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A mitochondria-targetable near-infrared fluorescent probe for imaging nitroxyl (HNO) in living cells

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ABSTRACT

Nitroxyl is reported to be associated with particular physiological or pathological states. Although some fluorescent probes for nitroxyl have been reported, none of them can be used for monitoring mitochondrial nitroxyl in living cells with spatial specificity. Herein, for the first time, we develop a mitochondria-targetable NIR fluorescent probe based on a merocyanine skeleton. The proposed probe is constructed by using 2-(diphenylphosphino)benzoyl group as the nitroxyl recognition moiety and the lipophilic indolium cation as the mitochondria-targeted site. The probe displays an intense fluorescence turn-on response to nitroxyl via the aza-ylide formation and its subsequent Staudinger ligation to release the NIR fluorophore. The probe is highly sensitive and selective towards nitroxyl over other biologically relevant reactive oxygen and nitrogen species. Cell imaging and colocalization experiments prove that the probe is suitable for visualizing nitroxyl in the mitochondria of living cells.

1. Introduction

Nitroxyl (HNO), the one-electron reduced and protonated form of well-known signalling agent nitric oxide (NO), has been found to exhibit the distinct biological and chemical properties from those of NO [1]. Recent studies show that HNO can be produced under biological conditions and is associated with particular physiological states. For example, HNO can react with thiols in aldehyde dehydrogenase, leading to the inhibition of the activity of the enzyme [2]. HNO is found to be a novel regulator of cardiovascular function and may provide useful tools for treating cardiovascular diseases such as heart failure [3]. In addition, HNO exhibits myocardial antihypertrophic and superoxide-suppressing actions [4]. Taken together, these findings indicate that HNO plays a pivotal role in biology. In order to advance our insight into the physiological and pathological roles of HNO, there is a strong desire to develop chemical tools which allow for sensitive and selective detection of HNO in vivo.

The mitochondrion is an essential organelle within eukaryotic cells, utilizing oxygen to digest carbohydrates and fats and releasing reactive oxygen species (ROS) [5]. Studies have shown that mitochondrion is the main area where endogenous NO is synthesized by inducible nitric oxide synthase (NOS) enzymes in mammalian cells [6]. Though endogenous generation of HNO has not been directly observed in live organisms, recent research shows mitochondrial NO reduction to HNO may occur with the aid of cytochrome c, manganese superoxide dismutase, and xanthine oxidase [7]. Additionally, HNO can be produced by the isolated neuronal NOS in the absence of the cofactor tetrahydrobiopterin (THB) or by oxidation of hydroxylamine (NH_2OH) with heme-containing proteins [8]. Based on these discoveries of HNO chemistry and biology, it is urgently required to develop methods to monitor mitochondrial HNO for further understanding its biosynthesis and function mechanism.

Unfortunately, it is well-known that HNO is a highly reactive and short-lived species and can rapidly dimerize ($k = 8 \times 10^6\ M^{-1}\ s^{-1}$) to yield highly labile hyponitrous acid, which dehydrates to nitrous oxide (N_2O) [9]. Therefore, direct detection of HNO in biological systems remains a challenge. Fluorescence techniques have served as a popular and robust tool for in situ visualizing biologically active important species in living systems due to excellent sensitivity, non-invasiveness, and well-defined spatiotemporal resolutions [10]. As a result, several HNO fluorescent probes have been

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exploited based on HNO-induced reduction of Cu(II)-complex to its corresponding Cu(I)-analogue [11]. However, these probes are prone to be interfered by abundant biological reductants such as glutathione (GSH) and ascorbic acid. To circumvent this limitation, some fluorescent probes for HNO using 2-(diphenylphosphino) benzoyl group as the recognition moiety have been developed recently [12]. These foregoing probes are reductant-resistant, thus its selectivity being improved significantly. Although these probes are reported to be able to image HNO inside live cells, none of them can specifically monitor HNO in mitochondria of living cells apparently due to the lack of a mitochondria-targetable group. Besides the lack of mitochondrial specificity, another major limitation associated with most of these HNO probes is that the excitation/emission wavelengths of them mainly are located in the UV-visible region [12a-c, 12f, 12g], whereas near-infrared (NIR) (650–900 nm) fluorescent probes are advantageous for cellular studies due to minimum photodamage to biological systems, deep tissue penetration, and lower background autofluorescence in living systems [13]. To the best of our knowledge, no mitochondria-targetable fluorescent probes for HNO in living cells have been reported to date.

Herein, we design and synthesize a mitochondria-targetable NIR fluorescent probe (**MitoHNO**) for HNO sensing under physiological conditions. The proposed probe is designed by using a novel merocyanine skeleton as the NIR fluorescent platform and 2-(diphenylphosphino)benzoyl group as the HNO recognition moiety (Scheme 1). In addition, the probe contains a lipophilic indolium unit as the mitochondria-targeted site. In the presence of HNO, **MitoHNO** exhibits a dramatic fluorescence turn-on response at λ_{max} = 727 nm. The probe has been proved to be able to detect mitochondrial HNO with high sensitivity and selectivity and applied for living cell imaging studies.

2. Experimental section

2.1. Materials and instruments

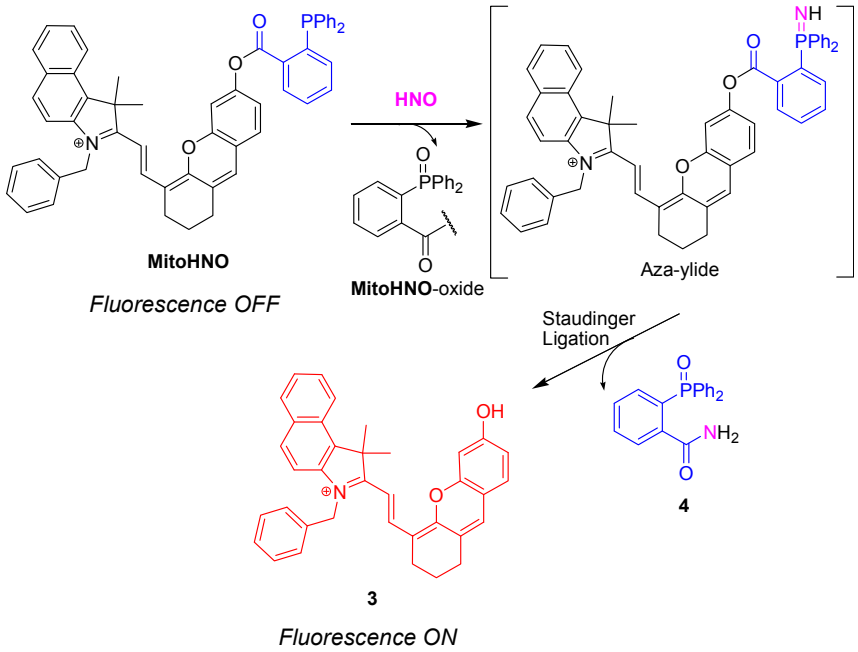
Unless stated otherwise, all chemicals were obtained from commercial suppliers and used without further purification. Solvents were dried by standard procedures before use. Flash chromatography was performed using Qingdao Ocean silica gel (200–300 mesh). Double-distilled water was used throughout all experiments. 4′,6-Diamidino-2-phenylindole (DAPI) was obtained from Sigma. MitoTracker Green FM was obtained from Molecular Probes (Eugene, OR). Human hepatocellular carcinoma SMMC-7721 cells were obtained from Cell Engineering Research Centre and Department of Cell Biology of Fourth Military Medical University (China).

The fluorescence spectra and relative fluorescence intensity were measured with a Shimadzu RF-5301 spectrofluorimeter equipped with a Hamamatsu (Japan) 928 photo multiplier tube. UV/Vis spectra were made with a Shimadzu UV-2550 spectrophotometer. High-resolution mass spectra (HRMS) were collected using a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics Corp., USA) with electrospray ionization (ESI) mode. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer, using tetramethylsilane (TMS) as the internal standard. Fluorescence imaging was performed by an Olympus FV1000 confocal laser scanning microscope (Japan). IR spectra were recorded on a Bruker Vertex 70 FT-IR spectrometer using a diamond ATR attachment. The pH was measured with a Sartorius PB-10 pH meter. Melting point was measured with X-4 melting-point apparatus with microscope (China).

2.2. Synthesis

2.2.1. Synthesis of compound *1*

2,3,3-Trimethyl-4,5-benzo-3H-indole (3.14 g, 15 mmol) and benzyl bromide (2.57 g, 15 mmol) were dissolved in toluene (20 mL) (Scheme 2). The reaction mixture was stirred at 100 °C for 20 h, cooled and added dropwise to ether (60 mL). The precipitate was filtered and washed with ether to afford the crude product, which was purified by recrystallization from methanol/ether (1:10, v/v) to give compound **1** as a beige solid (3.99 g, 70% yield). m.p. 161–162 °C. IR (ATR): $\bar{\upsilon}$ = 3397, 3053, 2976, 2712, 2662, 2588, 1923, 1820, 1642, 1582, 1524, 1464, 1390, 1279, 1213, 1151, 1008, 911, 804,



Scheme 1. Proposed reaction mechanism of **MitoHNO** toward HNO.