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Fluorescent Probes

A 3,7-Dihydroxyphenoxazine-based Fluorescent Probe for Selective Detection of Intracellular Hydrogen Peroxide

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Abstract: A novel *N*-borylbenzyloxycarbonyl-3,7-dihydroxyphenoxazine fluorescent probe (NBCD) for detecting H_2O_2 in living cells is described. The probe could achieve high selectivity for detecting H_2O_2 over other biological reactive oxygen species (ROS). In addition, upon addition of H_2O_2, NBCD exhibited color change from colorless to pink, which makes it a "naked-eye" probe for H_2O_2 detection. NBCD could not only be used to detect enzymatically generated H_2O_2 but also to detect H_2O_2 in living systems by using fluorescence spectroscopy, with a detection limit of 2 μM. Importantly, NBCD enabled the visualization of epidermal growth factor (EGF)-stimulated H_2O_2 generation inside the cells.

Hydrogen peroxide (H_2O_2), an important reactive oxygen species (ROS), has been correlated with various functions in the regulation of many physiological processes.[1] For a long time, H_2O_2 has been considered as a harmful metabolic product and an important molecule involved in pathophysiological pathways associated with various diseases.[2] Recently, H_2O_2 has been found to act as a signaling molecule in many biological processes such as cell proliferation, migration, and differentiation.[3] Moderate levels of H_2O_2 play a crucial role in physiology, aging, and various diseases in living organisms.[4] Overproduction of H_2O_2 at the cellular level has been linked to cancer, neurodegenerative diseases, DNA damage, mutation, and genetic instability.[5] Thus, the significant physiological importance of H_2O_2 has led to numerous studies aimed at developing high-efficiency fluorescent probes for detecting H_2O_2 in living systems.

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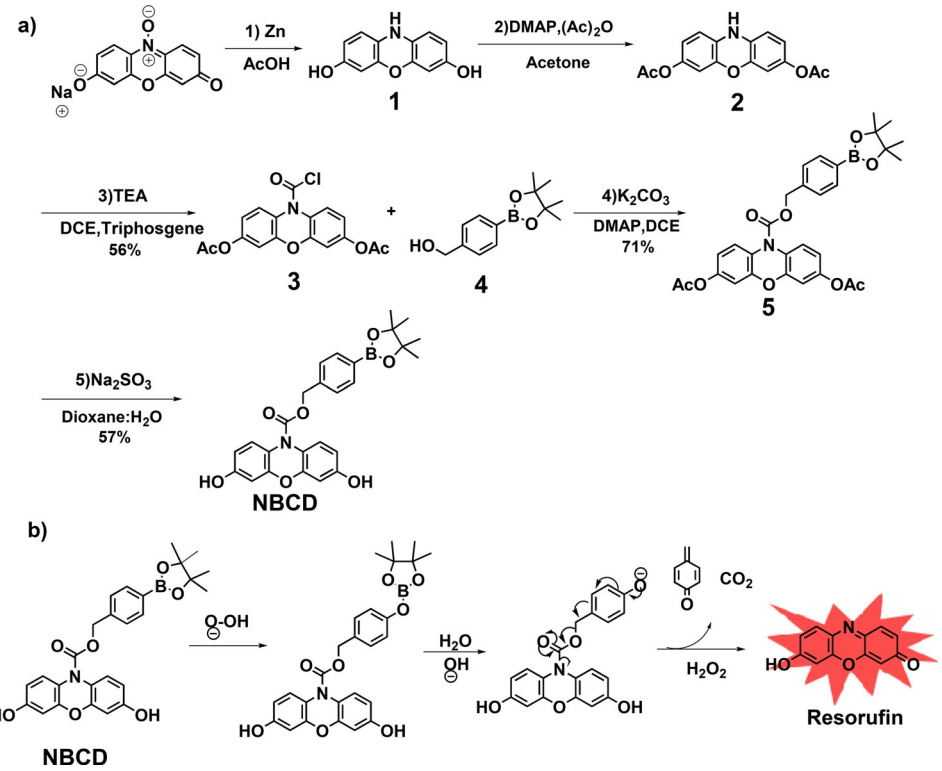
Several types of probes for the detection of H_2O_2 have been reported, such as fluorescein,[6] coumarin,[7] and luciferin[8]. As one of the important fluorescent reporters, resorufin, a highly fluorescent molecule at pH 8 ($\phi_F = 0.75$),[9] has excitation/emission maxima of ~570/585 nm, and there is much less interference from autofluorescence in most biological samples. N-Protected 3,7-dihydroxyphenoxazine[10] and O-alkyl resorufin derivatives[1c, 11] have been described as chemical probes for enzymes in the literature. As a precursor of a fluorescent probe, the former has an advantage of lower fluorescence background, which is less than that of the latter because of an interrupted conjugated system. A combination of *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) and horseradish peroxidase (HRP) was used in tests of enzymes that produce H_2O_2.[12] However, Amplex Red could only detect H_2O_2 indirectly because it could not be oxidized by H_2O_2 itself.[10] Hitomi and co-workers presented a metal-based fluorescent probe for H_2O_2, named MBFh1, containing an iron complex and a non-fluorescent 3,7-dihydroxyphenoxazine derivative.[10] Unfortunately, MBFh1 is decomposed readily under cell culture conditions.[1c] The application of these two resorufin-based probes is limited to only cell-free systems, even though the detection of H_2O_2 in a cellular system is of great importance. To our knowledge, an *N*-protected 3,7-dihydroxyphenoxazine-derived chemical probe has not yet been described for the selective detection of H_2O_2 in living cells. Therefore, we aimed to design a novel chemical probe with good stability and high selectivity for the detection of H_2O_2 in living cells, taking advantage of the attributes of unique fluorescent properties of resorufin. Herein, we report a novel *N*-protected 3,7-dihydroxyphenoxazine probe (NBCD) comprising a 3,7-dihydroxyphenoxazine as the reporter and a borate-based carbamate leaving group[8a, 13] as the H_2O_2 response site. This probe features a good stability and high selectivity, and detection of H_2O_2 was achieved not only in a cell-free system but also in living cells.

The synthesis of the designed probe NBCD was initiated from resazurin sodium salt (Scheme 1a and Scheme S1, Supporting Information). Reduction of resazurin sodium salt with zinc powder and glacial acetic acid afforded 3,7-dihydroxyphenoxazine (**1**). It reacted with acetic anhydride and *N*,*N*-dimethyl-4-aminopyridine (DMAP) to generate 3,7-diacetoxyphenoxazine (**2**),[10] and the subsequent treatment with triphosgene yielded *N*-carbonyl-3,7-diacetoxyphenoxazine (**3**). Further reaction of **3** with borylbenzyl alcohol (**4**) followed by hydrolysis led to the desired *N*-borylbenzyloxycarbonyl-3,7-dihydroxyphenoxazine (NBCD). A possible mechanism of resorufin genera-



Scheme 1. a) Synthesis and activation of NBCD. b) Possible mechanism of resorufin generation by the reaction of NBCD with H_2O_2.

tion by the reaction of NBCD with H_2O_2 is proposed in Scheme 1 b.

NBCD was expected to be able to display red fluorescence in the presence of H_2O_2 because of the release of resorufin, allowing for its use as a probe for detecting H_2O_2. Experimentally, upon treatment with H_2O_2, NBCD displayed a remarkable red fluorescence. Subsequently, the UV/Vis spectroscopic properties of NBCD were assessed under in vitro conditions (20 mM HEPES buffer containing 0.5% DMSO, pH 7.3). Upon addition of H_2O_2 (20 equiv) to a solution of NBCD, a gradual increase in the absorption peak in the UV region (450–610 nm) over 45 min was observed, which suggested the generation of a new chemical entity (Figure 1 a). Moreover, there was a good linearity between the absorption intensity and time in the range of 0 to 45 min (Figure 1 a, inset). NBCD exhibited no absorption in the UV region (450–610 nm) in the absence of H_2O_2, and a concentration-dependent absorption response of NBCD was observed at 30 min after the addition of H_2O_2 (Figure S1, Supporting Information). The absorption intensity of NBCD increased significantly as a function of H_2O_2 concentration. Under pseudo-first-order conditions (5 μM NBCD, 5 mM H_2O_2), the observed rate constant for H_2O_2 is $k_{obs} = 2.266 \times 10^{-3}\ s^{-1}$ (Figure S2, Supporting Information).

Next, we investigated the fluorescence emission property of NBCD (5 μM) in 20 mM HEPES buffer (pH 7.3) that is close to physiological conditions. When excited at 530 nm, NBCD exhibited no fluorescence emission at around 590 nm in the absence of H_2O_2. The subsequent addition of H_2O_2 to the solution of NBCD led to a gradual increase in bright red fluorescence intensity at around 590 nm, which is visible with a clear color change from colorless to pink (Figure 1 b, inset, and Figure S3). Moreover, there was a good linearity between the fluorescence intensity (590 nm) and incubation time in the range of 0 to 60 min (Figure 1 b inset). These results demonstrated that NBCD could fluorescently detect H_2O_2 with excellent sensitivity. We also investigated the concentration-dependent fluorescence response of NBCD at 30 min after the addition of H_2O_2 in range of 0–200 μM (Figure S4). The absorption and fluorescence spectra of the reaction mixture were identical to those observed with resorufin. The generation of resorufin was also confirmed chemically, and other byproducts were also found by NMR spectroscopy and mass spectrometry (Figure S5 A and S6 A, B). Moreover, the conversion efficiency of NBCD was quantified (Figure S5 B).

In addition, we investigated the chemoselectivity of NBCD toward H_2O_2 under the above-mentioned analytical conditions and compared its relative reactivity of probe towards various ROS. Selectivity data were collected at several time points over 60 min. As expected, only H_2O_2 significantly increased the fluorescence intensity (590 nm). Fluorescence emission changes were hardly observed in the presence of other ROS, including hydroxyl radical ($^{\bullet}OH$), *tert*-butylhydroperoxide (TBHP), *tert*-butoxy radical ($^{\bullet}OtBu$), superoxide (O^{2-}), hypochlorite (ClO^-), and nitric oxide (NO) (Figure 2). Thus, these experimental results demonstrated that NBCD possessed excellent chemoselectivity towards H_2O_2 over other analytes.

As H_2O_2 is an important co-product of many oxidases, the activity of the responsible enzymes or the concentration of

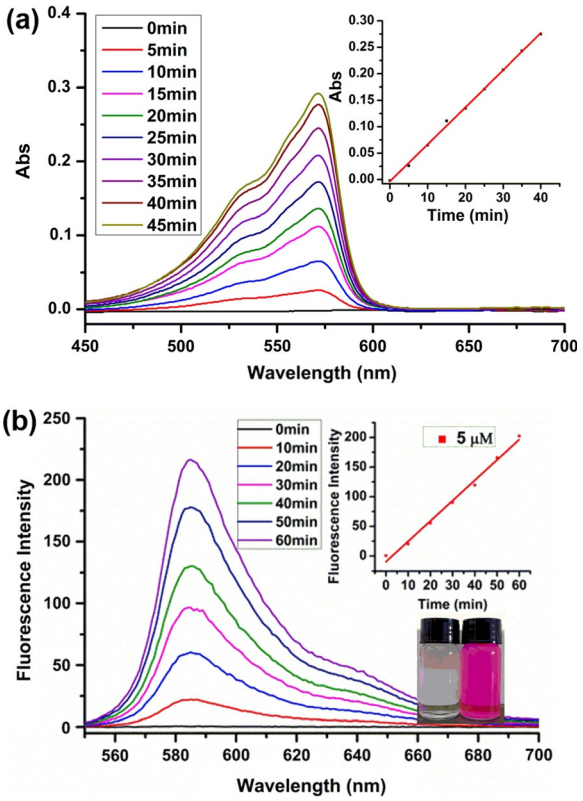


Figure 1. (a) Time-dependent absorption spectra during the reaction of NBCD (10 μM) with 20 equiv. of H_2O_2 over 45 min. Inset: Linear relationship between absorbance and time. (b) Time-dependent fluorescence intensity changes of NBCD (5 μM) incubated with H_2O_2 (5 μM) over 60 min. Inset: Linear relationship between fluorescence intensity and time; photographs of the cuvettes containing NBCD before (left) and after (right) addition of H_2O_2. Experiments were conducted at 37 °C in 20 mM HEPES buffer containing 0.5% DMSO (pH 7.3).

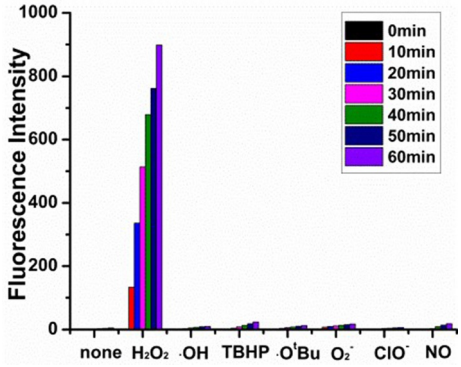


Figure 2. Fluorescence responses of NBCD (5 μM) towards ROS in 20 mM HEPES, containing 0.5% DMSO, pH7.3, at 37 °C. Bars represent the relative responses at 0, 10, 20, 30, 40, 50, and 60 min after addition of each oxidant. Data were acquired at 10 μM H_2O_2, excess NO, and at a concentration of 500 μM for all other oxidants, with λ_{exc} = 530 nm and λ_{em} = 590 nm.

these substrates could be evaluated by monitoring H_2O_2. Therefore, we investigated the ability of NBCD for detection of enzymatically generated H_2O_2 to extend its application.

We used NBCD to detect H_2O_2 generated by glucose oxidase and D-glucose. Addition of D-glucose to a glucose oxidase solution containing NBCD quickly induced a progressive increase of the fluorescence peak centered at around 590 nm because of the generation of H_2O_2. However, addition of D-glucose to a mixture solution of glucose oxidase containing NBCD in the presence of catalase caused almost no fluorescence emission at around 590 nm because H_2O_2 was consumed by catalase. These results proved the ability of NBCD to assess enzymatically generated H_2O_2 (Figure 3a). We also investigated the fluorescence responses of NBCD towards different amounts of D-Glucose (0–50 μM) after the reaction for 30 min. The addition of D-Glucose to the solution of NBCD and glucose oxidase resulted in a gradual increase of the fluorescence peak at around 590 nm with a good linearity between the fluorescence intensity and the concentration of D-glucose (0–50 μM) (Figure 3b and Figure S7).

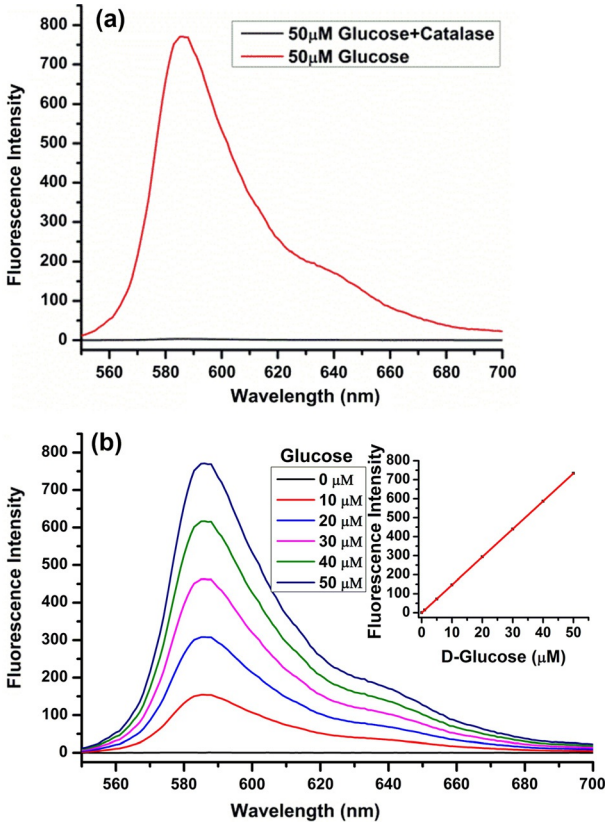


Figure 3. (a) Fluorescence detection of enzymatically generated H_2O_2 by NBCD. Red trace: 10 μM NBCD, 50 μM D-glucose, 8 μg mL^{-1} glucose oxidase, λ_{ex} = 530 nm. Black trace: as in panel in the presence of catalase 80 μg mL^{-1}. (b) Fluorescence responses of NBCD (10 μM) towards different concentrations of D-glucose at 30 min. Inset: Linear relationship between fluorescence intensity and concentration of D-Glucose; conditions: 8 μg mL^{-1} glucose oxidase, λ_{ex} = 530 nm at 37 °C in 20 mM HEPES buffer containing 0.5% DMSO (pH 7.3).

The dependence of the fluorescence intensity on pH was investigated by the reaction of NBCD with H_2O_2 at different pH conditions in a range of pH from 4 to 9 (Figure S8). No fluorescence emission was observed in the range of pH 4–6 since deprotection is impeded by the protonation of phenol, while the fluorescence intensity significantly increased at pH 6–9 and

reached the maximum near pH 8. In addition, NBCD had excellent stability in different pH buffers and cell culture media (Figure S9 A, B).

Based on the attractive properties of NBCD including the excellent ON/OFF ratio, high selectivity, and satisfactory stability, NBCD was further evaluated for its application in fluorescence imaging of H_2O_2 inside living systems. The experiment was initiated with the cytotoxicity study of NBCD in cells. The cell viability was investigated by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT assay) after incubation of cells with NBCD at various concentrations for 24 h. Little cytotoxicity was observed at the maximum tested concentration of 100 μM (Figure S10 A). Then, H_2O_2 imaging experiments were investigated using a previously reported method.[1c] HeLa cells were incubated with 5 μM NBCD for 15 min at 37 °C, followed by washing for four times to remove NBCD from the solution outside of the cells. The cells were then incubated with H_2O_2 at various concentrations for 40 min. The fluorescence imaging showed clearly red fluorescence inside the cells (Figure 4 b), which proved the good sensitivity of NBCD for H_2O_2 inside living cells. We also investigated the time-dependent fluorescence response of NBCD over 45 min after the addition of H_2O_2 (Figure S11). To demonstrate that the red fluorescence was caused by the reaction of NBCD with H_2O_2, control experiments were performed by co-incubation of catalase with H_2O_2 that showed an extremely low level of fluorescence (Figure 4 c). Moreover, fluorescence could still be observed upon exposure to only 2 μM H_2O_2, which further demonstrated the good sensitivity of NBCD (Figure S12), although the quantification of fluorescence intensity indicated that only small amounts of NBCD could pass through the cell membrane (Figure S14).[1c]

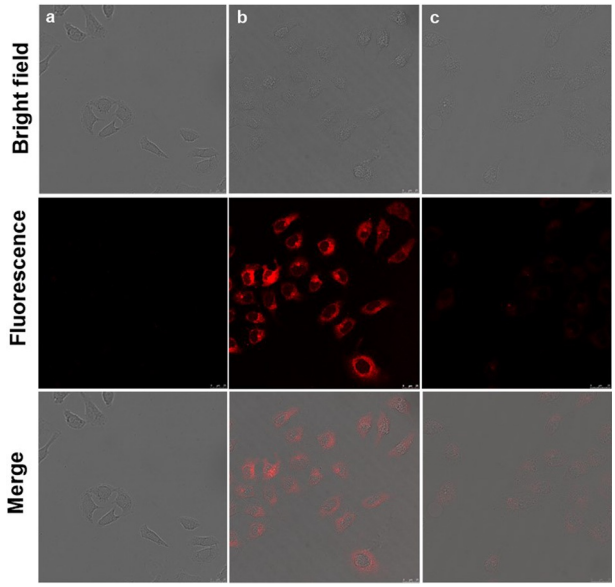


Figure 4. Confocal fluorescence and bright field images of live HeLa cells after incubation with 5 μM NBCD for 15 min, followed by washing and further incubation without H_2O_2 (a), with 500 μM H_2O_2 (b), with 500 μM H_2O_2 together with catalase (c) for 40 min. Scale bars, 25 μm.

Finally, we evaluated the ability of NBCD for application in fluorescence imaging of intracellular H_2O_2 generated after stimulation by the epidermal growth factor (EGF). In the experiments, the detection of EGF-stimulated H_2O_2 inside A431 cells was investigated according to the literature.[1c] Red fluorescent signals were observed inside the cells once the cells were stimulated with EGF, which proved the good sensitivity of NBCD for EGF-stimulated H_2O_2 inside living cells (Figure S13).

In conclusion, we have described the design, synthesis, properties and biological applications of a novel *N*-borylbenzyloxycarbonyl-3,7-dihydroxyphenoxazine fluorescent probe for the detection of H_2O_2 in both in vitro systems and in living systems. NBCD displayed high H_2O_2 selectivity towards various ROS, owing to the adoption of a boronate moiety. Moreover, NBCD exhibited an apparent color change from colorless to pink upon addition of H_2O_2, and thus could be used as a "naked-eye" probe for H_2O_2. NBCD could be used to detect H_2O_2 in living cells by using fluorescence spectroscopy with a detection limit of 2 μM. This probe is stable under cell culture conditions and could also detect enzymatically generated H_2O_2. Most importantly, NBCD also enabled the visualization of H_2O_2 generated by EGF stimulation inside the cells. The excellent chemoselectivity for H_2O_2, good stability in the biological pH range, and low cytotoxicity assure that this novel *N*-protected 3,7-dihydroxyphenoxazine probe could well serve as an effective tool to investigate the detailed biological function of H_2O_2 in living systems.

Experimental Section

Additional preparation details and fluorescent confocal microscopy images, UV/Vis spectra, fluorescence spectra, details of the synthesis, ^{1}H NMR, ^{13}C NMR, and MS spectra of compounds are included in Supporting Information.

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Keywords: fluorescent probes · hydrogen peroxide · imaging agents · N-protected 3,7-dihydroxyphenoxazine · reactive oxygen species · sensors

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