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REVIEW

New progress in spectroscopic probes for reactive oxygen species

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Abstract
Reactive oxygen species (ROS) play an important role in many critical physiological processes. However, overproduction and accumulation of ROS in vivo can damage some biomolecules and lead to a variety of diseases. Therefore, it is necessary to develop efficient methods for the detection of ROS. Spectroscopic probes have been extensively employed in this respect because of their high sensitivity and superior spatiotemporal sampling capability. In this review, representative spectroscopic probes for the common ROS developed in the recent 5 years are summarized, and discussed according to design strategies and recognition groups.

Keywords Spectroscopic probes · Reactive oxygen species · Design strategy · Recognition group

1 Introduction

Reactive oxygen species (ROS) are a series of highly reactive oxygen-containing species, including hydrogen peroxide (H_2O_2), hypochlorite (OCl^-), peroxynitrite ($ONOO^-$), superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (·OH), nitric oxide (NO), singlet oxygen ($^1O_2$) and so on. They are beneficial to many critical biological processes, such as defense of pathogens invasion, signal transduction and cell redox homeostasis [1–3]. However, overproduction and the accumulation of ROS in vivo may damage some biomolecules (e.g., proteins, lipids and DNA) and lead to a variety of diseases (e.g., neurodegenerative disorders, arteriosclerosis and even cancer) [4–7]. On the other hand, many ROS have similar properties, such as strong oxidizing ability, low concentration and short lifetime. Therefore, the sensitive and selective detection of ROS in biological systems is of great significance for the prevention, diagnosis and treatment of these diseases.

Spectroscopic probes are a class of reagents that interact with the analytes leading to the changes of their spectroscopic (absorption, fluorescent, or chemiluminescent) properties; on the basis of these changes, the analytes can thus be detected [8–10]. Spectroscopic probes have become powerful and extensively employed tools for the detection of biological substances owing to their noninvasiveness, high sensitivity and superior spatiotemporal sampling capability [8–10]. So far, a large number of spectroscopic probes for ROS have been developed in the past decades [11–15]. Considering the high reactivity, low concentration, short lifetime and rapid conversion of ROS, probes that are capable of real-time and in situ detecting and monitoring are of great importance for studying the biological functions of ROS. In particular, the spectroscopic probes with unique design strategies and specific recognition groups to distinguish the different ROS are much more desirable. In this review, an effort will be made to summarize, and discuss the newly developed spectroscopic probes for the common ROS in the recent 5 years, with various design strategies and recognition groups.

2 Spectroscopic Probes for Various ROS

2.1 Probes for Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a relative stable ROS endogenously produced from $O_2^{\cdot-}$ under the catalysis of superoxide dismutase. Detection of H_2O_2 primarily depends on the oxidation of boronate ester as a recognition moiety of a spectroscopic probe [4, 16]. For example, phenylboronate ester can be oxidized to its corresponding phenol form, and this

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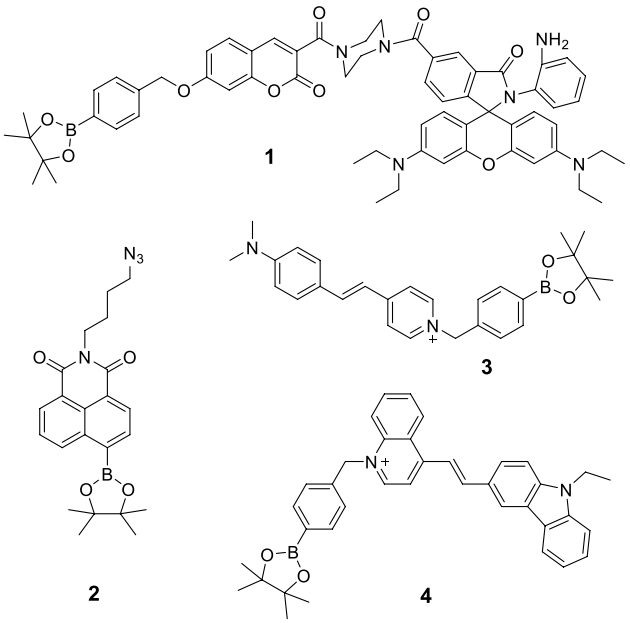
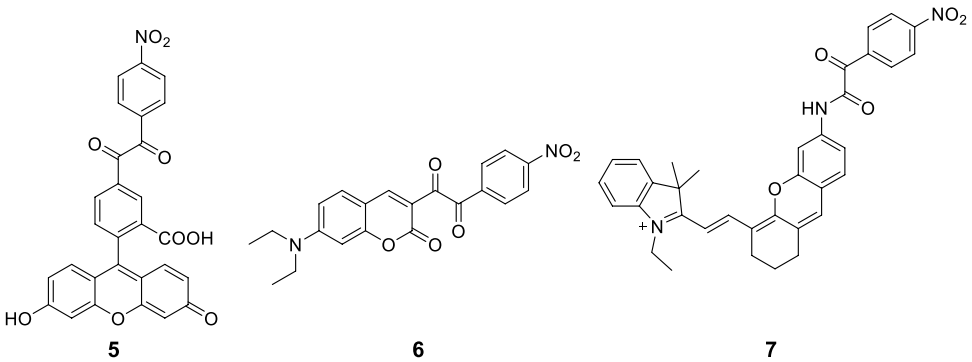
reaction usually causes the fluorescence of the probe to be turned on or altered. A great number of such probes have been reported for H_2O_2 detection.

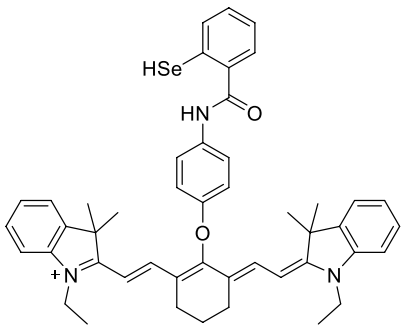
Using boronate ester group for H_2O_2 and *o*-phenylenediamine group for NO, Yuan et al. prepared a fluorescent probe **1** with dual recognition sites [17]. The probe reacted with both H_2O_2 and NO in pH 7.4 PBS buffer (20% CH_3CN) generating distinct fluorescence signals, which made it suitable for dual-color imaging of H_2O_2 and NO in living cells and for studying the interaction of the two species. Yi's group developed a naphthalimide-based ratiometric probe **2** for H_2O_2 [18]. In response to H_2O_2, the ratio of fluorescence intensities of probe **2** at 555 and 403 nm (I_{555}/I_{403}) increased greatly due to the oxidation of boronate ester. Besides, this probe contained an azide group to link to various molecules via the click reaction. For imaging H_2O_2 in nucleus, probe **2** was further modified with a nuclear localization signal peptide. By combining a rotatable mitochondria-targetable fluorophore and a boronate ester moiety, Lin's group developed a dual-detection fluorescent probe **3**, which can be used to monitor the level of H_2O_2 and the viscosity change in mitochondria [19]. Due to twisted internal charge transfer (TICT) process, the probe exhibited a weak emission band when excited at 500 nm; however, an obvious fluorescence enhancement at 607 nm was observed at higher viscosity, resulting from the limitation of intramolecular rotation. On the other hand, upon treatment with H_2O_2, the fluorescence of probe **3** increased dramatically at 510 nm with excitation at 400 nm. Recently, a mitochondria-targetable probe **4** has been developed for H_2O_2 assay by Shao's group [20]. However, several probes with boronate ester moiety were also reported to detect other ROS such as $ONOO^-$ and OCl^- (see below). This indicates that the boronate ester moiety is still not very selective for H_2O_2.



Taking advantage of the unique chemical reactivity of benzil with H_2O_2, Nagano's group developed a selective probe **5** [21]. Upon treating the weakly fluorescent probe with H_2O_2, benzil group was transformed to benzoic anhydride through a Baeyer–Villiger type reaction and finally hydrolyzed to give the highly fluorescent product 5-carboxyfluorescein. Probe **5** was demonstrated to be sensitive and selective for H_2O_2 and was applied to imaging H_2O_2 in RAW 264.7 and A431 cells. Similarly, a coumarin-based two-photon probe **6** was developed [22]. Moreover, Tang's group reported a mitochondria-targetable near infrared (NIR) fluorescent probe **7** for H_2O_2 [23]. The probe itself was nonfluorescent. However, an obvious fluorescence enhancement at 704 nm could be observed upon treating with H_2O_2. The probe could monitor the overproduction of H_2O_2 during ischemia–reperfusion injury in both HepG2 cells and kidney of mouse.



Based on the unique five-membered ring opening-closing response of ebselen to glutathione(GSH)/H_2O_2, Tang's group developed a NIR reversible fluorescent probe **8** for monitoring the changes of GSH/H_2O_2 levels in vivo [24]. The probe was weakly fluorescent at 794 nm upon 768 nm excitation due to the photoinduced electron transfer (PET) quenching process. In response to H_2O_2, the probe was oxidized to form a Se–N bond and a five-membered ring product, which was highly fluorescent. On the other hand, upon treating the oxidized product with GSH, the five-membered ring could be opened to its original form. The probe showed good reversibility with at least four repeated redox cycle triggered by GSH/H_2O_2. Yu's group developed a selenium-containing probe **9** for H_2O_2 [25]. Different from probe **8**, an aggregation-induced emission response mechanism was involved in probe **9**. Probe **9** exhibited very weak fluorescence emission due to PET process. However, oxidation of the Se atom produced a selenium oxide product, which was highly fluorescent with an emission band at 476 nm in its aggregation state.



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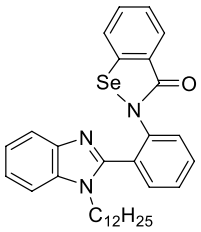

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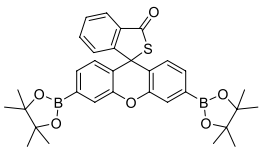
2.2 Probes for Hypochlorite

Hypochlorite (OCl^-) is produced by the reaction of H_2O_2 and Cl^- under catalysis of myeloperoxidase in biosystems. It has been known as a powerful microbicidal agent in the immune system. Similar to other ROS, however, overproduction of OCl^- may damage biomolecules such as lipids, proteins or DNA and involve in a variety of diseases. The detection of OCl^- mainly depends on its strong oxidization for sulfur, selenium, hydrazide, *p*-methoxyphenol, and aldoxime groups.

The spirocyclic thiolactone skeleton of rhodamine or fluorescein was reported to selectively react with Hg^{2+} and OCl^- [8]. Because natural living biosystems usually contains no or extremely low concentration of Hg^{2+}, the thiolactone skeleton of rhodamine or fluorescein can serve as a specific recognition unit for OCl^- [26]. The spirocyclic structure breaks the conjugated system of fluorophore, being nonfluorescent; however, upon treatment with OCl^-, the thiolactone skeleton undergoes a chlorination reaction to form an "S–Cl" intermediate, which is then hydrolyzed and accompanied by fluorescence recovery of the fluorophore. Xu et al. designed a "dual-lock" probe **10**, with two recognition sites, boronate ester and thiolactone [27]. It is specific for OCl^- without noticeable activity toward other ROS including H_2O_2 and $ONOO^-$, because only OCl^- can react with both boronate ester and the spirocyclic thiolactone structure to result in fluorescence turn-on response. Ma and colleagues developed a mitochondria-targeting probe **11** by conjugating a rhodamine thiolactone skeleton with the commonly used mitochondria-targetable group of triphenylphosphonium [28]. The probe exhibited good mitochondria-targeting ability and has been first applied to monitoring the generation of OCl^- in macrophages infected by bacteria.



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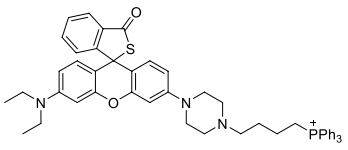

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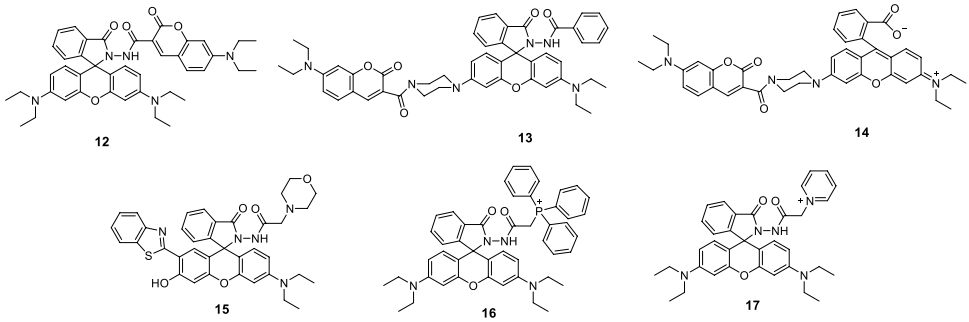
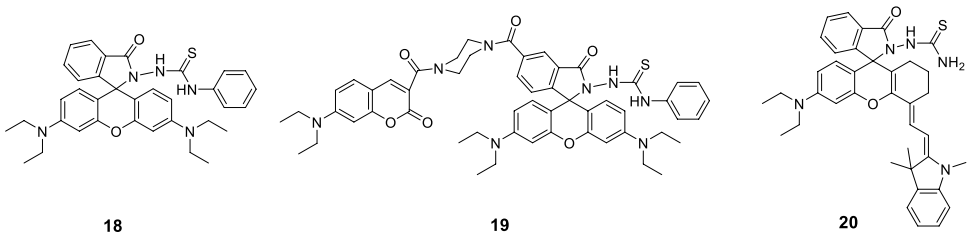
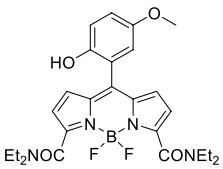
OCl^- can selectively oxidize dibenzoyl hydrazine into dibenzoyl diimide, which is further decomposed by nucleophilic agents such as H_2O. On the basis of this reaction, Ma's group proposed for the first time that dibenzoyl hydrazine may be employed as a specific recognition group for OCl^- detection [29]. Since then, many similar probes with a hydrazide moiety have been developed for the assay of OCl^-. For instance, by directly conjugating a coumarin fluorophore and a rhodamine fluorophore to the two end of a diacylhydrazine group, Long et al. developed a ratiometric probe **12** [30], which exhibited fluorescence of coumarin at 501 nm; upon treatment with OCl^- in pH 8.5 phosphate buffer (40% DMF as co-solvent), the diacylhydrazine moiety was oxidized and hydrolyzed, leading to the fluorescence increase of rhodamine at 578 nm. The ratio of fluorescence intensities at 578 nm and 501 nm (I_{578}/I_{501}) exhibited a large change with OCl^-. Zhang et al. developed **13** as a ratiometric fluorescent probe [31], which could respond to OCl^- in pH 8 phosphate buffer (40% DMF), exhibiting obvious fluorescence ratio (I_{580}/I_{470}) increase. Interestingly, the product of probe **13** reacting with OCl^-, compound **14**, can be used directly as a probe to detect hypochlorous acid (HOCl) in pH 5 phosphate buffer (40% DMF), with great fluorescence ratio (I_{470}/I_{580}) increase. Moreover, by conjugating with morpholine, and triphenylphosphonium or pyridinium, the lysosome-targeting probe **15**, and mitochondria-targeting probes **16** and **17** were developed by Lin' group and Yu' group, respectively [32, 33].



Yuan et al. developed the thiosemicarbazide based OCl^- probes **18**-**20** [34, 35]. Probe **18** was nonfluorescent due to the spirocyclic structure of rhodamine, but exhibited a fluorescence enhancement at around 590 nm in response to NaOCl in pH 7.4 PBS (50% DMF). The turn-on response of probe **18** resulted from the conversion of thiosemicarbazide into oxadiazole and the opening of its spirocyclic structure. By conjugating coumarin with rhodamine, they further changed the fluorescence turn-on probe **18** into a ratiometric fluorescent OCl^- probe **19** based on a fluorescence resonance energy transfer (FRET) mechanism. Probe **19** showed fluorescence ratio (I_{594}/I_{473}) increase after reacting with OCl^-. Probe **20**, a thiosemicarbazide-based NIR one, showed fluorescence increase at 746 nm in response to NaOCl in pH 7.4 PBS (30% CH_3CN), and was employed for imaging OCl^- in living cells and mice.



Yang's group developed a series of fluorescent probes **21**–**23** for the selective detection of hypochlorite [36–38]. They found that *p*-methoxyphenol could be rapidly oxidized to benzoquinone by NaOCl but stable toward most of the other common ROS. Taking advantage of this specific reaction, they designed probe **21** by conjugating *p*-methoxyphenol with a BODIPY skeleton [36]. The probe was nonfluorescent due to PET from *p*-methoxyphenol to BODIPY; after *p*-methoxyphenol was oxidized to benzoquinone, the PET process was prohibited and the obtained quinone product was highly fluorescent. Probe **21** has been successfully employed to monitor the formation of OCl^- in an abiotic but enzymatic system (myeloperoxidase/H_2O_2/Cl^- system) and in living macrophage cells upon stimulation. However, the quinone product of probe **21** could be further oxidized by OCl^- to give compounds of weaker fluorescence, which may interfere with OCl^- detection. To prevent the overoxidation of the quinone product, they further modified the structure of probe **21** to get probe **22** [37], in which two methyl groups were added to the 1- and 7-positions of the BODIPY core to increase the steric hindrance around *p*-methoxyphenol. Among the three analogues of probe **22**, **22**b showed the best performance, and had been used for detecting endogenous OCl^- in both human and mouse macrophages. By introducing two ortho chlorine substituents, they designed probe **23** with fluorescein [38]. The introduction of chlorine lowers the pK_a of the phenol and enhances its reactivity toward OCl^-; the phenol unit could quench the fluorescence by PET process. Upon exposure to OCl^- in aqueous solution, a large enhancement in the fluorescence intensity was observed due to the oxidation of phenol and release of quinone. The similar electron-rich structure, *p*-aminophenol, has also been proved to be a viable recognition group for OCl^- detection. For example, Guo et al. developed a *p*-aminophenol based probe **24** [39], which is essentially nonfluorescent since the electron-rich 4-aminophenol moiety can quench fluorescence via PET mechanism. Upon oxidation by OCl^-, two emission bands at 460 nm and 570 nm appeared. However, probe **24** suffers from obvious interference from $ONOO^-$.



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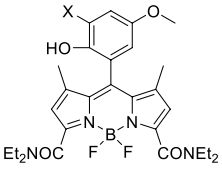

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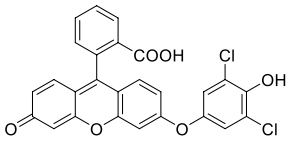
a X=H
b X=F
c X=Cl



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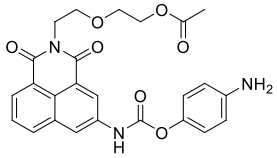

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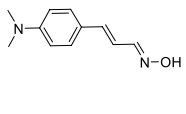
The C=N bond isomerization of aldoxime has been demonstrated to be the predominant quenching process of excited states in fluorophores with a bridged aldoxime structure; however, upon oxidation with OCl^- to give the corresponding aldehyde, carboxylic acid or nitrile oxide derivative, the C=N bond isomerization would be removed or inhibited, thereby leading to strong fluorescence emission. Based on this strategy, probes **25**-**27** were designed. Probe **25** was non-emissive due to nonradiative deactivation of C=N bond isomerization [40]. The conversion of the aldoxime into the nitrile oxide derivative by OCl^- efficiently led to fluorescence increase at 556 nm when excited at 450 nm. Similarly, this process serves as the basis for the operation of two other BODIPY based OCl^- probes **26** and **27** [41, 42].



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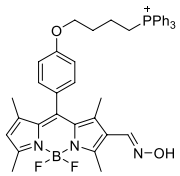

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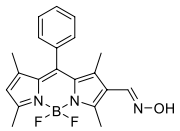

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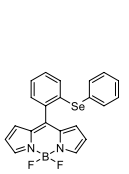
Two reversible BODIPY-based OCl^- probes **28** and **29** were developed by Wu's and Han's group, respectively [43, 44]. The reversible recognition mechanism relies on oxidation–reduction cycle of selenide and selenoxide. For example, due to PET from the diphenyl selenide group to the BODIPY moiety, probe **28** displays weak fluorescence. Upon addition of OCl^- to the probe solution, the diphenyl selenide group is oxidized to its corresponding selenoxide, which does not participate in PET quenching. As a result, the increase in fluorescence intensity at 526 nm takes place, which, however, can be eliminated by addition of the reducing agent GSH to the solution. This observation indicates that selenoxide is reduced to its original state (selenide). In a similar way, the oxidation–reduction cycle of selenide and selenoxide also takes place in probe **29** by reaction with the oxidation–reduction couple of OCl^- and H_2S. Both **28** and **29** are applied to imaging the redox cycles between OCl^- and GSH (or H_2S) in living cells. Another reversible OCl^- probe **30** was developed by Han's group with 1,8-naphthalimide as a spectroscopic unit [45]. Different from probe **28** and **29**, the PET process from selenide to fluorophore does not exist in probe **30**. However, there is an excited state configuration twist process in probe **30**, but not in its selenoxide form, which contributes to the fluorescence quenching of naphthalimide core. Probe **30** was successfully employed to image the redox cycles in living cells and mice. Moreover, Cheng et al. developed a selenium-containing NIR fluorescent probe **31** for OCl^- [46]. Its self-aggregation behavior causes fluorescence quenching. Nevertheless, oxidation of the probe to its selenoxide form by OCl^- leads to the disaggregation with fluorescence recovery at 786 nm when excited at 690 nm. This probe showed good selectivity towards OCl^-, and was applicable for detection of OCl^- in fetal bovine serum (FBS) and in live mice.



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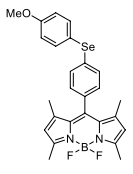

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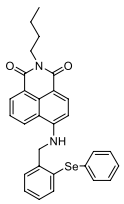

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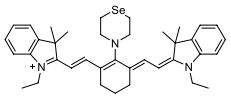

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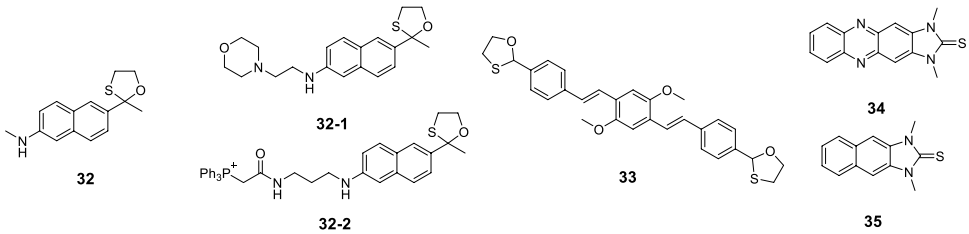
Two-photon imaging has many advantages such as great tissue penetrability, high spatial resolution, and low phototoxicity, which make two-photon fluorescent probe an attractive tool for detecting OCl^- in live cells and tissues. Yuan et al. designed a two-photon fluorescent probe **32** for detection of OCl^- with acedan as a fluorophore [47]. The reaction of OCl^- with **32** induces remarkable fluorescence increase at 500 nm upon excitation at 375 nm, which is attributed to the deprotection of nonfluorescent oxathiolane group by OCl^-. To monitor OCl^- level in the mitochondria and lysosome of

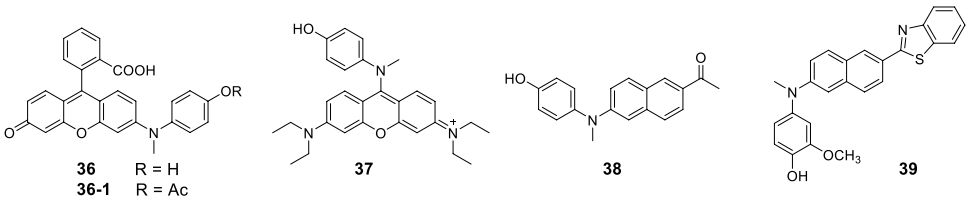
macrophage cells, the mitochondria and lysosome-targeting probes **32-1** and **32-2** were further prepared, bearing triphenylphosphonium and morpholine group, respectively. Cao et al. developed another oxathiolane-based two-photon probe **33**, which was successfully applied to monitoring endogenous OCl^- stimulated by lipopolysaccharide (LPS) in living microglia BV-2 cells [48]. Yoon et al. developed two two-photon probes **34** and **35** [49]. The addition of OCl^- leads to remarkable fluorescence increase at 505 nm for **34** and at 450 nm for **35**, which are attributed to the formation of the corresponding highly fluorescent imidazolium. Probe **34** was employed to monitor OCl^- in live cells and tissues by two-photon microscopy.



2.3 Probes for Peroxynitrite

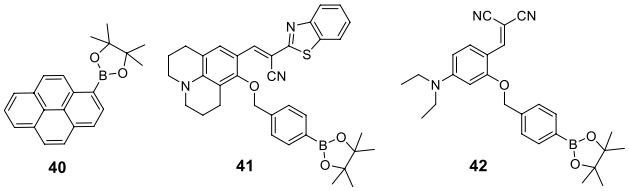
Peroxynitrite ($ONOO^-$) is a highly reactive oxidant, produced endogenously in living biosystems by a diffusion-limited reaction of the free radicals nitric oxide (NO) and superoxide ($O_2^{\cdot-}$). The oxidization and nitration activity of $ONOO^-$ plays a beneficial role in cell signal transduction pathways and immune systems. However, the overproduction of $ONOO^-$ is also harmful to humans. Oxidation reaction of methyl(4-hydroxyphenyl)amino is the major strategy for detection of $ONOO^-$.

Methyl(4-hydroxyphenyl)amino is a frequently used recognition group for design of $ONOO^-$ probes. Yang's group developed a turn-on fluorescent probe **36** by conjugating rhodol with methyl(4-hydroxyphenyl)amino [50]. Due to the specific $ONOO^-$-triggered oxidative *N*-dearylation reaction, the probe exhibits a remarkable fluorescence turn-on response to $ONOO^-$ without obvious interference from other ROS. Probe **36** also showed rapid response and high sensitivity towards $ONOO^-$. The acetate derivative **36-1** was prepared to improve cell membrane permeability. This probe was applied to imaging intracellular $ONOO^-$ in a variety of cell types by both single-photon and two-photon excitation fluorescence microscopy, and monitoring the endogenous peroxynitrite generation in live tissues from a mouse model of atherosclerosis. Zhang et al. developed a mitochondria-targetable fluorescent probe **37** for $ONOO^-$ by directly conjugating methyl(4-hydroxyphenyl)amino group with the electropositive fluorophore pyronin [51]. The probe showed good mitochondria-targeting performance and was applied to monitoring $ONOO^-$ in the mitochondria of macrophage cells. By conjugating methyl(4-hydroxyphenyl)amino group with two-photon fluorophore acedan, Hong et al. designed a two-photon probe **38**, which can detect $ONOO^-$ in pH 7.4 PBS [52]. Yoon et al. reported another two-photon probe **39** based on methyl(4-hydroxyphenyl)amino [53]. This probe was successfully used for two-photon imaging in rat hippocampal tissue.



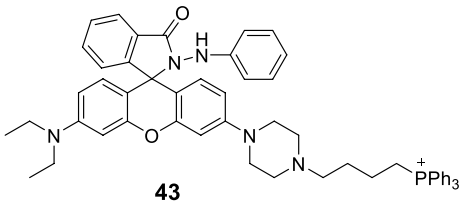
$ONOO^-$ may react with arylboronate esters to yield the corresponding phenol derivatives much faster than ClO^- and H_2O_2, which makes arylboronate ester a potential recognition group for $ONOO^-$ probes. Yu et al. designed such a probe **40** based on the pyrene fluorophore, which exhibited rapid turn-on response to $ONOO^-$ at 410 nm within 15 s [54]. However, the interferences from H_2O_2, ClO^- and OBr^- with treatment for 15 min are indeed not negligible. Kim et al. prepared probe **41** with an arylboronate ester as the recognition unit for $ONOO^-$ and a nitrile group for the following cyclization [55]. Upon adding $ONOO^-$ to the solution of probe **41** in pH 7.4 phosphate buffer (10% ethanol), the arylboronate ester was oxidized to the corresponding phenol, followed by a domino decomposition reaction to generate benzothiazolyl iminocoumarin

with bright fluorescence emission at 540 nm. No obvious response occurred when probe **41** was incubated with other ROS except H_2O_2, which induced a small fluorescence response after 120 min. In a similar mechanism, Zhou et al. developed a ratiometric probe **42**, with fluorescence ratio (I_{480}/I_{580}) increase in response to $ONOO^-$ in pH 7.4 PBS (10% CH_3CN) [56]. However, H_2O_2 would induce an obvious fluorescence enhancement under high concentrations (larger than 100 μM) and long treatment time (more than half an hour).



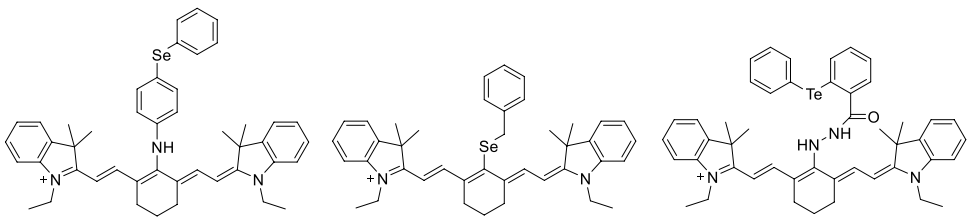
40 41 42

Based on the oxidation of phenylhydrazine, Ma's group developed a mitochondria-targeting probe **43** [57]. The probe itself was nonfluorescent because of the spirocyclic structure of rhodamine. However, upon reaction of the probe with $ONOO^-$, the oxidation of phenylhydrazine by $ONOO^-$ and the subsequent hydrolysis led to the opening of the nonfluorescent spirocyclic structure, and thus triggered a dramatic fluorescence intensity increase at 578 nm. Other ROS scarcely resulted in the fluorescence response. The probe has been applied to imaging $ONOO^-$ in mitochondria of RAW 264.7 cells under various stimuli.



43

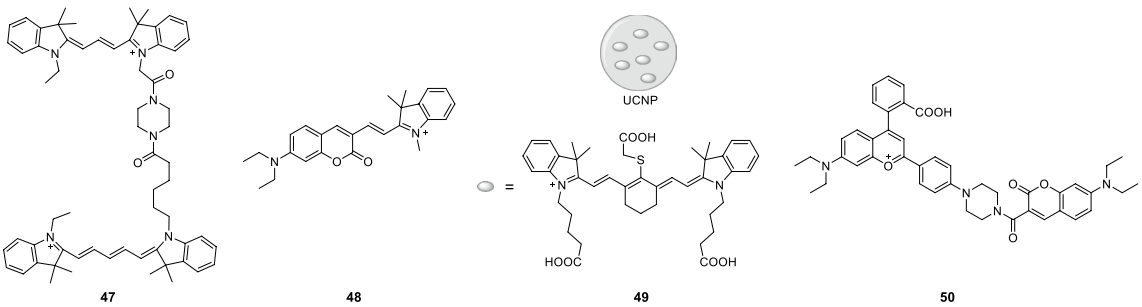
The oxidation–reduction cycle of selenide and selenoxide is also applied to design reversible fluorescent probes for $ONOO^-$. Han's group developed such a NIR reversible probe **44** [58]. The probe was weakly fluorescent with emission at 800 due to the PET process from the selenide moiety to the cyanine moiety. Upon the oxidation of selenide to selenoxide by $ONOO^-$, the PET process was inhibited and a fluorescence turn-on response took place with emission changing from 800 to 775 nm as a result of push–pull electronic effect. Other ROS, including ClO^- and H_2O_2, did not trigger obvious fluorescence response. On the other hand, the selenoxide form of probe **44** also displayed well selective turn-off response to thiols (Lys and GSH), which reduced selenoxide to form the original probe. The redox cycle of probe **44** could be repeated at least five times with no loss of fluorescence intensity. Tang's group designed another NIR reversible probe **45** for the selective detection of $ONOO^-$ and monitoring redox cycles in living cells by directly conjugating a benzylselenide group with the cyanine fluorophore [59]. Interestingly, contrary to probe **44**, probe **45** was highly fluorescent but its oxidized selenoxide form was weakly fluorescent. The redox cycle of probe **45**, whereby $ONOO^-$ and reduced ascorbate, could be repeated at least 8 times. The oxidation–reduction cycle of telluride and telluroxide was also employed by Han's group to design the tellurium-containing fluorescent probe **46** for monitoring intracellular $ONOO^-$/GSH redox cycles [60]. Oxidation of probe **46** by $ONOO^-$ resulted in large fluorescence increase at 820 nm, which was then eliminated by GSH and Cys. Probe **46** was demonstrated to be reversible for at least 5 repeated redox cycles.



44 45 46

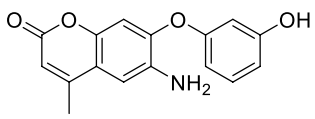
Another important strategy for the design of $ONOO^-$ probe is to take advantage of $ONOO^-$-triggered oxidative cleavage of fluorophores (e.g., cyanine or hemicyanine). Based on this strategy, probes **47-50** were designed for selective detection of $ONOO^-$. Qian's group developed a FRET-based fluorescent probe **47** by covalently linking Cy3 as energy donor and Cy5 energy acceptor to achieve a ratiometric detection of $OONO^-$ [61]. Upon exposure to $OONO^-$, Cy3 showed better stability for its shorter polymethine chain, whereas Cy5 was rapidly cleaved by $OONO^-$, accompanied by the fluorescence intensities increase at 560 nm and decrease at 660 nm. Probe **47** exhibited good mitochondria-targeting performance due to the positively charged structure of cyanine dyes, and was applied for the detection of $OONO^-$ in living cells. Probe **48** was developed by Yoon's group based on a coumarin-hemicyanine scaffold [62]. The probe emitted fluorescence at 635 nm when excited at 475 nm. Addition of $ONOO^-$ to the probe solution resulted in oxidative cleavage of the electron rich C=C bond linking the coumarin and hemicyanine moieties to form a new coumarin derivative, accompanied by emission decrease at 635 nm and a concomitant increase at 515 nm.

Peng et al. have developed multilayered upconversion nanoparticles (UCNPs) coated with cyanine dyes as a nanoprobe **49**, which was used to monitor the generation of $ONOO^-$ in a paracetamol-induced liver injury model [63]. The UCNPs were selected as the energy donor and chromophore Cy7 was chosen as the energy acceptor to quench the luminescence of the nanoparticles. The presence of $ONOO^-$ could readily result in the cleavage of Cy7 and then recover the luminescence of UCNPs. Cyanine or hemicyanine dyes have also been demonstrated to be very susceptible to other biological species, such as ROS (e.g., OCl^-) and reactive sulfur species (e.g., H_2S), which produce ineluctable interference for $ONOO^-$ detection. Thus, Cheng et al. screened a long wavelength fluorescent dye with specific and sensitive response to $ONOO^-$ as the energy acceptor, and integrated it into the FRET-based ratiometric fluorescent probe **50** with coumarin as the energy donor [64]. The probe exhibited good mitochondria-targeting performance and two-photon spectral properties, which makes it useful for detecting mitochondrial $ONOO^-$ and two-photon imaging.



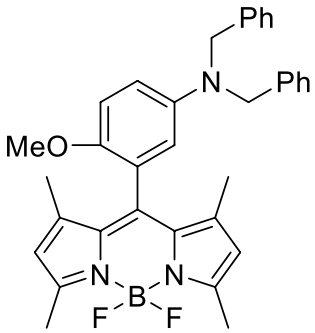
47 48 49 50

Zhang et al. have developed a three-channel fluorescent probe **51** to distinguish $ONOO^-$ from OCl^- [65]. This probe was prepared using a coumarin scaffold and emitted green fluorescence at 525 nm when excited at 355 nm. The oxidation of phenol by $ONOO^-$ and nucleophilic attack of amino group in close proximity could lead to the formation of aminophenol, as an orange-emitting intermediate (emission at 585 nm when excited at 475 nm). Subsequent two-electron oxidation of aminophenol by $ONOO^-$ would give an iminoquinone, as a red-emitting resorufin derivative (emission at 595 nm when excited at 576 nm). However, OCl^- could only oxidize probe **51** to the orange-emitting intermediate, resulted in different signal output. Probe **51** was successfully applied for the detection of endogenous $ONOO^-$ in living cells.



51

Guo's group presented a new strategy for $ONOO^-$ by exploiting the $ONOO^-$-triggered N-oxidation and N-nitrosation reactions of aromatic tertiary amine [66]. The as-designed probe **52** showed weak fluorescence in the solution when excited at 485 nm, resulting from the efficient PET quenching process. However, the reaction of **52** with $ONOO^-$ led to a significant fluorescence enhancement at 517 nm, indicating that PET has been blocked by $ONOO^-$-triggered *N*-oxidation and N-nitrosation. The probe was demonstrated to be highly sensitive and selective for $ONOO^-$ and applied to monitoring the endogenous $ONOO^-$ in living cells and tissues. They also developed mitochondria- and lysosome-targeting fluorescent probes by conjugating triphenylphosphonium and morpholine to the alkoxy group of probe **52**.

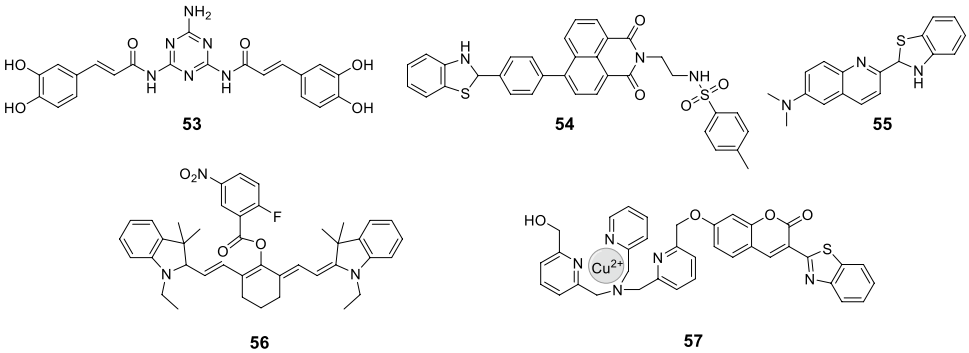


52

2.4 Probes for Superoxide Radical

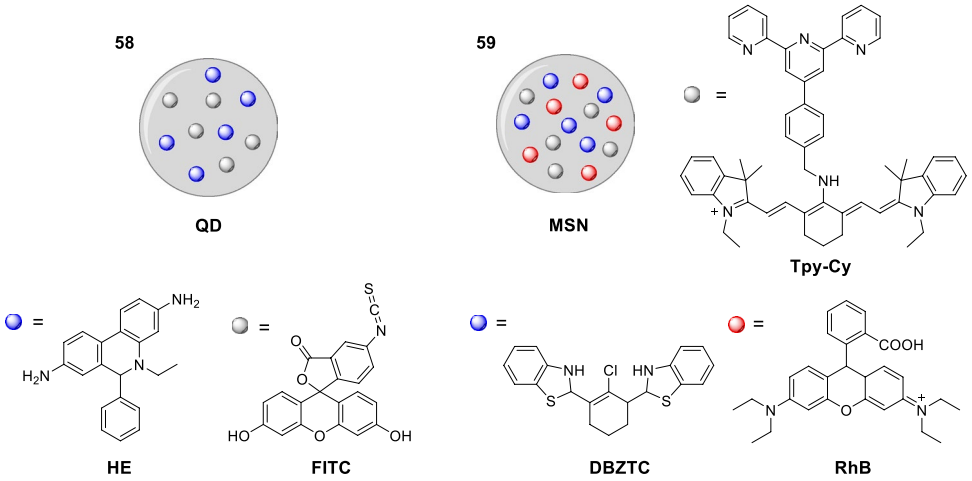
Superoxide radical ($O_2^{\cdot-}$) is the primary ROS generated mainly in mitochondria as a monoelectronic reduction product of O_2 from the mitochondrial electron transport chain, and acts as the precursor of many other ROS. The detection of $O_2^{\cdot-}$ primarily relies on its hydrogen abstraction, elimination of sulfonate and phosphinate, etc.

Tang's group has developed a reversible fluorescent probe **53** with both one-photon and two-photon fluorescence properties by covalently conjugating two caffeic acid molecules with a tripolycyanamide scaffold [67]. The intact probe was non-fluorescence; however, in response to $O_2^{\cdot-}$, a fluorescence intensity increase at 515 nm was observed with one-photon excitation at 491 nm and two-photon excitation at 800 nm. The reversible cycles of probe **53** could be mediated by $O_2^{\cdot-}$ and GSH with at least 3 repeated redox cycles. Moreover, the probe was applied for monitoring $O_2^{\cdot-}$ in cells, zebrafish, and ischemia–reperfusion injury mice model. Tang's group also developed a fluorescent probe **54** for $O_2^{\cdot-}$ [68], in which benzothiazoline acted as a PET quencher to fluorophore 1,8-naphthalimide. After benzothiazoline was dehydrogenated by $O_2^{\cdot-}$ to benzothiazole, the PET process was inhibited, resulting in the dramatical fluorescence intensity enhancement at 450 nm when excited at 360 nm (one-photon) or 700 nm (two-photon). In the meantime, probe **54** exhibited good endoplasmic reticulum-targeting performance. In another study, Liu' group reported a quinoline derivative-based two-photon fluorescent probe **55** [69]. Dehydrogenation of benzothiazoline by $O_2^{\cdot-}$ resulted in an enlarged conjugate system and a co-planar D-π-A structure with obvious intramolecular charge transfer (ICT) process, which accompanied by significant fluorescence intensity enhancement at 550 nm. Yu et al. developed a near-infrared mitochondria-targeting probe **56** with dual channel responses to $O_2^{\cdot-}$ and hydrogen polysulfides (H_2S_n) [70]. The hydrogen abstraction of hydrocyanine by $O_2^{\cdot-}$ resulted in fluorescence increase at 794 nm, indicating the formation of cyanine dye. The further bis-electrophilic reaction of H_2S_n and nitro-activated fluorobenzoiate triggered fluorescence increase at 625 nm, which was attributed to the formation of ketone cyanine. Probe **56** was applied to detecting the mitochondrial $O_2^{\cdot-}$ and H_2S_n successively in RAW264.7 cells and in mice. Au-Yeung et al. reported a strategy for $O_2^{\cdot-}$ detection based on the copper-mediated oxidative bond cleavage reaction [71]. They prepared a copper complex-caged coumarin, probe **57**, which was weakly emissive in Tris buffer but showed a strong fluorescence turn-on response at 488 nm upon addition of $O_2^{\cdot-}$, and was employed for imaging endogenous $O_2^{\cdot-}$ variations in various living cells.

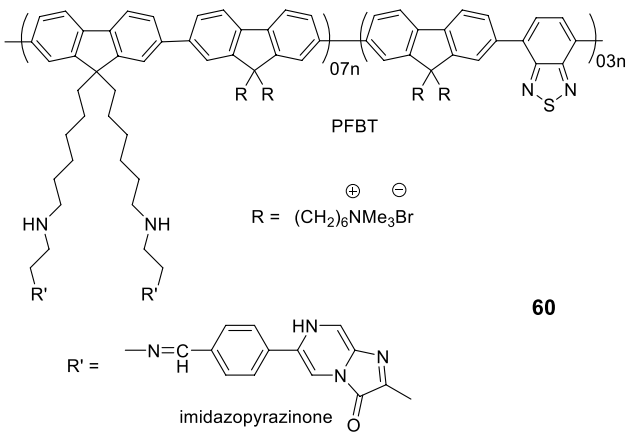


Tian's group has developed a mitochondria-targeting ratiometric nanoprobe **58** for both $O_2^{\cdot-}$ and pH [72]. In probe **58**, the widely used and commercially available fluorophore hydroethidine (HE) and fluorescein isothiocyanate (FITC) were chosen as responsive units for $O_2^{\cdot-}$ and pH, respectively. The silica shell encapsulated CdSe/ZnS quantum dots (QD) were selected as an inner reference as well as a carrier to assemble the responsive molecules and mitochondria-targetable molecules. Upon exposure to different concentrations of $O_2^{\cdot-}$ or different pH, fluorescence in green channel (600–670 nm, ascribed to the oxidation products of HE) enhanced gradually with the increase of $O_2^{\cdot-}$ concentrations, and fluorescence in blue channel (510–570 nm, from FITC) decreased gradually with the decrease of pH values. However, fluorescence from QD (710–800 nm) kept constant. Probe **58** was successfully applied for simultaneous imaging of $O_2^{\cdot-}$ and pH in mitochondria of living cells. Tang's group developed another ratiometric nanoprobe **59** for simultaneous imaging of $O_2^{\cdot-}$ and pH, in which the mesoporous silica nanoparticle (MSN) was loaded with rhodamine B (RhB) as reference and two fluorescent probes, DBZTC for $O_2^{\cdot-}$

measurement and Tpy-Cy for pH detection [73]. The probe has been applied for imaging the dynamics of pH and $O_2^{\cdot-}$ variations in autophagy and apoptosis in HeLa cells.

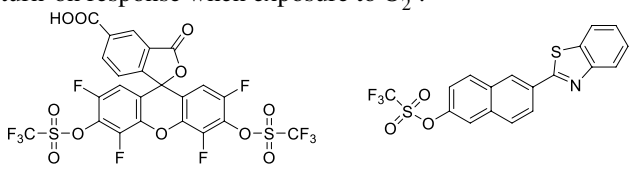


Tang's group developed a chemiluminescence resonance energy transfer (CRET) based $O_2^{\cdot-}$ nanoprobe **60** [74]. The conjugated polymers (PFBT), selected as both the CRET acceptor and signal amplification matrix, were incorporated into nanoparticles by nanoprecipitation. The imidazopyrazinone moiety acted as both recognition unit of $O_2^{\cdot-}$ and the CRET donor. The specific reaction between imidazopyrazinone and $O_2^{\cdot-}$ could produce chemiluminescence at 490 nm, which then transferred to the conjugated polymers, resulting in the magnified emission at 560 nm. The distinguished signal amplification is conducive to the ultrahigh sensitivity of probe **60**, which was applied for imaging $O_2^{\cdot-}$ level differences between normal and tumor tissues of mice without exogenous stimulation.



60

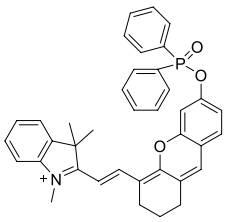
Elimination of sulfonate and phosphinate is an alternative strategy for design of $O_2^{\cdot-}$ probes. Yang's group prepared probe **61** by protecting the 3′- and 6′-position phenol OH group of 5-carboxy-2′,4′,5′,7′-tetrafluorofluorescein with trifluoromethanesulfonate group, which could efficiently quench the fluorescence of fluorescein [75]. The strong electron-withdrawing property of trifluoromethyl group could activate the sulfonate ester toward nucleophilic attack by $O_2^{\cdot-}$, yielding the original fluorophore and a turn-on response. Probe **61** showed high sensitivity and selectivity for $O_2^{\cdot-}$, whereas other ROS (e.g., ·OH, $ONOO^-$ and OCl^-) or nucleophilic reagent (*e.g.*, GSH) hardly produced fluorescence response. Probe **61** may be modified at the 5-carboxy with dimethyl iminodiacetate for better cellular uptake and retention, or with a triphenylphosphonium moiety to prepare mitochondria-targeting probe. Using the trifluoromethanesulfonate group, Zhang et al. developed a novel two-photon probe **62** [76]. The strong electron-withdrawing effect of trifluoromethyl induces an ICT process and leads to almost non-emission of probe **62**, which undergoes a fluorescence turn-on response when exposure to $O_2^{\cdot-}$.



61 62

Diphenyl phosphinate group is another frequently used recognition moiety for $O_2^{\cdot-}$ probes. Compared to sulfonate, diphenyl phosphinate may react faster with $O_2^{\cdot-}$, and the steric hindrance of the two phenyl can prevent interference from other nucleophilic reagent (e.g., GSH). Based on diphenyl phosphinate group, Zhang's group developed a NIR probe **63** with hemicyanine as fluorophore, which has been demonstrated to be nonfluorescent with the phenol

OH blocked [77]. However, the selective deprotection of the diphenyl phosphinate group by $O_2^{\cdot-}$ could result in remarkable fluorescence enhancement at 704 nm. Probe **63** also showed good applicability in living cells, zebrafish and mouse. Tang's group developed probe **64** with two-channel response [78]. Due to the hydrophobicity of tetraphenylethene moiety and other phenyl groups, probe **64** was hardly soluble and formed aggregates with emission at 615 nm in aqueous media. After reacting with $O_2^{\cdot-}$, new aggregates were formed with the emission shifting to 525 nm. Probe **64** can be used for imaging endogenous $O_2^{\cdot-}$ in living cells under different conditions.



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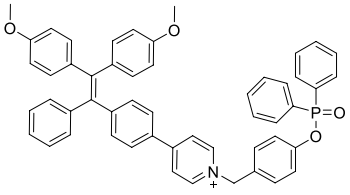
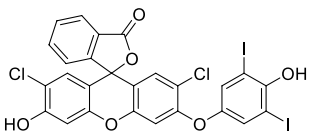


64

2.5 Probes for Hydroxyl Radical

Hydroxyl radical (·OH) is highly reactive, and easily damages biomolecules like DNA, proteins and lipids in cells and tissues. The reaction of ·OH with other molecules generally takes place via several ways, including monoelectronic oxidation, hydrogen abstraction and addition reaction. Therefore, the detection of ·OH also depends on these reaction types.

Similar to other ROS, oxidation reaction is also a major strategy for the design of ·OH probes. Yang's group developed probe **65** for ·OH based on the oxidation of *p*-hydroxyl phenol [79]. Different from the OCl^- probe **23**, introduction of two iodine atoms, instead of chlorine, at the ortho position of the phenolic hydroxyl group could lead to a selective response for ·OH over other ROS, including OCl^- and $ONOO^-$. In the absence of ·OH, probe **65** was nonfluorescent because of the quenching effect of diiodophenol. When exposure to either Fenton reagent or tetrachlorobenzoquinone/H_2O_2 system, rapid and dramatic enhancements in fluorescence intensity were observed. Probe **65** was applied to evaluating the scavenging capacities of seven reported ·OH scavengers. It was also modified to improve its cellular permeability and retention for further application in cell imaging. Liu et al. developed probe **66** based on a hybrid cyanine-phenothiazine platform, in which the sulfur atom of phenothiazine shows a higher electron cloud density, and can be efficiently oxidized by ·OH to yield sulfoxide, accompanying a fluorescence enhancement at 590 nm [80]. The probe has been applied to imaging ·OH in living cells and various types of bacteria, as well as in zebrafish.



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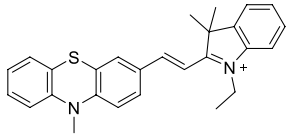
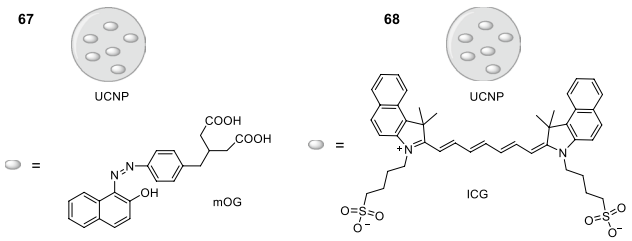


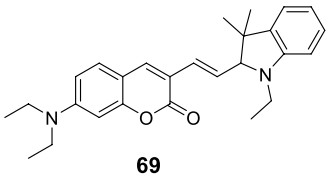
66

Liu's group reported an upconversion nanoprobe **67** [81], in which the upconversion nanoparticles (UCNP) were employed as both energy donor and carriers. On the other hand, an azo dye, modified Orange G (mOG), was used as a responsive unit to ·OH and assembled on the bared surface of UCNPs. In the absence of ·OH, the emission of UCNP could be quenched by mOG efficiently owing to the good overlap between the absorption of mOG and the emission of UCNP. Upon reacting with ·OH, the azo bond of mOG is oxidized and broken, resulting in the recovery of upconversion luminescence at 487 nm under excitation of 980 nm. Probe **67** was demonstrated to be highly sensitive to ·OH with a limit of detection at femtomolar level. Moreover, it was biocompatible for monitoring ·OH in living cells and tissues. Zhou's group developed nanoprobe **68** by modifying UCNP with NIR dye indocyanine green (ICG) as quencher [82]. After reacting with ·OH, ICG was decomposed, resulting in the upconversion emission increase at 654 nm and decrease at 540 nm. Probe **68** was successfully used for the detection of ·OH with colorimetric, upconversion luminescent, as well as photothermal stepped method.



Based on the hydrogen abstraction property of ·OH, Lin's group developed a ratiometric fluorescent probe **69** [83]. The probe showed the typical emission of coumarin dyes at 495 nm; however, after reacting with ·OH in H_2O/CH_3OH (1:1, v/v) media, a significant red-shift in emission to 651 nm was observed, resulting from the conversion of probe **69** to a hybrid coumarin-cyanine platform by ·OH-mediated hydrogen abstraction. Probe **69** was selective for ·OH and applied to detecting endogenous ·OH in live Hela cells. Similarly, they developed another hydrogen abstraction-based probe **70**, which exhibited a turn-on response to ·OH with emission at 604 nm in pH 7.4 PBS buffer (containing 50% DMF) and was used for cell imaging [84]. Kim et al. developed a rhodamine cyclic hydrazide probe **71** for ·OH detection [85]. The probe was colorless and showed negligible fluorescence

in Tris–HCl buffer, due to the spirocyclic structure of rhodamine. Upon treating with ·OH, a strong fluorescence emission at 550 nm was observed, due to hydrogen abstraction of the pyrazolidine moiety and opening of the spirocyclic structure. The probe was also confirmed to be selective for ·OH and applied to imaging ·OH in A549 and RAW264.7 cells.



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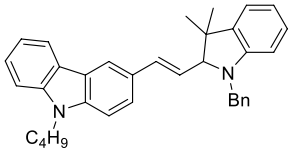

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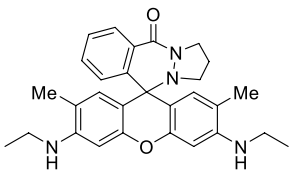
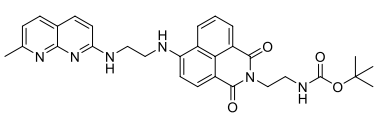


71

One characteristic reactivity of ·OH is the hydroxylation of aromatic rings, which is also employed for the detection of ·OH. This strategy can greatly improve the selectivity for ·OH. Yi's group has designed a ratiometric probe **72** for the detection of ·OH by connecting naphthalimide to naphthyridine [86]. When excited at 371 nm, the intact probe displayed a strong emission at 552 nm and a weaker emission band at 426 nm, which were attributed to naphthalimide group and naphthyridine group. However, the treatment of probe **72** with ·OH caused strong blue emission at 418 nm, resulting from the formation of the hydroxylated naphthyridine derivative without naphthalimide. Probe **72** showed selective ratiometric response to ·OH and was applied to imaging ·OH in RAW264.7 cells. Zhang's group developed probe **73** with dual reactive sites, which could respond to ·OH and OCl^- with distinguishable fluorescence signals [87]. The original probe was nonfluorescent because the spirocyclic structure broke the conjugated system of fluorescein. Upon treating with ·OH, the aromatic rings of probe **73** were first hydroxylated to form an intermediate product, which then simultaneously underwent an aromatic ring-breaking and a spirocycle-opening process by the ultrastrong oxidizability of ·OH. The final product has two conjugated aromatic rings, and gives a strong cyan fluorescence at 486 nm upon excitation at 410 nm. On the other hand, treatment with OCl^- caused the oxidative hydrolysis of hydrazide to form the fluorescein chromophore with emission at 520 nm upon excitation at 490 nm. Probe **73** could be used for distinguishable detection of ·OH and OCl^- without interference from other ROS, and real-time discrimination and quantitative analysis of the two ROS in living cells and zebrafish.



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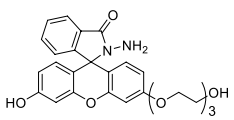

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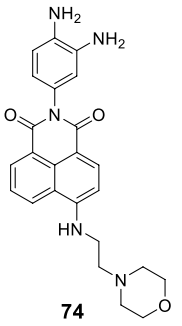
2.6 Probes for Nitric Oxide

Nitric oxide (NO) is known as a cellar messenger molecule, which plays an important role in various physiological and pathological processes. In mammalian cells, endogenous NO is mainly generated from L-arginine under catalysis of inducible nitric oxide synthases in mitochondria. The detection of NO is mainly based on the following three strategies: using *o*-phenylenediamine, transition metal complex, and the newly reported Se-N bond formation. There are also several probes reported in other types.

The electron-rich *o*-phenylenediamine is the most frequently used NO recognition group, and based on it numerous fluorescent probes for NO have been developed. Probe **74** is a lysosome-targeting two-photon probe, which shows high sensitivity and selectivity for NO over other ROS [88]. The electron-rich amino groups can effectively quench the fluorescence of naphthalimide by PET process. However, upon reacting with NO under aerobic conditions, the *o*-phenylenediamine moiety is converted to an electron-deficient benzotriazole group, which inhibits the PET process to generate a fluorescence turn-on response. With probe **74**, the capture of NO within lysosomes of macrophage cells has been achieved by both two-photon fluorescence microscopy and flow cytometry. Probe **75** is a mitochondria-targeting probe, which, upon reaction with NO, exhibits a large fluorescence enhancement resulting from the spirocyclic opening of rhodamine lactam structure [89], whereas probe **76** is a ratiometric probe constructed on a BODIPY-rhodamine scaffold [90]. Liu's group reported a two-photon probe **77** with PET mechanism [91]. The probe shows two-photon fluorescence response toward NO and can be applied to detecting NO in live cells and tissues. Although *o*-phenylenediamine group is widely used for NO detection, there still remain some shortcomings. One is that benzotriazole is pH sensitive, and its deprotonation at physiological pH can result in undesirable fluorescence decrease, thereby lowering the NO detection sensitivity. Moreover, *o*-phenylenediamine is reported to react with dehydroascorbic acid, ascorbic acid or methylglyoxal to induce fluorescence signal changes. To solve these problems, Guo's group developed a new strategy by direct conjugation of a fluorophore with one amino of *o*-phenylenediamine [92]. The as-designed probe **78** displays dual-channel response to NO at 536 nm and 618 nm helped by Cys and GSH,

respectively, as well as high sensitivity and selectivity for NO. Probe **78** is also confirmed to be well mitochondria-targetable.

Ma's group presented a new strategy for the detection of NO by utilizing the interaction of NO with selenide [95].



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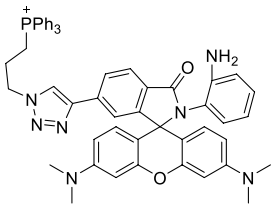

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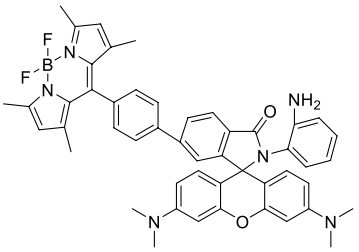

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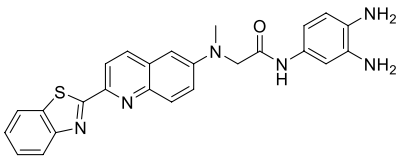

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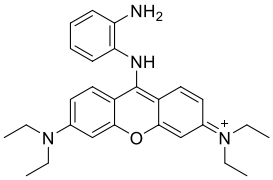

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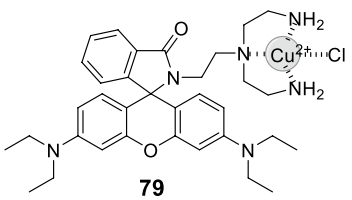
In transition-metal complex probes, the transition metal in close proximity can act either as a quencher to the appended fluorophore or as a promoter to facilitate reaction of NO with the probe. Duan's group developed a Cu(II) complex probe **79** [93]. The Cu(II) center is surrounded by three nitrogen donors and one chloride anion to form a planar square geometry, with available coordination sites in the axial position for NO. Upon reacting with NO, Cu(II) is reduced to Cu(I) with concomitant nitrosation of the amide nitrogen to open the spirolactam, resulting in obvious fluorescence enhancement at 580 nm. Qian's group reported an *o*-phenylenediamine-based probe **80** with a pendant quinoline moiety for binding Cu(II), which acts as a promoter for nitrosation of the two nitrogen atoms in *o*-phenylenediamine moiety to form two different *N*-nitrosation products and finally generate the same triazole product with fluorescence emission at 440 nm [94]. Despite the progress in the transition-metal complex probes, there still exist several disadvantages such as biological incompatibility, easy leakage from cells, or side effect of the metal ion.



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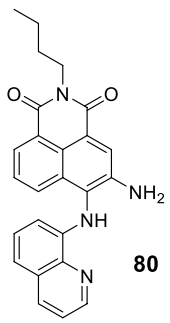

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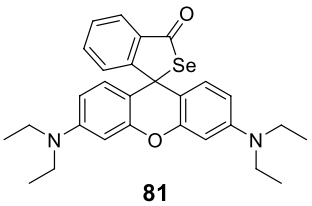
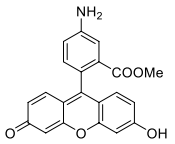
Compound **81** is such a probe, which can overcome the above-mentioned disadvantages of *o*-phenylenediamine- or transition metal complex-based probes to a large extent. Probe **81** itself emitted very weak fluorescence due to the spirocyclic structure of rhodamine B selenolactone. Upon reacting with NO, the probe exhibited a large fluorescence enhancement with emission at 580 nm, resulting from the opening of its spirocyclic structure by the formation of an unstable intermediate with Se-NO bond, which was subsequent hydrolyzed into rhodamine B. Probe **81** showed a high selectivity for NO and was successfully applied to imaging NO in living cells.



81

NO can trigger reductive deamination of an aromatic primary monoamine, thereby altering the electronic influence of the aromatic moiety on the fluorophore to modulate the fluorescence signal. This reaction was employed by Wang's group to develop probe **82** [96]. The fluorescence of the probe was efficiently quenched by the electron-rich aromatic primary monoamine group through PET process. However, treatment with NO resulted in deamination of the aromatic primary monoamine group with concomitant fluorescence

recovery at 524 nm. The probe was selective for NO and applied for endogenous NO detection in RAW 264.7 cells. In another study, Guo's group developed probe **83** and **84** with *p*-methoxyaniline as reaction site [97]. Both the probes showed fast fluorescence response and high selectivity, and were used for imaging NO in living cells.



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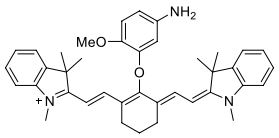

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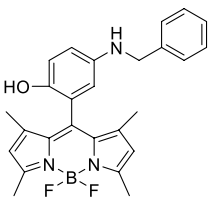
Based on the nitrosation reaction of the electron-rich aromatic secondary amine with NO under aerobic condition and the widely used PET switching mechanism, Guo's group presented a new strategy to construct fluorescent probe **85** for NO, in which the *N*-benzyl-4-hydroxyaniline moiety serves as not only NO reaction group but also PET quenching unit, and the BODIPY dye as fluorescence unit [98]. Treating probe **85** with NO could result in nitrosation of the secondary amine, thereby inhibiting the PET process and recovering the fluorescence of BODIPY. Probe **85** showed high selectivity and sensitivity to NO, and was further modified with triphenylphosphonium for monitoring NO in mitochondria. In addition, Liu's group developed a NIR two-photon fluorescent probe **86** with 4-methoxy-*N*-methylaniline moiety as NO recognition site [99]. Fluorescence turn-on response could take place upon nitrosation of the methylamino group by NO. The probe enabled the detection of NO in living cells and the xenograft tumor mouse model.



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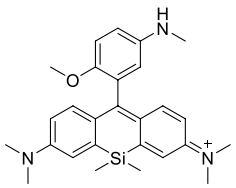

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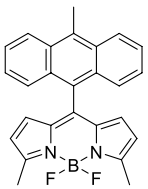
2.7 Probes for Singlet Oxygen

Singlet oxygen ($^1O_2$) is the lowest excited state of molecular oxygen. It is usually involved in photodynamic therapy for its biotoxicity to various kinds of molecules. The detection of $^1O_2$ mainly depends on cycloaddition of anthracene moiety [4, 8]. Based on this strategy, Ma et al. have proposed a highly selective and sensitive chemiluminescence $^1O_2$ probe, 4,5-dimethylthio-4′-[2-(9-anthryloxy)- ethylthio]tetrathiafulvalene [100]. The probe was prepared by the incorporation of electron-rich tetrathiafulvalene unit into the reactive luminophore of anthracene specific for $^1O_2$, and exhibited both strong chemiluminescence response to and high selectivity for $^1O_2$ only, rather than the other ROS, such as H_2O_2, OCl^-, $O_2^{\cdot-}$ and ·OH. This remarkable chemiluminescence property makes the probe possible to be used widely for $^1O_2$ detection in many chemical and biological systems. This applicability has been demonstrated by monitoring the $^1O_2$ generation in a metal-catalyzed decomposition system of tert-butyl hydroperoxide. Moreover, studies on the chemiluminescence reaction mechanism of the probe revealed that the introduction of an electron-rich moiety into the 9-position of anthracene can greatly activate its reactivity towards $^1O_2$. Using the trapping unit of anthracene, Filatov et al. have developed two BODIPY-anthracene dyads **87** and **88** [101]. The unique donor–acceptor structures of the two compounds make them act as efficient heavy atom-free photosensitizers to excite the ground state molecular oxygen to form $^1O_2$. On the other hand, both the two compounds exhibit obvious fluorescence enhancement in response to $^1O_2$. Kim et al. developed a far-red probe **89** for $^1O_2$ [102]. The probe is composed of a dimethylanthracene moiety as recognition group and a Si-rhodamine fluorophore. Upon reacting with $^1O_2$, a great fluorescence enhancement was observed as a result of anthracene cycloaddition by $^1O_2$ and inhibition of PET process. Probe **89** was applied to monitoring $^1O_2$ generated during photodynamic therapy.



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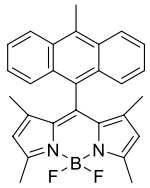

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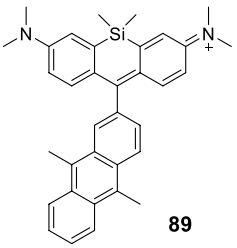

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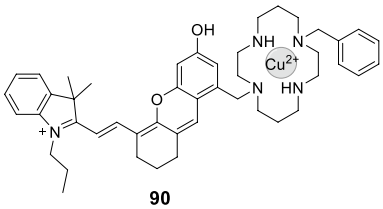
2.8 Probes for Nitroxyl

Nitroxyl (HNO) is the one-electron reduced and protonated product of NO. It shows very different biological functions from NO. A great of efforts have been made for detection of HNO. One major approach is based on the HNO-induced reduction of Cu(II) to Cu(I). Recently, Wrobel et al. developed such a NIR probe **90** for HNO [103]. The probe comprises a Cu(II)-cyclam complex and exhibits weak fluorescence emission due to the paramagnetic quenching of Cu(II). Upon reacting with HNO, Cu(II) is reduced to Cu(I) and released from the complex. As a consequence, fluorescence intensity increase at 715 nm is produced. The probe is selective for HNO and can detect exogenous HNO in live mammalian cells. Alternatively, by taking advantage

of triarylphosphine as recognition group, Lin's group developed a two-photon probe **91** for HNO [104]. The intact probe showed relatively weak fluorescence; however, in the presence of HNO, a drastic fluorescence enhancement at around 512 nm was observed, resulting from the reaction of HNO with triarylphosphine to give the corresponding phosphine oxide and release of the fluorophore. Probe **91** was applied for two-photon imaging of exogenous HNO in living cells and fresh rat liver slices.



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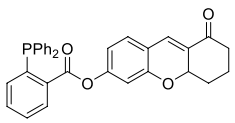

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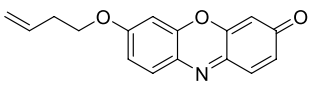
2.9 Probes for Ozone

Ozone (O_3) has also attracted much attention because of its key role in human health and disease. Ma's group developed probe **92** for O_3 by incorporating the but-3-enyl group (as specific recognition site for O_3) into a resorufin fluorophore [105]. The fluorescence of resorufin is efficiently quenched by 7-hydroxy substitution. However, upon reacting with O_3, the but-3-enyl group can be selectively cleaved, accompanied by release of resorufin and dramatic fluorescence enhancement at 585 nm. The probe is rather sensitive to O_3 with a detection limit at nanomolar level and has been used for the detection of O_3 in ambient air samples and living cells.



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3 Conclusions and Future Perspectives

In the review, we have summarized and discussed a series of representative spectroscopic probes for the common ROS developed in the recent 5 years according to design strategies and recognition groups. Most of these probes exhibit excellent analytical performance and are applied to the real biosamples. However, there are still several obvious problems to be solved in the future development of selective and sensitive probes for ROS. One of the major problems is that many current probes based on oxidation reaction suffer from oxidative interferences from other ROS. For example, the most frequently used recognition group for H_2O_2 also reacts with OCl^- and $ONOO^-$. To improve the selectivity, an alternative approach may be to employ a specific chemical reaction, such as the hydroxylation of aromatic compounds by ·OH, and nucleophilic reaction of $O_2^{\cdot-}$. For practical applications of the probes in biochemical studies, the probes should be water-soluble without additional organic solvent (e.g., CH_3CN, DMF, EtOH) as cosolvent and work well under physiological conditions. Particularly, because of the low concentration and short lifetime of some ROS in biological samples, it is still necessary to further develop ROS probes with higher sensitivity and stronger trapping ability (*e.g.,* introducing stronger electron donors to the 9,10-position of anthracene core is beneficial to construct a $^1O_2$ probe with faster trapping ability).

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