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 applications. In this work, a novel fluorescent probe (MNI-NTR) based on a nitrobenzene switch for the detection of NTR in aqueous solution has been designed and synthesized on a 1,8-naphthalimide scaffold. The probe exhibited a high selectivity for NTR over various analytes with a detection limit of 9.8 ng/ml, especially without the interference from hydrogen sulfide. 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 nitrobenzene and morpholine groups to the naphthalimide fluorophore.

1 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, degradation 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 cannot 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 synthesised 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.

2 Experimental Section

2.1 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 NBS™ 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.

2.2 Synthesis and characterization of compounds

2.2.1 Synthesis of N-ethylmorpholine-4-hydroxy-1, 8-naphthalimide (compound 1)

Compound 1 was synthesized following procedures described in the literature. 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, 1H, J = 8.0 Hz), 4.17 (t, 2H, J = 7.0 Hz), 3.55 (t, 4H, 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, 131.61, 129.77, 129.48, 125.96, 123.02, 122.21, 112.65, 110.57, 66.58, 56.12, 53,82, 36.91. HRMS (ESI) Calcd for C18H19N2O4 [MH+]: 327.1345, found 327.1358.

2.2.2 Synthesis of the fluorescent probe 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 hours 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 (0.37g, 80%). $^1$H NMR (400 MHz, CDCl$_3$) 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). $^{13}$C NMR (100 MHz, CDCl$_3$) δ (ppm): 14.14, 22.70, 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, 142.72, 148.00, 159.0, 163.76, 164.37. HRMS: m/z, calcd for C$_{25}$H$_{23}$N$_3$O$_6$, [MH]$^+$: 462.1665, found 462.1661.

3 Results And Discussion

3.1 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-NO$_2$ had already been implemented,[18] and the only structural difference between Na-NO$_2$ and our probe is the group connected to the nitrogen atom of the 1, 8-naphthalimide fluorophore. By substituting the butyl group of Na-NO$_2$ into morpholine group, MNI-NTR was easily synthesised and well characterized by $^1$H NMR, $^{13}$C NMR and HRMS. Comparing to Na-NO$_2$, 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.

3.2 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 minutes. 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 ($\lambda_{ex}$) of 445 nm, giving negligible background noise. Upon the addition of NTR, a fluorescence band ($F_{445}$) arose substantially with a maximum emission wavelength ($\lambda_{em}$) 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 ($F_{445}^{550}$) and the concentration of NTR ([NTR]) is given in Fig. 1b. Under the given conditions, $F_{445}^{550}$ 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\textsuperscript{[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 in PBS.

3.3 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 hour. 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\textsuperscript{[32]} to get parameters ($V_{\text{max}} = 0.017 \mu M/s$, $K_m = 43.7 \mu M$). 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.

3.4 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 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 ($H_2O_2$, 1 mM), sodium hypochlorite (NaOCl, 100 µM), reduced glutathione (GSH, 1mM, 5 mM), sodium sulfide (Na$_2$S, 1 mM, a 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. Thus, it could be concluded that MNI-NTR was indeed a highly selective fluorescent probe for the detection of NTR in PBS, especially a probe without the interference from H$_2$S.

4 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 \((f = 0.3948)\).

**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). \(^a\) 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.

<table>
<thead>
<tr>
<th>Excitation energy (^a)</th>
<th>(\mathbf{f}^b)</th>
<th>CI expansion coefficients (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S_0-S_1) 2.97eV (417 nm)</td>
<td>0.0000</td>
<td>90.6% (H-1 (\rightarrow) L)</td>
</tr>
<tr>
<td>(S_0-S_2) 2.91 eV (425 nm)</td>
<td>0.0000</td>
<td>90.9% (H (\rightarrow) L)</td>
</tr>
<tr>
<td>(S_0-S_3) 2.93 eV (423 nm)</td>
<td>0.0012</td>
<td>99.6% (H (\rightarrow) L + 1)</td>
</tr>
<tr>
<td>(S_0-S_4) 2.95 eV (420 nm)</td>
<td>0.3948</td>
<td>97.0% (H-1 (\rightarrow) L + 1)</td>
</tr>
</tbody>
</table>

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).

### 5 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 species including \(H_2S\) 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 the morpholine moiety and the nitrobenzene group to the naphthalimide fluorophore.

### Declarations
1. 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.

2. Conflicts of interest/Competing interests

The authors have declared that there is no competing financial and/or non-financial interests.

3. Funding

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4. Ethics Declaration statement

Not applicable.

5. Consent to Participate

Not applicable.

6. Consent for publication

Not applicable.

7. 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.

References


**Scheme 1**

Scheme 1 is available in the Supplemental Files section.

**Figures**
Figure 1

The response of the fluorescence and absorption spectra of **MNI-NTR** (5 μM) to various concentrations of NTR 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.
Figure 2. Kinetic traces of the relative fluorescence units (RFU550) in PBS (10 mM, pH=7.2, 5% DMSO as co-solvent) at 37°C, λex = 445 nm, λem= 550 nm. A series of concentrations of MNI-NTR (0 µM, 0.78 µM, 1.56 µM, 3.13 µM, 4.69 µM, 6.25 µM, 12.5 µM, 18.75 µM, 25 µM) were added to a 96-well microplate containing NTR (25 µg/mL) and NADH (0.5 mM).

Figure 2

See image above for figure legend.
Figure 3

Selectivity of MNI-NTR. Various analytes were added to the probe (5 μM) in PBS (10 mM, pH=7.2, 1% DMSO as co-solvent) in the presence of NADH (0.5 mM) at 37°C. Fluorescence intensity values at 550 nm were collected after waiting for 60 min, $\lambda_{ex} = 445$ nm.
Figure 4

The relevant molecular orbitals of MNI-NTR based on the optimized geometry at the ground state.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.tif