Small-Molecule Fluorescent Probes for Imaging and Detection of Reactive Oxygen, Nitrogen, and Sulfur Species in Biological Systems

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Intracellular redox homeostasis provides broad implications in physiological and pathological fields. The disruption of redox...
homeostasis is closely associated with some human diseases, such as cancer, neurodegenerative diseases, cardiovascular diseases, diabetes mellitus, and gastrointestinal diseases. Therefore, cells possess an elaborate regulation system to maintain their redox balance and the large or significant redox state changes can be buffered by the redox-active molecules. These molecules experience inter-reaction and interconversion to facilitate the dynamic balance of intracellular redox state, among which three types of representative molecules should be mentioned, including reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS).

ROS are produced intracellularly through multiple mechanisms during the metabolism process. Also, ROS mainly include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (O$_2^+$), hydroxyl radical (OH$^-$), lipid peroxy radical (ROO$^-$), ozone (O$_3$), hypochlorous acid/hypochlorite (HOCl/ClO$^-$), and hypobromous acid/hypobromite (HOBr/BrO$^-$). Since the oxidation state of oxygen in ROS ranges from 2 to 0, ROS are highly reactive and are considered as one of the key mechanisms in the redox signaling network. Therefore, the development of fluorescent probes for ROS, RNS, and RSS to elucidate their cross-talking in cells, tissues, and organisms is a crucial aspect of research in the field of redox biology.

Figure 2. Structures of fluorescent probes for H_2O_2.
regulatory molecules for cells. ROS cannot only eliminate undesirable xenobiotics but also have great effects on cell signaling, which is vital for the regulation of physiological and pathological processes. RNS refer to NO and NO-related molecules with nitrogen oxidation state ranging from 1+ to 4+, such as peroxynitrite (ONOO−), nitrogen dioxide (NO2), S-nitrosothiols (RSNO), and nitroxyl (HNO).10 RNS are able to react with a wide spectrum of biomolecules, including proteins, nucleic acid, and lipids, and may act as transporters of NO in the cell signaling events.11,12 RSS is defined as “redox-active sulfur-containing molecules that are able, under physiological conditions, to either oxidize or reduce biomolecules.”13 RSS mainly include biothiols (GSH, Cys, Hcy), H2S, thyl radical (RS*), disulfide (RSSR), sulfenic acid (RSOH), sulfur dioxide/sulfite/bisulfite (SO2/SO32−/HSO3−), persulfides (R−S−SH/H2S2), and polysulfides (RnSn/HnS2n n > 2). Sulfur in RSS possesses oxidation state ranging from 2− to 4+, endowing RSS with variable reactivity so that many redox reactions are mediated by RSS. In recent years, RSS biology has become an increasing focus of research.15–16

A growing body of research now shows that the multiple signaling transduction pathways are involved in the intracellular redox maintenance, which covers the active participation of ROS, RNS, and RSS (RONSS, for short).14,17 The cross-talking between RONSS plays a very important role in the physiological and pathological processes.14,17 The deep understanding or elucidation of the interaction among RONSS and its corresponding regulation effect on redox balance open up new area of research as well as new challenges in cell biology.

As the short lifetime, high reactivity, and transient conversion characters of RONSS, efficient analytical tool that can in situ monitor the generation, distribution, accumulation, and dynamic fluctuation of RONSS in biological systems can provide important information for their biofunction study. In recent years, because of the real-time, sensitive, and noninvasive characteristics, fluorescence imaging utilizing small-molecule fluorescent probes has become a powerful analytical tool to visualize the fluctuation and distribution of biomolecules.18–20 Quite a number of fluorescent probes specific for RONSS have been designed and proposed based on different natures of RONSS, such as nucleophilic, electrophilic, oxidation, and reduction, etc (Figure 1).15–18–20 Gratifyingly, some mechanisms and principles relating to the redox biochemistry have been revealed by these probes and researchers can understand more about the RONSS biofunctions than ever.

To display the recent progress in fluorescence imaging of RONSS, in this review, we aim to summarize the representative small-molecule fluorescent probes for RONSS in the last 2 years. The probes from our own group are also included. We mainly present the design, recognition, and response mechanisms of the probes. The probes which have not been applied in biological systems are not included.

**Fluorescent Probes for H2O2**

H2O2 is one of the most important ROS, which is mainly generated from mitochondria by the activation of NADPH oxidase complex. As a second messenger, H2O2 is tightly linked to diverse cellular processes.22–25 Whether it serves beneficial or harmful roles is closely associated with its distribution and concentration. In the last 2 years, a variety of fluorescent probes have been developed for detecting H2O2 in cells and animals. Collectively, four sensing strategies have been used, including the reaction of aryloboronates or phenylboronic acid to phenols,24–33 the conversion of oxonium or diketone to acid,34,35 oxidation of phenol to quinone,36 and carbon–carbon double bond cleavage.37

**Probes based on the reaction of aryloboronates or phenylboronic acid to phenols.** The most commonly used sensing moiety for H2O2 is aryloboronic acid, which has been accommodated in many fluorophores to obtain various probes. A mitochondria-targetable fluorescent probe 1 with two imaging channels for viscosity and H2O2 was proposed.28 Besides the boronic for H2O2, the skeleton of 1 (Figure 2) is composed of N,N-dimethylamino as an electron donor, pyridinium cation as a mitochondrial targeting group and an electron acceptor, as well as double bond and benzene as the connector. Fluorescence imaging showed that 1 was highly selective and could monitor the level of H2O2 and viscosity changes in intracellular mitochondria.

To study the synergetic variation of H2O2 in different organelles, probes 2 and 3 (Figure 2) were developed for mitochondria and endoplasmic reticulum (ER) localization, respectively.25 The merocyanine core of 2 and 1,8-naphthalimide fluorophore of 3 emit distinguishable fluorescence. By the dual-imaging of 2 and 3, we can see that during mitochondria-oriented apoptosis, mitochondria H2O2 levels continuously increased first, whereas the ER H2O2 levels rose subsequently after a delay. However, during ER-oriented apoptosis, ER is the major site for overproduction of H2O2, and delayed elevation of the H2O2 levels was found in the mitochondria.

The pH-switchable fluorophores with different pKₐ were used to construct probes for H2O2 that can be lighted on within certain acidity ranges. Probes 426 and 527 were developed for H2O2 under acidic and alkaline conditions, respectively. Probe 4 (Figure 2) possesses a spirobienzopyran fluorophore emitting strongly at lower pH, whereas 5 (Figure 2) has a Changsha near-infrared dye derivative giving ratiometric signal at higher pH. Both of these two probes showed high specificity to H2O2 and were used for live cell imaging. This type of probe provides a new strategy for the spatially confined imaging in certain cell regions.

Speed up the recognition reaction of aryloboronates-based probes to H2O2 is vital since H2O2 is highly reactive and diffuses rapidly in cells. Therefore, several fast responsive probes were designed to improve the temporal resolution for H2O2 detection. p-Pinacolboronylbenzyl was grafted on a quaternized quinoline unit which was masked on a carbazole group to compose a fluorescent probe 628 (Figure 2). Following the 1,6-rearrangement-elimination by H2O2, the photoinduced electron transfer (PET) was inhibited and the fluorescence of 6 was turned on within 5 min. On the basis of the push–pull molecular frame and fluorescence turn on mechanism of ICT (intramolecular charge transfer), the Wang group29 proposed a three-component fluorescent probe 7 (Figure 2) for H2O2 in Hela cells and in vivo. One highlight of 7 is its relatively short response time to H2O2 within 8 min because of the acceleration of nucleophilic reactions by benzonitrile. Another fast-responsive probe 8 was synthesized by Lin et al.30 Probe 8 (Figure 2) has a simple structure with 1,8-naphthalimide fluorophore and lysosome-targeting morpholine moiety. Probe 8 exhibited a dynamic response of ~2 min toward H2O2. The fourth H2O2-rapid-reaction probe 9 was based on a 4-hydroxybenzochalcone fluorophore.31 Probe 9 (Figure 2) reacts with H2O2 in ~3.5 min and can be used in tissue imaging.

The environmental sensitization for H2O2 detection was also performed. Environmental sensitization means that the fluorescence of the product of a H2O2-recognizable reaction

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can be dramatically enhanced in the presence of some specific environmental conditions or coexisting substances. A H$_2$O$_2$ specific and DNA-binding fluorescent probe 10 (Figure 2) was designed. Reaction with H$_2$O$_2$ induces 10 to release a fluorescent quinone-cyanine derivative with a one-donor-two-acceptor (D2A) structure which could switch-on NIR fluorescence.

Figure 3. Structures of fluorescent probes for O$_2^*$, $^1$O$_2$, $^•$OH, and total ROS.
fluorescence strongly through binding to the DNA minor groove. Bioimaging experiments demonstrated that probe 10 could detect and quantify H₂O₂, produced by EGF/Nox pathways and postmenopausal stress in both primary and senescent cells. Probe 11 (Figure 2) derived from aggregation-induced emission (AIE) was proposed for H₂O₂ in the serum sample.³³ Probe 11 was integrated with tetrphenylethylene (TPE) as the core structure, the phenylboronic ester as the reaction site and the imine group as the emission mediator. The product from the reaction between 11 and H₂O₂ is polar sensitive and gives strong fluorescence in solution with water contents above 90%. Since H₂O₂ is quantitatively generated from the D-glucose rearrangement reaction, a NIR (near-infrared) fluorescent probe 12 (Figure 2) with an oxonium group in its benzopyrylum-coumarin skeleton was developed.³⁴ Oxonium can be nucleophilically attacked by H₂O₂ to release the coumarin part, initiating a ratiometric response and large emission shift. Two-photon imaging by 12 realized the detection of exogenous and endogenous H₂O₂ in the living cells as well as in zebrafish.

α-Ketoamide moiety can be specifically switched to amine by H₂O₂ in aqueous solution. This reaction was utilized to design a new NIR fluorescent probe 13 (Figure 2), which was constructed by integrating α-ketoamide with hemicyanine fluorophore.³⁵ ICT mechanism was utilized to modulate the fluorescence change. It was proved that H₂O₂ was overgenerated during ischemia-reperfusion injury at both cell and organ levels by using 13.

Probes Based on the Conversion of Oxonium or Diketone to Acid. On the basis of the Baeyer–Villiger oxidative rearrangement reaction, a NIR (near-infrared) fluorescent probe 12 (Figure 2) with an oxonium group in its benzopyrylum-coumarin skeleton was developed.³⁴ Oxonium can be nucleophilically attacked by H₂O₂ to release the coumarin part, initiating a ratiometric response and large emission shift. Two-photon imaging by 12 realized the detection of exogenous and endogenous H₂O₂ in the living cells as well as in zebrafish.

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Probes Based on Oxidation of Phenol to Quinone. Probe 14 (Figure 2) with naphthalimide fluorophore and catechol sensing moiety was fabricated.³⁶ The oxidation of H₂O₂ converted the catechol into α-quinone, inhibiting the PET from the catechol moiety to the naphthalimide core to turn on fluorescence. Cell imaging confirmed that 14 could monitor endogenous H₂O₂ in lysosomes as well as in brain tissues and in living nematodes.

Probes Based on Carbon–Carbon Double Bond Cleavage. Utilizing the H₂O₂-Promoted carbon–carbon double bond cleavage, probe 15 (Figure 2) with enamine group as the responsive moiety was fabricated.³⁷ Upon reaction with H₂O₂, a peroxide intermediate was formed, which then was transformed to stable amide, accompanying with blue fluorescence emission. Probe 15 was used to image H₂O₂ in cells, mouse liver slice, and tumor tissues.

Fluorescent Probes for O₂**. O₂** is the “primary” ROS in living systems since it can be served as a precursor for many ROS, including H₂O₂, OH, O₂, and ClO−.³⁸ In addition, O₂** acts as a mediator in oxidative chain reactions.³⁹ Overproduction of O₂** initiates oxidative stress and disruption of the redox homeostasis. Normally, redox mechanism (oxidation ability of O₂**-) and nonredox mechanism (nucleophilicity or other reactive nature of O₂**) are used to develop reaction-based fluorescence probes for O₂**.

Probes Based on Redox Mechanism. Utilizing the oxidation properties of O₂**, a two-photon fluorescent probe 16 for the detection and imaging of O₂** was developed (Figure 3). When the benzoiazolizine group grafting onto quinoline scaffold was specifically dehydrogenized by O₂**, fluorescence was turned on because of the extension of π-conjugations and the ICT process. Inspired by the satisfactory characteristics of 16, in situ visualization of endogenous O₂** in lung inflammation samples was achieved.⁴⁰

Using the same mechanism, another two-photon probe 17 (Figure 3), which was obtained by combining benzothiazoline to 1,8-naphthalimide with a endoplasmic reticulum-targeting benzisulfamide, was designed to image O₂** in diabetic mice.⁴¹ Two-photon fluorescence imaging showed that the concentration of O₂** in abdominal or hepatic tissue of diabetic mice is higher than that in normal mice, and metformin pretreatment can depress O₂** level.

Probe 18 comprising covalently linked caffeoyl, fluorescein, and triphenylphosphonium (Figure 3) was developed for O₂** and pH in mitochondria.⁴² Catechol moiety in caffeoyl could be readily oxidized by O₂** to α-quinone, giving fluorescence which is differential from fluorescence. With the assistance of 18, it was found that inhibition of Darnamin-related protein 1 (Drp1) resulted in a signal through mitochondrial complexes I and II to enhance the O₂** and pH levels. Eventually, mitochondrial damage and apoptosis in breast cancer cells happened.

Probes Based on Nonredox Mechanism. Several probes were constructed based on the nucleophilicity of O₂**. Two probes of 19 and 20 (Figure 3) were developed through the nucleophilic and subsequent deprotection of the diphenyl phosphate group by O₂**. 19 is NIR-emissive and cell-membrane permeable, and was applied to image O₂** in zebrafish and mouse liver.⁴³ Probe 20 is a pyridine modified TPE compound with two-channel fluorescence. When the diphenyl phosphate linked cresol-like structure was selectively fractured by O₂**, the pyridine modified TPE was released. The original fluorescence emitted by donor–acceptor structure was lost and new aggregation emitted green fluorescence emerged. Intracellular O₂** fluctuation during apoptosis and inflammation was tracked and monitored by 20.⁴⁴ O₂** also can nucleophilically attack trifluoromethanesulfonate group. Therefore, probe 21 (Figure 3) was proposed using a donor–acceptor (D–A) structured naphthalene derivative as a potent two-photon fluorophore.⁴⁵ The deprotection of trifluoromethanesulfonate by intracellular and tissue O₂** resulted in high fluorescence signal and deep imaging depth of ∼150 μm.

A fluorescence/chemiluminescence dual sensing platform 22 (Figure 3) for O₂** was designed by covalently linking TPE and imidazopyrazinone in the naphthalimide fluorophore.⁴⁶ The reaction between O₂** and imidazopyrazinone induces apparent increase in both fluorescence and chemiluminescence. Besides the application in imaging O₂** in Raw264.7 cells and inflamed mice, acetylated O₂** in HL-7702 cells was uninterruptedly monitored by 22 with chemiluminescence and the results were further confirmed by fluorescence imaging.

Fluorescent Probes for 1O₂. 1O₂ is a highly reactive oxygen species (ROS) that oxidizes a broad range of biomolecules.⁴⁷,⁴⁸ On the one hand, 1O₂ can facilitate the treatment of cancer by destroying tumor cells. On the other hand, 1O₂ can regulate the intracellular signaling pathways. As the short half-life (t½ = 10⁻⁶ ~ 10⁻⁷ s) of 1O₂, the fluorescence for 1O₂ needs a fast dynamic response.

The first two-photon fluorescent probe 23 (Figure 3) for imaging of 1O₂ in tissues was proposed by Zhang et al.⁴⁹ This mitochondrial-targeting probe 23 was designed based on the PET mechanism, integrating 9-methanthrace, triphenylphosphonium, and naphthalimide core. When the 9-methanthrace group acting as an electron acceptor trapped 1O₂ to inhibit the PET process, great fluorescence enhancement was observed. 1O₂ generation in mitochondria during the photodynamic therapy (PDT) was detected by 23.
Figure 4. Structures of fluorescent probes for HClO/ClO\(^{-}\) and HBrO/BrO\(^{-}\).
A ratiometric photoluminescence probe 24 (Figure 3) was developed by connecting an Ir(III) complex and a coumarin 314 derivative. The specific ratiometric signal was activated through the abstraction of the α-H from the tertiary amine by \( \text{O}_2 \). Probe 24 was used as a bioimaging agent to evaluate endogenously produced \( \text{O}_2 \) in cells.

**Fluorescent Probes for *OH.** As one of the most reactive and aggressive oxidizing agents, *OH plays important roles in immunity metabolism as well as in the environment. The excessive production of *OH might cause serious damage to many biomolecules. Fluorescent probes for *OH are exploited based on its oxidation ability.

Sulfur atom in phenothiazines conjugated with cyanine can be oxidized by *OH to yield sulfoxide. On the basis of this, a highly selective and mitochondria-targeting probe 25 was synthesized. Probe 25 (Figure 3) showed a ratiometric variation in absorbance and dramatic enhancement in fluorescence. Interference of other hROS, including ONOO\(^-\), ClO\(^-\), and \( \text{O}_2 \), was negligible. Probe 25 was used to detect endogenous *OH in living cells and various type of bacteria. Finally, the toxicity of TiO\(_2\)NPs with different diameter was investigated in zebra fish embryos. The results showed that there was an inverse relationship between the TiO\(_2\)NPs diameter and their toxicity.

A fluorescent probe 26 (Figure 3) for the detection of *OH was designed based on hydride carbazole-cyanine dyes. The oxidation of the pyrrolidine by *OH produced azole and turned on fluorescence. Probe 26 exhibited properties in terms of fast response, good selectivity, and low cytotoxicity. Probe 26 was successfully employed to monitor endogenous *OH in living HeLa cells.

**Fluorescent Probes for Total ROS.** A series of Ge-pyronine dyes (GePs, 27) (Figure 3) were synthesized with emission wavelengths from deep-red to NIR. Replacing O with Ge increases \( 10^4 \sim 10^5 \) times higher affinity toward GSH compared with the parent O-pyronine. Thus, GSH addition to 9-position C results in a quick quenching in 10 s and the fluorescence recovers in 20s by adding ROS. The redox switch can be repeated for several cycles. Probe 27 was used for real-time monitoring of the reversible and dynamic interconversion between ROS oxidation and GSH reduction in living cells.

A lysosome-targetable NIR fluorescent probe 28 (Figure 3) for hROS was synthesized based on the Si-rhodamine skeleton. When the pyrrole NH of 28 was oxidized by hROS to generate N-hydroxylation, the PET process was hindered to turn on the fluorescence. Probe 28 is resistant to autoxidation and photoxidation and responds to hROS quickly. Probe 28 was used to real-time and long-term image the basal or stimulated response, good selectivity, and low cytotoxicity. Probe 28 was successfully employed to monitor endogenous *OH in living HeLa cells.

**Fluorescent Probes for HClO/ClO\(^-\).** HClO/ClO\(^-\) is widely used as a disinfectant in daily life. Moreover, intracellular HClO is one of the hROS, which is generated by \( \text{H}_2\text{O}_2 \) and Cl\(^-\) catalyzed by myeloperoxidase (MPO). During the process of aging and immune response, HClO has been proved to play an important role. The significant oxidation ability of HClO, which comes from Cl atom of an oxidation state of +1, builds the foundation of fluorescent probes for HClO. According to the reaction mechanism, we divided the probes into four categories, including oxidation cleavage of lactam or lactone, oxidation cleavage of double bonds (C=C, C=S, C=N, and N=N), oxidation of thioether or selenide, and other oxidation-related mechanisms.

Probes Based on Oxidation Cleavage of Lactam or Lactone. Probe 29 (Figure 4), a fluorescein-hydrazine derivative, was developed to detect HClO and *OH with different channels in biological systems. The cleavage of lactam by HClO recovered the conjugated system of fluorescein and gives green emission. Living cell imaging demonstrated 29 could simultaneously monitor the endogenous HClO and *OH in zebra fish. Another lactam-cleavage probe was 30 (Figure 4), which was a mitochondria targeting biotin—rhodamine—TPP conjugate. ClO\(^-\) interacted with benzoyl acetoxydrazide moiety to block out the spiroirpenning rhodamine within 10 s. Biotin moiety guarantees the tumor cell specificity.

A nonfluorescent probe 31 (Figure 4) was synthesized based on the ring masking by sulfur atom on rhodamine 6G. In the presence of HClO, thioester was broken and sulfur atom was replaced by an oxygen atom to recover highly fluorescent rhodamine. Probe 31 could detect endogenous HClO in neutrophils in a mouse infection model and in drosophila.

Probes Based on Oxidation Cleavage of Double Bonds. A pyrene-based water-soluble fluorescent probe 32 (Figure 4) was synthesized. When the C=C double bond connecting pyrene and merocyanine was broken by ClO\(^-\), water-insoluble pyrenecarboxaldehyde was generated, leading to a ratiometric fluorescence signal. Endogenous HClO in RAW264.7 cells was detected by 32. Another probe 33 with a C=C bond conjugated fisher aldehyde to BODIPY could also be broken by HClO. Probe 33 (Figure 4) was NIR emissive and lysosome targeting and demonstrated a fast response to HClO.

Oxidative deoximation reaction was used to fabricate probes for HClO. Probe 34 (Figure 4) possesses a napthalamide aldoxime core with large Stokes shift. HClO oxidatively removed the C=N bond, resulting in dramatic fluorescence enhancement. As to probe 35 (Figure 4), the dediaminomaleonitrile reaction by ClO\(^-\) recovered the planarity of the molecule, changed the D–A structure, and finally increased the fluorescence intensity.

The C=S bond was used as reactive site to design HClO-specific fluorescence probes. Taking advantages of imidazolium salts, a mitochondrial-targeting two-photon fluorescent probe 36 (Figure 4) was synthesized bearing imidazoline-2-thione to function as a C=O recognition unit. When the thione functional group was specially oxidized by ClO\(^-\), the fluorescent imidazolium was produced. Probe 36 was soluble in pure aqueous solution and fluorescence produced by other ROS and RNS was negligible. Moreover, 36 was successfully applied to image ClO\(^-\) in Hela and HepG2 cells, and in astrocytes. Based on the electrophilic addition of Cl\(^+\) to the sulfur of a C=S bond, a probe 37 (Figure 4) for the picomolar level of native HClO in lysosome was synthesized and showed high sensitivity to discriminating tumor tissue from normal tissue in mice.

On the basis of the oxygenolysis of an azo moiety, probe 38 (Figure 4) for selective and sensitive detection of HClO over other ROS and RNS in absolute PBS solution was synthesized. An open-ring structure with green fluorescence was observed after the azo moiety was oxidatively cleaved. Fluorescence imaging in mouse supported the ability of 38 in biological applications.

**Probes Based on Oxidation of Thioether or Selenide.** Through tethering acridine orange and phenothiazine with propane to generate PET process, a pH-independent probe 39 (Figure 4) was synthesized based on the HClO-promoted oxidation of phenothiazine to the phenothiazine sulfoxide and cation which prevented the PET pathway. Probe 39 showed high...
sensitivity (detection limit of 2.7 nM) and fast response (within 5 s) toward HClO in water. Probe 39 could efficiently detect HClO produced in stressed cells. Another example was probe 40 (Figure 4) with a thioether-containing moiety that can be specifically oxidized into sulfoxide, which ruled out the fluorescence quenching through inhibiting the PET process.

Figure 5. Structures of fluorescent probes for ONOO⁻.

Two closely related phenyl selenyl based boron-dipyrromethene (BODIPY) turn-on fluorescent probes (41, Figure 4) for ClO⁻ were synthesized. The probes showed high selectivity for ClO⁻, and emission intensity reached a maximum within 1 s. When selenium was oxidized by HClO to selenoxide, the PET pathway was foreclosed which evoked a significant enhancement
Probes Based on Other Oxidation-Related Mechanisms. Since phenol can be oxidized to quinone and the incorporating ortho halogen substituents can improve the selectivity, a fluorescent probe 42 (Figure 4) for HClO was synthesized. Oxidative O-dearylation of 2,6-dichlorophenol by HClO gave green fluorescence. Probe 42 exhibited high sensitivity (1 equiv of HOCI gave a >358-fold enhancement in fluorescence) and high selectivity (approximately 93-fold higher increase in fluorescence intensity over 1 equiv of ONOO− and OH•). Detection of endogenous HClO was operated in phagocytes as well as in living HepG2 cells. Moreover, in flow cytometry and a 96-well microplate assay for the quantitative detection of endogenous HClO, 42 showed superior sensitivity.

A coumarin-alkene-rhodamin fluorescence resonance energy transfer (FRET) probe 43 (Figure 4) was designed with ratiometric signal for HClO. The selective reaction between HClO and rhodamin thiohydrazide induced the dehydrogenation and spiro-ring opening, which realized the FRET process. Comparing to the traditional coumarin-rhodamin FRET platform, 43 exhibited high energy transfer efficiency (81.1%), making it suitable for detection in biological samples.

Phenyboronic acid group was found to be attacked and decomposed by ClO•−. Probe 44 (Figure 4) consisting of meso-(4-pyridyl)-substituted BODIPY as the chromophore group and phenylboronic acid as the sensing group was developed for ClO•−. Probe 44 was applied to imaging mitochondrial ClO•− in HepG2 cells.

Fluorescent Probes for HBrO/BrO•−. As an important ROS, HBrO generated from bromide plays a key role in the formation of sulfilimine cross-links in collagen IV. Because of the approximately 1000-fold lower level of Br•− than that of Cl•−, the concentration of HBrO is relatively lower than that of HClO, making it difficult to detect. Only a small number of fluorescent probes were developed.

On the basis of the catalytic effect of HBrO on the cyclization between the amino group and S-methyl group, an ultrasensitive probe 45 (Figure 4) was developed for HBrO. Probe 45 exhibits ultrasensitivity (down to 17 pm) and real-time response (within minutes) to HBrO. Endogenous HBrO in HepG2 cells and zebrafish was imaged by 45. Utilizing the same mechanism, 46 (Figure 4) was designed for mitochondria HBrO. Reaction with HBrO expanded the π-conjugation system of 46, and NIR fluorescence was emitted, providing a tool for the study of physiological and pathological functions of HBrO.

Probes Based on Dearylation to Produce Amido. ONOO• can trigger the N-dearylation reaction to remove the N-aryl. Rhodamine-based probe 47 (Figure 5) containing the N-aryl group as a fluorescence quencher and reaction site was constructed. Probe 47 was water-soluble and virtually non-fluorescent. One equiv of ONOO• gave a 93-fold enhancement of fluorescence within 2 s while 1 equiv of OH• or HClO did not interfere. Probe 47 was utilized to visualize endogenous ONOO• generated in mouse liver tissues under an acute alcohol binge condition and during hepatic ischem-reperfusion injury. A similar fluorescent probe 48 (Figure 5) was designed based on the BODIPY scaffold masking with 4-hydroxyaniline, which was changed to 4-methoxyaniline in the presence of ONOO•. Probe 48 displayed high selectivity to ONOO• over other ROS (2 equiv of ROS did not elicit noticeable fluorescence changes). Another fluorescent probe 49 (Figure 5) utilizes benzothiazole as a two-photon fluorophore. Probe 49 presents a low detection limit (0.35 nM) and high specificity toward ONOO• and was used to image endogenous ONOO• in biological samples.

Probes Based on Deboronation. Although arylboronate has been widely employed in the design of fluorescent probe for H2O2, in some cases it shows higher reaction rate with ONOO• than H2O2. This character was utilized in the development of ONOO• fluorescent probes.

Fluorescent probe 50 (Figure 5) could be unmasked by ONOO• to release N-methyl-benzothiazole with the initiation of ESIP, leading to fluorescence increase. Since fluorescein is an easily detectable fluorescent compound with high quantum yield, a fluorescein-boronate fluorescent probe 51 (Figure 5) was synthesized. The reaction rate of 51 with ONOO• was about 106 and 2700 times faster than that of H2O2 and HClO at 25 °C. The actual intracellular flux of ONOO• was estimated utilizing 51. In probe 52 (Figure 5), deboronation generated hydroxyl which intramolecularly cyclized to form a bright inimocoumarin fluorophore. The detection limit of 52 reaches 0.83 nM.

Probes Based on the Breakage of Unsaturated Bond. The ONOO•-induced diene oxidation has been utilized to design fluorescent probes. Since Cy3 has a shorter polymethine chain and better stability to ONOO• than Cy5, a fine-tuning fluorescent probe 53 (Figure 5) was obtained through integrating Cy3 and Cy5 which usually act as a popular FRET pair. This mitochondria-targeting probe operates in a FRET-based ratiometric mode and the detection limit is 0.65 nM. Moreover, its applicability has been demonstrated for fluorescence imaging of ONOO• in stimulated macrophage cells. Another specific probe 54 (Figure 5) was designed based on the BODIPY scaffold with trans-cinnamaldehyde molecules. It is ratiometric since the diene interconnection can be oxidized to form the BODIPY skeleton, accompanied by the fluorescence emission changes in the signal of red-to-green. High-throughput flow cytometry analysis indicated that 54 could quantify the ONOO• generation in the inflammation mouse model.

Probes Based on the Decomposition of α-Ketoamide Group. Since ONOO• is a biomarker for certain drug-induced liver injury (DILI), two fluorescent probes were designed and applied to mapping ONOO• during DILI and the corresponding remediation pathway. Probe 55 (Figure 5) could release 4-amino-1,8-naphthlic anhydride to restore ICT process and turn on fluorescence via the decomposition of α-ketoamide group. Probe 55 was used to evaluate the upregulation of ONOO• during acetaminophen (APAP) and tolcapone-induced hepatocyte injury. Another mitochondria-targeting probe 56 (Figure 5)
5) with rhodamine skeleton revealed that CYP450/CYP2E1-mediated enzymatic metabolism of APAP leads to ONOO$^-$ generation in liver cells.91

Probes Based on Other Mechanisms. The ONOO$^-$-triggered N-oxidation and N-nitrosation reaction were utilized to design a new kind of fluorescent probe for ONOO$^-$. In probe 57 (Figure 5), BODIPY was installed with 4-methoxy substituted aromatic tertiary amine to quench fluorescence through the PET mechanism. When 57 was oxidized to produce N-oxide and N-nitroso products, the PET was blocked to recover fluorescence. Moreover, the mitochondria- and lysosome-targetable derivatives were used to image ONOO$^-$ in the corresponding subcellular organelles and in kidney tissues of diabetic rats.92

A two-photon ratiometric fluorescent probe 58 (Figure 5) for detecting mitochondrial ONOO$^-$ was designed based on the FRET mechanism.93 Probe 58 combined a modified chromenylium fluorophore as the energy acceptor and the modified coumarin as the energy donor. Probe 58 is fast responsive (within 20 s) toward ONOO$^-$. Moreover, the minimal cytotoxicity of 58 enabled it to monitor endogenous ONOO$^-$ in both cells and LPS stimulated mouse model.

Mitochondria-targeting probe 59 (Figure 5) was installed by utilizing spirocyclic and nonfluorescent rhodamine as the fluorescence platform and phenylhydrazine as the recognition unit. The oxidative hydrolysis of phenylhydrazine by ONOO$^-$ could open the spirocyclic structure and turn on the fluorescence of rhodamine. Probe 59 is capable of monitoring the mitochondrial ONOO$^-$ level in live RAW 264.7 cells.94

Fluorescent Probes for NO. As a small uncharged free radical and ubiquitous messenger molecule, nitric oxide (NO) is implicated in many physiological and pathophysiological processes, such as endothelial dysfunction, cancer, and neurodegenerative diseases.95-98 In recent years, the turn-on fluorescent probes for NO were mostly designed based on the inhibition of the PET pathway, including the reaction between NO and o-phenylenediamine to generate triazole99-102 and N-nitrosation of aromatic secondary amine.103,104

Probes Based on the Formation of Triazole. As a two-photon fluorescent probe of NO, probe 60 (Figure 6) realized “NIR in”
“far-red out” through utilizing Nile Red as the TP fluorophore and o-phenylenediamine as the reaction site. The reaction between 60 and NO could generate triazole which caused the inhibition of the PET process and enhancement of fluorescence. Compound 60 was utilized to measure both exogenous and endogenous NO in living cells as well as NO generation in LPS-mediated inflamed tissue of mice at depth up to 170 μm.99 Considering that most probes were developed for imaging intracellular NO and could not reflect the release and transmembrane behavior of NO, an amphiphilic probe 61 (Figure 6) containing hydrophilic groups to keep the fluorophore and recognition site outside the cell and an alkyl chain as the membrane anchor was designed based on BODIPY scaffold.100 This highly water-soluble probe selectively monitored NO released out of the cells on the outer surface of RAW 264.7 murine macrophages and human vascular endothelial cells.

Probe 62 (Figure 6) was lysosome-targeting and was designed based on the o-phenylenediamine-locked Si-rhodamine skeleton.101 This NIR fluorescent probe with a detection limit as low as 36.3 nM is stable in acidic solution and could monitor exogenous and endogenous NO in real time. Via change the Si-rhodamine lactam to deoxylactam, a probe with better properties was obtained which can avoid the serious interference from Cys. Moreover, this probe exhibits an ultralow detection limit (0.12 nM) and huge fluorescence off-on response (6300-fold).102

Probes Based on N-Nitrosation of Aromatic Secondary Amine. Mitochondria-targeting probe 63 (Figure 6) was designed based on the BODIPY dye masking with the N-benzyl-4-hydroxyaniline group which act as the reaction site for NO as well as the electron donor for PET, and triphenylphos-
phosphonium cation was introduced to act as the mitochondria-targeting group. Cellular experiments demonstrated that 63 was fast responsive (within seconds), highly sensitive (nanomolar level), and selective for NO over other various other ROS. A similar probe was designed which consists of benzo[g]coumarin as the two-photon fluorophore. The probe could in situ monitor endogenous NO in live cells and in deep tissues. Moreover, this probe was utilized to monitor the NO generation in an ischemia reperfusion injury in the mouse model.

Probes Based on Other Mechanisms. Since the C4-position of Hantzsch ester substituted with a benzylic group could react with NO to cleave the C–C bond, a novel FRET off-on NO fluorescent probe 64 (Figure 6) was designed. In this probe, 1,4-dihydro-pyridine acts as a linker to connect fluorescein isothiocyanate and [4′-(N,N'-dimethylamino)phenylazo] benzoyl as well as a quencher which could be released via the bond cleavage and recovered fluorescence. The specificity, sensitivity, and effectiveness of the reaction enabled the fluorescence intensity linearly with the amount of NO. On the basis of this, this ratiometric fluorescent probe semiquantitatively detected NO in the inflamed mice model.

Fluorescent Probes for HNO. As a one-electron-reduced and protonated analogue of NO, nitroxy (HNO) has been considered as a product of NO and H$_2$S in mammals. Thus, the detection of HNO is really valuable to investigate the crossstalk between NO and H$_2$S in vivo.

Inspired by the specific reaction between NO and phosphine, several fluorescent probes were designed. The detection mechanism is the specific reaction between HNO and 2-(diphenylphosphino)-benzoate moiety and can give the correspondingaza-ylide, followed by the intramolecular attack to cleave the adjacent ester linker and released hydroxyl groups.

Probe 65 (Figure 6) was designed based on the BODIPY-coumarin conjugate platform. The reaction between HNO and the probe can produce the phenolate group which acts as a much stronger electron donor accompanied by the fluorescent enhancement at 463 nm and decreases at 592 nm. Furthermore, low cytotoxicity and good cell membrane permeability enabled 65 to be suitable for imaging of HNO in living cells.

Probe 66 (Figure 6) was designed based on the coumarin core which endowed the two-photon property. The triphenylphosphine group was utilized as the mitochondria-targeting group and diphenylphosphine group as the reaction site. The detection limit of this mitochondria-targeting probe was as low as ~18 nM which enabled one to detect the formation of exogenous and endogenous mitochondrial HNO.

Since the FRET route can be switched off by the closed spirolactone form of the rhodol, a FRET-based two-photon ratiometric fluorescent probe 67 (Figure 6) was developed. The reaction between HNO and the probe could generate the fluorescent xanthene and induced the occurrence of FRET. This high selective probe could image HNO with dual-channel pattern in living cells and rat liver tissues with satisfactory deep-tissue imaging depth. A similar ratiometric two-photon probe which contains benzo[h]chromene-rhodol skeleton was synthesized and successfully mapped the NO/H$_2$S cross-talk in vivo.

Fluorescent Probes for SNO. Considering the crucial functional roles of S-nitrosylation in normal physiology and disease, a fluorescent small molecular probe 68 (Figure 6) for the in situ imaging of GSNOs in live cells was designed. Since the 3-hydroxythymalimide fluorophore was protected by 2-(diphenylphosphino) benzoic to block the ESPT process, the probe was nonfluorescent. The reaction with GSNO could release the free phenol and turn on fluorescence. This probe exhibited good specificity toward GSNO which was chosen as a representative RSNO species. Because of the excellent biocompatibility, this turn-on fluorescent probe was successfully applied to image the dynamic change of protein S-nitrosylation in live endothelial cells.

**FLUORESCENT PROBES FOR RSS**

Fluorescent Probes for GSH. Glutathione (GSH) is the most abundant (1–10 mM) and representative nonprotein thiol inside the cell which can be oxidized to its dimeric form, GSSG. GSH plays crucial roles in the intracellular antioxidant defense and associates with a wide variety of biological phenomena including inflammation, oxidative stress, gene expression, signal transduction, and apoptosis. Therefore, the detection of GSH and its metabolism in biological samples is appealing. Generally, taking advantage of the nucleophilicity of GSH, the Michael addition reaction or the nucleophilic substitution was mainly employed to develop fluorescent probes for GSH tracing.

Probes Based on the Michael Addition. Inspired by extraordinarily fast reversible Michael addition–elimination reaction between silicon-substituted rhodamine, an electrophilic group, and the nucleophilic GSH, two reversible ratiometric fluorescent probes 69 and 70 (Figure 7) were designed to quantitatively analyze GSH by utilizing a FRET platform. Tetramethylrhodamine (TMR), a typical rhodamine, was selected as an energy donor and silicon-substituted rhodamine was served as an energy acceptor. After treated with GSH, the FRET process was interrupted due to the fluorescent quenching of acceptor. Meanwhile, the fluorescence of the response moiety was recovered when removing GSH, thus leading to a ratiometric readout. With high temporal resolution, these two probes were proved to be revolutionary tools to tracking and quantification GSH fluctuation under physical context. Another reversible ratiometric fluorescent probe 71 (Figure 7) was constructed to quantify GSH fluctuation in HeLa cells, in which probe solution exhibited two different emission signals ($\lambda_{Em} = 488$ nm and $\lambda_{Em} = 560$ nm) that can be attributed to the change of the conjugated system after the Michael addition or elimination reaction between the probe and GSH.

On the basis of the Michael addition accompanied by the hydrolysis process, the fluorescent probes for GSH detecting were developed by integrating the alkene or alkyne group into an electron-withdrawing group carbonyl. Upon incubation with GSH, the addition reaction performed first, and then the H$_2$ bond was formed between the N–H group of GSH and carbonyl to hydrolyze ultimately to a ring-opening compound with strong fluorescence, thus leading a fluorescent turn-on behavior.

Probes Based on the Nucleophilic Substitution. Utilizing nucleophilic substitution of GSH and proposed probes, a superior sulfonamide containing group was employed to construct fluorescent probes with outstanding response behavior. A FRET-based ratiometric fluorescent probe 72 was developed once sulfonamide group was introduced as a specific reaction site of GSH and the linker between the energy donor and acceptor. Upon exposure to GSH, the S–N bond in the probes were cut off and the FRET pathway was interrupted resulting in ratio signals. In view of electron-withdrawing character of the sulfonamide group, the fluorescence of probe 73 was quenched on account of the inhibited ICT process once sulfonamide was integrated into naphthalimide. The attack of GSH would locate GSH on the fluorophore and light up fluorescence through recovering ICT of naphthalimide.
Fluorescent Probes for Cys. As one of the sulphydryl-containing small molecular amino acids, cysteine (Cys) plays crucial roles in many physiological and pathological processes. Excess amounts or deficiency of Cys are associated with diseases, while normal levels of Cys are essential for maintaining the synthesis of various proteins and act as the source of sulfide in human metabolism. Therefore, the detection of Cys and its metabolism in biological samples is appealing. Overall, given the nucleophilicity of Cys, the Michael $\alpha,\beta$-unsaturated addition reaction of Cys was a widely used design strategy to construct Cys specific probes.

Probes Based on the Addition of Maleimide. In view of extraordinarily fast reaction rates and outstanding specificity toward Cys residues in proteins, the maleimide group was integrated into well-known fluorescent reporters, such as coumarin and naphthalimide, to afford Cys specific fluorescent probes. Through combination of an acrylate group and a maleimide group onto the opposite sites of a coumarin fluorophore, a well-designed turn-on fluorescent probe 74 was obtained for Cys detection. The two different recognition groups synergistically worked through different intramolecular cyclization processes. The thiol-Michael addition to the acrylate followed by the cyclization-deprotection step recovered its ICT fluorescence. Probe 74 could selectively detect and image Cys over Hcy and GSH in cells. The PET process caused by the response group maleimide endowed 75 with a low fluorescent background signal. Meanwhile, the strong electron-withdrawing naphthalimide enhanced the reactivity of the probe which enabled the ultrasanensitivity (2.0 nM). Moreover, this two-photon fluorescent probe could fast and selective image Cys in living cells.

Probes Based on the Addition of Acrylate or Acrylate Derivatives. The acrylate or acrylate derivative was another wildly used trigger for constructing Cys specific fluorescent probes. As a representative, a two-photon NIR turn-on fluorescent probe 76 was synthesized for specific detection of Cys over Hcy, GSH, and other amino acids. The acrylate was masked on the dicyanomethylene-4H-pyran skeleton which has strong fluorescence emission (150 nm), two-photon excitation (850 nm), and NIR emission (702 nm). All these enabled its application for biological imaging. A dual optical probe was reported based on the crotonyl group masking on the oxazolidinone-dioxide-functionalized conjugated phenol. After the Michael addition and a subsequent cyclization reaction of Cys, the fluorescence of the probe 77 was turned on as well as the solution color changed from colorless to red. Notably, the probe can be located in the mitochondria and can be attributed to the positive charge of the probe product.

Probes Based on the Addition of $\alpha,\beta$-Unsaturated Ketone. Nucleophilic addition of Cys to the $\alpha,\beta$-unsaturated ketone was employed to develop Cys fluorescent probes by integrating ketone to common dyes. For example, a tailor-made specific fluorescent probe 78 was developed based on the 4-chlorocoumarin-hemicyanine framework to discriminate Cys from other thiols. After incubation with Cys, the probe solution showed a maximal absorption peak blue-shift from 576 to 373 nm on account of the destruction of the conjugate structure that can be attributed to the substitution-rearrangement reaction of the primary amine of Cys, followed by the attack of liberated free thiol to the adjacent double bond, which is unavailable for other biothiols as well as NAC. Another representative $\alpha,\beta$-unsaturated ketone containing fluorescent probe 79 was developed through incorporating the ethanoylcoumarin and terephthalaldehyde, in which the former acted as the Cys reaction site and the latter was a sensitive nucleophilic addition reaction site for SO$_2$. Since SO$_2$ is the metabolite of Cys, this dual-site probe monitored the conversion of Cys and SO$_2$ in a ratiometric manner. Probe 79 also was the first probe to monitor endogenous SO$_2$ without stimulation of SO$_2$ donors. Bioimaging experiments demonstrated that 79 could quickly (within 100 s) and reversibly image Cys as well as SO$_2$ in two channels in A549 cells and zebrashift.

Probes Based on the Reduction of Aldehyde. Considering electron-withdrawing aldehyde could be reduced by Cys, a novel turn-on fluorescent probe 80 was designed for detection of Cys over Hcy/GSH as well as other amino acids. The working mechanism of this BODIPY-based glyoxal hydrazone probe was the inhibited ICT quenching through addition to the unsaturated aldehyde, followed by the formation of a 5-membered ring of thiazoline. Probe 80 was used to image intracellular Cys.

Fluorescent Probes for Hcy. The quite similar structure between Hcy and Cys makes the discrimination of Hcy over Cys a significant challenge. On the basis of 1,1'-binaphthyl fluorophore tethered to an aldehyde moiety, a Hcy specific fluorescent probe was designed through the cyclization reaction between Hcy and the aldehyde group in the probe. Once exposed to Hcy, a six-membered thiazinane was generated accompanying with fluorescence enhancement. H NMR analysis was carried out to strongly verify the fluorescence increment triggered only by Hcy, rather than Cys, GSH, and other biothiols.

Fluorescent Probes for Total GSH, Cys, and Hcy. Probes Based on the Substitution of Benzoxadazole. A generic strategy for the detection and measurement of GSH in the absence and presence of Cys/Hcy was presented. In this strategy, a 7-nitro-2,1,3-benzoxadazole (NBD) group was selected as the fluorophore to conjugate with the fluorophore through sulfhydryl-labile ether linkage. Probe 81 (Figure 7) is nonfluorescent due to ICT. The substitution reaction of GSH to 81 resulted in one emission because of the release of the strong fluorescent fluorophore and nonfluorescent sulfur ether. Meanwhile, the substitution reaction of Cys/Hcy will exhibit two distinctly separated emissions since the cleavage product of Cys/Hcy will experience intramolecular Smiles rearrangement to form a new highly fluorescent compound, which is unavailable for the cleavage product of GSH. The two different emission spectra can be analyzed to detect and differentiate GSH from Cys/Hcy, facilitating detection of GSH both in vitro and in vivo.

Probes Based on the Substitution of 2,4-Dinitrobenzenesulfonyl. The electron-withdrawing 2,4-dinitrobenzenesulfonyl group was widely used as a trigger to assemble biological thiols specific fluorescent probes. Biothiols could selectively induce the O–S bond to cleave and remove the 2,4-dinitrobenzenesulfonyl group which acted as a fluorescent quencher as well as response moiety. The release of the fluorophore resulted in a remarkable fluorescence light-up response. Notably, the concentration range-dependent fluorescent probe 82 for sensing of biological thiols was designed based on judicious selection of the corresponding sensitivity sites and the dye platform, which was hybrid from 7-hydroxyccoumarin and merocyanine. The 2,4-dinitrobenzenesulfonate moiety and the chloro group were chosen as the high- and low-sensitivity site of thiols, respectively. The removal of the 2,4-dinitrobenzenesulfonyl group will result in ICT quenching through addition to the unsaturated aldehyde, followed by the formation of a 5-membered ring of thiazoline. Probe 80 was used to image intracellular Cys.
sulfonate moiety by the low concentration range (0−35 μM) of thiols exhibited a turn-on mode of fluorescence signal. Moreover, the 4-chloro group might be replaced by a high concentration range (35−500 μM) of thiols, followed by intramolecular S_NAr substitution to generate a 4-amino product and induce a ratiometric readout.

**Probes Based on the Substitution of Pyrimidine.** Grafting the pyrimidine group with different substituents on resorufin or fluorescein could obtain a series of fluorescent probe and
provided detailed structure-reactivity relationships. Via this combinatorial strategy, fluorescent probe 83 for detection of biotiol was identified based on pyrimidine chemistry. It is the first time that the pyrimidine functionality was employed as the unique recognition unit to construct optical probe for biotiohs. Probe 83 could be utilized as the analytical molecular tool in the living cell fluorescence imaging. Moreover, 83 could selectively visualize the endogenous biotiol fluctuations in mouse model.

Probes Based on the Addition of $\alpha$-$\beta$-Unsaturated Malonitrile. By integrating $\alpha$-$\beta$-unsaturated malonitrile and chlorine atom to the coumarin platform, a simple molecule probe 84 was developed. The chlorine atom could be replaced by thiol group of biothiols and different products were produced through different cascade reaction, such as intramolecular rearrangement of the biothiols (Cys and Hcy) or cyclization reaction of the $\alpha$-$\beta$-unsaturated malonitrile (GSH). Cell imaging studies indicated that 84 could simultaneously and selectively sense Cys and GSH in HeLa cells.

Probes Based on the Substitution of Tetrafluoroterephthalonitrile. The currently smallest fluorescent probe 85, which could differentiate Cys, Hcy and GSH was discovered. The Cys thiol group replaced a fluorene on the probe and cyclized to form a six-membered ring with strong green fluorescence ($\lambda_{\text{ex/flu}}$ at 420 nm/500 nm) and two-photon property ($\lambda_{\text{exf=}}$ 860 nm). The reactions between GSH/Hcy and 85 adopted aromatic nucleophilic substitution, generating products without two-photon property but with strong blue fluorescence ($\lambda_{\text{ex/flu}}$ at 350 nm/450 nm). Addition of CTAB (cetyltrimethylammonium bromide) merely altered the fluorescence color of the reaction product of Hcy through facilitating the intramolecular ring formation ($\lambda_{\text{ex/flu}}$ at 420 nm/500 nm). The results indicated the differentiation of these three thiols.

Fluorescent Probes for H$_2$S. As the smallest and simplest biotiol in living systems, hydrogen sulfide (H$_2$S) is known to be the third gaseous signal molecule following nitric oxide (NO) and carbon monoxide (CO). Either protective or deleterious depending on its intracellular concentrations, H$_2$S is supposed to influence various physiological and pathological processes, including the modulation of blood vessel tone and cardioprotection, the endogenous stimulation of angiogenesis and mitochondrial bioenergetics. It has been reported that the physiological concentration of H$_2$S in mammalian serum is in the range of 30–100 $\mu$M, while those in the brain is in the range of 50–160 $\mu$M. Considering the challenges in quantitatively detection of this volatile small molecule in living systems, the biologically relevant concentrations of H$_2$S may notably fluctuate with diverse types of cells and different subcellular regions. In view of the important roles of H$_2$S as a cytoprotectant and gasotransmitter, monitoring the level and dynamics of H$_2$S in living cells and organisms contributes to better understanding of its basic cellular functions. Based on the nucleophilic attack and reducing capacity of H$_2$S, strategies employed for the selective and sensitive detection of this small reactive molecule are classified into four categories in this review: nucleophilic addition, H$_2$S-induced thiolysis, azide group reduction and other novel recognition mechanisms.

Probes Based on Nucleophilic Addition of H$_2$S to Conjugated Systems. Given the nucleophilicity of HS$^-$ at physiological pH, electrophilic centers in conjugated fluorescent molecules such as cyanine or other dyes can be feasibly attacked by H$_2$S-induced nucleophilic addition. Different signal transduction mechanisms including FRET, ICT (twisted intramolecular charge transfer), ICT, and PET were employed to develop fluorescent probes based on this reaction. Probe 86 as illustrated in Figure 8, was reported as the first TICT-based fluorescent probe for H$_2$S, using hemicyanine as recognition site for H$_2$S and BODIPY as the fluorescence reporting group. The nucleophilic addition of H$_2$S to the indole unit blocks the intramolecular TICT pathway and thus enhances the fluorescence. Notably, 86 specifically detected H$_2$S in a short response time (within 2 min for 50 $\mu$M H$_2$S), and visualization of exogenous as well as endogenous H$_2$S in living cells was achieved in this work. Carbazole platform was selected to develop a lysosome-targeting two-photon fluorescent probe 87 (Figure 8), using pyridine moiety as the site for $\text{H}^+$ as well as the targeting group for lysosome and the indole unit as the reaction site of H$_2$S. Targeting to acidic lysosome exhibited a red-shifted fluorescence signal at 625 nm, while the nucleophilic addition of lysosomal H$_2$S to the indolium C-2 atom produced a blue-shifted fluorescence readout at 550 nm. Accordingly, visualizing lysosomes and tracking lysosomal H$_2$S in living cells and mouse liver tissues, were achieved simultaneously with dual-channel fluorescent imaging in this work. The H$_2$S level in living systems is susceptible to levels of Cys, Hcy, and GSH, because of the close network of generation and metabolic pathways within biotiohs. A hybrid multisignal fluorescent probe 88 (Figure 8) was developed for simultaneously distinguishing and sequentially sensing Cys/Hcy, GSH, and H$_2$S for the first time. A red emission at 609 nm could be obtained upon the cut off of the ether bond by reaction with Cys/Hcy, GSH or low level H$_2$S, while another green emission at 546 nm was also observed for Cys/Hcy. Besides, high level H$_2$S generated a new blue emission at 485 nm. Accordingly, distinguishing fluorescence signals at three distinct emission bands (blue-green-red) with 61 and 63 nm interval distances between two adjacent bands, respectively, were collected upon responding to the respective biotiohs.

Probes Based on Nucleophilic Addition of H$_2$S-induced Thiolysis. Another nucleophilic attack strategy for H$_2$S-specific probes namely H$_2$S-induced thiolysis in this review, is based on the selective addition of HS$^-$ to a single electrophilic position on the probe scaffold, which is followed by the removal of the electrophilic functionalities, resulting turn on fluorescence signals. The fluorescence of these probes are typically protected with incorporated electron withdrawing motifs that can be cleaved in the presence of H$_2$S. Thereinto, 7-nitro-1,2,3-benzoxadiazole (NBD) amine has been incorporated to naphthalimide, styrly-BODIPY and cyanine skeleton to develop H$_2$S-specific fluorescent probes, based on their ability to undergo thiolysis by H$_2$S via a nucleophilic aromatic substitution ($S_{Ar}$) mechanism. A NIR fluorescent probe 89 (Figure 8), based on the H$_2$S-thiolysis of NBD amine masking on the cyanine skeleton, was recently reported by Li group to explore its application in living mice. Endogenous H$_2$S in colorectal cancer cells in murine tumor model could be monitored in situ in living mice, through intratumoral injection of 89. In addition to NBD, some electron withdrawing ester moieties are also incorporated as leaving groups that could be specifically removed by nucleophilic H$_2$S to release fluorescent scaffolds with turn on signals. As illustrated in Figure 8, an electrophilic cyanate as the recognition site was masked on the 3-hydroxyflavone to develop an ESPT-based fluorescent 90 (Figure 8) for detection of H$_2$S in vitro.
and imaging in living cells. Probe 90 was characteristic of high sensitivity with low detection limit (0.25 μM) and relatively quick response time (<60 s) toward H2S over other biothiols.

**Probes based on Azide Group Reduction.** Electron-withdrawing azide moieties can be chemo-selectively reduced to electron-donating amino products by H$_2$S, which are supposed to significantly change the electronic distribution of the whole molecule once installed onto fluorophores.\textsuperscript{161–163} Probe 91 (Figure 8)\textsuperscript{162} was reported to sense endogenous H$_2$S formation in cancer cells within 15 min without adding any external stimulators was reported recently, based on azide reduction. With the sensitivity of 91 toward H$_2$S as low as 24.3 nM, distinguishing cancer cells from normal cells was achieved based on the level of endogenous H$_2$S formation.

**Probes Based on Other Reaction Mechanisms.** Some novel reaction strategies for specific detection of H$_2$S have been investigated in the recent 2 years.\textsuperscript{164–166} Nucleophilic aromatic substitution of hydrogen (S$_n$Ar\textsuperscript{164}) reactions that can be catalyzed by sulfide, were employed to develop a phenalenone-based fluorescent probe 92 (Figure 8)\textsuperscript{164} for monitoring of aqueous sulfides in living cells. The mechanism was found to be that the sulfide added to the double bond, then underwent isomerization and rearrangement, followed by hydrolyzation and elimination to afford the final product. A fluorescent turn-off probe 93 (Figure 8)\textsuperscript{166} was developed utilizing the phenylselenium group as a leaving group masking on the BODIPY core. Substitution of the phenylselenide group at the 3- and 5-position of BODIPY by H$_2$S accounted for the decrease in fluorescence intensity. Probe 93 was employed for imaging of intracellular H$_2$S in living cells in this work.

**Fluorescent Probes for H$_2$S$_{pr}$.** With the in-depth understanding of physiological roles taken by RSS in cytobiology, increasing attention has been given to hydropersulfides (R-SSHs), which exhibit important biological activities in the modulation of enzyme functions and the synthesis of sulfur-containing vitamins and cofactors.\textsuperscript{166} More recent studies on H$_2$S redox biology even suggest that R-SSHs account for most biological mechanisms which were originally attributed to H$_2$S.\textsuperscript{167–169} H$_2$S$_{pr}$ can be considered as oxidized forms or redox partners of H$_2$S, which may coexist in biological systems as redox couple to collectively regulate sulfur-containing redox balance. As for the chemical reactivity, H$_2$S$_{pr}$ are expected to be stronger nucleophiles but weaker reductant than H$_2$S. To explore the varied roles of H$_2$S$_{pr}$ in biological systems, it is critical to employ effective analytical tool for detecting and monitoring the formation and consumption of these RSS species.

Phenyl 2-(benzothio)benzoate\textsuperscript{170,171} and 2-fluoro-5-nitrobenzoic ester\textsuperscript{172–174} are the two most used recognition units specific for H$_2$S$_{pr}$. The first dual-channel fluorescent probe 94 (Figure 8)\textsuperscript{170} capable of visualizing H$_2$S and H$_2$S$_{pr}$ with different fluorescence signals was developed in 2016, to reveal the mutual relationship and cross-talk between H$_2$S and H$_2$S$_{pr}$ in cells. Reaction with H$_2$S$_{pr}$ released the green fluorescence of rhodol at 542 nm, while recognizing H$_2$S produced the emission signals of both major blue coumarin major at 445 nm and minor green rhodol at 542 nm. Therefore, simultaneous appearance of the two well separated emissions confirmed the presence of H$_2$S$_{pr}$ whereas single green emission was attributed to H$_2$S. Notably, 94 was successfully applied for the ratio detection ($F_{542 \text{ nm}}/F_{430 \text{ nm}}$) of relative H$_2$S$_{pr}$ and H$_2$S in mixture solutions with varying Na$_2$S$_2$/Na$_2$S concentrations. Bioimaging experiments proved that 94 could selectively detect H$_2$S and H$_2$S$_{pr}$ from distinct emission channels in HeLa cells. The first ratiometric fluorescent probe 95 (Figure 8)\textsuperscript{172} for the detection of H$_2$S$_{pr}$ was developed with 2-fluoro-5-nitrobenzoic group as a reaction unit. Via the formation of H$_2$S$_{pr}$-mediated benzodithiolone, followed by the release of the electron-donating amino group to modulate the ICT system, 95 could image exogenous and endogenous H$_2$S$_{pr}$ in living cells.

Other reaction strategies can also be used to detect H$_2$S$_{pr}$ selectively. The unique reversible reaction of sulfane sulfur with other sulfur atoms was employed to develop a reversible fluorescent probe 96 (Figure 8)\textsuperscript{175} for sulfane sulfur, based on FRET mechanism. The sulfur atom in SSip-1 could reversibly bind to sulfane sulfur, followed by the generation of intramolecular spirocyclization of xanthene dye to turn on fluorescence. Concentration fluctuation of sulfane sulfur in living cells was reversibly visualized with 96 in this work. A multiplex NIR fluorescent probe 97 (Figure 8)\textsuperscript{177} for the in situ detection of endogenous O$_2^{2-}$ and H$_2$S$_{pr}$ was designed based on heptamethylene cyanine dye. The hydrogen in the probe could be extracted by O$_2^{2-}$ to recover the conjugated system and emit low fluorescence. H$_2$S$_{pr}$ could reoptimize the PET process to provide stronger fluorescence through reducing the nitro group to amino group. Probe 97 could selectively image O$_2^{2-}$ and H$_2$S$_{pr}$ in cells and in vivo.

**Fluorescent Probes for Cys-SSH.** Supposed to be the source of the whole profile of R-SSHs derivatives in living organisms at the micromolar level,\textsuperscript{176} Cys-SSH is reported to play a leading role to synthesize sulfur-containing cofactors, regulate cellular signaling, modulate enzyme activities, and adjust cellular redox milieu.\textsuperscript{167,178} The first near-infrared ratiometric fluorescent probe 98 (Figure 8)\textsuperscript{179} for the detection of Cys-SSH in living cells and in vivo was developed in 2016, based on the selenium–sulfur exchange reaction. The diselenide group masking on the heptamethine cyanine skeleton can be rapidly reduced by Cys-SSH within minutes, thus triggering the ICT process and exhibiting a larger spectral blue shift in emission from 797 to 749 nm. Quantitative detection of CBS enzyme-mediated Cys-SSH biosynthesis was achieved by ratiometric imaging analyses with fluorescence intensity ratio ($F_{480 \text{ nm}}/F_{430 \text{ nm}}$) in living cells. Meanwhile, 98 incorporated with liver-targeting galactose-terminated ligand was successfully applied to visualize Cys-SSH in peritoneal cavity of mouse BALB/c and in hepatic carcinoma models. The first reversible ratiometric fluorescent probe 99 (Figure 8)\textsuperscript{180} responsive to R-SSHs, including H$_2$S$_2$, Cys-SSH, and GSSH, was reported recently, based on the decrease of FRET efficiency after recognition. Once reversibly reacting with the pyrionine fluorophore, the conjugated structure of the xanthene ring was interrupted and induced the dual-emission change at 479 and 584 nm. Probe 99 could dynamically visualize the exogenous and endogenous R-SSHs in living cells by ratio imaging ($R = F_{430-480}/F_{550-630}$ nm).

**Fluorescent Probes for SO$_2$.** Sulfur dioxide (SO$_2$) is widely present as an air pollutant in the environment which exists in the forms of sulfite (SO$_4^{2-}$) and bisulphite (HSO$_3^{-}$) anions in aqueous solution. Besides, SO$_2$ can be generated in the body during the oxidation of hydrogen sulfide or sulfur-containing amino acids metabolism. Recently, SO$_2$ is recognized as a gaseous signal transmitter modulating numerous biological processes. Abnormal levels of SO$_2$ and its derivatives (SO$_3^{2-}$ and HSO$_3^{-}$) are closely associated with neurological disorders, cancers, and some other diseases.\textsuperscript{181–184} Recently, there are some fluorescent probes reported for imaging SO$_2$ and its derivatives in living systems. Generally, these probes are mainly designed by exploiting the nucleophilic addition of SO$_2$ derivatives to the

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unsaturated bond. The $\alpha,\beta$-unsaturated bond in the probe structure is easily attacked by the $\text{SO}_2$ derivatives to generate new blue-shifted fluorescence emission, thus resulting in the ratiometric fluorescence signal. The probes 100–104 are designed by applying this strategy. The alternative strategy of designing ratiometric fluorescence probes for $\text{SO}_2$ is modulating the FRET process. The probes 105–107 present the emission of the FRET acceptor whose conjugation structure is easily disturbed by nucleophilic addition of $\text{SO}_2$ derivatives to quench the fluorescence emission. Therefore, upon reaction with $\text{SO}_2$, the fluorescence switch to blue-shifted emission which attributes to the FRET donor, giving the ratiometric fluorescence signal for $\text{SO}_2$ quantification.

A new ratiometric fluorescent probe 108 was proposed for reversible detection of $\text{HSO}_3^-$ by conjugating benzothiazole and functionalized cyanine dye. Nucleophilic addition of $\text{HSO}_3^-$ to the probe resulted in distinct ratiometric fluorescence response, and subsequent treatment of the addition product by peroxides would restore the original probe in high yield. Fluorescence imaging experiments indicated that this probe could sense $\text{HSO}_3^-$ reversibly in living cells as well as in aqueous solution. Besides, another fluorescent probe 109 was developed by conjugating naphthopyran and benzothiazolium to reversibly image bisulfate/$\text{H}_2\text{O}_2$ redox cycle in live cells and zebrafish.

Probe 110 was designed to detect $\text{SO}_3^{2-}$ and $\text{SO}_4^{2-}$/$\text{HSO}_4^-$ simultaneously. The probe itself was non-fluorescent. Upon treatment with $\text{SO}_3^{2-}$, the addition of $\text{SO}_3^{2-}$ to the probe structure could restrict the ICT pathway due to the disruption of the conjugation and result in the fluorescence turn-on response at 442 nm. Besides, the presence of $\text{SO}_4^{2-}$/$\text{HSO}_4^-$ induced the aggregation-induced emission to enable the turn-on fluorescence response at 511 nm. Fluorescence microscopic images indicated that this probe could differentially sense...
intracellular SO$_2^{2-}$ and SO$_4^{2-}$ inside live HeLa cells. Probe 111 (Figure 9) was designed to detect SO$_2$ and ClO$^-$ simultaneously. This probe consisted of a phenanthroimidazole-modified fluorophore and a benzylidine malononitrile moiety as the reaction site of ClO$^-$ and SO$_2$. Nucleophilic addition of SO$_2$ to this probe resulted in fluorescence emission at 410 nm within 60 s, while oxidation of ClO$^-$ to this probe resulted in fluorescence emission at 500 nm within seconds. This probe was applied in detecting SO$_2$ and endogenous ClO$^-$ in cells as well as visualizing the homeostasis of SO$_2$/ClO$^-$. Nucleophilic addition of HSO$_3^-$ to this probe resulted in fluorescence characteristic.

**Fluorescent Probes for Sec and H$_2$Se.** Besides reactive sulfur species (RSS), reactive selenium species are other intracellular antioxidant systems, mainly including selenocysteine (Sec) and hydrogen selenide (H$_2$Se). Sec, the counterpart of cysteine, is the essential building block of selenoproteins which are involved in various biological processes and associated with cancers, inflammations, neurodegenerative disorders, and other significant diseases. H$_2$Se, the counterpart of H$_2$S, is considered an important metabolite of selenium-containing compounds. Similarly, endogenous H$_2$Se has been found to modulate various pathophysiological phenomena and associated with significant diseases.

Figure 10. Structures of fluorescent probes for Sec and H$_2$Se.

Therefore, as a chalcogen like sulfur, selenium endows reactive selenium species reaction activities similar to RSS and fluorescent probes of Sec and H$_2$Se may greatly contribute to the deep understanding of their biological functions.

Probes 113 and 114 (Figure 10) are designed by integrating the 2,4-dinitrobenzenesulfonamide moiety to a certain fluorophore, which can be attacked by Sec to release the fluorophore and restore the fluorescence emission. Probe 113 is based on the aminocoumarin platform, which exhibits near-zero background fluorescence as well as high specificity for Sec over competing amino acids and biothiols. Fluorescence imaging experiments indicated that this probe could monitor endogenous Sec in human cancer cells and zebrafish. Probe 114 is developed based on the NIR fluorophore, dicyanomethylene-benzopyran. This probe was applied to image Sec in serum samples, live cells, and animals.

Probe 115 (Figure 10) was designed for Sec sensing by exploiting the selenium—sulfur exchange reaction. This probe consists of heptamethine cyanine as NIR fluorophore, bis(2-hydroxyethyl) disulfide as the recognition unit, and D-galactose as the liver targeting moiety. The probe was used to evaluate the antioxidant and anti-inflammatory effect of Sec during liver inflammation. By comparing Sec levels in normal and acute hepatitis mice models, Sec was proved to be critical to maintain the redox states of the liver.

Probe 116 (Figure 10) was designed to detect H$_2$Se based on the NIR merocyanine dye core. H$_2$Se can easily break the Se—N bond in the 2,1,3-benzoselenadiazole moiety via nucleophilic addition to generate diamino merocyanine fluorophore and restore the fluorescence emission. This probe is used to image endogenous H$_2$Se in live cells and in vivo with high selectivity over H$_2$S, Sec, and biological thiols. Fluorescence imaging experiments indicated that this probe could sensitively monitor H$_2$Se in live cells and in vivo. Moreover, the generation and accumulation of H$_2$Se in HepG2 cells under hypoxic conditions and in the solid tumor were also revealed with the aid of this probe.
CONCLUDING REMARKS

In this review, we summarized the small-molecule fluorescent probes for imaging and detection of reactive oxygen, nitrogen, and sulfur species (RONSS) that developed since 2016. The probes consisted of various fluorophores and recognition moieties, providing an effective tool box for RONSS detection in living organisms. Undoubtedly, the further exploration of RONSS biology will be readily pushed forward by these new probes. Meanwhile, some issues need further thinking. Since the same recognition moiety accommodated to different fluorophores will exhibit specificity to different targets, the structure-reactivity relationships of the reported probes need deep investigation to draw the inherent rules, which can guide the purposeful design and synthesis of probes. Although the detection of endogenous analytes in vivo is generally accessed, the imaging depth is still limited because of low light penetration ability and stability of the probes. NIR and two-photon probes with low background interference help solve part of this but not all of it. The significant improvements may be afforded by using NIR-II window (1000–1700 nm)-emissive probes, which is characteristic of good imaging resolution and depth. The research progress in this area should be paid attention. In some cases, influence of the cellular microenvironment on probe response characteristics cannot be neglected. The preliminary experiment in cell extracts or simulated cell physiological conditions may contribute to the identification of the difference, guiding the observation in living cells or in vivo. Finally, considering the short lifetime and high reactivity of RONSS, the concentration driven reversible probes with an ideal dissociation constant ($K_d$) that can real-time quantitative monitoring of RONSS are still highly needed, in spite of such probes being proposed for GSH. Collectively, these issues provide not only a challenge but an entry to a new area of study for researchers. We believe that, with the development of new small-molecule fluorescent probes, the physiological and pathological functions of RONSS in biological systems will be better interpreted and understood.

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