Enhanced Fluorescence Turn-on Imaging of Hypochlorous Acid in Living Immune and Cancer Cells

Sandip V. Mulay,[a, b] Minsuk Choi,[c] Yoon Jeong Jang,[b] Youngsam Kim,[a, b] Sangyong Jon,[c] and David G. Churchill*[a, b]

Abstract: Two closely related phenyl selenyl based boron-dipyromethene (BODIPY) turn-on fluorescent probes for the detection of hypochlorous acid (HOCl) were synthesized for studies in chemical biology; emission intensity is modulated by a photoinduced electron-transfer (PET) process. Probe 2 intrinsically shows a negligible background signal; however, after reaction with HOCl, chemical oxidation of selenium forecloses the PET process, which evokes a significant increase in fluorescence intensity. The fluorescence intensity of probes 1 and 2 with HOCl involves an ~18 and ~50-fold enhancement compared with the respective responses from other reactive oxygen/nitrogen species (ROS/RNS) and low detection limits (30.9 nM for 1 and 4.5 nM for 2). Both probes show a very fast response with HOCl; emission intensity reached a maximum within 1 s. These probes show high selectivity for HOCl, as confirmed by confocal microscopy imaging when testing with RAW264.7 and MCF-7 cells.

Introduction

Biosensing with small molecular probes enables for monitoring of biochemical and biomolecular processes in organisms and allows for potential applications in various fields from fundamental biological research to clinical diagnostics.[1] Organoselenium-based molecular probes show significant promise in helping to reliably detect biological analytes such as reactive oxygen/nitrogen species (ROS/RNS) and biothiols in a qualitative and quantitative way in living biological systems.[2] These biological species are thought to play an important role for normal physiological processes; an overproduction or deficiency of them could be responsible for cancer and neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease. Among ROS, hypochlorous acid (HOCl) is a potential antimicrobial agent that plays a significant role in the human immuno-defense system.[3, 4] Endogenous hypochlorous acid/hypochlorite is generated from hydrogen peroxide (H2O2) and chloride ions (Cl–) in a myeloperoxidase (MPO)-catalysed reaction.[4, 5] However, abnormal HOCl levels in living organisms can lead to diseases such as atherosclerosis,[6] rheumatoid arthritis,[7] lung injury,[8] cancer,[9] neurodegenerative disorders,[10] kidney[11] and cardiovascular disease.[12]

Fluorescence techniques are used extensively for biological cellular imaging because they are highly sensitive, rapid in response and non-invasive.[13] Over the past two decades, extensive effort has been invested in the development of selective fluorescent probes for the imaging of cellular ROS.[14] To measure the exact concentration of HOCl in living systems, the development of fluorescent probes with excellent selectivity, high sensitivity and real-time detection are required. Many strategies were used to detect HOCl based on its high reactivity, such as oxidation of S, Se and Te to the corresponding chalcogenide oxide,[15] oxidation/hydrolysis of dibenzoylhydrazine into dibenzoyl diimide[16] and oxidation of hydroquinone to quinone.[17] In addition, oxime,[18] thiol compounds (thioether,[19a–d] internal thiostere[19w–y] and thiosemicarbazide[19z]) and other groups[20] connected to probe-reporting moieties were used in the detection of HOCl. Although there are a number of fluorescent probes reported to-date for the detection of HOCl, very few among them can be used for real-time detection in biological investigations because of basic science issues such as low selectivity, sensitivity, poor photostability and/or chemostability and low probe molecular weight. Recently our group has made a new entry with the report of novel heterocyclic annulated boron-dipyrromethene (BODIPY) systems (selenium and tellurium atoms) help form a six-membered heterocycle between the 1-position of BODIPY and ortho position of the meso phenyl ring) for the detection of HOCl.[15h] To the best of our knowledge, there are only two reports in which selenium is directly attached to the fluorophore and that are used for selective detection of HOCl[15e, h].
Herein, we present an effort at optimizing the turn-on signal for this system. Two related “turn-on” selective HOCl responsive fluorescent probes in which a phenyl selenide moiety is incorporated at the 2-position of the meso unsubstituted BODIPY system is able to quench the fluorescence of the fluorophore by photoinduced electron transfer (PET). It is well known that substitution with heavy atoms at the 2- and 6-positions of the BODIPY results in decreased fluorescence quantum yields. Based on this background, we believed that introducing chlorine at the 6-position, in addition to the phenyl selenium group would help to further decrease the fluorescence quantum yield. After oxidation of the selenide, which brings the molecule to a selenoxide, both probes become fluorescent (Figure 1). Importantly, probes 1 and 2 can both be applied to detect HOCl, selectively (in RAW264.7 and MCF-7 cells) but the difference in background emission is an important basic science issue that is underscored in this paper.

Results and Discussion

The syntheses of the 2-substituted phenylselenyl BODIPY probes 1 and 2 is outlined in Scheme 1. Both probes were synthesized from the known BODIPY compound (3) in excellent yield. Probe 1 was achieved by treatment of 3 with 1.0 equivalent of phenylselenyl chloride. The monochlorination of 3 with N-chlorosuccinimide (NCS) was effected in hexafluoro-2-propanol (HFIP) to give 4, and the subsequent reaction with phenylselenyl chloride effected the formation of the probe 2. The structures of 1 and 2 were characterized by spectroscopic techniques ( multinuclear NMR spectral data, mass spectrometry; Figure S1–S17, Supporting Information).

The UV/vis and fluorescent spectroscopic properties of probes are shown in Figure 2 and Figure S18 (Supporting Information). The analysis of UV/vis absorption spectra (5 μM solution of probes 1 and 2 in EtOH/10 mM PBS pH 7.4, 1:2 v/v) show absorbance maxima at 512 and 526 nm, respectively. After addition of NaOCl (5.0 equiv) to these probe solutions, the absorbance peaks shifted towards lower wavelengths (492 and 511 nm, respectively). Based on our hypothesis, (the 2-phenylselenyl group quenches the fluorescence of BODIPY by a PET process), probes 1 and 2 show an extremely low background signal. In the case of probe 2, the added effect of the chloride group at the 6-position makes the starting probe less fluorescent at the outset.

To check the selectivity of these probes (1 and 2) with ROS/RNS, screening experiments of probes with ROS/RNS (e.g., NaOCl, H2O2, tBuOOH, O2•-, ‘OH, tBuOCl, NO•, and ONOO•) under physiological conditions (EtOH/10 mM PBS pH 7.4, 1/2, v/v) in 2.0 and 5.0 mM solutions of the probes were performed, respectively. As displayed in Figure 2, 1 and 2 show excellent selectivity with NaOCl (5.0 equiv), whereas there is no fluorescence intensity with other ROS/RNS even when competing analytes were present at much higher concentrations (100 equiv). After addition of NaOCl to the probe solution, clear emission maxima were observed at 507 and 526 nm. Through the presence of the chloride group, enhancements of fluorescence intensity were found to be ~50-fold for 2, whereas it was ~18-fold for compound 1.

Titration of probes 1 and 2 with NaOCl was then carried out. The spectral determinations of these probes with various concentrations of NaOCl (0 to 6 μM) and (0 to 20 μM) were recorded for 1 (2 μM) and 2 (5 μM), respectively. The fluorescence titration experiments supported that the emission intensities of these two BODIPY-derived probes gradually increased from 0 to 3.0, and 0 to 4.0 equivalents, upon addition of NaOCl, and
was found to be linearly proportional to the concentration of NaOCl (Figure 3, inset shows triplicate trials). The emission intensity reached saturation when ~3.0 and ~4.0 equivalents of NaOCl were added to a solution of 1 and 2, respectively. According to the titration analysis, the detection limits (3σ/s, in which σ is the standard deviation of 10 blank measurements, s is the slope of the linear equation) were determined to be 30.9 nM for 1 and 4.5 nM for 2, lower than that for the reported probes in which a phenyl selenyl was directly attached to the fluorophore (Figures S19–20, Supporting Information).

Incorporation of the phenyl selenyl group at the 2-position in 1 and the chlorine at the 6-position in 2, allows the fluorescent quantum yield (Φ_F) to be dramatically increased to 0.41 for 1 and 0.57 for 2.

To study the reaction rate, time-dependent fluorescent analysis was carried out with 2.0 and 4.0 equivalents of NaOCl for probes 1 and 2, respectively. These probes showed a very fast response with NaOCl and reached a maximum fluorescent intensity within a few seconds (Figure 4). These time-dependent experiments indicate that the reaction between 1 and 2 with NaOCl (oxidation of selenium to selenoxide) is extremely fast, which could be used for real-time imaging of cellular HOCl.

To demonstrate the effects of pH on 1 and 2, the fluorescence emission intensity of both probes were measured under a wide range of pH values (Figure S21, Supporting Information). These pH studies show that pH does not affect the fluorescence of 1 and 2. Furthermore, upon addition of NaOCl (1.0 equivalents for 1 and 2.0 equiv for 2) to the 2.0 and 5.0 μM solutions of various pH values, both probes showed a strong response in acidic conditions, whereas under basic conditions, fluorescence intensity decreased dramatically. These results indicated that 1 and 2 are stable within the overall range of pH values tested, and performs satisfactorily under acidic and physiological conditions. The concentration of HOCl in cancer cell...
cells is greater compared to normal cells; considering this, the current probes could be useful to detect the concentration of HOCl in such cells (vide infra).

It was previously reported that HOCl oxidizes selenium to selenoxide rapidly in aqueous media, as well as in organic solvents. To confirm the sensing mechanism of NaOCl, based on this hypothesis (oxidation of selenium to selenoxide), the $^1$H and $^{77}$Se NMR spectra of probe 2 before and after treatment with NaOCl were recorded as the direct product of reaction (without purification) and the $^1$H NMR spectrum showed a small amount of unreacted probe 2. The $^{77}$Se NMR spectrum, after addition of NaOCl, shows a sharp peak at $\delta = 208.1$ ppm (2) which disappeared, leading to a new concomitant downfield peak at $\delta = 821.0$ ppm (Figure S22, Supporting Information). Also, the $^1$H NMR spectrum shows a downfield shifting of the phenyl protons from $\delta = 7.15$–7.21 ppm to 7.48–7.53 and 7.67–7.70 ppm (Figure S23, Supporting Information). HRMS data of 1 and 2 with NaOCl (1.5 equiv) were recorded. The molecular formula of the oxidized probe (1) was $\text{C}_{19}\text{H}_{18}\text{BF}_3\text{N}_2\text{OSeNa}$ (m/z: calcd: 443.0321; found: 443.0610, Figure S24, Supporting Information), and for 2 was $\text{C}_{30}\text{H}_{20}\text{BClF}_3\text{N}_2\text{OSeNa}$ (m/z: calcd: 477.0232; found: 477.0212, Figure S25, Supporting Information). These NMR and HRMS results strongly support that selenoxide formation has taken place (Scheme 2).

In order to understand the redox cycling capacity of selenium in probes 1 and 2, the solutions of the probes, oxidised with hypochlorite, were treated with glutathione, $N$-acetyl-$\text{L}$-cysteine, homocysteine and $\text{L}$-cysteine, well-known reducing biothiols, to determine whether the selenium oxide species Se=O reverts to its original reduced divalent state. Figure S26 (Supporting Information) shows the reaction of the oxidised products with biothiols. The results obtained showed a remarkable decrease in fluorescence intensity with cysteine and glutathione. This result indicates the reversibility of the probes, which is an important attribute for further probe modification that can lead to new generation probes that monitor the dynamic variations of hypochlorite in living systems.

To investigate the photomechanism, DFT and time-dependent DFT (TD-DFT) calculations were utilized. Even though the optimized structures of 1 and 2 show that the phenylselenium and the BODIPY moieties are in a perpendicular arrangement, electron transfer between selenium and the BODIPY core would be efficient due to the directly attached selenium on the 2-position of the BODIPY core. The largest intense transition of 2 from HOMO−1 to LUMO (configuration interaction (CI) = 61.5%), including an oscillator strength ($f$) of 0.4984, is defined as the dominant transition. In the case of oxidized system 2, it showed the largest intense transition from HOMO to LUMO (CI = 51.9%) with an oscillator strength of $f = 0.4086$ (Table S1, Supporting Information). Based on this data of the electron transitions, an electronic distribution of states was considered to explain the photomechanism. The electronic distribution of the HOMO and HOMO−1 of 2 is exhibited on the BODIPY core corresponding to fluorescence enhancement. Furthermore, 1 and oxidized 1 showed very similar results to 2 and oxidized 2. The largest intense transition of 1 is from the HOMO−1 to LUMO state (CI = 59.3%) involving an oscillator strength of $f = 0.6524$ (Table S1, Supporting Information). The results of the oxidized form of 1, its transition from HOMO to LUMO (CI = 73.3%) had an oscillator strength $f = 0.5943$. The electronic distributions were also obtained with similar features. From these results, fluorescence enhancement arises from blocking the PET process, by oxidation of selenium to selenoxide in both probes (1 and 2).

Finally, biological experiments were carried out to support our results that strong green fluorescence is observed upon the reaction of hypochlorite and the selected probe, as shown by the fluorescence and confocal microscopy images of the breast cancer cell line MCF-7 (Figures S27–28, Supporting Information).
mation) and murine live macrophage cell line RAW264.7 (Figure 5). MCF-7 cells were treated with 1 or 2 for 30 min, followed by incubation with 10 equivalents of sodium hypochlorite (NaOCl) for 30 min. Whereas the cells treated with probe only show a very week green fluorescence (Figure S27b for 1 and Figure S28b for 2, Supporting Information), the NaOCl incubated cells show a much stronger green fluorescence (Figure S27c for 1 and Figure S28c for 2, Supporting Information). To confirm the ability of probes 1 and 2 to detect the actual induced ROS in cells, additional experiments were then performed. The cells were incubated with hydrogen peroxide (H₂O₂) for 30 min to induce ROS production within cells; then, 1 and 2 were treated for 30 min (Figure S27d for 1 and Figure S28d for 2, Supporting Information). In addition, another experiment in murine live macrophage was performed in the RAW264.7 cell line under the stimulation of lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) that was able to produce endogenous HOCl concentration. For this experiment, probe 2 was selected due its to low limit of detection (LOD) and background fluorescence. To confirm the ability of 2 for the detection of induced HOCl in live cells, RAW264.7 cells were pre-stimulated with LPS (1 μg mL⁻¹) and PMA, (1 μg mL⁻¹) and then treated with 2. As shown in Figure 5d, this combination showed a much stronger intracellular green fluorescence than when the cells were treated with LPS/PMA or 2 only (Figure 5b and 5c), which showed nonfluorescence.

From these results, 1 and 2 demonstrated cell membrane permeability and detection of hypochlorous acid in live cells.

Furthermore, cell viability testing was performed to confirm biocompatibility of 2. The RAW264.7 and MCF-7 cells were pre-incubated with various concentrations of 2 (1, 5, 10, 20, 50 μM). WST-1 cell proliferation assays were carried out after cells were pre-incubated in 2 for 1 or 2 h. As shown in Figure 6 and Figure S29 (Supporting Information) (MCF-7 cell viability results), there is no substantial decrease in cell viability when using 2. These results reveal no significant cytotoxicity of 2 at concentrations of up to 50 μM and help give a fuller understanding for their potential biological applications.

Conclusion

Herein, we have synthesized novel turn-on phenyl selenide-based BODIPY probes, bearing a substituent difference, for the selective detection of hypochlorous acid over other ROS/RNS. The probes display excellent selectivity, sensitivity, as well as short time responses (seconds) for hypochlorite. These probes displayed remarkable fluorescence enhancement (~18 and ~50-fold), and low detection limits (30.9 and 4.5 nm) for 1 and 2, respectively. In addition, these probes showed a turn-on response with hypochlorous acid in living cells (RAW264.7 and MCF-7), confirmed by fluorescence and confocal microscopy imaging. Our results suggest that incorporating a Cl at the 6-position in the BODIPY framework (probe 2) helps further decrease background fluorescence, lowers the detection limit, and involves high-yielding synthetic steps, compared to probe 1, which could serve as a better probe for live cell imaging.
Experimental Section

Materials and Instrumentation

All materials used herein were used as received from commercial suppliers (Aldrich, Tokyo Chemical Industry) and were used without further purification. TLC was performed on DC Kieselgel 60 F254 silica gel plates. The spots were visualized by staining with KMnO4 or by using a bench-top UV lamp. Column chromatographic purifications were carried out on silica gel (230–400 mesh ASTM).

Synthesis of probe 1

To a stirred solution of 3 (50 mg, 0.20 mmol) in dry CH2Cl2 (10 mL), PhSeCl (38 mg, 0.20 mmol, 1.0 equiv) was added. The reaction mixture was stirred at room temperature for 30 min (reaction monitored by TLC). Then, the solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography using hexane and CH2Cl2 (3:1) to afford 1 as a red solid (55 mg, 68%). 1H NMR (600 MHz, CDCl3): δ = 2.28 (s, 3H, H3), 2.29 (s, 3H, H3), 2.57 (s, 3H, H3), 2.60 (s, 3H, H3), 6.12 (s, 1H, H9), 7.14 (s, 1H, H1), 7.13–7.21 ppm (m, 5H, H10, H11, H2); 13C NMR (100 MHz, CDCl3): δ = 11.5 (C1, C11), 11.9 (C11), 14.1 (C11), 15.0 (C11), 115.1 (C1), 120.3 (C1), 120.7 (C1), 126.0 (C1), 128.8 (C1), 129.4 (C1), 132.3 (C1), 132.7 (C1), 134.5 (C1), 143.3 (C1), 145.1 (C1), 158.9 (C1), 159.4 ppm (C1); 19F NMR (76.3 MHz, CDCl3): δ = −204.7 ppm; 19B NMR (128.4 MHz, CDCl3): δ = −0.73 ppm (t, JBF = 33.4 Hz); 27Se NMR (76.3 MHz, CDCl3): δ = −146.3 ppm (q, JSe = 33.2 Hz); HRMS (ESI): m/z: calcd for C19H22BF2SeNa+: 427.0672; found: 427.0677 [M+Na]+.

Synthesis of probe 2

To a stirred solution of 4 (100 mg, 0.35 mmol) in dry CH2Cl2 (10 mL) was added PhSeCl (68 mg, 0.35 mmol, 1.0 equiv). The reaction mixture was stirred at room temperature for 30 min (reaction monitored by TLC). Then, the solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography using hexane and CH2Cl2 (3:1) to afford 2 as a red solid (141 mg, 91%). 1H NMR (400 MHz, CDCl3): δ = 2.21 (s, 3H, H3), 2.27 (s, 3H, H3), 2.56 (s, 3H, H3), 2.61 (s, 3H, H3), 7.11 (s, 1H, H9), 7.14–7.23 ppm (m, 5H, H1, H10, H11, H12); 13C NMR (100 MHz, CDCl3): δ = 9.7 (C1, C2), 12.0 (C3, C4), 12.5 (C2, C3), 14.3 (C2), 116.4 (C3), 121.2 (C2), 121.4 (C3), 126.3 (C3), 129.1 (C1, C11), 129.4 (C1), 131.2 (C1), 132.2 (C1), 133.0 (C1), 136.7 (C1), 147.0 (C1), 153.5 (C1), 161.1 ppm (C1); 77Se NMR (76.3 MHz, CDCl3): δ = −208.1 ppm; 19B NMR (128.4 MHz, CDCl3): δ = 0.52 ppm (t, JBF = 33.2 Hz); 27F NMR (376.5 MHz, CDCl3): δ = −146.1 ppm (q, JSe = 32.2 Hz); HRMS (ESI): m/z: calcd for C19H22BF2ClSeNa+: 461.0283; found: 461.0270 [M+Na]+.

Cell culture and fluorescence imaging

RAW 264.7 murine macrophages and MCF-7 human breast cancer cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) and Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum ( Gibco) and 1% penicillin/streptomycin (100 U/mL). Gibco). The RAW 264.7 and MCF-7 cells seeded at a density of 1 × 104 cells on sterilized 18 mmØ cover-slips in a 12-well cell culture plate and was maintained at 37 °C in a 5% CO2 humidified incubator. In ROS-inducing experiments, RAW 264.7 cells at 50% confluence were stimulated with LPS (1 µg mL−1) and PMA (1 µg mL−1) in serum-free DMEM for the durations indicated. Fluorescent images were acquired on a ZEISS LSM 780 laser scanning confocal microscope and Nikon eclipse 80i fluorescence microscope were used. The excitation wavelength was 405 and 488 nm; the detection wavelength was 410–498 and 490–553 nm, respectively. Prior to imaging, the culture medium was removed and the cells were washed with D-PBS three times.

Cell viability assays

The cell viability of RAW 264.7 and MCF-7 cells in the presence of the probes was determined by a WST-1 assay kit (Roche) through the use of the manufacturer’s protocols. RAW 264.7 and MCF-7 cells were seeded with 1 × 105 cells/100 µL in a 96-well cell culture plate (n = 5). The cells were pre-treated with the probe at various concentrations (1, 5, 10, 20 and 50 µM) for 30 min. A cell proliferation reagent WST-1 was added (10 µL) to each well at 2 h at 37 °C in a 5% CO2 humidified incubator. Absorbances were measured using a VersaMax ELISA microplate reader at 450 nm and the reference wavelength was 590 nm.

DFT calculations

Optimized structures and HOMO–LUMO distributions of the states were estimated using DFT/TDDFT calculations using the Gaussian 09 program. The B3LYP functional with a 6–31g* basis set was used and the 6–311g* basis set was used for Se only. All calculations were performed in the gas phase. CI coefficients were obtained by the square of the values.
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