement as a turn-on response, and the detection limit was 1.12×10^{-7} M.

Wang *et al.* developed a turn-on fluorescent probe **8** for H_2S detection in aqueous solutions, blood serum and whole blood.[11] The detection mechanism of the probe was illustrated in Fig. 2a. When the azido group was attached to a strongly electron-withdrawing dansyl fluorophore, the reductive reaction was accelerated. Probe **8** showed a fast response to H_2S, within seconds, which made quantitative H_2S detection possible regardless of the fact that it is rapidly metabolized in biological systems. The detection limit was 1 μM in buffer/Tween and 5 μM in bovine serum. The H_2S concentrations in C57BL6/J mouse model blood were determined to be 31.9 ± 9.4 μM using **8**. The addition of H_2S led to a 40-fold increase in the fluorescence of probe **8**.



Pluth *et al.* reported two reaction-based chemiluminescent probes for H_2S (**9a** and **9b**).[12] Since chemiluminescence does not require excitation by any source, there is little chance of photodegradation of the probe. Furthermore, chemiluminescent probes can avoid biological background interference. Therefore, chemiluminescent methods can provide high signal-to-noise ratios in H_2S detection. **9a** and **9b** combined an H_2S-mediated azido group with a luminol derived platform. After reduction by H_2S, chemiluminescence resulted from the oxidation of the

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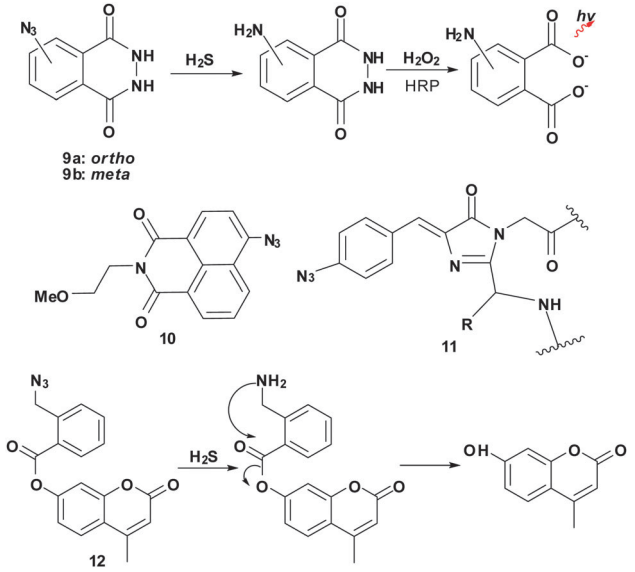
phthalhydrazide moiety, which was oxidized by H_2O_2 using horseradish peroxidase (HRP) as a catalyst. Probe **9b** was used to detect enzymatically-produced H_2S from both isolated CSE enzymes and C6 cell lysates. Probes **9a** and **9b** had strong luminescence responses towards H_2S, with 128- and 48-fold increases, respectively. The detection limits of **9a** and **9b** were 0.7 ± 0.3 and 4.6 ± 2.0 μM, respectively. The group also synthesized fluorescent probe **10** based on azido-naphthalimide.[13] When probe **10** reacted with H_2S, the azide was reduced to an amine following a turn-on fluorescent response. Probe **10** could detect H_2S in HeLa cells; however, this was subject to interference from GSH. Probe **10** gave a quantum yield of 0.096 ± 0.001, and the detection limit was 5–10 μM.

Ai *et al.* reported encoded fluorescent proteins (FPs)-based probe **11** to detect H_2S.[14] FPs can be self-sufficient in generating intrinsic chromophores from polypeptide sequences. They incorporated *p*-azidophenylalanine (*p*AzF) into peptides of FPs to obtain azide-derived chromophores. This genetically encoded probe could be used to monitor H_2S concentration changes in HeLa cells.

Han *et al.* designed and synthesized fluorescent probe **12** based on utilization of *o*-(azidomethyl)benzoyl as the hydroxyl protecting group.[15] The detection mechanism of the probe was illustrated in Fig. 2d. When H_2S triggered the reduction of the azido moiety, the fluorophore 7-hydroxy-4-methylcoumarin was deprotected, and fluorescence was emitted. Probe **12** could be used for imaging H_2S in HeLa cells, and the detection limit was 10 μM.



Han *et al.* reported a colorimetric and ratiometric fluorescent probe **13** for detecting H_2S.[16] The detection mechanism of the probe was illustrated in Fig. 2b. It was anticipated that the modulation of various electron-donating groups on a cyanine dye could affect its intermolecular electron density distribution. Therefore, controlling the electron-donating ability of different substituent groups would result in ICT-induced blue or red shifts in the emission spectrum of cyanine. When H_2S reduced the azide to an amine, the near-infrared heptamethine cyanine platform showed a shift in its emission spectra from 710 nm to 750 nm. The quantum yields of probe **13** and its product changed from 0.11 to 0.12. The probe could evaluate H_2S by fluorescence ratio signals in aqueous solution and fetal bovine serum. Probe **13** could monitor H_2S release by ADT-OH. This probe was able to sense different H_2S levels in RAW 264.7 cells using confocal microscopy ratiometric imaging. The detection limit was 0.08 μM.

Li *et al.* reported two coumarin-based fluorescent probes **14** and **15** for the detection of H_2S.[17] Probes **14** and **15** were both caged by an azido group. When reduced by H_2S, probe **15** showed a larger increase in fluorescence intensity, because the electron-donating ability of $-NEt_2$ was stronger than that of –OH, and was controlled by stronger ICT effects. **14** and **15** gave quantum yields of 0.16 ± 0.013 and 0.58 ± 0.02, respectively, and probe **15** could detect H_2S in rabbit plasma and PC-3 cells.

Cho *et al.* reported a two-photon (TP) H_2S probe **16** for deep tissue imaging.[18] The probe employed 7-(benzo[*d*]thiazol-2-yl)-9,9-(2-methoxyethoxy)ethyl-9*H*-fluorene as the fluorophore. After reduction by H_2S, the TP absorption cross section of **16** was 302 GM at 750 nm in HEPES buffer. The probe was able to detect endogenous H_2S in HeLa cells and could visualize the overall H_2S distribution at depths of 90–190 μm in a rat hippocampal slice. The products of **16** had Φ = 0.46. The detection limit was 5–10 μM. Cho *et al.* next reported another two-photon ratiometric probe **17** for H_2S detection in mitochondria.[19] The detection mechanism of probe **17** was illustrated in Fig. 2b and d. 6-(Benzo[*d*]thiazol-2′-yl)-2-(methylamino)naphthalene was selected as the fluorophore, and 4-azidobenzyl carbamate was chosen as the H_2S response site. The mitochondrial targeting group was a triphenylphosphonium salt. After reduction by H_2S, the fluorophore was released. Under test conditions, probe **17a** showed a shift in emission from 464 nm to 545 nm, and the quantum yields of probe **17a** and its product changed from 0.24 to 0.12. **17b** exhibited a shift in emission from 420 nm to 500 nm, and in quantum yield from 0.23 to 0.50. There was a larger Stokes shift between the probe **17a** and its precursor due to the fact that **17a** had a more stable charge-transfer excited state. The two-photon ratiometric probe can be used to detect mitochondrial H_2S levels in living cells and tissues. The probe **17a** demonstrated the relationship between the cystathionine β-synthase expression level and the H_2S level in astrocytes. Furthermore, the experiments showed that a genetically mutated Parkinson's disease (PD)-related gene could affect H_2S production in the brains of PD patients.

Peng *et al.* and Xu *et al.* reported a two-photon fluorescent probe **18** with near-infrared emission for the detection of H_2S.[20,21] A styrene group was introduced into the fluorophore to extend the conjugation system of the dicyanomethylenedihydrofuran. After reduction by H_2S, the two-photon absorption cross section of **18** was 50 GM at 820 nm in DMSO. The probe could give a 354-fold fluorescence increase upon detection of H_2S. Furthermore, this probe can detect H_2S in commercial fetal bovine serum, MCF-7 cells, HUVEC cells, rat liver cancer slices and ICR mice. The reported detection limit was 3.05 μM.

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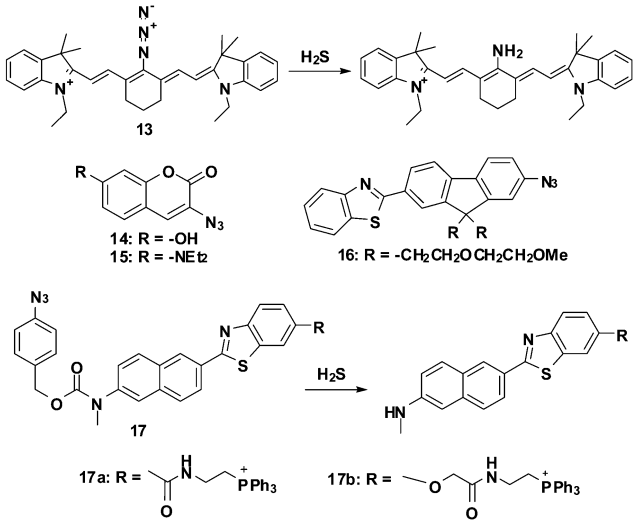


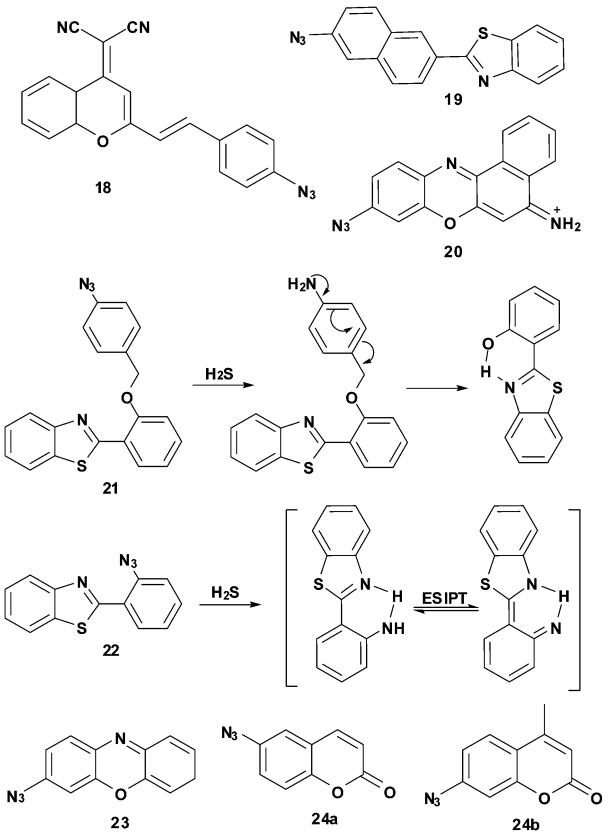
Zhang *et al.* synthesized a two-photon fluorescent probe **19** for the detection of H_2S.[22] This probe employed a naphthalene derivative as the two-photon fluorophore. The detection mechanism of probe **19** was shown in Fig. 2b. Probe **19** had a donor–π–acceptor (D–π–A) structure, and the recognition unit azide acted as the electron withdrawing group which could break the D–π–A structure. When H_2S reduced the azide to an amine, the D–π–A structure recovered and the probe emitted strong fluorescence. The TP absorption cross section of product **19** was estimated to be 110.98 GM at 760 nm, and probe **19** showed a 21-fold TP excited fluorescence increase. The probe could be used to detect endogenous H_2S in HeLa cells, and the detection limit was 20 nM.

Ma *et al.* reported a cresyl violet-based ratiometric fluorescent probe **20**.[23] The probe also had a donor–π–acceptor structure. When the azido group was reduced to an amino group, the D–π–A structure changed from electron-withdrawing to electron-donating. The result led to a spectroscopic blue or red shift in emission, which could provide a ratiometric method of H_2S detection with Φ = 0.44 and 0.54, respectively. This probe could be used to detect H_2S in MCF-7 cells and zebrafish by ratiometric imaging. The detection limit of the probe was 0.1 μM.

Chang *et al.* reported a ratiometric fluorescent probe **21** based on an excited-state intramolecular proton transfer (ESIPT) mechanism for H_2S detection.[24] When the azido group was reduced to an amino group, the *p*-aminobenzyl moiety underwent an intramolecular 1,6-elimination to release the ESIPT dye 2-(2′-hydroxyphenyl)-benzothiazole (HBT). The ratio of emission intensity varied 43-fold. The probes based on the ESIPT mechanism often resulted in large Stokes shifts. The ESIPT mechanism could be exploited to design probes based on their unique, environment-sensitive nature. The probe was used to detect H_2S in HeLa cells, and the detection limit was 2.4 μM.

Guo *et al.* reported a fluorescent probe 2-(2-azidophenyl) benzothiazole (**22**) based on the ESIPT mechanism for H_2S detection.[25] The probe exhibited a 1150-fold increase in fluorescence with Φ = 0.4138. The probe was used to detect H_2S in B16 cells, and the detection limit was 0.78 nM.

Tang *et al.* reported fluorescent probes **23** and **24** for H_2S detection.[26,27] Probe **23** was synthesized based on the fluorophore phenoxazinon, and showed a 23-fold increase in fluorescence. The probe can detect H_2S in PBS buffer, fetal bovine serum, and HeLa cells. With coumarin as a fluorophore, these authors reported two-photon fluorescent probes **24a** and **24b** for H_2S detection. **24b** showed better selectivity and sensitivity than probe **24a**. The product of **24b** gave Φ = 0.88 ± 0.02. Probe **24b** could detect H_2S from both exogenous addition and possible enzymatic production, and imaging of H_2S was achieved in the cardiac tissues of normal rats and atherosclerotic rats.



Zeng *et al.* integrated a naphthalimide azide derivative into the anchoring site of carbon nanodots, and developed a fluorescence resonance energy transfer (FRET) ratiometric fluorescent probe **25**.[28] FRET is the interaction between two excited state fluorophores correlated with distance. The process involves the nonradiative transfer of excitation energy from an excited donor to a proximal ground-state acceptor, and it is convenient in the design of ratiometric probes involving the ratio of two emission intensities at different wavelengths. In FRET systems, the emission wavelength of the donor is the excitation wavelength of the acceptor. Therefore, regulation of the precise energy match between carbon nanodots and the naphthalimide azide derivative would be beneficial for H_2S detection. Probe **25** could detect H_2S in HeLa and L929 cells, and the detection limit was 10 nM.

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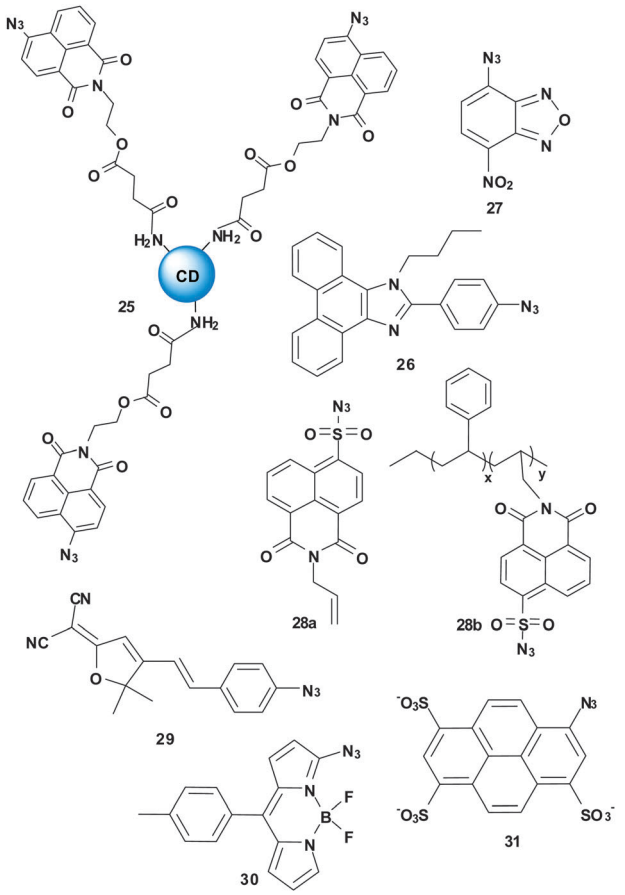

Lin *et al.* reported probe **26**, phenanthroimidazole, for H_2S detection.[29] The azido group could withdraw the electrons from phenanthroimidazole, which made the fluorescence weak. Upon treatment of the probe with H_2S, the fluorescence of the probe could reach saturation within 3 min. The product of **26** gave $\Phi = 0.62$. The probe could be used to detect H_2S in HeLa cells with a detection limit of 8.79×10^{-7} M.

Chen *et al.* reported 7-nitrobenz-2-oxa-1,3-diazole as a colorimetric and fluorescent probe **27** for H_2S detection.[30] When the azide was reduced to an amine, probe **27** showed a change in colour from pale-yellow to deep-yellow. The increase of the fluorescence intensity was up to 16-fold. The probe was used to image H_2S in living MCF-7 cells, and the detection limit was 680 nM.

Wu *et al.* reported the polymer-based fluorescent probe **28** for H_2S detection.[31] The monomer **28a** could be further functionalized to polymer **28b**. Na_2S induced a 5-fold and a 3-fold increase in the fluorescence intensity of **28a** and **28b**, respectively. Probe **28b** could detect H_2S in HeLa cells.

Xu *et al.* reported a fluorescent probe **29** for H_2S detection based on dicyanomethylenedihydrofuran.[32] **29** gave $\Phi = 0.018$, and the probe was able to detect H_2S in living human umbilical vein endothelial cells.

Talukdar *et al.* designed a colorimetric and fluorometric probe **30** for the detection of H_2S.[33] The BODIPY-azide could be reduced to a BODIPY-amide by H_2S with a turn-on fluorescent response. The detection mechanism was as shown in Fig. 2a. The fluorescence of **30** was quenched by the electron-rich α-nitrogen of the azido group. The probe displayed a fast response time in serum albumin (within 30 s) with a 28-fold fluorescence increase, and the detection limit was 259 nM. The probe was used to detect H_2S in HeLa cells.

Hartman *et al.* reported the 8-azidopyrene-1,3,6-trisulfonate probe **31** for H_2S detection with a 24-fold fluorescence increase.[34] The probe had a high water solubility at concentrations >100 mM, and could measure H_2S in serum.

Fluorescent probes based on reducing nitro groups to amines

There exists a major obstacle in the design of fluorescent probes by exploiting nitro fluorophores, because the nitro group has always been considered to be a strong quencher of fluorophores. However, the nitro group can be reduced by Na_2S to produce the corresponding amino group under mild conditions, which opens a door to the design and synthesis of new types of fluorescent probe containing a nitro group for H_2S detection. Taking advantage of this reductive reaction, two main types of H_2S fluorescent probes have been developed. One type adopts the photoinduced electron transfer (PET) mechanism, which benefits from the strongly electron-withdrawing nature of the nitro group (Fig. 3a). The other type uses the internal charge transfer (ICT) mechanism, which results from a donor–π–acceptor structure caused by a strong push–pull electronic effect (Fig. 3b).

Chen *et al.* designed and synthesized a near-infrared fluorescent probe **32** for H_2S detection in HEPES buffer and in fetal bovine serum.[35] The probe involved the PET mechanism in sensing H_2S (Fig. 3a). The fluorescence of heptamethine cyanine would be quenched *via* a photoinduced electron transfer (PET) process from the excited fluorophore to the strongly electron-withdrawing nitro group (donor-excited PET; d-PET).[3,36,37] On the other hand, while the nitro group was reduced to an amino group, there might exist an acceptor-excited PET (a-PET) process from the amino group to the excited fluorophore, since the amino group is a strongly electron-donating group with lone-pair electrons.[38,39] However, the probe displayed an increase in fluorescence emission, and the quantum yield increased from 0.05 to 0.11. This phenomenon was attributed to substituent effects; that is, it was not favorable for the substituent in the *meta*-position of the aromatic ring to act as an electron donor. The probe has been used to track H_2S in RAW264.7 cells.

Li *et al.* reported colorimetric and ratiometric fluorescent probes **33a–c** based on an ICT strategy for the detection of

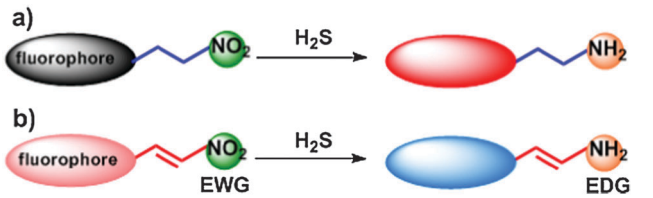

Fig. 3 Summary of strategies for the design of fluorescent probes based on reducing nitro groups to amines.

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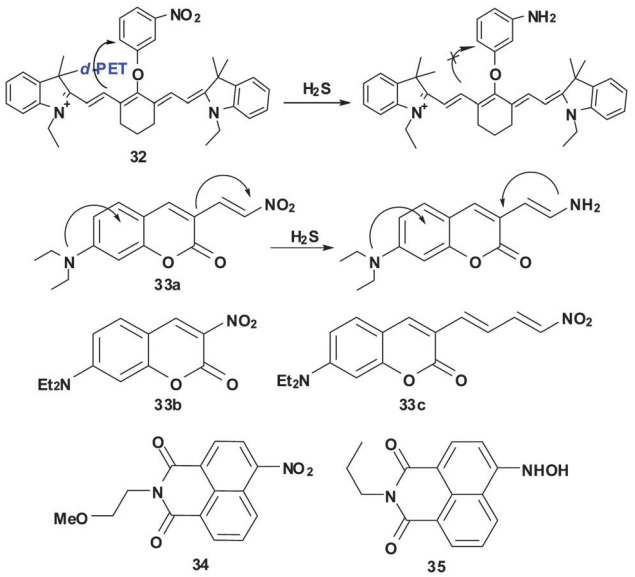
H_2S.[40] Probe **33a** demonstrated a typical push–pull electronic system, crossing the coumarin fluorophore. After reaction with H_2S, the push–pull electron system was blocked by a push–push electron system, which disturbed the ICT mechanism, leading to spectral shifts. The D–π–A structures of probes **33a–c** could result in different sensitivities and selectivities. The reaction of probe **33a** with H_2S resulted in an emission shift from 602 nm (Φ = 0.023) to 482 nm (Φ = 0.236). The detection limit using **33a** was 2.5 μM.

Pluth *et al.* reported a 4-nitronaphthalimide fluorescent probe **34** for the detection of H_2S.[13] The functionalization of the 4-position with amino and nitro in the naphthalimide fluorophore platform resulted in fluorescence turn-on. Probe **34** was also responsive to Cys and GSH. Probe **34** gave Φ = 0.096 ± 0.001, and the probe was used to detect H_2S in HeLa cells.

Wang *et al.* reported hydroxylamine naphthalimide (**35**) as a fluorescent probe for H_2S based on the ICT mechanism.[41] During the reduction of nitro groups to amines, hydroxylamine derivatives are produced as intermediates. The hydroxylamine moiety can be reduced more easily than the nitro group. Moreover, the hydroxylamine moiety is an electron-withdrawing group, and can quench the fluorescence of naphthalimide. **35** gave Φ = 0.12, and the probe could be used to detect H_2S in astrocyte cells.



Fluorescent probes based on reducing selenoxide to selenide

As the active site of the antioxidant enzyme glutathione peroxidase (GPx), organoselenium compounds modulate cellular antioxidant defense systems in defense against damage by reactive oxygen species (ROS) *via* the reduction of reactive oxygen species by biothiols. The oxidation–reduction reaction of the selenoenzyme depends on a unique ping-pong mechanism between selenoxide and selenide.[42] Taking advantage of mimics of the catalytic cycle could aid in the development of fluorescent probes for the reversible detection of H_2S. Since selenoxide and selenide are electron-withdrawing and electron-donating groups, respectively,

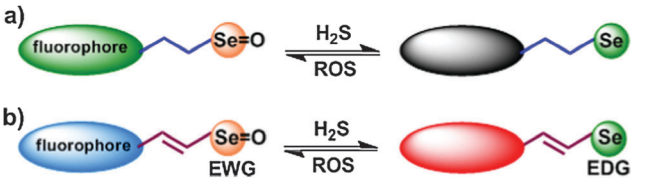


Fig. 4 Summary of strategies for fluorescent probes based on reducing selenoxide to selenide.

fluorescent probes can be smoothly achieved by a photoinduced electron transfer (PET) mechanism (Fig. 4a) and an internal charge transfer (ICT) mechanism (Fig. 4b).

Han *et al.* developed a series of fluorescent probes (**36**, **37**, **38**) containing organoselenium moieties that could be used for monitoring the redox cycles between H_2S and ROS. The reversible fluorescent probe **36** could detect the redox cycle between HClO and H_2S. The mechanism was as shown in Fig. 4a. The probe employed a BODIPY fluorophore as the signal transducer and 4-methyoxylphenylselanyl benzene (MPhSe) as the modulator.[43] The fluorescence of **36** was quenched as a result of PET between the modulator and the transducer, but oxidation of Se prevented the PET, causing the fluorescence emission to be "switched on". The quantum yield increased from 0.13 to 0.96. The probe could be used to detect the redox cycle induced by HClO and H_2S in RAW264.7 cells.

After integration of the modulator (4-methyoxylphenylselenide, MPhSe) into the BODIPY platform through a styrene bridge, probe **37** could function as a near-infrared reversible ratiometric fluorescent probe for the redox cycle between HBrO oxidative stress and H_2S.[44] The mechanism was as shown in Fig. 4b. This approach could facilitate the D–π–A conjugation system and tune the red-shift of the emission efficaciously due to the strong electron-donating properties of the selenide group. After selenide was oxidized to selenoxide by HBrO, the fluorescence of the probe would blue-shift because of the electron-withdrawing effect of selenoxide. The quantum yield increased from 0.00083 to 0.206 (at 635 nm). The probe has been successfully used to detect the HBrO/H_2S redox cycle in the mouse macrophage cell line RAW264.7.

The probe **38** was based on a 1,8-naphthalimide fluorophore.[45] There exists a PET process in **38** (selenide-form), which was confirmed by time-dependent density functional theory calculations. However, there also was an excited state configuration twist process in the selenide-form, but not in its selenoxide form (**38**). This excited state configuration twist would cause fluorescence quenching. The quantum yield of **38** increased from 0.04 to 0.45. The probe was capable of detecting HOCl oxidative stress and H_2S reducing repair in RAW 264.7 cells and in mice.

Fluorescent probes based on the nucleophilic reactions of H_2S

H_2S is a reactive nucleophilic species that can participate in nucleophilic substitution *in vivo*. The major challenge in H_2S detection is to distinguish H_2S from other biological nucleophiles,

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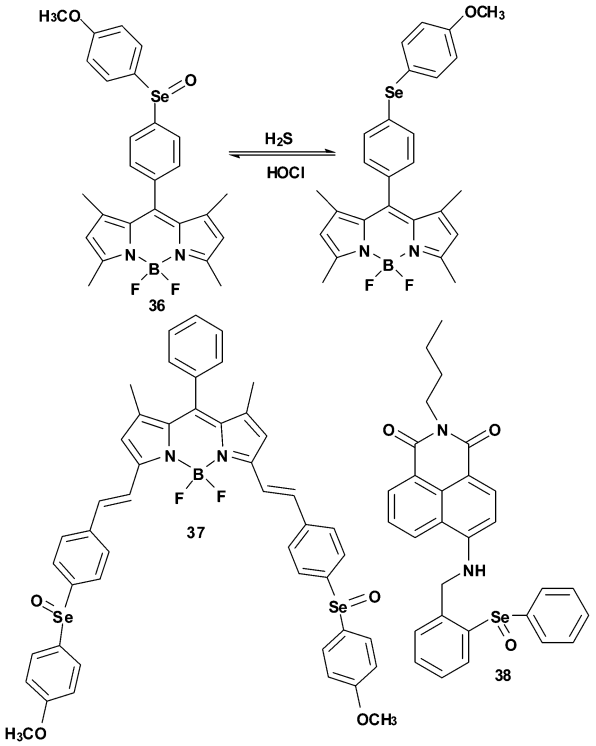

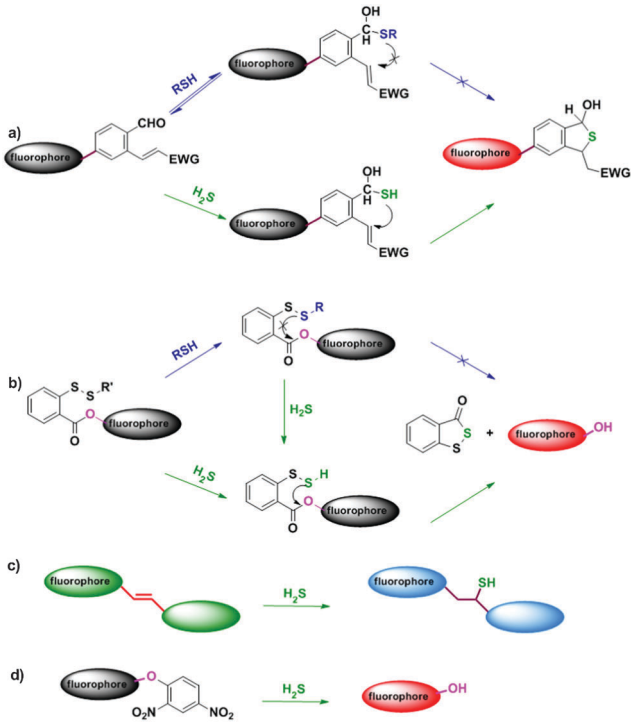

Fig. 5 Summary of strategies for fluorescent probes based on the nucleophilic property of H_2S.

such as cysteine and glutathione, which are present at micromolar or millimolar concentrations inside most cells. The pK_a of H_2S is ~7.0 in aqueous solution, whereas other bio-thiols have higher pK_a values (Cys: ~8.3, GSH: ~9.2). Therefore, H_2S is considered to be a stronger nucleophile than other biothiols in physiological media. H_2S can undergo dual nucleophilic reaction as a non-substituted biothiol, however mono-substituted thiols can only undergo one nucleophilic reaction. Based on the nucleophilic and dual nucleophilic properties, fluorescent probes containing bis-electrophilic entities have been devised for H_2S detection. As shown in Fig. 5a and b, H_2S can react with the more electrophilic moiety of the fluorescent probe to form an intermediate containing free mercapto (–SH). If another electrophilic site is present at a suitable position, such as an *ortho*-ester group or an α,β-unsaturated acrylate group, the –SH group can undergo Michael addition (Fig. 5a) or a spontaneous cyclization (Fig. 5b) to trigger the fluorescent switch turn-on. The fluorescent probes based on these strategies, as shown in Fig. 5a and b, can also react with other biothiols such as Cys and GSH. However, the intermediates cannot continue to the next cyclization reaction. Therefore, the fluorescent signal does not suffer from the interference caused by other biothiols. As a strong nucleophile, H_2S also can interrupt the π-conjugation of the probe, thereby leading to a change in the emission wavelength of the probe (Fig. 5c). The removal of the strong electron-withdrawing group by H_2S can release the fluorophore (Fig. 5d); this strategy is expected to give turn-on fluorescent probes.

Fluorescent probes based on Michael addition reaction

Qian *et al.* reported two H_2S-selective fluorescent probes **39** and **40**.[46] Probe **39** employed BODIPY as the fluorophore, and probe **40** exploited 1,3,5-triaryl-2-pyrazoline as the fluorophore. The H_2S recognition moiety consisted of an aromatic framework which was substituted by an α,β-unsaturated acrylate methyl ester and *ortho*-aldehyde (–CHO). The aldehyde group could reversibly react with H_2S to form a hemithioacetal intermediate, which was suitable for Michael addition to the proximal acrylate to yield thioacetal (Fig. 5a). This tandem reaction could block the PET process, and the probe showed a turn-on response to H_2S. Probes **39** and **40** to H_2S gave Φ = 0.208 and 0.058, and these probes could detect H_2S in HeLa cells.

Li *et al.* reported an ICT-based turn-on fluorescent probe **41** for H_2S detection.[47] The aryl ring substituted by an *ortho*-aldehyde and an α,β-unsaturated acrylate methyl ester was conjugated to the BODIPY fluorophore *via* styrene. The quantum yield increased from 0.006 to 0.13. The probe could detect H_2S in RAW 264.7 macrophage cells, and the detection limit was 2.5 μM.

Zhao *et al.* reported a BODIPY-based probe **42** for H_2S detection.[48] The probe was designed by replacing the *ortho*-acrylate ester with an α,β-unsaturated phenyl ketone. The probe was able to respond to sulfide in bovine plasma, and the reaction was completed within 120 s at room temperature. **42** gave Φ = 0.10. This fast-response probe suggested that the average sulfide concentration in the blood plasma of four mice was 56.0 ± 2.5 μM. The average sulfide concentration in four C57BL6/J mouse brain tissues was estimated to be 7.1 ± 1.4 μmol g^{-1} protein.

Xian *et al.* reported a fluorescent probe **43** for H_2S detection.[49] The design strategy was based on a Michael addition of

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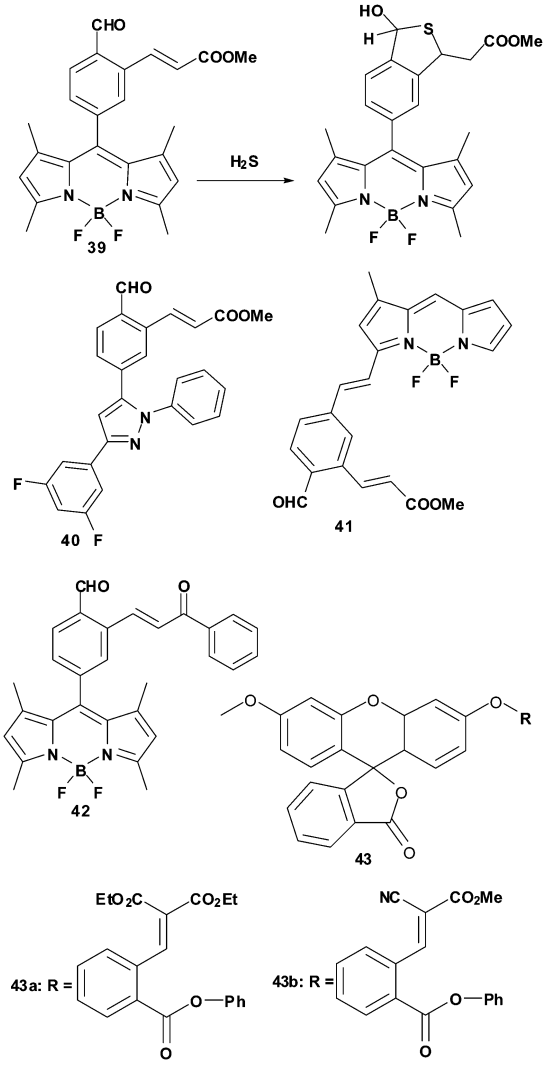
H_2S followed by an intramolecular cyclization to release the fluorophore. Because biothiols could react readily with Michael acceptors at physiological pH in a rapid equilibrium process, monosubstituted biothiols would not consume the probes in reversible reactions. Probe **43a** and **43b** led to 11-fold and 160-fold turn-on responses, respectively. The probe **43b** could detect H_2S in COS7 cells, and the detection limit was found to be 1 μM.



Fluorescent probes based on dual nucleophilic reactions

Xian *et al.* designed a series of turn-on probes (**44**, **45**) to detect H_2S based on the dual nucleophilic property.[50,51] These fluorescent probes contained reactive disulfide groups. H_2S could react with the disulfide group to give a free –SH containing intermediate; the intermediate next underwent a spontaneous cyclization to release the fluorophore. Other monosubstituted biothiols, such as Cys and GSH, did not lead to interference. The probe **44** could be used to detect H_2S in bovine plasma COS7 cells. The fluorescence quantum yield increased from 0.003 to 0.392. Xian *et al.* expanded this strategy to prepare and test the probes **45a–e**. The fluorophores, methoxy fluorescein, 7-hydroxycoumarin, resorufin, and 2-methyl TokyoGreen were chosen as the fluorescent signal transducers. **45d** and **45e** could be used to detect the production of H_2S from the H_2S donor YZ-4-074 in HeLa cells. The intensities increased 130-, 275-, 68-, 20-, and 60-fold for **45a–e**, respectively, and the detection limits were determined to be 60, 79, 47, 266, and 47 nm for **45a–e**.

Qian *et al.* reported a ratiometric fluorescent probe **46** based on the excited state intramolecular proton transfer (ESIPT) mechanism for H_2S detection.[52] The fluorophore 2-(2′-hydroxyphenyl) benzothiazole exhibited ratiometric detection capability, following a large Stokes shift. The recognition reaction completed within 2 min. The probe showed a 30-fold fluorescence increase, and could be used to detect H_2S in HeLa cells. The detection limit was 0.12 μM.

Guo *et al.* reported a methylfluorescein-based probe **47** for the detection of H_2S.[53] 2-(iodomethyl) benzoate was chosen as the H_2S trapping group. Upon exposure to H_2S, the H_2S-induced substitution–cyclization reaction took place smoothly to release the fluorophore. **47** showed $\Phi = 0.379$, and the probe could detect H_2S in COS-7 cells. The detection limit was 0.10 μM.

Tang *et al.* reported a near-infrared ratiometric fluorescent probe **48** for the detection of H_2S.[54] 2-Carboxybenzaldehyde was selected as the H_2S sensing group, with the aldehyde and the ester at the dual nucleophilic addition positions. After reaction with H_2S, the released fluorophore cyanine benefited from tautomerism between the enol and the ketone forms to allow ratiometric detection. There was a 2500-fold increase in ratio response. The probe could target mitochondria and detect the H_2S in HepG2 and A549 cells; the detection limit was 5.0–10 nM.

Fluorescent probes based on a double bond addition reaction

He *et al.* designed a ratiometric fluorescent probe **49** for H_2S detection.[55] Probe **49** could be considered as a hybrid fluorophore of coumarin and merocyanine through an ethylene group. The probes benefited from the fast HS^- nucleophilic addition to the merocyanine moiety in a medium of nearly neutral pH. After HS^-, the main form of H_2S under physiological conditions, added to the indolenium C-2 atom of **49**, the π-conjugation of the probe was disturbed, eliminating the emission of merocyanine but retaining that of coumarin, causing a shift in the fluorescence spectrum. The probe showed rapid responses to the changes of H_2S concentration in solution and cells, which were completed within 30 and 80 s, respectively. The intensity ratio of **49** increased over 120-fold. This probe was applied for the detection of changes in the level of H_2S in the mitochondria of MCF-7 cells.

Guo *et al.* reported a ratiometric fluorescent probe **50** based on a flavylium derivative and a commercially available pyronine dye **51** for H_2S detection.[56] Probe **50** gave a turn-off response due to the interruption of the π-conjugation of the pyronine ring. The probe could detect H_2S based on the selective nucleophilic attack of H_2S on the electrically positive benzopyrylium moiety, which would interrupt the π-conjugation, thereby leading to changes in the emission profile. Probe **50** could provide a ratiometric fluorescent response within 10 s. There

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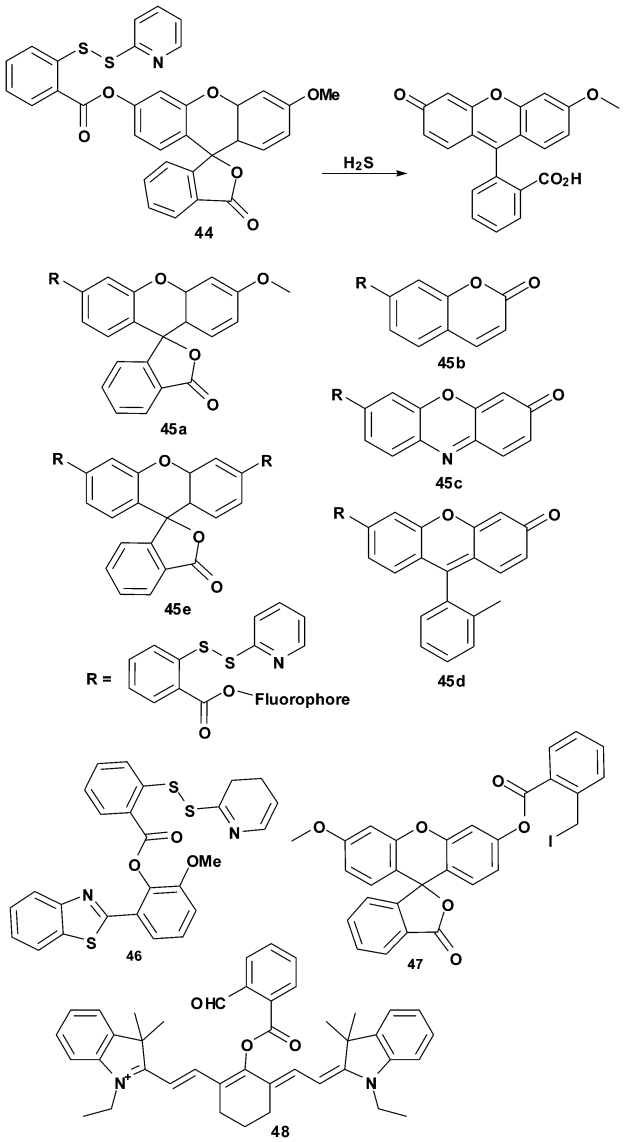
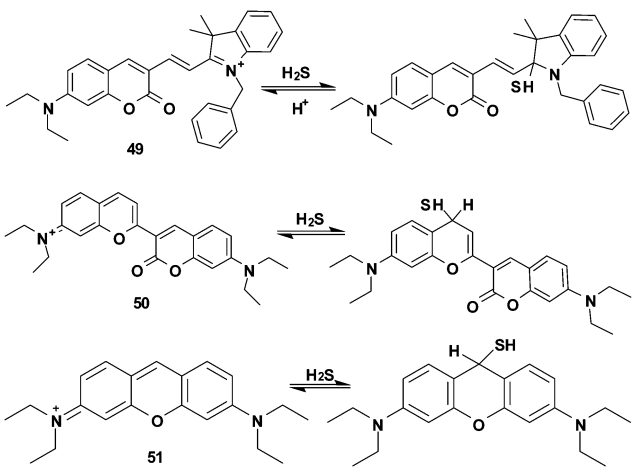


was a 1200-fold increase in the ratiometric value. Probe **50** could be used to detect H_2S in HeLa cells and in human serum; the detection limit was 0.14 μM.



Fluorescent probes based on a thiolysis reaction

Lin *et al.* synthesized a near-infrared fluorescent probe **52** based on a thiolysis reaction for H_2S detection.[57] The dinitrophenyl group has often been used to protect tyrosine in peptide synthesis. Thiols could remove the dinitrophenyl group under basic conditions. The probe was prepared through condensation of the BODIPY with a Fisher aldehyde, and then caged by 1-fluoro-2,4-dinitrobenzene. The probe is non-fluorescent due to the d-PET process from the excited dye to the strong electron-withdrawing group. After reaction with H_2S, the fluorophore was released with an 18-fold increase in fluorescence. This probe was used to detect H_2S in bovine serum and MCF-7 cells, and the detection limit was 5×10^{-8} M.

Xu *et al.* reported a lysosome-targeting fluorescent probe **53** for H_2S detection.[58] The probe was prepared by introducing a dinitrophenyl ether group into the 4-position of 1,8-naphthalimide, and 4-(2-aminoethyl)morpholine into the *N*-imide position as the lysosome-targeting group. The lysosome-targeting probe is of significance for the study of the distribution and function of H_2S in lysosomes of living cells. The fluorescence intensity of **53** increased 42-fold. The probe could be used to detect H_2S in the lysosomes of MCF-7 cells. The detection limit was 0.48 μM. Xu *et al.* also developed a 1,8-naphthalimide-derived probe **54** as a two-photon fluorescent probe for H_2S detection based on the thiolysis of dinitrophenyl ether.[59] The fluorescence intensity of **54** increased 37-fold. The probe **54** was applied to detect H_2S in bovine serum and MCF-7 cells, and the detection limit was 0.18 μM.

Feng *et al.* reported a 3-hydroxyflavone-based ESIPT probe **55** for H_2S detection.[60] The emission intensity increased 660-fold when detecting H_2S. The probe could be used to detect H_2S in biological serum and in simulated wastewater samples. The detection limit was 0.10 μM.

Yi *et al.* reported fluorescent probes **56a** and **56b** for H_2S detection, which employed fluorescein and naphthofluorescein as fluorophores.[61] Based on the thiolysis of (7-nitro-1,2,3-benzoxadiazole) ether, the probes could release the fluorophores and give a turn-on response to H_2S. The fluorescence intensity of **56a** and **56b** increased 77-fold and 1000-fold, respectively. The detection limit of **56b** was determined to be 16 μM in solution.

Fluorescent probes based on copper sulfide precipitation

After forming stable metal complexes with Cu(II), organic chelators have an efficacious quenching effect on fluorophores due to the fact that the paramagnetic Cu(II) center can accept the excited state electrons of the fluorophores. It is expected that the removal of Cu^{2+} from the complex will result in fluorescence recovery. Such fluorescent probes are often assembled in a fluorophore–chelator–metal ion manner (Fig. 6). According to the hard and soft acids and bases (HSAB) theory, S^{2-} has a strong affinity for Cu(II). The CuS precipitate is relatively stable, with $K_{sp} = 1.26 \times 10^{-36}$. After the addition of H_2S in solution,

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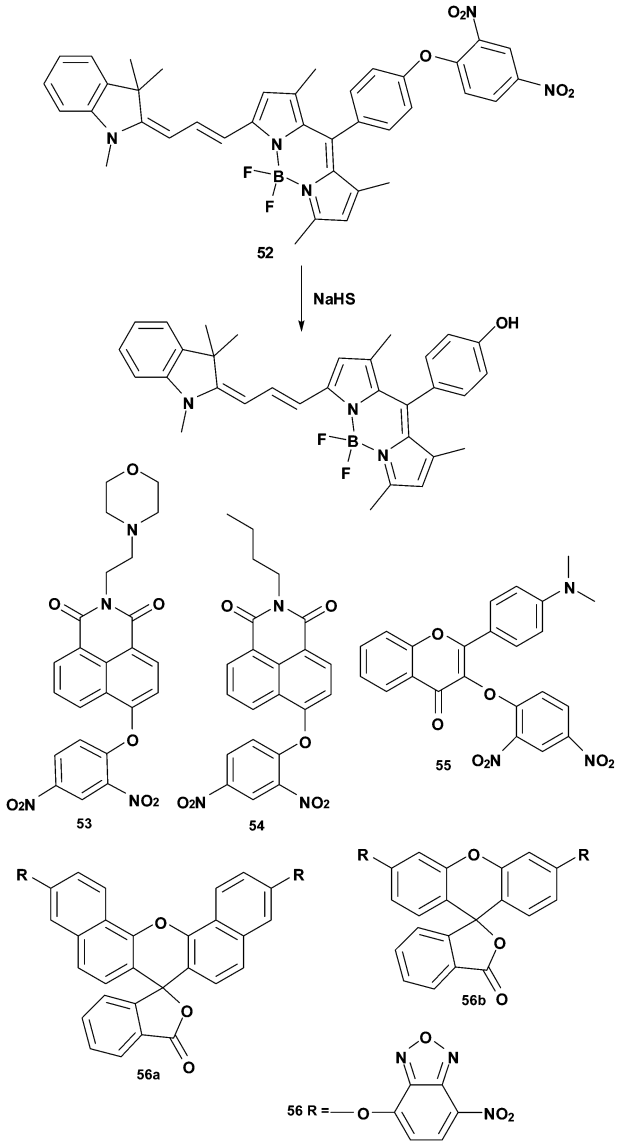

Cu(II) will be eliminated from the complex, and the corresponding fluorescent probes will again become emissive.

Chang *et al.* developed a fluorescent probe **57** for the selective sensing of S^{2-}.[62] The probe was designed based on a Cu^{2+} complex of fluorescein containing a dipicolylamine chelator. The detection limit was 420 nM in aqueous solution.

Li *et al.* reported a conjugated polymer fluorescent probe **58** based on a disubstituted polyacetylene containing a dipicolylamine chelator in the side chains for the detection of Cu^{2+} and S^{2-}.[63] The fluorescence of the probe could be quenched by Cu^{2+}. Based on the displacement strategy using S^{2-} to remove Cu^{2+}, the quenched fluorescence of the probe could recover. The detection limit was 5.0×10^{-7} mol L^{-1}.

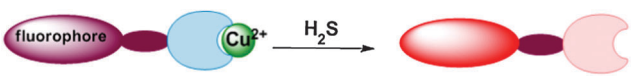

Fig. 6 Summary of the design strategy for fluorescent probes based on copper sulfide precipitation.

Lin *et al.* reported a near-infrared fluorescent probe **59** for H_2S detection.[64] The probe was composed of a cyanine dye, a piperazine linker and an 8-aminoquinoline ligand. After removal of Cu^{2+} by H_2S, the probe gave a turn-on response. The probe **59** showed Φ = 0.11, and the detection limit was 280 nM.

Nagano synthesized a series of azamacrocyclic Cu^{2+}-complex fluorescent probes **60a–d** for H_2S detection.[65] The probe **60b** exhibited high sensitivity and selectivity in the detection of H_2S, and the recognition reaction finished within seconds. It showed a 50-fold fluorescence increase upon the addition of H_2S. Probe **60b** could be used to detect H_2S produced by 3-mercaptopyruvate sulfurtransferase (3-MST), pseudoenzymatic H_2S release, and intracellular H_2S in HeLa cells.

Bai *et al.* reported an 8-hydroxyquinoline-appended fluorescein derivative **61** for H_2S detection.[66] The fluorescence of the probe was quenched by Cu^{2+}, which resulted in an off–on type probe for S^{2-} detection with a 5-fold increase in the fluorescence intensity. The probe was able to reversibly indicate S^{2-} and Cu^{2+} changes in turn, and could detect H_2S in HeLa cells.

Li *et al.* reported a water-soluble fluorescent probe **62** based on a 1,1′-bi-2-naphthol derivative for H_2S detection.[67] The metal ligand of the probe was 1,4,7,10-tetraazacyclododecane. This probe could recognize Cu^{2+} and S^{2-} in an on–off–on mode. The detection limit for the determination of S^{2-} was 1.6×10^{-5} M.

Shen *et al.* designed a fluorescent probe based on phenanthrene-fused dipyrromethene analogue **63** for H_2S detection.[68] Upon treatment with H_2S, the probe showed a 14-fold fluorescence increase. The probe could be used as a turn-on fluorescent probe for detecting H_2S in HeLa cells.

Zang *et al.* designed a water-soluble fluorescent probe for H_2S detection based on a 3-(1*H*-benzimidazol-2-yl)-2-hydroxybenzoate sodium derivative, **64**.[69] The probe was capable of the detection of Cu^{2+} and S^{2-} ions at physiological pH. The displacement mechanism the probe employed was supported by the fluorescence lifetime data. Free probe **64** exhibited Φ = 0.1063. The detection limitation of S^{2-} was determined to be 2.51×10^{-6} M.

Long *et al.* developed a fluorescent probe **65** for the detection of H_2S based on the displacement method.[70] The released compound **65** gave a 20-fold fluorescence intensity increase. The probe could be used to detect H_2S in MCF-7 cells. The detection limit was 0.18 μM.

Zhu *et al.* developed a colorimetric probe **66** based on boron-dipyrromethene-Cu^{2+} for the detection of H_2S in aqueous media.[71] The probe displayed a 50 nm red-shift in absorption upon the addition of H_2S in solution. The ratio of the absorbance showed a 34-fold ratiometric increase. The detection limit was 1.67×10^{-7} M.

Tang *et al.* reported the 2-(2′-aminophenyl)benzimidazole derivative fluorescent probe **67** for recognition of Cu^{2+} and S^{2-} in aqueous solution.[72] The probe displayed an excited-state intramolecular proton transfer (ESIPT) mechanism, and the detection limit was 9.12×10^{-7} M.

Ramesh *et al.* synthesized a near-infrared ratiometric indole functionalized rhodamine derivative probe **68** for the detection

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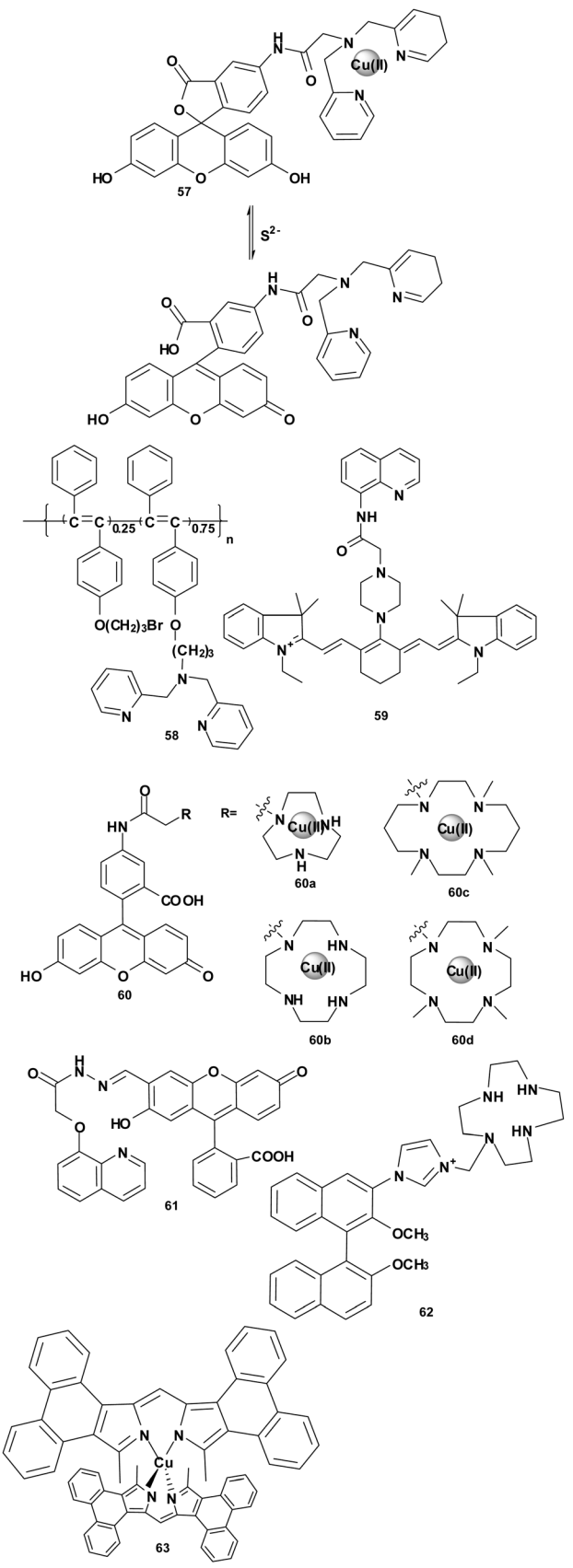

of Cu^{2+} and S^{2-}.[73] The probe employed the resonance energy transfer (RET) mechanism for the detection of Cu^{2+}, the process of which involved a donor indole and an acceptor Cu^{2+} bound rhodamine moiety. The Cu^{2+} complex gave an on–off response in the presence of S^{2-}. The Cu-complex probe **68** showed a 600-fold decrease of fluorescence intensity upon the addition of S^{2-}. The probe could detect Cu^{2+} and S^{2-} in HeLa cells.

Fluorescent probes for sulfane sulfurs

Sulfane sulfurs are the uncharged form of sulfur (S^0), and are attached to proteins through a covalent bond between the S^0 atom and other sulfur atoms, such as elemental sulfur (S_8), persulfides (R–S–SH), polysulfides (R–S–S_n–S–R), thiosulfate ($S_2O_3^{2-}$), polythionates ($^-SO_3$–S_n–SO_3^-), disulfides and so on.[74–76] As members of the family of reactive sulfur species, sulfane sulfurs exhibit important physiological functions including cellular signal transduction and physiological regulation. The emerging evidence suggests that sulfane sulfurs may be the real signal transduction molecules for cellular events. The rapid production and clearance of H_2S in several biochemical pathways also depends on the metabolic process of sulfane sulfurs.

Xian *et al.* reported fluorescent probes **69**, **70** for the detection of sulfane sulfurs.[77] The detection mechanism was similar to that of probe **44**, which is also illustrated in Fig. 5b. Sulfane sulfur compounds are reactive and labile, and there often exist thiosulfoxide tautomers. Therefore, a sulfur atom of a sulfane sulfur could react with nucleophilic groups such as the mercapto group to produce a reactive intermediate, which immediately underwent an intermolecular cyclization reaction to release the fluorophore. The fluorescence intensity of both probes increased 50-fold and 25-fold upon the detection of sulfane sulfurs. Probe **69** was used to detect sulfane sulfide both in H9c2 and HeLa cells. The detection limits were 32 nM (for **69**) and 73 nM (for **70**).

Fluorescent probes for SO_2 and its derivatives

Sulfur dioxide (SO_2) has long been considered as an air pollutant. People who are exposed to SO_2 may suffer from respiratory diseases and cancer. SO_2 is rapidly hydrated to sulfite (SO_3^{2-}) and bisulfite (HSO_3^-) in neutral solutions (3 : 1 M/M). However, endogenous SO_2 can be produced from the degradation of sulfur-containing amino acids. SO_2, and its derivatives, may have physiological roles in the regulation of cardiovascular function in synergy with NO. Sulfite is also used in food as an antioxidant to prevent bacterial growth. The design strategies of fluorescent probes for SO_2 detection are mainly inspired by the nucleophilic properties of SO_2 (including SO_3^{2-} and HSO_3^-). The reaction mechanisms of these fluorescent probes can be sorted according to the nucleophilic addition to aldehydes/ketones (Fig. 7a) and the nucleophilic addition to double bonds (Fig. 7b). SO_2 and its derivatives selectively add to aldehydes/ketones or to double bonds, leading to changes in the electron-withdrawing effects

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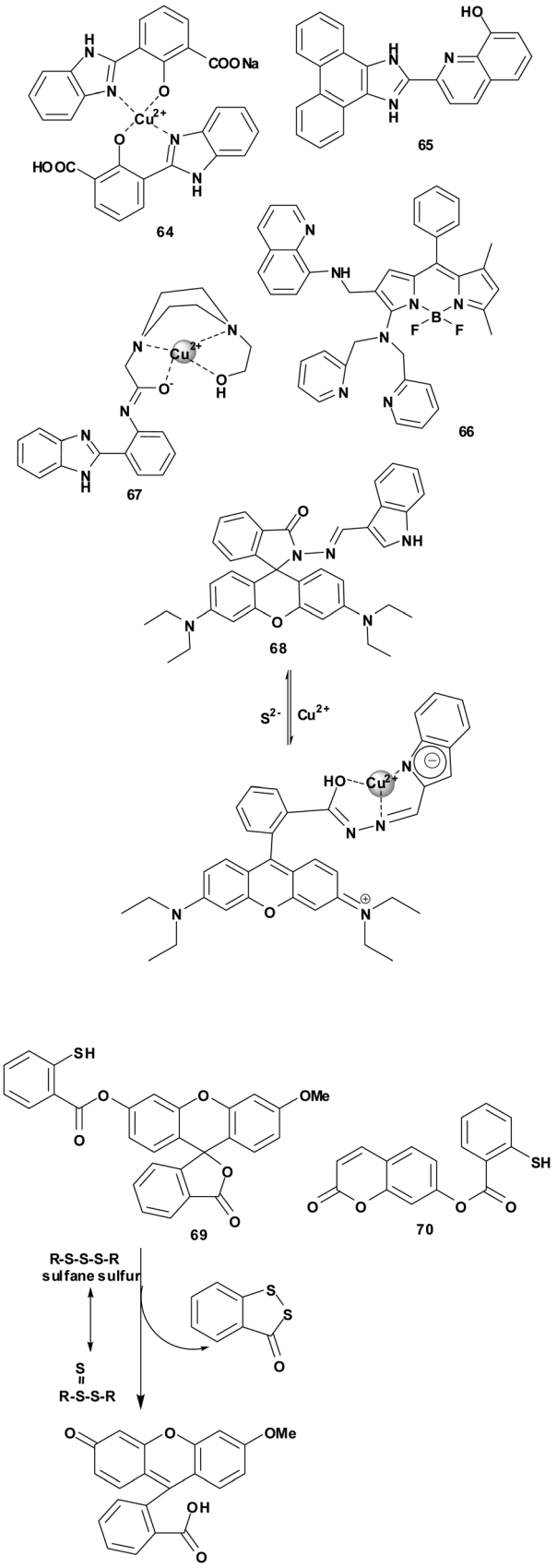

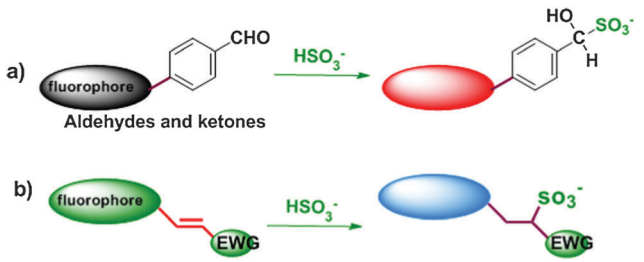

Fig. 7 Summary of strategies for fluorescent probes based on the nucleophilic properties of SO_2 and its derivatives.

or the π-conjugated systems of the probes, which can cause a response in terms of the fluorescence signal.

Fluorescent probes based on aldehyde/ketone addition

Chang *et al.* reported fluorescent probe **71** for sulfite detection.[78] Sulfite could selectively deprotect the resorufin levulinate moiety of the probe. When sulfite reacted with the carbonyl carbon of the levulinate, a sulfite-added tetrahedral intermediate was formed. The intermediate would next undergo an intermolecular cyclization reaction to release the fluorophore. The fluorescence increase was up to 57-fold. The probe was used to detect sulfite in aqueous solution. The detection limit was 4.9×10^{-5} M.

Guo *et al.* synthesized four coumarin-based fluorescent probes **72a–d** for the detection of bisulfite.[79] Bisulfite could selectively attack the aldehyde moieties of the probes. The quantum yields of products of **72a–d** were 0.33, 0.62, 0.52 and 0.26, respectively. These probes were used to detect sulfite in granulated sugar. The detection limit was 1.0×10^{-6} M.

Yang *et al.* developed a rhodamine-based fluorescent probe **73** for the detection of bisulfite.[80] Bisulfite could react with the aldehyde to form an aldehyde–bisulfite adduct. Probe **73** also changed from a spirolactam (nonfluorescent) to a ring-opened spirolactam structure, increasing the fluorescence emission. The probe could detect bisulfite in aqueous media. The detection limit was 8.9×10^{-7} M. These authors also presented a ratiometric fluorescent probe 4-(1*H*-benzimidazol-2-yl)benzaldehyde (**74**) for bisulfite detection.[81] The aldehyde moiety reacted with bisulfite to produce a bisulfite adduct, which resulted in different electron-withdrawing effects and triggered the ICT process. The probe could detect bisulfite in aqueous media. The detection limit was 0.4 μM.

Feng *et al.* developed a coumarin-based fluorescent probe **75** for the detection of bisulfite.[82] The nucleophilic addition reaction with aldehyde would switch-on the fluorescent probe for bisulfite, initiating the ICT process. The aldehyde–bisulfite adduct produced had a quantum yield of 0.43. The probe could detect bisulfite in solution and in HeLa cells. The detection limit was 3.0 μM.

Guo *et al.* designed a C=N isomerization-based fluorescent probe **76** for bisulfite detection.[83] The C=N isomerization could be inhibited by the intramolecular N–H···N=C hydrogen bond. The formation of the hydrogen bond would block the C=N rotations, and fix the molecular structures in place,

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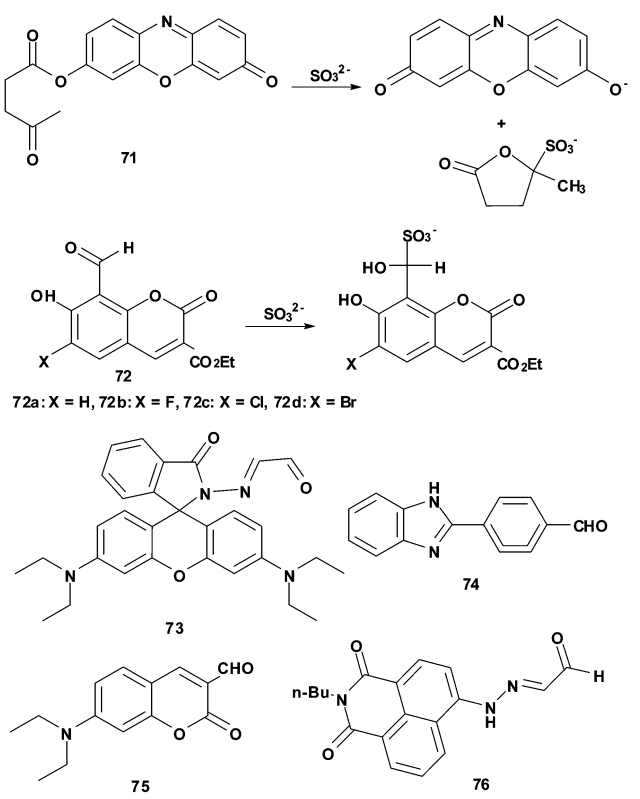


resulting in minimizing the nonradiative energy of the excited state. The quantum yield was 0.374. The probe could detect bisulfite in locally granulated sugar; the detection limit was 0.1 μM.

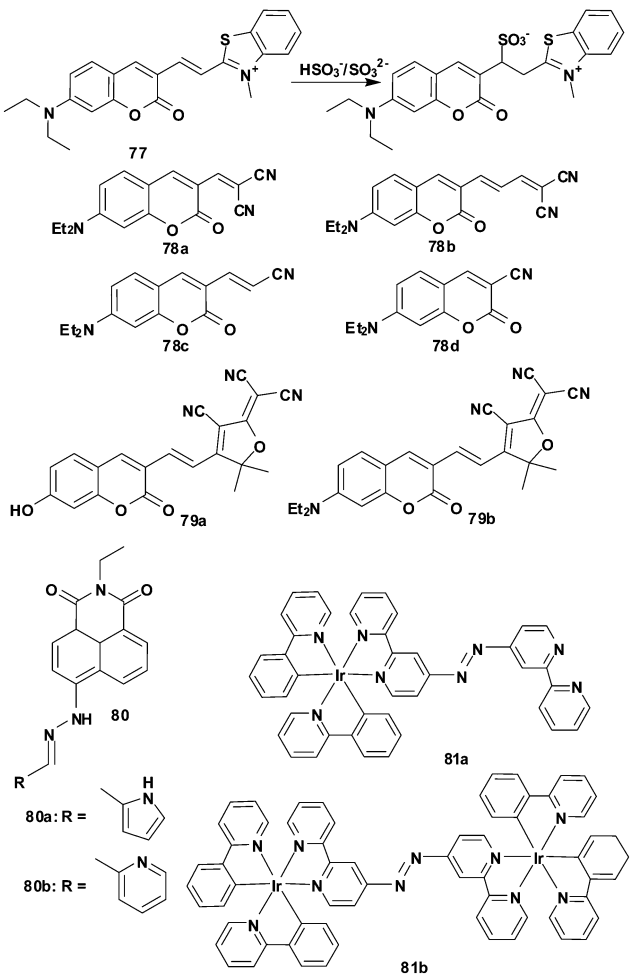
Fluorescent probes based on double bond addition

Guo *et al.* reported a coumarin–hemicyanine fluorescent probe **77** for bisulfite and sulfite detection.[84] The nucleophilic attack of SO_3^{2-}/HSO_3^- on the double bond interrupted the π-conjugated structure of probe **77**. As a result, the emission profiles before and after adding SO_3^{2-}/HSO_3^- shifted due to the distinct emissions of the coumarin–hemicyanine fluorophore and the produced coumarin fluorophore. The probe gave a 1110-fold increase in the ratiometric signal. The probe could be used to detect SO_3^{2-}/HSO_3^- in HeLa cells. The detection limit was 0.38 μM.

Li *et al.* reported colorimetric and ratiometric fluorescent probes **78a–d** for the detection of sulfite.[85] These probes were based on the fluorophore 7-diethylamine coumarin, which conjugated with the cyano group through double bonds. Probes **78a** and **b** provided the best responses to SO_3^{2-}. SO_3^{2-} underwent a Michael addition to the α,β-unsaturated double bond, which interrupted the intramolecular charge transfer (ICT) process of the probe. The probes could detect sulfite in solution. Probe **78a** had a 232-fold intensity ratio increase. The detection limit was 58 μM. The same group also presented near-infrared fluorescent probes **79a** and **b** for the colorimetric and ratiometric detection of SO_2 derivatives.[86] The probes were composed of coumarin fluorophores and 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran, which had three cyano groups. Probe **79a** possessed better water solubility and electron-withdrawing properties. The signal ratio at two different wavelengths could increase 775-fold within 90 seconds. The probe was used to detect SO_2 derivatives in U-2OS cells, and the detection limit was 0.27 nM.

Weng *et al.* reported fluorescent probes (**80a** and **b**) based on 4-hydrazinyl-1,8-naphthalimide for SO_3^{2-}/HSO_3^- detection.[87] The electron donating pyrrole moiety in probe **80a** was responsible for fluorescence quenching through the photoinduced electron transfer (PET) process. The SO_3^{2-}/HSO_3^- could form hydrogen bonds with the pyrrole moiety, and the PET process was blocked. Probe **80a** gave a 65-fold fluorescent increase upon the detection of sulfite. The probe could be used to monitor the SO_2 released from an SO_2 donor in real time and could detect SO_3^{2-}/HSO_3^- in GES-1 cells. The detection limit was 0.56 μM.

Chao *et al.* developed azo group-bridged dinuclear iridium(III) complexes **81a** and **b** as phosphorescent probes for the detection of SO_3^{2-}/HSO_3^-.[88] When the azo group was incorporated into ruthenium(II) complexes, it became more reactive and could react with SO_3^{2-}/HSO_3^-. Moreover, the azo group is an electron-withdrawing group which could quench the luminescence of luminescent metal complexes employing the metal-to-ligand charge-transfer (MLCT) mechanism. After the SO_2 derivatives underwent nucleophilic addition to the azo group, probe **81b** became luminescent. The phosphorescence responses



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showed a 26-fold and 27-fold increase for sulfite and bisulfite. Probe **81b** was able to detect external and endogenous SO_3^{2-}/HSO_3^- in HepG2 cells. The detection limits were 0.24 μM for SO_3^{2-} and 0.14 μM for HSO_3^-.

Conclusions and prospects

In this feature article, we have summarized the synthetic and design strategies in the development of reaction-based fluorescent probes which are classified by the reaction types between analytes (H_2S, sulfane sulfurs and SO_2 derivatives) and probes. According to the reaction types, the probes are illustrated through and explained by examples: (a) H_2S reductive reactions: reducing azides to give amines, reducing nitro/azanol to give amines, and reducing selenoxide to give selenide; (b) H_2S nucleophilic reactions: Michael addition reaction, dual nucleophilic reaction, double bond addition reaction, and thiolysis reaction; (c) copper sulfide precipitation reaction. The detection of sulfane sulfurs is mainly based on the nucleophilic addition reaction, and the detection of SO_2 derivatives is based on the nucleophilic and reductive properties of SO_2 derivatives.

Rapid recent developments in fluorescent probes for reactive sulfur species will likely prove to further facilitate analysis by fluorescence bioimaging technology. Compared with traditional methods, the detection of reactive sulfur species *via* fluorescence spectrometry can lower the external influence on the endogenous species distribution, can reduce the time of sample preparation, and can achieve real-time detection, in line with the high reactivity of these species. Despite the fact that reaction-based probes deliver us unique and versatile approaches for examining a wide range of reactive species in chemical and biological systems, there also exist many obstacles in terms of new reaction types, selectivity, sensitivity, response time, and fundamental applications. Although the reported detection limits are down to micromolar or even nanomolar levels, fluorescent probes for the detection of original reactive sulfur species and their distribution under normal physiological conditions are very rare. The distribution of reactive sulfur species in cells can be elucidated by probes which have targeting functions, and the quantitative analysis of reactive sulfur species in cells can be resolved by flow cytometry. For example, endogenous H_2S variation is always restricted to a shape range within a short time; we term it as the "H_2S spark" because this is the spontaneous and spatio-temporally localizated nature of H_2S biological release. Therefore, to design a probe which can sense the "H_2S spark" will be very meaningful. A similar situation also occurs with other reactive small molecules such as NO, H_2O_2, CO, NH_3, SO_2, and sulfane sulfurs.

Organisms possess a complex environment; the generation and decay of bioreactive species involves various stages. It is desirable to exploit distinctive design schemes that involve reversible chemoselective reactions and catalytic processes which can simulate enzymes. The reversible strategies suffer from many challenges, such as photostability, perturbation of target molecules, cell leakage, and response frequency. Along these lines, the launch of multiresponse probes for bioimaging detection is also expected, because the reactive small molecules show signaling crosstalk during physiological and pathological processes. Compared with single analyte probes, multiresponse probes will provide facilities for the improvement of diagnostic and therapy tools, and offer important future directions for biological events.

Above all, the development of perfect probes is a challenging task. It requires to master basic concepts and strategies. The development of probes should address key biological problems. Future reaction-based design principles must pay attention to endogenous reactive small molecules under normal physiological conditions. Regardless of the position of the probes in the spectrum (visible region or near-infrared range), desirable probes should respond well to a minor concentration change, give dependable results, and meanwhile avoid interference from native cellular species, particularly biomolecular species such as glutathione and cysteine, and so on. All these will require the probes to exhibit good selectivity, high sensitivity, good photostability, low cytotoxicity, suitable water solubility, and the ability to work within the physiological pH range. Real applications of fluorescent probes, especially in clinical diagnostic imaging, is the ultimate goal.

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