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A highly sensitive long-wavelength fluorescence probe for nitroreductase and hypoxia: selective detection and quantification†

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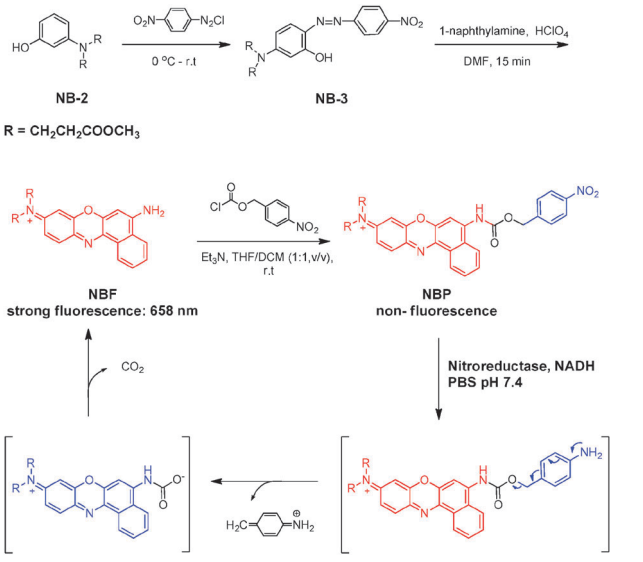
A novel long-wavelength fluorescence probe NBP has been developed for the detection of nitroreductase (NTR) and hypoxia. NBP could be activated by NTR at 0.1 μM to release the fluorophore NBF and significant changes in fluorescence emission at 658 nm were observed. This feature makes it advantageous for imaging hypoxic cells with minimal endogenous interference.

Hypoxia, regions of low oxygen tension, is characteristic of solid tumors.[1] Hypoxia renders tumor cells higher resistance towards cancer therapies and is associated with metastasis.[2] Compared to normal cells, hypoxic cells are found to display an elevated level of reductive enzymes, such as nitroreductase, DT-diaphorase and azoreductase, which result in reductive stress.[3,4] Thus, hypoxia and these enzymes represent a viable target for tumor cell detection and treatment. Nitroreductases (NTRs), a family of flavin-containing enzymes, reduce nitroaromatics to corresponding nitroso, hydroxylamine or amino derivatives, which are often mutagenic or carcinogenic.[5,6] Recently, NTRs were employed in development of gene- or virus-directed enzyme prodrug cancer therapies and drug screening.[7,8] Therefore, the detection of NTRs activity and hypoxia is significant for drug development and tumor diagnosis.

Hypoxia or NTRs have been detected by many approaches.[9] Because of their high sensitivity and spatiotemporal resolution, fluorescence probes are favorable tools for noninvasive detection of enzymes and disease diagnosis.[10] Recently, several fluorescence probes against hypoxia have been developed based on various reductases such as nitroreductase, azoreductase and quinone reductase.[11] A few classic examples include a *p*-nitro benzyl moiety functionalized SNARF dye reported by Nakata and co-workers, the azo bond tethered BHQ–Cy5 pair reported by Nagano *et al.* and an indolequinone unit employed as the hypoxia-sensitive moiety by Komatsu *et al.*[12] Near-infrared probes offer various advantages, including deeper penetration and minimized autofluorescence.[13] Nile Blue is widely used in biological imaging because of its excellent photochemical stability, long wavelength excitation/emission maxima and modest Stokes shifts.[14] Herein, we describe an NIR fluorescence probe (**NBP**) based on Nile Blue for nitroreductase and tumor hypoxia with minimized interference from absorption and autofluorescence of bio-species.

Our strategy for the construction of this NIR probe **NBP** relies on tethering the *p*-nitrobenzyl moiety to a Nile Blue fluorophore *via* a self-collapsible carbamate linkage (Scheme 1).[15] The *p*-nitrobenzyl moiety as a nitroreductase-sensitive moiety has been frequently used in prodrugs and detection of tumor hypoxia.[4,16] To improve the cell permeability of the probe, we introduced two methyl esters into the Nile Blue scaffold. Under hypoxic conditions, the *p*-nitrobenzyl moiety of the probe **NBP** was reduced to form the *p*-aminobenzyl derivative, which is unstable and spontaneously collapses to release the Nile Blue fluorophore **NBF**, and the fluorescence was restored.



Scheme 1 Synthetic scheme of the fluorescence probe **NBP** and the deduced mechanism of **NBP** activated by the enzyme NTR.

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The probe **NBP** was readily synthesized using *p*-aminophenol **NB-2** as the starting material (Scheme 1) and its structure was confirmed by ^{1}H- and ^{13}C-NMR and HRMS. The reactive azo compound **NB-3** was synthesized by the reaction of substituted *p*-aminophenol **NB-2** with 1 equivalent of an aryl diazonium salt. The condensation reaction of **NB-3** with 1-aminonaphthalene was accomplished by stirring in perchloric acid at 160 °C to afford the Nile Blue fluorophore (**NBF**). **NBF** was stirred with 4-nitrophenyl chloroformate in anhydrous THF–DCM for 2 hours to yield the probe **NBP**.[17]

The fluorescence properties of fluorophore **NBF** with respect to pH were investigated (Fig. S1, ESI†). **NBF** exhibits a pK_a of 9.6, which is two units lower than the physiological pH. Therefore, **NBF** can be used in biological systems. Spectroscopic evaluation of **NBP** and **NBF** was carried out in PBS buffer (pH 7.4, 0.01 M) with 1% DMSO as cosolvent. The probe **NBP** has a strong absorption peak at 525 nm and is essentially non-fluorescent. The fluorophore **NBF** shows maximal absorption and a strong fluorescence peak at around 613 nm and 658 nm, respectively (Fig. S2, ESI†). When **NBP** was incubated with the enzyme NTR, a drastic enhancement of fluorescence intensity at 658 nm was observed.

Next, we studied the reactivity of the probe **NBF** toward NTR reduction.[18] The fluorescence spectra were collected in the presence of NADH (50 μM, as a cofactor of NTR) in PBS buffer (pH 7.4, 0.01 M, 1% DMSO) at 37 °C. Under these conditions, the free probe **NBP** (1 μM) is nearly non-fluorescent when excited at around 580 nm. Upon addition of 12.5 μg mL^{-1} of oxygen-sensitive NTR, a time dependent fluorescence enhancement was observed and the maximal fluorescence intensity was enhanced at around 658 nm in 1 h (Fig. 1a). This demonstrated that the *p*-nitrobenzyl moiety of the probe **NBP** was reduced and the fluorophore **NBF** was released as expected. The formation of **NBF** was also confirmed by HPLC analysis (Fig. S3, ESI†). We also found that the fluorescence enhancement is related to the initial concentrations of **NBP** (0–1 μM) (Fig. 1b). As shown in Fig. 2a, the fluorescence enhancement of **NBP** was dose-dependent with respect to NTR. A linear response was found at 2.5–12.5 μg mL^{-1} and the detection limit was deduced to be 180 ng mL^{-1} (Fig. 2b). Therefore, **NBP** is a sensitive fluorescence probe for detection of nitroreductase not only because of the detection limit but also because of the low concentration

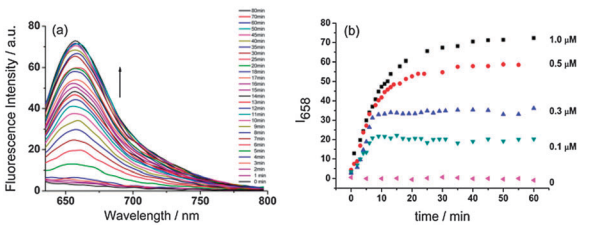

Fig. 1 Fluorescent spectra of **NBP** to NTR. (a) **NBP** (1 μM) was cultured with 12.5 μg mL^{-1} NTR and NADH (50 μM). (b) Fluorescence response (I_{658}) with varied concentrations of **NBP**: 0 (control), 0.1, 0.3, 0.5 and 1 μM. The measurements were performed in 0.01 M PBS buffer (pH 7.4, 1% DMSO) with 12.5 μg mL^{-1} nitroreductase and 50 equivalents of NADH at 37 °C over an incubation time of 70 min. The fluorescence intensity data were collected after a certain time at around 658 nm. The excitation wavelength was 580 nm.

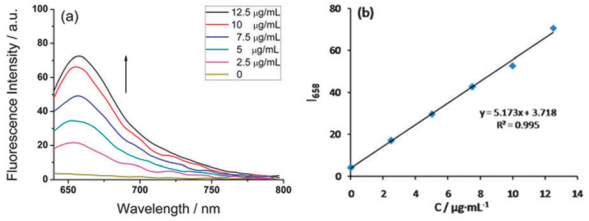

Fig. 2 Fluorescent spectra of **NBP** with respect to concentrations of nitroreductase. (a) 1 μM **NBP** was incubated with different concentrations of nitroreductase and 50 eq. NADH. (b) A linear correlation between fluorescence response and concentrations of nitroreductase. The excitation wavelength was 580 nm.

of the probe. This implies its potential for application in biological systems.

To further study the versatility of **NBP**, we investigated the selectivity of **NBP** toward other biological reducing agents such as thiols, which have been reported to reductively activate various antitumor prodrugs.[19] The probe **NBP** (1 μM) was treated with the various biological reductants in PBS buffer (pH 7.4, 0.01 M, 1% DMSO) at 37 °C, such as dithiothreitol (DTT), glutathione (GSH), cysteine (Cys) and homocysteine (Hcy). As shown in Fig. 3, biological thiols (2 mM) induced nearly no fluorescence responses within 70 min at 37 °C. And the impact of NADH on the probes **NBF** and **NBP** was also studied. Upon addition of 50 equiv. of NADH (50 μM), the fluorescence intensity of **NBF** decreased slightly (Fig. S4, ESI†) and the fluorescence intensity of **NBP** remained negligible, even after 70 min at 37 °C (Fig. S5, ESI†).[20] In contrast, incubation of the probe **NBP** (1 μM) with NTR (12.5 μg mL^{-1}) and NADH (50 μM) together induced distinguished fluorescence enhancement at around 658 nm. These results suggested that the fluorescence changes of **NBP** were only induced by nitroreductase and NADH, which can be used to selectively monitor NTR without interferences from other biological reductants.

The MTT assay showed that **NBP** did not exhibit cytotoxicity at 1 μM and at 37 °C for 48 h (Fig. S6, ESI†). The application of the probe **NBP** for intracellular hypoxia detection was also investigated. A549 cells were incubated under normoxic (20% pO_2) and hypoxic (5% and 1% pO_2) conditions for 8 h at 37 °C. Then the

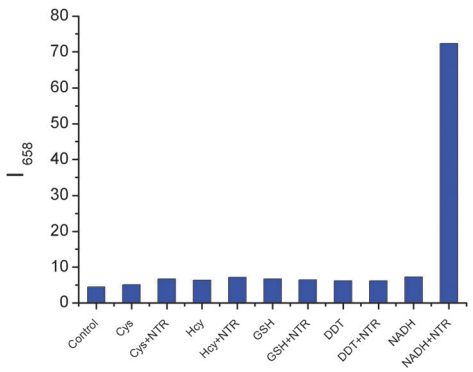

Fig. 3 Fluorescence response of **NBP** to the biological reductants. Control: the free probe **NBP**. Fluorescence response of **NBP** (1 μM) treated with various biological thiol reductants (2 mM) and NADH (50 eq.) in PBS buffer (0.01 M, pH 7.4 with 1% DMSO) at 37 °C. The fluorescent intensity data at around 658 nm were collected after reaction for 70 min. Fluorescent excitation was provided at 580 nm.

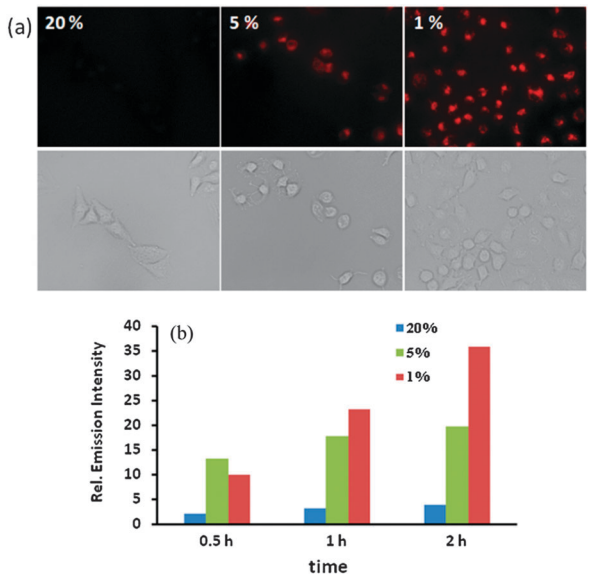

Fig. 4 (a) Fluorescence and bright-field images of A549 cells at different oxygen concentrations. The cells were incubated under normoxic (20% pO_2) and different hypoxic (5%, 1% pO_2) conditions for 8 h, and then treated with **NBP** (1 μM) for 2 h. (b) Mean fluorescence intensity change of A549 incubated with **NBP** (1 μM) for 0–2 h after incubation under normoxic (20% pO_2) and different hypoxic (5%, 1% pO_2) conditions for 8 h. *Image J* software gave the average fluorescence intensity value of A549 cells.

cells were washed with PBS buffer (pH 7.4) and were treated with 1 μM **NBP** in FBS-free DMEM for 2 h, 1 h and 0.5 h. Before taking images, A549 cells were washed three times with PBS buffer. As shown in Fig. 4a, A549 cells under normoxic conditions showed nearly no fluorescence enhancement. Whereas the cells incubated under hypoxic conditions showed drastic fluorescence enhancement and the fluorescence enhancement of cells is further promoted by 1% pO_2 compared to 5% pO_2. Using *Image J*™ software, we also obtained the average fluorescence intensity value at various time points (Fig. S7, ESI†). As shown in Fig. 4b, the fluorescence intensity of A549 cells increased dramatically but a slight enhancement was observed for the normoxic environment. These results demonstrated the potential of probe **NBP** to sensitively detect hypoxia in tumor cells.

In summary, we have developed a novel selective and sensitive fluorescence probe **NBP** for the detection of nitroreductase and hypoxia. Incubated with NADH, the enzyme NTR activated the probe **NBP** to release the fluorophore **NBF** and the intensity of emission enhanced dramatically at around 658 nm. Fluorescent imaging of A549 cells also showed that the probe **NBP** can be used to detect tumor hypoxia. The near-infrared fluorescence and modest Stokes shifts of probe **NBP** minimized the interference from absorption and autofluorescence of bio-species, which makes the probe applicable for tumor diagnosis.

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