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Reaction-Based Fluorescent Probes for the Detection and Imaging of Reactive Oxygen, Nitrogen, and Sulfur Species

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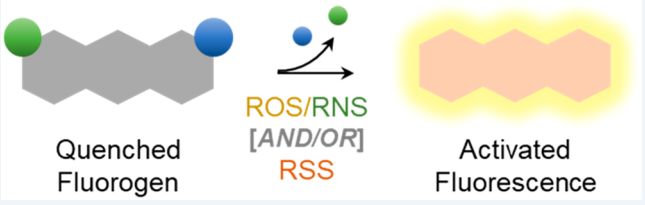
CONSPECTUS: This Account describes a range of strategies for the development of fluorescent probes for detecting reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive (redox-active) sulfur species (RSS). Many ROS/RNS have been implicated in pathological processes such as Alzheimer's disease, cancer, diabetes mellitus, cardiovascular disease, and aging, while many RSS play important roles in maintaining redox homeostasis, serving as antioxidants and acting as free radical scavengers. Fluorescence-based systems have emerged as one of the best ways to monitor the concentrations and locations of these often very short lived species. Because of the high levels of sensitivity and in particular their ability to be used for temporal and spatial sampling for in vivo imaging applications. As a direct result, there has been a huge surge in the development of fluorescent probes for sensitive and selective detection of ROS, RNS, and RSS within cellular environments. However, cellular environments are extremely complex, often with more than one species involved in a given biochemical process. As a result, there has been a rise in the development of dual-responsive fluorescent probes (AND-logic probes) that can monitor the presence of more than one species in a biological environment. Our aim with this Account is to introduce the fluorescent probes that we have developed for in vitro and in vivo measurement of ROS, RNS, and RSS. Fluorescence-based sensing mechanisms used in the construction of the probes include photoinduced electron transfer, intramolecular charge transfer, excited-state intramolecular proton transfer (ESIPT), and fluorescence resonance energy transfer. In particular, probes for hydrogen peroxide, hypochlorous acid, superoxide, peroxynitrite, glutathione, cysteine, homocysteine, and hydrogen sulfide are discussed. In addition, we describe the development of AND-logic-based systems capable of detecting two species, such as peroxynitrite and glutathione. One of the most interesting advances contained in this Account is our extension of indicator displacement assays (IDAs) to reaction-based indicator displacement assays (RIAs). In an IDA system, an indicator is allowed to bind reversibly to a receptor. Then a competitive analyte is introduced into the system, resulting in displacement of the indicator from the host, which in turn modulates the optical signal. With an RIA-based system, the indicator is cleaved from a preformed receptor−indicator complex rather than being displaced by the analyte. Nevertheless, without a doubt the most significant result contained in this Account is the use of an ESIPT-based probe for the simultaneous sensing of fibrous proteins/peptides AND environmental ROS/RNS.



INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are groups of reactive neutral and anionic small molecules that are produced within many cell types. Common biologically relevant ROS/RNS include singlet oxygen ($^1O_2$) superoxide ($O_2^{\bullet-}$), hydroxyl radical (HO·), peroxyl radical (ROO·), hydrogen peroxide (H_2O_2), hypochlorous acid/hypochlorite (HOCl/ClO^-), nitric oxide (NO), nitroxyl (HNO), and peroxynitrite ($ONOO^-$). Although ROS/RNS are involved in many biological signaling pathways, over-production of ROS/RNS (oxidative stress) can result in oxidative damage to a wide range of biomolecules such as nucleic acids, carbohydrates, lipids, and proteins, which can lead to loss of molecular and cellular functions. Importantly, excess production of ROS/RNS has been implicated in pathological processes such as Alzheimer's disease (AD), cancer, diabetes mellitus, cardiovascular disease, and aging.[1,2]

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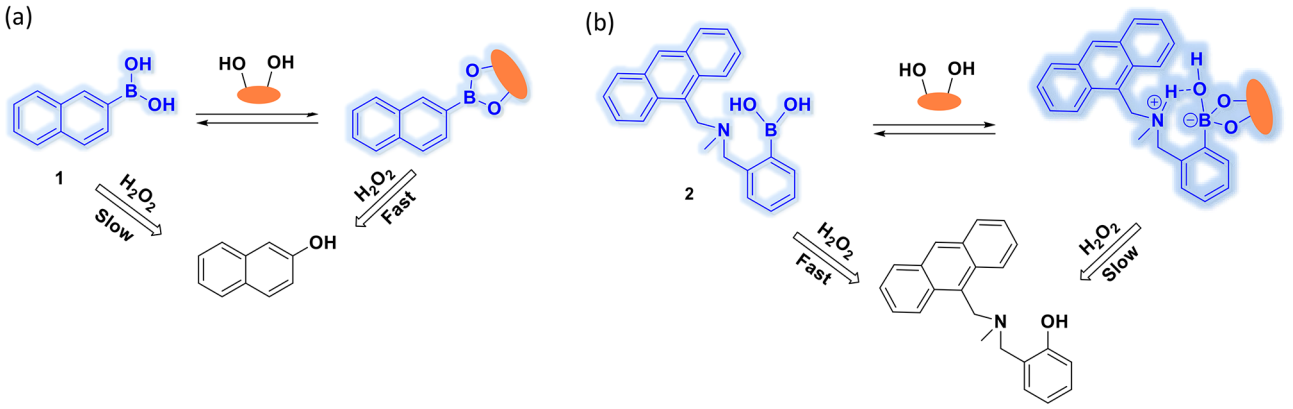
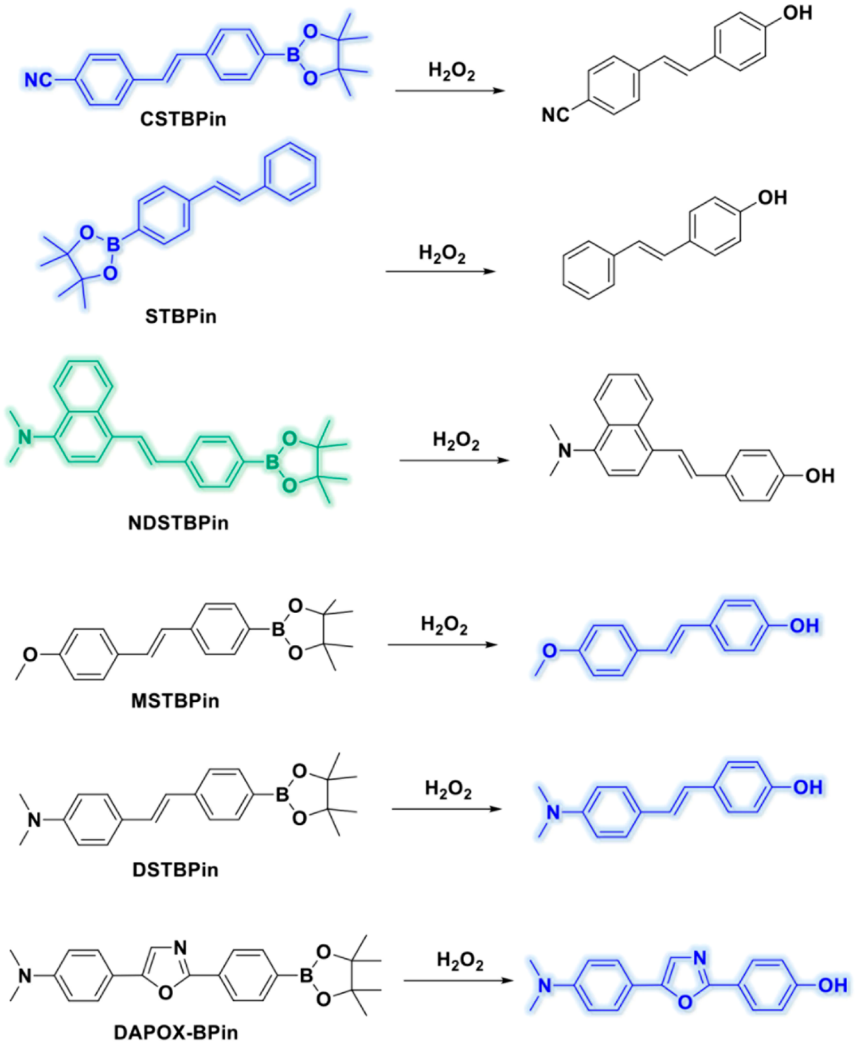
Scheme 1. (a) 1 and 1−D-Fructose Complex for Detecting H_2O_2; (b) 2 and 2−D-Fructose Complex for Detecting H_2O_2



Scheme 2. Boronate Probes for Detecting H_2O_2



Biological thiols such as glutathione (GSH) and cysteine (Cys) play important roles in maintaining redox homeostasis, serving as antioxidants and acting as free radical scavengers (e.g., GSH acts a $ONOO^-$ scavenger).[3] As a result, elevated levels of GSH are often produced to protect cells that are exposed to oxidative stress. Therefore, the susceptibility of a cell toward $ONOO^-$ is largely dependent upon the concentration of intracellular GSH present. As a consequence, imbalances in thiol concentration have been implicitly linked to a number of diseases, such as inflammatory diseases, cancer, and neurodegenerative disorders.[4,5]

The development of fluorescent small-molecule probes to monitor the intracellular concentrations of bioanalytes is a powerful tool to monitor specific cellular processes related to certain diseases.[6−16] Because biological pathways often involve multiple reactive chemical species, dual-responsive fluorescent

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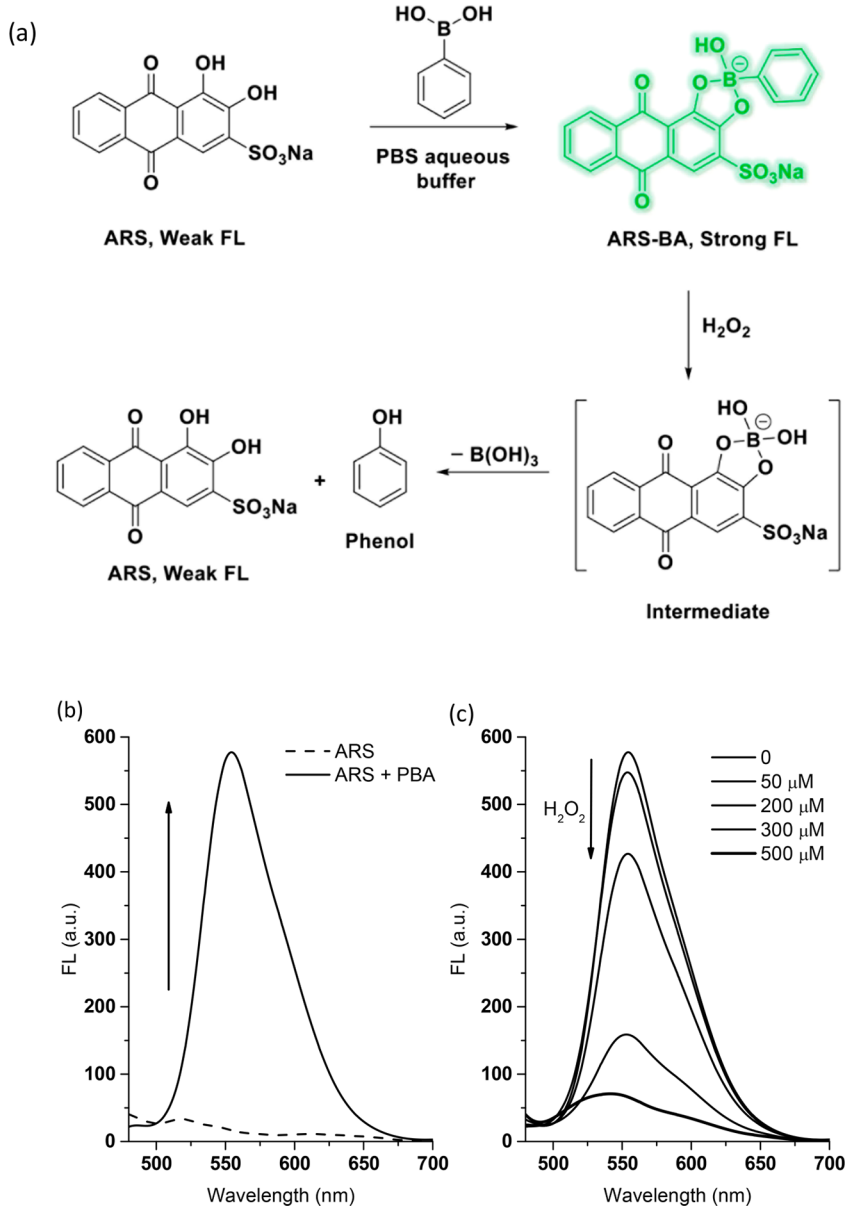
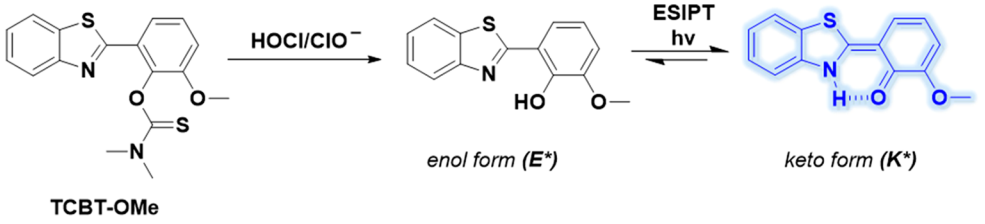
Scheme 3. Mechanism and Fluorescence Spectra of the ARS−PBA Complex for Detecting H_2O_2[a]



[a]Adapted from ref 36. Published by The Royal Society of Chemistry.

Scheme 4. ESIPT-Based Probe TCBT-OMe for Detecting HOCl/ClO^-



probes (AND-logic probes) have been developed for two different analyte inputs in order to communicate a detectable signal output.[17−20] At this point we should raise a note of caution: in the design of fluorescent probes, it is important to consider the reaction mechanisms involved, products and intermediates formed, and their cellular distribution and concentration in addition to environmental factors that may influence the performance and utility of the probes.[21,22]

1. FLUORESCENT PROBES FOR ROS/RNS

1.1. Fluorescent Probes for H_2O_2

Hydrogen peroxide can diffuse freely across cell membranes and into and out of cells, where it is known to play critical roles in several physiological processes, such as apoptosis, cell proliferation, cell signaling, and differentiation.[23] Therefore, the accumulation of excess H_2O_2 has also been implicated in

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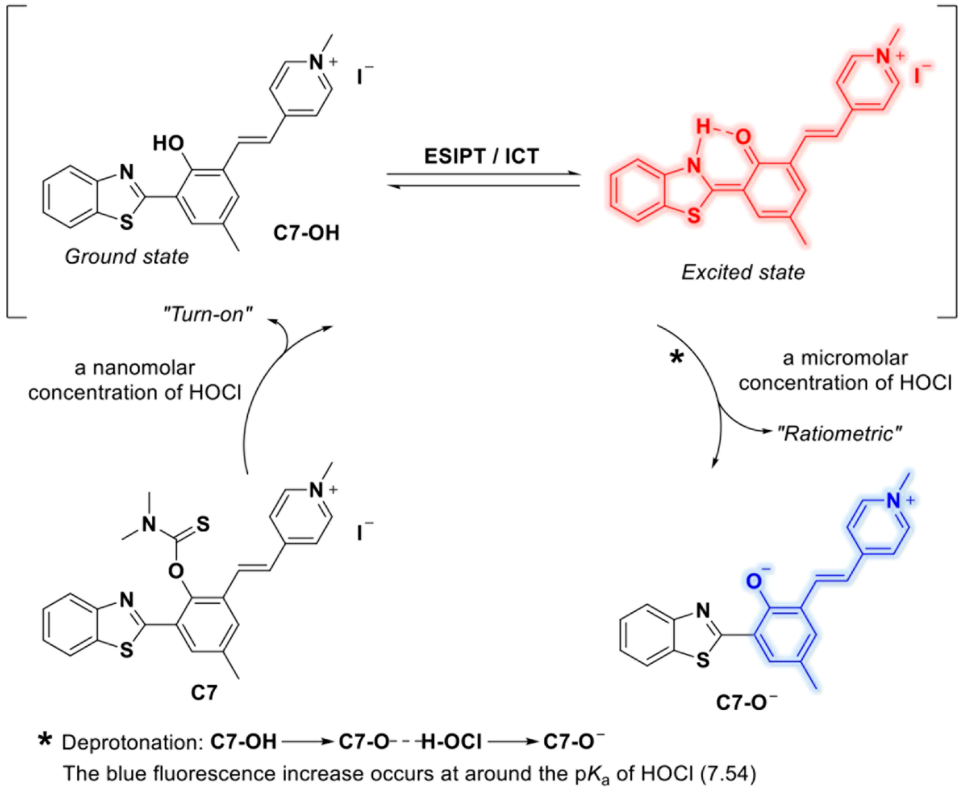
Scheme 5. ESIPT-Based Probe C7 for Detecting HOCl



numerous pathological conditions, including aging, neurodegenerative diseases, and cancer.[24,25]

Background knowledge accumulated from over 26 years of research on boronic acid-based molecular fluorescent saccharide sensors in the James group inspired our first fluorescent sensor system for detecting H_2O_2. This probe design relies on the increased Lewis acidity of boronate esters over their corresponding boronic acids.[26,27]

As illustrated in Scheme 1, the rates of reaction of boronic acids **1** and **2** with H_2O_2 in the presence/absence of D-fructose are opposed. Boronic acid **1** displays enhanced reactivity toward H_2O_2 in the presence of D-fructose. Since the boronate ester formed contains a more electrophilic boron center, it reacts more rapidly with H_2O_2, resulting in accelerated oxidative cleavage to afford the corresponding naphthol. Alternatively, boronic acid **2** displays reduced reactivity toward H_2O_2 in the presence of D-fructose because it forms a boronate ester whose reactivity toward H_2O_2 is reduced by the presence of a water-inserted N−B bond that reduces the electrophilicity of its boron center. The Anslyn group has spent many years unraveling the nature of solvent-inserted N−B bonds and has established that they are best represented as species containing a negatively charged sp^3 boron atom (cf. reduced electrophilicity), a positively charged tertiary ammonium group, and a hydrogen bond between the solvent-inserted oxygen and ammonium group (see Scheme 1b).[28,29] The sensing performance of boronic acid **2** was particularly interesting since addition of D-fructose results in an increase in the intensity of its fluorescence response in the visible region. The dual fluorescence response of probe **2** suggests that using similar systems with more soluble probes may enable mapping of both intracellular H_2O_2 AND saccharide concentrations.[30]

"Turn on" fluorescent sensors are generally more useful for intracellular sensing applications because "turn off" fluorescent sensors can be quenched (nonspecifically) by off-target endogenous species to afford false-positive results. This led us to start developing sensors based on turn-on intramolecular charge transfer (ICT) fluorescence systems, which was achieved by the synthesis of a series of H_2O_2-responsive boronate ester-based fluorescence probes (Scheme 2). Fluorophores that alter their emission via changes in ICT usually have an electron donor (D) on one end of the fluorophore and an electron acceptor (A) on the other end.[31] The probes **DSTBPin** and **MSTBPin** exhibited an off−on fluorescence response when exposed to H_2O_2, with phenol formation resulting in loss of an ICT excited state. Conversely, probe **CSTBPin** exhibited an on−off reduction in fluorescence when treated with H_2O_2, caused by the presence of an ICT excited state in its phenol cleavage product. Interestingly, small reductions in fluorescence intensity were observed when the long-wavelength probe **NDSTBPin** was treated with H_2O_2. This was attributed to the extended conjugation in that system, which reduced the ICT effect. These changes in fluorescence output demonstrate the importance of electron-withdrawing and electron-donating groups for determining the fluorescent output of these types of probes.[32]

The development of a sensor for a bioanalyte often requires appropriate selection of both an efficient receptor/reactive group and a matched chromophore, but this approach often requires long synthetic routes, and once the chromophore fragment has been prepared, it is not easy to change its properties. One approach for overcoming the limitations of this type of receptor-based system is to use the indicator displacement assay (IDA) approach championed by Anslyn and coworkers.[33] In an IDA system, an indicator is allowed to bind reversibly to a receptor. Then a competitive analyte is

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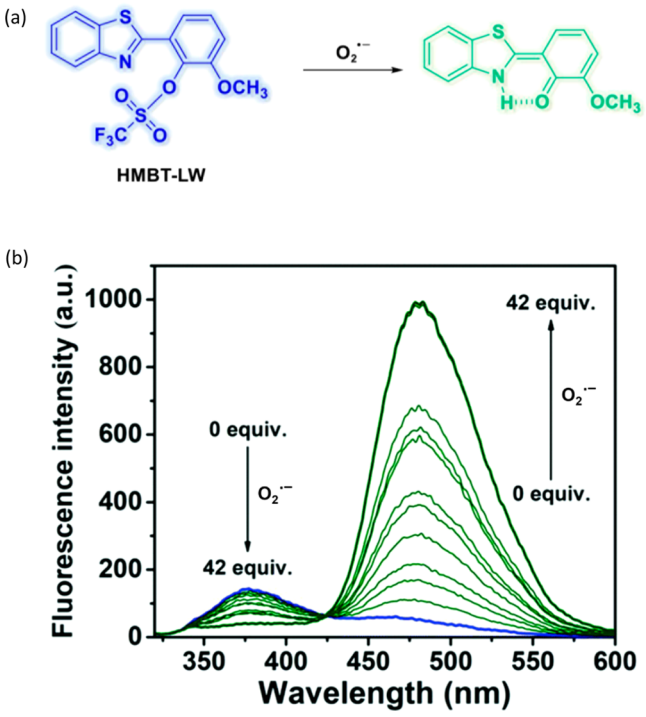
Scheme 6. (a) ESIPT-Based Probe HMBT-LW for Detecting $O_2^{\bullet-}$; (b) Changes in Fluorescence Emission Intensity of HMBT-LW (5 μM) with Increasing $O_2^{\bullet-}$ in Phosphate-Buffered Saline (PBS) (10 mM v/v, 1:1 DMSO/PBS, pH 7.4) after 3 min at λ_{ex} = 310 nm[a]



[a]Adapted with permission from ref 41. Published by The Royal Society of Chemistry (RSC) on behalf of the Centre National de la Recherche Scientifique (CNRS) and the RSC.

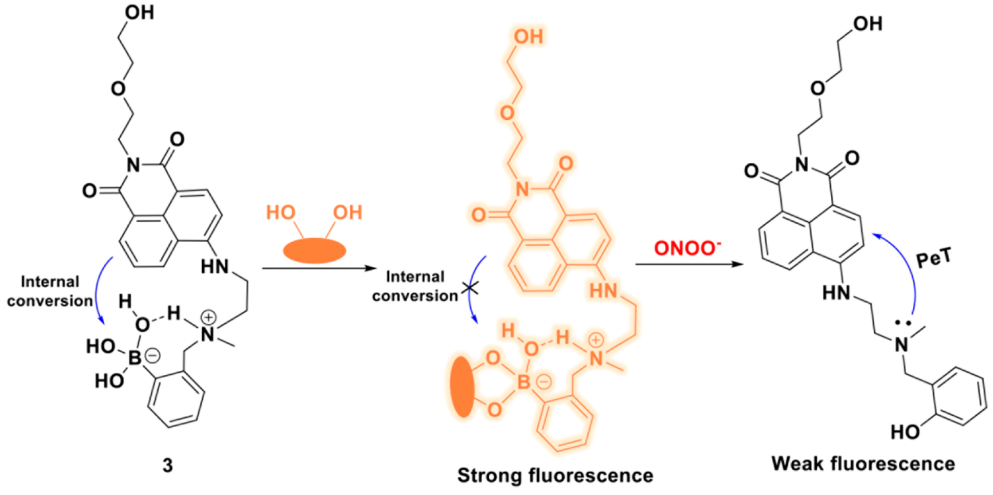
introduced into the system, resulting in displacement of the indicator from the host, which in turn modulates the optical signal. We extended the IDA approach to include reaction-based systems and developed a reaction-based indicator displacement assay (RIA) suitable for use in detecting H_2O_2. This system relies on complexation of phenylboronic acid (PBA) with the indicator Alizarin Red S (ARS) to afford a fluorescent complex whose aryl unit is oxidatively cleaved upon exposure to H_2O_2 (turn off) in aqueous media. The reaction of boronic acid receptors with ARS was recently investigated by Ishihara, who reported that all "systems examined are the chelated tetrahedral anions" over a 5−10 pH range.[34,35] Therefore, Scheme 3 has been updated to reflect the formation of a tetrahedral ARS−PBA complex as the major species present in solution. These RIA systems can be used to detect the presence of H_2O_2 through monitoring of changes to their colorimetric, fluorometric, and electrochemical outputs. For example, reaction of the ARS−PBA probe with H_2O_2 resulted in a red-shifted visible color change, an on−off fluorescence response (due to ARS release), and a turn-on electrochemical signal caused by the formation of phenol. Our research has shown that this type of RIA system is effective for monitoring of H_2O_2 (and H_2O_2-related species) in environmental and physiological scenarios.[36]

1.2. Fluorescent Probes for HOCl/ClO$^-$

The reaction between chloride anions and H_2O_2 is catalyzed by myeloperoxidase (MPO) in leukocytes to generate HOCl/ClO$^-$, which is deployed for its microbiocidal properties. Unfortunately, excess production of HOCl/ClO$^-$ can also lead to uncontrolled damage to a range of biomolecules, such as amino acids, proteins, carbohydrates, and lipids.

We recently became interested in exploiting the properties of excited-state intramolecular proton transfer (ESIPT) to develop fluorescent sensors.[7,9] Briefly, ESIPT fluorophores normally exist in an *enolic* (**E**) form in the ground state, and photoexcitation results in redistribution of their electronic charge, increasing the acidity of the hydrogen-bond-donor group and enhancing the basicity of the hydrogen-bond-acceptor unit. This results in extremely fast enol-to-keto phototautomerization, with the photoexcited enol form (**E***) rapidly converting into the excited keto form (**K***), which displays increased fluorescence. Therefore, we decided to develop an ESIPT-based fluorescent probe, **TCBT-OMe**, containing a HClO/ClO$^-$-responsive dimethylthiocarbamate linker for HClO/ClO$^-$ detection (Scheme 4) over biologically relevant concentration ranges (limit of detection (LOD) = 0.16 nM). Excellent selectivity was obtained for HClO/ClO$^-$ over other ROS/RNS (ONOO$^-$, H_2O_2, ROO·, ·OH, $O_2^{\bullet-}$, $^1O_2$, NO) and a range of free amino acids (glycine, asparagine, cysteine, homocysteine, glutathione, arginine, histidine, serine, glycine,

Scheme 7. Probe 3 for Detecting ONOO$^-$ in the Presence of D-Fructose



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Scheme 8. ARS−NBA Complex for Detecting $ONOO^-$

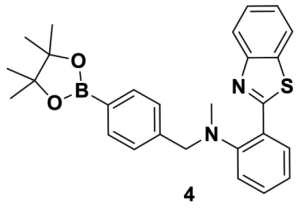
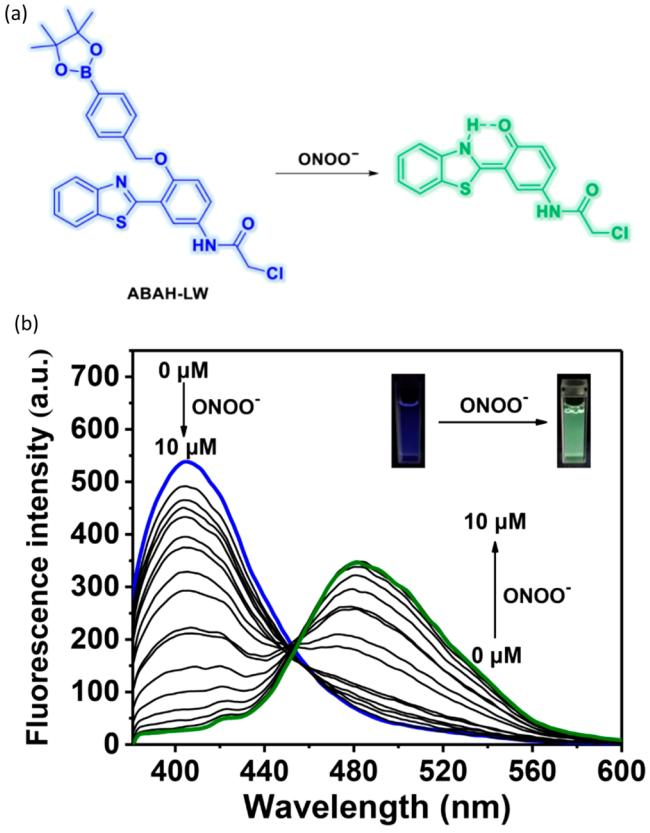


Figure 1. Probe **4** for detecting $ONOO^-$.

Scheme 9. (a) Probe ABAH-LW for Detecting $ONOO^-$; (b) Changes in Fluorescence Emission of ABAH-LW (3 μM) with Increasing Addition of $ONOO^-$ in PBS (pH 8.2, Containing 8% DMSO, 1 mM CTAB) after 1 min at λ_{ex} = 370 nm[a]



[a]Adapted with permission from ref 47. Copyright 2018 Royal Society of Chemistry.

and threonine), and **TCBT-OMe** could be used to monitor endogenous and exogenous $HClO/ClO^-$ in HeLa cells.[37]

More recently, a series of turn-on benzathiazole probes **C1**−**C7** containing reactive dimethylthiocarbamate units for the in vivo detection of $HClO/ClO^-$ at biologically relevant

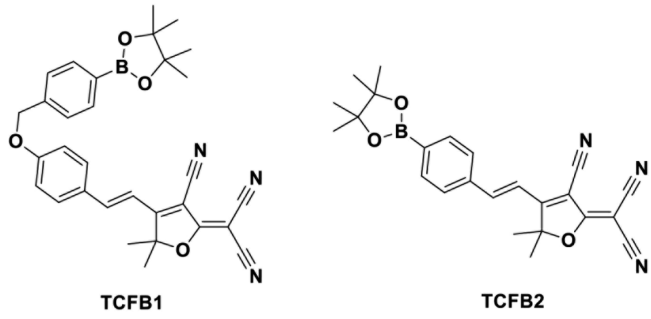
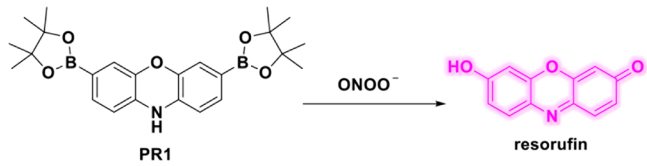


Figure 2. TCFB1 and **TCFB2** for detecting $ONOO^-$.

Scheme 10. PR1 for Detecting $ONOO^-$



concentrations (nanomolar) were developed (Scheme 5).[38] These probes displayed a range of blue-to-red "fluorescence rainbow" emissions, thus facilitating a broad choice of potential colors for in vivo colorimetric imaging of $HClO/ClO^-$. The fluorescence of probe **C7** can target mitochondria and displays different fluorescence responses to low and high concentrations of HOCl: an obvious turn-on signal in response to nanomolar concentrations of HOCl (biologically relevant concentrations, LOD_1 = 18 nM) and a subsequent ratiometric response to HOCl at the micromolar level (high-risk pathogenic concentrations, LOD_2 = 0.47 μM)

Importantly, the ratiometric response of the fluorescent output of these sensors provides enhanced reliability compared with simple fluorescence and avoids any cross-talk caused when several probes are combined for detection.[39]

1.3. Fluorescent Probes for $O_2^{\bullet-}$

Often known as the "'primary'" reactive oxygen species, superoxide is responsible for the production of other ROS/RNS species such as H_2O_2 and $ONOO^-$. In addition, $O_2^{\bullet-}$ is recognized as a potential envoy that regulates the cell-signaling network.[40] The production of $O_2^{\bullet-}$ occurs primarily in mitochondria, caused by leakage of electrons to O_2 from the electron transport chain, resulting in high reactivity and a short half-life, making $O_2^{\bullet-}$ difficult to detect. However, because of its high reactivity, it is associated with a range of pathological conditions such as aging, ischemia−reperfusion injury, and inflammation.

The importance of $O_2^{\bullet-}$ in biological systems led us to explore the possibility of developing an ESIPT-based fluorescent probe

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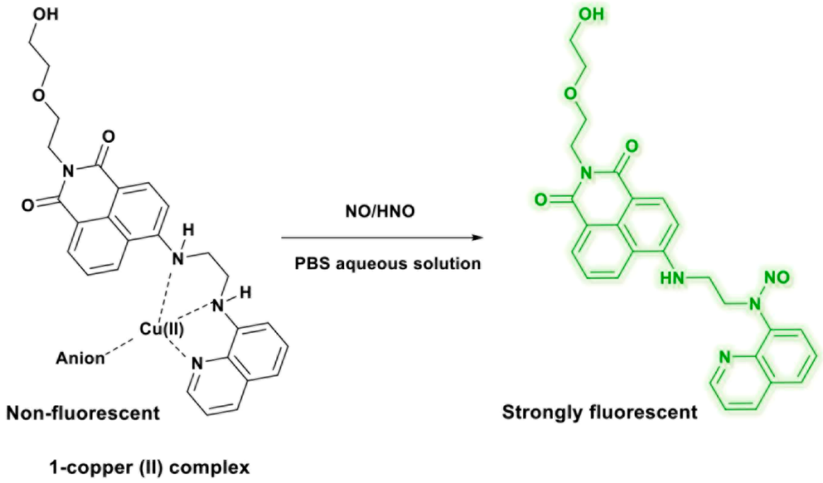
Scheme 11. 1−copper(II) Complex for Detecting NO and HNO



for its rapid detection both in vitro and in vivo. We developed **HMBT-LW** as a simple ESIPT-based fluorescent probe that was shown to exhibit good sensitivity and selectivity for the rapid detection of low concentrations of $O_2^{\bullet-}$ (LOD = 7.4 μM) (Scheme 6).[41]

1.4. Fluorescent Probes for $ONOO^-$

Peroxynitrite, a reactive nitrogen species, is produced via the rapid reaction of $O_2^{\bullet-}$ with NO and is known for its destructive properties in cellular systems, where it readily causes irreversible damage to lipids, proteins, and DNA. As a result, uncontrolled generation of $ONOO^-$ has been implicated as a key pathogenic factor in numerous diseases, including ischemia−reperfusion injury, neurodegenerative diseases, inflammation, and cancer. Consequently, the development of a fluorescent probe for the selective cellular detection of $ONOO^-$ in the presence of other ROS/RNS may potentially facilitate advances in diagnostics resulting in improved treatments for numerous medical conditions.

Expanding on our original approach previously described in Scheme 1, we employed a similar approach for the intracellular detection of $ONOO^-$ using boronic acid[42,43] as a sensing motif in the presence of monosaccharides (e.g., D-fructose). Once again, the presence of $ONOO^-$ results in oxidative cleavage of the boronic ester fragment of the probe **3**−D-fructose complex, which triggers a significant on−off fluorescence response (Scheme 7). The probe **3**−D-fructose complex demonstrated excellent selectivity over most other ROS and RNS (H_2O_2, NO_2^-, NO_3^-, ROO·, $O_2^{\bullet-}$, HO·, and NO), enabling it to be used to successfully visualize exogenous (HeLa) and endogenous (RAW 264.7) $ONOO^-$ in living cells, thus providing the opportunity to investigate diseases such as inflammation that are known to generate increased levels of $ONOO^-$.[44]

ARIA probe containing a boronic acid (NBA) as the receptor and ARS as the reporter fluorophore was also developed for the fluorescent detection of $ONOO^-$(Scheme 8).[45] The parent ARS−NBA system displayed a negligible response toward H_2O_2 and other ROS/RNS as a result of protection of the boron center by the N−B solvent−insertion interaction.[28] However, a significant UV−vis absorption and fluorescence response was observed in the presence of more reactive $ONOO^-$, illustrating that an RIA system could be developed and employed for the in vitro and in vivo sensing of ROS and RNS.[45]

Our initial sensors were designed to incorporate a N−B solvent-insertion interaction to reduce the reactivity of the boronic acid unit in order to enable selectivity for more reactive $ONOO^-$ over less nucleophilic ROS. However, during our investigations we discovered that the reactivity of $ONOO^-$ compared with other ROS (i.e., H_2O_2) was sufficiently greater that this additional protection element was not necessary. Therefore, while the N−B solvent insertion does enhance the selectivity of the probes for $ONOO^-$ over other ROS, we have found that simple boronate esters react significantly faster with low concentrations of $ONOO^-$ to make this additional protecting group strategy unnecessary. Therefore, we set out to develop an ESIPT-based fluorescent sensor for detecting $ONOO^-$ where rapid $ONOO^-$-facilitated oxidation of a boronate ester "protecting" unit of probe **4** is used to trigger the ESIPT turn-on process (Figure 1). Oxidative cleavage of the boronate ester fragment of probe **4** results in an increase in fluorescence, allowing this probe to be used for the visualization of $ONOO^-$ in the RAW 264.7 and HeLa cell lines.[46] More recently, we developed **ABAH-LW** as an ESIPT ratiometric boronate-based probe that was used for the successful visualization of $ONOO^-$ in the endoplasmic reticulum of HeLa cells (Scheme 9).[47]

An important feature of turn-on fluorescent sensors used for detection of bioanalytes in cellular systems is the excitation and emission wavelengths of the fluorescence process. In particular, long-wavelength systems in the near-infrared (NIR) region are desirable because they have lower background fluorescence and enable the use of low-energy light sources that result in less tissue damage during imaging. In addition, long-wavelength fluorophores also allow for deeper penetration and visualization of cellular masses and tissues, making them more suitable for whole-animal imaging experiments. Because of these factors, we developed two long-wavelength boronate fluorescent probes, **TCFB1** and **TCFB2**, for detecting $ONOO^-$ in cellular systems (Figure 2). **TCFB1** was shown to have a low sensitivity toward $ONOO^-$ and exhibited poor solubility in aqueous solution, but **TCFB2** could be used to monitor exogenous and endogenous $ONOO^-$, producing a significant fluorescence turn-on response to $ONOO^-$ in numerous cell lines (Hep-G2, RAW 264.7, HeLa, and A459).[48]

The Chang group developed **PR1**, a seminal long-wavelength probe for detecting H_2O_2 (Scheme 10).[49] We recently improved the synthesis of **PR1** and demonstrated that it can be used as a

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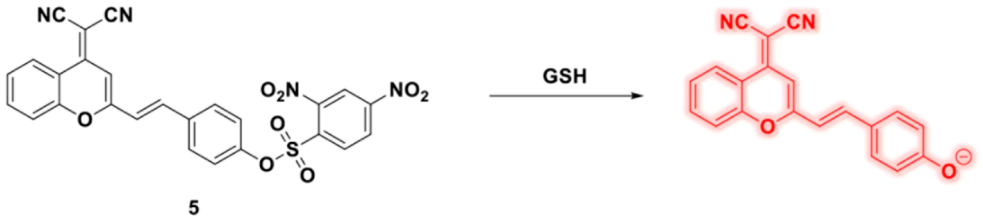
Scheme 12. Probe 5 for Detecting GSH



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sensitive and selective sensor for $ONOO^-$ as well as H_2O_2. As part of our research, we carried out detailed investigations of the ability of **PR1** to visualize pathways responsible for the generation of $ONOO^-$, with fluorescence imaging demonstrating the cellular location of **PR1** in polarized J774.2 macrophages.[50]

1.5. Fluorescent Probes for NO/HNO

Nitric oxide is a signaling molecule produced from L-arginine by a family of nitric oxide synthases (NOSs). Many physiological processes are mediated by NO, including neurotransmission, immune regulation, smooth muscle relaxation, and blood pressure regulation, and uncontrolled production of NO can lead to nitrosative stress with the formation of $ONOO^-$.

The one-electron reduction of NO produces nitroxyl radical (HNO), which has a unique chemical and biological profile compared with NO. Since it is a small neutral molecule ($pK_a \approx 11.4$), it can freely cross cell membranes and can be involved in redox reactions with a range of biological oxidants and reductants. Since HNO has been shown to have some beneficial physiological properties, it is considered to be a possible pharmacological agent in applications to increase cardiac output, afford protective effects against myocardial ischemia injury, and act as an anticancer agent.

Consequently, we designed a selective and sensitive fluorescent probe with a turn-on fluorescence response for NO and HNO under physiological conditions (Scheme 11). The fluorescence response of this water-soluble complex **1**–**copper(II)** is caused by NO^+-mediated nitration of its amino group, which stops photoinduced electron transfer (PeT)[31] through release of copper(I) from the receptor. This biocompatible probe could be used for imaging of exogenous and endogenous NO and HNO in live cells (HeLa and RAW 264.7, respectively).[51]

2. FLUORESCENT PROBES FOR REACTIVE (REDOX-ACTIVE) SULFUR SPECIES

2.1. Fluorescent Probes for Biological Thiols (GSH, Cys, and HCys)

Glutathione is a natural tripeptide (γ-L-glutamyl-L-cysteinylglycine) that is present as an antioxidant in cells at millimolar concentrations; it exists mainly as the reduced form (GSH) rather than the oxidized disulfide form (GSSG). However, under conditions of oxidative stress, elevated levels of GSH are often seen, and the susceptibility of cells toward damage from ROS/RNS species is strongly correlated to the intracellular GSH level. In this respect, dysregulation of GSH homeostasis has been identified as an indicator of numerous diseases, including the onset of AIDS, cancer, neurodegenerative diseases, and liver damage.

Cysteine is a biological-thiol-containing α-amino acid that plays an important part in several biological processes, including cellular detoxification, protein synthesis, and metabolism.

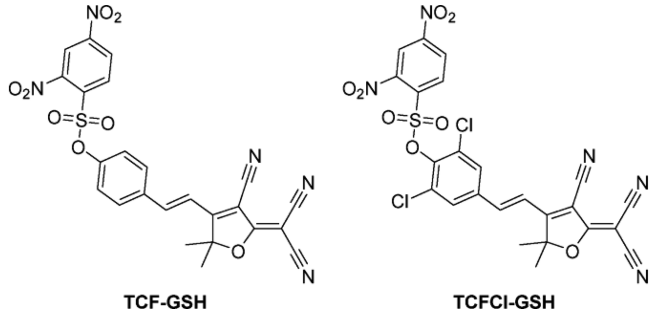

Figure 3. TCF-GSH and **TCFCl-GSH** for detecting GSH.

Nevertheless, atypical levels of Cys are also linked with several disease states, including neurological disorders, motor neuron diseases, and cancer.

The naturally occurring aminothiol homocysteine (HCys) is biosynthesized through demethylation of the proteinogenic α-amino acid methionine (Met). The structural similarity of HCys to Met means that it can potentially be accepted as a substrate by methionyl t-RNA synthetase (MetRS) for protein synthesis. However, the error-editing capacity of MetRS prevents unwanted incorporation of HCys into proteins through a cleavage pathway that results in the formation of HCys thiolactone (HTL). However, HTL can then undergo an irreversible nonenzymatic reaction with the lysine residues of proteins via a process known as protein N-homocysteinylation that results in structural modifications, protein aggregation, and loss of enzymatic function. Hyperhomocysteinemia (i.e., raised levels of HCys) has been linked with numerous diseases, including neurodegenerative and cardiovascular diseases and pregnancy disorders.

Given the biological importance of these thiols, we were interested in developing suitable fluorescent probes for their measurement and localization in cellular systems. Consequently, we developed an NIR and colorimetric molecular probe based on a dicyanomethylene-4*H*-pyran (DCM) chromophore that facilitates the selective detection of GSH in living cells (HeLa) (Scheme 12). The fluorescence turn-on response of this probe is generated from its reaction with GSH, which results in cleavage of its 2,4-dinitrobenzenesulfonyl (DNBS) unit and release of a fluorescent chromophore.[52]

Two other tricyanofuran (TCF)-based turn-on fluorescence probes, **TCF-GSH** and **TCFCl-GSH**, that rely on cleavage of a DNBS unit were prepared as sensors for biological thiols (Figure 3). While **TCF-GSH** was sensitive toward the presence of GSH (LOD = 0.28 μM), this probe proved to be toxic to live cells, and its fluorescence intensity decreased at higher concentrations of GSH (>50 μM). To overcome this problem, **TCFCl-GSH** was developed, which was shown to detect GSH at biologically relevant concentrations (LOD = 0.45 μM). **TCFCl-GSH** proved to be nontoxic and produced a clear turn-on response with good photostability when exogenous GSH was added to

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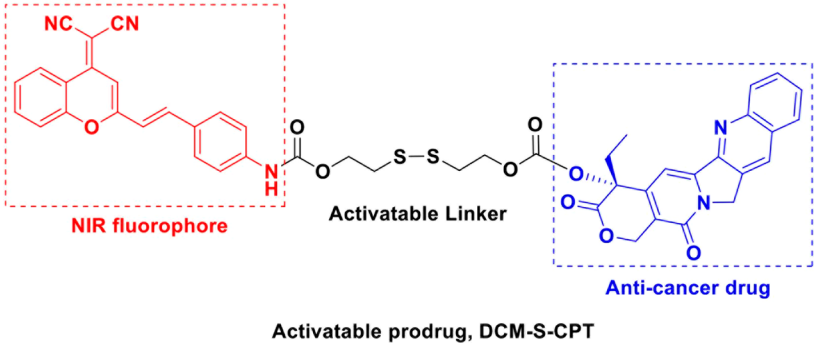


Figure 4. Theranostic prodrug **DCM-S-CPT**.

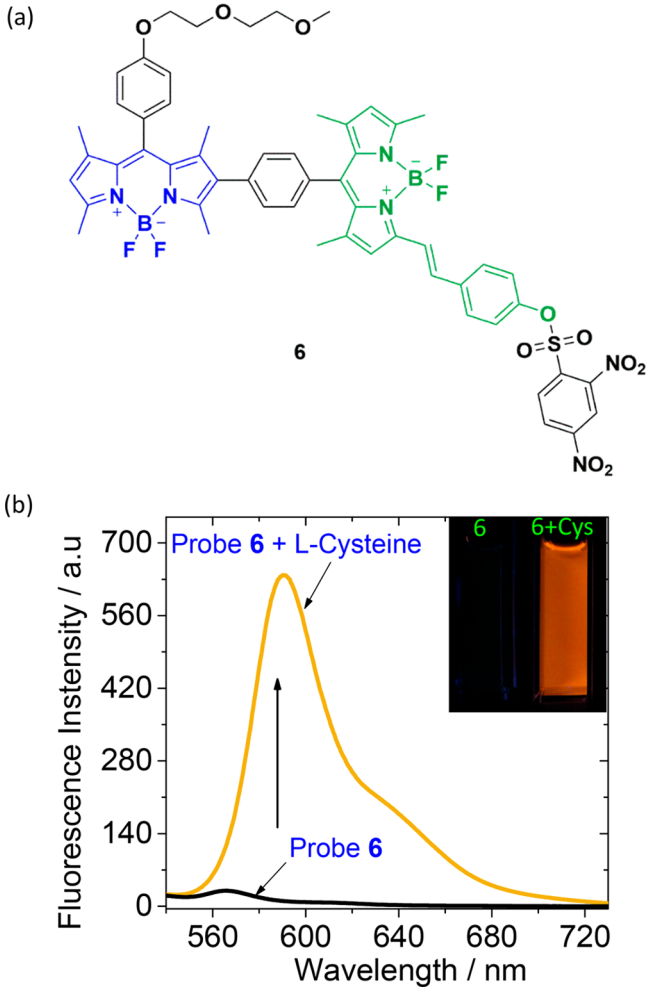
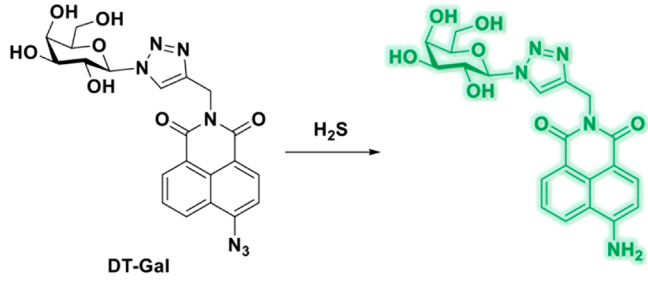


Figure 5. (a) Probe **6** for detecting thiols. (b) Fluorescence emission spectra of probe **6** (10 μM) before and after addition of L-cysteine (300 μM) in 4:1 v/v MeOH/H_2O at λ_{ex} = 505 nm at 37 °C. Adapted with permission from ref 56. Copyright 2012 Royal Society of Chemistry.

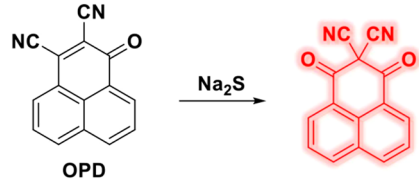
HeLa cells. **TCFCl-GSH** could be used to evaluate changing levels of GSH in live cells. For example, the addition of H_2O_2 or cisplatin, which are known to deplete endogenous GSH, reduced the fluorescence intensity, whereas addition of *N*-acetylcysteine, a known GSH-generating drug, restored the biological thiol levels and fluorescence intensity.[53]

Expanding the use of the DCM chromophore, the potential of using a novel NIR theranostic prodrug, **DCM-S-CPT**, for cancer chemotherapy in living animals was explored (Figure 4).

Scheme 13. DT-Gal for Detecting H_2S



Scheme 14. OPD for Detecting Na_2S



Connection of the DCM fragment to the camptothecin (CPT) drug fragment through a covalent disulfide linker turns off the fluorescence and cytotoxicity of **DCM-S-CPT**. However, the presence of excess GSH in cancer cells can result in selective cleavage of the disulfide bond of the linker of **DCM-S-CPT**, which releases the cytotoxic drug cargo and the fluorophore from PEG−PLA-loaded nanoparticles. This tumor-cell-specific release mechanism enabled NIR fluorescent monitoring to be used to track the ability of this **DCM-S-CP** prodrug system to deliver its cytotoxic payload to tumor-bearing nude mice in vivo. PEG−PLA nanoparticles loaded with **DCM-S-CPT** were found to exhibit enhanced antitumor activity and greater plasma half-life in mice than CPT alone.[54]

The excellent photophysical properties of boron−dipyrromethene (BODIPY)[55] were exploited to develop molecular probe **6** as a red-emitting fluorescence resonance energy transfer (FRET) sensor for the selective detection of Cys and HCys (Figure 5). The BODIPY unit of probe **6** (shown in blue) acts as a FRET donor, while its O-protected 4-hydroxyphenyl-BODIPY fragment (shown in green) acts as a FRET acceptor to modulate its fluorescence response. Treatment of **6** with an exogenous thiol results in cleavage of the DNBS unit from the fluorophore fragment, which then affords a turn-on fluorescence response. Addition of Cys (or HCys) turns on red emission at 590 nm upon excitation at 505 nm, thus producing a pseudo-Stokes shift of 77 nm that is significantly greater than the small Stokes shift

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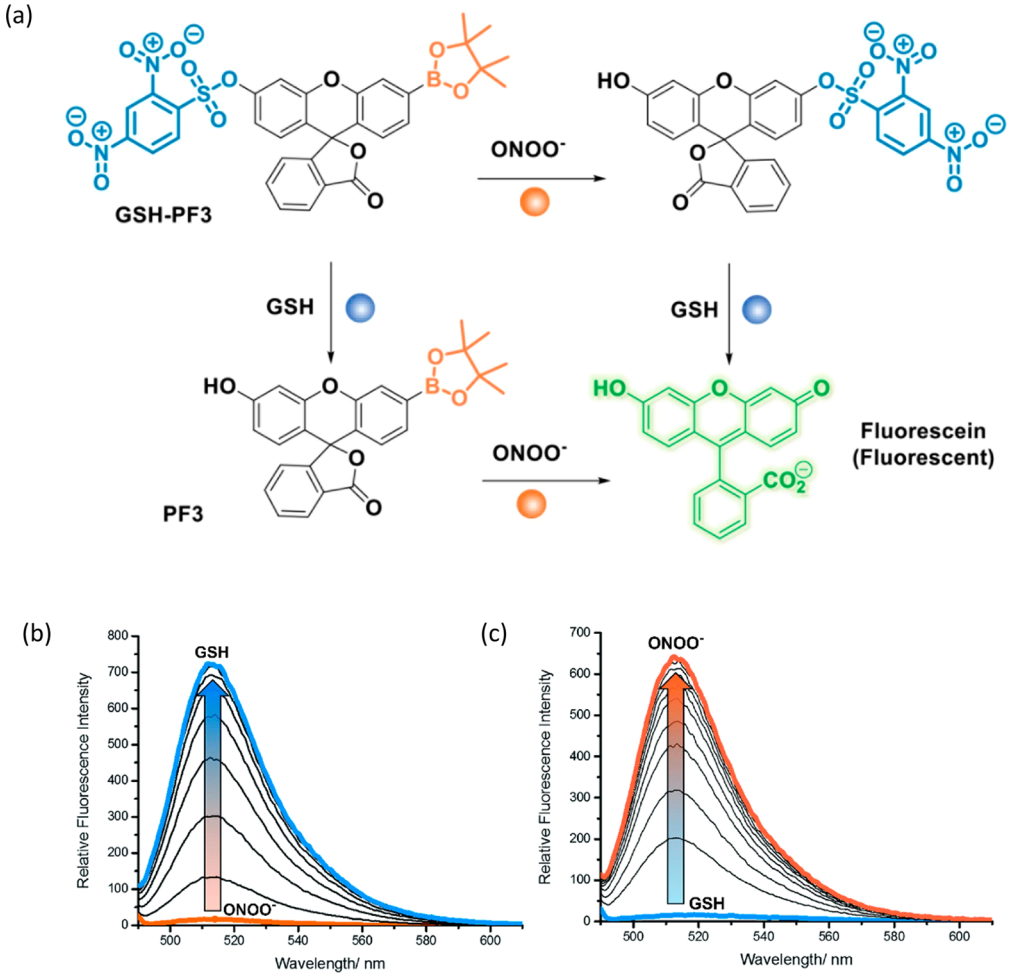
Scheme 15. (a) GSH-PF3 for Detecting $ONOO^-$ AND GSH; (b, c) Fluorescence Spectra of GSH-PF3 (0.5 μM) upon Addition of (a) $ONOO^-$ (10 μM) Followed by GSH (0−80 μM) with a 5 min Wait between Additions and (c) GSH (200 μM) Followed by Addition of $ONOO^-$ (0−10 μM) with a 10 min Wait between Additions (52 wt % Methanol, pH 8.21, λ_{ex} = 488 nm, 25 °C)[a]



[a]Adapted with permission from ref 60. Copyright 2018 Royal Society of Chemistry.

(ca. 10 nm) normally observed for such BODIPY-based FRET systems.[56]

2.2. Fluorescent Probes for H_2S/Na_2S

Hydrogen sulfide has been reported as an endogenous gaseous transmitter that regulates several physiological and pathological processes, including neurotransmission, vasodilation, inflammation, atherosclerosis, oxidative stress, and inhibition of insulin signaling.

The biological importance of H_2S prompted us to develop the galactosyl(azido)naphthalimide-based fluorogenic probe **DT-Gal**, in which the galactosyl unit is used as a targeting group for liver cells and the azido unit acts as a functional group for selective reaction with H_2S (Scheme 13). The potential of **DT-Gal** to act as a target-specific probe for imaging of H_2S in HepG2 liver cancer cells has been demonstrated,[57] as abnormal H_2S levels are known to be important in the pathogenesis of a number of liver diseases.

1-Oxo-1*H*-phenalene-2,3-dicarbonitrile (OPD) was also developed as a selective and sensitive fluorescent probe for detection of aqueous Na_2S over other thiols and inorganic sulfur compounds (Scheme 14). The fluorescence increased with the sulfide concentration from 1.0 to 30 μM with an LOD of 52 nM.[58]

3. AND-LOGIC-BASED FLUORESCENT PROBES FOR DETECTING ROS/RNS, RSS, AND OTHER SPECIES

Standard fluorescent probes require a single analyte to produce a fluorescence response. However, biological pathways are complex and dependent on the presence/action of multiple reactive chemical species. As a result, a number of fluorescent probes for dual or multianalyte detection have been developed that can be used as molecular logic gates for medical diagnostic applications. Consequently, we are interested in the development of AND-logic-based fluorescent probes that require the simultaneous or sequential action of two or more bioanalytes to produce a fluorescence response. Importantly, dual-analyte probes have the advantage that they are capable of simultaneously detecting short-lived species that may be produced only transiently in a cell, which is often not possible if two different sensing probes are employed for the independent sensing of each analyte. In addition, we believe that these dual-probe systems are particularly interesting since they provide an unequivocal method to monitor bimolecular events that may be responsible for progression of a specific disease.[59]

Our initial aim was to develop an AND-logic-based fluorescent probe for the simultaneous detection of $ONOO^-$ and GSH that would allow monitoring of intracellular GSH

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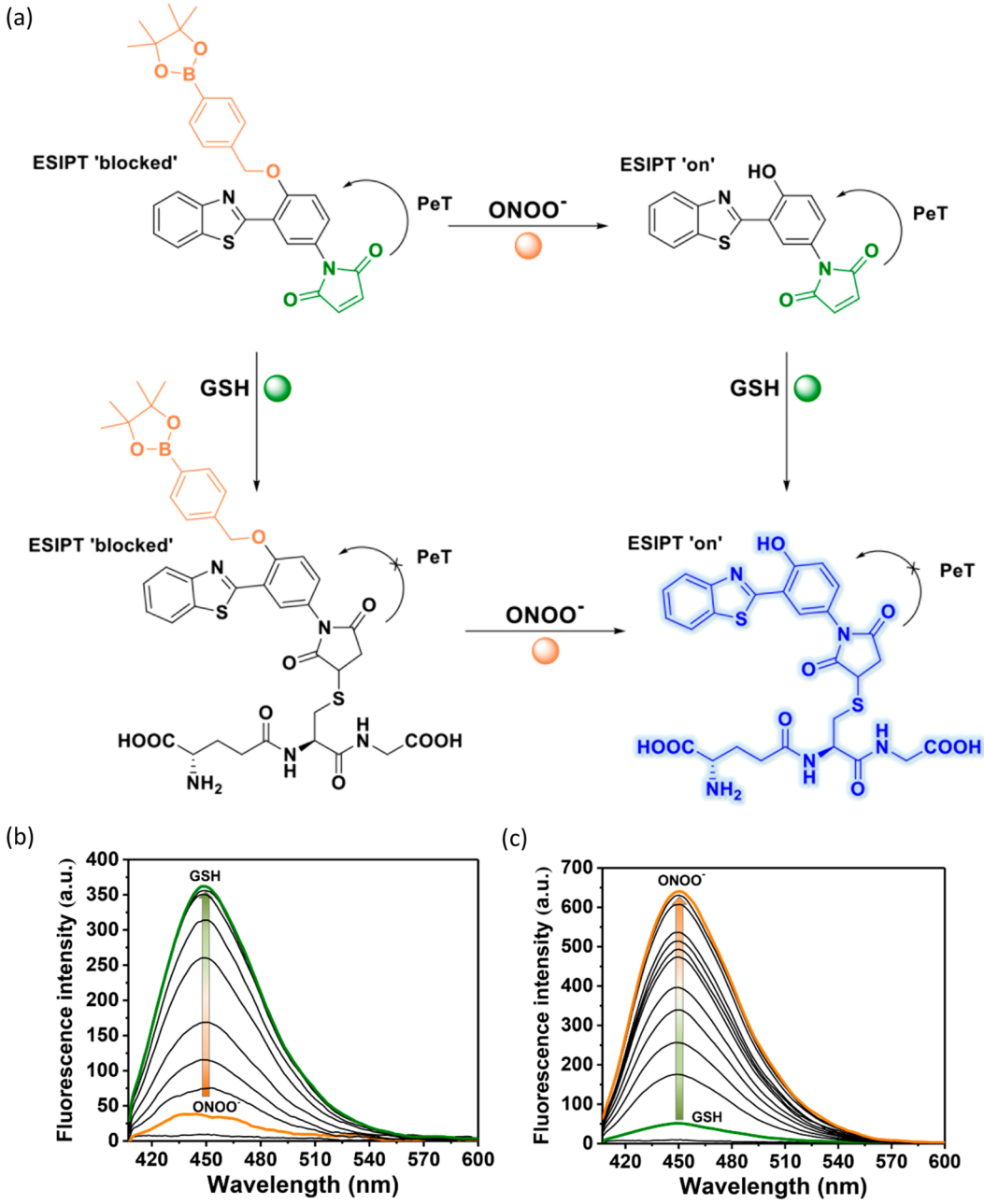
Scheme 16. (a) GSH-ABAH for Detecting $ONOO^-$ AND GSH; (b, c) Fluorescence Spectra of GSH-ABAH (2 μM) upon Addition of (a) $ONOO^-$ (4 μM) Followed by GSH (0−2 μM) after a 1 min Wait and (c) GSH (5 μM) Followed by $ONOO^-$ (0−14 μM) after a 1 min Wait (8% DMSO, 1 mM CTAB, pH 8.20, λ_{ex} = 390 nm, 25 °C)[a]



[a]Adapted with permission from ref 61. Copyright 2018 Royal Society of Chemistry.

levels in cells under oxidative stress. The probe **GSH-PF3** containing a cleavable DNBS linker, which was prepared from commercially available fluorescein in three steps, produced a minimal fluorescence response when GSH and $ONOO^-$ were added independently. However, when both analytes were present simultaneously, **GSH-PF3** produced a significant fluorescence enhancement (40-fold) (Scheme 15). **GSH-PF3** exhibited exceptional selectivity for detection of GSH AND $ONOO^-$ analytes in cellular systems, producing a strong fluorescence response only when both GSH AND $ONOO^-$ are present.[60]

The ESIPT-based AND-logic fluorescent probe **GSH-ABAH** with good cellular permeability was next prepared for the simultaneous detection of $ONOO^-$ AND biological thiols (Scheme 16). A fluorescence response was not observed when either SIN-1 ($ONOO^-$ donor) or GSH was added to RAW264.7 cells, but simultaneous addition of both analytes resulted in a strong fluorescence response.[61]

Since Chang's **PR1** sensor was a good system for $ONOO^-$, we decided to add additional cleavable linkers to that probe to develop a new series of AND probes for $ONOO^-$ and other selected bioanalytes. Accordingly, a new fluorescent resorufin-based "pinkment" probe[62] was used as an easily functionalizable scaffold to produce a range of AND-based fluorescent probes for detecting $ONOO^-$ and a second analyte (Scheme 17). A **pinkment-OH** core unit was used to prepare **pinkment-OTBS**

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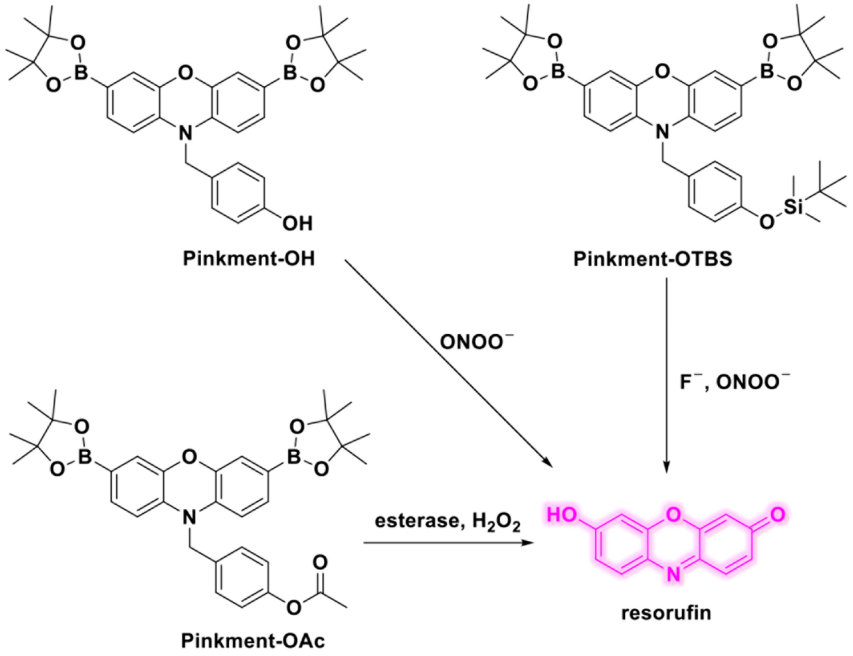
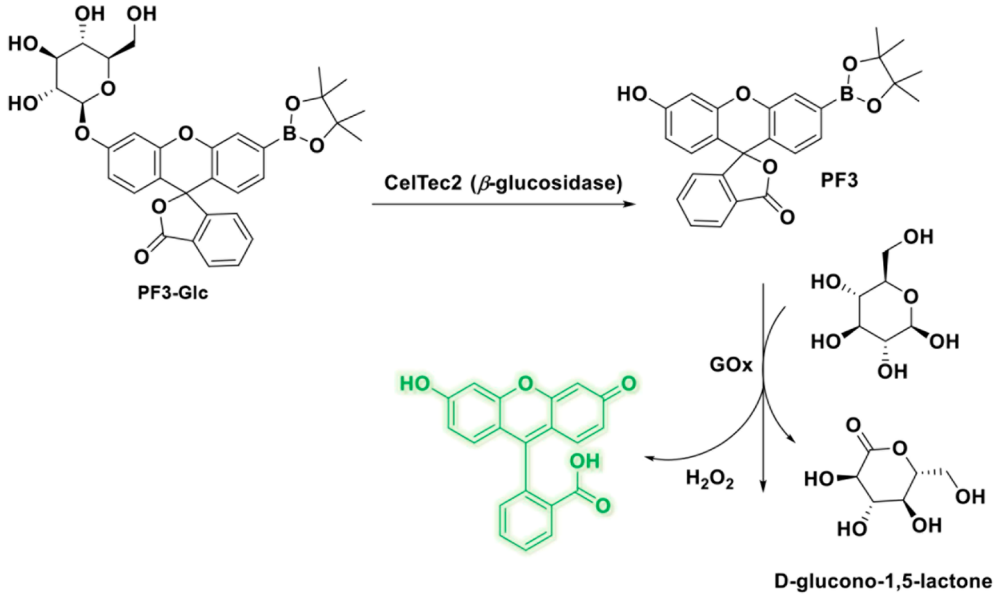
Scheme 17. Pinkment-OH for Detecting $ONOO^-$, Pinkment-OTBS for Detecting $ONOO^-$ AND Fluoride, and Pinkment-OAc for Detecting $ONOO^-$ AND Esterase Activity



Scheme 18. Dual-Enzyme-Activated PF3-Glc



and **pinkment-OAc** as proof-of-concept systems that were shown to function as dual probes for detecting $ONOO^-$ AND F^- or H_2O_2 AND esterase activity, respectively.[63]

More recently, an enzyme-mediated fluorescein-based dual-analyte probe, **PF3-Glc**, consisting of β-glucosidase (β-glc) and H_2O_2 trigger units was developed as part of an AND-based system. β-glc catalyzes hydrolysis of the glycosidic bond of **PF3-Glc** to afford monoboronate fluorescein **PF3** (nonfluorescent) and glucose, which is oxidized by glucose oxidase (GOx) to afford D-glucono-δ-lactone with the concomitant generation of H_2O_2. The H_2O_2 that is produced then facilitates oxidative cleavage of the boronic ester fragment of **PF3** to afford fluorescein, whose formation results in an 80-fold increase in the fluorescence intensity of the system (Scheme 18).[64]

A series of environmentally sensitive 3-hydroxyflavone (3-HF) ESIPT boronate-based fluorescent probes were developed that exhibit a ratiometric response toward $ONOO^-$ in a micellar environment (Figure 6a). Environmental sensitivity of a fluorescence probe is often considered to be an unwelcome property for sensing applications, but we have used the sensitivity of 3-HF toward hydrophobic environments to differentiate between micellar and aqueous environments. In addition the progression of AD is known to be associated with the formation of insoluble amyloid-β (Aβ) plaques, so we have used our ESIPT-based 3-HF probes to image different aggregation states of Aβ in the presence of $ONOO^-$. For example, the probe **3-HF-OMe** was found to produce a ratiometric fluorescence response when bound to Aβ aggregates

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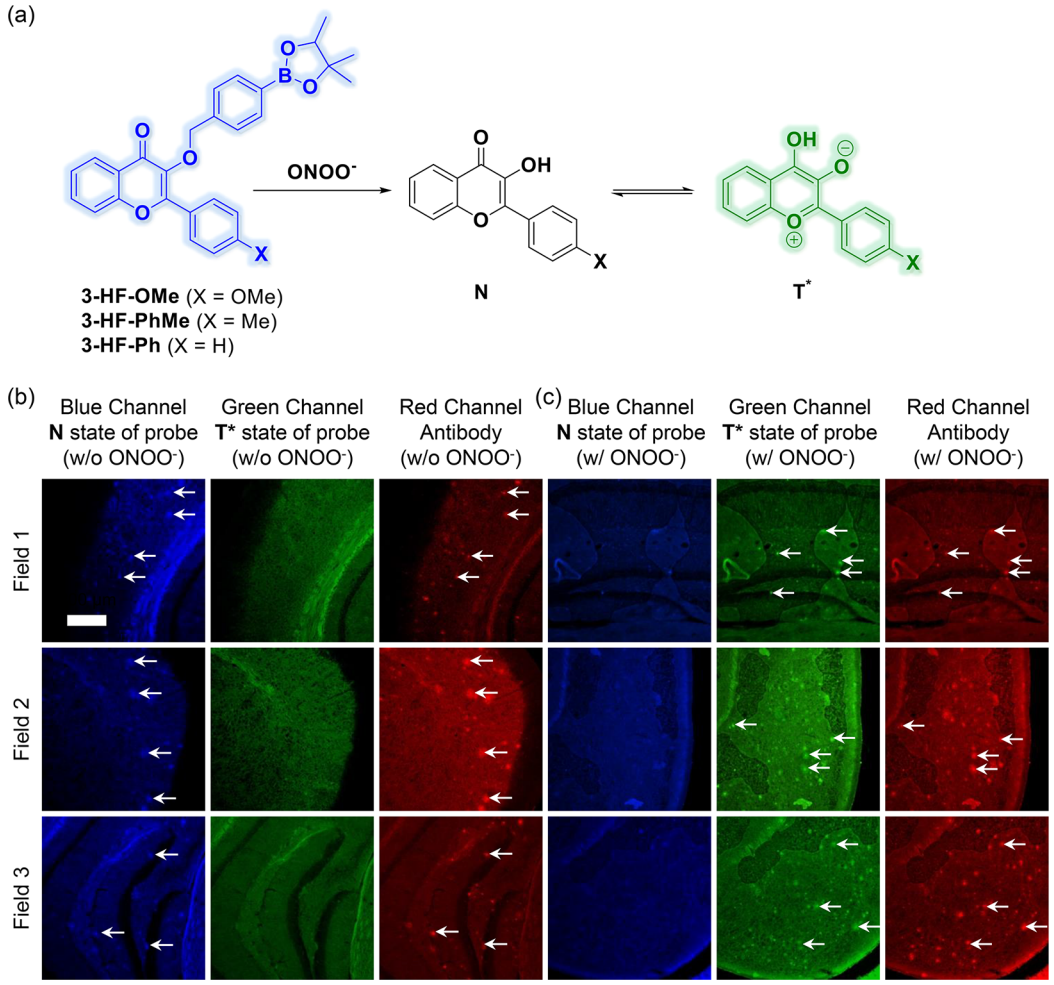

Figure 6. (a) ESIPT probes **3-HF-X** (X = OMe, Me, H) for detecting $ONOO^-$. The normal (**N**) and phototautomeric (**T***) forms are shown. (b, c) Fluorescence imaging of a brain section of a transgenic mouse treated with **3-HF-OMe** (20 μM) (b) without and (c) with $ONOO^-$ (30 μM). The excitation/emission wavelengths for the blue (**N**-state), green (**T***-state), and red (anti-Aβ antibody) channels are 404/425−475, 404/500−550, and 561/640−730 nm, respectively. The white arrows indicate stained Aβ aggregates. Reprinted from ref 65. Copyright 2018 American Chemical Society.

in the presence of $ONOO^-$, thus affording a novel protein-based host−guest system. Fluorescence imaging studies of **3-HF-OMe** revealed high N-state fluorescence in mice brain sections (Figure 6b, blue channel) that were shown to contain Aβ aggregates through correlation studies with anti-Aβ42 antibodies (Figure 6b, red channel). Subsequent treatment of these brain sections with $ONOO^-$ generated the T* state of **3-HF-OMe** (Figure 6c, green channel) with regions of fluorescence once again correlated with anti-Aβ antibody fluorescence studies (Figure 6c, red channel). These highly promising biomacromolecular imaging results demonstrate how other ESIPT-based probes might potentially be used for simultaneous sensing of fibrous proteins/peptides AND environmental ROS/RNS. This system can be considered as a "reactive species" AND "environment" based fluorescent probe.[65]

CONCLUSIONS

This Account has described research carried out in our research groups over the past six years toward the development of fluorescent sensors to probe redox biology and provide a better understanding of the various disease states that generate excess ROS. In section 3 we have described the development of dual-activated sensors, which we believe will prove instrumental in providing an understanding of the complex role that different ROS species play in the complex pathways that underpin disease-specific redox processes.

We anticipate that the use of dual-activated probes to evaluate cellular changes associated with many different disease states will become increasingly important, with probes that are responsive to different environmental conditions present in cells becoming more prevalent. We hope that this Account of the research carried out in our groups has demonstrated that responsive fluorescent probes can serve as effective tools to investigate dynamic redox chemistry in living systems and will provide inspiration to develop fluorescent tools that will facilitate improved disease diagnostics and therapies for the treatment of diseases.

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Notes

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