Cyclic Organoselenide BODIPY-Based Probe: Targeting Superoxide in MCF-7 Cancer Cells

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ABSTRACT: All aerobic cells contain reactive oxygen species (ROSs) in balance with biochemical antioxidants. Oxidative stress is developed when this balance gets disturbed because of excessive production of ROSs or depletion of antioxidants. Here, in this work, we have developed the first cyclic diselenide BODIPY-based (organoselenium-containing) probe for the selective detection of superoxide. The probe demonstrates excellent selective response for superoxide over other ROSs with nine-fold increase in fluorescence intensity. The detection limit was found to be 0.924 μM. The plausible "turn-on" mechanism has been proposed based on the spectroscopic and quantum chemical data. Usefulness of the probe for selective detection of superoxide was confirmed in mammalian breast cancer cell lines.

INTRODUCTION

In recent decades, organoselenium chemistry has received enhanced attention because of its various important roles, excellent redox properties,1 and relatively low level of cytotoxicity2 compared to inorganic selenium compounds.3 Organoselenium-containing compounds are of increasing importance in enzymology, medicine, and bio-organic chemistry.4 Specially, selenium-containing heterocyclic compounds possess several biological activities, which include anti-inflammatory, antitumor, antifungal, and antioxidant properties.5,6 Additionally, selenium plays an important role at the active site of glutathione peroxidase (GPx)7−10 in the conversion of natural hydrogen peroxide to water.11 Organoselenium-based molecular probes provide reliable detection of biologically important analytes such as reactive oxygen species (ROSs),12−15 reactive nitrogen species,16,17 biothiols,18 amino acids, and so forth in a qualitative and quantitative way because of their redox properties. Production of ROSs is associated with various human disorders9−22 such as Alzheimer’s, Parkinson’s, and cancers where the excess production of ROSs is the major causative factor of the diseases.23,24 ROSs25 include HOCl, ‘OH, H2O2, O2•−, ’BuOOH, ’BuO•, and so forth.26 Selective and sensitive detection of such species with fluorescence microscopy requires discrete synthetic molecular designs and facile synthesis, which makes this field challenging and important.27 Superoxide (O2•−) with a short half-life is an important ROS and the detection of which at actual time is crucial but complicated. It is well known that superoxide can elaborate chemistry by combining with NO to produce peroxynitrite which is a versatile and highly potent oxidant as compared to NO and superoxide. This can attack and damage a broad range of biological species.28,29 Cells possess an antioxidant defence system to regulate their internal redox equilibrium and give essential biological function of endogenously produced ROSs, for example, signal transduction, neurotransmission, and blood pressure modulation.20,21,24 Detection of biologically important species using fluorescence techniques is particularly attractive because of its high sensitivity, fast response time, simplicity of implementation, and high potential for imaging in live cells or tissues.30 BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) has
well-known characteristics including intense fluorescence with high quantum yield, good photochemical stability, a large extinction coefficient, and so forth.\textsuperscript{31–33} Biosensing with small molecular probes enables monitoring of biochemical and biomolecular processes in organisms at the cellular level and allows potential applications in various fields from fundamental biological research to clinical diagnostics.\textsuperscript{34} In recent years, numerous selenium-containing small molecular probes for the detection of ROSs and biothiols have been reported in the literature.\textsuperscript{31,12,35–38} However, to the best of our knowledge, there are very few reports on selenium-based probes for selective detection of superoxide.\textsuperscript{39,40} Here, we have designed and synthesized the first cyclic diselenide (heterocyclic)-containing BODIPY-based probe for selective and sensitive detection of superoxide.

\section*{RESULTS AND DISCUSSION}

Synthesis of a cyclic diselenide-containing probe is outlined in Scheme 1. Dipyrrmethane 2 was synthesized from the corresponding peri-substituted diselenide-containing naphthaldehy de 1 and pyrrole (25 equiv) with a catalytic amount of trifluoroacetic acid (TFA). The crude product was purified by column chromatography and was characterized by \(^1\)H, \(^{13}\)C, and \(^{77}\)Se NMR spectroscopy, mass spectrometry, and elemental analysis (Figures S1–S10). In the \(^1\)H NMR spectrum of dipyrrmethane 2, the signal for aldehydic proton disappeared and a new signal at \(\delta 5.4 \) ppm for aliphatic CH proton and three signals for the pyrrole ring were observed. Cyclic diselenide-containing BODIPY probe 3 was synthesized from the reaction of dipyrrmethanediselenide intermediate 2 with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (1 equiv) in DCM followed by addition of triethylamine (10 equiv) and boron trifluoride diethyl etherate (BF\(_3\)OET\(_2\)) (10 equiv) under a nitrogen atmosphere. The crude compound 3 was purified by column chromatography and characterized by \(^1\)H, \(^{13}\)C, and \(^{77}\)Se NMR spectroscopy and mass spectrometry (Figures S11–S19). In the \(^1\)H NMR spectrum of probe 3, the signal for the aliphatic dipyrrmethane proton at \(\delta 5.4 \) ppm disappeared. Additionally, in the \(^{77}\)Se NMR spectrum, two signals were observed for chemically different selenium, one at \(\delta 410 \) ppm and another significantly downfield at \(\delta 449 \) ppm, when compared with dipyrrmethane (\(\delta 411 \) and \(\delta 434 \) ppm, respectively), which further confirmed the formation of cyclic diselenide-containing BODIPY probe 3.

The successful synthesis of the first cyclic diselenide BODIPY-based probe increases the curiosity about the reactivity of the probe for selective and sensitive detection of biologically important analytes. Therefore, the spectroscopic properties of probe 3 were studied in DMSO/water (30:70). The probe was screened with various ROSs such as O\(_2^\bullet^-\), H\(_2\)O\(_2\), BuOOH, OCl\(^-\), OH\(^-\), and BuO\(^+\) in water using a UV-visible spectrophotometer and spectrofluorometer. Probe 3 shows strong green turn-on fluorescence upon addition of KO\(_2\) over the other ROSs (H\(_2\)O\(_2\), BuOOH, OCl\(^-\), OH\(^-\), and BuO\(^+\)). It is already reported in the literature that KO\(_2\) in aqueous medium can be used as superoxide.\textsuperscript{41}

The probe was incubated with different ROSs for 15 min and the quantitative absorbance and emission spectra were recorded. The probe absorbed at 506 nm and emitted at 521 nm with almost negligible emission intensity (Figures S22, 1, and 2). The molar extinction coefficient of the probe was found to be 16,000 M\(^{-1}\).cm\(^{-1}\). High increase in fluorescence intensity of the probe in presence of superoxide was observed (from 1.375 for the probe to 647.949 for the probe with superoxide). The quantum yield of the probe and its oxidized form was calculated according to the literature.\textsuperscript{36} The fluorescence quantum yield of probe 3 was increased from 2 to 17 percent (nine-fold increase in fluorescence intensity, calculated from quantum yield).

The interference study of probe 3 was carried out in the presence of superoxide along with addition of other ROSs. It was observed that there is no remarkable change in fluorescence intensity of the probe with superoxide in presence of other ROSs. This suggests that there is no interference of other ROSs in the detection of superoxide using probe 3 (Figure S23).

In increasing concentration study, with gradual increase in superoxide concentration, linear increase in fluorescence intensity of probe 3 was observed (Figure 3). The detection limit of the probe was determined by plotting the graph of
emission intensity versus concentration of superoxide. The detection limit of 3 was found to be 0.924 μM (Figure S24).

The kinetic study of the probe was conducted by adding superoxide (20 μL, 666.67 μM) to the probe solution (3 mL, 20 μM), and the spectrum was recorded for 1 h (Figure 4). It shows steady increase in fluorescence intensity up to 15 min and then saturated. This study suggests that the probe has good fluorescence stability for a longer time.

In order to understand the redox capacity of selenium in probe 3, the probe was oxidized with superoxide and further treated with biothiols such as glutathione, N-acetyl-L-cysteine, homocysteine, and L-cysteine. The result obtained shows significant decrease in fluorescence intensity of the probe (Figure S25).

Finally, to support the “turn-on” event that occurred for the detection of superoxide with probe 3, the reaction of probe 3 with KO₂ (33.33 equiv) was carried out; the mass spectrum and the ¹H NMR spectrum were recorded. ¹H NMR spectroscopy revealed that the protons attached to the pyrrole and naphthalene of the probe have been shifted after reaction with KO₂ (Figures S20 and S). Additionally, from the mass spectrum analysis, the isotopic pattern of selenium with one oxygen was observed at m/z 491 [MO − 1]+ (Figure S21), which suggested the monoxidation of probe 3. However, there were two probable Se sites of oxidation. In order to ascertain the most active site for the oxidation, density functional theory (DFT) calculations at the B3LYP/6-311G*(d,p) level of theory were performed. Figure 6 displays the optimized structures of the probe. NBO charge analysis suggested a positive charge density of 0.286e and 0.263e at Se1 and Se2, respectively. Additionally, ⁷⁷Se NMR of probe 3 showed peaks at 449 and 410 ppm for Se1 (proximal Se) and Se2 (distal Se), respectively (Figure S13), indicating that Se1 was more electron deficient than Se2. Therefore, because of higher positive charge density on Se1, it was expected that the nucleophile (O₂−) would attack on it and Se1 will get oxidized to form Se=O and attain oxidation state Se(IV). This data supported the proposed plausible mechanism as shown in Scheme 2 and concluded that because of oxidation of selenium by superoxide, there was no availability of electrons at the selenium center for transfer from cyclic diselenide to the BODIPY core. Hence, the photoinduced electron transfer process was blocked.³⁹ This was further confirmed by the analysis of frontier molecular orbitals (FMOs) of the probe and its oxidized form (Figure 7). The highest occupied molecular orbital (HOMO) of the probe revealed higher electron density on the naphthalene core, while in the low unoccupied molecular orbital (LUMO), electrons were localized at the BODIPY core. Migration of electrons from the HOMO to LUMO suggested the quenching state of the probe.⁴² Upon oxidation, FMOs suggested retention of electron density on the HOMO and LUMO of the BODIPY core; thereby, the probe showed magnificent increase in fluorescence intensity.

Cytotoxicity Assay. To check the utility of the probe for selective detection of superoxide in cells, the MCF-7 mammalian breast cancer cell line was chosen. First, the MTT cell viability assay was carried out to understand the cytotoxicity of the probe. It was performed with a range of probe concentrations (1, 5, 10, 20, and 100 μM). The probe showed good permeability and no cytotoxicity was observed.
This study confirmed that the probe can be internalized without cellular damage (Figure 8).

**Cell Imaging.** To determine the selectivity of probe 3 inside living cells, treatment of the MCF-7 cell line (human breast cancer cells) with the probe was carried out. MCF-7 cells were incubated with phorbol-12-myristate-13-acetate (PMA) which produces superoxide inside the cells. As a control, only MCF-7 cells were observed under blue laser of fluorescence microscopy, which did not show any native fluorescence inside the cells. In the PMA control, no remarkable fluorescence inside the cells was observed. MCF-7 cells were seeded in a 24-well plate (∼1 × 10⁴ cells per well) in Dulbecco’s modified Eagle’s medium (DMEM) on glass coverslips. The cells were treated with probe 3 dissolved in DMSO and PMA (1 μM) was added to induce ROS production. The cells were observed under a Leica DMi8 microscope in the DIC and fluorescence mode, and a strong green fluorescence signal was observed inside the cells (Figure 9). These results confirmed the ability of the probe for selective detection of superoxide inside the cells.

### CONCLUSIONS

The first cyclic diselenide-containing BODIPY-based probe was synthesized and characterized. The molecule was found to be a potential small molecular probe for selective and sensitive (nine-fold increase in fluorescence intensity) detection of superoxide inside the cells.
superoxide. The detection limit of the probe was found to be 0.924 μM and the quantum yield of the oxidized product was calculated as 17 percent. The plausible mechanism of the turn-on event has been proposed and supported with spectroscopic data and quantum chemical calculations. The quantum chemical calculations indicated that the proximal Se gets oxidized in comparison with distal Se. Finally, the utility of the probe for selective detection of superoxide in mammalian breast cancer cell lines was explored, which suggested that the probe can be used for the in vivo detection of superoxide. This study will explore the possibilities of using organoselenium molecules in medicine for monitoring of cancer.

■ EXPERIMENTAL SECTION

Materials. All chemicals used for synthesis and photophysical study (analytical grade) were procured from commercial sources and used without further purification. Column chromatography was performed on silica gel (60–120). The 1H NMR spectra were recorded in CDCl3 with Bruker 300 and 400 MHz instruments. The frequency for the 13C nucleus was 75.47 MHz for the 300 MHz instrument. The frequency for the 77Se nucleus was 76.34 MHz for the 400 MHz instrument. Tetramethylsilane was used as an internal standard for 1H NMR and 13C NMR spectroscopy and Ph2Se2 was used as the external standard for 77Se NMR spectroscopy. All ROSs were purchased from Sigma-Aldrich and used without further purification. Mass spectra were obtained using Maxis Impact (BRUKER). UV spectra were recorded with Shimadzu UV2450. The fluorescence spectra were obtained using Shimadzu RF5301PC. The fluorescence microscopic images were obtained using Leica DMi8.

Synthesis of Compound 2. The reaction mixture of naphthalene-1,8-diselenide-2-carboxaldehyde 1 (0.400 g, 1.280 mmol) and 2.2 mL of pyrrole (2.146 g, 32 mmol) with a catalytic amount of TFA was stirred for 12 h under a nitrogen atmosphere. The reaction was monitored by thin-layer chromatography (TLC) and the crude product was purified by column chromatography using DCM/pet ether (1:1). Yield of 2 was 0.433 g (79%). Spectroscopic data for compound 2: 1H NMR (300 MHz, CDCl3): δ 8.01 (bs, 2H), 7.47-7.41 (m, 2H), 7.34 (d, 1H), 7.23-7.18 (m, 1H), 7.03 (d, 1H), 6.68 (q, 2H), 6.16 (q, 2H), 6.04 (bs, 2H), 5.41 (s, 1H). 13C NMR (75.4 MHz, CDCl3): δ 141.0, 140.2, 138.0, 136.6, 135.9, 131.4, 129.3, 128.6, 127.2, 125.0, 123.9, 123.2, 121.8, 118.7, 118.3, 117.6, 116.8, 109.0, 108.7, 44.6. 77Se NMR (76.3 MHz, CDCl3): δ 0.924 μM and the quantum yield of the oxidized product was calculated as 17 percent. The plausible mechanism of the turn-on event has been proposed and supported with spectroscopic data and quantum chemical calculations. The quantum chemical calculations indicated that the proximal Se gets oxidized in comparison with distal Se. Finally, the utility of the probe for selective detection of superoxide in mammalian breast cancer cell lines was explored, which suggested that the probe can be used for the in vivo detection of superoxide. This study will explore the possibilities of using organoselenium molecules in medicine for monitoring of cancer.

Figure 7. HOMO and LUMO of (a) probe 3 and (b) oxidized form of probe 3 in gas phase calculations (B3LYP method and 6-311 G * (d,p) basis set).

Figure 8. Cell viability assay of MCF-7 cells preincubated for 1 h with probe 3 (1, 5, 10, 20, 50, and 100 μM concentrations).

Figure 9. Fluorescence microscopic images of breast cancer MCF-7 cells. Bright-field and fluorescence images of (a) MCF-7 cells, (b) MCF-7 cells with PMA, (c) MCF-7 cells incubated with probe 3, and (d) MCF-7 cells incubated with probe 3 and PMA (PMA generates superoxide in the cell).
CDCl3): δ 411, 434. ESI–MS: calcld for C18H14N2Se2, 429.95; found m/z, 428.9410 (M − H)⁺.

**Synthesis of Probe 3.** Naphthalene-1,8-diselenenide-2-dipryromethane 2 (0.500 g, 1.168 mmol) and DDQ (0.265 g, 1.168 mmol) were stirred in dry DCM. The reaction was continued until the complete consumption of dipryromethane. The progress of the reaction was monitored by TLC. After 1 h of stirring, 1.6 mL of triethylamine (1.180 g, 11.680 mmol) was added followed by addition of 1.4 mL of BF3·OEt2 (1.657 g, 11.680 mmol). The reaction was stirred for 3 h. The reaction mixture was concentrated under vacuum and the crude product was purified by column chromatography in DCM:pet ether (1:1). The yield of probe 3 was 0.148 g (26%) (mp 202 °C). Spectroscopic Data for probe 3: 1H NMR (300 MHz, CDCl3): δ 7.96 (bs, 2H), 7.62 (d, 1H), 7.56 (d, 1H), 7.44 (d, 1H), 7.38-7.35 (t, 1H), 7.22 (d, 1H), 6.99 (d, 2H), 6.50 (d, 2H). 13C NMR (76.3 MHz, CDCl3): δ 145.5, 143.6, 141.8, 138.0, 137.8, 133.6, 131.1, 129.1, 128.6, 127.5, 124.5, 123.6, 122.4, 118.9. 77Se NMR (76.3 MHz, CDCl3): δ 449 (Se1), 410 (Se2). ESI–MS: calcld for C18H14BF2N2Se2 + Na, 498.92; found m/z, 498.9219 (M + Na)⁺.

**Photophysical Study.** The spectrophotometric titration of probe 3 was measured in DMSO–water, as the concentration of stock solution was 20 μM. All (0.1 M) ROSs (O2•−, H2O2, BuOOH, OCl−, ClO•, OH•, and BuO•) were prepared in distilled water. Spectral data were recorded after 15 min of incubation of the probe with ROSs. The excitation wavelength of the probe was 506 nm.

**Screening and Sensitivity of Probe 3 with ROSs.** Screening of probe 3 was carried out with various ROSs (O2•−, H2O2, BuOOH, OCl−, OH•, and BuO•). In this experiment, 3 mL of probe 3 (20 μM) with ROSs (33.33 equiv) in water was taken. The photometric titration experiment was performed by adding probe 3 (20 μM) with increasing concentration of KO2 (0-666.67 μM) in each vial. UV–vis and fluorescence measurements were taken after incubation of 15 min.

**Interference Study.** This experiment was performed with 3 mL of probe 3 (20 μM) and 666.67 μM KO2 (0.1 M) in every vial which previously contained 666.67 μM of other ROSs, for example, H2O2, BuOOH, OCl−, OH•, and BuO• (0.1 M), in water. Fluorescence readings were recorded after incubation of 15 min.

**Determination of the Detection Limit.** The detection limit was calculated on the basis of the increasing concentration curve of fluorescence. The fluorescence emission spectrum of probe 3 was taken three times and the standard deviation of the blank measurement was calculated. To calculate the slope, the fluorescence intensity at 521 nm was plotted as a concentration of KO2. The detection limit was calculated using the following equation

\[ \text{detection limit} = 3\sigma / k \]

where σ stands for standard deviation of 10 blank measurements and k is the slope between the fluorescence intensity and concentration of KO2.

**Computational Study.** The quantum chemical calculations were performed based on DFT using Gaussian 09 software at the B3LYP/6-311G(d,p) level of theory. The structure of the probe and its oxidized form were optimized in the gas phase. The NBO analysis and HOMO–LUMO calculations were carried out in Gaussian 09 software.

**Cell Viability Assay.** MCF-7 human breast cancer cells were seeded at a density of ~1 × 10⁴ cells in a 96-well cell culture plate and incubated overnight. The toxicity of the probe was determined using various concentrations (1, 5, 10, 20, 50, and 100 μM). DMSO and cell-free media were used as controls. The plate was incubated for 1 h at 37 °C and MTT dye (10 μL, 5 mg/mL) was added to each well and further incubated for 4 h. Finally, after addition of DMSO-SDS mixture (100 μL), the cells were incubated overnight. The absorbance was measured using a SpectraMax M2 plated reader at 560 nm with background scattering at 690 nm.

**Cell Culture and Fluorescence Imaging.** MCF-7 human breast cancer cells were seeded in a 24-well plate at a density of 1 × 10⁴ cells well in DMEM on glass coverslips and incubated overnight at 37 °C in 5% CO2. Later, MCF-7 cells were treated with the probe (100 μM) dissolved in DMSO followed by the addition of PMA (1 μM) to induce ROS production. Finally, the cells were incubated for 1 h and fixed using 4% para-formaldehyde for 20 min. DABCO (1,4-diazobicyclo-2,2,2-octane) medium was used to mount the coverslips. Fluorescent images were obtained on a Leica DMI8 microscope in the DIC and fluorescence mode.

**ASSOCIATED CONTENT**

► Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02074.

Schemes, experimental data, NMR, mass, absorption spectra, fluorescence spectra, and biological details (PDF)

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**Notes**

The authors declare no competing financial interest.

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