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A novel water-soluble BODIPY dye as red fluorescent probe for imaging hypoxic status of human cancer cells

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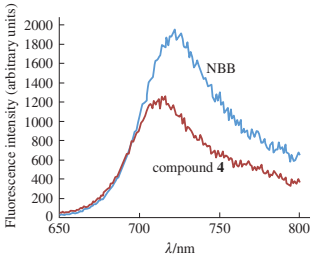
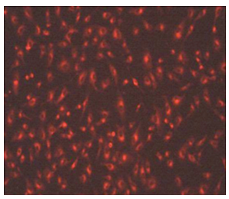
The development of effective methods for real-time monitoring of nitroreductase (NTR) activity is of great significance for medical diagnosis and cancer research. Here, we present a novel water-soluble BODIPY-based chemodosimeter (NBB) for imaging the hypoxic status of human non-small-cell lung cancer A549 cells. We assume that 'on–off' response of NBB is activated by the NTR-mediated reductive release of *meso*-phenol BODIPY dye resulting in unusual fluorescence.





A549 cells incubated with NBB probe

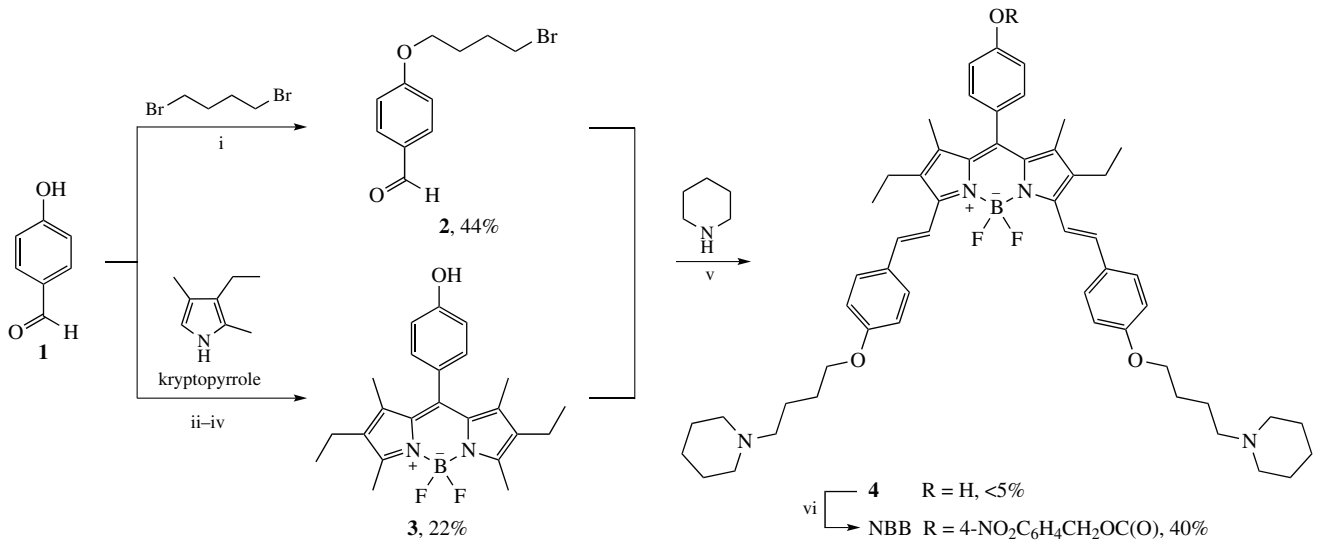
Keywords: BODIPY, fluorescent probe, hypoxia, lung cancer, nitroreductase.

Hypoxia is a pathological condition in which tissues suffer from a lack of oxygen required for normal cell metabolism.[1] In some solid tumors, the median oxygen concentration is about 4%, and locally it can even decrease to less than 0.1%.[2] In oncology, tumor hypoxia is often associated with a weak response to treatment and a poor clinical outcome.[3,4] The identification of tumor with large hypoxic area is necessary for both selective hypoxia-directed chemotherapy and the optimal treatment of oxygen-sensitive tumor by photodynamic therapy or radiotherapy. Therefore, the development of contrast agents, especially those based on fluorescence modality,[4] for imaging the hypoxic status of tumor cells in various biological systems, *i.e.*, living cells, *ex vivo* or *in vivo,* is of considerable clinical significance.[5]

As overexpression and activity of nitroreductase (NTR) enzymes are directly related to the hypoxic status of living cells, these redox enzymes along with azoreductase and DT-diaphorase are currently considered as important relevant biomarkers for detection of this oxygen-deprivation state.[6,7] Real-time monitoring of NTR activity can also serve as an indirect but no less effective method for assessing the oxygen content in living organisms.[8] In the presence of NTR, nitroaromatic compounds are reduced under hypoxic conditions to the corresponding hydroxylamine or amino derivatives.[9] Consequently, several fluorescent probes based on NTR-responsive reactions have been devised by taking advantage of this enzymatic reduction and the fluorescence quenching by nitroaryl moiety *via* oxidative photoinduced electron transfer (PET).[10] A number of fluorogenic nitroaryl derivatives have been successfully used for imaging hypoxia in tumor cells through an effective fluorescence 'off–on' mode.[9,11–15] However, just a few of them included fluorescent scaffold such as 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) dye.[16–19] Furthermore, these probes have not been optimized with respect to spectral and physico-chemical properties, *e.g.*, emission maximum in the red spectral range, water solubility or cell permeability.

Here we report the synthesis of a novel water-soluble red fluorescent probe, referred to as NBB, based on a distyryl-substituted BODIPY scaffold[20] containing three phenol functions. The *meso*-phenol moiety of this compound is assumed to act as a fluorescence switch through the protection–deprotection of the hydroxyl group with *p*-nitrobenzyloxycarbonyl moiety recognizable by NTR.[21,22] The two other phenol moieties are available for a further orthogonal functionalization of NBB with (piperidin-1-yl)alkyl groups to enhance water solubility and cellular uptake. We successfully applied this novel NTR-responsive BODIPY-based fluorescent probe for imaging the hypoxic status of human non-small-cell lung cancer A549 cells.

NBB was prepared from commercially available reagents in four steps (Scheme 1). At first, we synthesized benzaldehyde derivative **2** bearing the 4-bromobutoxy group as a *para*-substituent under conventional conditions. Dual-functional reagent **2** is ready for both the Knoevenagel condensation with BODIPY dye **3** and the concomitant N-alkylation of piperidine with 4-bromobutyl arm. The synthesis of BODIPY dye **3** bearing the *p*-hydroxyphenyl moiety as a *meso*-aryl substituent was carried out according to Lindsey's general procedure.[23] The condensation of *p*-hydroxybenzaldehyde **1** with kryptopyrrole in CH_2Cl_2 using the sequential treatment with trifluoroacetic acid (TFA), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), triethylamine and boron trifluoride–diethyl ether complex ($BF_3 \cdot Et_2O$) provided compound **3** in 22% yield. Next, the Knoevenagel condensation was conducted with an excess of



Scheme 1 *Reagents and conditions*: i, K_2CO_3, DMF, room temperature, 24 h; ii, TFA, CH_2Cl_2, room temperature, 50 min; iii, DDQ, Et_3N, room temperature, 50 min; iv, $BF_3 \cdot Et_2O$, room temperature, 10 h; v, *p*-TsOH, toluene, Δ; vi, *p*-nitrobenzyl chloroformate, Et_3N, CH_2Cl_2, 0 °C, 30 min.

benzaldehyde **2** (5 equiv.), in the presence of piperidine and *p*-toluenesulfonic acid (*p*-TsOH), in boiling dry toluene until complete evaporation of the solvent. Desired bis-(piperidin-1-yl)-alkylated distyryl-substituted BODIPY dye **4** was isolated in a modest non-optimized yield. Finally, the O-acylation of the remaining phenol moiety with *p*-nitrobenzyl chloroformate in the presence of Et_3N as HCl-trapping agent in dry CH_2Cl_2 afforded target NBB after purification by column chromatography over silica gel. All spectroscopic data, especially those from NMR spectroscopy and mass spectrometry, corresponded to the suggested structure (see Online Supplementary Materials). The purity of NBB sample was confirmed by RP-HPLC analysis with UV–VIS detection at different wavelengths.

The photophysical properties of NBB probe were determined in water containing 10% DMSO to minimize the formation of non-fluorescent H-type aggregates (Figures S1 and S2 in Online Supplementary Materials). The UV–VIS absorption spectrum of this distyryl-substituted BODIPY derivative exhibits a strong absorption band centered at 659 nm ($\varepsilon = 19 \times 10^3$ dm^3 mol^{-1} cm^{-1}). The fluorescence spectrum of NBB demonstrates a broad red emission band centered at 686 nm (λ_{ex} = 610 nm) and a good symmetry. The FWHM (full-width at half maximum) was 373 cm^{-1} and the Stokes' shift was 697 cm^{-1}. The relative fluorescence quantum yield in 10% DMSO was found to be 0.15. The fluorescence spectra of NBB were measured in different solvents, especially in MeCN–phosphate buffered saline (pH 7.4) (PBS) of various composition (Figures S3–S6 in Online Supplementary Materials), and revealed a weaker emission in the far-red region, centered at 722 nm (λ_{ex} = 610 nm), in pure PBS (Figure 1). This unexpected spectral behavior suggests that NBB is prone to excimer formation in an aqueous medium,[24,25] which is superior to the quenching effect of the PET-active *p*-nitrobenzyl moiety. It is interesting to note that, according to the same spectral measurements, the ability of distyryl-substituted BODIPY dye **4** bearing the free *meso*-phenol fragment to form a far-red emitting excimer was much more limited (see Figures 1 and S3). Thus, a strategy for detecting NTR enzymes through the deprotection of NBB phenol, which yields an intensiometric 'on–off' response, may be devised.

The practical utility of NBB as a red fluorescent imaging agent is determined by its ability to interact with NTR enzyme in aqueous medium with high specificity and sensitivity. In this context, the fluorogenic response of NBB (1.0 μM) towards

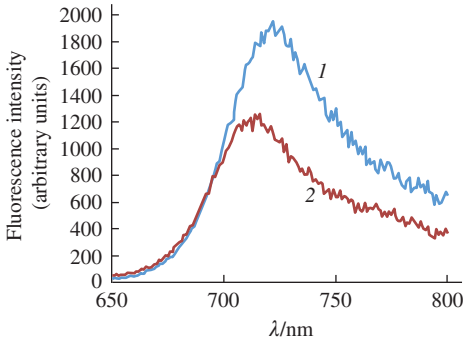


Figure 1 Fluorescence emission spectra (λ_{ex} = 610 nm) of (*1*) NBB and (*2*) compound **4** recorded in PBS.

recombinant NTR† (0.1 U) in the presence of the NADH cofactor was examined in PBS. Although the decrease in red fluorescence, probably due to the disruption of excimer formation, has been observed, all the attempts to find compound **4** in crude mixture after the enzymatic reaction through RP-HPLC-MS analysis, in both full scan and SIM modes, were unsuccessful, and only the starting probe NBB was detected (data not shown). Despite these inconsistent results, the potential of NBB probe was assessed in the real biological model of hypoxia (*vide infra*).

NBB probe was used to visualize hypoxia in living cells, namely A549 lung cancer cell line. The activity of reductases in this cell line increases significantly with decreasing oxygen level.[16] After a preliminary analysis of the toxicity of NBB, the incubation of A549 cells with this probe at 10 and 20 μM for 24 h did not lead to significant changes in their morphology, spreading and attachment. In addition, the significant cytoplasmic accumulation of NBB probe in the cells was observed (Figure 2).

Fluorescence microscopy does not allow a quick quantitative analysis of the accumulated probe to be performed and has evident limitations in analyzing a large number of samples. Therefore, to work with cells in hypoxia, a protocol applicable to fluorescence microplate reader was used. Previously, the method was modified to provide a more stable and reliable fluorescence signal in molecular biology experiments.[26,27] The advantage of this method is the rapid processing of NBB-containing cells with a PBS–DMSO (9 : 1, v/v) solution. The hypoxic and normoxic

† Commercial nitroreductase expressed in *Escherichia coli* (Sigma-Aldrich, #N9284).

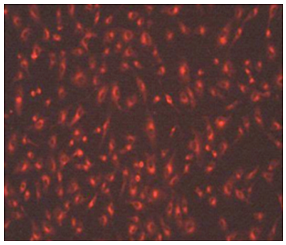


Figure 2 Fluorescence image of A549 cells incubated with NBB probe (20 μM) for 60 min (λ_{ex}= 530–585 nm, λ_{em} = 615 nm).

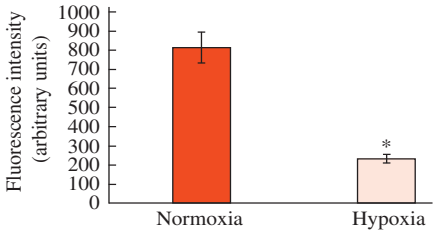


Figure 3 Fluorescence intensity of A549 cells incubated with NBB probe under normoxia and hypoxia conditions (λ_{ex} = 610 nm, λ_{em} = 680 nm, * $p < 0.05$ *vs.* normoxia).

cells were stained and samples were prepared (see Online Supplementary Materials). The hypoxic samples had the weaker fluorescence intensity compared with that of the normoxic samples (Figure 3). Thus, NBB probe efficiently stains living cells and acts as a fluorogenic 'on–off' sensor for hypoxia.

In summary, we have developed a novel NTR-responsive fluorogenic probe based on a distyryl-substituted BODIPY scaffold, operating according to the unusual fluorescence 'on–off' response mode. This probe was shown to be applicable for visualizing the NTR distribution in lung cancer cells although we were unable to reveal the mechanism of NBB probe activation by recombinant NTR expressed in *E. coli*, which leads to a decrease in the red fluorescence signal. The probe can easily be prepared and displays both promising analytical characteristics, such as significant excitation/emission in the red-to-near-infrared spectral range, and good solubility in water. Undoubtedly, this bioreductive fluorescent imaging agent may have the potential for practical use in other biological systems and in *in vivo* imaging studies.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.11.020.

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