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Redox-Responsive Fluorescent Probes with Different Design Strategies

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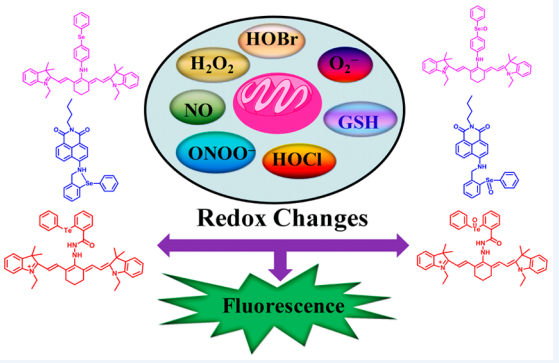
CONSPECTUS: In an aerobic organism, reactive oxygen species (ROS) are an inevitable metabolic byproduct. Endogenously produced ROS have a significant role in physiological processes, but excess ROS can cause oxidative stress and can damage tissue. Cells possess elaborate mechanisms to regulate their internal redox status. The intracellular redox homeostasis plays an essential role in maintaining cellular function. However, moderate alterations in redox balance can accompany major transitions in a cell's life cycle. Because of the role of ROS in physiology and in pathology, researchers need new tools to study redox chemistry in biological systems.



In recent years, researchers have made remarkable progress in developing new, highly sensitive and selective fluorescent probes that respond to redox changes, and in this Account we highlight related research, primarily from our own group. We present an overview of the design, photophysical properties, and fluorescence transduction mechanisms of reported molecules that probe redox changes. We have designed and synthesized a series of fluorescent probes for redox cycles in biological systems relying on the active center of glutathione peroxidase (GPx). We have also constructed probes based on the oxidation and reduction of hydroquinone and of 2,2,6,6-tetramethylpiperidinooxy (TEMPO). Most of these probes exhibit high sensitivity and good selectivity, absorb in the near-infrared, and respond rapidly. Such probes are useful for confocal fluorescence microscopy, a dynamic imaging technique that could allow researchers to observe biologically important ROS and antioxidants in real time. This technique and these probes provide potentially useful tools for exploring the generation, transport, physiological function, and pathogenic mechanisms of ROS and antioxidants.

We also describe features that could improve the properties of redox-responsive fluorescent probes: greater photostability; rapid, dynamic, cyclic and ratiometric responses; and broader absorption in the near-IR region. In addition, fluorescent probes that include organochalcogens such as selenium and tellurium show promise for a new class of fluorescent redox probes that are both chemically stable and robustly reversible. However, further investigations of the chemical and fluorescence transduction mechanisms of selenium-based probes in response to ROS are needed.

INTRODUCTION

In an aerobic organism, reactive oxygen species (ROS) are derived from the reduction of molecular oxygen in the process of the metabolism. And ROS include peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), and hypochlorous acid/hypochlorite ($HOCl/^-OCl$), hypobromous acid/hypobromite ($HOBr/^-OBr$), superoxide ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and peroxyl radical ($ROO\cdot$).[1−3] For instance, $ONOO^-$ is a powerful and toxic oxidant derived from nitric oxide and superoxide.[4] In vivo, $ONOO^-$ can contribute to cell signaling and homeostasis regulating; on the other hand, overproduction of $ONOO^-$ would induce oxidative injury, which is linked with many diseases such as diabetes, cancer, and neurodegenerative disorders.[5] Endogenous HOCl is produced from H_2O_2 and chloride ions (Cl^-) catalyzed by the heme enzyme myeloperoxidase (MPO) in the neutrophils. At physiologic pH levels, HOCl exists as a mixture of the undissociated acid and the hypochlorite ion due to its pK_a of 7.53. In vivo, HOCl plays an essential role in the antimicrobial system.[6] However, HOCl may also lead to extensive oxidative stress via oxidizing or chlorinating the biomolecules, such as the lipids, nitrogen-containing compounds, and nucleic acids.[7] HOBr has the similar chemical and physical properties to those of HOCl, and the endogenous HOBr can be generated from H_2O_2 with Br^- catalyzed by eosinophil peroxidase (EPO).[8] Similarly, HOBr can react with thiols, thioethers, and amino acids, promoting oxidative tissue injury.

Cells possess an elaborate antioxidant defense system to regulate their internal redox equilibrium.[9,10] Given the essential biological functions of endogenously produced ROS, for instance, signal transduction, neurotransmission, and blood pressure modulation, ROS are not completely scavenged.[11,12] However, increased production of ROS or decreased levels of antioxidants would induce oxidative stress through the

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oxidation of biomolecules, such as the lipids of cellular membranes, DNA, and proteins, leading to tissue damage.[13−15] And the imbalance of the cellular redox indicates a crucial pathogenic mechanism in conditions such as inflammatory diseases, cancer, and neurodegenerative disorders. In other words, intracellular redox homeostasis governs numerous essential biological processes and presents broad implications in human health and diseases[2,9] (Figure 1). From this

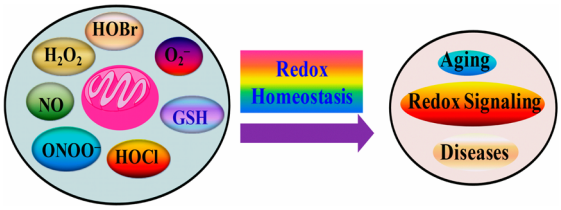

Figure 1. Primary ROS and reductant in cells, and the pathophysiological implications of their redox balance.

perspective, the elucidation of the biological functions of intracellular redox changes between ROS and antioxidants has become an important area of research. Fluorescence imaging presents the advantages of higher sensitivity, better selectivity, greater convenience, and less invasiveness compared with other approaches, and has thereby been considered to present a potentially powerful methodology for studying various species in biosystems.[16,17]

In recent years, a large amount of reaction-based fluorescent probes for ROS have been reported, whereas the development of reversible probes is relatively slow.[18,19] Several fluorescent probes based on fluorescent protein have been developed to monitor dynamic redox changes.[20−25] However, techniques requiring gene manipulation and redox-responsive probes based on small molecules are scarce. Chang's group developed the first small molecular fluorescent probe for monitoring reversible oxidation and reduction activity in living systems.[26] Relying on the active center of glutathione peroxidase (GPx), we have recently designed and synthesized a series of fluorescent probes for redox changes in biological systems.[27−32] Afterward, the area of research in redox-responsive small molecular fluorescent probes captured greater researchers' attention. Specifically, the primary design strategies are mimicking the GPx activity, oxidation, and reduction of hydroquinone or 2,2,6,6-tetramethylpiperidinooxy (TEMPO). Although Churchill et al. have reviewed recent advances in fluorescent probes containing selenium and tellurium for biologically important analytes,[33] the small molecular fluorescent probes for redox changes have not been systematically summarized to our knowledge. In this Account, from the perspective of different reaction mechanisms of probes responding to redox changes, we will present an overview of redox-responsive small molecular fluorescent probes mainly reported by our group. And we hope this account would provide a favor to those who are interested in this continually growing research field.

REDOX RESPONSIVE FLUORESCENT PROBES BASED ON GPx MIMICS

GPx is a selenoenzyme that serves by protecting various organisms from oxidative damage, through catalyzing the reduction of hydroperoxides by glutathione (GSH).[34] To clarify the catalytic mechanism of GPx and exploit antioxidant drugs, mimicking the function of GPx is currently capturing considerable interest.[34,35] During the development of GPx mimics, organoselenium compounds, which include monoselenides, diselenides, and ebselen analogues, have become the major GPx mimics on account of their ability to catalyze the reduction of hydroperoxides by glutathione (GSH) or other thiols.[34−37] (Figure 2).

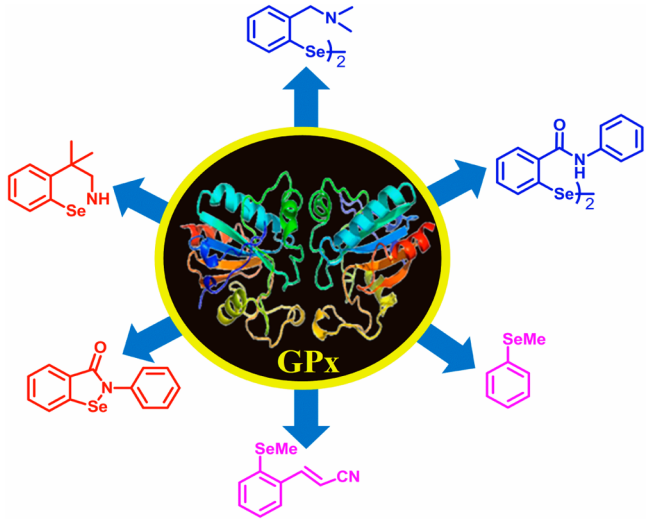

Figure 2. X-ray crystal structure of GPx (PDB code: 1GP1).[38] Structures of several GPx mimics reported in the literature adapted with permission from ref 34. Copyright 2010 American Chemical Society.

In this section, on the basis of GPx mimics, we highlight the fluorescent probes for the change of redox couples, mainly $ONOO^-$/thiols, HOCl/thiols, HOBr/thiols and GSH/ROS. And the design strategies of the probes contain oxidation and reduction of selenium, oxidation and reduction of tellurium, and cleavage of diselenide bonds.

OXIDATION AND REDUCTION OF ORGANOSELENIUM COMPOUNDS

In 1990, Detty and co-workers developed mitochondrial targeting cationic chalcogenapyrylium dyes containing selenium and/or tellurium. The dyes were oxidized by singlet oxygen upon irradiation and gave yellow-green fluorescence with a maximum emission wavelength of 580 nm in the mitochondria. This was the first example of fluorescence switching due to ROS through oxidizing selenium or tellurium.[39] In the course of our ongoing studies on developing redox-sensitive fluorescent probes, we have mimicked the GPx activity center and exploited the first near-infrared (NIR) reversible fluorescent probe **1** for $ONOO^-$ based on heptamethine cyanine dye (Figure 3A).[27] In this probe, heptamethine cyanine dye with NIR fluorescence was selected as the fluorophore, and 4-(phenylselanyl)aniline is used as a fluorescence modulator. Similar to the GPx catalytic cycle, probe **1** can be oxidized by $ONOO^-$ to produce the corresponding selenoxide **1′**, which upon reduction by thiols produces selenide **1** again. The fluorescence of probe **1** is quenched through a rapid photoinduced electron-transfer (PET) process between the modulator and the heptamethine cyanine dye in the excited state. However, the PET process of probe **1** is blocked and the fluorescence intensity increased by ~23.3-fold and the quantum yield increased from 0.05 to 0.12 after the addition of $ONOO^-$

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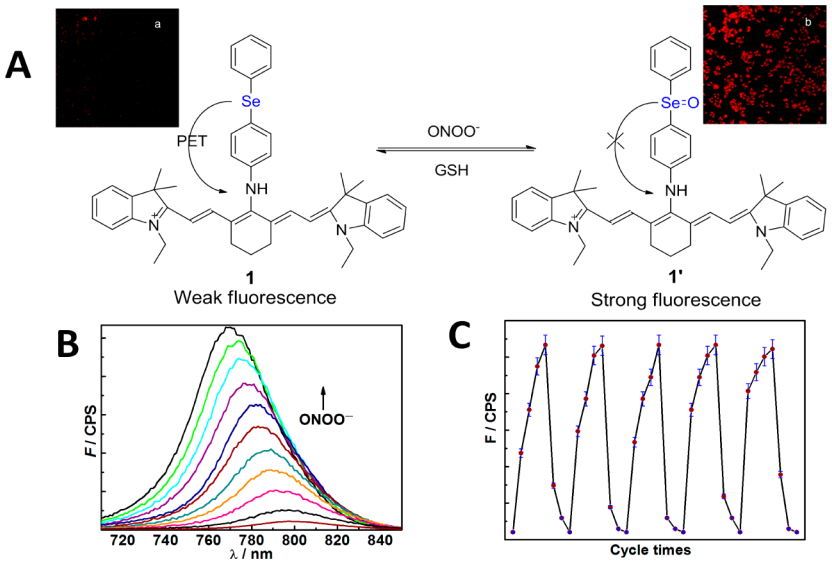

Figure 3. (A) Structures of probe **1** and its oxidized form **1′**, and the proposed fluorescence switch mechanism. (B) Fluorescence spectra of probe **1** after the addition of different $ONOO^-$ concentrations. (C) Fluorescence responses of probe **1** to $ONOO^-$/GSH redox cycles. Reproduced with permission from ref 27. Copyright 2011 American Chemical Society.

(Figure 3B). As a result, the fluorescence of the probe is switched "on" and "off" when responding to $ONOO^-$ and GSH, respectively (Figure 3C).

Probe **1** exhibited excellent selectivity in monitoring $ONOO^-$ screening to other ROS. Besides, only the thiols can turn off the fluorescence. The maximun absorption and emission wavelength of the probe is 758 and 800 nm, respectively, which avoids the influence of autofluorescence in biological systems. Moreover, the probe is of low toxicity toward cultured RAW264.7 cells due to the half maximal inhibitory concentration (IC_{50}) value being 500 μM. And the probe is capable of monitoring $ONOO^-$ oxidative stress and GSH reducing repair through reversible fluorescence response in living RAW264.7 cells.

In addition, we have theoretically studied the PET mechanism of the probe **1** using density functional theory (DFT) and time-dependent density functional theory (TD-DFT) calculations that possessing moderate efficiency and accuracy.[27] According to the calculated Frank−Condon factor (f) reported by ref 27, the low-lying excited singlet state S_1, S_2 of selenide **1** is a dark state (f = 0.013) and the S_3 state is a bright one (f = 1.617). In contrast to selenide **1**, the S_1 state of selenoxide **1′** is a radiative state (f = 1.014). In other words, the bright state equilibrium populations of **1′** will be increased so that the fluorescence is enhanced. These calculations conform to the experimental results and the PET mechanism of **1** is fully rationalized from the electronic states perspective, see Figure 4.

As mentioned above, selenium is a versatile center of GPx functional mimics. Taking advantage of the redox property of selenium, we have developed a reversible fluorescent probe **2** for the redox events between HOCl and hydrogen sulfide (H_2S) (Figure 5).[31] In probe **2**, BODIPY dye possessing robust photo and chemical stabilities, high absorption coefficients, and fluorescence quantum yields was utilized as the dye moiety, and 4-methyoxylphenylselanyl benzene was selected as the fluorescence regulater. Probe **2** emits faint fluorescence, which is due to a fast PET process from the electron-rich Se moiety to the BODIPY dye. Upon addition of HOCl, the selenide is oxidized to be the electron-deficient selenoxide.

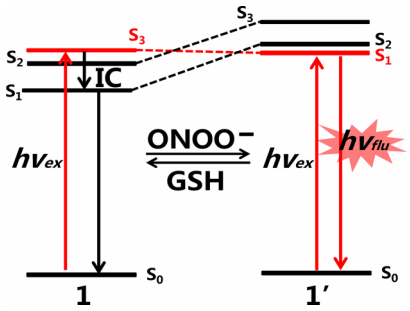

Figure 4. Schematic view of the reversible fluorescence transduction of **1** for $ONOO^-$ and GSH. The excited states involved in the excitation and the emission processes were based on the calculated results reported by ref 27.

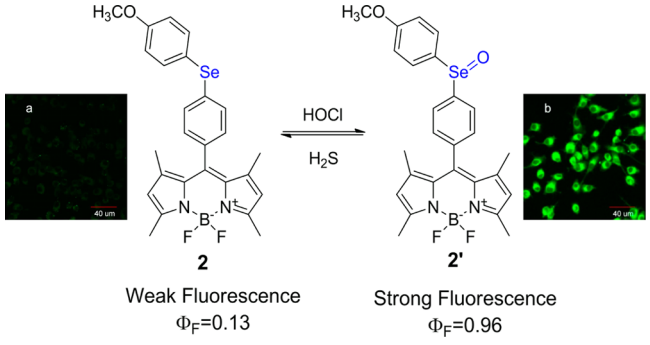

Figure 5. Structure of probe **2** and sensing mechanism for $HOCl/H_2S$ redox couple. Reference 31. Reproduced by permission of The Royal Society of Chemistry.

Accordingly, the PET process is blocked and the fluorescence is turned "on" with the quantum yield increased from 0.13 to 0.96. The maximum wavelength of the absorption and fluorescence lies at 500 and 510 nm, respectively.

As a reversible fluorescent probe for the oxidation and reduction cycles, probe **2** exhibits excellent selectivity to the HOCl and H_2S. In addition, this probe has been applied to

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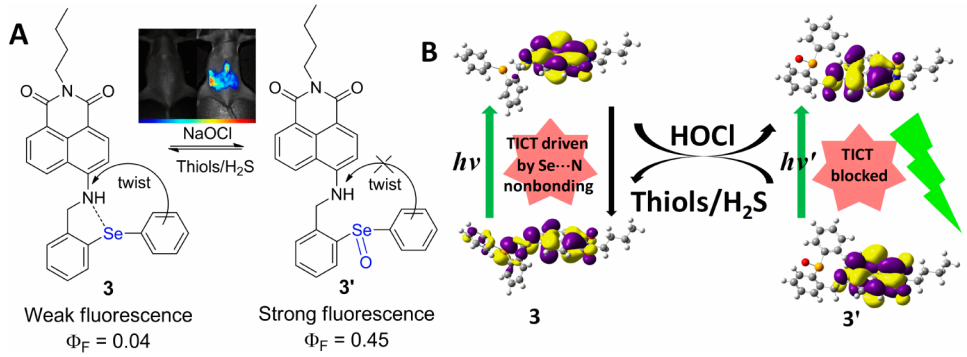

Figure 6. (A) Structures of probe **3** and its oxidized product **3′**. Reference 28. Reproduced by permission of The Royal Society of Chemistry. (B) Frontier molecular orbitals of **3** and **3′**, and the proposed detecting mechanism for HOCl/thiols redox couple. Reference 40. Reproduced by permission of the PCCP Owner Societies.

visualize HOCl/H_2S redox cycle in living RAW264.7 cells using the confocal fluorescence microscopy.

Meanwhile, we have also designed and developed a reversible fluorescent probe **3** for HOCl by mimicking the GPx center (Figure 6A).[28] The fluorescence response mechanism of this probe is different from those of our previous reported probes. The Se and N nonbonding interaction is utilized to modulate the fluorescence of selenide **3** and its oxidized form **3′**. In the probe **3**, the selenium containing moiety twists in the excited state driven by the Se···N nonbonding interaction and the fluorescence is quenched. When the selenium is oxidized by HOCl, the Se···N nonbonding interaction is blocked by the presence of an oxygen atom. As a result, the configuration twisting is prevented and the fluorescence is enhanced with the quantum yield increased from 0.04 to 0.45. The mechanism of fluorescence transduction in probe **3** responding to HOCl has been fully rationalized by combining DFT/TD-DFT calculations and experimental study (Figure 6B).[40]

Probe **3** exhibits several advantages in terms of selectivity, sensitivity, and response time. Concretely, only HOCl is capable of oxidizing probe **3**; H_2S and high concentrations of GSH can reduce the oxidized form **3′**. The limit of detection for HOCl is 5.86×10^7 M, and the probe can respond to HOCl in 8 min. In addition, the probe has low toxicity to cultured cell lines under experimental conditions. And we have applied this probe to visualizing HOCl oxidative stress and reducing repair with GSH and H_2S in living cells and living mice.

Another NIR reversible fluorescent probe **4** for the oxidation−reduction cycles mediated by HOBr and H_2S has been developed by our group (Figure 7).[30] In the same manner, the reversibility of the probe is achieved by means of the versatile selenium. The probe **4** is based on the high performing BODIPY dye and the fluorescence spectra lie in the NIR region with an emission maximum at 711 nm. After addition of HOBr, the fluorescence intensity localized at 711 nm decreased and a new emission band centered at 635 nm increased. This phenomenon is explained by electron donating ability of "selenoxide" being weaker than that of "selenide", which shortened the donor−π−acceptor conjugated system. The significant variation in fluorescence wavelength enables **4** to monitor the HOBr/H_2S redox cycles ratiometrically, which can eliminate the influences of polarity, probe concentration, and photobleaching.

The probe is highly sensitive and specific to the detection of HOBr/H_2S redox cycle. The detection limit of the probe **4** for HOBr is determined to be 9.7×10^{-7} M in the testing

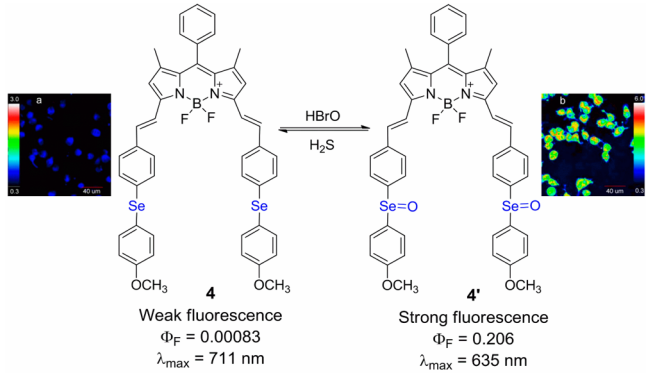

Figure 7. Structure and sensing mechanism of probe **4** for HOBr/H_2S redox couple. Reference 30. Reproduced by permission of The Royal Society of Chemistry.

conditions. Furthermore, the fluorescent probe **4** has been utilized to real-time imaging of the HOBr/H_2S redox cycles in living RAW264.7 cells, which indicates the promising application of this probe.

Developing novel fluorescent probes for redox changes with subcellular localization will provide new access to exploring the generation, metastasis, and physiological functions of the redox homeostasis in specific organelles. Recently, we have reported a lysosome-targetable fluorescent probe **5** with selenide as the modulator for monitoring HOCl/GSH redox couple, Figure 8.[41] This probe exhibited a fast, selective, and sensitive fluorescence response to HOCl, as well as capability to detect HOCl in lysosomes of living cells.

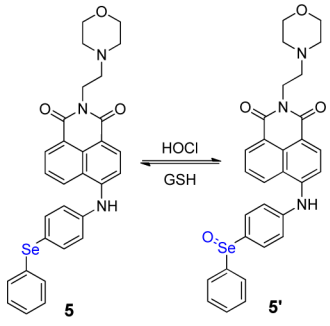

Figure 8. Structures of probe **5** and its oxidized form. Adapted with permission from ref 41. Copyright 2015 Elsevier B.V.

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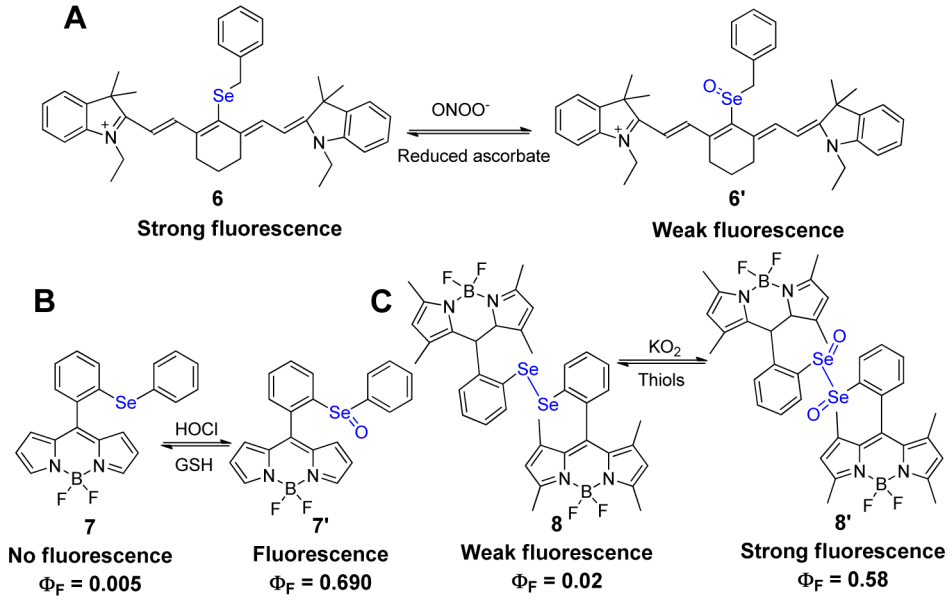

Figure 9. Structures of the reported probes **6**–**8** for detecting oxidation and reduction cycles. (A) Redox cycle of probe **6**. Reference 42. Adapted by permission of The Royal Society of Chemistry. (B) Structures of probe **7** and its oxidized form **7′**. Adapted with permission from ref 43. Copyright 2013 American Chemical Society. (C) Probe **8** and the detection mechanism for O_2^-/thiols couple. Adapted with permission from ref 44. Copyright 2014 American Chemical Society.

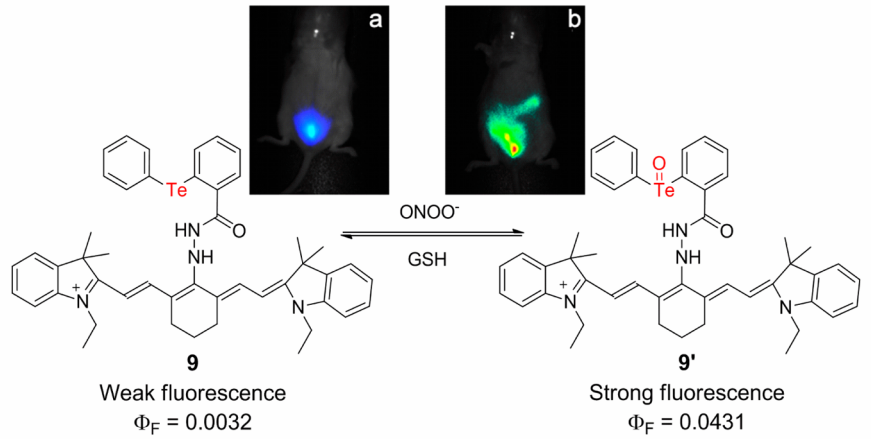

Figure 10. Structure of the fluorescent probe **9** for $ONOO^-$/GSH redox couple. Reproduced with permission from ref 32. Copyright 2013 American Chemical Society.

Utilizing the same design strategy of the redox properties of selenide, Tang et al. synthesized a reversible NIR fluorescent probe **6** for the changes between $ONOO^-$ and reduced ascorbate with an "on–off–on" fluorescence response[42] (Figure 9A). Wu and Liu developed a BODIPY-based fluorescent probe **7** for the detection of redox changes between HOCl and GSH[43] (Figure 9B). Churchill et al. developed a diselenide-based reversible fluorescent probe **8** for the selective and sensitive detection of superoxide and thiols (Figure 9C).[44]

OXIDATION AND REDUCTION OF ORGANOTELLURIUM COMPOUNDS

Compared with organoselenium compounds, organotellurium compounds exhibit more compelling antioxidant activity and display considerably higher GPx-like activity.[45−47]

Choosing a heptamethine cyanine dye as signal transducer due to its NIR absorption and emission profiles, and integrating 2-(phenyltellanyl)benzohydrazide as a fluorescent modulator into the fluorophore relied on its unique redox property. We have recently designed a NIR reversible fluorescent probe **9** for detecting the changes of $ONOO^-$/thiols redox couple (Figure 10).[32]

Basing on the PET mechanism, the probe **9** displays an "off–on–off" reversible fluorescence response to $ONOO^-$ and thiols redox cycles by oxidation and reduction of the tellurium atom. As a reversible fluorescent probe, **9** exhibits high sensitivity, selectivity to $ONOO^-$ and thiols, NIR excitation and emission wavelength (λ_{ex} = 793 nm, λ_{em} = 820 nm), and mitochondrial target. The IC_{50} obtained by MTT assay is 160 μM, which demonstrated that the cytotoxicity of the probe is low. Furthermore, the probe can be applied to visualization of the changes of oxidation and reduction cycles between the outbreak of $ONOO^-$ and the GSH repair in living RAW264.7 cells, as well as in living mice.

On the basis of the redox properties of the tellurium atom, Nagano et al. designed and synthesized a reversible NIR

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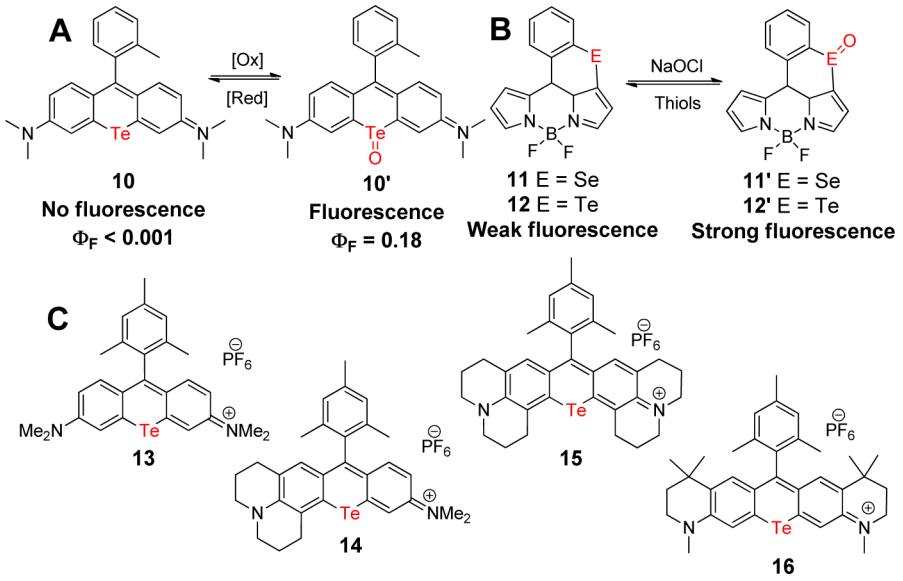

Figure 11. Structures of the fluorescent probes **10**–**16** for oxidation and reduction cycles. (A) Structures of probe **10** and its oxidized form **10**′. Reference 48. Adapted by permission of The Royal Society of Chemistry. (B) Structures of probes **11** and **12**, and the sensing mechanism for HOCl/thiols couple. Adapted with permission from ref 49. Copyright 2014 American Chemical Society. (C) Structures of tellurorhodamine dyes **13**–**16**. Adapted with permission from refs 50 and 51, respectively. Copyright 2013 and 2014 American Chemical Society.

fluorescent probe **10** for ROS, (Figure 11A).[48] However, the probe exhibits poor specificity, and ·OH, $ONOO^-$, and HOCl can oxidize the probe. Churchill et al. synthesized selenium and tellurium containing compounds **11** and **12** from their respective bis(*o*-formylphenyl) dichalcogenide intermediates to detect hypochlorite and thiols selectively (Figure 11B).[49] Detty and co-workers reported tellurorhodamine dyes with high stabilities **13**–**16** by introducing different substituents at the 9-position (Figure 11C).[50,51] The dyes can be oxidized by $^1O_2$ via self-photosensitization to the corresponding telluroxides and be readily reduced by GSH, which allow monitoring of redox cycling and provides more robust reversible systems for continuous redox processes in the cells.

CLEAVAGE OF DISELENIDE BOND

Intracellular thiol plays an essential role in protecting cell against oxidative stress, of which GSH is the most abundant nonprotein thiol.[52] The intracellular thiol concentration can fluctuate dynamically during regulating the redox status.

Diaryl diselenides is one of the major categories of GPx mimics reported in the literature.[34,53] Taking advantage of the result stating that the cleavage of diselenide bonds by thiols is 5 orders of magnitude faster than that of disulfide bonds,[54] we have designed and synthesized a diselenide containing fluorescent probe **17** for rapid detection of thiols, (Figure 12A).[29] As the rapid and reversible recognition center for thiols, the diselenide bond is utilized to connect two fluorescein scaffolds. Probe **17** emits weak fluorescence due to the formation of a ground-state intramolecular dimer complex between the two fluorescein dyes. Upon the diselenide bond being cleaved by GSH, the fluorescein is released and the fluorescence is enhanced (Figure 12B).

The rate constant *k* for the reaction of **17** with GSH obtained using a stopped-flow spectrophotometer is 2.0×10^7 M^{-1} s^{-1} at pH 7.4 and 25 °C, which is 3–4 orders of magnitude higher than the previously reported rates for thiol quantification fluorescent probes (Figure 12C).[55,56] This fluorescent probe is found to be selective, sensitive,k and reversible for thiols. And

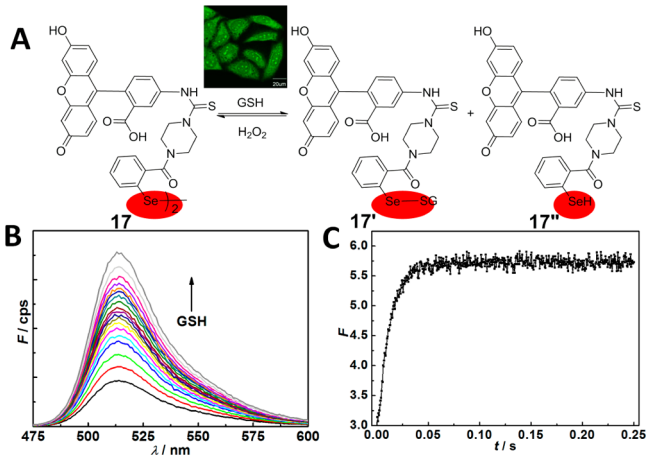

Figure 12. (A) Structure of probe **17** and the proposed reaction mechanism. (B) Fluorescence spectra of probe **17** with different concentrations of GSH. (C) Time course of fluorescence response of probe **17** to GSH using a stopped-flow spectrophotometer. Reference 29. Reproduced by permission of The Royal Society of Chemistry.

the IC_{50} of the probe cultured in HeLa cells for 24 h is 76 μM, which indicates low toxicity under the experimental conditions. In addition, we have applied **17** to successfully visual thiols, redox changes mediated by thiols, and ROS in living HeLa cells.

FLUORESCENT PROBES FOR REDOX CHANGES BASED ON HYDROQUINONE AND QUINONE

The neurotransmitter dopamine (DA) has been reported to be able to be oxidized to DA-*o*-quinone. Subsequently, DA-*o*-quinone can be scavenged by GSH and CySH by Michael addition to yield a number of soluble glutathionyl or cysteinyl conjugates, respectively.[57]

By integrating the redox-responsive hydroquinone unit into the *meso* position of BODIPY dye through a phenylene spacer, Benniston et al. developed a fluorescent probe **18** for reversibly detecting reduced and oxidized species (Figure 13A).[58] After

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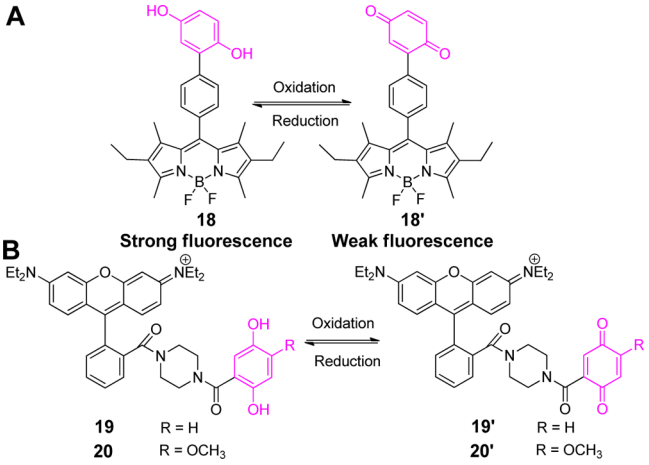

Figure 13. Structures of probes **18**–**20** for oxidation and reduction cycles, and their detection mechanisms. Adapted with permission from ref 58. Copyright 2008 John Wiley & Sons. And reference 59. Copyright (2010) Elsevier B.V., respectively.

the addition of sodium percarbonate in a biomembrane mimic environment, the hydroquinone moiety of the probe was oxidized to be quinone and the fluorescence was efficiently quenched. The reversibility of probe **18** is assessed by means of treatment with sodium ascorbate. These results demonstrated the feasibility of the probe sensing the reversible cycling of the redox couple. Taking advantage of a similar strategy, Krämer and co-workers attached hydroquinones to the fluorophore rhodamine B, and developed fluorescent probes **19** and **20** for redox change, (Figure 13B).[59] The sensors are water-soluble, readily internalized by live cells, and their oxidation potentials are tunable. Moreover, the fluorescence of the probes is redox-dependent, thus exhibiting potential applications in real-time mechanistic studies of metal complex or enzyme catalyzed oxidation reactions by single molecule fluorescence microscopy.

We designed and synthesized a NIR fluorescent probe **21** for detecting H_2O_2 oxidative stress and thiol-reducing repair.[60] In probe **21**, the chemical redox-responsive unit dopamine is integrated into the heptamethine cyanine scaffold, (Figure 14). The absorption spectrum of the probe is centered at 630 nm, and the fluorescence maximum is 755 nm (Φ = 0.13). The

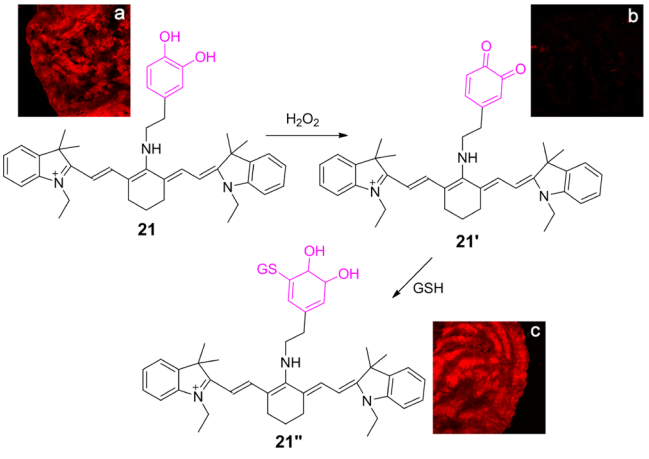

Figure 14. Structure and sensing mechanism of probe **21** for redox changes. Reference 60. Reproduced by permission of The Royal Society of Chemistry.

probe exhibited an on–off fluorescence switch for H_2O_2 selectively, and the fluorescence can be turned on once again via the Michael addition between the oxidized probe and thiols. However, it is infeasible for the probe to detect the oxidation and reduction cycles continuously. The IC_{50} of the probe in HL-7702 cells is 360 μM, which indicates the probe exhibits low toxicity to cultured cell lines. In addition, the fluorescent probe has been applied in sensing the redox change in HL-7702, HepG2, and RAW264.7 cell lines. The probe is also capable of sensing H_2O_2 oxidative stress and thiol repair in fresh rat hippocampus tissues.

FLUORESCENT PROBES FOR REDOX CHANGES BASED ON TEMPO

TEMPO can trap other free radicals and form alkoxyamine species in biological systems. In general, TEMPO-containing compounds possess lower fluorescence quantum yields compared with their related diamagnetic adducts. Thus, TEMPO can serve as fluorescence modulator. An enhancement in fluorescence would be observed upon the reaction of nitroxide radicals with free radical species.[61]

Taking advantage of the redox sensitive property of TEMPO moiety, Takeoka and co-workers synthesized a mitochondrial targeted fluorescent probe **22** for redox reactions (Figure 15A).[62] The maximum excitation and emission wavelength of probe **22** are 460 and 495 nm, respectively. Probe **22** has been applied in investigating the mechanism of ROS production successfully during the electron transport chain in the mitochondria. Subsequently, we reported two reversible fluorescent probes, **23** and **24**, for HOBr and ascorbic acid redox couple (Figure 15B).[63] In the probe, TEMPO as the reversible fluorescence controller is integrated into the heptamethine cyanine with NIR absorption and fluorescene. After the TEMPO moiety is oxidized to be an oxoammonium cation by HOBr, the fluorescence is quenched by donor-excited PET (d-PET) between the excited state fluorophore and oxoammonium cation. However, fluorescence is increased following the oxoammonium cation being reduced by ascorbic acid, which resulted from the prevention of the d-PET process. Furthermore, the probe shows low toxicity to the RAW264.7 cell line and can be used in continuous monitoring of intracellular HOBr oxidative stress and thiols repair.

CONCLUSIONS AND PROSPECTS

In this Account, we have reviewed recent advances in small molecular fluorescent probes for detecting the redox changes from the perspective of different response mechanisms. The aforementioned fluorescent probes mainly used their reversible oxidation and reduction reaction to respond to the redox changes. In general, design strategies include mimicking the active center of GPx, basing on hydroquinone and quinone, as well as utilizing TEMPO. Over the past few years, our work has focused on developing small fluorescent probes for redox changes based on GPx mimics. Most of the probes exhibit high sensitivity, good selectivity, near-infrared absorption, and rapid response. Employing rational synthetic routes, Churchill et al. have reported a number of fluorescent probes for redox cycles by incorporating organochalcogen atoms (Se, Te). Detty et al. have developed tellurorhodamine dyes with high chemical stabilities and provide more robust reversible systems for continuous redox processes. These probes can achieve the in situ, real-time, and dynamic fluorescence imaging analysis,

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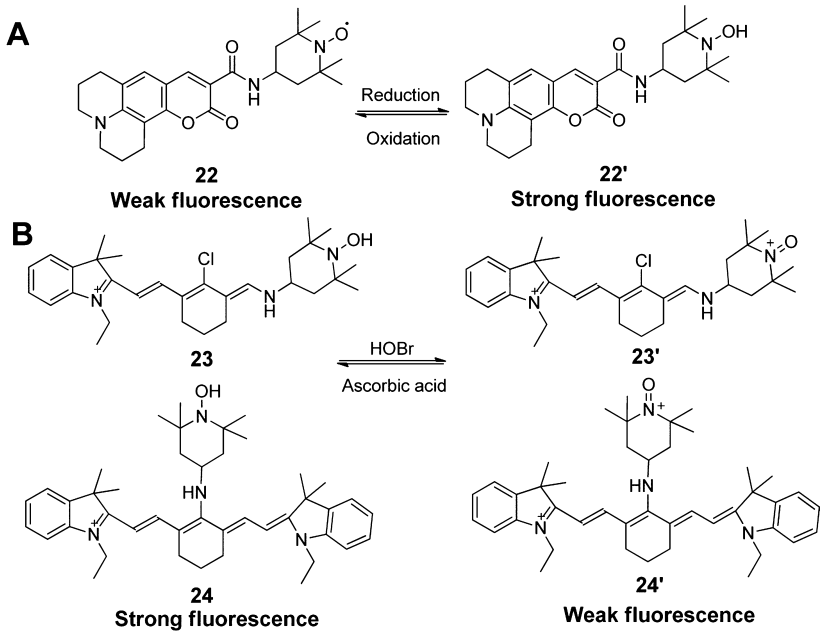

Figure 15. Structures of probes **22**–**24** and their reversible detection mechanisms for redox changes. (A) Structures of probes **22** and **22**′ reported by Takeoka and co-workers. (B) Structures of probes **23** and **24** for HOBr/ascorbic acid redox cycle. References 62 and 63. Adapted by permission of The Royal Society of Chemistry.

which would provide strong theoretical ideas and experimental basis for exploring the generation, metastasis, physiological functions, and pathogenic mechanisms of intracellular ROS and antioxidants.

The development of redox-responsive fluorescent probes certainly may facilitate specific detection equilibration of redox couples in real-time. However, besides selectivity, sensitivity, water solubility, and cell permeability, designing ideal fluorescent probes with high performance for imaging redox balance in biological systems should take into account the following factors: First, the probes should be able to monitor redox changes in real time. The active species participating in redox changes possess characteristics of short lifetime and high reactivity, which renders detection difficult. Accordingly, further efforts should place an emphasis on developing probes with the virtue of rapid, dynamic and cyclic response. Second, eliminating the interference from environmental conditions and biological systems is necessary. Ratiometric fluorescent probes building in correction of two excitation or emission wavelengths is capable of satisfying this requirement. Finally, NIR fluorescent probes with good tissue penetration and low autofluorescence should be explored. However, most of our reported redox-sensitive NIR fluorescent probes are based on cyanine dyes, which are known to possess poor photostability and low fluorescence quantum yield. Consequently, the development of NIR fluorescence dyes with photostability, chemical stability, and high quantum yields is indispensable.

The issue raised by reviewers about the different selectivity of different selenium based fluorescent probes requires clarification. We can only explain this special performance of the probes from two potential factors: the different internal activity of ROS and the different structure of selenium based probe, which can be concluded from Skaff and co-workers' research.[64] From the second-order rate constants k for reactions of the same selenium containing compound with different ROS, we can note that k is diverse. On the other hand, we can find a reversed k for reaction of the same ROS with different selenium containing compound. However, the details of how to influence the selectivity are very complicated and remain to be studied. Mugesh and co-workers have been engaged in developing functional mimics of GPx and investigating the catalytic mechanism.[34] They demonstrated some effects of substituents on the GPx activity of organoselenium compounds. At this point, the investigation of the chemo-mechanisms for selenium based probes responding to different ROS has an important significance in designing new probes for various ROS/antioxidants couples. In addition, the detailed research on fluorescence sensing mechanisms is crucial to design high-performance fluorescent probes. And time-resolved fluorescence analysis, ultrafast fluorescence depletion spectroscopy, and femtosecond transient absorption spectroscopy are the most effective experimental methods to investigate the fluorescence mechanism.[65] Besides, the DFT/TD-DFT calculations can give the clearer picture of the photophysical processes in the electronic excited states than the experimental methods, which have captured increasing attention.[66−69] Recently, utilizing the DFT/TD-DFT method, we have investigated the excited state hydrogen-bonding dynamics,[68] as well as the excited-state proton transfer (ESPT) of the fluorescent probes for fluoride[70−72] and cyanide.[73] The investigation of the excited state photophysical processes is capable of expanding our understanding of the fluorescence mechanism via experimental and theoretical methods, thereby providing a basis for designing available fluorescent probes.[74,75]

In summary, the ongoing research in developing redox-responsive fluorescent probes with excellent properties will provide an effective tool to investigate dynamic redox chemistry in living systems.

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Notes

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