

Selenium-Based Fluorescence Probes for the Detection of Bioactive Molecules

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Cite This: *ACS Org. Inorg. Au* 2022, 2, 262–288

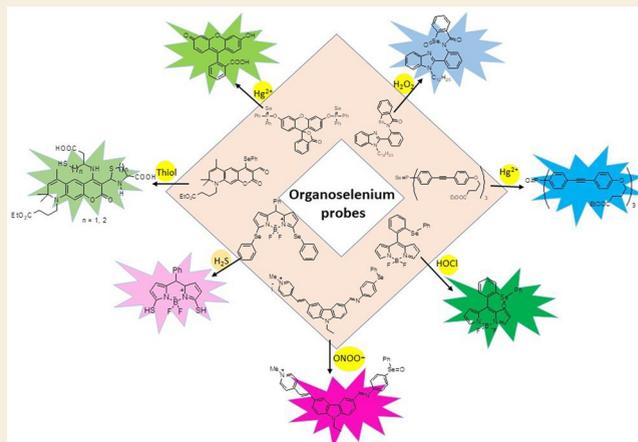
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ABSTRACT: Chemistry of organoselenium reagents have now become an important tool of synthetic organic and medicinal chemistry. These reagents activate the olefinic double bonds and used to archive the number of organic transformations under mild reaction conditions. A number of organoselenium compounds have been identified as potent oxidants. Recently, various organoselenium species have been employed as chemical sensors for detecting toxic metals. Moreover, a number of selenium-based fluorescent probes have been developed for detecting harmful peroxides and ROS. In this review article, the synthesis of selenium-based fluorescent probes will be covered including their application in the detection of toxic metals and harmful peroxides including ROS.



KEYWORDS: Organoselenium compounds, Fluorescence probes, ROS, Metal ions, Thiols

1. INTRODUCTION

Since the discovery of the first organoselenium compound in 1836,¹ various organoselenium reagents have been developed as important motifs for the synthesis of numerous biologically important synthetic and naturally occurring scaffolds.^{2–8} The scope of the biochemistry of selenium has been expanded with the discovery of selenocysteine in a number of mammalian enzymes. At the present date, 25 selenoproteins such as glutathione peroxidase,⁹ iodothyronine deiodinase,¹⁰ and thioredoxin reductase¹¹ have been reported. Selenium is an essential dietary element, and various organoselenium reagents are known for GPx and deiodinase activity.^{12–15} Insufficient selenium in the diet inactivates selenoproteins, which results in oxidative stress and further to cognitive decline that contributes to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease. Selenium, when incorporated into the catalytic site of glutathione peroxidase, inhibits lipid peroxidation caused by ferroptosis. The organoselenium compounds have also been known to protect neurons in ischemic stroke and other brain illnesses where ferroptosis is associated.^{16,17}

Selenium is usually found in the divalent state with two covalent bonds along with two lone pairs of valence electrons. In the chalcogen series, selenium exhibits a soft/hard donor property, due to different oxidation states and the polarizability (which depends on its chemical environment). The hard/soft

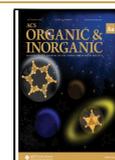
nature of the selenium helps it to recognize specific analytes, and thus organoselenium compounds have been explored as fluorophores. Several bioactive molecules such as thiols, reactive oxygen species (ROS), reactive nitrogen species (RNS), etc., play an important role in the biological system.^{18–20} These analytes have been detected using various methods, but the most simple, inexpensive, rapid, and sensitive method of detection is fluorimetry. Recently, a number of selenium-containing fluorescent probes have been developed as potential sensors for the selective detection of bioactive molecules (metal ions, carboxylate anions, ROS, RNS, thiols).^{21–34} These fluorescent probes include various fluorophores including rhodamine, extended cyanine groups, coumarin, naphthalimide, benzimidazole, boron-dipyrromethene (BODIPY) systems, and dipyrzopyridine. The probes work through various photomechanisms including photo-induced electron transfer (PET) and internal charge transfer (ICT). This review article highlights the recent developments

Received: November 5, 2021

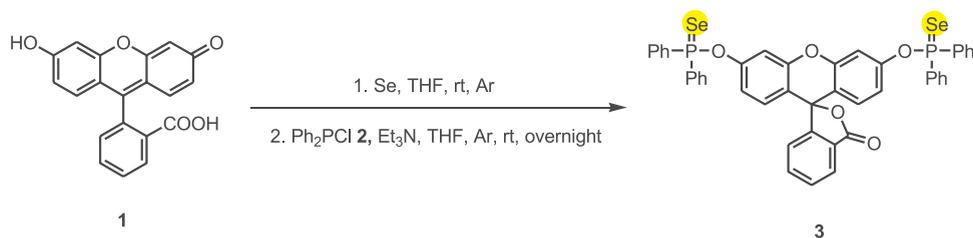
Revised: January 27, 2022

Accepted: January 27, 2022

Published: February 18, 2022



Scheme 1. Synthesis of Fluorescent Probe 3 from Fluorescein 1



of selenium-cored fluorescence probes and their applications as a sensor for small molecules in the living cells and tissues.

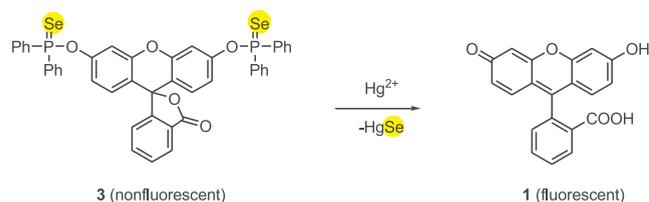
2. DETECTION OF Hg²⁺ IONS

Mercury metal ions have been recognized as highly toxic candidates even at very low concentrations and are responsible for some serious biological disorders in human body.^{35–39} These metal ions can easily pass through cell membranes via epidermal, respiratory, and gastrointestinal tissues. The long-term exposure to mercury metal ion causes irreversible damage to the central neurological and endocrine systems.⁴⁰ Recently, Parkin and co-workers have demonstrated that the selenophilicity of mercury plays an important role to detect Hg²⁺ ions in biological samples.⁴¹ Although there are few analytical methods such as plasma spectroscopy, atomic absorption, atomic emission, and electrochemical methods are available to detect Hg²⁺ ions, but the fluorescence method showed obvious advantages in biological systems in term of high sensitivity and specificity.^{42–44}

In 2009, Tang and co-workers investigated an organo-selenium fluorescent probe 3 based on a deselenation reaction in aqueous solution to detect mercury ion.⁴⁵ The probe 3 mainly consists of two distinguishable chemical units, fluorescein as a fluorophore and *p*-phenylphosphinoselenonic group as a reactive target for Hg²⁺ ions. The synthesis of fluorescent probe 3 was achieved by the reaction of fluorescein 1 with chlorodiphenylphosphine 2 and selenium powder in the presence of triethylamine (Scheme 1).

The probe 3 exhibited high selectivity for Hg²⁺ ions over many other relevant metal ions as well as high sensitivity with a very low detection limit of 1.0 nM. The mechanism for the detection of Hg²⁺ by probe 3 can be explained on the basis of the deselenylation reaction (Scheme 2). According to the

Scheme 2. Deselenation of Fluorescent Probe 3 with Mercury Ions



mechanism, a nonfluorescent probe 3 reacts with Hg²⁺ ions to form fluorescent fluorescein 1 and HgSe. The formation of HgSe was confirmed by X-ray analysis. After the stoichiometric response of selenoprobe 3 to mercury ions, the intensity of fluorescence at λ_{em} was found to be directly proportional to the amount of mercury ion, up to 2 equiv. Furthermore, the probe was found to be highly effective, sensitive, and selective in

mercury ions imaging in RAW 264.7 cells. This would be helpful in studying the toxicity of Hg²⁺ ions along with its bioactivity in the living organisms.

In 2011, Leray and co-workers⁴⁶ reported a fluorescent probe 8 to detect Hg²⁺ ions, based on a phosphorus selenium linkage. The synthesis of probe 8 was achieved by an easily accessible precursor (4-bromophenyl)ethynyltrimethylsilane 4 in three steps as described in Scheme 3. Initially, the starting material 4 was converted into selenium-containing compound 5, which was then transferred to corresponding terminal alkyne 6 with the removal of trimethylsilyl functionality under basic conditions. Finally, terminal alkyne 6 was treated with iodobenzene derivative 7 under coupling reaction conditions to form probe 8 in 44% yields (Scheme 3).

The fluorescence was increased with the increase in the concentration of Hg²⁺ ions. The effect of change in fluorescence of probe 8 with the addition of Hg²⁺ ions in aqueous solution was because of mercury-induced irreversible deselenization reaction (Scheme 4). The fluorescent phosphane oxide 9 is formed as a result of this reaction.⁴⁷

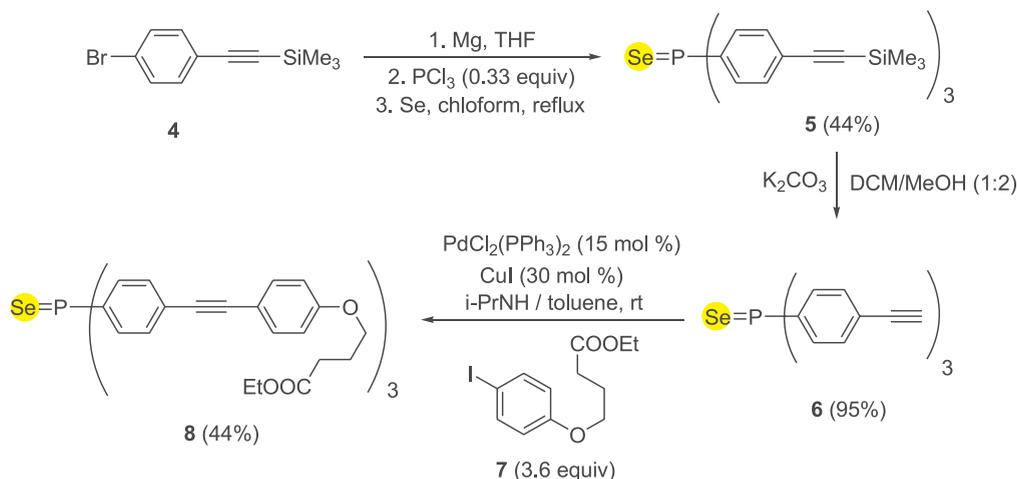
Furthermore, to prove the conversion of phosphane selenide to oxide, a known triphenylphosphane selenide 9 was prepared and treated with mercury salt (Scheme 4). The formation of phosphane oxide 11 was confirmed by ³¹P NMR spectroscopy. It was clear from mechanistic studies that the turn on of the fluorescence was due to the formation of phosphane oxide from phosphane selenide. The selectivity and sensibility toward mercury were estimated to be 0.18 ppb, which is comparable to the targeted level of the World Health Organization.

In 2010, Ma and co-workers⁴⁸ investigated a fluorescent probe 12 with a unique selenospirocyclic structure, which can produce fluorescence with the reaction of both Hg²⁺ and Ag⁺ ions through deselenation. The synthesis of fluorescence probe 12 was achieved in single step by the reaction of rhodamine B 11 with phosphorus oxychloride followed by the addition of selenium powder in the presence of LiAlH₄ (Scheme 5).

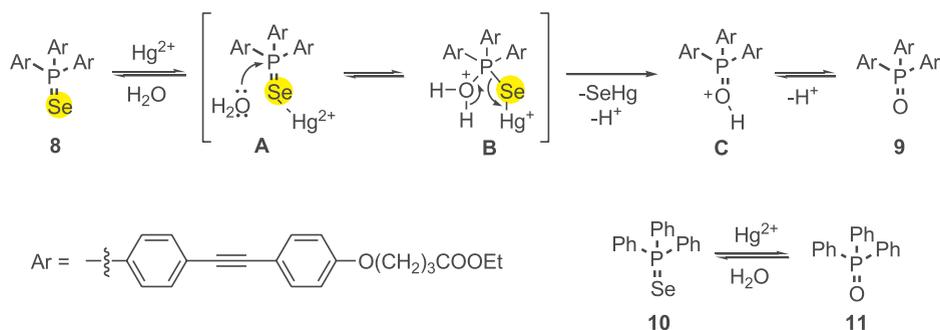
The possible reaction mechanism for “OFF-ON” fluorescence of 12 with both Hg²⁺ and Ag⁺ ions can be explained on basis of irreversible deselenation (Scheme 6). According to the mechanism, the selenium atom of 12 binds with Hg²⁺ and Ag⁺ ions to form an intermediate A. Furthermore, the intermediate A could lead to hydrolytic cleavage of the selenolactone bond to form 13 with emission of fluorescence (Scheme 6). The mechanism was supported by electrospray ionization mass spectral analysis of reaction products.

The probe 13 is advantageous over the other fluorescent probes because of (a) its membrane-permeable capability and (b) its reaction to both Ag⁺ and Hg²⁺ ions in the presence of Cl⁻. These properties make it potentially useful for imaging not only Hg²⁺ ions but also Ag⁺ ions in live cells. The behavior of probe 13 with Ag⁺ ions could be explained on the basis of higher affinity of Se than Cl⁻ for Ag⁺, because the solubility

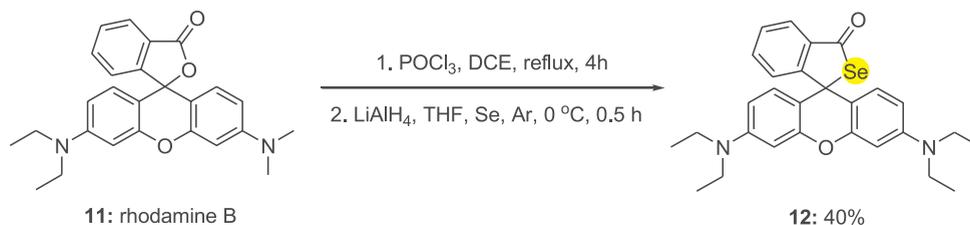
Scheme 3. Synthesis of Fluorescent Probe 8 Starting from Functionalized Alkyne 4



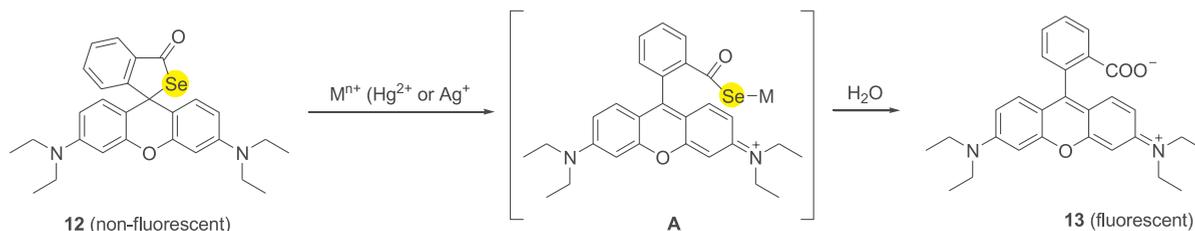
Scheme 4. Mechanism for the Deselenation of Compound 8 with Mercury Ions



Scheme 5. Synthesis of Fluorescent Probe (RBse) 12 from Rhodamine 11



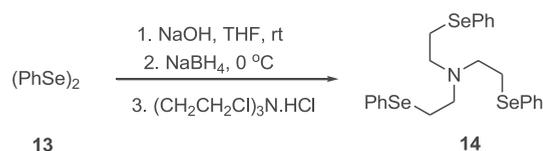
Scheme 6. Deselenation of RBSe 12 with Mercury or Silver Ions



product of Ag_2Se ($K_{\text{sp}} = 1.6 \times 10^{-56}$) is much lower than that of AgCl ($K_{\text{sp}} = 1.56 \times 10^{-10}$).^{49–51} Furthermore, Hg^{2+} and Ag^+ ion imaging in HeLa cells demonstrated its applicability. The detection limits were found to be 23 nM for Hg^{2+} and 52 nM for Ag^+ ions. The reaction of Ag^+ ions with the probe inside the cells is much slower than that of Hg^{2+} ions, which can be attributed to the high concentration of chloride ions present in the cell inhibiting the formation of sufficient free Ag^+ ions.

Singh and his research group⁵² investigated an organo-selenium-based NSe_3 type of tripodal system 14 as a Hg^{2+} ion selective fluorescence “turn-on” probe. The “turn-on” fluorescence behavior of selenotripod 14 is important because it is dependent on Hg–Se bonds and serves as reporting unit for this system (Scheme 7). Even in the presence of several other chalcophilic metal ions, the selenotripod 14 was found to be highly sensitive and selective toward Hg^{2+} ions. The system responds immediately and has shown a subnanomolar

Scheme 7. Synthesis of NSe₃-type Tripodal System 14 from Diphenyl Diselenide 13



detection limit (0.1 nM) for Hg²⁺ ions. The same probe was sensing efficiently both aqueous and nonaqueous Hg²⁺ ions at 2 nM concentration.

Furthermore, the same research group reported the synthesis of sterically encumbered tetra-substituted organoselenium species 15⁵³ and hexakis(alkylseleno)benzenes 16,⁵⁴ respectively, and their application in sensing Hg²⁺ ions specifically (Figure 1).

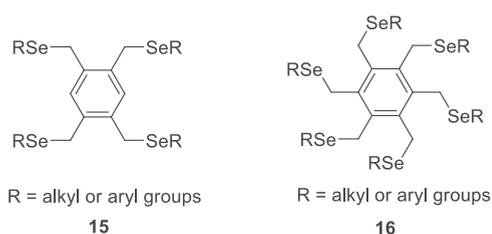


Figure 1. Showing tetra-substituted organoselenium species 15 and hexakis(alkylseleno)benzenes 16.

3. DETECTION OF REACTIVE OXYGEN SPECIES

During aerobic respiration and cellular metabolism, oxygen is used to produce energy in the form of adenosine triphosphate (ATP) in cells. During the oxidative metabolism, oxygen is partially reduced to reactive oxygen species (ROS). ROS include peroxynitrite (ONOO⁻), superoxide (O₂⁻), hypochlorite/hypochlorous acid/ (OCl⁻/HOCl), hypobromite/hypobromous acid (OBr⁻/HOBr), hydrogen peroxide (H₂O₂), hydroxyl radical (OH⁻), and peroxy radical (ROO[·]). The endogenously produced ROS are quite essential for biological functions like signal transduction, neurotransmission, and blood pressure modulation. However, an enhanced level of ROS or low level of antioxidants induces oxidative stress resulting in damage of biomolecules such as DNA, proteins, and lipids of cellular membranes. In long-term damage, redox perturbation leads to inflammatory diseases,

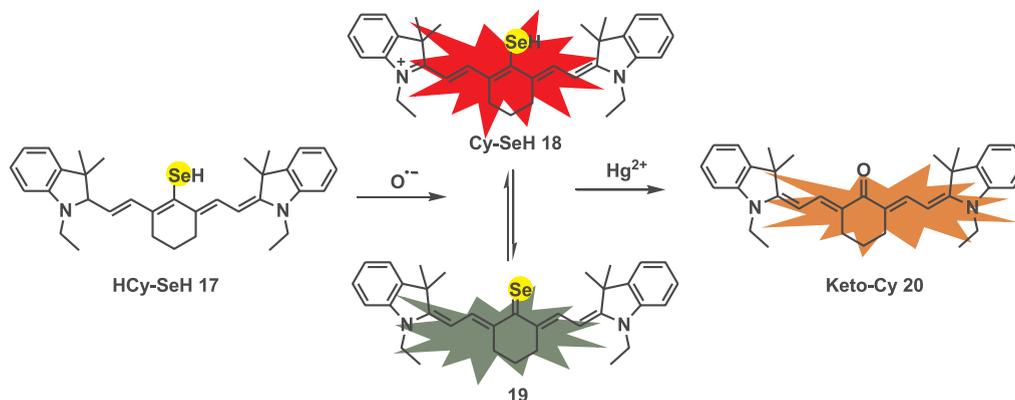
cardiovascular diseases, cancer, neurodegenerative disorders, HIV activation, and aging. In short, the intracellular redox balance is very crucial for biological processes. Selenium is an important element in the human body. Under physiological conditions, a rapid and reversible redox transformation can easily happen between selenide and its selenoxide derivative. Many selenide-based fluorescent probes were synthesized to check intracellular redox status due to its sensitivity toward biological redox systems.^{27,55,56}

Lingxin Chen et al.⁵⁷ designed a near-infrared three-channel fluorescent probe HCy-SeH 17 for the associated detection of mercury (Hg²⁺) and super oxide (O₂⁻). As the cytotoxicity of heavy metal ion Hg²⁺ is discussed in an earlier section, it is well-known that it also induces severe oxidative stress. While mercury poisoning can be treated with many different drugs such as sodium selenite, it still continuously damages human health. The anticipated reason behind this is intracellular oxidative stress caused by Hg²⁺ ion. To synthesize the probe HCy-SeH, heptamethine cyanine is used as fluorophore and a selenol group as a response unit. Heptamethine cyanine dye Cy₇.Cl on reacting with disodium selenide gives an intermediate Cy₇.SeH 18, which is further reduced by NaBH₄ to obtain HCy-SeH as the final product. The response toward the probe in biological samples is generated because of two chemical reactions, hydrogen abstraction reaction in the presence of O₂⁻ and another selenium antagonism reaction.

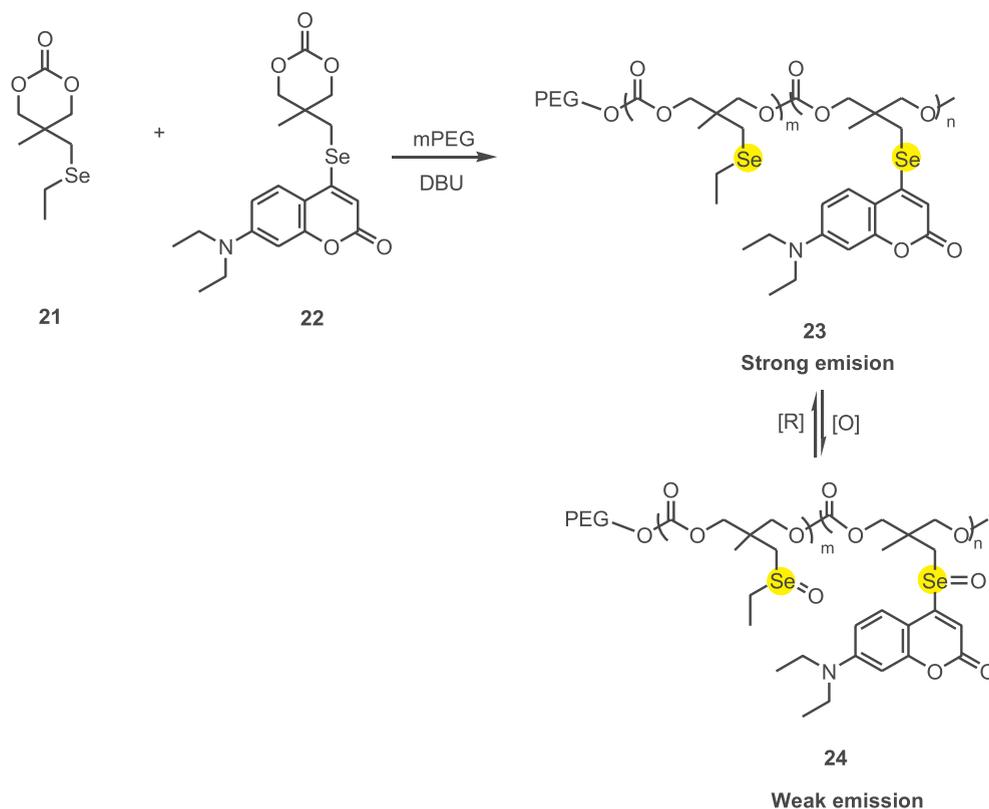
The π-electron system of polymethine in cyanine is distributed during the formation of hydrocyanine, which results in no fluorescence emission from HCy-SeH 17. After the oxidation of probe by O₂⁻ Cy₇.SeH 18 recovers its conjugated system and emits fluorescence. Cy₇.SeH shows red emission with an absorption peak centered at 755 nm and emission peak centered at 800 nm. Cy₇.SeH coexists with its Lewis conjugated base Cy=Se 19, which exhibits green emission with an absorption peak and emission peak centered at 405 and 567 nm, respectively. Finally, Hg²⁺ on reacting with the intermediates Cy₇.SeH 18 and Cy=Se 19 gives Keto-Cy 20 with a max absorption at 510 nm and an emission peak at 607 nm. The probe displayed high sensitivity and selectivity for the detection of super oxides and mercury ions (Scheme 8).

The flow cytometry analysis and fluorescence imaging together established that the probe can be useful to detect in situ O₂⁻ and Hg²⁺ in HEK 293 cells. The results established that Hg²⁺ ion accumulation disturbs the cellular antioxidant system and encourages overproduction of O₂⁻. The probe upon application to mice models for chronic mercuration

Scheme 8. Mechanism for the Detection of Mercury Ions and Super Oxide Species by HCy-SeH 17



Scheme 9. Synthesis of Copolymer 23 by the Reaction of Selenium-Based Compounds 21 and 22 in the Presence of a Base



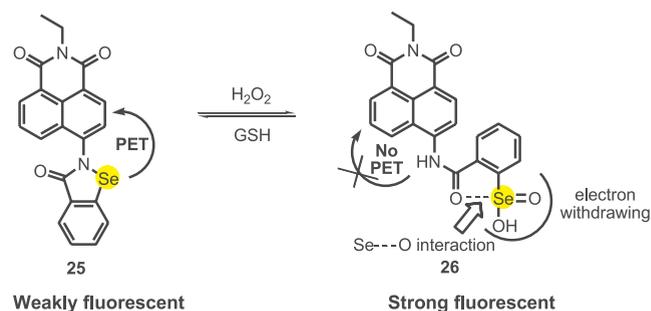
confirmed the kidney damage leading to renal fibrosis due to mercury poisoning. On using sodium selenite as Hg^{2+} ion antagonist, the chronic mercuration was observed to reduce, but the damage due to increased level of $\text{O}_2^{\bullet-}$ were not diminished. The probe is a powerful tool for associated in vitro and in vivo detection of $\text{O}_2^{\bullet-}$ and Hg^{2+} ions. It can further be used for clinical surgery pre-evaluation.

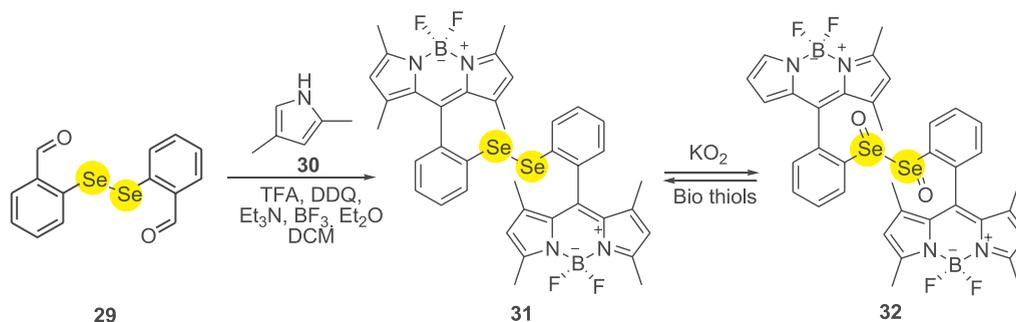
In 2019, Li et al.⁵⁸ reported an amphiphilic copolymer nanoparticle 23 as a redox-responsive vehicle together for the chemotherapy and photodynamic therapy. The amphiphilic block copolymers were synthesized by the anionic ring-opening copolymerization of the cyclic carbonate attached to an ethylseleno group monomer M1 21 with a small fraction of the coumarin-based chromophore monomer F1 22, using PEG as the macroinitiator (Scheme 9). The copolymer forms micellar nanoparticles with the diameter of 40–110 nm and were found to be sensitive toward H_2O_2 present in cells under physiological condition with no cytotoxicity against human breast epithelial HBL-100 cells. The ROS and the GSH in the cell trigger a reversible transformation between the selenide and selenoxide inducing fluorescence. The cells treated with block copolymer (PF) nanoparticles exhibits qualitative correlation between the level of ROS in the cells and intensity of the fluorescence.

Besides, PTX and cisplatin were loaded in the copolymer nanoparticles concurrently, and by adding cross-links through Se–Pt coordination the nanoparticles loaded with both drugs were stabilized. On triggering the oxidation/reduction reaction, the selenide and its selenoxide derivative 24 were easily transformed to each other, leading to the reversible changes in morphology of the PF nanoparticles. Under diverse redox stimulation, the dual drug-loaded PF nanoparticles exhibit a pulsatile drug release profile. However, at relatively

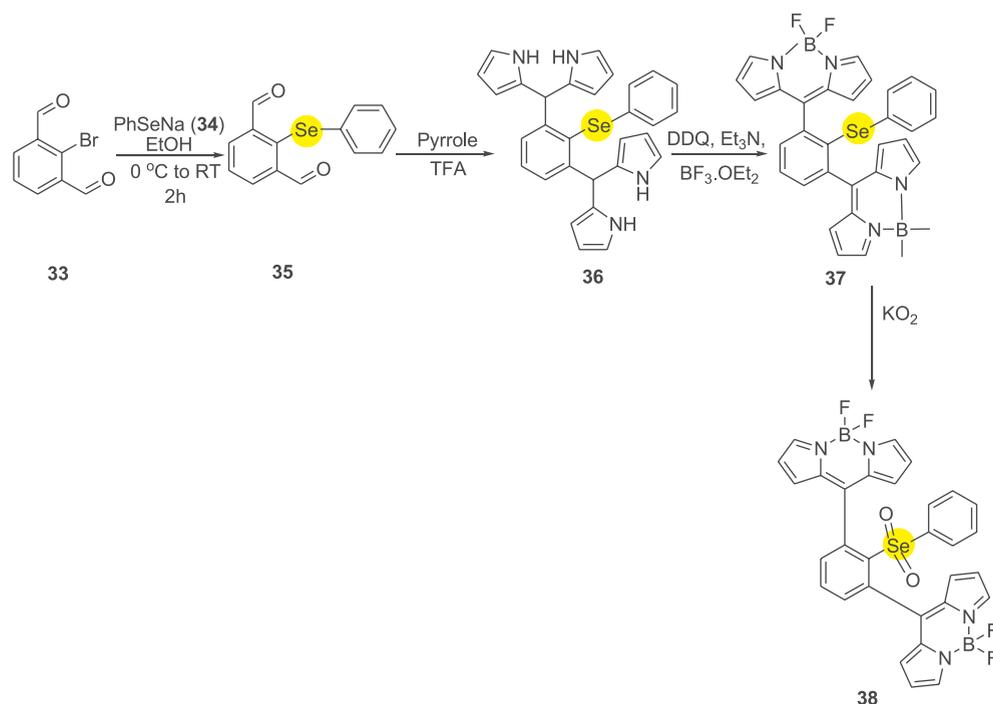
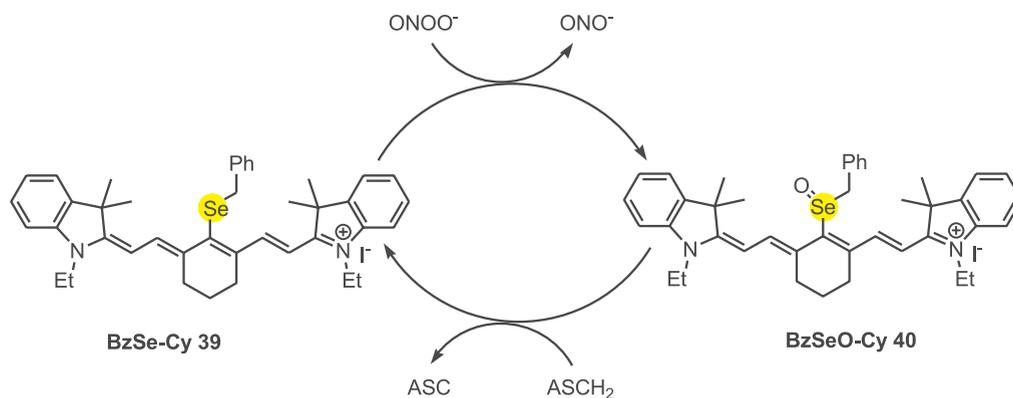
high ROS levels, the continuous drug release was detected. The reversible redox response aspect makes dual drug-loaded PF nanoparticles appropriate for killing malignant cells without damaging the healthy cells. Thus, a redox-sensitive fluorescent, amphiphilic copolymer is considered to be a good theranostic nanoplatform which can release drugs in the targeted cells with high level of ROS and thus simultaneously can be used for monitoring the redox status of the cell.

Mugesh and co-workers⁵⁹ designed naphthalamide-based ebselen fluorescent probe 25. The probe NapEp 25 was synthesized by reacting 4-bromo-*N*-ethyl-1,8-naphthalimide to benzo[*d*][1,2]-selenazol-3(2*H*)-one. The probe is nonfluorescent in aqueous solution, but on adding H_2O_2 of various concentrations (50–500 nM), a strong concentration-dependent fluorescence is observed at 465 nm (Scheme 10). It is well reported that the selenium moiety in ebselen can be oxidized with ROS such as H_2O_2 , super oxide ($\text{O}_2^{\bullet-}$), and peroxyxynitrite (ONOO^-). Although the probe showed fluorescence in the

Scheme 10. Change in Florescence of Probe NapEp 25 with H_2O_2 and GSH

Scheme 12. Synthesis of Diselenide-Based BODIPY Probe 31 from Bis(*O*-formyl-phenyl)diselenide 29

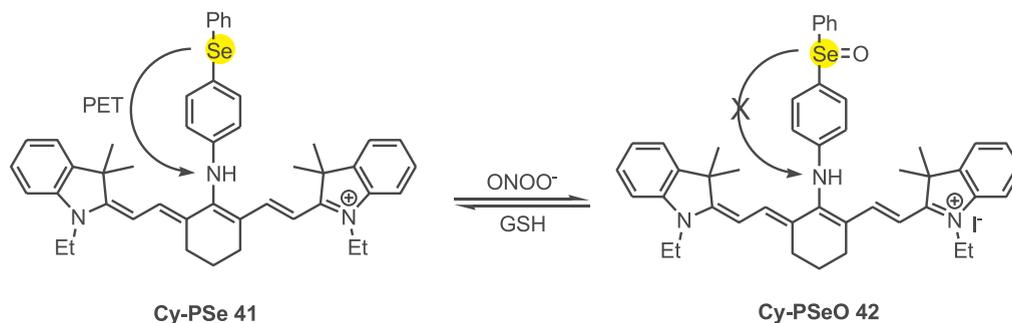
Scheme 13. Synthesis of Monoselenium diBODIPY Probe 37 Starting with 2-Bromoisophthalaldehyde 33

Scheme 14. Redox Cycle for the Change in the Fluorescence of Probe BzSe-Cy 39 with ONOO⁻ and ASCH₂

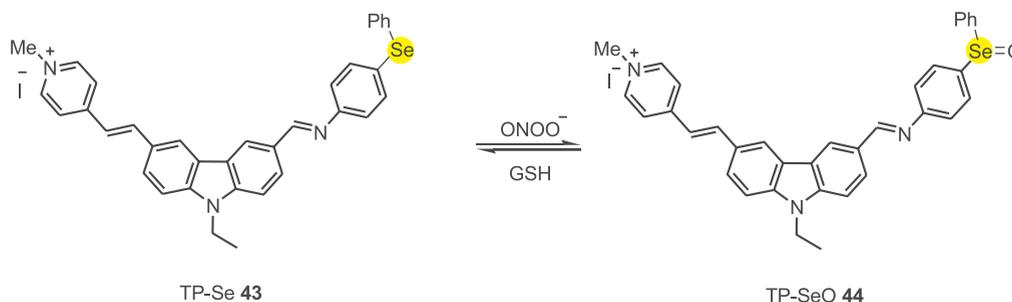
Peroxyntirite (ONOO⁻) is a powerful oxidizing agent which plays an important role in biological processes, especially physiological and pathological.^{64–67} The change in concentration of ONOO⁻ can lead to useful as well as adverse effects in the cell. A balanced level of ONOO⁻ regulates some important cellular functions such as organ homeostasis and

maintains cellular integrity, while an increased level of ONOO⁻ leads to DNA damage and induces lipid peroxidation in cell membranes.⁶⁸ The fluorescent probes have some advantages over the other reported methods to detect the ONOO⁻; for example, few fluorescent probes can be used for selective detection of ONOO⁻. Additionally, these fluorescent

Scheme 15. Change in Florescence of Probe Cy-PSe 41 with Peroxy Nitrite Species and GSH



Scheme 16. Change in florescence of Probe TP-Se 43 with Peroxy Nitrite and GSH



probes can respond reversibly to the change in ONOO^- concentration which would help determine the generation and metabolism of ONOO^- as well as its dynamic damaging effect on living cells.

Tang and co-workers⁶⁹ have developed a near-infrared reversible organoselenium fluorescent probe **39** (BzSe-Cy) for sensing ONOO^- and imaging of redox cycles in living cells. BzSe-Cy **39** is a selenium containing molecule that responds reversibly to the change in concentration of ONOO^- or ASCH_2 . On reacting with ONOO^- , BzSe-Cy **39** shows substantial decrease in the fluorescence intensity, while in the presence of ASCH_2 the fluorescence intensity increases. The same fluorescence probe was also employed for other reactive oxygen and nitrogen species but exhibited only high sensitivity and selectivity with ONOO^- . The mechanism for the change in the fluorescence of probe with ONOO^- and ASCH_2 can be explained on the basis of redox cycle as depicted in Scheme 14. Initially, BzSe-Cy **39** reacts with ONOO^- and oxidized to BzSeO-Cy **40**, which is weakly fluorescent upon 770 nm excitation due to a photoinduced electron transfer process. Further, BzSeO-Cy **40** was reconverted to BzSe-Cy **39** with the reaction of reduced ascorbate to continue the redox cycle (Scheme 14).

Furthermore, the probe was effective in imaging several cycles of oxidative stress and reductive repair in RAW 264.7 cells. This fluorescent probe could be used as important tool to study the generation, metabolism, and the dynamic damaging procedure of ONOO^- in living cells.

Furthermore, Han and co-workers⁷⁰ have developed an NIR reversible fluorescent probe **41** containing cyanine (Cy), an NIR fluorescent dye, as signal transducer and 4-(phenylselenenyl)aniline as modulator. The NIR reversible fluorescent probe **41** is highly sensitive and selective in monitoring peroxynitrite redox events. Under stimulated physiological conditions, probe **41** exhibited absorption and emission λ_{max} at 758 and 800 nm, respectively. On adding 1

equiv of ONOO^- to the buffer solution with Cy-PSe, the fluorescence intensity increases by 23.3-fold and the quantum yield increased from 0.05 to 0.12. The mechanism for the mode of action of fluorescent probe **41** can be explained on the basis of the ping-pong mechanism.^{71,72} The probe **41** (Cy-PSe) detects ONOO^- in living cells through a fast PET process.

The PET process between the modular and the transducer quenches the fluorescence of Cy-PSe, but the oxidation of Se prevents the PET, which “switched on” the fluorescence emission (Scheme 15). Furthermore, selenoxide Cy-PSeO **42** can be reduced to selenide by GSH effectively and rapidly; at this point, the probe begins to function. The probe does not show the influence of autofluorescence in biological systems and has no cytotoxicity toward cells. The study shows that it can be useful in real-time imaging of living cells.

Wenjun Xie et al.⁷³ synthesized a single fluorescent Two Photon-Selenium probe (TP-Se) **43** for reversible detection of ONOO^- in mitochondria. An electron donating carbazole and an electron withdrawing cationic pyridinium moiety were chosen as the TP fluorophore due to the excellent intermolecular charge transfer. The methylpyridinium moiety was used as mitochondrial targeted functional group to find the location of mitochondria as well as to increase the solubility of probe.

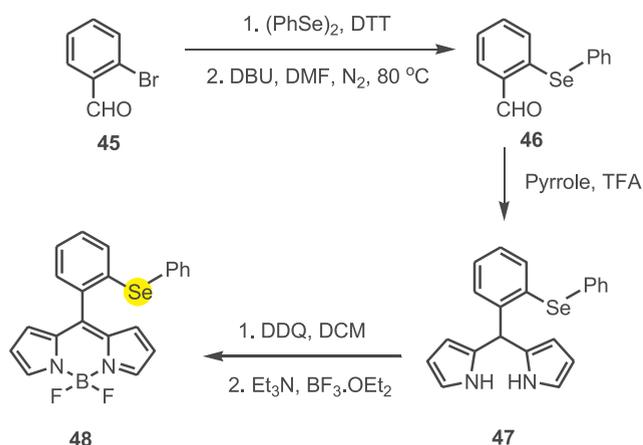
On reacting with peroxynitrite, the probe switch on the fluorescence emission due to the formation of selenoxide, TP-SeO **44** (Scheme 16). The Se oxidation prevents the PET between the modulator and the transducer. Before reacting with ONOO^- , the fluorescence of TP-Se probe was quenched as a consequence of PET. TP-Se **43** loaded RAW 264.7 cells in the presence of 3-morpholinosydnonimine, a peroxynitrite donor resulted in a considerable increase in intracellular fluorescence within 10 min with the detection limit as low as 31 nM. When cells were treated with GSH S-transferase as the ROS scavenger, the intracellular fluorescence decreased as the

TP-Se sense the redox reaction. The subcellular localization of TP-Se, HeLa cells, and a MitoTracker Red FM mitochondrial dye upon colocalization study exhibited mitochondrial ONOO⁻ under TP excitation in living cells. As a result, the TP-Se probe can be used to visualize mitochondrial peroxynitrite levels with minimal background fluorescence and cytotoxicity.

Hypochlorous acid (HOCl), a kind of ROS, is a powerful antimicrobial agent and works as an important architect in the immune system.⁷⁴ Hypochlorous acid is mainly produced in various leukocytes by the myeloperoxidase catalyzed reaction of a chloride ion with hydrogen peroxide.⁷⁵ Generally, HOCl is produced during the defense mechanism in the immune system, but its excess amount leads various diseases including arthritis,⁷⁶ cancer,⁷⁷ and neurodegeneration.⁷⁸

Wu and co-workers⁷⁹ have reported a highly sensitive and selective fluorescent probe HCSe to detect HOCl based on specific HOCl-mediated oxidation of diphenyl selenide functionality. The probe can be synthesized in three steps, starting with easily available 2-bromobenzaldehyde **45** (Scheme 17). In the first step, 2-(phenylselenyl) benzaldehyde

Scheme 17. Synthesis of Probe 48 Starting from 2-Bromobenzaldehyde 45 in Two Chemical Steps

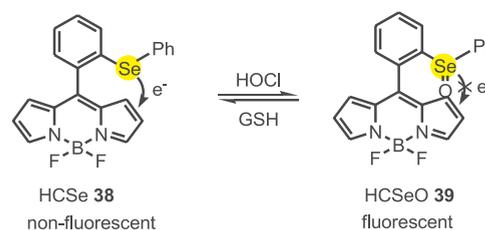


46 was synthesized by the reaction of 2-bromobenzaldehyde **45** with diphenyl diselenide in the presence of dithiothreitol (DTT). Further, 2-(phenylselenyl) benzaldehyde **46** reacts with pyrrole in the presence of trifluoroacetic acid (TFA) to form corresponding dipyromethane. Finally, probe **48** was obtained by the oxidation of 2-(phenylselenyl)-phenyldipyromethane **47** using DDQ followed by the reaction of BF₃·OEt₂ in the presence of triethylamine.

In probe **48**, BODIPY (borondipyromethane, a fluorescent dye) was used as signal transduction unit and diphenyl selenide as modulator to respond the amount of HOCl. The “ON-OFF” fluorescence of probe **48** can be explained by the redox cycle of selenium-based probe with HOCl and GSH. The probe **48** exhibits a strong green fluorescence immediately with addition of HOCl because it oxidizes the selenide functionality of **48** to the selenoxide functionality **49**. Furthermore, the fluorescence of **49** was quenched with the addition of GSH (glutathione) because **49** is reduced to nonfluorescent **48** (Scheme 18).

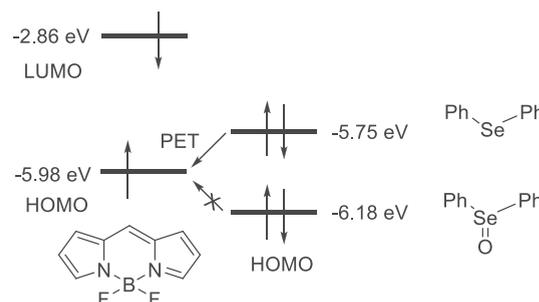
The mechanism for the detection of hypochlorous acid by fluorescence probe **48** can be explained on the density functional theory (DFT) calculations. As shown in Scheme

Scheme 18. Redox Cycle of Selenium-Based Fluorescent Probe 48 with HOCl and GSH



19, the HOMO energy level (−5.75 eV) of the diphenyl selenide moiety is higher than that of the fluorophore BODIPY

Scheme 19. Showing the Energy Diagram for the Reaction of Selenium-Based Probe 48 with HOCl



(−5.98 eV), meaning the intramolecular electron transfer from the diphenyl selenide moiety to the BODIPY moiety is energetically favorable. As a result, the fluorescence of the BODIPY moiety is quenched via a PET process.

Next, the oxidation of selenide moiety (HCSe) to selenoxide moiety (HCSeO) occurs in the presence of HOCl. Since the diphenyl selenoxide moiety shows a HOMO energy level (−6.18 eV) lower in comparison to the BODIPY unit, the PET process is restricted and BODIPY restored the fluorescence.

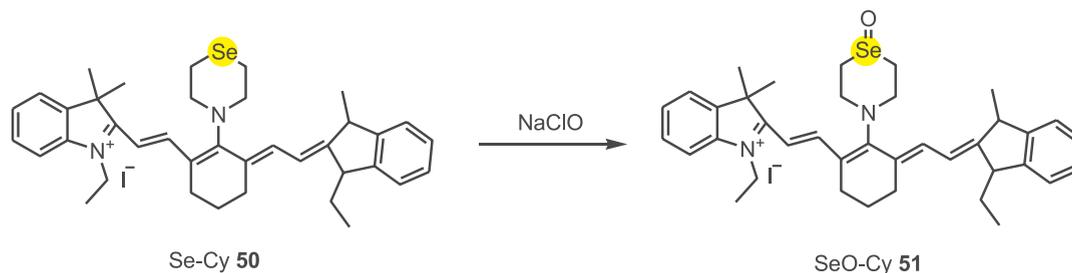
Furthermore, the probe was successfully applied for imaging hypochlorous acid in RAW264.7 cells by confocal fluorescence microscope. The detection limit of HCSe was found to be 7.98 nM, which makes it sufficiently sensitive to be used to evaluate the important roles of HOCl in biological systems.

Xiaojun Peng et al.⁸⁰ developed a selective near-infrared (NIR) selenium containing fluorescent probe Se-Cy7 **50** on the aggregation behavior of a heptamethine cyanine dye for selective and rapid response for HClO. In aqueous solution, the probe exhibits weak fluorescence, but on addition of NaClO, the emission spectra are obtained at 786 nm. On addition of 2.0 equiv of NaClO, 9.4-fold fluorescence enhancement was observed and the process finished within few seconds. At the same time, the detection limit of 0.31 mM for HClO (3s/k) makes the probe suitable for quantitative detection of HClO.

It was found that in aqueous solution SeCy **50** showed weak fluorescence due to the stern aggregation, but on reacting with HClO it was converted to SeOCy **51**. With the formation of SeOCy **51**, the aggregated probe is gradually released and enhance the fluorescence intensity (Scheme 20). The probe was found to be effective in visualizing HClO in living animals.

The mouse injected with SeCy **50**, when injected with 2 equiv of NaClO in the same region, showed enhancement in fluorescence intensity gradually within 40 min. Thus, it was

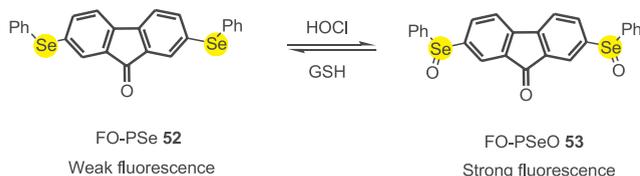
Scheme 20. Reaction of Fluorescent Probe Se-Cy 50 with NaClO to Form Highly Fluorescent Selenoxide 51



established that SeCy 50 can detect NaClO *in vivo* without interference of background signals.

Tang et al.⁸¹ designed (FO-PSe) 52 a novel turn-on probe for the detection of HOCl. A two-photon (TP) fluorophore, 9-fluorenone was covalently conjugated with two selenium-containing moieties. The two-photon (TP) imaging procedure is considered better in comparison to their one-photon (OP) counterparts, as it can penetrate deep without photodamaging the sample by the use of two lower energy photons for excitation. The probe 52 exhibits weak fluorescence due to photoinduced electron transfer (PET) from the diphenyl selenide group to 9-fluorenone, but when HOCl is added, the selenium of the probe is oxidized to FO-PSeO 53 preventing the PET and thus turn-on fluorescence (Scheme 21).

Scheme 21. Change in Fluorescence of Probe FO-PSe 52 with HOCl and GSH



The FO-PSe 52 shows an absorption band centered at 415 nm, while the emission maximum corresponding to it appeared at near 520 nm; the excitation maximum was found to be 415 and 800 nm for OP and TP, respectively. The probe was reported to be highly selective to HOCl, as other ROS and RNS hardly showed any significant fluorescence. The detection limit of HOCl was estimated to be 0.35 μM , which indicates that the probe can be used to determine the quantitative level of HOCl. The reversibility of the probe was established when cells on pretreating with glutathione (GSH) show weaker fluorescence compared to the cells stimulated with HOCl. The probe was also found to monitor HOCl in living cells under physiological conditions. The mouse macrophage cell line

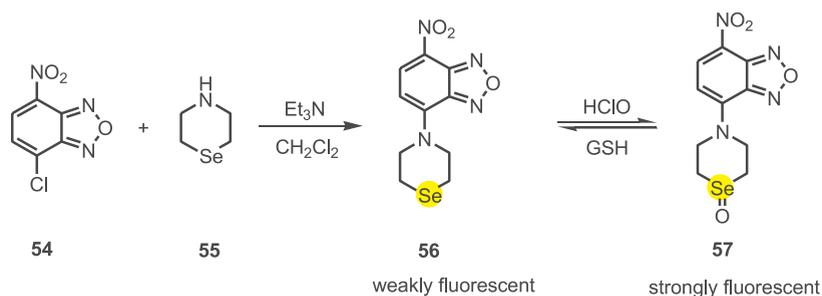
RAW 264.7 shows almost no fluorescence in the absence of stimulant, whereas strong fluorescence was detected after treatment with phorbol 12-myristate 13-acetate (PMA). This indicates that probe 52 can be used to selectively visualize HOCl in cells. The application of FO-PSe was exhibited in intravital imaging in zebrafish. A strong response was observed when HOCl was added to the probe preloaded zebrafish, and after further treatment with vitamin C, fluorescence of the zebrafish decreased sharply. The probe FO-PSe also tracked the changes in HOCl levels deep inside mice and zebrafish. The probe is an ideal tool for investigating HOCl levels in live cells as well as *in vivo*, by using TP deep tissue imaging.

Shouzhao Yao⁸² et al. synthesized a simple and reversible fluorescent probe for ClO^- by reacting 7-nitrobenz-2-oxa-1,3-diazole (NBD) 54, a fluorophore, and selenomorpholine 55 as the recognition unit. The NBD-selenide probe 56 shows a weak fluorescence due to the PET process from the selenide to NBD in the excited state. In the presence of hypochlorite, the selenide oxidizes to selenoxide 57, which hampers the PET process and thus boosts the fluorescence (Scheme 22). The probe when tested against other ROS was found to be a highly selective, sensitive, and fast (<10 s) visual-based fluorescent probe for the detection of hypochlorite even at the concentration of 3.3 nM.

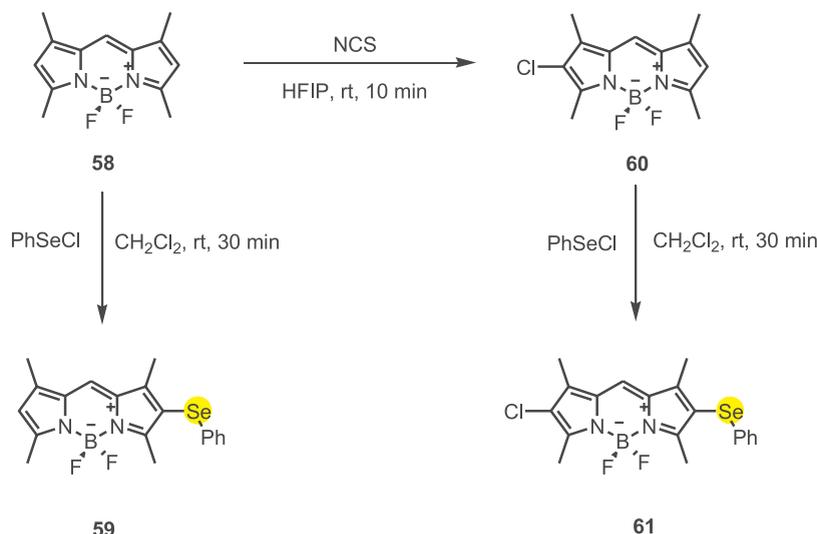
The sensor's biological application is the real-time imaging of cellular redox cycles in HeLa cells. On incubating the HeLa cells with probe (10 μM) at 37 $^\circ\text{C}$ for 30 min, a dark fluorescence was recorded. On adding 30 μM NaClO into the medium and on incubating for an extra 10 min a strong fluorescence appeared. Further, the cells on loading with 100 μM GSH for 10 min exhibit no fluorescence. These findings indicate that the probe is a chemical sensor with good permeability and good reversibility that could monitor real-time redox status in cells.

Peng Li et al.⁸³ designed Lyso-NI-Se, a lysosome-targetable fluorescent probe for imaging HOCl in living cells. The probe is synthesized using naphthalamide as fluorophore, the selenide

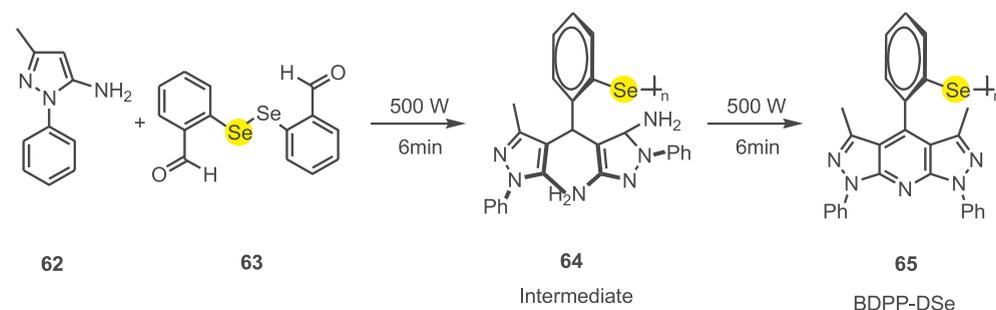
Scheme 22. Synthesis of Fluorescent Probe 56 and Its Reaction with HClO and GSH



Scheme 23. Synthesis of Fluorescent Probes 59 and 61 from Compound 58



Scheme 24. Synthesis of Fluorescent Probe BDPP-DSe 65 in Two Chemical Steps



group as fluorescent modular, and the (aminoethyl)-morpholine moiety as a function that led the probe to lysosomes. In phosphate buffer, Lyso-NI-Se exhibits a broad absorption band centered at 452 nm. Upon addition of NaClO, a fluorescence band rises significantly with maximum emission wavelength of 540 nm in a few seconds. The detection limit for HOCl was calculated to be 1.85×10^{-8} M. The probe turns on due to the restricted PET from the selenide pendant to the naphthalimide fluorophore. The intensity of the fluorescence decreases sharply on addition of GSH. The probe does not show any enhancement in the presence of other biologically relevant ROS or RNS. The Lyso-NI-Se highlighted a fast, selective, and sensitive fluorescence response to HOCl and was successfully used to monitor exogenous HClO in lysosomes of living cells.

Churchil and co-workers^{84,85} synthesized two correlated “turn-on” fluorescent probes selectively for HOCl by incorporating phenyl selenide moiety to meso-unsubstituted BODIPY. On substituting BODIPY at the 2 and 6 positions with heavy metal, the fluorescence quantum yield decreases. Thus, in probe 59 the 2 position of BODIPY was substituted with phenyl selenide, and in probe 61, along with phenyl selenide at 2 position, Cl at the 6 position was introduced. In another probe Mes-BOD-SePh, the mesitylene at the 9 position of BODIPY along with a chloro and phenyl selenide at the 2 and 6 positions were also synthesized and studied via live cell imaging.

Probe 59 was synthesized by reacting BODIPY compound 58 with 1.0 equiv of phenyl selenyl chloride. The

monochlorination of BODIPY with N-chlorosuccinimide (NCS) occurs in the presence of hexafluoro-2 propanol (HFIP) to provide 60, which on further reaction with phenylselenyl chloride gives probe 61 (Scheme 23). Both probes show absorbance maxima at 512 and 526 nm, respectively, and after adding 5.0 equiv of NaOCl, the absorption peak shifts toward longer wavelengths, i.e., 492 and 511 nm, respectively. The detection limits reported were 30.9 nM for 59 and 4.5 nM for 60.

Density function theory (DFT) and time dependent DFT (TD-DFT) calculation explained the photomechanism of the probes. The electrons transfer from selenium to BODIPY core is efficient due to the direct attachment of both. The largest intense transition of probe 61 from HOMO-1 to LUMO (configuration interaction CI = 61.5%), together with oscillator strength (f) of 0.4984, is demarcated as the dominant transition. In the case of the oxidized state of probe 61, the largest intensity transition from HOMO to LUMO (CI = 51.9) had an oscillator strength (f) of 0.4086. The electronic distribution of HOMO and HOMO-1 of 61 is displayed on the phenylselenium moiety that delivers a PET to the BODIPY core. On the other hand, for the oxidized form of 61, the HOMO and LUMO level showed overlapped electronic distribution on the BODIPY core resulting in fluorescence enhancement. Thus, it was concluded that fluorescence enhancement is due to the oxidation of selenium to selenoxide, which blocks the PET process. The probe can be applied as a “turn-on” response selectively, as real-time imaging for hypochlorous acid at very low concentration in RAW264.7

and MCF-7 confirmed by fluorescence and confocal microscopy imaging.

Along with aforementioned probes, Churchill et al.⁸⁶ reported for the first time a dipyrazolopyridine (DPP) based probe bearing a diselenide group for chemosensing. Under solvent-free condition 5-amino-3-methyl-1-phenylpyrazole **62** reacts with bis(*ortho*-formylphenyl)diselenide **63** to give compound **64** as an intermediate, and on further irradiating the intermediate compound **64** at 500 W for 15 min, the final BDPP-DSe **65** was obtained (Scheme 24).

The absorption and emission spectra of BDPP-DSe probe **65** were 331 and 436 nm, respectively. The probe was found to be sensitive toward the HOCl in comparison to other ROS, and the detection limit was calculated to be 3.6×10^{-7} M. Probe **65** shows a turn-on response in the presence of NaOCl along with a 180-fold increase in fluorescence intensity. The turn-on response is attributed to the oxidation of selenium to form BDPP-DSeO. This chemical change halts the PET mechanism between phenyl selenide group and BDPP, thus resulting in the fluorescence. Upon testing with other competitive ROS, the probe does not show any optical change and backs the selectivity for the NaOCl. A confocal microscopy imaging study using BDPP-DSe **65** was carried out in living breast cancer cells that demonstrated detection of HClO in living cells; thus the study concludes that the probe is cell membrane permeable. The probe can be potentially applied in various fields such as neurosciences, health sciences, as well as cancer and brain related diseases.

In another work, Churchill et al. developed two new probes, Probe [A] and Probe [B] (Figure 2) using anhydride and

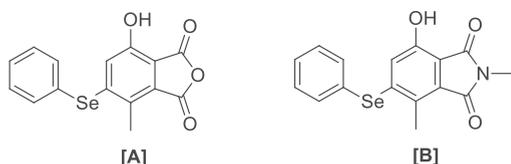


Figure 2. Structures of probes [A] and [B].

amide phenyl selenide conjugates.⁸⁷ Both probes show no fluorescence due to the presence of the phenyl selenium group at the 2 position, as it is involved in the PET process, but as soon as OCl^- is added, selenoxide is formed which blocks electron transfer from the phenyl selenium donor that results in blocking the PET process and turns on fluorescence in less than 1 s.

The HRMS data backed the formation of selenoxide after addition of OCl^- to the Probe [B]. Upon addition of L-cysteine into oxidized probe [B], turn-off fluorescence within 5 min is observed. Density functional theory (DFT) geometry optimization calculations confirmed the photo mechanism. The oxidized probe [A] was found to degrade in cell assays,

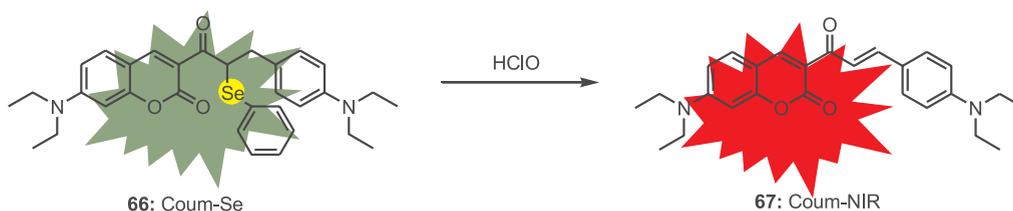
whereas probe [B] shows high stability at physiological pH. The UV-Vis absorption spectrum for [B] shows absorbance maxima at 396 nm, and upon addition of 1 equiv of OCl^- the absorption peak undergoes a red shift to 416 nm. No major change in absorption maxima is observed upon addition of other ROS/RNS. The probe was confirmed to be nontoxic after studying U-2 OS cells and HeLa cells. It was found that probe [B] undergoes aggregation induced emission (AIE) in lipid droplets (LDs); thus it can be used as a probe for LDs.

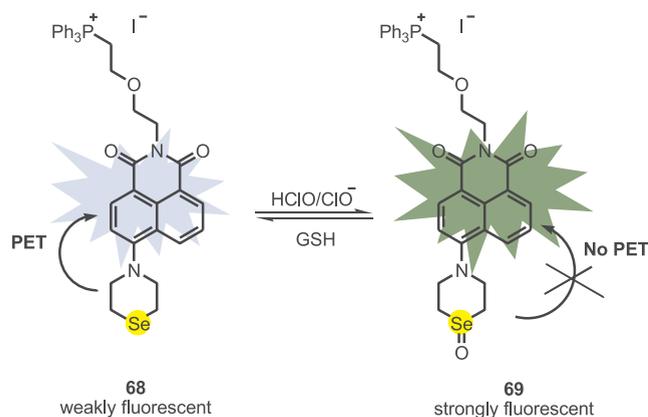
Tang et al.⁸⁸ designed an innovative fluorescent probe Coum-Se **66** composed of an α -phenylseleno carbonyl moiety and coumarin as the fluorophore. The Coum-Se probe displays a single emission band at around 495 nm. In the presence of HClO, the initial band gradually disappears, and concurrently a new peak appears at 618 nm. The fluorescence ratio (I_{618}/I_{495}) depends on the dose and increases 241-fold in the presence of 5 equiv of HClO. The Coum-Se **66** probe emits green fluorescence, while in the presence of HClO, selenium oxidizes to selenoxide and further undergoes a spontaneous *syn* elimination to generate Coum-NIR **67**, with an extended conjugation system which releases striking red fluorescence (Scheme 25). Upon adding HClO, both the intensities rapidly change and reach a plateau in 5 s as a response time. The probe is highly sensitive toward HOCl, as it shows no fluorescence ratio variation in the presence of bio-related ROS and RNS other than HClO. The calculated detection limit of the probe was found to be 4.6 nM.

The two-photon excitation Coum-Se **66** probe can be used for bioanalytical application in live cells, as it has shown minimal cytotoxicity against RAW264.7. It visualizes HClO fluctuation in RAW 264.7 macrophages upon stimulation with lipopolysaccharide (LPS) and phorbol myristate. The ratiometric response was further analyzed by ratiometric visualization of biogenic HClO in live animals. During LPS-induced acute hepatitis, the Coum-Se **66** was employed to monitor the real-time HClO bust. The result showed remarkable red fluorescence and a drop in green fluorescence with increasing dose of LPS in the mouse liver. The Coum-Se **66** can be used to map the ratiometric levels of HClO in live animals during any biomedical process.

Wei Guo and co-workers⁸⁹ reported a naphthalimide-based reversible fluorescent probe Nap-Se **68** by integrating naphthalimide with a fluorophore and selenomorpholine. The triphenylphosphonium group attached to the naphthalimide unit improves the water solubility and guides the probe to mitochondria. The probe works on the principle of PET; thus Nap-Se **68** itself displays weak fluorescence. Upon addition of 1.0 equiv of ClO^- , the PET process is hindered due to the formation of selenoxide **69** and is observed as a surge in fluorescence intensity (Scheme 26). Within 2 s a plateau was reached suggesting a fast response of the probe toward ClO^- . Upon addition of 1 equiv of GSH, the intensity of the probe

Scheme 25. Elimination of Selenide Moiety from Probe **66** as Selenoxide Elimination with HClO



Scheme 26. Redox Cycle of Selenium-Based Fluorescent Probe 68 with HClO with GSH

decreases gradually to its original state in 30 min showing reversibility of the probe. The estimated detection limit of the probe toward ClO^- was reported to be 4.8 nM.

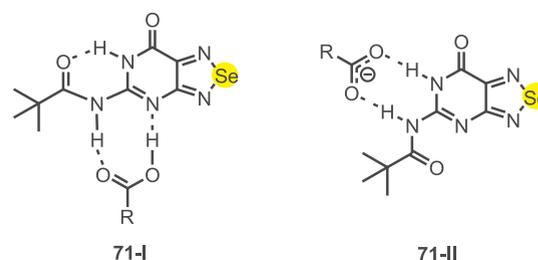
Raw 264.7 macrophages loaded with Nap-Se **68** display weak green emission. The cells stimulated with LPS and PMA followed by addition of Nap-Se **68** display enhanced emission in the green channel. Further, imaging the costained HeLa cells with Nap-Se and Mito Tracker Red FM using confocal laser scanning microscopy shows that green fluorescence from Nap-Se **68** was well overlapped with the red fluorescence signal from Mito Tracker. The study indicates that the Nap-Se was found to be primarily located in mitochondria. The probe can be explored for further study of redox biology.

4. DETECTION OF CARBOXYLATE ANIONS AND CARBOXYLIC ACIDS

In past two decades, the recognition of carboxylic acids and carboxylate anions by synthetic receptors has been receiving particular attention due to their applications in biological pharmaceutical science.^{90–92} Recently, various sensors have been developed for recognizing a specific anion over a wide range of anions and other guests.^{93–96} Goswami and his co-workers⁹⁷ have reported the synthesis of selenium-cored chromogenic fluorescent sensor **71** to distinguish carboxylate anions from carboxylic acids and other anions. The synthesis of sensor **71** was achieved in the solid phase by irradiating 2,5,6-triamino-3H-pyrimidin-4-one dihydrochloride **70** and the selenium dioxide mixture in a microwave oven followed by pivaloylation using pivalic anhydride (Scheme 27).

The receptor **71** mostly exists in the **71-I** state in which the lactam NH proton of the pyrimidine moiety forms a strong intramolecular hydrogen bonding with the carbonyl moiety of the amide. Furthermore, in the presence of carboxylate anions the intramolecular hydrogen bonds break, and the flexible

amide proton present in the same direction as lactam NH recognizes carboxylate (**71-II**) (Scheme 28).

Scheme 28. Mode of Binding of Sensor 71 to (I) a Carboxylic Acid Moiety and (II) the Carboxylate Moiety

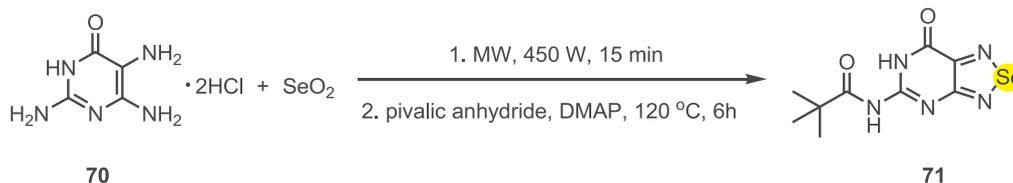
The fluorescence behavior of **71** was recorded with the addition of carboxylic acid and carboxylate ions. There was no change in the fluorescence intensity with the addition of monocarboxylic acid, while a significant increase in the fluorescence was observed with the addition of carboxylate anions. These studies clearly indicate that **71** can be used as a fluorescence probe for detection of carboxylate anions over the other interfering anions and carboxylic acids. Furthermore, these results were supported by the NMR studies.

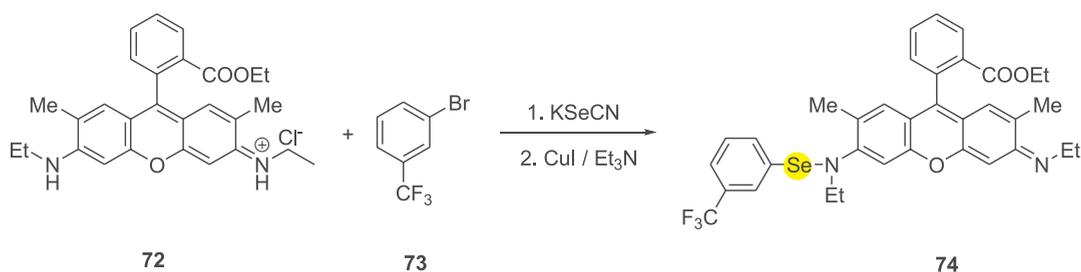
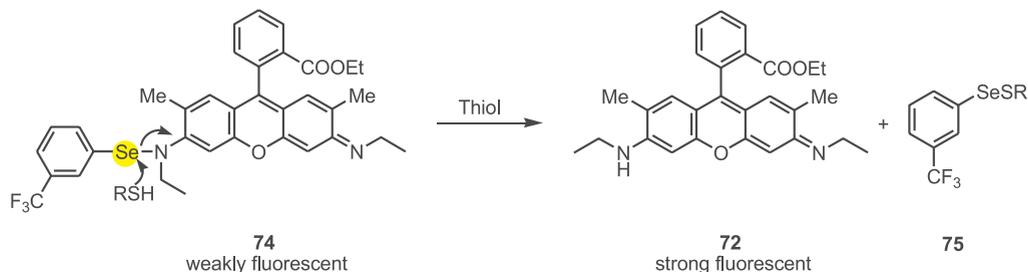
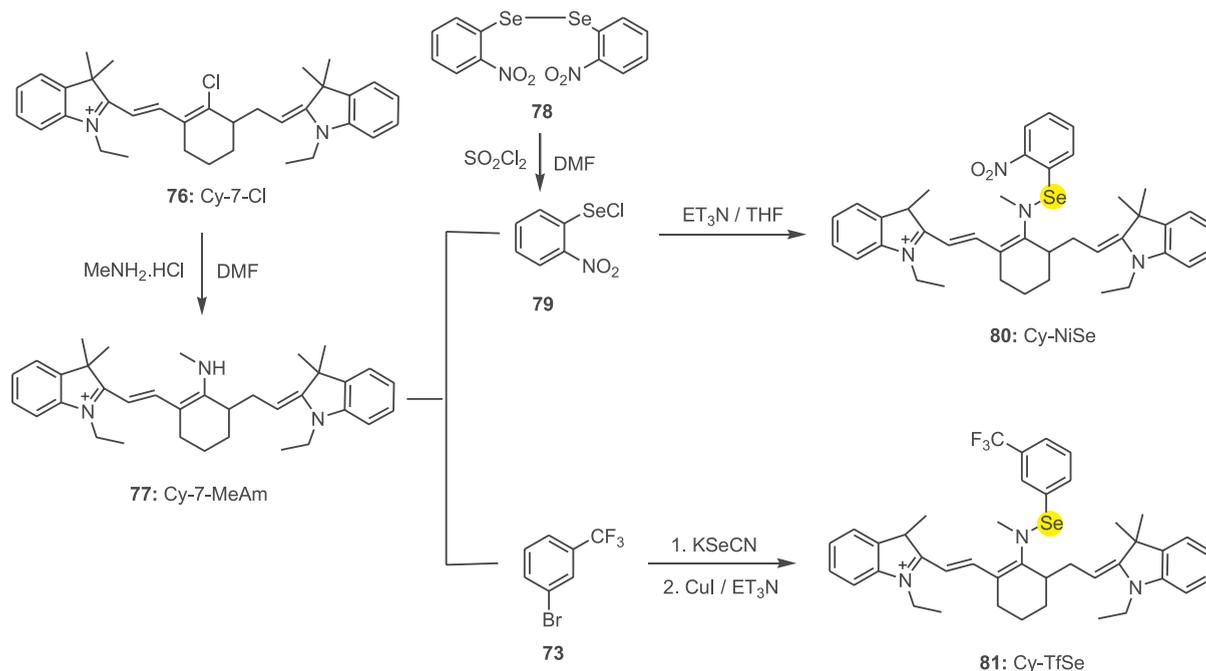
5. DETECTION OF THIOLS

Thiols are important constituents of cellular proteins and other simple molecules such as glutathione (GSH), cysteine (Cys), and homocysteine (Hcy), which are responsible for several biological events.^{98,99} The abnormal behavior of cellular thiols leads to a number of diseases including cancer, AIDS, and Alzheimer's and cardiovascular diseases. In addition, cysteine (Cys) is a nonessential amino acid, and its deficiency is responsible for some syndromes such as slow growth in children and liver damage.^{21,100–102} The elevated level of homocysteine (Hcy) in plasma leads to Alzheimer's disease and cobalamin (vitamin B12) deficiency.^{103,104} More importantly, glutathione (GSH) is the most abundant intracellular nonprotein thiol¹⁰⁵ which exist in reduced (GSH) as well as oxidized (GSSG) states and plays a vital role in maintaining intracellular redox balance.¹⁰⁶

In 2007, Tang and co-workers¹⁰⁷ developed a rhodamine-based fluorescent probe **74** containing a Se–N bond for the detection of thiols. The synthesis of the rhodamine-based fluorescent probe **74** was achieved by the reaction of rhodamine 6G **72**, 3-bromobenzotrifluoride **73**, and KSeCN in the presence of CuI using triethylamine as the base under inert atmosphere (Scheme 29).

The mechanism to detect thiols by rhodamine-cored probe **74** depends on the strong nucleophilicity of the sulfhydryl to cleave the Se–N bond of probe **74**. The probe in aqueous solution is weakly fluorescent, while after the Se–N bond

Scheme 27. Synthesis of Sensor 71 by the Reaction of Pyrimidine 70 with Selenium Dioxide

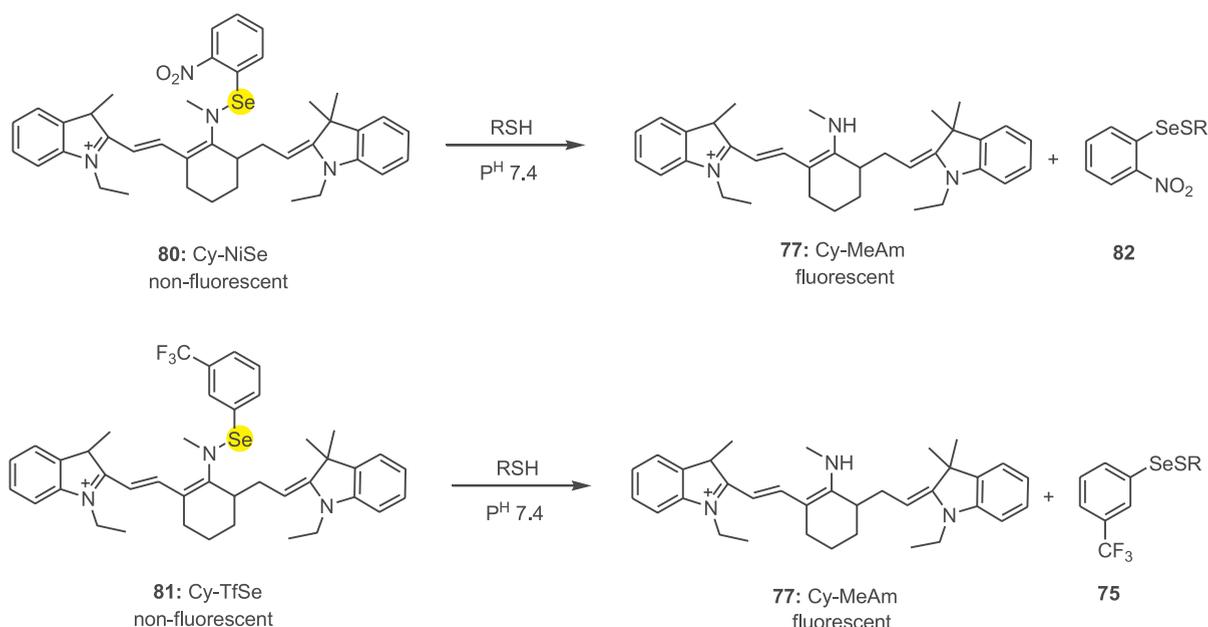
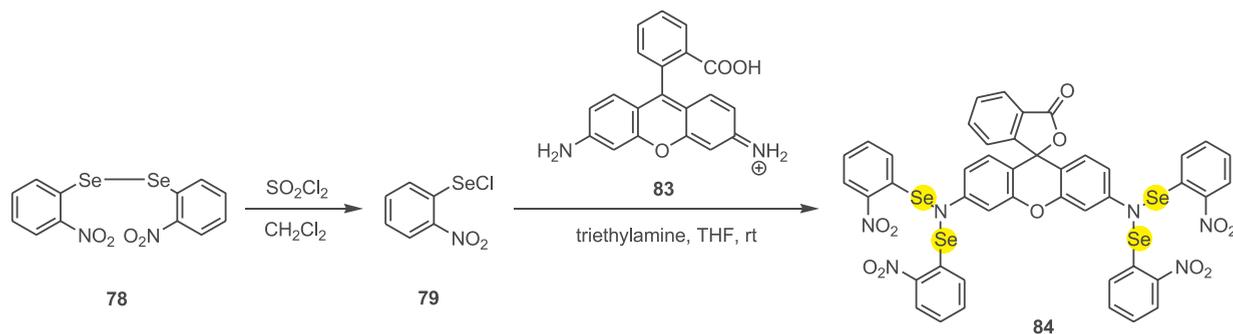
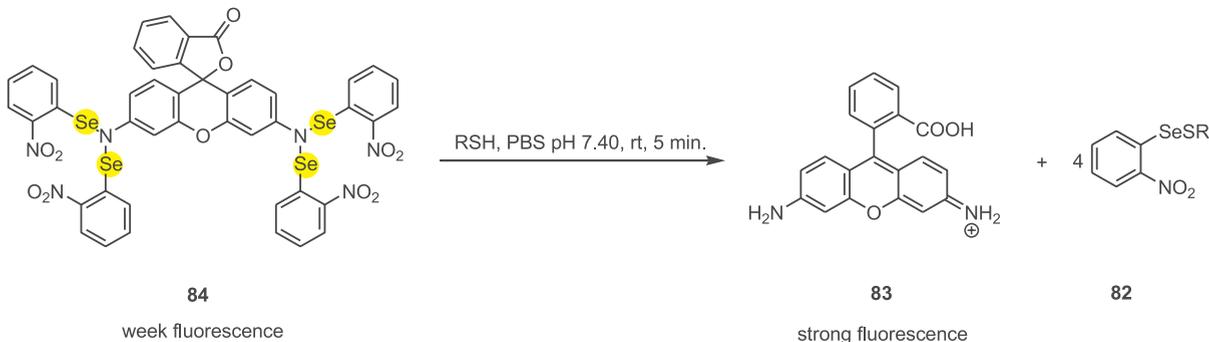
Scheme 29. Synthesis of Fluorescent Probe 74 by the Reaction of Rhodamine 72 and Arylbromide 73 with KSeCN Followed by CuI/Et₃N**Scheme 30. Reaction of Fluorescent Probe 74 with Thiol****Scheme 31. Synthesis of Fluorescent Probes 77, 80, and 81**

breaks in the presence of sulfhydryl it gives the corresponding selenenyl sulfide 75 and the strongly fluorescent dye Rhodamine 6G 72 (Scheme 30). Probe 74 exhibited high selectivity toward GSH in the presence of other analytes. In addition, the probe was reacted with both nonprotein and protein thiols and has shown 2–3 times higher sensitivity for protein thiols than nonprotein thiols. The detection limit was calculated to be 1.4 nM, which indicated that the probe could detect GSH qualitatively and quantitatively. Furthermore, the probe was effective for thiols imaging in both HL-7702 cells and HepG2 cells with high selectivity and sensitivity.

In 2012, Wang and co-workers¹⁰⁸ developed two highly sensitive and selective near-infrared (NIR) fluorescent probes

80 (Cy-NiSe) and 81 (Cy-TfSe) for the detection of thiols on the basis of Se–N bond cleavage in cells and tissues. Initially, intermediate 77 (Cy7-MeAm) was synthesized by the reaction of 76 (Cy7-Cl) with methanamine hydrochloride in 87% yield. The other intermediate *o*-nitrophenyl selenochloride 76 was obtained by diselenide 78 using thionyl chloride.

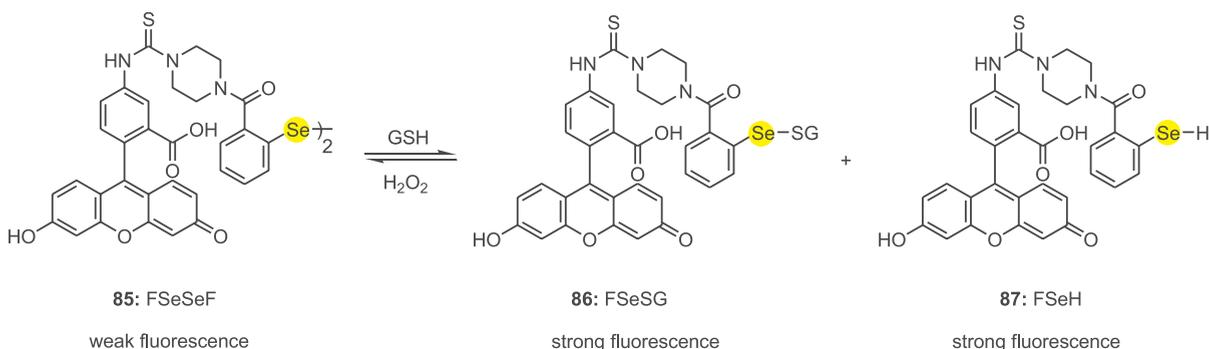
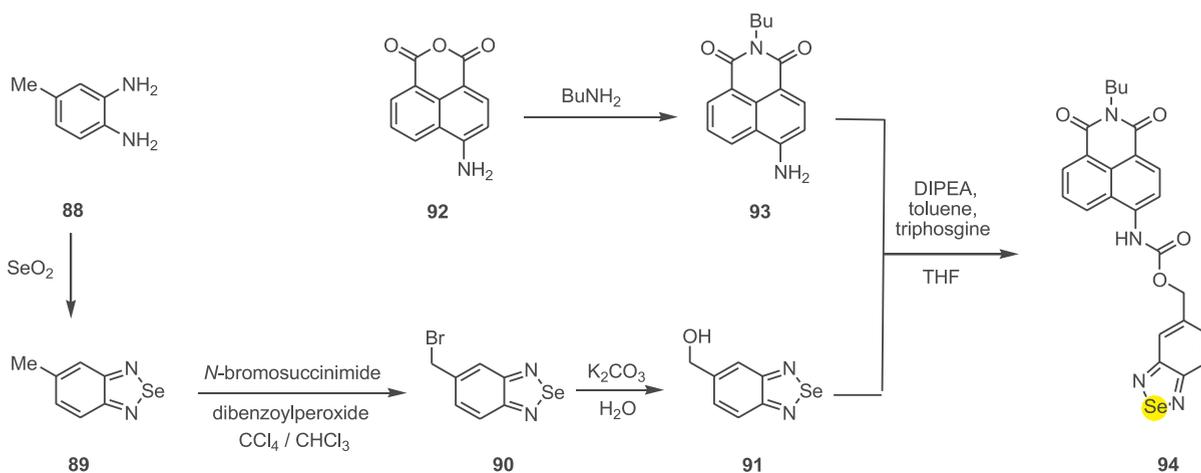
In the next step, selenochloride 79 reacts with 77 (Cy7-MeAm) to form probe 80 (Cy-NiSe) in the presence of triethylamine. Finally, intermediate 77 (Cy7-MeAm) reacts with 3-bromobenzotrifluoride 73 and KSeCN to form the other probe 81 (Cy-TfSe) in the presence of triethylamine and CuI (Scheme 31).

Scheme 32. Conversion of Nonfluorescent Probes Cy-NiSe 80 and Cy-TfSe 81 to Fluorescent Probe Cy-MeAm upon Treatment with Thiol**Scheme 33. Synthesis of Fluorescent Probe 84 Starting from Diselenide 78 in Two Chemical Steps****Scheme 34. Conversion of Weak Fluorescent Probe 84 to Strong Fluorescent Rhodamine 83 on the Treatment with Thiol**

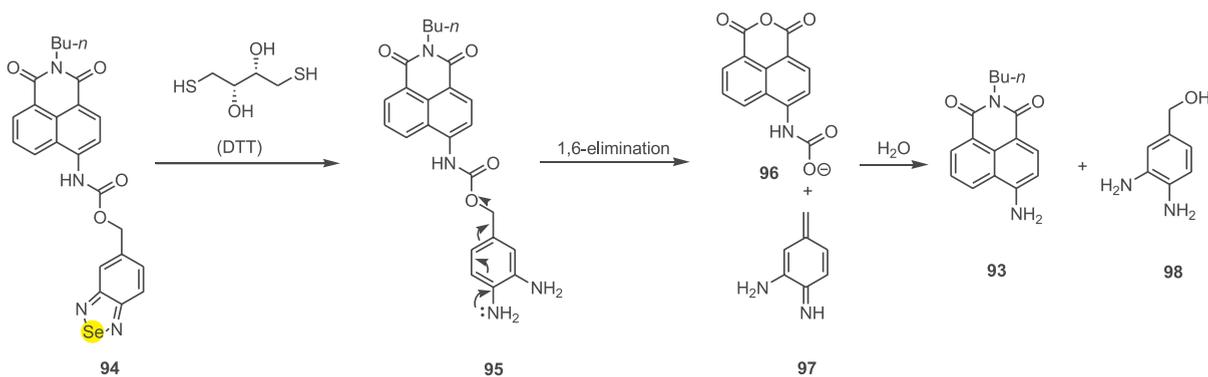
The mechanism for change in the fluorescence of probes **80** and **81** with the addition of thiols is depicted in [Scheme 32](#). In the probes, cyanine dye was used as the signal transducer while strongly electron-withdrawing 2-nitrophenylselene and 3-(trifluoro-methyl)phenylselene groups were used as modulators. Initially, probes did not show any fluorescence because of the donor-excited photoinduced electron transfer (d-PET) between fluorophore and modulators. Further, both probes **80** and **81** exhibited strong fluorescence with the addition of thiol because of the restriction of the d-PET process between the

fluorophore and modulators due the cleavage of the Se–N bond to form cyanine dye **77**. Furthermore, both probes **80** and **81** have been applied successfully for the detection of thiols in living RAW 264.7 cells and fresh rat liver tissue. These findings will be of great benefit for biomedical researchers investigating the effects of thiols in biological systems.

Tang and co-workers¹⁰⁹ have reported a highly sensitive and specific organoselenium fluorescent probe **84** for the detection of thiols. The probe **84** (Rh-Se-2) was synthesized by the reaction of diselenide **78** and thionyl chloride followed by the

Scheme 35. Change in Fluorescence of Diselenide-Based Probe FSeSeF 85 with GSH and H₂O₂Scheme 36. Synthesis of Fluorescent Probe 94 Starting from *ortho*-Diamine 88

Scheme 37. Mechanism for the Change in Fluorescence of the Probe 94 to Detect DTT



reaction of 83 (rhodanine) in the presence of triethylamine (Scheme 33).

The mechanism to detect thiols by rhodamine-cored probe 84 was quite similar to Rhodamine 6G-cored probe 74. Probe 84 did not show fluorescence originally in the aqueous solution but exhibited strong fluorescence with the addition of thiols due to the formation of 83 (Rhodamine, fluorescent dye) by the nucleophilic attack of thiol sulfur to cleave the Se–N bond (Scheme 34). Since glutathione is abundantly present in cells, it was selected as the representative thiol for the spectral experiments.

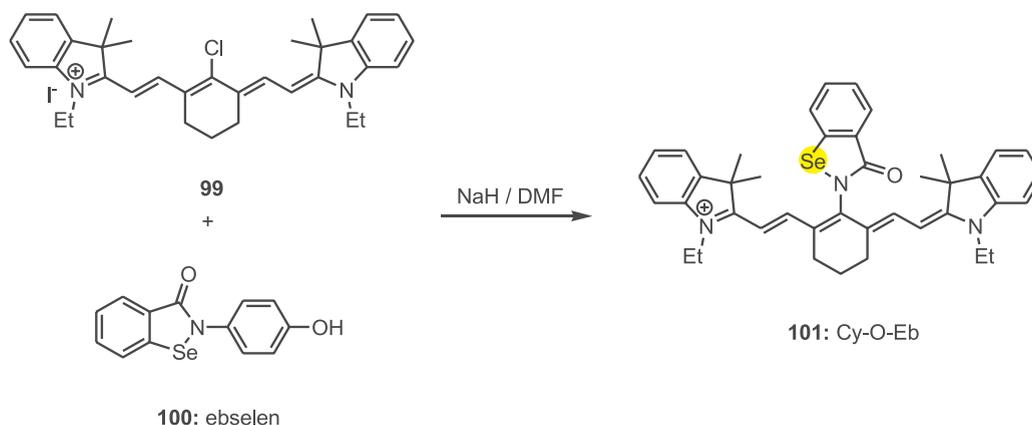
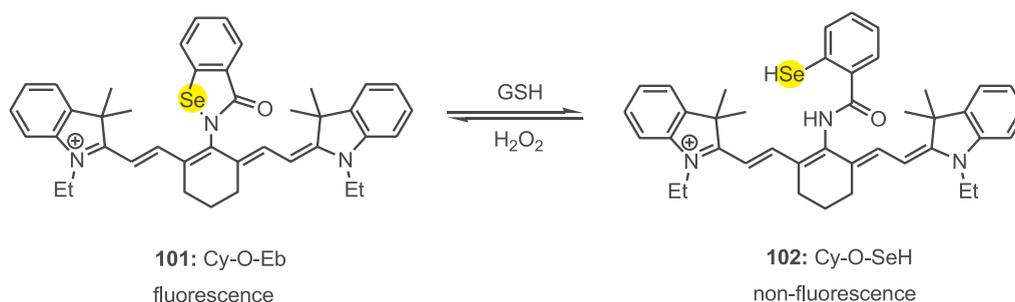
Interestingly, probe 84 can even differentiate between thiols and nonthiols with a clear change in color, easily observed by the naked eye. Furthermore, the confocal microscopy experiments using HL-7702 cells and HepG2 cells confirm that the

probe 84 can visualize the variation in concentration of thiol in both normal and aberrant cell types. Thus, this can be considered a simple and dependable fluorometric system for the analysis of intracellular thiol.

Han and co-workers¹¹⁰ reported a fluorescent probe 85 (FSeSeF) based on diselenide for the fast detection of thiols and redox changes occurring due to the presence of thiols and ROS in living cells. This probe works according to the ping-pong mechanism of the GPx activity of diselenide compounds (Scheme 35). Probe 85 with a diselenide bond is a weak fluorescent molecule. This diselenide bond is considered a rapid, reversible recognition center for thiols and two fluoresceins as signal reporters.

Interestingly, 85 exhibited strong fluorescence with the addition of GSH due to the cleavage of diselenide bond of 85

Scheme 38. Synthesis of Fluorescent Probe Cy-O-Eb 101 by the Reaction of Ebselen 100 with Compound 99 in the Presence of a Base

Scheme 39. Mechanism of Change in Fluorescence of Probe 101 with GSH/H₂O₂

to form both selenenyl sulfide **86** (FSeSG) and selenol **87** (FSeH) containing one fluorescein unit. According to the ping-pong mechanism of GPx activity of the diselenide compounds, in the presence of H₂O₂ molecules **86** (FSeSG) and **87** (FSeH) can be restored back to **85** (FSeSeF); thus the redox changes in the cells due to the presence of thiols and ROS can be easily detected. These mechanistic studies were supported by API-ES mass spectroscopy and ⁷⁷Se NMR. The detection limit for H₂O₂ was found to be 2.25 × 10⁻⁷ M. Furthermore, probe **85** was successfully applied for the detection of thiols in living cells (HeLa cells).

1,4-Dithiothreitol (DTT) is a synthetic thiol^{111,112} and is commonly used as a powerful reducing agent.^{113,114} DTT is a highly toxic substance which in the presence of transition metals lead to oxidative damage of biomolecules.^{115,116} It also increases the toxicity of some arsenic and mercury containing compounds.^{117,118} In 2010, a highly selective fluorescent probe **94** was developed for the detection of DTT using the internal charge transfer (ICT) mechanism.¹¹⁹

Probe **94** can be divided into three distinct parts—(a) a fluorophore, 4-aminonaphthalimide, (b) an organoselenium moiety as DTT receptor, and (c) the 3,4-diaminophenyl methanol, a self-immolative spacer selected for connecting the fluorophore with the receptor. In order to synthesize probe **94**, two different intermediates **90** and **91** were synthesized and then reacted in the presence of DIPEA and triphosgene in toluene to form probe **94** (Scheme 36).

The mechanism for the change in fluorescence of probe **94** to detect DTT has been depicted in Scheme 37. According to the mechanism, nonfluorescent probe **94** reacts with DTT and cleaves the piarselenole-based carbamate protecting group via an internal charge transfer process and regenerates 4-

aminonaphthalimide **93** with green fluorescence. Interestingly, the probe has a high selectivity for DTT when compared to other biothiols such as cysteine and glutathione, which was attributed to DTT's strong reducing ability. The probe displays a 66 nm red-shift of fluorescence emission, and upon adding DTT, the color changes from colorless to jade-green and thus acts as a "naked-eye" probe for DTT. The live cell image experiments further demonstrate its importance in sensing DTT as well as changing the redox environment in living cells.

Tang and co-workers¹²⁰ developed a fluorescent probe **101** for the detection of intracellular GSH/H₂O₂, illustrated in Scheme 39. In **101** the heptamethine cyanine platform (Cy) was used as a fluorophore since it has a high extinction coefficient, low cytotoxicity, and good compatibility with cells. The ebselen (Eb) moiety was integrated as a fluorescent modulator in the probe due to its unique response toward GSH/H₂O₂. The probe was synthesized by the reaction of **99** with ebselen **100** in the presence of sodium hydride in DMF (Scheme 38).

The mechanism of change in fluorescence of probe **101** with GSH/H₂O₂ can be explained by redox cycle (Scheme 39). The strong fluorescent probe **101** can be reduced with the treatment of GSH by cleavage of Se-N which exhibits weak fluorescence. Furthermore, the reduced product **102** is quite unstable and reacts with H₂O₂ to oxidize to more fluorescent **101**. In addition, the mechanism was also supported by mass spectroscopic analysis.

Finally, the probe was successfully implemented for real-time imaging of intracellular redox state changes during the apoptosis process carried out in mitochondria. The results showed that HepG2 and HL7702 cells exhibited better endurance toward H₂O₂ in comparison to normal cells when

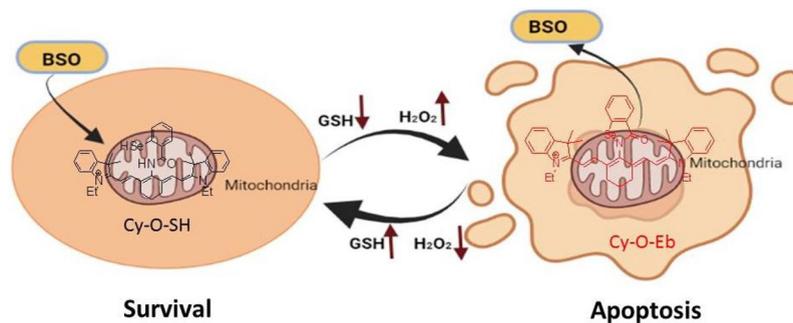
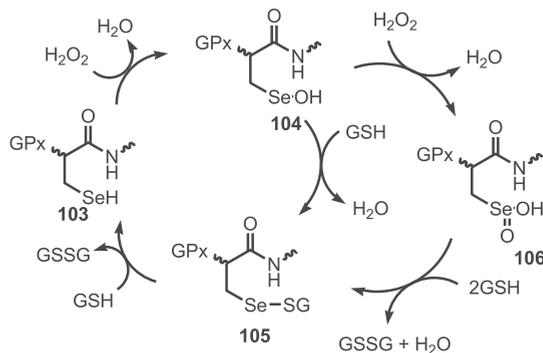


Figure 3. Illustration of the BSO-induced tumor apoptosis and the reversal of apoptosis in HepG2 cells.

H_2O_2 was gradually increased by BSO stimuli. BSO-induced tumor apoptosis and apoptosis reversal in HepG2 cells are schematized in Figure 3. Furthermore, upon applying the probe on the wound margin in zebrafish the H_2O_2 changes in the affected area were successfully monitored.

Glutathione peroxidase enzyme (GPx) in the presence of glutathione (GSH) and catalyzes the reduction of harmful peroxides.^{121–124} Mugesh and his research group^{125–133} have developed various selenium-cored probes to regulate the reversible redox cycle with peroxides and thiols. The catalytic cycle initiates with the oxidation of active selenol **103** by peroxides resulting in corresponding selenenic acid **104**, which reacts with a thiol to generate an important intermediate selenenyl sulfide **105**. The Se–S bond in the selenenyl sulfide **105** is further attacked by a second GSH to give active selenol **103** along with the thiol cofactor (GSSG) in its oxidized form (Scheme 40). With the decrease in thiol level, the selenenic

Scheme 40. Mechanism for the Reduction of Harmful Peroxides in the Presence of Glutathione (GSH)



acid oxidizes to selenenic acid **106** which interferes with the main catalytic pathway. In this cycle the fast reaction of the selenenic acid and GSH results in selenenyl sulfide **105** which further reacts with another GSH to give selenol. These reactions ensure that the selenium moiety in the enzyme is not irreversibly inactivated.

Churchill et al.¹³⁴ developed a phenyl-selenium substituted coumarin probe **110** for rapid and highly selective detection of glutathione (GSH) compared to cysteine (Cys)/homocysteine (Hcy) without background fluorescence. Probe **110** was synthesized from 3-(diethylamino)phenol **107** by treating with diphenyl malonate to get 7-(diethylamino)-4-hydroxy-2H-chromen-2-one **108**, which upon further reaction with dimethylformamide and phosphorus oxychloride gives 4-chloro-7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde

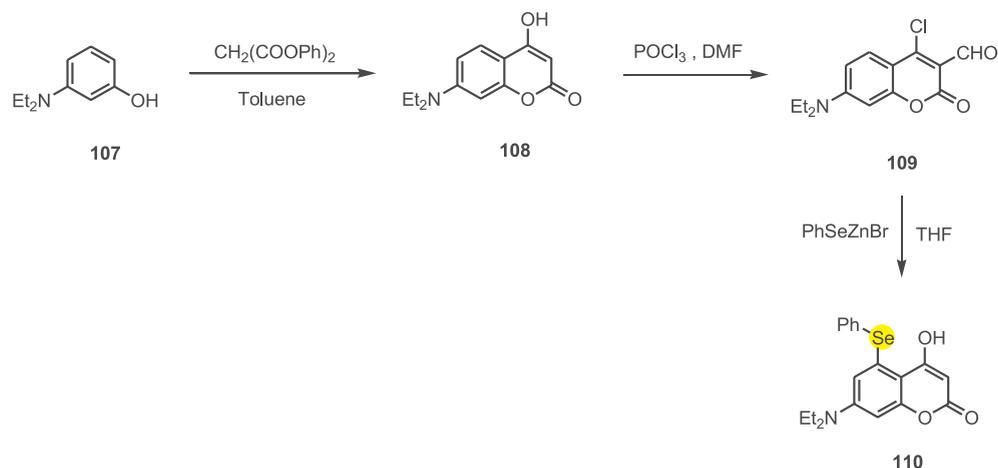
109. Upon reacting intermediate **109** with phenylselenenyl zinc bromide, probe **110** is obtained (Scheme 41).

The phenylselenide group at the fourth position of coumarin has the capability to quench the fluorescence via the PET process as well as also being a good leaving group. The adjacent aldehyde group increases the electrophilicity at the fourth position as a Michael acceptor and helps to cyclize in the presence of sulfhydryl and primary amine groups of biothiols resulting in fluorescence (Scheme 42). The substitution reaction at the fourth position of coumarin with sulfhydryl of GSH occurs so fast that maximum fluorescence intensity is attained in a subsecond time frame and exhibits a 100-fold fluorescent increase in comparison to the fluorescence generated from other amino acids, including Cys and Hcy.

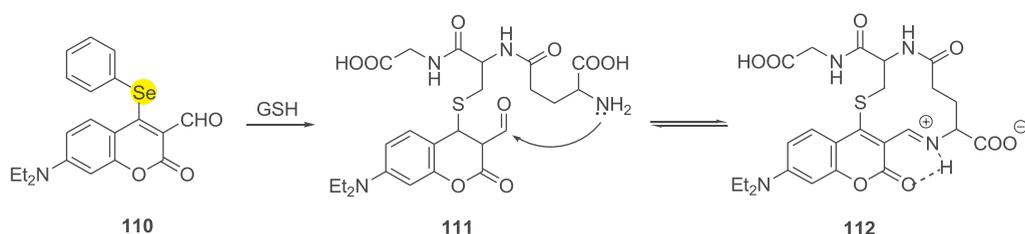
To envision the optimized structures and to calculate molecular orbital energy levels, density functional theory (DFT) calculations were performed for **110**, **111**, and **112**. The results showed that **112** forms a stable hydrogen bond between the adjacent carbonyl group and iminium, resulting in a red-shift. The calculated frontier orbital energy difference for **112** was found to be smaller in comparison to that for **110** and **111**. Confocal microscopy imaging of living cell systems indicates that the probe identifies GSH in Hep3B cells quickly and explicitly, and the limit of detection was determined to be 2.7×10^{-7} M. Furthermore, cell viability testing showed low cytotoxicity toward the probe; thus the probe has the potential for biological uses.

In 2018, Churchill and co-workers¹³⁵ in continuation of the above work developed an improved version of coumarin-based fluorescent probes to detect biothiols. To block the rotational freedom, the alkylamino group of the above probe was replaced with N-heterocyclic derivative (DACP-1) **113**. This improved the fluorescence emission intensity; however the emission wavelength remains the same. To get a more bathochromic shift in absorption and emission wavelength, an additional π -conjugation was introduced in the newly attached N-heterocyclic ring (DACP-2) **114**. The new double bond gives DACP-2 **114** better optical attributes for chemosensing in comparison to DACP-1 **113**. An ethyl butanoate group at the amino position improves water solubility of both probes. A phenylselenium group at the 4-position of both probes completely quenches the fluorescence via PET to give a nonfluorescent species. The probe exhibits selectively in the red region for GSH and in the green region for Cys/Hcy (Scheme 43). In comparison to closely related species such as amino acids and Cys/Hcy, DSCP-1 **113** and DACP-2 **114** showed a strong improvement in fluorescence when treated with GSH. The fluorescence quantum yields increase for the red channel (GSH) [<0.001 to 0.52 (DACP-1)

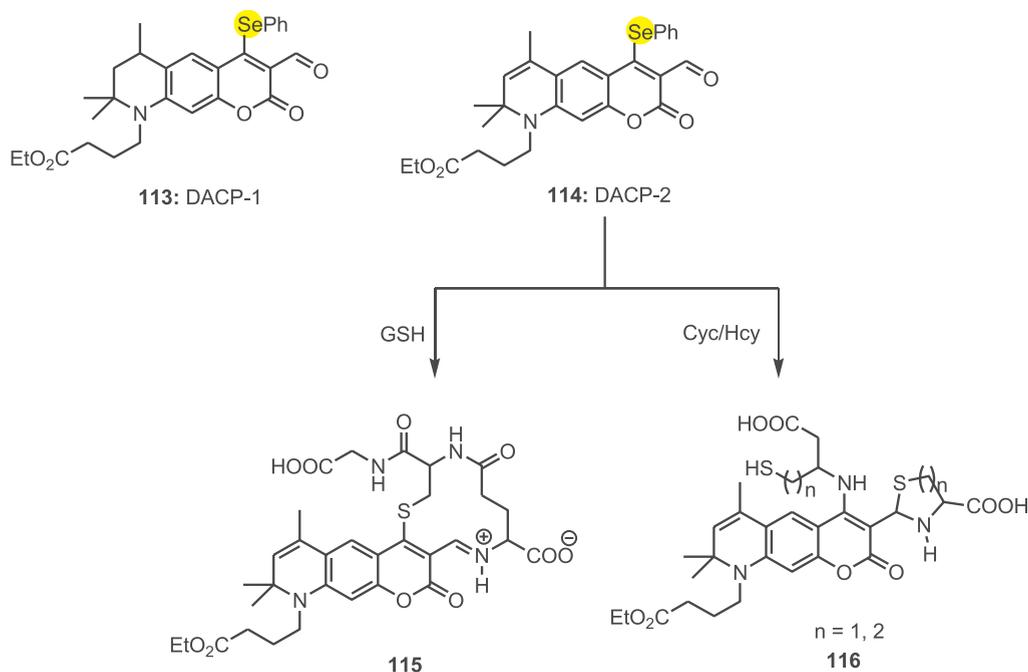
Scheme 41. Synthesis of Fluorescent Probe 110 Starting from 3-Hydroxyaniline 107 in Three Chemical Steps



Scheme 42. Reaction of Fluorescent Probe 110 with GSH



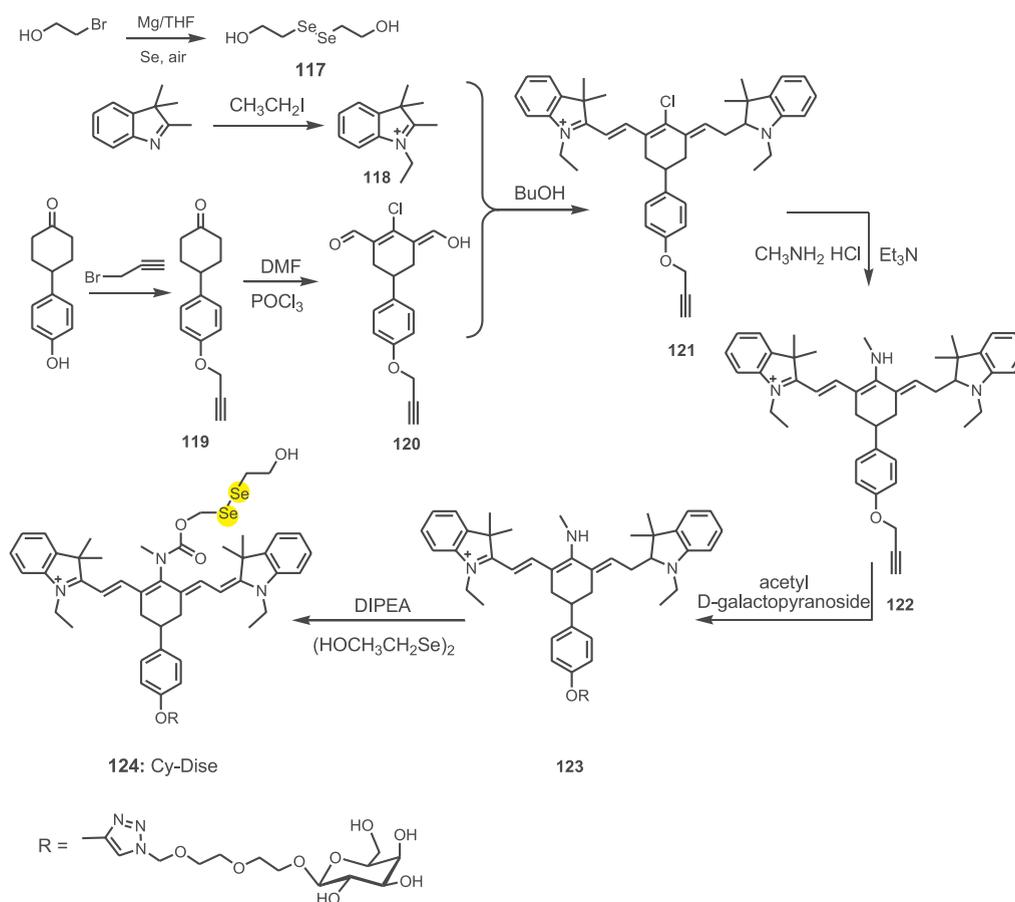
Scheme 43. Reaction of Fluorescent Probe 114 with GSH and Cys/Hcy



and 0.48 (DACP-2)] and the green channel (Cys) [<0.001 to 0.030 (DACP-1) and 0.026 (DACP-2)]. Selective detection was further confirmed by confocal microscopy imaging experiment on live human lung cancer cells and fibroblasts as well as mice tumor model imaging. The detection limits of DACP-1 and DACP-2 for Cys were found to be 0.31 and 1.27 μM , respectively.

For the first time, Chen and co-workers¹³⁶ synthesized a novel near-infrared fluorescent probe Cy-Dise **124** for selectively detecting cysteine hydropersulfide (Cys-SSH) in living cells and in vivo. Cy-Dise **124** involves three moieties: bis(2-hydroxyethyl) diselenide, a response module; heptamethine cyanine, a signal transducer; and D-galactose, a targeting unit. The response module bis(2-hydroxyethyl) diselenide **117** was synthesized by reacting selenium and 2-bromoethanol.

Scheme 44. Synthesis of Fluorescent Probe Cy-Dise 124



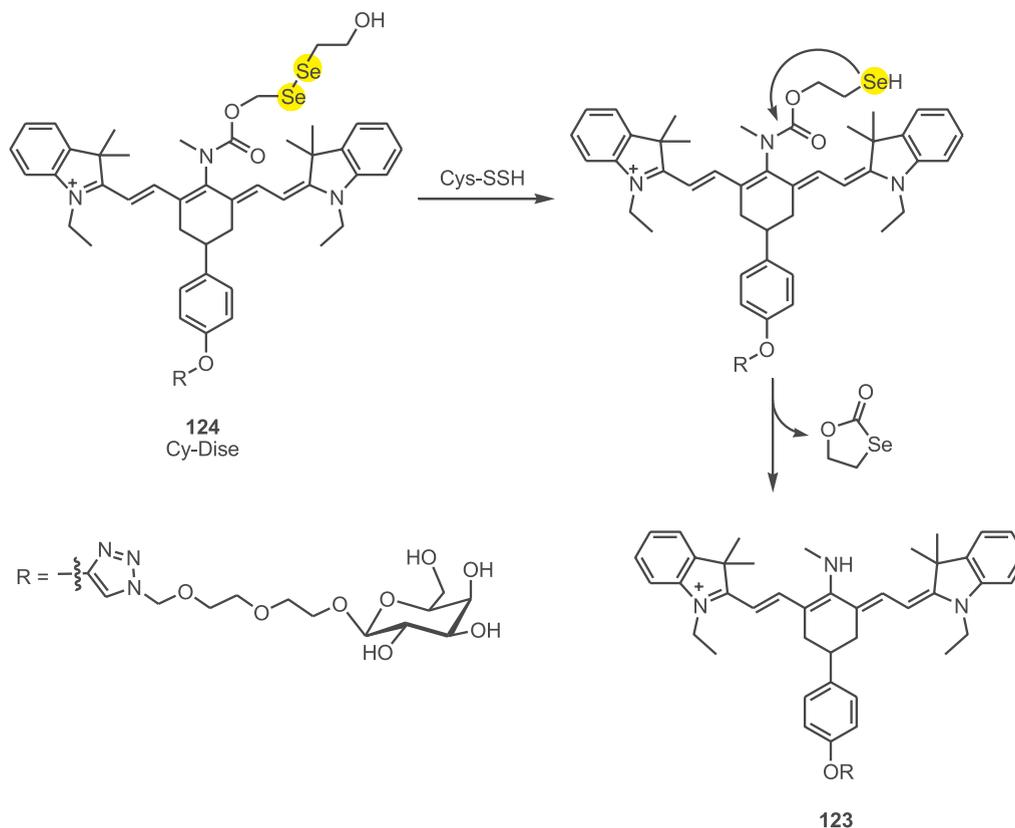
The heptamethine cyanine dye **121** was synthesized using propargyl bromide and 4-(4-hydroxyphenyl) cyclohexanone **119**. Further, reacting compound **119** with DMF and POCl₃ yields **120**. An intermediate compound, **120**, and 1-ethyl-2,3,3-trimethyl-3*H*-indolenium iodide salt **118** afford compound **121**. A nucleophilic substitution reaction by methylamine hydrochloride at the *meso* position of compound **121** results in compound **122**. Acetyl-D-galactopyranoside is attached to compound **122** via click chemistry yielding compound **123**. In the last step, compound **123** is treated with triphosgene subsequently with the addition of **117** to obtain Cy-Dise **124** (Scheme 44).

The integration of bis(2-hydroxyethyl) diselenide with a carbonyl group in the central nitrogen atom results in a red shift in the emission spectrum. Exchanging the response modulator by Cys-SSH leads to a blue shift in the emission spectrum. The ICT-based red–blue shift in the emission spectrum helps in detection of Cys-SSH in living cells and in vivo. The release of fluorophore **123** occurs due to the intermediate formed immediately upon reduction of diselenide and fast intramolecular cyclization along with the cleavage of the neighboring carbamate bond (Scheme 45). In the presence of Cys-SSH, the maximum absorbance changes from 790 to 614 nm accompanied by a color change from green to blue. The emission max wavelength also shifts from 797 to 749 nm. The intensity of fluorescence is directly related to the Cys-SSH concentration and thus has outstanding sensitivity and selectivity. The quantitative detection capability of Cy-Dise for Cys-SSH has been established by a ratio imaging analysis of HL-7702 cells, HepG2 cells, and primary hepatocytes. The

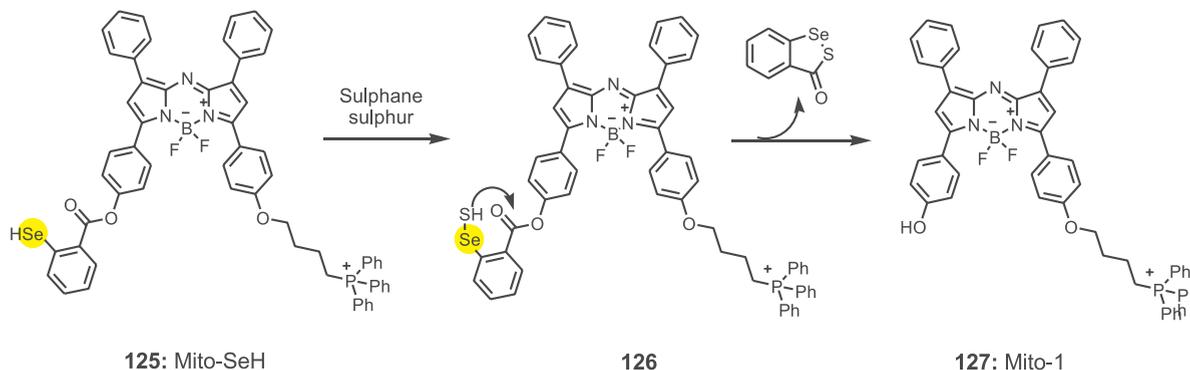
experimental detection limit was determined to be 0.12 μM. The investigations in Sprague-Dawley (SD) rats further exhibited the potential use of the probe for recognizing Cys-SSH in the liver. The galactose part helps the probe to aim at the liver. The role of Cys-SSH in biological functions can be studied by using Cy-Dise **124**.

Following the previous work on Cy-Dise, Chen et al.¹³⁷ developed another NIR fluorescent probe, CyO-Dise for qualitative and quantitative detection of GSH, during hyperthermia and hypothermia processes in vitro and in vivo. The CyO-Dise probe consists of bis(2-hydroxyethyl) diselenide, heptamethine cyanine, and D-galactose, and instead of an amide junction as in Cy-Dise, a more reactive ester bridge was used to improve the reaction kinetics and thermodynamics of diselenide toward GSH. In the presence of GSH, the selenium–sulfur reaction starts and the nucleophilic addition reactivation of the –SeH group is accelerated leading to the release of fluorophore. Based on this, the probe CyO-Dise detected GSH within 35 s. With the help of CyO-Dise, it was observed that short-term temperature stress increases cellular GSH concentration while long-term temperature stress results in an irregular decrease in cellular GSH. Thus, temperature stress can be useful to overcome the drug resistance of the HepG2/DDP cells by dipping the drug efflux and triggering mitochondrial apoptosis pathway. The probe was found useful for imaging the GSH levels of HepG2 and HepG2/DDP xenografts in vivo. The adjuvant therapy of a chemotherapy drug and temperature stress has been found to be effective for the inhibition of cancer cells. The strategy may be applied for

Scheme 45. Reaction of Fluorescent Probe Cy-Dise 124 with Cys-SSH



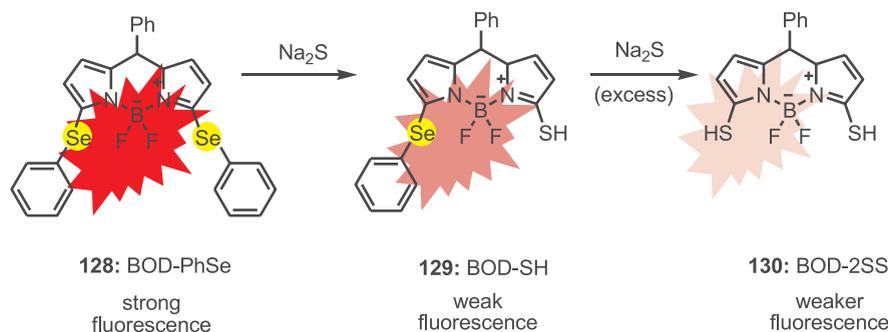
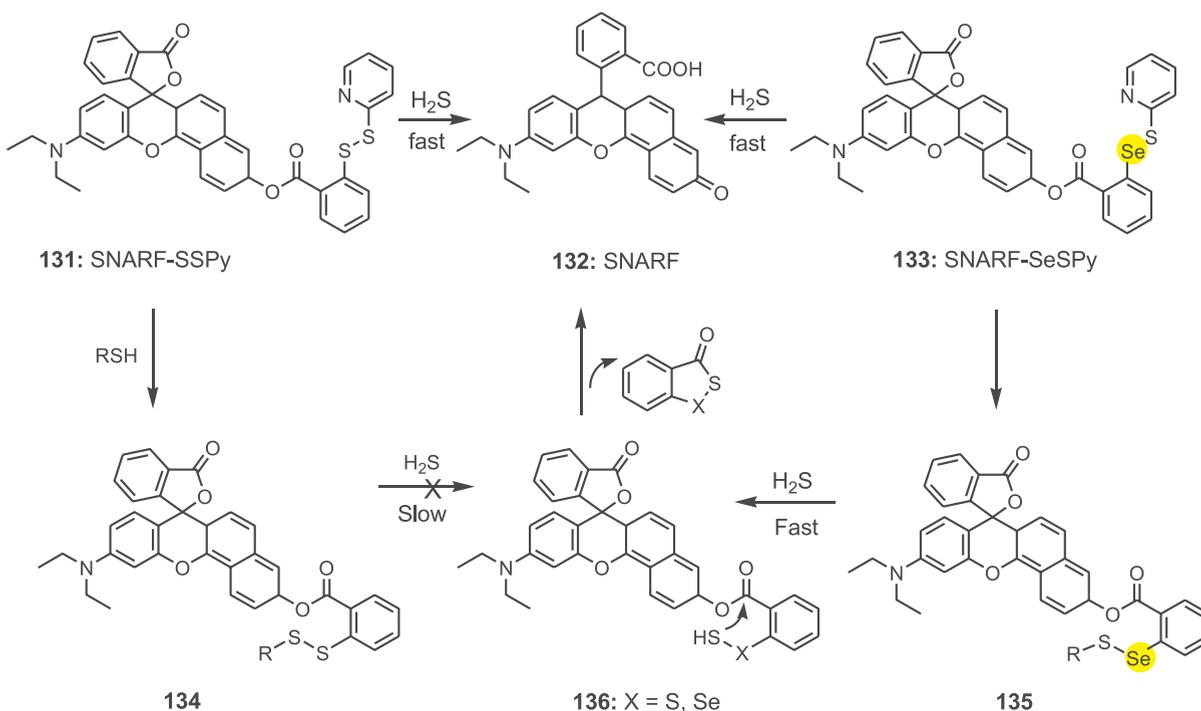
Scheme 46. Reaction of NIR Fluorescent Probe Mito-SeH 125 with Sulfane Sulfur



the development of new chemical tools for accurate cancer diagnosis and evaluation of the efficacy of treatment.

Recently, Chen et al.¹³⁸ reported an NIR fluorescent probe Mito-SeH **125** for *in vivo* and *in vitro* selective imaging of sulfane sulfur in mitochondria under hypoxia stress. Probe **125** comprises of three moieties: azo-BODIPY fluorophore as module, selenol group 2-hydro-selenobenzoate as a strong sulfur-acceptor, and a lipophilic alkyltriphenyl phosphonium cation as the mitochondrial targeting unit. The probe was synthesized by reacting Mito-1 with 2,2'-diselanediyldibenzoic acid in the presence of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP). After stirring for 12 h, the mixture was neutralized with dilute HBr and extracted with DCM. After evaporating the organic solvent, the residue was dissolved in ethanol, and dithiothreitol was added and stirred for 5 h. The product was obtained after evaporating the solution.

Sulfane sulfur generally tautomerizes to the thiosulfoxide in biological systems and is known to be used in extraordinary regulatory functions. The reactive sulfur atom in thiosulfoxide, in the presence of probe **125**, form a Se-SH adduct **126**, and undergoes a fast intramolecular cyclization to liberate the fluorophore Mito-1 **127** (Scheme 46). Mito-SeH **125** was found to be selective and highly sensitive with the detection limit of 3.1 nM toward sulfane sulfur. The probe shows low cytotoxicity, and the flow cytometry study confirmed the potential of the probe to detect endogenous sulfane sulfur in cells. The probe also has the capability of specifically locating and responding to the mitochondrial sulfane sulfur. The correlation between degrees of hypoxia and fluorescent intensities of Mito-SeH reveals that the sulfane sulfur stress response is highly dependent on oxygen levels. The probe was used to detect real-time hypoxia-induced sulfane sulfur changes *in vivo* and *in vitro*. The Mito-SeH can be used to further

Scheme 47. Change in Fluorescence of Probe BOD-PhSe 128 with Na₂SScheme 48. Sensing Mechanism of Probes SNARF-SSPy 131 and SNARF-SeSPy 133 toward H₂S

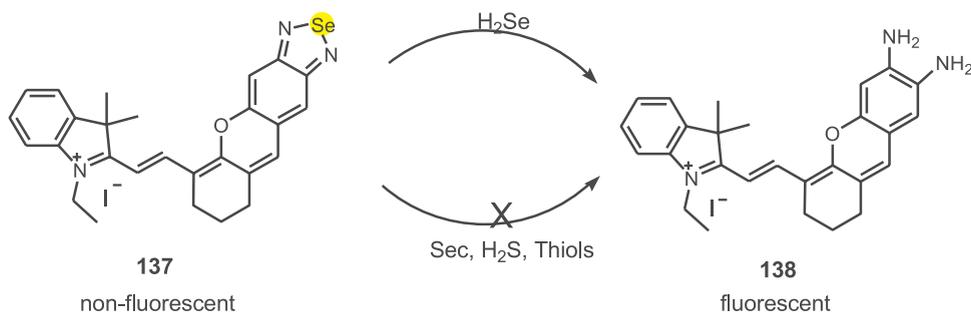
investigate the biological roles of sulfane sulfur in cells and in vivo.

Hydrogen sulfide (H₂S) has emerged as an endogenous gaseous transmitter of signaling molecules that regulate various functions like cardiovascular, neuronal, and immune system in human body. The concentration of sulfide in biological systems ranges from 10 to 100 μM in blood plasma, and a small change in the concentration leads to diseases such as Down's syndrome, diabetes, arterial and pulmonary hypertension, and Alzheimer's disease. Guo et al. developed a phenylselenium-substituted BODIPY fluorescent turn-off sensor for the detection of H₂S.¹³⁹ The probe BOD-PhSe 128 was synthesized by reacting 2,5-dichloro BODIPY to benzeneselenol in the presence of base. The reaction completed in 10 h, and the product was reported in good yield.

The detection of H₂S is based on the substitution reaction of the phenylselenide group of the probe at the 3-position with H₂S to form BOD-SH 129. In excess H₂S, further substitution of the phenylselenide group at the 5-position of the probe occurs, forming BOD-2SH 130 with the decrease in fluorescence emission intensity (Scheme 47). The probe displayed an excellent selectivity and sensitivity with a 0.0025

μM detection limit toward H₂S. The probe with good membrane permeability as well as no cytotoxicity demonstrates the intracellular H₂S monitoring and imaging via fluorescence microscopy in living cells.

In 2020, Yu and co-workers¹⁴⁰ reported two red-emitting turn-on fluorescence probes SNARF-SSPy 131 and SNARF-SeSPy 133. Both probes contain semi-naphthorhodafluor (SNARF) attached to pyridyl disulfide (eSSPy) and pyridyl selenenyl sulfide (eSeSPy) by an ester linker. Both probes could undergo nucleophilic reaction to liberate the SNARF 132 fluorophore. However, in the presence of high concentrations of thiols, SNARF-SSPy 131 with a disulfide (S–S–) linkage undergoes the first nucleophilic substitution to form intermediate 134. SNARF-SeSPy 133 which contains a selenenyl sulfide (–Se–S–) linkage reacts with thiols to produce intermediate 135 and then further reacts with H₂S to produce –Se–SH intermediate 136, and finally the intramolecular cyclization takes place to release the SNARF 132 fluorophore (Scheme 48). The major reaction products of SNARF-SeSPy 133 with H₂S were found to be fluorophore SNARF 132 and cyclic acyl selenylsulfides.

Scheme 49. Conversion of Nonfluorescent Probe 137 to Fluorescent Probe 138 by the Reaction with H₂Se

When both probes were treated with high concentrations of thiols (Cys, Hcy, GSH) and H₂S, SNARF-SSPy exhibited an insignificant fluorescence change while SNARF-SeSPy showed a significant increase in fluorescence. This observation shows that SNARF-SeSPy could detect H₂S in the pool of biological thiols and exhibit good anti-interference. It was found to respond H₂S with high selectivity and sensitivity with the detection limit of 34 nM along with low cytotoxicity. H₂S imaging in living cells and zebrafish confirmed that SNARF-SeSPy can track exogenous and endogenous H₂S in vitro and in vivo; thus it could be applied for monitoring the same.

H₂Se is a vital metabolite found in the reduced form of selenium and obtained from dietary Se compounds. Endogenous H₂Se is involved in several biological and pathological processes. It is produced by reducing selenite with the help of GSH and other reduction systems. Tang et al.¹⁴¹ developed NIR-H₂Se 137, a novel fluorescent probe for detecting H₂Se. The probe consists of a 2,1,3-benzoselenadiazole (BS) moiety and near-infrared (NIR) merocyanine dye 138. The NIR-H₂Se exhibits emission maximum at 735 nm with feeble fluorescence due to the quenching of BS owing to the heavy atom effect of Se (Scheme 49).

Under physiological conditions, NIR-H₂Se selectively recognizes H₂Se over selenocysteine (sec), H₂S, Cys, Hcy, GSH, ROS, and metal ions. The probe also exhibits a rapid response and sensitivity toward H₂Se with the calculated detection of the limit to be 7.0 nM. The study shows that under hypoxic condition the H₂Se content increases and ROS level decreases, whereas in normoxic conditions, the H₂O₂ content increases and H₂Se level rises slightly during HepG2 cell apoptosis induced by Na₂SeO₃. This suggests that non-oxidative stress is the pathway by which Se acts as an anticancer agent in hypoxic solid tumors. The probe can be applied to study the further role of H₂Se and Se in the anticancer mechanism.

7. CONCLUSION AND FUTURE VISION

The organoselenium probes are gaining the attention of researchers because of their selectivity and sensitivity to recognize different analytes such as metal ions, biologically important anions, ROS, and thiols. In this review article, the synthesis and application of various selenium-based fluorescent probes have been summarized. The review article includes the detection of toxic metals such as mercury and silver ions by selenium-cored fluorescent probes. The scope of the selenium-based probes is not limited to the detection of toxic metals but successfully employed for the detection of various reactive oxygen species ROS and thiols.

The different selenium fluorophores are synthesized either by changing the fluorophore or by altering the Se-containing

active reaction site. These changes improve the specificity of recognition and the “on–off” property of the probes. The different recognition modes discussed in this review include deselenation of fluorophore in the presence of analytes and the reaction of fluorophore with analytes. Organoselenium compounds exhibit high reactivity specifically toward Se–N, Se–O, and Se–P bonds during oxidation and elimination reactions and on reaction with nucleophiles and electrophiles, which make the probes a desirable sensor for neutral molecules (thiols) and ions (metal ions and anions). The selenium in organoselenium compounds is of low oxidation potential, which makes it sensitive toward various ROS. Superoxides, hydrogen peroxides, hypochlorous acid, and so forth which come under ROS exhibit the same chemical properties; however their reactivity depends on the electronic structure and chemical nature. Thus, by changing the steric and electronic environment around Se, selectivity and sensitivity of selenoprobes for ROS can be explored. Along with selectivity and sensitivity, water solubility and cell permeability are also important in the application of the probe in biological systems. This review article would provide insight about the synthesis of different molecular probes for the recognition of various analytes. The review would be quite helpful for researchers who are working in different areas of material and medicinal chemistry.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

R.M. acknowledges the support from the Department of Science and Technology, New Delhi, India, for WOS-A project SR/WOS-A/CS-107/2018. F.V.S. is thankful to VIT Chennai for providing infrastructure for preparing the review article.

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