6-26-2020

Biosensor and Chemosensor Fluorophores that contain Chalcogenide Centers

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Cover Page Footnote

MINI-REVIEW

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Abstract

In this timely review, we discuss the important attributes of various chalcogen-containing small-molecule probes that have been synthesized in the Molecular Logic Gate Laboratory at the Korea Advanced Institute of Science and Technology. Specifically, we discuss some of the important chemical and photophysical properties of these probes, including reversibility, responsiveness (response time), cellular localization, sensitivity to analytes, selectivity (toward a specific analyte in question), and some bioavailability criteria. Chalcogenides undergo reversible redox-type reactions with reactive oxygen species (ROS). These controlled solution reactions enable a sensible and clear response as they act to immediately affect the chemical and electronic properties of the chalcogen moiety. Often, the lone pair belongs to chalcogens, which communicate electronically with the rest of the probes. Importantly, chemically oxidized chalcogenides can revert to their original reduced (divalent) form through the addition of natural or unnatural biothiols (or other reductants). This phenomenon is considered reversibility from the standpoint of probes. It can also be called "resetability." In this manner, a variety of fluorophore frameworks can be used to detect ROS and thiols. Further studies can help experimentally determine the lipophilicity and even the cellular localization of probes, which are important in assessing their value as diagnostic agents in biological sciences and their possible therapeutic potential.

Keywords: chemosensing, fluorescence, live cell imaging, reactive oxygen species, biothiols, selenium, reversible probe

Introduction

Optical data acquisition and information imaging have been increasingly developed in recent years and decades; this area of research helps transform our definitive understanding of biology and disease progression. Such new methods, which often relate to molecular design, help fuel research interest seen in various fields and are reflected by many high-profile reports and accomplishments, such as that of Dubochet, Frank, and Henderson who codeveloped high-resolution cryoelectron microscopy, which was awarded the Nobel Prize in Chemistry in 2017. The ability to determine the structures of biomolecules, such as unstructured proteins in their native matrix (i.e., water, albeit solid H_2O) and at the molecular level without the need for purification, as well as crystallization, which is a challenging and difficult process, is an important pursuit [1]. The

understanding of the key chemical constituents of neurodegenerative diseases needs to be at the molecular level and is still to be completed. The ways that synthetic molecules can interface with natural systems need to be determined to help delineate the chemical processes involved in complex disorders with perplexing etiology, such as dementia. *Optical imaging* using suitable synthetic molecular probes is a versatile method for obtaining molecular-level information on analytes. The versatility of this method can be attributed to its noninvasiveness toward the biomolecule host, as well as its ability to provide real-time detection economically with contrast media that are well-defined and reproducibly synthesized with sufficient yields. These excellent and unique attributes enable the comparison of the method with other methodologies, such as high-performance liquid chromatography, electrochemical methods, and positron emission tomography.

Chalcogenides are "ore-forming" main group (representative) elements from Group 16 (Column VIA) of the periodic table (i.e., oxygen [O], sulfur [S], selenium [Se], tellurium [Te], and polonium). For example, S and Se show similarities in terms of preferred electronic configuration, chemical behavior, and product formation. Currently, chalcogenides have attracted considerable interest from researchers around the world. For example, organoselenium compounds are widely researched as materials for solar energy harvesting, imaging, and biological and environmental sensing [2–6].

S is present in the human body and often encountered in the form of thiols and sulfides in small biomolecules, such as the amino acids cysteine (Cys, $pK_a = 8.5$) and homocysteine (Hcy, $pK_a = 10.0$) and the tripeptide glutathione (GSH). Cys and selenocysteine, as well as methionine, are members of the "21" naturally occurring amino acids. These species are known as biothiols [7]. Endogenous biothiols that contain sulfhydryl groups play important roles in biological systems. For example, GSH is the most abundant intracellular biothiol and plays an essential role in the antioxidant system. GSH exists in equilibrium between its oxidized disulfide form and its reduced thiol form (Figure 1). The ratio between oxidized and reduced forms is an important biological indicator, which is used as a means to determine oxidative stress. This ratio is often repeated in research articles. Sometimes scientific and medical reports will describe "total GSH" levels. Furthermore, the GSH level is closely related to oxidative stress, which means that it can also be used as a clean molecular indicator among other indicators of various conditions, such as the presence, status, and stage of neurological diseases, dementia, cancer, and diabetes [8–10].

As an interesting historical note, in the early 20th century, most scientists believed that Se was toxic to people, even in trace amounts; moreover, knowledge of the element's diverse organic and biological chemistry was extremely limited. Perhaps the biological utility of what is commonly considered a stinky element was difficult to fathom. However, in 1957, Schwarz and Foltz identified Se as a trace element and an essential element in biology. Its isosteric nature as a congener to S and a non-metalloid version of Te makes it an intriguing element to explore in synthetic bioorganic chemistry and beyond. Se acts as a micronutrient in many living organisms [11]. Further research led to the discovery in 1973 that two bacterial enzymes also contain Se; these proteins are formate dehydrogenase and glycine reductase. At about the same time, Se was discovered to play a major role in the active site of the antioxidant enzyme glutathione peroxidase [12,13]. Since then, many chemical and biological researchers have worked on elucidating the chemistry of Se, which has led to the discovery that chalcogenides, particularly Se, have extensive and important functions in biological
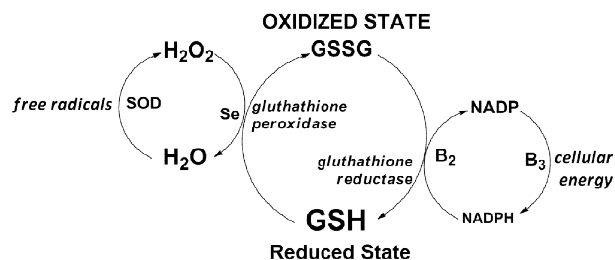

Figure 1. Illustration of the Function and Interrelationship between Glutathione Peroxidase and Glutathione Reductase

systems as enzymatic antioxidants, imparting anti-inflammatory, antitumor, antifungal, and antibacterial properties to living systems [14].

Organochalcogenide chemical systems are not new and have been used extensively as functional materials, tools, and synthetic intermediates in pharmaceutical research. These systems are known to exhibit diverse biological activities, both in vivo and in vitro, including antibacterial, antidiabetic, antiarrhythmic (cardiac dysrhythmia), and antitumor activities. This class of compound has also been researched in the context of incurable diseases, such as neurodegenerative diseases (e.g., Parkinson's and Alzheimer's diseases) [15,16]. Furthermore, continuous research on phenylchalcogenide-related molecular probes for bio-imaging is undertaken. Much of this interest is driven by the chemical versatility of Se in covalent bonding. Moreover, the ability of Se to participate in redox chemistry/biology enables small Se-containing molecules to become widely regarded as candidate antioxidant compounds in medicinal chemistry. Organochalcogenide derivatives have recently attracted considerable attention in synthetic and medicinal chemistry research. The development (i.e., design) process is important and challenging [17], with step efficiency and environmental benignity, as well as practical and affordable chemical methodology, being major concerns.

Our research group has synthesized a series of molecular probes bearing organic chalcogenide sites (i.e., S, Se, and Te) [18–21]. These chalcogenide probes can indicate the presence and concentration of important analytes, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), including hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), superoxide ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), nitric oxide (NO), and peroxynitrite ($ONOO^-$), as well as biothiols. In such research, selectivity is an important issue and an ongoing challenge.

ROS and RNS are O- or nitrogen-containing species that exist in nature and within the human body in a range of low concentrations. In many cases, ROS and RNS are produced by the body as a defense mechanism

against various pathogens and play important roles in homeostatic signaling and control [22]. Thus, an excess of ROS/RNS in the body can indicate human diseases, such as inflammatory diseases (e.g., arthritis), hepatic ischemia–reperfusion injury, atherosclerosis, neuronal degeneration and death (e.g., Alzheimer's disease), lung injury, and cardiovascular diseases [23,24]. Several functional groups, such as *p*-methoxyphenol, oxime, and dibenzylhydrazine hydroquinone, and chalcogen-containing systems exhibit reactivity that can be exploited to detect analytes, such as ROS and RNS. These reactive moieties are embedded in fluorophores to help modulate the fluorescence intensity in response to ROS/RNS concentration. Furthermore, the oxidation of selenides and tellurides, often found in the R–Se–R form, is present but is not always the case. These chemical systems exhibit rapid ($Se^{II} \rightarrow Se^{IV}$) response and chemical/oxidation state reversibility ($Se^{IV} \rightarrow Se^{II}$) upon the addition of biothiols. Thus, because their C–E bond strength is moderate, these chemical systems are reliable motifs for probes and can provide real-time detection of ROS and RNS. That is, in many cases, reversible or resettable characteristics can be achieved. However, these properties still need to be better optimized.

The following sections contain a discussion about chalcogen-based chemosensors, focusing on small molecules classified as fluorophores. These fluorophores contain organochalcogen centers (i.e., R–S–, R–Se–, or R–Te–) that can oxidize specific analytes. These small synthetic molecular systems undergo various photochemical reactions that can be used to detect small-molecule analytes important in biology.

Fluorescence characteristics and switchability of Se-containing organic compounds. Fluorescence is a process by which light energy is emitted from an electronically excited molecule that can absorb and emit photons. Then, the resulting molecule undergoes electronic relaxation and does not produce heat. Molecules that exhibit this ability are called fluorophores. The movement of electrons in this process can be visualized using a simplified electron-state diagram called a Perrin–Jablonski diagram (Figure 2). An energy
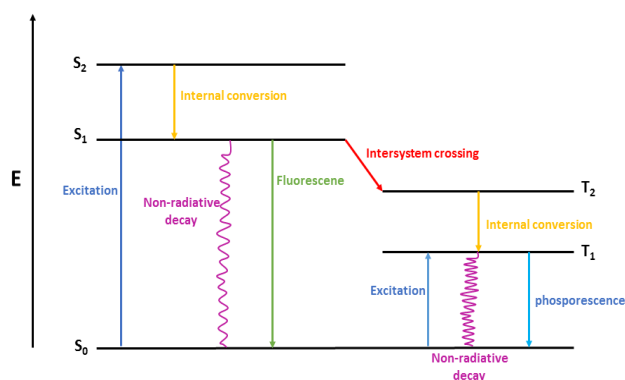

Figure 2. A Simplified Perrin–Jablonski Diagram

source, such as a UV lamp, is commonly used to provide photons that promote a frontier electron (not core) to a higher vibrational–electronic energy level, that is, the first excited state (S_1). Then, the electron rapidly relaxes to the lowest energy level, resulting in photon emission. Furthermore, electrons undergo spin conversion into a forbidden triplet state (T_1), instead of the lowest singlet excited state, a process known as intersystem crossing. Emissions from the triplet state (phosphorescence) result in lower energy than fluorescence emissions from longer wavelengths. However, Se in fluorophores is mainly involved in a chemical system that exhibits fluorescence. Therefore, phosphorescence, which may be an important existing property in many cases, is not discussed in this review.

Common analytical techniques used to detect analytes in solution. Numerous photomechanisms are currently exploited as a means to utilize the fluorescence of fluorophores to detect biologically important analytes. These photomechanisms include photoinduced electron transfer (PET), fluorescence ratiometry, Förster resonance energy transfer (FRET), and aggregated-induced emission (AIE) (Figure 3). Among these well-known phenomena, PET is most commonly used. PET is the process of electron transfer from the lowest unoccupied molecular orbital of a quencher moiety to the highest occupied molecular orbital of a fluorophore moiety, resulting in diminished fluorescence capability of the fluorophore. The two versions of PET, which depend on the oxidation potentials between fluorophores and quenchers, are reductive PET (acceptor–PET) and oxidative PET (donor–PET). The PET strategy provides sensitive and reliable responses toward specific analytes. However, this system can be hindered by background interference from sample media and environmental effects.

To overcome these obstacles, approaches to data interpretation involve comparing the relative intensities of two markedly different wavelengths. Such a strategy is called "ratiometric" probing. This method relies on changes in fluorescence spectra, that is, fluorescence shifts, upon the reaction of a probe with an analyte and has become widely adopted. The two types of fluorescence shifts are hypsochromic shift (i.e., to longer wavelength/higher energy) and bathochromic shift (i.e., to shorter wavelength/lower energy).

The FRET phenomenon relies on two different fluorophores and their proximity. The interaction between two fluorophores whereby electronic dipole–dipole coupling occurs between two chemical groups that are separated in space but are tethered together in a certain manner. The donor fluorophore transfers its excited electronic energy to the acceptor fluorophore and does not emit a photon. Thus, to achieve this experimentally, the emission spectra of the fluorophore
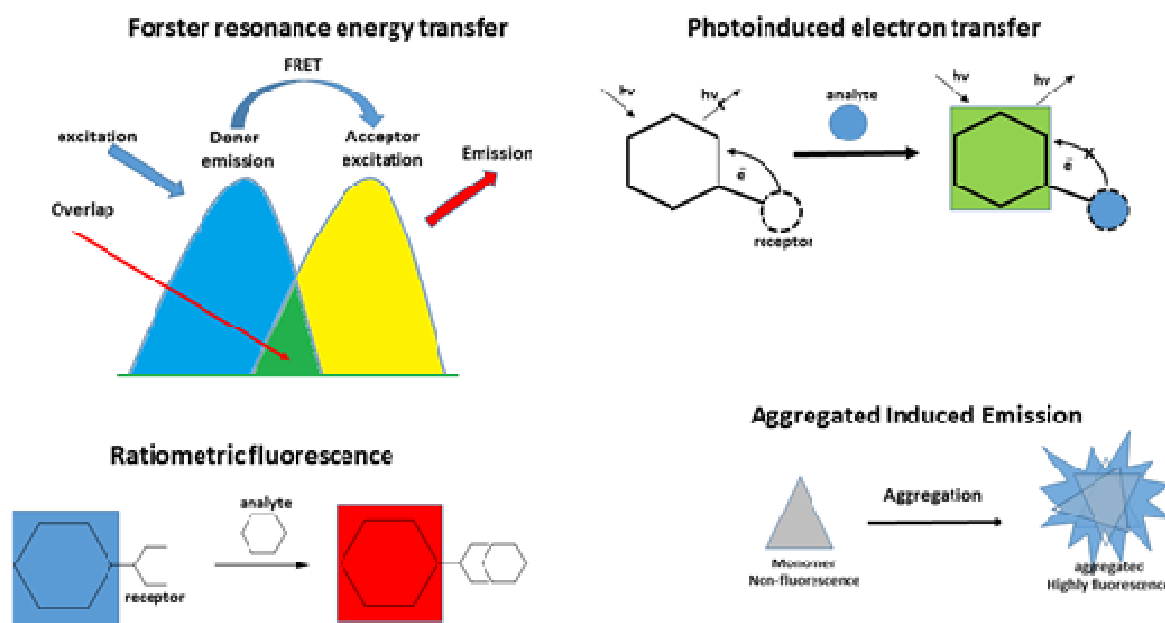
Figure 3. Common Fluorescence Phenomena that Enable the Detection of Analytes

donor and acceptor should overlap. The good performance of the system is constrained to the distance between the fluorophore donor and acceptor and, in some cases, the rigidity of the system as well. As FRET is sensitive to the distance between the interacting fluorophores, the distance should preferably be 20 Å but inner and outer extremes should range between approximately 5 Å and approximately 100 Å to achieve the optimal performance [25].

AIE is a photophysical phenomenon that occurs within fluorophores in which separated and proximal molecules are not fluorescent in solution but are sometimes highly emissive when packed in an aggregated state.

Crystal structure studies can help show how such molecules might be aggregating. However, a crystal is a highly ordered solid, whereas an aggregate is not constrained to the same format. This phenomenon was first reported by Tang *et al.* in 2001 based on silole derivatives, which exhibited "turn-on" fluorescence in the condensed state [26] and was aided by the propeller design of the molecule. Many chemical researchers have investigated the mechanisms of AIE, which include (i) conformational planarization, (ii) J-aggregation, (iii) excited-state intramolecular proton transfer, and (iv) twisted intramolecular charge transfer. But none of these proposed mechanisms is universally applicable. Nevertheless, many various aspects of the AIE phenomenon have been presented in the literature. In 2015, Tang *et al.* hypothesized that, in the aggregated or solid state, the molecules interlock, which restricts the intramolecular rotation, vibration, and relaxation

through radiative pathways. Moreover, the molecules should avoid dense "face-to-face" packing, which would favor strong π–π stacking interactions that promote radiationless relaxation that leads to aggregation-caused luminescence quenching [27].

Aside from their redox potentials, Se-containing organic compounds also have a measurable, albeit subtle, steric effect based on the substitution of one congener for another congener, for example, S for O, Se for S, and Te for Se. How this form of substitution factors into AIE will be elucidated in the future. However, what probably trumps the steric effect is the reactivity and interplay with ROS to yield the element monoxides R–E(O)–R, as well as taking advantage of the E–C bond strength in a given system.

For such biological applications, chalcogens can be preferred to transition metals because of their potential redox activities, with changes in their valence states from 2 to 4 and from 4 to 6, as well as their reliable geometric formations upon single chemical oxidation. Chalcogens can be reliably monooxygenated and chemically reduced in solution and possibly in the aggregated state as well. For example, Se has a good nuclear magnetic resonance (NMR) spectroscopic handle. A complicating feature of this research is that the reduced chalcogenide centers, including Se, can serve as donor atoms for metal chelation. Regardless, the dramatic change in fluorescence based on the changes in redox reactivity is an advantage of this subdiscipline. As first reported by M. R. Detty in 1990, optical characteristics are sometimes profoundly changed by the additional +2 oxidation state of Se in

which the active center is found within an aromatic ring or as a substituent found directly attached to a given fluorogenic system or a π-delocalized framework [14]. Although a chalcogen that can be oxidized by chemical means, for example, hydrolysis, to [$R_2Se=O$] or perhaps [$R_2Se(OH)_2$] may be contained within an aromatic ring; alternatively, it may be present as a substituent. There is also the possibility of the C–E bond to cleave and to do so selectively and perhaps homolytically in some cases. C–Se bond cleavage, while it may seem in many cases an unwanted event, can be exploited and investigated with selective biothiol detection in mind for example. Interestingly, the Se(O)R group is a well-known leaving group in organic chemistry. Therefore, Facile Se–C breakage based on the connecting carbon being electrophilic can become a design parameter in small synthetic fluorogenic molecular design (chemosensing). Given the often low concentrations of analyte available, the involvement of, for example, the chemistry of S in biology is dynamic and diverse. Therefore, we focus herein on how chalcogen atoms can be embedded in, or connected as substituents to, a fluorophore or medicinal chemistry compound (they may be one and the same). The results can be contextualized in terms of cellular biology models, organism studies, and research on the chemistry of aging and neurodegenerative disease (long term).

For these novel compounds to be used as sensors, organochalcogens are attached to a fluorophore as a means to modulate the selectivity of fluorescence. The presence of the organochalcogen within the fluorophore serves as a means to modulate the selectivity of fluorescence and to receive the analyte. Usually, the organochalcogen is used as a fluorescence quencher in the so-called fluorescence "turn-off" state. Then, after the addition of the analyte, the fluorophore regains its fluorescence and undergoes fluorescence "turn-on" switching. Currently, many fluorescent chemosensors use "turn-on" methods, with advantages in terms of their reliability, as well as freedom from background noise, compared with "turn-off" fluorescent sensors. In this case, strategies, such as PET, ratiometric approaches, FRET, and AIE, can be employed to control the "turn-on" and "turn-off" behavior of a

luminescent system. The details of the mechanisms of each of these phenomena can be found in other sources.

ROS detection by chalcogen oxidation. Our group has been involved in synthesizing and analyzing small-molecule Se- and main-group-containing organic and organometallic systems (i.e., main group) for their sensing and therapeutic potentials, as well as their selective fluorescence imaging and low toxicity properties [28–33]. As far as we are concerned, the eventual goal is to probe aspects of neurodegenerative diseases in humans and animal disease models in a more precise manner based on the present "state-of-the-art" research in organochalcogen-based platforms, motifs, and fragments.

Chalcogen centers can detect the presence and concentration of important analytes, such as ROS and RNS. Various fluorophores have been investigated. The use of boron dipyrromethene (BODIPY) as a fluorophore to detect ROS is a growing field. Chalcogenide systems containing selenide as a receptor exhibit reversibility upon reaction with ClO⁻ and biothiols; these have been investigated extensively in our laboratory. In 2013, our laboratory reported the synthesis of a highly specific and sensitive fluorescent probe for the detection of ClO⁻ in pure water (rapid "turn-on", λ_{ex} = 400-fold, and λ_{em} = 560 nm) [34]. The reaction involves the oxidation of a 2-thienyl-sulfide-2-benzoic acid pendent group attached to the 8-position of the BODIPY acceptor probe. The thienyl moiety is considered the electron donor. Further research has indicated that the probe can be used to image OCl⁻ in neuronal cell cultures (neuroblastoma) (Figure 4).

In 2014, Churchill *et al.* reported the synthesis of a diselenide-containing BODIPY dimeric probe. The probe was observed to be sensitive and selective toward $O_2^{\bullet-}$. The system was proposed to form the selenoxide moieties [−Se=O, −Se=O] (Figure 5) [35]. The probe exhibits reversible fluorescent properties upon the addition of biothiol concentration (unpublished results). Cell imaging studies of living breast cancer cells (MCF-7/ADR) were undertaken, which helped demonstrate the ability of the probe to detect both exogenous and endogenous superoxide concentrations.

SH-SY5Y Cell   **SH-SY5Y Cell + HOCl**

Figure 4. Reaction of *meso*-thienyl-BODIPY with ClO⁻

In the same year, Churchill *et al*. also synthesized an annulated BODIPY chalcogenide (Se and Te) system (Figure 6). This was the first example of such Se-mediated addition at the 1-position of the BODIPY system. The telluride version of the annulated BODIPY complex was also tested and discovered to be highly sensitive and selective toward ClO⁻ (detection limit of 3.7 μM and fluorescence enhancement of 62-fold) under physiologically relevant conditions (water/ethanol, 99:1, *v/v*, 0.1 M phosphate-buffered saline (PBS), pH 7.5). Furthermore, reversibility tests using treatment with biothiols demonstrated that the probe could be used for up to six simulated redox cycles [33].

In 2016, Churchill *et al*. synthesized a phenylselenyl-based BODIPY "turn-on" fluorescent probe for the detection of hypochlorous acid (HOCl) [36]. The probe exhibits a negligible background signal because of the incorporation of a second and different heavy atom, that is, Cl, within the probe. Upon reacting with HOCl, the chemical oxidation of Se cancels the PET process, causing a significant increase in fluorescence intensity

centered at λ_{max} = 526 nm. The probe shows an instant response to HOCl (reaction time = 15 ms) and provides a low detection limit (i.e., 4.5 nM). The probe exhibited cell membrane permeability and detection of HOCl (both exogenous and endogenous) in live RAW 264.7 macrophage cells (Figure 7).

As a continuation of their research, Churchill *et al*. attempted to improve the phenylselenyl-based BODIPY probe by adding a mesitylene moiety at the *meso* position [37]. They reasoned that the addition of the mesitylene moiety would add rigidity to the structure, thus increasing the fluorescence of the compound upon reaction with HOCl. As expected, the new probe (Mes–BOD–SePh) exhibited identical properties to that of its predecessor in terms of chemical selectivity toward HOCl and the photophysical properties of the probe. The probe exhibited excellent selectivity, sensitivity, and rapid response under physiological conditions (approximately 2 min). Mes–BOD–SePh showed fluorescence enhancement of up to 110-fold with a detection limit of 19.6 nM (Figure 8).
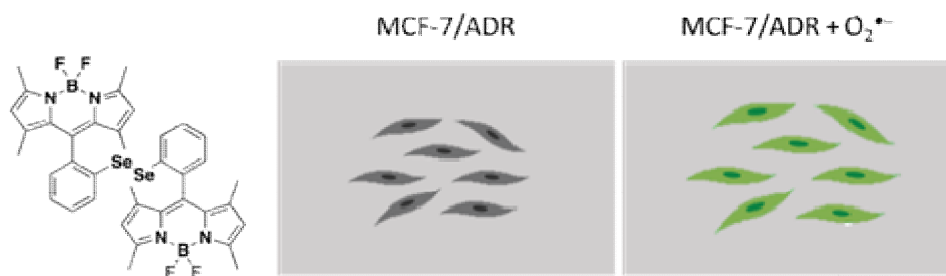

Figure 5.   Reaction of Diselenide-containing BODIPY with O₂•⁻
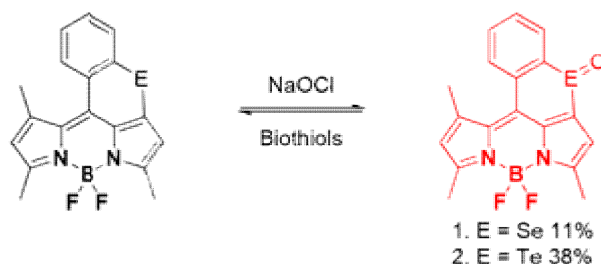

Figure 6.   Reaction of Annulated BODIPY with NAOCl
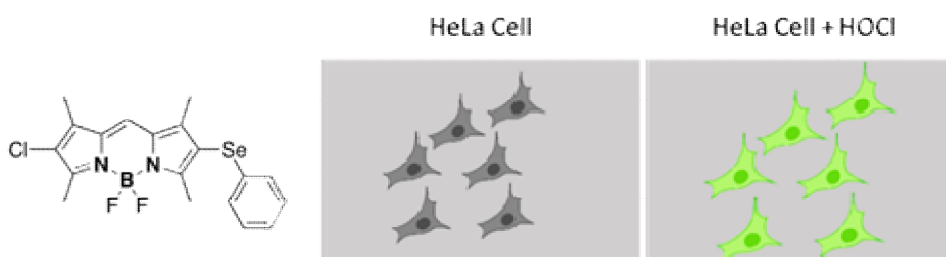

Figure 7.   Reaction of Phenylselenyl-based BODIPY with HOCl
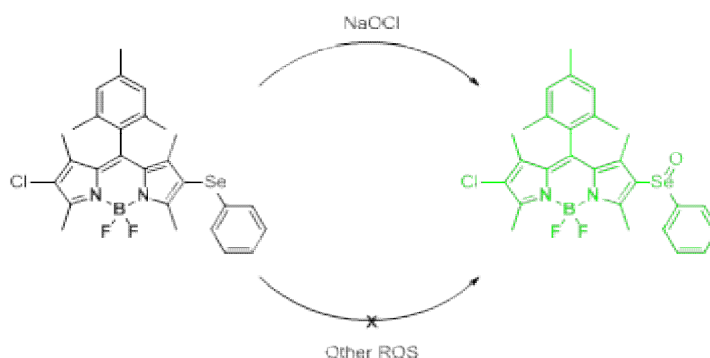
Figure 8. Reaction of Mes–BOD–SePh with HOCl and its Selectivity toward ClO⁻ Compared with Other ROS and RNS
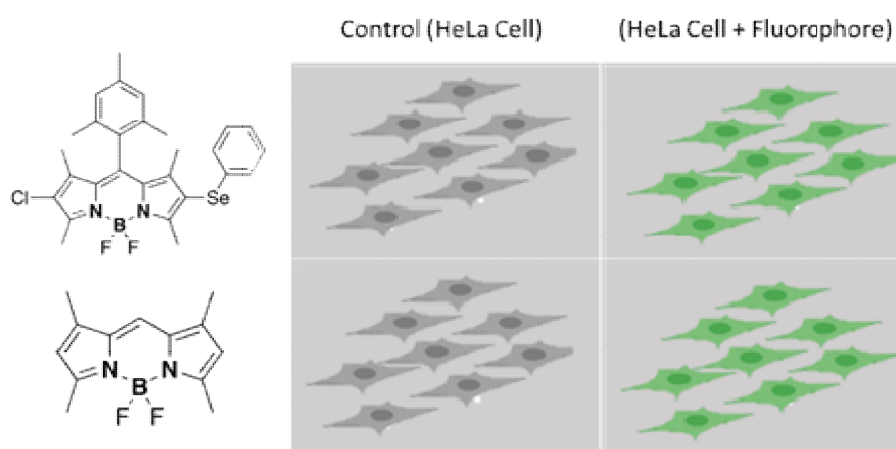

Figure 9. Fluorescence Images of Human Adipose Stem Cells. The Cells were Incubated in (a) Control Medium, (b) Commercially Available Lipid Droplet Staining Agent (BODIPY 493/503), and (c) Mes–BOD–SePh

However, when the probe was introduced to live human adipose stem cells, we observed a strong green fluorescence even without the addition of HOCl. The incorporation of the rigid and bulky mesitylene group at the *meso* position of the BODIPY framework not only improves fluorescence and probe photostability but also helps selectively transport the probe in lipid droplets (LDs). This is another example of how fluorophore chemical substituent effects can be used. They can give rise to markedly different probe behavior under physiological conditions and cell imaging experiments (Figure 9).

Although the BODIPY framework has the 5–6–5 fused S-indacene framework, there are other types of identical systems that can be explored. In 2016, Churchill *et al*. synthesized a diselenide dipyrazolopyridine probe in the laboratory using a domestic microwave oven. The probe exhibited a selective "turn-on" fluorescence response toward NaOCl with an approximate 180-fold fluorescence increase [32]. Confocal imaging studies were undertaken; these showed that the probe can be used to detect HOCl in living cells, as demonstrated using MCF-7 cells (Figure 10).

In 2017, a dithiophenylmaleimide BODIPY (**BDP-NGM**)-based fluorescent chemosensing system was reported [31a]. The system was tested and confirmed to be a "turn-on" probe in the presence of ROS/RNS and observed to show rapid response to peroxynitrite with excellent selectivity and sensitivity. The probe showed fluorescence enhancement of up to 18-fold with a detection limit of 100 nM. Although the "turn-on" increase was mild and the detection limit was unimpressive in this case, we still feel that this new modality represents a fresh and novel approach. In this system, the 3,4-bis(phenylthio)maleimide group acts as a quencher. Therefore, in the absence of ONOO⁻, the inherent fluorescence imparted to the BODIPY system is quenched because of the PET process and shows negligible fluorescence and poor quantum yield. Thus, this is the first maleimide-based probe for the endogenous detection of peroxynitrite involving a "turn-on" response in live RAW 264.7 cells, as supported and confirmed by confocal microscopy imaging (Figure 11). We experienced difficulty in synthesizing the analogous Se version, that is, one SePh would attach, but not both.
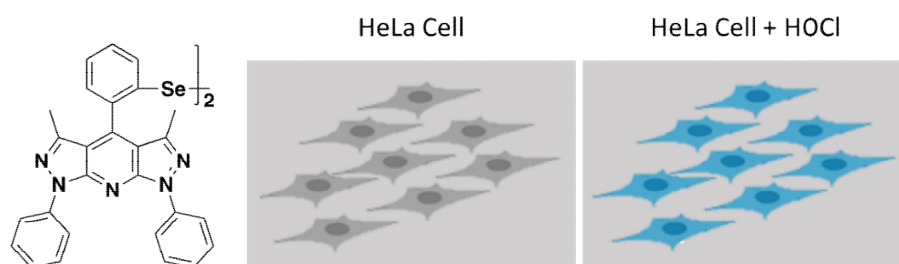
Figure 10. Reaction of the Diselenide Probe with HOCl and Color Change of MCF-7 Cells
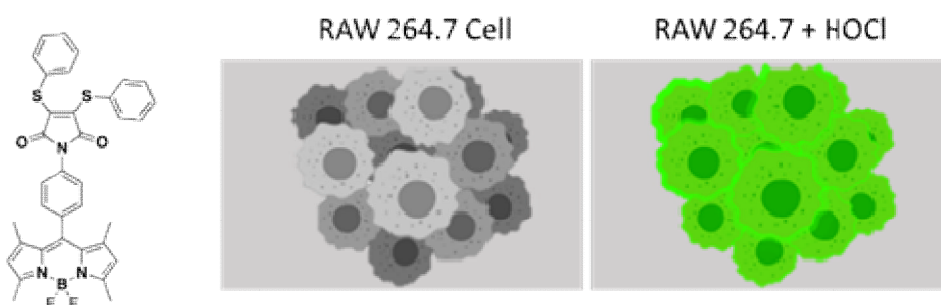

Figure 11. Reaction of BDP-NGM with ONOO⁻

Although the BODIPY framework is classified as a robust fluorophore, the preparation of some of these systems involves major drawbacks in terms of low product yield and difficulty in synthesis. In other words, obtaining a robust and outstanding probe can be hard work. Therefore, we also explored hemicyanine fluorophores incorporating a diphenyl selenide moiety (**HemiSe**) [38]. This fluorophore is relatively easy to functionalize through condensation reactions, as shown in Figure 12.

HemiSe exhibits selective and sensitive "turn-on" fluorescence in the presence of $O_2^{\bullet-}$. The time–response profile of the probe upon reaction with $O_2^{\bullet-}$ revealed that the entire reaction was completed in 13 min, with a 20-fold increase in emission intensity and a detection limit of 11.9 nm. In this chemical system, the oxidation of the diphenyl selenide unit acts as a PET fluorescent quencher in which, in the absence of $O_2^{\bullet-}$, the fluorescence of **HemiSe** is quenched because the intramolecular PET process enabled by the electron-rich Se group is active. Before chemical oxidation, the probe shows negligible fluorescence. Surprisingly, instead of reacting with selenide to form selenoxide, there is strong evidence to support the idea that $O_2^{\bullet-}$ reacted with the double bond within **HemiSe** to form a hydroperoxide product capable of blocking the PET effect. The product exhibits a blue emission band centered at 439 nm. The proposed product is illustrated based on the reactivity of other nucleophiles with the cyanine system. There is an extremely modest shift of the peak in the ^{77}Se NMR spectrum of **HemiSe** from 366.5 ppm (before the addition of $O_2^{\bullet-}$) to 372.8 ppm

(after the addition of $O_2^{\bullet-}$). This small change indicates that no chemical oxidation of selenide to selenoxide occurs. Moreover, the oxidation of Se results in a large spectral shift (movement to a position of approximately 800 ppm) (Figure 13). The probe can also be used for the endogenous detection of $O_2^{\bullet-}$, as demonstrated by its "turn-on" response in RAW 264.7 cells, confirming the practicality of this probe as a tool for biological sciences. However, it has come to light that superoxide is short-lived in aqueous media as it reacts with itself (or perhaps other radical species). Therefore, solutions with KO_2, for example, should be prepared in DMSO.

We did not explore the mouse model because of the blue fluorescence emission and poor aqueous solubility (an organic cosolvent is required). Hence, we further developed a new fluorophore skeleton based on mycophenolic acid (MPA) that was involved in laboratory research. With this framework in mind, two fluorescent probes, that is, **Probe–1** and **Probe–OCl**, were prepared and characterized (Figure 14). Interestingly, the synthetic intermediate of MPA exhibits fluorescence for the specific detection of HOCl [28,39]. Both fluorescent probes exhibited sensitive, selective, and rapid "turn-on" fluorescence responses (<1 s) when exposed to HOCl compared with other oxidants because of the oxidation of selenide to selenoxide. Both probes also show an increase in fluorescence quantum yield (approximately 3.8-fold in **Probe–1** and approximately 11-fold in **Probe–OCl**). Furthermore, both probes are completely soluble in aqueous media without the help of any cosolvent.
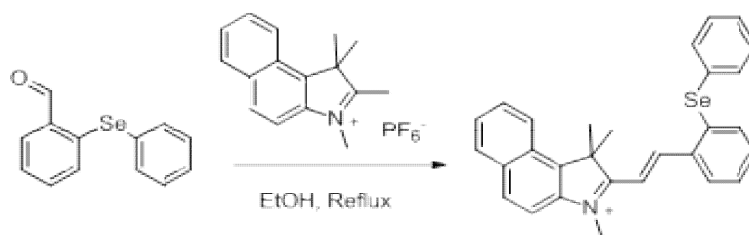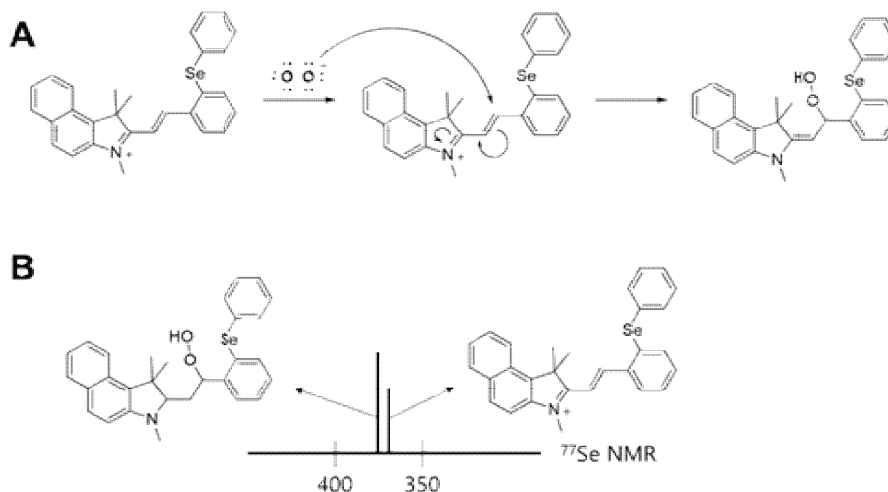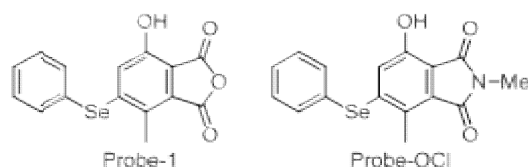
Figure 12. Synthesis of HemiSe

Figure 13. (A) Proposed Mechanism of the Reaction between HemiSe and O$_2^{\bullet-}$ and (B) Comparison of the ^{77}Se NMR Spectra of HemiSe Before (Right Peak) and After (Left Peak) the Addition of O$_2^{\bullet-}$ to CD$_3$OD

Figure 14.   Probe–1[67] and Probe–OCl for HOCl

However, further studies revealed that **Probe–1** is thermodynamically unstable in water over long periods of time. Moreover, chemical decomposition is observed to be the culprit under the action of ClO$^-$. Therefore, the incorporation of a [MeN] unit to generate the derivative methylmaleimide helped improve the signal stability. The result was **Probe–OCl**, which exhibits high sensitivity, specificity, and rapid "turn-on" fluorescence characteristics for HOCl under physiological pH. Furthermore, **Probe–OCl** undergoes AIE and accumulates in LDs. We reanalyzed the Mes–BOD–SePh derivative to compare the localization of the two probes (Figure 15). **Probe–OCl** was reported to be a probe for LDs, as seen in cuvette aggregation and induced fluorescence based on the addition of ClO$^-$.

Regrettably, **Probe–OCl** did not detect HOCl in living cells. We hypothesized that the incorporation of phenylselenide contributes to the AIE effect that occurs with **Probe–OCl**. Hence, in recent research from the Churchill laboratory, as well as from other laboratories, phenylselenide within **Probe–OCl** was changed to methacrylate, which was attached to the hydroxyl position. Then, phenylselenide was replaced by Br. The change in receptor alters the selectivity of the compound toward Cys with the necessary reaction time decreasing to 30 min. However, because the reaction of methacrylate with Cys involves a displacement reaction through Michael addition, the new compound cannot be used reversibly, which is disappointing yet inevitable. Further study of this compound with living cells is now underway, and the results will be published in due course.
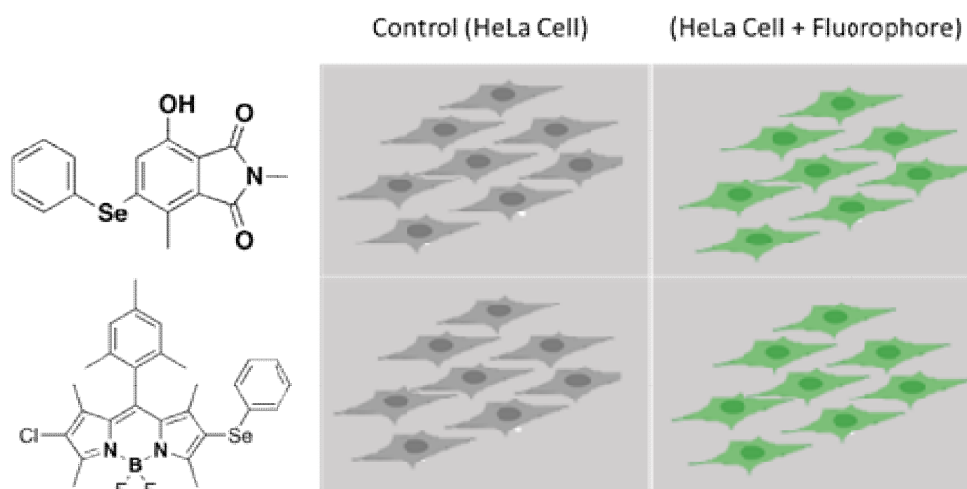
Figure 15. Fluorescence Imaging in Live U2OS Cells Labeled with Probe–OCl and Mes–BOD–SePh. DRAQ5: Cell-permeable far-red Fluorescent DNA dye; DAPI: 4',6-diamidino-2-phenylindole, a Fluorescent Stain that Binds Strongly to Adenine–thymine-rich Regions in DNA; Alexa Fluor 488: Green-fluorescent dye with Excitation Ideally Suited to the 488 nm Laser Line
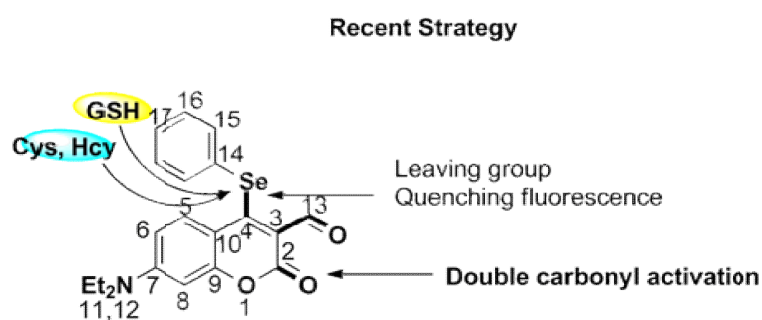

Figure 16. Fluorescence Detection Strategy for Thiols

Detection of biothiols. Biothiols, which are generally small molecules in biology that contain sulfhydryl groups, such as Cys, Hcy, and GSH, play essential roles in biological systems as endogenous species. GSH is a vital antioxidant and the most abundant intracellular biothiol. These species exist in equilibrium between its oxidized disulfide form and its reduced thiol form. Nothing is stopping the biothiols from cross-coupling R–S–S–R, except for mass action. An increase in the concentration of GSH can be related to oxidative stress; in some cases, this correlates with various conditions, such as Alzheimer's disease (dementia) and Parkinson's disease, as well as cancer, HIV/AIDS, sickle cell anemia, and the onset of liver damage [40]. Because of the important roles of biothiols, many researchers worldwide have sought to develop probes that can discriminate between biothiols and amino acids, that is, probes that react with biothiols and become fluorescent and probes that do not react or differently react with non-RSH-containing species. Recently, nucleophilic substitution reactions of strong nucleophilic sulfhydryl

groups have enabled the discrimination of GSH over Cys and Hcy through intramolecular displacement and intramolecular cyclization [41]. Given the current efforts and literature, we developed a probe that has two reactive sites, that is, an aldehyde and electrophilic carbon adjacent to the phenylselenide group, for the selective detection of GSH (Figure 16). The analyte comes in contact with the electrophilic carbon center, and the pendant amino group reacts to form the reversible covalent bond with the –CHO group. Alternatively, the aminothiol group comes in contact with the –CHO group and acts on the electrophile at a slightly later time.

In this work, we incorporated a phenylselenide group at the 4-position of coumarin [42]. The "heavy" Se atom of this group can quench the fluorescence of coumarin by PET. Furthermore, it can behave as a leaving group in response to GSH. Here, the aldehyde group plays a dual role: it enhances the electrophilicity of the 4-position as a Michael acceptor and enables the

cyclization reaction with docking of the primary amine groups once the sulfhydryl end has undergone the nucleophilic substitution reaction. The probe exhibits yellow fluorescence upon reaction with GSH and blue fluorescence upon reaction with Cys/Hcy. We hypothesize that the 4-position of compound **1** (Figure 17), which is "doubly activated by two carbonyl groups" (see Figure 16), undergoes rapid substitution with a strong nucleophilic sulfhydryl group such as that in GSH. Compound **1** shows rapid analyte detection, that is, 150 ms for GSH, because of the ability of the doubly activated α,β-unsaturated system to respond to GSH. Confocal microscopy imaging of living Hep3B cells indicates that the probe specifically detects GSH in cells. Furthermore, cell viability testing demonstrated the low cytotoxicity of the probe and its potential for biological use. However, compound **1** still has several limitations: its fluorescence emission wavelength is not redshifted (approximately 550 nm) after cyclization, it is not water-soluble, and it exhibits a low fluorescence quantum yield. All of these aspects limit the use of compound **1** for in vivo applications [42].

With these limitations in mind, in 2017, Churchill *et al.* sought to develop a more reliable probe for in vivo application based on the structure and functionalities of compound **1** (Figure 18). The improvements involved modifications of several parts of compound **1**. First, we tried to block the rotational freedom of the alkylamino group by preparing the *N*-heterocyclic derivative. This derivatization hereby fixed the amino group and yielded the desired redshift in the absorption/emission by eliciting a planar intramolecular charge transfer

mechanism (Figure 18). Then, an additional π-conjugation was added via the newly introduced heterocyclic ring to enhance the bathochromic shift in absorption and emission wavelengths; this helped improve the optical attributes required for chemosensing. Both versions were tested and compared. To further improve the water solubility of the new probe (compound **2**), an ethyl butanoate group was incorporated at the amino position. The ester group was not chemically reduced before the tests. This pendant amino group was incorporated with the intent of improving cell permeability. The new probe (compound **2**) exhibits a strong fluorescence enhancement with GSH (red fluorescence) and selectivity toward other amino acid molecules tested, including Cys/Hcy (green fluorescence) [30].

The new probe (compound **2**) detects GSH and Cys/Hcy in the red and green channels, respectively, in live A549 cells. However, once a probe was used for detection, it cannot be reused. Moreover, the probe is stuck in the "on" position/state. This lack of reversibility is a current hot topic regarding the limitation of molecular probes. Furthermore, the selective fluorescent detection of Cys through the "green channel" with human fibroblast cells was confirmed by confocal microscopy imaging experiments. In vivo optical fluorescence imaging in mouse models was employed. The results indicated that compound **2** could be used to detect GSH in mouse models. In conclusion, the new probe can be implemented in future biomedical research for the selective detection of GSH in diagnostic applications
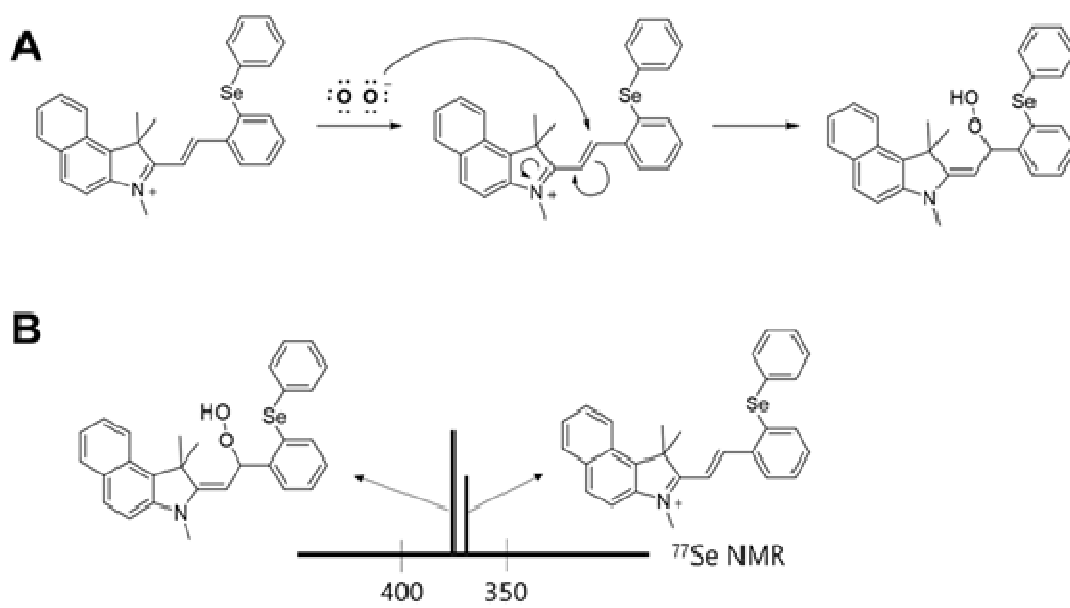

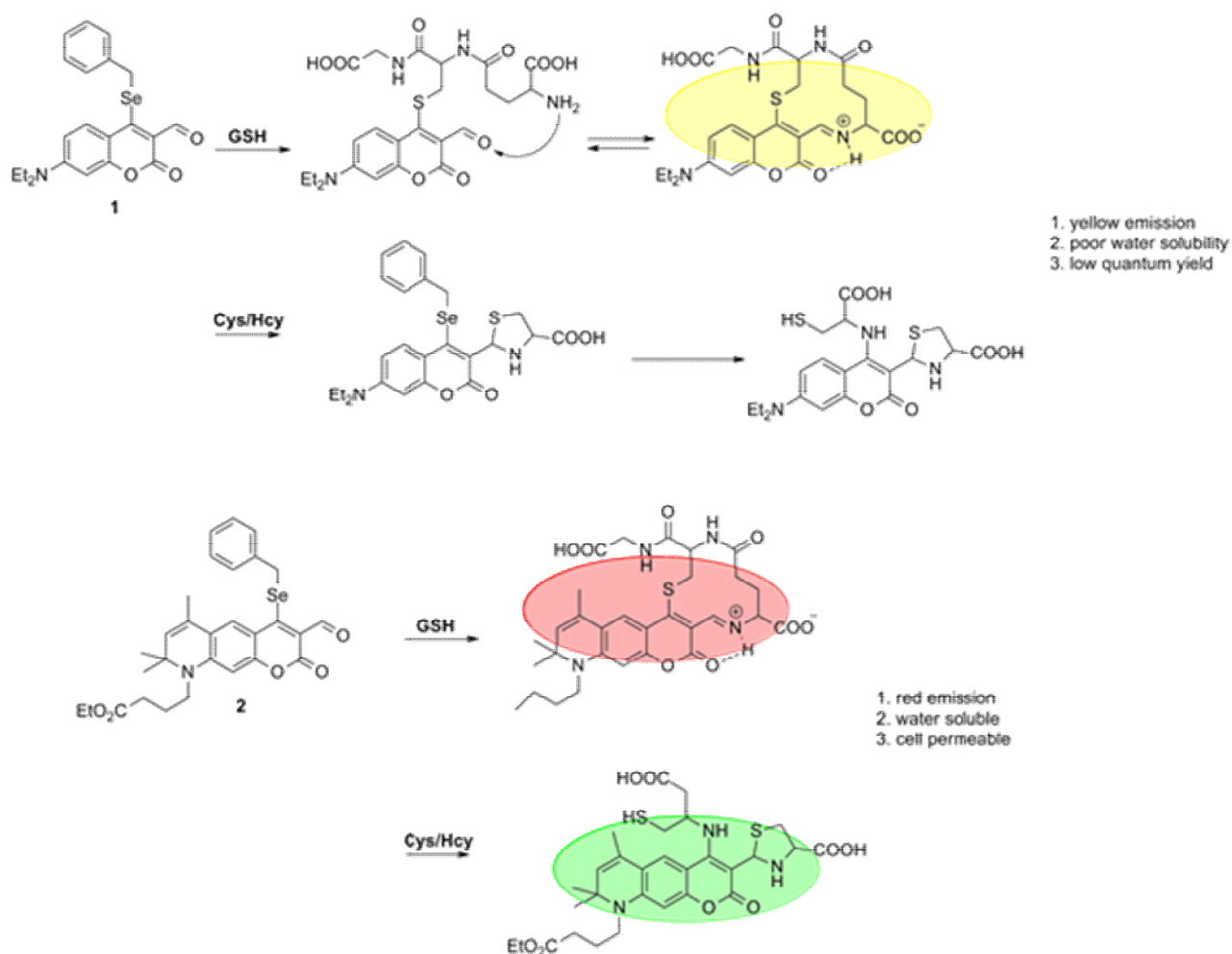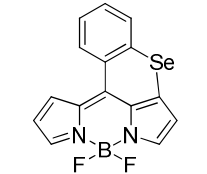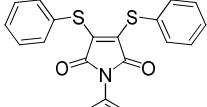**Figure 17. Mechanism of the Reaction of Compound 1 with Biothiols**
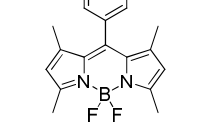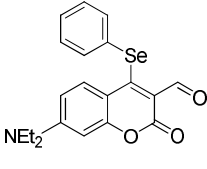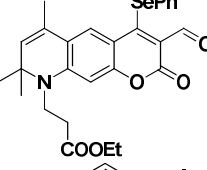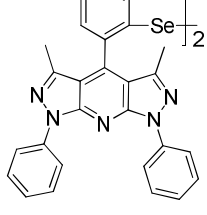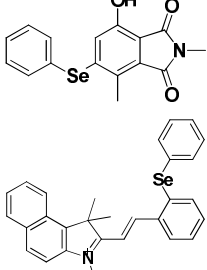
Figure 18. Compounds 1 and 2 from the Churchill laboratory for the Selective Detection of GSH

Table 1.   Fluorescent Molecules Discussed in this Review

Compound	Molecular weight	λ_{ex} (nm)	λ_{em} (nm)	Stokes shift (nm)	Φ_F (fluorescence quantum yield)	Log P	Analyte the probe was found to be selective toward
	438.0	511	526	13	0.57	−1.01 (calculated)	Hypochlorous acid (HOCl)[36]
	542.0	512	526	14	0.45	1.80 (calculated)	In vitro: HOCl In vivo: Lipid droplet through aggregated-induced emission (AIE)[37]
	836.2	504	514	10	0.58	2.12 (calculated)	Superoxide (O_2^-)[35]

Table 1. Fluorescent Molecules Discussed in this Review (*Continue*)

Compound	Molecular weight	λex (nm)	λem (nm)	Stokes shift (nm)	ΦF (fluorescence quantum yield)	Log P	Analyte the probe was found to be selective toward
	346.0	597	610	13	0.23	−3.76 (calculated)	Hypochlorous acid (HOCl)[33]
	635.2	500	512	12	0.42	3.63 (experimental)	Peroxynitrite (ONOO⁻)[31a]
	329.0	518	GSH: 550 Cys or Hcy: 471	GSH: 28 Cys or Hcy: −47 (blueshift)	N.A.	4.90 (calculated)	Glutathione (GSH): yellow fluorescence Cysteine (Cys) or homocysteine (Hcy): blue fluorescence[42]
	538.1	GSH: 550 Cys or Hcy: 410	GSH: 590 Cys or Hcy: 510	GSH: 40 Cys or Hcy: 100	N.A.	5.56 (calculated)	Glutathione (GSH): red fluorescence Cysteine (Cys) + homocysteine (Hcy): yellow fluorescence[30]
	508.5	311	436	125	N.A.	1.49 (calculated)	Hypochlorous acid (HOCl)[32]
	348.0	416	523	107	0.37	0.842 (experimental)	In vitro: HOCl In vivo: Lipid droplet through aggregated-induced emission (AIE)[39]
	468.1	360	439	79	0.45	1.99 (experimental)	Superoxide (O₂⁻)[38]

Summary

There is a worldwide interest in synthesizing and characterizing functional next-generation biologically relevant molecular probes with excellent attributes. In this mini-review, we graphically showcase a variety of different classes of (chemosensor) probe molecules that were synthesized in our chemical laboratory at the Korea Advanced Institute of Science and Technology. Moreover, we briefly introduced organochalcogenides, that is, the oxidant species, which are commonly referred to as ROS/RNS. We also discussed the fluorescence characteristics and photomechanisms briefly. The photophysical mechanisms of FRET and PET were briefly introduced. The aspects of the preferred signal were used. The probes and the illustrations of the cells

help the readers learn about the diverse cell types investigated. We discussed that some probes undergo Se–C cleavage as reliable motifs, different from the oxidation of Se. We look forward to publishing more articles in this area, as well as learning more from the efforts of other research laboratories worldwide.

Acknowledgments

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