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A novel mitochondria-targeted fluorescent probe for imaging hydrazine in living cells, tissues and animals

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ABSTRACT

The cytotoxicity of hydrazine (N_2H_4) is closely associated with mitochondria damages, and real-timely detecting mitochondrial N_2H_4 is of great importance for the in-depth study of the pathophysiological functions of N_2H_4 in living system. Although some of N_2H_4 fluorescent probes have been developed, the selective fluorescence imaging of mitochondrial N_2H_4 has rarely been reported. Herein, we present a novel mitochondria-targeted fluorescent probe (**Rho-N_2H_4**) for imaging N_2H_4 in living system. In the **Rho-N_2H_4** system, a rhodamine derivative **Rho** with red emission was employed as the fluorophore, and acetyl group was utilized as response site for hydrazine. Upon treatment with N_2H_4, **Rho-N_2H_4** displayed a drastically fluorescent signal at 645 nm. With the aid of **Rho-N_2H_4**, the fluorescence imaging of mitochondrial N_2H_4 in living cells was achieved. Assisted by high-definition 3D imaging, **Rho-N_2H_4** can be applied for the imaging of N_2H_4 in living tissues with a penetration depth of about 50 μm with red emission manner. Furthermore, *in vivo* experiments demonstrated that **Rho-N_2H_4** can be capable of imaging N_2H_4 in living animals. We expect that **Rho-N_2H_4** could act as a promising tool for in-depthly unveiling the physiological and pathological roles of mitochondrial N_2H_4 in living system.

1. Introduction

Hydrazine (N_2H_4) has extensive applications in industrial production and pharmaceutical fields [1–5]. However, as the exposure to excessive amounts of N_2H_4 during manufacture and drug treatment can cause severely toxic effects to human [6–10]. It has been reported that the cytotoxicity of N_2H_4 is closely associated with mitochondrial damages. N_2H_4 displayed complicated effects on the metabolic activity of mitochondria, which involves: 1) the inhibition monoamine oxidases within mitochondria to disorder foreign amine compounds to enter the body and limit metabolic transformation of amine neurotransmitters; 2) the swelling of mitochondria and low matrical density to disrupt the internal homeostasis; 3) the interference α-ketoacid oxidation in Krebs cycle located in mitochondria. The N_2H_4-induced effects are closely related with hepatotoxicity, kidney and brain diseases [11–16]. Nevertheless, the pathophysiological effects of N_2H_4 on mitochondria are still not completely well-defined. Thus, detecting mitochondrial N_2H_4 is of great significance to reveal the roles of N_2H_4 in associated diseases.

To date, a variety of techniques such as chromatographic, electrochemical and titrimetry have been engineered for N_2H_4 detection [17–22]. Nevertheless, these approaches need destroy the living bio-samples, are complex and costly operations. Besides, they cannot real-timely monitor N_2H_4 in the native biological environment. By sharp contrast, fluorescence imaging has become a powerful technique for real-time imaging of various molecules in native biological systems because of its noninvasive manner, high sensitivity, real-time visualization and simplicity [23–28]. To date, some fluorescent probes have been designed for N_2H_4 in living cells [29–33]. However, mitochondrial-targeted fluorescence probes for N_2H_4 are very scarce [34]. Therefore, the development of mitochondria-targeted fluorescent probe for monitoring N_2H_4 is in an urgent need.

Herein, a novel mitochondria-targeted fluorescent probe (**Rho-N_2H_4**) for imaging N_2H_4 was designed and synthesized. The probe showed high sensitivity and selectivity for N_2H_4 detection. **Rho-N_2H_4** was successfully employed to image mitochondrial N_2H_4 in living cells. Moreover, **Rho-N_2H_4** was successfully employed for imaging N_2H_4 in living tissues and mice. We expect that **Rho-N_2H_4**

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could be a powerful tool to in-depthly study physiological and pathophysiological functions of mitochondrial N_2H_4.

2. Experiment section

2.1. Materials and instruments

Unless otherwise stated, all solvents and reagents involved in synthesis were obtained from commercial suppliers without further purification. All compounds were purified with silica gel (200–300 mesh) column, which were purchased from the Qingdao Ocean Chemicals. Then the compounds were characterized by NMR and High Resolution Mass Spectrum (HRMS). ^{1}H NMR and ^{13}C NMR were performed with the Bruker spectrometer (400 MHz), using TMS as an internal standard. HRMS was recorded with Bruker 9.4T Apex-ultra hybrid Qh-FTICR. The absorption spectra were recorded with a Shimadzu UV-2700 spectrophotometer, and the fluorescence spectra were measured with a Hitachi F-4600 spectrofluorimeter with a 1 cm standard quartz cuvette. Cell imaging experiments *in vitro* were performed with Nikon A1 Confocal Laser Scanning Microscope (CLSM). Experiments *in vivo* were received with Caliper Life Sciences animal imaging system.

2.2. Synthesis of ***Rho-N_2H_4***

As displayed in Scheme 1, the starting compound **Rho** was synthesized according to previously reported methods [35]. **Rho** (537 mg, 1 mmol) and acetic anhydride (204 mg, 2 mmol) were added in a solution of 5 mL dichloromethane. After stirring for 5 min, 0.1 mL triethylamine was dropped slowly in the solution, and stirring at temperature for 4 h. The reaction mixture was concentrated under vacuum, and then the crude product was purified by silica column chromatography (CH_2Cl_2:CH_3OH = 50:1) to afford white solid. (83.7 mg, yield 30%). The new compounds **Rho-N_2H_4** was well characterized by the ^{1}H NMR, ^{13}C NMR and HRMS. Besides, the LC–MS data further identified the purification of **Rho-N_2H_4**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65-8.67 (d, J = 5.0 Hz, 1H), 8.07-8.09 (d, J = 5.0 Hz, 1H), 7.64 (m, 2H), 7.55 (d, J = 2.1 Hz, 1H), 7.41 (dd, J = 8.8, 3.2 Hz, 2H), 7.18 (d, J = 6.9 Hz, 1H), 6.79-6.82 (d, J = 6.0 Hz, 1H), 6.67 (m, 2H), 6.45 (dd, J = 8.9, 2.2 Hz, 1H), 3.42 (q, J = 7.0 Hz, 4H), 2.39 (s, 3H), 1.23 (s, 6H). ^{13}C NMR (100 MHz,$CDCl_3$) δ 169.87, 169.46, 153.70, 152.44, 149.94, 149.63, 147.50, 134.99, 129.59, 128.93, 126.98, 125.12, 124.89, 124.16,124.08, 122.60, 121.98, 121.47, 118.57, 112.76, 108.97, 104.95, 97.69, 84.42,77.52, 77.21, 76.89, 44.50, 21.22, 12.57, 1.06. HRMS (ESI) m/z: [M] $^+$ calcd for $C_{30}H_{25}NO_5^+$, 480.1805; found, 480.1870.

2.3. Preparation of testing solutions

The stock solutions of **Rho** and **Rho-N_2H_4** were 1 mM in ethanol. 5 μM of probes were prepared with Britton-Robinson (B-R) buffer (5% ethanol, pH = 7.4) respectively. Solutions of testing in different pH (4.0–9.0) were prepared by B-R buffer according to standard methods. Solutions with amounts of interference species were prepared with 150 μM of Na_2S, Na_2SO_3,Na_2SO_4,Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Mg^{2+},Ni^{2+}, Na^+, NO_2^-, NO_3^-, H_2O_2, O_2^-, OH^-, $ONOO^-$, NO_2, Hcys, GSH, Cys, HClO, N_2H_4, respectively.

2.4. Determination of the fluorescence quantum yield

Fluorescence quantum yield (Φ) was determined by using Rhodamine 6G (Φ_r = 0.95 in ethanol) as a fluorescence standard. For measurement of the fluorescence quantum yield of **Rho**. The solution of 1 μM **Rho-N_2H_4** reacted with 100 μM N_2H_4 was used to measure the absorbance and fluorescence according to previous report [36], and the quantum yield was calculated using the following equation:

$$\Phi_S = \Phi_r (A_r F_s / A_s F_r) (n_s^2 / n_r^2)$$

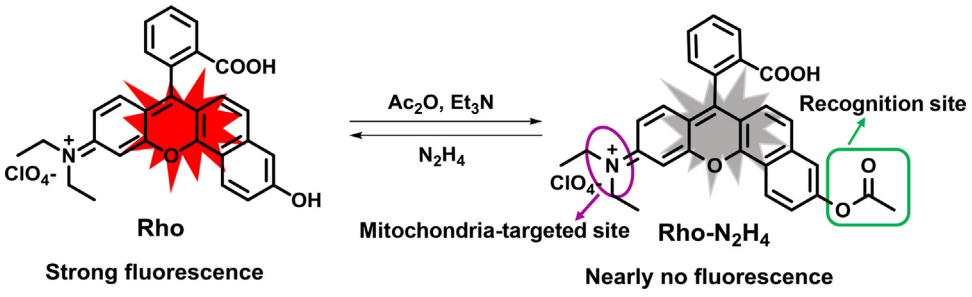
Where A_s and A_r are the absorbance of the sample and Rhodamine 6G respectively, F_s and F_r are the integrated fluorescence intensities, and n is the refractive index of the solvent.

2.5. Cell culture and cytotoxicity assay

HeLa (a human cervical carcinoma cell line) and 4T-1 cells (a murine mammary carcinoma cell line) were all cultured in 90% Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in an atmosphere of 37 °C and 5% CO_2. The MTT methods was adopted to assess the cytotoxicity of **Rho-N_2H_4**. Before experiments, HeLa and 4T-1 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. Then the fresh culture contained **Rho-N_2H_4** over a range of concentrations (0–20 μM) (n = 5) to substitute the previous media, and further incubation for 24 h. After that, 10 μL of MTT (5 mg/mL in PBS) was added into per well and incubated another 4 h. Finally, the media were discharged, and 100 μL of DMSO were loaded to solve the formazan crystals. The plate was shaken for around 10 min, and each well was analyzed by the microplate reader and detected at the absorbance of 490 nm.

2.6. Cellular imaging and subcellular localization study

HeLa and 4T-1 cells were seeded into 35 mm glass bottom dishes for 24 h incubation to render the confluence up to 80%. Then, fresh culture contained 5 μM of **Rho-N_2H_4** were added into dishes for culture another 30 min. After being washed with PBS twice, cells were treated with 25 μM and 50 μM of N_2H_4 for further incubation 40 min respectively. Lastly, cells were rinsed with PBS three times, and prepared for imaging with CLSM. For colocation experiments, after incubation with N_2H_4, both cells were further stained with Mito Tracker® Green FM (1 μL of 2 μM) for another 10 min. Fluorescence images of **Rho-N_2H_4** were



Scheme 1. Illustration of the mitochondria-targeted fluorescent probe for imaging hydrazine.