Sensors and Actuators B 254 (2018) 411–416

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

A reaction based one- and two-photon fluorescent probe for selective imaging H_2O_2 in living cells and tissues

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ARTICLE INFO

Article history:
Received 7 June 2017
Received in revised form 15 July 2017
Accepted 17 July 2017
Available online 19 July 2017

Keywords:
Fluorescence probe
Enamine
H_2O_2
Two-photon
Imaging

ABSTRACT

Hydrogen peroxide (H_2O_2) exists extensively in mammalian cells as an active and significant by-products in bio-metabolism and plays vital roles in normal physiological activities and the occurrence of many diseases. Thus, development of fluorescence probes for monitoring H_2O_2 is profound for the diagnosis and treatment of these diseases. Other than previous reported fluorescence probes for H_2O_2, we proposed a two-photon fluorescence probe **GC-2** possessing an original response enamine group, which can undergo the cleavage of carbon-carbon double bond by H_2O_2 and result in the appearance of blue fluorescence due to Intramolecular Electron Transfer (ICT). The probe **GC-2** exhibited satisfactory sensitivity to H_2O_2 with the limitation of detection (LOD) of 2.1×10^{-7} M, and displayed good selectivity to H_2O_2 from other ROS and amine acids under physiological pH conditions. Remarkably, our probe **GC-2** showed admirable one- and two- photon fluorescence imaging capability in living cells and tissues with low cytotoxicity. In a word, the rational designed probe has tremendous potential for monitoring H_2O_2 in living systems utilizing the one- and two- photon fluorescence imaging.

1. Introduction

Hydrogen peroxide (H_2O_2), a kind of Reactive Oxygen Species which are inevitable by-products in metabolism, plays crucial roles in biological system [1]. H_2O_2 in physiological level is capable of adjusting fundamental bioactivities [2–4]. However, there are increasingly available evidence indicating that the over-production of H_2O_2 in the body could cause pathological changes such as DNA damage, aging, and neurodegenerative diseases [5–8]. And the aberrant generation of H_2O_2 is closely related to the occurrence of malignant tumor, Parkinson's disease, Alzheimer disease and other diseases [8–11]. A substantial challenge in illuminating the relationship between H_2O_2 and these diseases is the realization of monitoring H_2O_2 in complex biological environments.

Nowadays, electrochemical method, spectrophotometry, chemiluminescence and fluorescence probes are employed to monitor the content of H_2O_2 for the prevention, diagnosis and treatment of related diseases [12]. Among these, fluorescence probes exhibited advantages of good selectivity, high sensitivity and simple operation [13,14]. Thus, the development of sensitive and selective sensors for H_2O_2 has stimulated intense interest in the analytical chemistry and biochemistry.

The principle of two-photon (TP) excited fluorescence is that two-photon are simultaneously absorbed to an excited-state via a virtual state [15]. Compared to traditional one-photon (OP) excited fluorescence, TP fluorescence imaging contains multiple advantages of efficient light detection, reduced fluorescence photobleaching, and improved image resolution [16]. And due to the employed excitation light is at long waveband, TP fluorescence can be imaged with deeper tissue penetration and reduced phototoxicity. Recently, several fluorescence probes for detecting H_2O_2 have been reported [17–21]. Most of response group in these fluorescence probes are based on benzenesulfonyl ester or boronate ester chemistry [22–25]. However, their reaction rates and fluorescence background levels need to be further improved for biological applications [26]. The development of novel response group specific for H_2O_2 is still in demand.

Herein we presented a novel one- and two-photon fluorescence probe **GC-2** for H_2O_2. To the best of our knowledge, this is the first report that enamine was employed as response group in H_2O_2 fluorescence probe. After specifically reacted with H_2O_2 under physiological condition, the chromophore 1,8-naphthalimide could turn on the blue fluorescence due to Intramolecular Electron Transfer (ICT). From the experiment we can conclude that the sensitivity and selectivity of the probe is admirable. Moreover, the probe **GC-**

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http://dx.doi.org/10.1016/j.snb.2017.07.133
0925-4005/

2 can be applied in one- and two-photon fluorescence imaging in living cells and tissues with low cytotoxicity.

2. Experiment 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. The One Drop spectrophotometer (OD-1000+, Nanjing, China) was applied to measure the UV–vis absorption spectra. Fluorescence spectra were determined on PerkinElmer LS55 spectrophotometer in 1×1 cm quartz cells with both excitation and emission slit widths of 5 nm. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance at 300-MHz; TMS was used to measure the values of δ in ppm. Mass data (ESI) was recorded on the quadruple mass spectrometry. High Resolution Mass Spectrometry (HRMS) was recorded on Agilent 6200 Series TOF and 6500 Series Q-TOF. The one-photon and two-photon laser confocal fluorescence microscopy (FluoView^{TM}, FV1000, Olympus, Japan) was used to obtain laser confocal cell and tissue imaging.

2.2. Synthesis of probe *GC-2*

The commercial compound **GC-1** (268 mg, 1 mmol) was dissolved in 20 mL toluene. Then acetylacetone (0.5 mL) and *p*-toluenesulfonic acid (PTS, 17.2 mg, 0.1 mmol) were added to the solution. The mixture was heated to reflux for 48 h. After the reaction completed, the solvent was removed under vacuum. The crude residue was purified by silica column chromatography using AcOEt/petroleum ether (2:1) to afford the probe **GC-2** as yellow solid (196 mg, 56%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 13.37 (s, 1H), 8.64 (d, J = 7.2 Hz, 1H), 8.56–8.48 (m, 2H), 7.80 (t, J = 7.9 Hz, 1H), 7.46 (d, J = 7.9 Hz, 1H), 5.46 (s, 1H), 4.21–4.16 (m, 2H), 3.24 (s, 3H), 2.16 (s, 3H), 1.75–1.67 (m, 2H), 1.49–1.42 (m, 2H), 0.98 (t, J = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.0, 191.1, 164.1, 158.7, 133.6, 131.6, 131.3, 129.2, 128.7, 127.3, 126.6, 126.4, 124.9, 123.1, 120.5, 119.2, 109.6, 100.9, 40.3, 31.4, 30.2, 29.6, 29.3, 20.3, 13.8. ESI–MS: 373.2 $[M+Na]^+$, 349.2 $[M-H]^-$. HRMS: Calcd. for $[M-H]^-$, 349.1552; Found, 349.1571.

2.3. Synthesis of compound *GC-3*

Compound **GC-1** (268 mg, 1 mmol) was added to 10 mL acetic anhydride. The mixture was warmed to 60 °C for 3 h. After the reaction completed, the solvent was removed and the residue was purified by silica column chromatography using AcOEt/petroleum ether (1:1) to afford compound **GC-3** as solid (247 mg, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62–8.56 (m, 2H), 8.35 (s, 1H), 8.16 (d, J = 8.4 Hz, 1H), 7.86 (s, 1H), 7.78–7.72 (m, 1H), 4.17 (t, J = 7.5 Hz, 2H), 2.37 (s, 3H), 1.77–1.69 (m, 2H), 1.50–1.41 (m, 2H), 0.98 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 163.5, 138.4, 132.1, 131.2, 126.8, 126.2, 123.5, 119.8, 40.3, 30.2, 24.6, 20.4, 13.8. ESI–MS: 333.1 $[M+Na]^+$, 309.1 $[M-H]^-$. HRMS: Calcd. for $[M-H]^-$, 309.1239; Found, 309.1259.

2.4. Spectroscopic properties and optical response to H_2O_2

The absorption and fluorescence spectra of **GC-2** with H_2O_2 reactions were performed at 37 °C in a 10 mL total volume of PBS buffer (10 mM, pH 7.4) in tubes for 30 min.

2.5. Sensing mechanism study of *GC-2* with H_2O_2

GC-2 (30 mg) and H_2O_2 (10 mL, 30%) were oscillated in shaking table at 37 °C for 1 h. The filtrate was collected and was extracted with dichloromethane for three times. The collected organic layers were removed under reduced pressure. The residue was purified with silica gel column. MS-ESI: 309.1 $[M-H]^-$.

2.6. Cell experiments

Human liver carcinoma HepG2 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2 cells were cultured in McCoy's 5a Medium containing 10% fetal bovine serum (FBS, Hyclone, containing 80 U/mL penicillin and 0.08 mg/mL streptomycin) in a 5% CO_2/95% air (v/v) incubator.

2.7. In vitro cytotoxicity assay

HepG2 cells were cultured in 96-well plates at the density of 50,000–100,000 cells per well and cultured at 37 °C in a 5% CO_2/95% air (v/v) incubator for 24 h. The cultures were removed and 200 μL different probe concentrations of probe **GC-2** (0, 10, 20, 30, 40, 50 μM) were added to continue culturing for 12 h. Then per well was added MTT solution (20 μL, 5 mg/mL) and cultured for another 4 h. The culture medium was replaced with 150 μL of DMSO to dissolve the crystallized product formazan. Finally the absorbance in each well of the 96-well plates was measured at 490 nm with a multi-well plate reader. The cell viability was calculated using the following formula: Cell viability = (the optical density of test wells – the optical density of medium control wells)/(the optical density of untreated wells – the optical density of medium control wells) × 100%. Each concentration was measured in triplicate and used in three independent experiments.

2.8. Cell imaging

HepG2 cells were plated on 15 mm observation dish and incubated with medium containing 10% fetal bovine serum for a day. The medium was replaced with **GC-2** (10 μM) and PBS and the cells were incubated for 30 min as the control group. For the second group, the cells were incubated with **GC-2** (10 μM) for 30 min, and then the H_2O_2 (100 μM) was added and incubated for another 30 min. For the third group, the cells were incubated with LPS for 12 h, and then **GC-2** (10 μM) was added and incubated for 30 min. Then each well was washed with PBS (10 mM, pH 7.4) for three times. Fluorescent images were obtained on one- and two-photon laser confocal scanning microscope (LCSM) with an objective lens (×60) (λ_{ex} = 405 and 750 nm for one-photon and two-photon respectively).

2.9. Tissue imaging

Mice with six weeks to eight weeks old were purchased for slice imaging. Liver obtained from a healthy mouse, then embedded it in the embedding medium (PVA) and frozen in −80 °C refrigerator for 1 h, freezing-microtome cut off the liver into 150 μm slice. First, stained the slice with probe **GC-2** (100 μM) for 1 h. Second, the whole solution was replaced by H_2O_2 (500 μM) and further standing for another 1.5 h, then washed slice with PBS (10 mM, pH 7.4) for three times. Finally, the z-scan mode of laser scanning confocal microscope was utilized to record the fluorescence intensities at different depths of the slice. Lipopolysaccharide (LPS, 2 mg/mL, 100 μL) was injected in tumor to induce inflammation and produced more ROS, and injected 100 μL **GC-2** (100 μM) after 14 h. The sectioned tumor slice was then subjected to confocal tissue imaging.