A 7-Hydroxybenzoxazinone-Containing Fluorescence Turn-On Probe for Biothiols and Its Bioimaging Applications

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Abstract: In this work, a novel 7-hydroxybenzoxazinone-based fluorescent probe (PBD) for the selective sensing of biothiols is reported. Upon treatment with biothiols, PBD shows a strong fluorescence enhancement (up to 70-fold) and a large Stokes shift (155 nm). Meanwhile, this probe exhibits high resistance to interference from other amino acids and competing species. PBD features good linearity ranges with a low detection limit of 14.5 nM for glutathione (GSH), 17.5 nM for cysteine (Cys), and 80.0 nM for homocysteine (Hcy), respectively. Finally, the potential utility of this probe for biothiol sensing in living HeLa cells is demonstrated.

Keywords: fluorescence probe; biothiols; 7-hydroxy-3-phenyl-benzoxazinone; large Stokes shift; 2,4-dinitrobenzenesulfonate

1. Introduction

Sulfhydryl-containing amino acids, such as cysteine (Cys), glutathione (GSH), and homocysteine (Hcy), play key roles in maintaining appropriate physiological and biological processes [1–5]. Malfunction of Cys levels has been associated with hair depigmentation, slowed growth rate, liver damage, edema, and so on [6]. Elevated levels of Hcy result in cardiovascular and Alzheimer’s disease [7,8]. GSH is the most abundant intracellular non-protein thiol and its concentration is within millimolar range. GSH deficiency is linked with cancer, liver damage, and neurodegenerative disease [9–11]. Owing to their significant roles, it is of great importance to develop rapid, simple and reliable methods for monitoring the levels of biothiols in biological systems.

Among various detection methods, fluorescent-probe-based detection has been accepted as one of the most efficient methods due to its advantages of having operational simplicity, being low cost, working under in situ real time conditions and involving no invasive bioimaging [12–16].

Great efforts have been directed to the development of fluorescent probes toward biothiols (Cys, Hcy, and GSH) [17–23]. Many probes have been constructed based on traditional fluorescent dyes, such as fluorescein [24], naphthalimide [25], coumarin [26], cyanin [27], BODIPY (boron-dipyrromethene) [23], xanthene [28], 2-(2′-hydroxyphenyl)benzothiazole [29], and dicyanomethylene−4H-pyran derivatives [30]. In spite of some sensors with good sensing performance, many of these probes still suffer from poor solubility, low sensitivity, low quantum yield, and a time-consuming detection process. Thus, it would be beneficial to develop fluorophores with a new structural skeleton. Up until now, fluorescent dyes with a 7-hydroxybenzoxazinone skeleton have received little attention [31–33]. We prepared 7-hydroxy-3-phenylbenzoxazinone (PBOH) and evaluated its optical property. PBOH exhibits strong yellow-green fluorescence and a large Stokes shift (155 nm). We envisioned that masking the hydroxyl group in PBOH with a 2,4-dinitrophenylsulfonyl...
group (DNBS) could generate a new fluorescent probe (PBD) for the selective detection of biothiols (Scheme 1). As they are well-known, arenesulfonates and arenesulphonamides can be readily cleaved by thiolate anions, and, therefore, the 2,4-dinitrobenzenesulfonyl (DNBS) group has often been chosen as the trigger group for thiols [24,34–38]. More importantly, the DNBS group can totally quench fluorescence, thus reducing background interference, because of its strong electron-withdrawing ability. We speculate that biothiols induce cleavage of the ester bond in the DNBS-PBOH conjugates, which can release free PBOH and thereby restore the strong emission, thus realizing a selective fluorescence off-on recognition of biothiols. The new probe PBD indeed exhibits a significant off-on response to biothiols. The investigation of PBD suggested that it possesses a strong anti-interference ability while sensing biothiols, with a low detection limit of 14.5 nM for GSH, 17.5 nM for Cys, and 80.0 nM for Hcy, respectively. In addition, PBD was successfully applied for biothiol detection in living HeLa cells.

![Design diagram of fluorescent probe PBD (2,4-dinitrobenzenesulfonate of 7-hydroxy-3-phenylbenzoxazinone) for biothiol detection.](image)

**Scheme 1.** Design diagram of fluorescent probe PBD (2,4-dinitrobenzenesulfonate of 7-hydroxy-3-phenylbenzoxazinone) for biothiol detection.

### 2. Results and Discussion

PBOH showed strong fluorescence ($\Phi = 0.60$) with a maximum at 540 nm and an absorption band centered at 385 nm in EtOH-PBS buffer (Phosphate Buffered Saline) solution (10 mM, pH = 7.4, 4:6, v/v) (Stokes shift = 155 nm). Owing to the quenching effect of the DNBS group, PBD alone exhibited extremely weak fluorescence with a low quantum yield at 540 nm ($\Phi = 0.011$), and its absorption maximum was blue-shifted to 340 nm under the same aqueous conditions (Figure S1). As expected, upon addition of biothiols, a remarkable fluorescence intensity enhancement at 540 nm was observed. As such, we constructed a turn-on fluorescent probe for biothiols.

#### 2.1. Time-Dependence and pH Effect

The sensory effect of PBD is exemplified by its reaction with GSH. The emission intensity was recorded at different time points to evaluate the response time between PBD and various amounts of GSH. As shown in Figure 1, after addition of GSH, the fluorescence emission peak at 540 nm reached a plateau in about 16 min and maintained stability for a long time.

Kinetic analysis was conducted next. Ten equivalents of GSH, Cys, and Hcy were added into the solutions of PBD (10 µM), respectively. Through monitoring the fluorescence emission at 540 nm, the reactions were found to obey a typical pseudo-first-order and the rate constants were determined to be 0.25 min$^{-1}$, 0.26 min$^{-1}$, and 0.20 min$^{-1}$ for GSH, Cys, and Hcy respectively (Figure S5).

Furthermore, we evaluated the pH effect on the sensory response of PBD (Figure S3). PBD itself was inert to pH change, indicative of the excellent stability of the probe over a broad pH range. By contrast, the probe exhibited a marked fluorescence response to GSH within the pH range 7 to 9, thus enabling detection of biothiols across a relatively wide pH range.

The following experiments of PBD were performed in EtOH-PBS buffer (10 mM, pH 7.4, 4:6, v/v) and 405 nm was chosen as the excitation wavelength in the fluorescent measurements. For convenience, the fluorescent intensity was recorded at 16 min after the addition of GSH.
were investigated by treating PBD (200 µM) with GSH in PBS buffer (10 mM, pH 7.4, 4:6, v/v). Fluorescence enhancement could be about 70-fold when 10 equivalents of GSH were added to the solution of PBD. As shown in Figure 2, upon treatment with GSH, the testing solution gave rise to a strong yellow-green fluorescence which is consistent with the emission spectrum of dye PBOH. A calibration plot of the signal to the GSH concentrations from 0 µM to 10 µM showed good linearity ($R^2 = 0.9933$), indicating that PBD can quantitatively detect GSH within this range. The detection limit of PBD toward GSH was found to be 14.5 nM based on the 3σ/slope method [39,40], thus enabling a highly sensitive detection of GSH. We also evaluated the response effect of Cys and Hcy to PBD. Under the same conditions, PBD exhibited similar optical behavior to Cys and Hcy with the detection limits being 17.5 nM and 80.0 nM, respectively (Figure S4).

The selectivity of PBD for biothiols (100 µM) was evaluated by screening its response to potential competing species (200 µM), including various natural amino acids (Val, Glu, Leu, Met, Asn, Ala, His, Trp, Phe, Ser, Arg, Lys, Gln, Asp, Ile, Thr, Tyr, Pro, and Gly), sulfur species (Na$_2$S, KSCN, Na$_2$SO$_3$, Na$_2$SO$_4$, Na$_2$S$_2$O$_3$, and Na$_2$S$_2$O$_4$), oxygen species (H$_2$O$_2$), and common salts (KF, NaCl, KBr, KI, Na$_2$CO$_3$, NaHCO$_3$, NaNO$_2$, NaNO$_3$, and AcONa). Figure 3 illustrates that treatment of PBD solution with 10 equivalents of GSH resulted in a 70-fold enhancement at 540 nm. Cys gave rise to a similar fluorescence increase. Hcy induced an increment half that of GSH. High concentrations (200 µM) of interfering species induced negligible changes. Following on from this, competition studies were investigated by treating PBD with GSH (100 µM) in the presence of 20 equivalents of other interfering species.
analytes. As shown in Figure 4a,b, addition of GSH to the probe solution resulted in a robust signal enhancement in spite of the coexistence of competing species, demonstrating that probe PBD displays strong immunity to interference while sensing biothiols.

Figure 3. Fluorescence spectra of PBD (10 µM) with biothiols (100 µM) and various tested analytes (200 µM). $\lambda_{ex} = 405$ nm.

Figure 4. (a,b) Selectivity and competition of PBD (10 µM) to GSH (100 µM) and other analytes (200 µM). Black bars represent the fluorescence intensity (540 nm) of a single analyte with probe PBD. Red bars represent the fluorescence intensity (540 nm) of PBD to GSH in the presence of various tested analytes. $\lambda_{ex} = 405$ nm.

2.3. Sensing Mechanism Study

The fluorescence turn-on response of PBD to biothiols may be attributed to a thiol-anion-mediated $S_NAr$ process which led to the cleavage of 2,4-dinitrophenylsulfonyl (Scheme 2). A comparison of the fluorescence spectral profiles of the reaction solution and the precursor compound provides evidence for the proposed mechanism. Upon addition of GSH to non-fluorescent PBD, a turn-on fluorescent signal at 540 nm was observed and the emission intensity nearly reached the intensity of PBOH. In addition, we successfully isolated the fluorescent product of the reaction by column chromatography. As expected, the $^1$H-NMR spectrum of the isolated fluorescent product is consistent with that of PBOH. Thus, the above results confirmed that dye PBOH was released via the cleavage of sulfonylate in PBD mediated by GSH.

Scheme 2. Schematic sensing mechanism of PBD toward biothiols.
To confirm the sensing mechanism, we also carried out $^1$H-NMR titration experiments to track product formation. After the addition of excessive Cys to PBD in DMSO-$d_6$/D$_2$O (8/2, v/v), the signals of PBD ($H_a$ and $H_b$) gradually declined. At the same time, three peaks corresponding to the protons ($H_{a'}$, $H_{b'}$, and $H_c'$) attributable to PBOH began to appear, indicating that PBOH had been released. With the continuation of this chemical reaction, the signals of PBOH ($H_{a'}$, $H_{b'}$, and $H_c'$) gradually increased. The signals of PBD ($H_a$ and $H_b$) had almost completely disappeared at 20 min. These results demonstrate the cleavage of the DNBS group and the production of fluorophore PBOH (Figure 5).

![Figure 5](image-url)

Figure 5. Partial $^1$H-NMR spectra of PBD before the addition (a) and the change after addition of Cys, 2 min (b), 5 min (c), and 20 min (d), and of PBOH (e) in DMSO-$d_6$/D$_2$O (8/2, v/v).

To elucidate further the off-on sensing mechanism of probe PBD towards biothiols, DFT (Density functional theory) calculations were performed. The frontier orbital diagram indicates that the LUMO energy level of DNBS ($-3.930$ eV) is much lower than that of fluorophore the PBOH ($-1.021$ eV) (Figure 6), which implied that the photo-induced electron transfer (PET) process can happen from the fluorophore moiety to the DNBS group. Owing to the effective fluorescence quenching effect of DNBS, PBD is essentially nonfluorescent. After removal of the DNBS group, the fluorescence is recovered.

![Figure 6](image-url)

Figure 6. Frontier orbital diagrams of PBOH and 2,4-dinitrophenylsulfonyl (DNBS). Orbital energies were calculated using Gaussian 03 program at B3LYP/6-31 g (d, p) level.

2.4. Live Cell Imaging

Having demonstrated its excellent responsiveness and anti-interference in sensing biothiols, we next examined whether PBD can be used to detect intracellular thiols in living cells. As shown in Figure 7, after incubating HeLa cells with PBD for 20 min, strong yellow-green fluorescence could be observed inside the cells under excitation at 405 nm. In the control experiments, cells were pretreated with N-ethylmaleimide (NEM) for 30 min to remove free thiols, and subsequently incubated with
PBD for another 20 min. After being washed with PBS buffer three times, the cells showed almost no fluorescence under a confocal fluorescence microscope. The results showed that PBD is cell-permeable and can sense intracellular biothiols in living cells.

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Figure 7. Confocal fluorescence images of HeLa cells. (Left) bright-field image; (middle) fluorescence image; and (right) overlayed image. (a) Cells treated with N-ethylmaleimide (NEM) (1 mM) for 30 min followed by incubation with PBD (10 µM) for 20 min. (b) Cells incubated with PBD (10 µM) for 20 min at 37 °C. (c) Untreated cells.

3. Materials and Methods

3.1. Materials.

All chemicals used were of analytical grade and were obtained from commercial suppliers. TLC (Thin-layer chromatography) silica gel plates (GF-254) and silica gel (200-300 mesh) for column chromatography were obtained from Qingdao Marine Chemicals, China. Deionized water was employed throughout all experiments. ^1H-NMR and ^13C-NMR spectra were acquired on a Bruker AVANCE II 400 spectrometer (Bruker, Switzerland), respectively. HRMS (High Resolution Mass Spectrometry) spectra were measured with a 6510-Q-TOF spectrometer (Agilent, USA). UV–Visible absorption spectra were recorded by a TU-1901 spectrometer (Beijing, China). Fluorescent measurements were performed on a Lengguang F97Pro FL Spectrophotometer (Shanghai, China). The pH measurement was carried out on a Leici PHS-3C pH meter (Shanghai, China). All spectra were recorded at room temperature.

3.2. Synthesis and Characterization of Compound Information

The four-step synthetic route is outlined in Scheme 3. The details of the synthesis and structural characterization are shown in the Supporting Information (Figures S8–S14).
In sum, we have developed a new off-on fluorescent probe PBD for biothiols by modifying a fluorescent dye 7-hydroxy-3-phenyl-benzoxazinone with a DNBS unit as a biothiol-specific recognition moiety. In response to biothiols, PBD displays significant fluorescence enhancement (up to 70-fold) and a large Stokes shift (155 nm). The probe exhibits a low detection limit as well as excellent selectivity and anti-interference ability toward biothiols over competing species. Finally, this probe has been used to sense biothiols in live HeLa cells.

Supplementary Materials: The supplementary materials are available online.

Author Contributions: B.L. and D.Z. conceived and designed experiments; B.L. performed experiments; B.L. and R.A. analyzed the data; Y.Z. conceived experiments and analyzed the data.

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Conflicts of Interest: There are no conflicts of interest to declare.

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**Sample Availability:** Samples of the compounds PBOH and PBD are not available from the authors.

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