Sensors and Actuators B 248 (2017) 50–56

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

A mitochondrial-targeted two-photon fluorescent probe for imaging hydrogen sulfide in the living cells and mouse liver tissues

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ARTICLE INFO

Article history:
Received 11 January 2017
Received in revised form 20 March 2017
Accepted 27 March 2017
Available online 29 March 2017

Keywords:
Hydrogen sulfide
Mitochondria
Two-photon
Fluorescent probe
Living cells
Living tissues

ABSTRACT

Hydrogen sulfide (H_2S), as signaling molecule, plays a crucial role in many biological processes in living organisms. At the organelle level, we need to study tools to explain the complexity of physiological H_2S in mitochondria. Toward this goal, we have developed a new example of a fast responsive and mitochondrial-targeted two-photon fluorescent H_2S probe (**MT-HS**) with a large turn-on fluorescence signal (40-fold fluorescence enhancement). The addition of H_2S to **MT-HS** results a dramatic fluorescence enhancement around 540 nm. The probe could image exogenous and endogenous H_2S in living cells and the probe was located in mitochondria with high colocalization coefficient compared with Mitochondrial-Tracker. The large fluorescence enhancement of the two-photon probe **MT-HS** renders it attractive for imaging H_2S in living tissues with deep tissue penetration.

1. Introduction

Although hydrogen sulfide (H_2S) is generally considered to be a poisonous gas, it following nitric oxide (NO) and carbon monoxide (CO) is considered the third most important multifunctional signaling molecule and associated with diverse physiological processes [1–3]. H_2S is enzymically synthesized by enzymes (cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS)) in the metabolic pathway that regulates tissue turnover of the sulphur-containing amino acids L-methionine, L-homocysteine and L-cysteine to H_2S [4]. The study showed that the concentration of hydrogen sulfide was different in various areas of the organism, such as brain tissue contains approximately 50–160 μM, serum contain 50–100 μM and blood about 10–50 μM [5,6]. At appropriate concentrations H_2S can mediate healthy physiological effects including neuromodulation [2], regulation of blood pressure [7], angiogenesis [8], oxygen sensing [9], anti-inflammatory action [10]. However, high concentration of H_2S will cause both acute and chronic toxicity to the organism [11]. Numerous human diseases such as Alzheimer's [12], Down syndrome [13], diabetes, and liver cirrhosis [14,15] have shown correlation to the abnormal level of H_2S. Studies have implied that H_2S biology is associated with certain organelles such as mitochondria. H_2S protects cardiac muscle from myocardial ischemia/reperfusion injury by preserving mitochondrial function [16]. Mitochondria play a key role in cell death pathways. For example, mitochondrial dysfunction caused by oxidative stress leads to numerous neurodegenerative and cardiovascular diseases [17]. Therefore, it is importance to develop efficient methods for the detection of mitochondrial H_2S in biological systems for better understanding its physiological and pathological functions in mitochondria.

Several chemical tools including colorimetric [18], electrochemical assay [19], polarographic analysis [20], and sulfide precipitation[21] have been developed for the detection of H_2S. These methods were not suitable to observe the production of H_2S *in vitro* and *in vivo* as they require complicated sample preparation and extraction of H_2S by means of destruction of tissues or cells. In recent years, fluorescence imaging has emerged as a powerful means to track biomolecules in living systems, thanks to significant advances in selectivity, sensitivity, as well as real-time imaging. Some groups using fluorescent probes have made great progress in the detection of H_2S [22–24]. However, most of the probes are distributed throughout the cell and cannot detect H_2S in a particular organelle. Although some mitochondrial-targeted fluorescent H_2S probes have been reported [25–27], some of those probes were based on one-photon microscopy (OPM), and they need a short

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http://dx.doi.org/10.1016/j.snb.2017.03.135
0925-4005/

excitation wavelength, that may lead to damage in cells and tissues and photobleaching of probes, and thus, limit the applications in tissues. By contrast, two-photon microscope (TPM) can overcome this kind of defects and has the following advantages: reduce photodamage to biosamples; increase tissue penetration; lower background fluorescence; and facilitate three-dimensional imaging of living tissues. So this is still a strong need and requires challenging work to develop new mitochondrial-targeted two-photon fluorescent H_2S probe.

Herein, we report **MT-HS** as mitochondrial-targeted two-photon fluorescent H_2S probe which employed 1,8-naphthalimide as fluorescent chemoprobes and azido group as the responding site. We investigated its optical properties, response rate and selectivity toward various reactive sulphur, reactive oxygen species (ROSs) and reactive nitrogen species (RNSs). The results suggest that it possesses a large turn-on fluorescence signal (40-fold fluorescence enhancement) and fast response rate (5–6 min). The cell imaging confirmed that it can be used effectively as an indicator to monitor the level of endogenous or exogenous H_2S in mitochondria, and the probe **MT-HS** is suitable for fluorescence imaging of H_2S in living tissues with deep penetration by using two-photon microscopy.

2. Experimental

2.1. Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. High resolution mass spectrometric (HRMS) analyses were measured on a Finnigan MAT 95 XP spectrometer; NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using TMS as an internal standard; Absorption spectra were obtained on a Shimadzu UV-2700 Power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; The fluorescence imaging of cells were performed with Nikon A1MP confocal microscopy; The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

2.2. Synthesis

With an inert atmosphere of nitrogen, to the mixture of compound 2 (350 mg, 0.54 mmol, 1.0 eq) in DMF (3 mL) was added NaN_3 (42 mg, 0.65 mmol, 1.2 eq). The reaction was stirred for 10 h at room temperature. 25 mL H_2O was added and the mixture was extracted with 50 mL dichloromethane thrice. The combined extracts were washed with water, brine and dried over Na_2SO_4. Concentration and chromatograph on silica gel (30:1 CH_2Cl_2/MeOH). This resulted in 200 mg (65%) of **MT-HS** as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.47–8.40 (m, 1H), 8.40–8.35 (m, 1H), 8.25–8.18 (m, 1H), 7.96–7.88 (m, 3H), 7.87–7.74 (m, 13H), 7.72 (d, J = 8.0 Hz, 1H), 4.23 (t, J = 7.3 Hz, 2H), 3.59 (d, J = 14.3 Hz, 2H), 3.20 (d, J = 6.0 Hz, 2H), 2.46 (t, J = 7.3 Hz, 2H), 1.65 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.2, 152.4, 144.3, 143.0, 141.3, 139.9, 137.9, 135.8, 130.4, 129.5, 129.4, 128.84, 128.14, 127.74, 127.64, 127.1, 125.5, 125.2, 124.6, 121.8, 120.6, 120.5, 113.5, 112.5, 110.2, 110.1, 102.7, 101.9, 67.0, 47.5, 47.2, 25.9; HRMS (ESI) m/z calcd for $C_{46}H_{48}N_6O_5$ $[M+H]^+$: 612.2165; found 612.2156.

2.3. Preparation of solutions of probe *MT-HS* and analytes

Unless otherwise noted, all the measurements were made according to the following procedure. A stock solution (1.0 mM) of **MT-HS** was prepared by dissolving the requisite amount of it in DMSO. In a 10 mL tube the test solution of compounds **MT-HS** was prepared by placing 0.1 mL of stock solution, 2.9 mL of DMSO, 3 mL of 0.1 M PBS buffer (different pH) and an appropriate volume of Na_2S sample solution. After adjusting the final volume to 10 mL with distilled-deionized water, standing at room temperature 3 min, 3 mL portion of it was transferred to a 1 cm quartz cell to measure absorbance or fluorescence. The stock solutions of analytes for selectivity experiments were prepared respectively by dissolving NaBr, NaF, $NaHPO_4^-$, $NaHSO_3$, $Na_2S_2O_3$, Na_2SO_3, NO (NONOate), N_2H_4, $NaNO_2$, $NaNO_3^-$, Hcy, GSH, Cys, CH_3CO_3H, TBHP, DBBP, HClO, H_2O_2, ·OH ($FeSO_4$ and H_2O_2), in twice-distilled water. The slight pH variations of the solutions were achieved by adding the minimum volumes of NaOH (0.1 M) or HCl (0.2 M).

2.4. Cytotoxicity assays

In vitro cytotoxicity was measured using the colorimetric methyl thiazolyl tetrazolium (MTT) assay in HeLa cells. Cells were seeded into the 24-well tissue culture plate in the presence of 100 μL Dulbecco's modifed eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO_2 atmosphere for overnight and then incubated for 24 h in the presence of **MT-HS** at different concentrations (0, 1, 5, 10, 20, 30 μM). Then cells were washed with PBS buffer and 100 μL supplemented DMEM medium was added. Subsequently, 10 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. Violet formazan was dissolved in 100 μL sodium dodecyl sulfate solution in the water-DMSO mixture. Absorbance of the solution was measured at 570 nm using a microplate reader. The cell viability was determined by assuming 100% cell viability for cells without **MT-HS**.

2.5. Cells culture and imaging of exogenous H_2S in HeLa cells

Before the experiments, the well prepared cells were washed with PBS (pH = 7.4) buffer three times. Subsequently, incubating with probe **MT-HS** (10 μM) (containing 0.1% DMSO as a cosolvent) for another 20 min at 37 °C, the HeLa cells were rinsed with PBS three times, and the cells were incubated with Na_2S for 30 min at 37 °C, and then washed with PBS three times, and the fluorescence images were acquired through a Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera.

2.6. Cells culture and imaging of endogenous H_2S in HeLa cells

Before the experiments, the well prepared cells were washed with PBS (pH = 7.4) buffer three times. HeLa cells were treated with 10 μM **MT-HS** for 20 min and then washed with PBS three times to remove the probe left in solution and optimize the background signal, the cells incubated with 100 μM cysteine for 2 h in an atmosphere of 5% CO2 and 95% air. For the control experiments, the cells were treated with 10 μM **MT-HS** for 20 min and then washed with PBS three times, and then cultivated for 2 h under the same conditions. For negative control group, the HeLa cells incubated with probe **MT-HS** (10 μM) for 20 min at 37 °C, washed by PBS buffer and subsequently incubated with 100 μM cysteine and DL-propargylglycine (PAG, 200 μM) for 2 h prior to imaging. Before imaging, cells were washed with PBS three times, and the fluorescence images were acquired through a Nikon A1MP confo-