A high-selectivity fluorescent probe for hypoxia imaging in cells and a tumor-bearing mouse model†

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Nitroreductase (NTR) with a high expression level in tumors has been considered as a biomarker of highly aggressive hypoxia tumors. Thus, it is important to develop powerful tools for tumor hypoxia detection. Here, we developed a two-photon fluorescent probe hTP-NNO2 for NTR detection. The probe with one-step synthesis exhibited high yield. hTP-NNO2 showed high selectivity and sensitivity for NTR and the detection limit was as low as 43 ng mL⁻¹. hTP-NNO2 also showed low cytotoxicity and high stability, indicating that hTP-NNO2 is suitable for NTR detection in real-time and in situ under physiological conditions. hTP-NNO2 was used for NTR imaging in hypoxia cells and the fluorescence intensity of hTP-NNO2 increased with decreasing oxygen concentration. Benefiting from the advantages of two-photon fluorescent probes, we performed NTR detection in deep brain tissue with an imaging depth of up to 100 μm. hTP-NNO2 was further successfully applied for NTR detection in zebrafish and tumors. These results indicated that we developed a promising fluorescence imaging tool for NTR detection in vitro and in vivo.

1. Introduction

Hypoxia is a pathological oxygen deficiency state in organisms, where the oxygen concentration is in the range from 4% to 0%.1,12 Hypoxia is conducive to the progression and metastasis of malignant tumors.3,5 Hypoxia can also increase the possibilities of gene mutations and lead to overexpression of many tumor-associated factors, particularly hypoxia-inducible factor 1 alpha (HIF-1α), and of many HIF-mediated genes.6,7 In addition, hypoxia is detrimental to the tumor prognosis because it leads to resistance to standard therapies and it promotes a more malignant phenotype.9–11 Lots of previous studies have shown that the hypoxic state of tumors is closely related to the process of tumor progression.5,8 Therefore, effective tools for tumor hypoxia detection are needed urgently for evaluating tumor progression. Nitroreductase (NTR) is an endogenous enzyme that is overexpressed in a hypoxia microenvironment especially in tumors, and the expression level of NTR is closely associated with the tumor progression and the hypoxia degree.1,12 We can assess the hypoxia level by measuring the concentration fluctuations of NTR.13–15 Therefore, effective tools for NTR detection are needed urgently for evaluating tumor progression and diagnosis.

Traditional methods for hypoxia detection include electron paramagnetic resonance imaging (EPRI), pO2 electrodes, positron emission tomography/computed tomography (PET/CT) and so on.17–19 However, these methods cannot achieve real-time and in situ imaging for NTR detection due to complex sample pretreatment, destruction of tissues or cells. Fluorescence imaging technology has been employed as a powerful tool for the detection of active species due to its advantages including rapid response, superior selectivity, outstanding spatial and temporal resolution, less invasiveness, and real-time and in situ detection.20,21 So far, many fluorescent probes have been developed for NTR detection, and most of them are one-photon fluorescent probes.22–25 Two-photon fluorescent probes use two near-infrared photons as an excitation source, and have obvious advantages over one-photon fluorescent probes, such as deeper tissue imaging depth, higher spatial and temporal resolution and longer observation time.26,27 The developed two-photon fluorescent probes for NTR detection have short fluorescence emission wavelengths, which causes the interference due to shallow penetration depth and autofluorescence of intrinsic bio-

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molecules. Thus, there is an urgent need to develop simple and convenient two-photon fluorescent probes with near-infrared fluorescence for NTR quick detection.

Here, we successfully designed and synthesized a two-photon fluorescent probe, hTP-NNO₂, for NTR detection with near-infrared fluorescence emission at 675 nm. We selected p-nitrobenzene as the response site and the synthesis methods of the probe based on a two-photon fluorophore TP-NH were simple and convenient. hTP-NNO₂ was successfully used for NTR detection in vitro and in living cells at different oxygen concentrations. We also performed NTR detection using a two-photon laser confocal microscope in deep brain tissue. We could capture cellular activities at each layer throughout the tissue, and the imaging depth was up to 100 µm. Probe hTP-NNO₂ was further employed for NTR detection in zebrafish and solid tumors.

2. Experimental section

2.1 Synthesis method of compound hTP-NNO₂

Compound TP-NH (0.0355 g, 0.1 mmol) and Cs₂CO₃ (0.16 g, 0.5 mmol) were dissolved in DMF (30 mL) for 30 min at 0 °C. Then 4-nitrobenzyl bromide (0.0432 g, 0.2 mmol) was added and the solution was stirred overnight. After concentration, the obtained crude product was purified by silica column chromatography (200–300 mesh) to afford the final product (0.0387, 78.9%). ¹H NMR (500 MHz, DMSO-D₆) δ (ppm): 1.635–1.670 (t, 3H), 1.841–1.885 (s, 6H), 3.229–3.311 (s, 3H), 4.423–4.529 (s, 1H), 4.772–4.885 (s, 2H), 4.901–5.038 (q, 2H), 6.821–6.934 (s, 1H), 7.400–7.435 (d, 2H), 7.537–7.589 (m, 4H), 7.645–7.694 (d, 1H), 7.768–7.830 (d, 1H), 8.003–8.052 (d, 1H), 8.055–8.110 (d, 1H), 8.139–8.186 (d, 1H), 8.188–8.253 (d, 2H), 8.256–8.319 (d, 1H). LC-MS (API-ES): m/z C₃₂H₃₂N₃O₂⁺ calcd 490.61, found [M⁺] 490.46.

2.2 Cell culture

Human lung carcinoma (A549) cells and human neuroblastoma (SH-SY5Y) cells were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured with DMEM supplemented with 10% FBS. SH-SY5Y cells were cultured with RPMI-1640 supplemented with 10% FBS. All cells were incubated at 37 °C under a humidified atmosphere containing 5% CO₂. The cells were passaged by scraping and seeding on 20 mm Petri dishes according to the manufacturer’s instructions. A multi gas incubator (Sanyo) was used for producing 1–21% O₂ concentration by means of N₂ substitution. 0.1% O₂ concentration was generated using an AnaeroPack™ (Mitsubishi Gas Chemical Company, Co. Inc., Japan).

2.3 Establishment of A549 transplanted tumor nude mice

5-Week-old specific pathogen-free nude mice were provided by Changzhou Cavens Lab Animal Co. Ltd. The mice were housed in individual ventilated cages, and given free access to a SPF laboratory diet and water. Mice were group-housed in a 12:12 light–dark cycle. 2 × 10⁶ cells were suspended in media and injected subcutaneously into nude mice. A549 xenografts were established in nude mice until the tumor volumes typically reached about 200 mm³. All experiments were performed in accordance with the guidelines established by the Committee of Animal Research Policy of Binzhou Medical University.

3. Results and discussion

3.1 Design and synthesis of hTP-NNO₂

Traditional one-photon fluorescent probes are limited by shallow penetration depth, photobleaching and interferences from environmental factors. Thus, we are committed to design a two-photon fluorescent probe for NTR detection. A series of nitroaryl compounds can be reduced to aminoaryl derivatives by NTR with a cofactor, reduced nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). We selected p-nitrobenzene as a response unit and the two-photon fluorophore TP-NH with near-infrared (NIR) fluorescence emission at 675 nm was used as a chromophore for NTR detection (Scheme 1). As expected, we observed weak fluorescence from the probe hTP-NNO₂ because the response unit p-nitrobenzene can quench the fluorescence of hTP-NNO₂. After reaction with NTR using NADH as a cofactor, the response unit p-nitrobenzene changed to an aminobenzene group and hTP-NNO₂ converted to TP-NH through a 1,6-rearrangement elimination reaction to emit a fluorescence signal at 675 nm. Compared to the developed two-photon fluorescent probe for NTR detection, the probe hTP-NNO₂ with simple synthesis steps showed high yields (78.9%). In addition, the probe hTP-NNO₂ with NIR fluorescence wavelength at 675 nm had a deeper tissue imaging depth and showed less endogenous autofluorescence with an imaging depth of up to 100 µm. The detailed synthesis route to hTP-NNO₂ is shown in Scheme S1.†

3.2 Spectral properties of the probe hTP-NNO₂ towards NTR

The spectral properties of the probe hTP-NNO₂ towards NTR were determined under simulated physiological conditions.
mL fluorescent probe. The curve of the reaction kinetics of exhibited a good spectral response towards NTR.

The regression equation. These results proved that the probe standard deviation of blank measurement and group in hTP-NNO₂ was cleaved and TP-NH was released increasing NTR concentration, indicating that the response relative fluorescence intensity and NTR concentrations (0 (Fig. 1b). There was a good linear relationship between the relative fluorescence intensities and the NTR concentrations. The experiments were repeated three times and the data are shown as mean±S.D.

d) The Lineweaver–Burk plot of the enzyme-catalyzed reaction. hTP-NNO₂ (5, 10, 12.5, 15, 17.5, 20, 25, and 50 μM). (e) Selectivity detection of hTP-NNO₂ (10 μM) towards various interfering species. 1, blank; 2, Fe³⁺ (1 mM); 3, Ca²⁺ (1 mM); 4, vitamin C (1 mM); 5, H₂O₂ (100 μM); 6, NaClO (1 mM); 7, homocysteine (100 μM); 8, tyrosine (100 μM); 9, dithiothreitol (1 mM); 10, H₂S (1 mM); 11, glutathione (10 mM); 12, serine (100 μM); 13, cysteine (100 μM); 14, NADH (0.5 mM); 15, NADH (0.5 mM) + NTR (20 μg ml⁻¹); 16, glutathione peroxidase (10 U); and 17, matrix metalloprotein-9 (100 U L⁻¹). (d) The dose–response relationship of fluorescence spectra of hTP-NNO₂ (10 mM) towards NTR (0–20 μg ml⁻¹). The probe showed a sensitive spectral response towards NTR. The relative absorbance spectra of hTP-NNO₂ increased with increasing NTR concentrations, and the absorption peak was centered at 515 nm (Fig. 1a). Then the relative fluorescence spectra were obtained at different concentrations of NTR (0–20 μg ml⁻¹) with 0.5 mM NADH as a cofactor. The fluorescence intensity of hTP-NNO₂ increased with increasing NTR concentration, indicating that the response group in hTP-NNO₂ was cleaved and TP-NH was released (Fig. 1b). There was a good linear relationship between the relative fluorescence intensity and NTR concentrations (0–20 μg ml⁻¹). The linear regression equation is \( F_{515 \text{ nm}} = 3.482 \times 10^4 \times 1 / \text{HPS} + 0.14 \text{M} \cdot \text{s}^{-1} \), where \( r = 0.9997 \) (Fig. 1c). The detection limit was determined to be 43 ng ml⁻¹ (3σ/k), where \( σ \) is the standard deviation of blank measurement and \( k \) is the slope of the regression equation. These results proved that the probe exhibited a good spectral response towards NTR.

Reaction kinetics was an important parameter for a new fluorescent probe. The curve of the reaction kinetics of hTP-NNO₂ towards NTR was obtained using NADH as a cofactor. We found that the reaction rate increased gradually with the increasing concentration of hTP-NNO₂, as shown in Fig. 1d. A Lineweaver–Burk double-reciprocal plot of \( 1 / V \) (V is the initial reaction rate) versus the reciprocal of the probe hTP-NNO₂ concentration was used to describe the reaction kinetics of hTP-NNO₂ and the kinetic equation is \( V = \frac{V_{\max} \cdot [\text{probe}]}{K_m + [\text{probe}]} \). After calculation, \( K_m \) and \( V_{\max} \) were found to be 45.84 μM and 0.14 μM s⁻¹, respectively. These results indicated that hTP-NNO₂ possesses high sensitivity towards NTR.

The selectivity of probes was another important parameter for NTR detection. To assess the selectivity of hTP-NNO₂ towards NTR, hTP-NNO₂ was tested in a wide variety of interfering species. The concentrations of other interfering substances were higher than their original level in the cells. As shown in Fig. 1e, physiologically relevant ions such as Fe³⁺, Ca²⁺, Na⁺, K⁺ and Mg²⁺ cannot cause fluorescence changes. Other active species such as vitamin C, H₂O₂, H₂S, glutathione and cysteine also cannot interfere with the NTR detection. hTP-NNO₂ showed apparent fluorescence changes after being reacted with NTR in the presence of NADH. Thus, the probe hTP-NNO₂ showed high selectivity and sensitivity for NTR detection. These results demonstrated that hTP-NNO₂ can be used for NTR detection in a complex biological environment and that hTP-NNO₂ is a promising tool for NTR detection in vivo.

### 3.3 NTR detection in living cells

Encouraged by the good spectral properties of the probe hTP-NNO₂ towards NTR in a simulated buffer system, we further explored NTR detection in living cells. We firstly performed MTT assays to evaluate the cytotoxicity of hTP-NNO₂. As shown in Fig. S2, A549 and SH-SY5Y cells were incubated...
with different concentrations of the probe hTP-NNO₂ (0–70 μM). The cells showed high viability, indicating that the probe had low cytotoxicity. Next, we detected the NTR concentration fluctuations in the cells at different oxygen concentrations. The cells were incubated with different oxygen concentrations (0.1%, 1%, 3%, 5%, 10% and 21%) for 6 h, and then the cells were incubated with 10 μM probe hTP-NNO₂ for 25 min at 37 °C before imaging. As shown in Fig. 2a, our probe showed very weak fluorescence signals at a normal oxygen concentration (21%). The fluorescence signals increased gradually with decreasing oxygen concentrations. hTP-NNO₂ showed stronger fluorescence signals in hypoxia cells especially in the cells that were incubated with 0.1% oxygen concentration. The corresponding quantitative analysis of fluorescence of Fig. 2a is shown in Fig. 2c. Flow cytometry analysis has been considered as a high-throughput assay technology for the analysis of a large number of samples. Therefore, we used flow cytometry analysis for NTR detection to confirm the results obtained using a confocal microscope, and these results are shown in Fig. 2b. The fluorescence intensity, as shown in Fig. 2b, was

![Fig. 2](image_url)
increased with decreasing oxygen concentration. These results were consistent with the results obtained using a laser scanning confocal microscope. The corresponding quantitative analysis of fluorescence of Fig. 2b is shown in Fig. 2d.

3.4 NTR detection using a two-photon confocal microscope

Benefiting from the advantages of two-photon fluorescent probes such as longer observation time, higher spatial and temporal resolution and deeper tissue imaging depth, we further performed NTR detection by using a two-photon confocal microscope. The cells, as shown in Fig. 3, were cultured at different oxygen concentrations (21% and 0.1%) for 6 h. Then, the cells were incubated with hTP-NNO₂ (10 μM) for 25 min for NTR detection. As shown in Fig. 3a, we can observe weak fluorescence signals under normoxic conditions (the control group). The cells incubated with 0.1% oxygen concentration (the hypoxia group) showed strong fluorescence signals (Fig. 3b). We could capture fluorescence signals at each layer throughout the cell to observe their cellular activities. Thus, our probe is a promising tool for NTR detection and observing cellular activities at each layer of deep tissues using a two-photon confocal microscope.

3.5 NTR detection in deep brain tissue

After the NTR detection using a two-photon confocal microscope, we further performed NTR detection in deep brain tissue. We selected the brain as the model organ for NTR detection because brain tissue is extremely sensitive to hypoxia. The hypoxia mouse models were raised in normobaric hypoxic (FI02 11%) chambers for 1 day, and the results are shown in Fig. 4. The brain tissue sections were incubated with probe (10 μM) for 30 min. The mice in the control group were placed under normoxic conditions, and we could capture weak fluorescence signals (Fig. 4a). The mice that were placed under hypoxia conditions exhibited bright fluorescence signals (Fig. 4b). These results indicated that the NTR level under hypoxia conditions was much higher than that under normoxic conditions. Moreover, the imaging depth of hTP-NO₂ can be as high as 100 μm, which made it possible to capture more detailed information in different tissue depths. We further performed H&E staining to evaluate the damage in brain tissue. As shown in Fig. 4c and d, the brain tissues in the control group were tightly arranged and the nuclei of the brain cells were regular. However, the brain tissues under hypoxia conditions were disordered and their shape was deformed. These results indicated that hTP-NNO₂ can capture more detailed information in deep brain tissue.

3.6 NTR detection in vivo

The probe with NIR fluorescence emission can achieve NTR detection with minimal interferences from background autofluorescence in biological systems, minimal photodamage to tissue. We selected the brain as the model organ for NTR detection because brain tissue is extremely sensitive to hypoxia. The hypoxia mouse models were raised in normobaric hypoxic (FI02 11%) chambers for 1 day, and the results are shown in Fig. 4. The brain tissue sections were incubated with probe (10 μM) for 30 min. The mice in the control group were placed under normoxic conditions, and we could capture weak fluorescence signals (Fig. 4a). The mice that were placed under hypoxia conditions exhibited bright fluorescence signals (Fig. 4b). These results indicated that the NTR level under hypoxia conditions was much higher than that under normoxic conditions. Moreover, the imaging depth of hTP-NO₂ can be as high as 100 μm, which made it possible to capture more detailed information in different tissue depths. We further performed H&E staining to evaluate the damage in brain tissue. As shown in Fig. 4c and d, the brain tissues in the control group were tightly arranged and the nuclei of the brain cells were regular. However, the brain tissues under hypoxia conditions were disordered and their shape was deformed. These results indicated that hTP-NNO₂ can capture more detailed information in deep brain tissue.

Fig. 3 NTR detection using a one-photon confocal microscope and a two-photon confocal microscope. (a) NTR detection under normal oxygen conditions (21%). (b) NTR detection under hypoxia conditions (0.1%). Cells were treated with 10 μl of the probe for 25 min at 37 °C. We used a two-photon confocal microscope to capture fluorescence signals at different imaging depths (0, 2, 4, 6, 8, 10, 12, and 14 μm).

Fig. 4 NTR detection using a confocal microscope under controlled (a) and hypoxia conditions (b). (c) and (d) are the corresponding HE staining images of a and b.
biological samples and deep tissue penetration. It is indispensable to assess the applicability of hTP-NNO2 for NTR detection in vivo. We firstly performed NTR detection in zebrafish, and the results are shown in Fig. 5a. Zebrafish were incubated with different concentrations of oxygen from 21% to 1% for 4 h, and then they were treated with hTP-NNO2 (10 μM) for 20 min. We can observe that the fluorescence intensities increased with a decrease in oxygen concentrations, and the quantitative analysis of fluorescence is shown in Fig. 5d. As is well known, NTR is overexpressed in tumors, and therefore we further performed NTR detection in A549-bearing mice. 5-Week-old nude mice were injected with $2 \times 10^6$ cells subcutaneously, and the A549 xenografts were established in nude mice until the tumor volumes typically reached about 200 mm$^3$. The A549 xenografts of nude mice were intratumorally injected with hTP-NNO2 (1 μM, 50 μL in 1:99 DMSO/saline v/v). The fluorescence signals were collected using a Bruker In vivo Imaging System. As shown in Fig. 5b, the fluorescence signals increased with time, without fluorescence leakage in 30 min. The corresponding quantitative analysis of fluorescence is shown in Fig. 5f.

Fig. 5  NTR detection in vivo. (a) NTR detection in zebrafish. (b) A549 tumor-bearing mice were intratumorally injected with hTP-NNO2 (1 μM, 50 μL in 1:99 DMSO/saline v/v) for in vivo NTR detection for 0–30 min. (c) Sub-distribution fluorescence imaging in the heart, liver, spleen, lungs, kidneys and tumors. (d) Corresponding quantitative analysis of fluorescence of a. (e) Corresponding quantitative analysis of fluorescence of b. (f) Corresponding quantitative analysis of fluorescence of c.
fluorescence is shown in Fig. 5e. In addition, the organs, including the heart, liver, spleen, lungs, and kidneys, and tumors were isolated for fluorescence imaging, and the results are shown in Fig. 5c. We can observe fluorescence signals only in the tumors without leakage to their adjacent organs. Thus, hTP-NNO2 is a promising tool for NTR detection in tumors.

4. Conclusions
In summary, we have developed a two-photon fluorescent probe hTP-NNO2 for NTR detection in vitro and in vivo. The probe showed fast response, superior sensitivity and excellent selectivity for NTR detection in complex biological samples. The probe hTP-NNO2 was successfully applied for NTR detection under hypoxia conditions in zebrafish and A549 xenografts of nude mice without fluorescence leakage to their adjacent organs. Furthermore, the two-photon fluorescent probe was suitable for NTR detection in deep brain tissue and the imaging depth of hTP-NNO2 was as high as 100 μm. We anticipate that the probe hTP-NNO2 can be used as a valuable analytical tool for investigating NTR-related physiological and pathological processes in tumors.

Ethics statement
All surgical procedures were conducted in conformity with the Care and Use of National Guidelines for the laboratory animals, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University, Yantai, China. Approval Number: BZ2014-102R.

Conflicts of interest
There are no conflicts to declare.

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