A Highly Selective Fluorescent Probe for the Detection of Nitroreductase Based on a Naphthalimide Scaffold

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Abstract

The development of fluorescent probes for nitroreductase (NTR) has received intense attention because of its biological significance and wide application. In this work, a novel fluorescent probe for the detection of NTR in aqueous solution was designed and synthesized on a 1,8-naphthalimide scaffold. In the presence of NTR and nicotinamide adenine dinucleotide (NADH) under physiological conditions, the probe was converted into a 4-hydroxy-1,8-naphthalimide derivative and exhibited a sharp fluorescence enhancement at 550 nm, with a high selectivity for NTR over various analytes. The detection limit for NTR was determined to be 9.8 ng/ml by this probe. Due to its low signal background, this probe showed > 70-fold fluorescence enhancement. Theoretical calculations revealed that the reason for the fluorescence quenching of this probe is the photoinduced electron transfer (PET) from both the nitrobenzene and morpholine groups to the naphthalimide fluorophore.

Keywords Fluorescence · Probe · Naphthalimide · Nitroreductase · Photoinduced electron transfer

Introduction

Nitroreductase (NTR) is a kind of flavin-containing enzyme with an effective function of reducing aromatic nitro compounds into the corresponding hydroxy-amino or amino derivatives [1–3]. Meanwhile, the completion of the -NO₂ reduction catalyzed by NTR relies on the presence of electron donors, such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) [4]. In particular, because of its oxygen sensitivity, the NTR level in human tumors is a biomarker for evaluating hypoxia [5]. Beside the overexpression in hypoxic human tumors, NTR could also be produced by a variety of bacteria, especially Escherichia coli, for various applications such as bioremediation, degration of pervasive nitroaromatic pollutants [6], and promoting chemotherapy [7]. Moreover, NTR also plays important roles in detoxification, pro-drug activation, cancer radiation therapy and gene therapy [8–14]. In this regard, the detection of NTR is of great importance.

So far, a variety of small-molecule fluorescent probes for the detection of NTR have been reported [15–21]. The principle for detecting the level of NTR by these probes is based on the measurement of the drastic fluorescence change of the sample before and after the NTR-catalyzed chemical reaction. The strategy for the conversion of NTR probes into the reaction products can be classified into two categories: one is the reduction of -NO₂ into -NH₂ without any other variation, and the other is a domino decomposition reaction induced by the reduction of -NO₂ for the activation of a fluorophore [5]. It is worth noting that the above strategy for NTR fluorescent probes had also been used extensively in the case of fluorescent probes for hydrogen sulfide (H₂S) [22–25], which indicates that the existence of H₂S under physiological conditions could act as a potential interference that can not be ignored in the accurate determination of NTR activity. Thus, we believe that when testing the selectivity of a new NTR fluorescent probe, the interference of H₂S must be checked. However, fluorescent probes for NTR were commonly reported without the H₂S interference investigation.
[6]. Hence, it is still important to develop fluorescent probes with high selectivity for nitroreductase (NTR), especially without the interference from H₂S.

In this work, we designed and synthesized a NTR fluorescent probe (MNI-NTR) based on a naphthalimide scaffold and investigated the fluorescence quenching mechanism of the probe by theoretical calculations. MNI-NTR was easily synthesized and well characterized. The results show that the probe MNI-NTR is non-fluorescent and exhibits a high selectivity for NTR without the interference from H₂S. With the presence of NTR and NADH, MNI-NTR is converted into a 4-hydroxy-1,8-naphthalimide derivative under physiological conditions, showing a strong fluorescence peak at 550 nm. Theoretical calculations reveal that the reason for the fluorescence quenching of MNI-NTR is the photoinduced electron transfer (PET) from the -NO₂ and morpholine groups to the naphthalimide fluorophore.

**Experimental Section**

**Materials and Instruments**

Unless otherwise stated, all reagents were obtained from commercial source of analytical reagent grade and used without further purification. ¹H and ¹³C nuclear magnetic resonance (NMR) data were measured by a Bruker 400 MHz NMR spectrometer. High resolution mass spectra (HRMS) were obtained by an Agilent Q-TOF 6540 spectrometer. Fluorescence spectra were obtained from Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon) and steady-state UV–Vis absorption spectra were measured by a Lambda 35 UV–Vis absorption spectrometer (Perkin Elmer) with a 1.0 cm quartz cuvette. 96-well black flat bottom polystyrene microplate (Corning® Product #3650, USA) with a microplate reader Varioskan Flash (Thermo Fisher Scientific, USA) was used in kinetic measurements and assay. Nitroreductase (NTR) is an Abcam product. Ultrapure water was used throughout.

**Synthesis and Characterization of Compounds**

**Synthesis of N-Ethylmorpholine-4-Hydroxy-1, 8-Naphthalimide (Compound 1)**

Compound 1 was synthesized following procedures described in the literature [7]. ¹H NMR (400 MHz, d⁶-DMSO)δ(ppm): 8.53 (dd, 1H, J = 8.2 Hz), 8.46 (dd, 1H, J = 7.2 Hz), 8.35 (d, J = 8.4 Hz, 1H), 7.77–7.73 (m, 1H), 7.13 (d, J = 8.0 Hz), 4.17 (t, 2H, J = 4.6 Hz), 2.60–2.50 (m, 6H). ¹³C NMR (d⁶-DMSO, 100 MHz) δ(ppm): 164.20, 163.47, 161.26, 134.13, 131.93, 130.77, 129.48, 125.96, 123.02, 122.21, 112.65, 100.75, 100.42, 78.90, 49.18, 37.03, 36.91. HRMS (ESI) Calcd for C₁₈H₁₉N₂O₄ [MH⁺] 327.1345, found 327.1358.

**Synthesis of 2–(2–Morpholin-4-yl-Ethyl)–7–(4–Nitro–Benzyloxy)–3a, 9b–Dihydro–Benzo [De] Isoquinoline–1, 3–Dione (MNI-NTR)**

To a 250 mL three-necked flask were added compound 1 (0.33 g, 1 mmol), potassium carbonate (1.00 g) and p-nitrobenzyl bromide (0.43 g, 2 mmol). After 3 times of vacuum/argon replacements, 150 mL of anhydrous acetonitrile was added, after which the mixture was stirred and heated to reflux for 4 h under argon protection. Then, the reaction solution was evaporated under reduced pressure and the crude product was purified by column chromatography to obtain a white solid (0.37 g, 80%). ¹H NMR (400 MHz, CDCl₃) δ(ppm): 8.63 (d, J = 8.0 Hz, 2H), 8.54 (d, J = 8.0 Hz, 1H), 8.33 (d, J = 8.4 Hz, 2H), 7.78 – 7.72 (m, 3H), 7.10 (d, J = 8.0 Hz, 1H), 5.49 (s, 1H), 4.34 (t, J = 7.0 Hz, 2H), 3.70 (t, J = 4.2 Hz, 4H), 2.73 (t, J = 6.8 Hz, 2H), 2.63 (s, 4H).

¹³C NMR (100 MHz, CDCl₃) δ (ppm): 14.27, 29.71, 31.93, 37.03, 53.77, 56.17, 66.94, 69.46, 106.42, 115.93, 122.55, 124.15, 126.41, 127.86, 128.45, 131.83, 133.14, 134.72, 148.00, 159.0, 163.76, 164.37. HRMS: m/z, calcd for C₂₅H₂₃N₃O₆, [MH⁺]: 462.1665, found 462.1661.

**Results and Discussion**

**Design and Synthesis of the Fluorescent Probe**

The N-substituted 1, 8- naphthalimide fluorophore enjoys good photochemical and thermal stability, and it is easy to make a structure modification with a satisfactory reaction yield. Thus, this scaffold is effectively and extensively used in the design of fluorescent probes [26–30]. In this work, the probe MNI-NTR comprises p-nitrobenzyl as the unique recognition group and 1,8-naphthalimide as fluorophore. In the presence of NADH, MNI-NTR undergoes an elimination reaction triggered by NTR to produce the fluorescent molecule N-ethylmorpholine-4-hydroxy-1, 8-naphthalimide (Compound 1) (Scheme 1). A similar idea of the NTR fluorescent probe Na-NO₂ had already been implemented [18], and the only structural difference between Na-NO₂ and our probe is the group connected to the nitrogen atom of the 1, 8-naphthalimide fluorophore. By substituting the butyl group of Na-NO₂ into morpholine group, MNI-NTR was easily synthesised and well characterized by ¹H NMR, ¹³C NMR and HRMS. Comparing to Na-NO₂, compound 1 was devised as the fluorescent product, because it is a more hydrophilic dye with a higher fluorescence quantum yield than N-butyl-4-hydroxy-1, 8-naphthalimide, which might
be helpful for improving the performance of the probe in aqueous solution.

**Absorption and Emission Spectral Response of the Probe MNI-NTR Towards NTR**

The absorption and fluorescence emission response of MNI-NTR towards NTR was investigated at 37 °C in phosphate buffer saline (PBS) (Fig. 1). Various amounts of NTR were taken by a pipette into centrifuge tubes containing solutions of MNI-NTR (5 μM) and NADH (0.5 mM) to catalyse the proposed chemical reaction for 60 min. Then, the absorption and emission spectra of the above samples were measured. As shown in Fig. 1a, MNI-NTR emitted almost no fluorescence in aqueous buffer under the excitation wavelength (λex) of 445 nm, giving negligible background noise. Upon the addition of NTR, a fluorescence band (F445) arose substantially with a maximum emission wavelength (λem) of 550 nm, and the fluorescence enhancement was more than 70-fold in the presence of 30 μg/mL NTR. The relationship between the fluorescence signal of the reaction solution at 550 nm (F550) and the concentration of NTR ([NTR]) is given in Fig. 1b. Under the given conditions, F550 was linearly proportional to [NTR] in the range of 0–3.0 μg/mL (linear correlation coefficient of 0.9929), and the detection limit for NTR was estimated to be 9.8 ng/mL based on S/N = 3. Meanwhile, as shown in Fig. 1c, the titration of MNI-NTR with NTR also caused the rise of the absorption band peaked at 445 nm, and the color of the solution changed from transparent to light yellow. The absorption and emission spectra is consistent with compound 1 in phosphate buffer [31], which suggests that compound 1 is indeed the fluorescent product of the NTR catalyzed reaction. In the presence of NADH (0.5 mM) in PBS, the yield of compound 1 was 60% by the NTR (25 μg/mL) catalyzed decomposition of MNI-NTR (5 μM), which was calculated by Lambert–Beer’s Law with the absorption value at 445 nm and the extinction coefficient of compound 1.

**Kinetic Study**

The enzymatic kinetic study of the above reaction was performed to evaluate the probe’s affinity for NTR and the catalytic efficiency. A series of concentrations (from 0 to 25 μM) of MNI-NTR were added to a 96-well microplate containing NTR and NADH, and the fluorescence signals were collected by a microplate reader for 1 h. Figure 2 suggests that the reaction at all the concentrations of MNI-NTR was completed within half an hour. Furthermore, beside the above kinetic data, the solutions containing various concentrations of compound 1 were prepared and a standard fluorescence curve was obtained under the same conditions, which was used to fit the Michaelis–Menten equation of the enzymatic reaction [32] to get parameters (Vmax = 0.017 μM/s, Km = 43.7 μM). The results of the kinetic study indicate that MNI-NTR is a reactive fluorescent probe for NTR with moderate affinity and low catalytic efficiency in PBS (10 mM, pH = 7.2,) with 5% DMSO as co-solvent.

**Selectivity of MNI-NTR**

We examined the selectivity of the fluorescent probe MNI-NTR in PBS, and the experimental results are shown in Fig. 3. A variety of substances, including a number of
Fig. 1 The response of the fluorescence and absorption spectra of MNI-NTR (5 μM) to various concentrations of NTR (0, 0.5 μg/mL, 1.0 μg/mL, 2.0 μg/mL, 3.0 μg/mL, 6.0 μg/mL, 10.0 μg/mL, 15.0 μg/mL, 20.0 μg/mL, 25.0 μg/mL, 30.0 μg/mL) in PBS (10 mM, pH 7.2, 1% DMSO as co-solvent) at 37 °C. 

a Fluorescence spectra. b The fluorescence titration curve. c Absorption spectra
reactive oxygen species (ROS), reactive sulfur species (RSH) and other species commonly involved in evaluating the selectivity of a NTR probe, were tested in the experiment. The addition of hydrogen peroxide (H$_2$O$_2$, 1 mM), sodium hypochlorite (NaOCl, 100 μM), reduced glutathione (GSH, 1 mM, 5 mM), sodium sulfide (Na$_2$S, 1 mM, an H$_2$S donor), dithiothreitol (DTT, 1 mM), homocysteine (1 mM), arginine (1 mM), cysteine (1 mM), vitamin C (Vc, 1 mM), human serum albumin (HSA, 1 mg/mL), calcium chloride (CaCl$_2$, 1 mM), and magnesium chloride (MgCl$_2$, 1 mM) all failed to make the fluorescence of MNI-NTR enhanced. Only NTR could induce a remarkable fluorescence augmentation, while negligible fluorescence was observed when the probe was treated with other species. In terms of probe selectivity, our results showed that this probe is one of the best in the published literature. Meanwhile, this probe showed the largest signal amplification among all the NTR probes based on the 1,8-naphthalimide scaffold (Table S1), mainly due to its low background. Furthermore, the fluorescence of MNI-NTR kept quenched under various pH values; whereas in the presence of NTR, the signal remarkably increased within the biologically relevant pH range (Fig. S1).

Theoretical Study on Fluorescence Quenching Mechanism of MNI-NTR

The experiment shows that MNI-NTR displays no fluorescence. To reveal the fluorescence quenching mechanism of MNI-NTR, density functional theory (DFT) and time-dependent density functional theory (TDDFT) calculations were performed by Gaussian 09 software. Taking into account the solvent effect of water, the configuration of MNI-NTR at the ground state was optimized using the polarization continuum model (PCM) with the B3LYP hybrid functional and TZVP basis set [33, 34]. Based on the ground-state stable conformation without and imaginary vibrational frequency, the vertical excited energies of MNI-NTR were obtained (Table 1). The oscillator strengths ($f$) of the three lowest singlet excited states ($S_1$, $S_2$ and $S_3$) are 0.0000, 0.0000 and 0.0012 respectively, so the three states...
are non-radiative, while the singlet excited state $S_4$ is a light state ($f = 0.3948$).

At the same time, we also obtained the electron transfer related molecular orbitals, as shown in Fig. 4. The calculation results in Table 1 and the shapes of the molecular orbitals plotted in Fig. 4 suggest that there is electron transfer in the excited state from both the nitrobenzene group and the morpholine group to the 1, 8-naphthalimide fluorophore. Thus, it can be concluded that fluorescence quenching of MNI-NTR is due to photoinduced electron transfer (PET).

### Conclusion

In summary, we have developed a highly selective fluorescent probe (MNI-NTR) for the detection of NTR by attaching the morpholine moiety and the nitrobenzene group to a naphthalimide fluorophore. MNI-NTR is non-fluorescent, and in the presence of NADH in PBS, the fluorescence signal is significantly enhanced by NTR via a decomposition reaction, which produces a fluorescent dye with a fluorescence band peaked at 550 nm. Other

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**Table 1** Vertical excited energies of MNI-NTR at the optimized ground state geometry calculated at the TD-DFT/B3LYP/TZVP level (water was employed as solvent in all the calculations)

| Transition | Excitation Energy (eV) | Oscillator Strength | CI Expansion Coefficients |
|------------|------------------------|--------------------|--------------------------|
| $S_0$-$S_1$ | 2.97 (417 nm)          | 0.0000             | 90.6% ($H - 1$ $\rightarrow$ $L$) |
| $S_0$-$S_2$ | 2.91 (425 nm)          | 0.0000             | 90.9% ($H$ $\rightarrow$ $L$)    |
| $S_0$-$S_3$ | 2.93 (423 nm)          | 0.0012             | 99.6% ($H$ $\rightarrow$ $L + 1$) |
| $S_0$-$S_4$ | 2.95 (420 nm)          | 0.3948             | 97.0% ($H - 1$ $\rightarrow$ $L + 1$) |

*a* The calculated excitation energy  
*b* Oscillator strength  
*c* H stands for HOMO and L stands for LUMO. Only the main contributions of each transition and their relevant MOs are listed
species including H$_2$S is not able to turn on the signal. Theoretical calculations by DFT/TDDFT reveal that the fluorescence quenching of MNI-NTR is due to PET from both the morpholine moiety and the nitrobenzene group to the naphthalimide fluorophore.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10895-022-02974-7.

**Author Contributions** Han Li and Jintao Feng contributed equally to this work. The first draft of the manuscript was written by Jintao Feng, Han Li and Zhen Wang. Zongjin Qu and Cuixia Yao contributed to the study conception and design. Material preparation, data collection and analysis were performed by Zongjin Qu, Han Li, Yan Jia and Peng Li. All authors read and approved the final manuscript.

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**Availability of Data and Material/Data Availability** The authors declare that [the/all other] data supporting the findings of this study are available within the article.

**Declarations**

**Ethics Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent for Publication** Not applicable.

**Conflicts of Interest/Competing Interests** The authors have declared that there is no competing financial and/or non-financial interests.

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