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Probing the formation of a Seleninic Acid in Living Cells by a Fluorescence Switching of a Glutathione Peroxidase Mimetic

Harinarayana Ungati, Vijayakumar Govindaraj, Megha Narayanan and Govindasamy Mugesh*

Abstract: *Glutathione peroxidase (GPx) is a selenoenzyme that protects cells against oxidative damage. Although the formation of a seleninic acid (-SeO_2H) has been proposed for the enzyme and its mimetics in oxidative stress, such species has not been identified in the cells. Herein, we report, for the first time, that the formation of seleninic acid can be monitored in living cells by using a redox active ebselen analogue having a naphthalimide fluorophore. The probe reacts with H_2O_2 to generate a highly fluorescent seleninic acid. The electron withdrawing nature of the -SeO_2H moiety and strong Se···O interactions, which prevent the photoinduced electron transfer, are responsible for the fluorescence.*

Hydrogen peroxide (H_2O_2) plays important roles in redox biology and cell signalling.[1] However, enhanced levels of H_2O_2 induce oxidative stress, resulting in damage to biomolecules such as DNA, proteins, and lipids.[2] In the long term, these damages lead to various disorders, such as cardiovascular diseases, cancer, neurodegeneration, HIV activation, and aging etc.[3] Glutathione peroxidase (GPx) is a selenoenzyme that protects the cells from oxidative stress by catalysing the reduction of H_2O_2 and organic peroxides using glutathione (GSH) as a cofactor.[4] The catalytic cycle of GPx involves initial oxidation of the selenol (E-SeH) by peroxides to produce a selenenic acid (E-SeOH). Under physiological conditions, the selenenic acid readily reacts with GSH to generate the enzyme-bound selenenyl sulfide (E-Se-SG), which upon reaction with a second GSH, regenerates the selenol with the release of GSSG (Figure 1A).[4] However, the selenenic acid has been shown to undergo further oxidation to the corresponding seleninic acid (E-SeO_2H) at higher peroxide and lower GSH levels, which represents severe oxidative stress in the cells.[5]

As the redox chemistry of selenium in GPx plays a key role in the cytoprotection, synthetic selenium compounds that functionally mimic the natural enzyme have been reported.[5] These mimetics include the well-known ebselen (**1**, Figure 1B) and its analogues, camphor-based selenenyl amide, various diselenides, azaselenonium chloride, allyl and alkyl selenides, and a cyclic selenelate ester.[6] They catalytically reduce H_2O_2 and organic peroxides in the presence of GSH or aromatic thiols. Although the formation of seleninic acid such as **2**-**5** has been observed for ebselen and other related compounds under different experimental conditions (Figure 1B),[5,6] it is not clear whether such species is produced in cells, particularly under oxidative stress conditions. Herein, we report for the first time that a selenium-based probe that generates a highly fluorescent seleninic acid can be used to understand the oxidative stress in the cells during its GPx-like redox regulation.

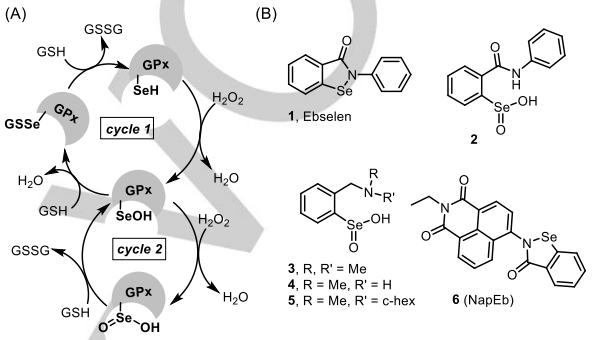

Figure 1. (A) The proposed mechanism of GPx, indicating the species formed under normal (cycle 1) and oxidative stress (cycle 2) conditions. (B) Chemical structures of ebselen (**1**), proposed GPx-mimetic-based seleninic acids (**2**-**5**) and the ebselen-based fluorescent probe (**6**) synthesized for this study.

The napthalimide-based ebselen derivative **6** was prepared from 4-bromo-*N*-ethyl-1,8-naphthalimide and benzo[*d*][1,2] selenazol-3(2*H*)-one as described in the Supporting Information. The photophysical properties of **6** were studied in 100 mM phosphate buffer at pH 7.4. A strong UV-Vis absorption band at 350 nm was observed, which shifted by ≈25 nm higher wavelength upon treatment with H_2O_2, indicating a facile reaction of **6** with H_2O_2. The fluorescence measurements indicated that **6** is essentially non-fluorescent in aqueous solution (Figure 2A), probably due to quenching of the napthalimide fluorescence by the cyclic selenazole moiety. To understand whether a photoinduced electron-transfer (PET) mechanism is responsible for its non-fluorescent nature, density functional theory (DFT) calculations were carried out by using B3LYP/6-311+G(d,p) basis set to determine the orbital energy levels.[7] The model compounds **7** and **8** were used as acceptor (A) and donor (D), respectively (Figure 2B). The highest occupied molecular orbital (HOMO) energy of the donor (-6.12 eV) falls between the HOMO and LUMO energy levels of the acceptor, which allows an efficient PET process to occur, leading to quenching of the fluorescence. Interestingly, a strong concentration-dependent fluorescence was observed at 465 nm upon addition of various amounts of H_2O_2 (0-12 µM, Figure 2A). A linear increase in the fluorescence was observed even at lower concentrations of H_2O_2 (50-500 nM, Figure 2C), indicating that the selenium moiety in **6** is highly redox sensitive. It is known that the selenium centre in ebselen (**1**) undergoes oxidation not only with H_2O_2, but also with other reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) and peroxynitrite ($ONOO^-$).[5] When **6** was treated with $O_2^{\bullet-}$ and $ONOO^-$, an increase in the fluorescence was observed, although the

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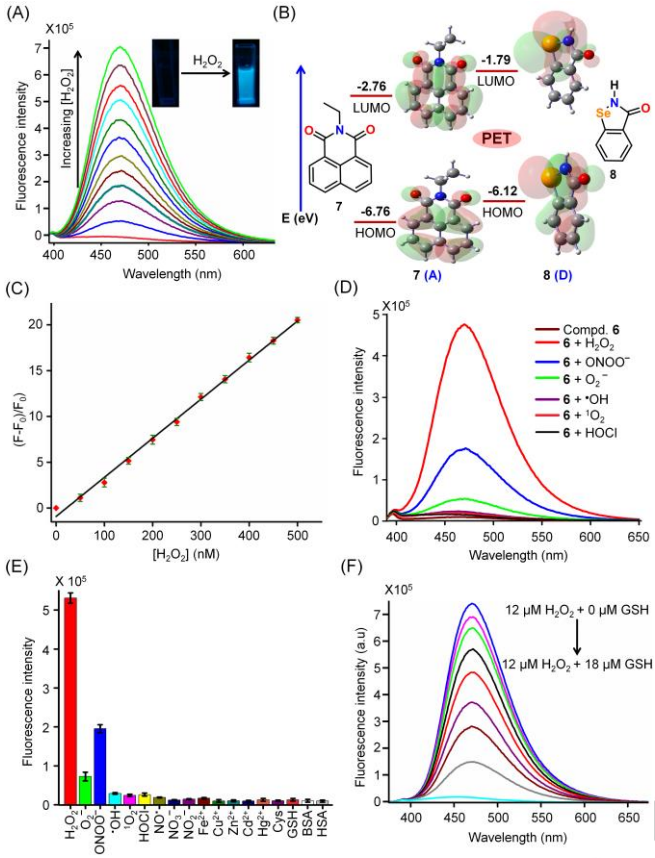

Figure 2. (A) Enhancement of fluorescence upon treatment of **6** (λ_{ex} = 350 nm λ_{em} = 475 nm) with H_2O_2 (2-12 μM) in phosphate buffer at pH 7.4. (B) The HOMO-LUMO energy levels of **7** (acceptor, A), and **8** (donor, D) obtained by DFT calculations. (C) A linear increase in the fluorescence observed for **6** at low concentrations (nM) of H_2O_2. (D) The fluorescence behaviour of **6** in the presence of various ROS after 10 min. (E) Fluorescence response of **6** for various biologically relevant species after 10 min. (F) Effect of GSH on the fluorescence bahaviour of **6** (10 μM) after treatment with H_2O_2.

increase was much lower than that observed for H_2O_2 within the given time. In contrast, no enhancement in the fluorescence was observed when **6** was treated with •OH, L-cysteine, GSH, cysteine proteins (bovine and human serum albumins, BSA and HSA, respectively), divalent metal ions (Fe^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}), nitric oxide (NO), nitrite (NO_2^-), nitrate (NO_3^-) and singlet oxygen ($^1O_2$) (Figure 2E). A rapid decrease in the fluorescence was observed when GSH was added to **6** after treatment with H_2O_2 (Figure 2F), indicating that the oxidized selenium species generated by treatment of **6** with H_2O_2 (Figure 2B) is converted to a non-fluorescent species by GSH.

We next investigated the nature of species produced in the reactions of **6** with H_2O_2 and GSH. It is known that ebselen (**1**) reacts with H_2O_2 to produce the corresponding selenoxide, which undergoes a spontaneous hydrolysis to generate the seleninic acid (**2**).[8] The crystal structure of GPx4 also revealed the formation of a seleninic acid.[5c] When the reaction of **6** with H_2O_2 (1 equiv) was followed by HPLC, a single peak at 14.1 min was observed (Figure 3B). The isolation and characterization by ^{77}Se NMR spectroscopy and mass spectrometry confirmed the formation of the seleninic acid **10**, which was the only major species in the reaction mixture even at higher concentrations (up to 30 equiv.) of H_2O_2. The rapid formation of **10** should occur through the initial oxidation of **6** by H_2O_2, followed by the hydrolysis of the resulting selenoxide **9**, as observed for ebselen. While the formation of the selenenic acid **13** was not observed due to its rapid cyclization to **6**,[8,9] only a small amount of the overoxidized selenonic acid **14** was observed when a large excess of H_2O_2 (more than 30 equiv.) was used. The reaction of **6** with GSH readily generated the selenenyl sulfide **11** (HPLC: 12.6 min). However, the reaction of **11** with GSH to generate the selenol **12** was very slow (Figure S17, SI). Interestingly, a remarkably higher fluorescence was observed for the seleninic acid **10** as compared to that of **6** and **11**. When GSH was added, the regeneration of **6** was observed by HPLC (Figure S18A, SI), confirming that **6** can act as an ON/OFF fluorescence probe. Crucially, the reactions of **6** with $O_2^{\bullet-}$ and $ONOO^-$ also produced **10** (Figure S18B, SI), indicating that the fluorescence increase upon treatment of **6** with $O_2^{\bullet-}$ and $ONOO^-$ (Figure 2D,E) is due to the formation of the seleninic acid **10**, which is in agreement with the reactivity of ebselen towards these species.[5]

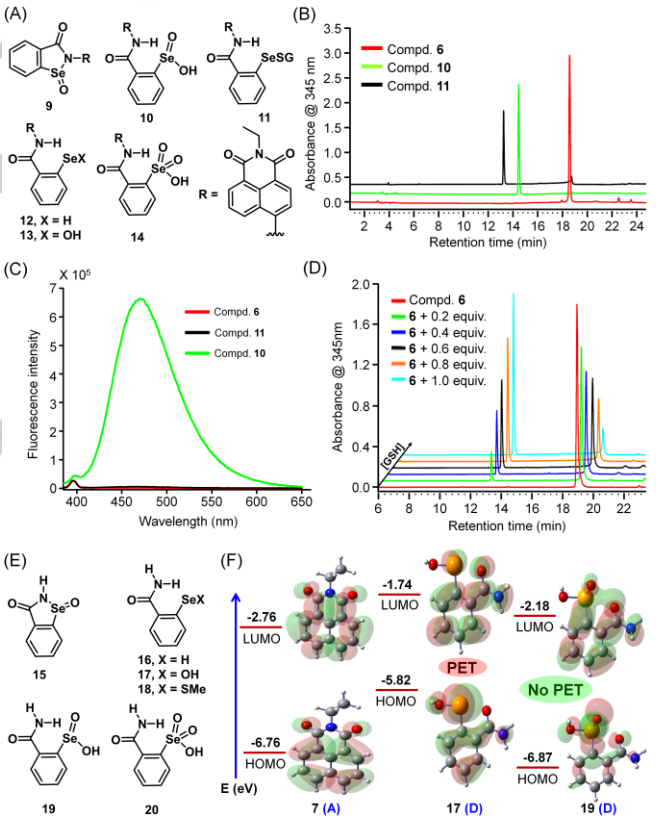

Figure 3. (A) Chemical structures of **9**-**14**. (B and C) HPLC chromatograms and fluorescence spectra of **6**, **10** and **11** (20 μM, λ_{ex} = 350 nm, λ_{em} = 475 nm). (D) HPLC chromatograms of **6** with various amounts of GSH. (E) Chemical structures of **15-20** used for the computational studies. (F) The HOMO-LUMO energy levels of **7** (acceptor, A), **17** and **19** (donor, D).

To understand the HOMO-LUMO energy levels of the intermediates, the model compound **7** was used as acceptor (A) and **15-20** were used as donors (D) (Figure 3E,F and Figure

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S29, SI). As the HOMO energy levels for **19** and **20** (D) were lower than that of **7** (A) (Figure 3F), the introduction of seleninic and selenonic acid moieties can block the PET, leading to fluorescence enhancement. Therefore, we carried out structure optimization and Natural Bond Orbital (NBO) analysis on **10** and **14**.[10] These calculations indicate that the carbonyl oxygen of the amide moiety interacts strongly with selenium in **10** (Figure 4A). Although the Se···O interaction in **10** ($E_{Se\cdots O}$: 5.4 kcal.mol^{-1}) is stronger than that of **14** (1.8 kcal.mol^{-1}), the cooperative effect of the Se···O interaction and the electron-withdrawing nature of the seleninic and selenonic acid moiety can prevent the PET-mediated fluorescence quenching in **10** and **14** by decreasing the electron donation from the donor to the acceptor (naphthalimide) moiety. As the experimental results indicate that the oxidation of seleninic acid **10** to the selenonic acid **14** is not a favoured process, the increase in the fluorescence upon reaction with H_2O_2 can be ascribed to the seleninic acid (**10**) (Figure 4B).

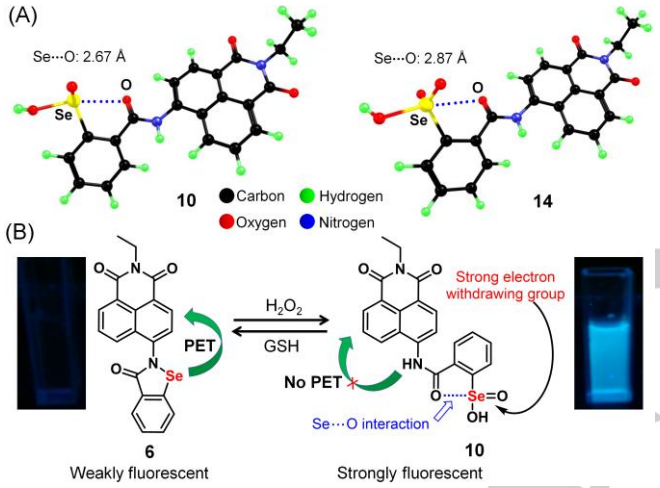

Figure 4. (A) Optimized structures of **10** and **14**, showing the non-covalent Se···O interactions. (B) The reversible generation of the highly fluorescent seleninic acid **10**.

The interesting fluorescent properties of **6** in the presence of H_2O_2 prompted us to investigate its cytotoxicity and fluorescence response in HepG2 (human liver carcinoma) cells and HUVEC (human umbilical vein endothelial cells). The cell viability was determined by using the standard MTT assay. Interestingly, **6** did not exhibit any cytotoxicity up to 50 μM concentration in HepG2 cells (Figure S30, SI), indicating that the low toxicity of the selenazole group present in ebselen was retained upon functionalization with a naphthalimide moiety. To understand the redox activity of **6** in cells, we studied the fluorescence behaviour of **6** in both HepG2 cells and HUVEC by using laser scanning microscopy and fluorescence microplate reader techniques. When the HepG2 cells were treated with 100 nM of **6**, very weak fluorescence was observed even in the presence of 100 μM of H_2O_2. A slight increase in the fluorescence was observed at 0.5 and 1 μM concentrations, and a significant increase was observed when 10 μM of **6** was used (Figure 5A-C). An increase in the fluorescence was also observed in primary HUVECs upon addition of 50 or 100 μM H_2O_2 (Figure 5C), indicating that **6** can enter cultured normal mammalian cells. The increase in the fluorescence can be ascribed to the reaction of **6** with H_2O_2 as shown in Figure 2A,C-E. To confirm the nature of selenium species produced in the cells, we analysed the cell lysates by HPLC. Interestingly, the treatment of **6** with cells pre-treated with H_2O_2 indicated the formation of the seleninic acid **10** as the major product along with small amounts of **6** and the selenenyl sulfide **11** (Figure 5D), suggesting that the formation of **10** can be observed under physiological conditions. As the formation of the selenonic acid **14** was not observed, the increase in the fluorescence upon inducing oxidative stress in the cells can be ascribed entirely to the formation of seleninic acid (**10**).

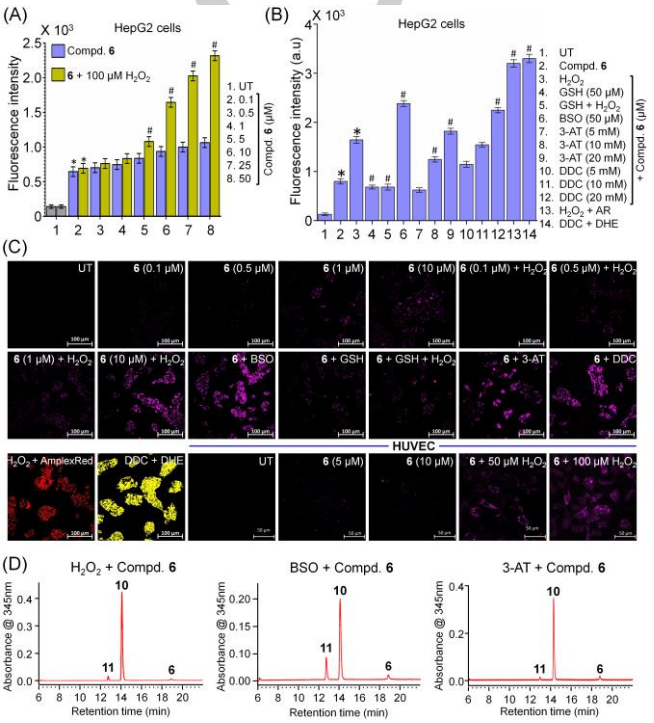

Figure 5. (A) The fluorescence measured by a plate reader after 30 min of treatment of HepG2 cells with **6** at various concentrations in the absence and presence of 100 μM of H_2O_2. *p < 0.05 compared with the untreated control cells; # p < 0.05 compared with cells treated with H_2O_2 and compd. **6**. (B,C) The fluorescence measured by a plate reader (B) and confocal microscopy images (C) after 30 min of treatment of cells with **6** and various substrates and inhibitors. (D) HPLC analysis of the intracellular forms of the probe **6**. The identity of each peak was confirmed by co-eluting the sample with authentic samples of **6**, **10** and **11** (Fig. S19).

It is known that ebselen and other selenium compounds utilize cellular GSH for their GPx-like activity. To understand the redox response of **6** upon reduction in the GSH level, the cells were treated with buthionine sulfoximine (BSO), which is known to block the GSH biosynthesis by inhibiting γ-glutamylcysteine synthetase.[11] The HepG2 cells were treated with 50 μM BSO prior to treatment with **6**. As shown in Figure 5B and C, a significant enhancement in the fluorescence was observed upon depletion of GSH, indicating that **6** responds well to the increase in the oxidative stress. The HPLC analysis indicated the formation of the seleninic acid **10** under this condition (Figure

5D). The increase in the fluorescence due to a decrease in the GSH:H_2O_2 ratio can be brought back to the control levels by treating the cells with GSH. When the cells were treated with GSH before addition of H_2O_2, no enhancement in the fluorescence was observed (Figure 5B and C). The HPLC analysis indicated that the selenenyl sulfide **11** and seleninic acid **10** are the major species produced when the GSH:H_2O_2 ratio are 1:1 and 1:3, respectively (Figure S21). These observations indicate that **6** undergoes redox reactions with H_2O_2 and GSH and the fluorescence intensity depends on the relative ratio of these two species in the cells.

To understand whether **6** can respond to changes in the intracellular H_2O_2 level without treating with exogenous H_2O_2, the intracellular H_2O_2 level was increased by inhibiting catalase (CAT), an enzyme that converts H_2O_2 to water and dioxygen, by using 3-amino,1,2,4 triazole (3-AT). The fluorescence response was enhanced significantly, indicating that **6** is sensitive to changes in the intracellular H_2O_2 concentrations. The formation of the seleninic acid **10** was confirmed by HPLC analysis of the cell lysates (Figure 5D). The increase in the H_2O_2 level in HepG2 cells upon inhibition of CAT was also confirmed independently by using the irreversible H_2O_2-specific probe Amplex Red, which reacts with H_2O_2 with a 1:1 stoichiometry in the presence of horseradish peroxidase (HRP) to produce a highly fluorescent resorufin.[12] However, unlike Amplex Red, **6** acts as an ON/OFF fluorescence probe by redox switching in the presence of H_2O_2 and GSH. In other words, **6** exhibits significant fluorescence only at higher concentrations of H_2O_2 or lower concentration of GSH and hence it can be used to detect severe oxidative stress in the cells. Interestingly, **6** is also sensitive to the superoxide level in the cells. A significant increase in the fluorescence was observed for **6**, when the $O_2^{\bullet-}$ concentration was increased intracellularly by inhibiting superoxide dismutase (SOD) using diethyldithiocarbamate (DDC). The increase in the $O_2^{\bullet-}$ level was confirmed further by treating the cells with superoxide-specific dihydroethidium (DHE) (Figure 5C).

In summary, we synthesized a redox active naphthalimide -based selenazole, which can react with H_2O_2, $O_2^{\bullet-}$ or peroxynitrite to produce a highly fluorescent seleninic acid. The interesting fluorescence behavior of the seleninic acid can be ascribed to the electron withdrawing nature of the -SeO_2H moiety and strong Se···O interactions, which together prevent the photoinduced electron transfer (PET) by lowering the HOMO energy level. The reversible, redox active and self-switching fluorescent probe reported in this paper is useful not only for controlling the level of ROS in mammalian cells, but also for designing fluorescent probes to understand the nature of species produced during the GPx-like activity of selenium-based compounds in cellular environment.

Acknowledgements

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Keywords: antioxidant • ebselen • fluorescent probe • glutathione peroxidase • selenium

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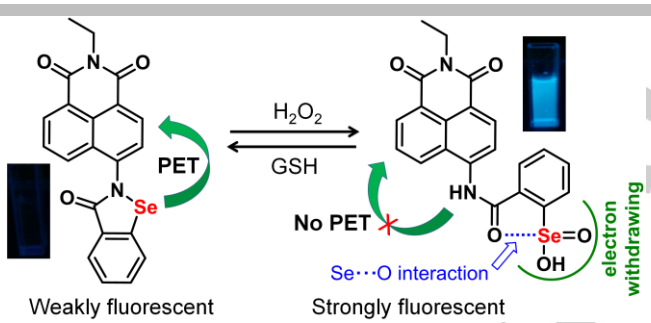
COMMUNICATION

A redox active ebselen analogue having a naphthalimide moiety can be used as a fluorescence probe to understand the formation of a seleninic acid in living cells. The fluorescent property of the seleninic acid can be ascribed to the strong Se···O noncovalent interactions and electron withdrawing nature of the seleninic acid moiety, which prevent the PET by lowering the HOMO energy level.



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