Sensors and Actuators B 250 (2017) 259–266

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Research Paper

A two-photon fluorescent probe for endogenous superoxide anion radical detection and imaging in living cells and tissues

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ARTICLE INFO

Article history:
Received 3 February 2017
Received in revised form 5 April 2017
Accepted 8 April 2017
Available online 10 April 2017

Keywords:
Endogenous O2⁻ detection and imaging
Two-photon fluorescent probe
Turn-on response
Cell imaging
Tissue imaging

ABSTRACT

Superoxide anion radical (O_2^-), the "primary" reactive oxygen species (ROS) in living systems, is linked to a variety of physiological and pathological processes. Therefore, developing an effective strategy to monitor the fluctuation of O_2^- in biological systems is of great importance. This paper describes a new turn-on two-photon fluorescent probe for endogenous O_2^- detection and imaging, which was rationally designed and synthesized *via* a non-redox strategy. In the presence of O_2^-, the probe exhibited notable fluorescence enhancement (~235-fold) with a low detection limit down to 1 nM, indicating a high signal-to-background ratio and excellent sensitivity. In addition, short response time, good biocompatibility, low cytotoxicity, long-term stability against light illumination, specificity to O_2^- over general reductants, and pH stability were demonstrated, indicating that the requirements for cellular O_2^- determination are met. Furthermore, the probe was successfully applied in two-photon fluorescence imaging of endogenous O_2^- in living cells and tissues and showed high imaging resolution and a deep-tissue imaging depth of ~150 μm, illustrating the promising potential for practical applications in complex biosystems and providing a valuable theoretical basis and technical support for the study of physiological and pathological functions of O_2^-.

1. Introduction

Superoxide anion radical (O_2^-), the "primary" reactive oxygen species (ROS) in living systems, is involved in cellular signaling transduction and implicated in various diseases [1–4]. Firstly, O_2^- can be served as a precursor for many ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) [5]. Secondly, O_2^- can act as a mediator in oxidative chain reactions. For example, the concentrations of cellular O_2^- and nitric oxide (NO) are in balance and always interact with each other. Excessive production of O_2^- will inhibit NO bioavailability, leading to endothelial dysfunction [6,7]. Thirdly, overgeneration of O_2^- is related to many oxidative-stress-induced diseases, such as degenerative disorders and ischemia-reperfusion (IR) injury in surgery [8,9]. In a word, it is important to develop an efficient analytical strategy for endogenous O_2^- detection in live cells and tissues.

Fluorescence analytical methods are ideal choices for explorations of endogenous O_2^- fluctuations because of their convenient preparation, high sensitivity, rapid response, and capability for real-time imaging [10–14]. In the last decade, many fluorescent probes for O_2^- detection have been reported, including hydroethidine (HE) and its mitochondria-targeting analogue (Mito-HE or MitoSOX) [15,16]. However, these hydroethidine-based probes usually suffer from the limitation of non-specific staining with other oxidants [15–17], resulting in poor selectivity and sensitivity. Meanwhile, interferences from common cellular reductants (e.g. Fe^{2+}, ascorbic acid, and glutathione) also hamper the usage of these O_2^- fluorescent probes in cell imaging [18–20]. Thus, designing a fluorescent probe without need for a redox reaction is urgently desired to render a high degree of selectivity for O_2^- in complex biological systems. Furthermore, many fluorescent probes are limited in biological applications by the shallow tissue-penetration depth, photobleaching, and autofluorescence of biosamples resulting from the short excitation wavelength (usually < 500 nm).

Two-photon (TP) fluorescent probes, which are excited by near-infrared (NIR) laser pulses, offer a means to overcome the above-mentioned disadvantages. Compared with one-photon excited fluorescent probes, TP fluorescent probes exhibit higher

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http://dx.doi.org/10.1016/j.snb.2017.04.041
0925-4005/

imaging resolution, larger depth penetration, lower background fluorescence, reduced photodamage, and better three-dimensional spatial localization [21–23]. Therefore, TP-based biosensing and bioimaging systems are promising candidates for various biological applications. Considering all of these advantages, design of a TP fluorescent probe for endogenous O_2^- detection without need for a redox reaction is crucial. However, to our best knowledge, only a few TP fluorescent probes for O_2^- detection based on a non-redox strategy have been reported and the detection limit of the reported probe was estimated to be 23 nM [24]. Thus, an ideal TP fluorescent probe for *in vivo* imaging of O_2^- with superior sensitivity and optical performance is still needed.

Herein, we propose a new turn-on two-photon fluorescent probe (probe **1**) based on a non-redox mechanism to detect O_2^- in living systems. Compared with the reported TP fluorescent probe based on a non-redox strategy [24], probe **1** exhibits the similar selectivity and better sensitivity with the detection limit as low as 1 nM. The fluorescent probe consists of a donor-π-acceptor (D-π-A) structured naphthalene derivative as a potent TP fluorophore and a trifluoromethanesulfonate group as an O_2^- recognition moiety (Scheme 1). The D-π-A structured naphthalene derivative has proven to be a powerful platform for TP probe design for a variety of analytes [25–27] due to its effective TP excitation action cross-section, low cytotoxicity, good biocompatibility and photostability [26,28,29]. Inspired by previous work [24,30,31], we protected the phenol OH group of the naphthalene derivative by converting it to a trifluoromethanesulfonate group. As a result, the synthesized probe **1** exhibits weak fluorescence emission, leading to a low fluorescence background signal. In the presence of aqueous O_2^-, remarkable one-photon and TP-excited fluorescence enhancement can be observed with detection limit as low as 1 nM. Probe **1** displays rapid response and good selectivity toward O_2^- over other ROS, reactive nitrogen species (RNS), and common cellular reductants, as well as excellent stability over a broad range of pHs. More importantly, probe **1** can be applied in direct TP imaging of endogenous O_2^- in living cells and tissue slices with satisfactory sensitivity and depth penetration.

2. Experimental

2.1. Reagents and instruments

All solvents and chemicals were purchased from commercial suppliers and were used without further purification unless otherwise stated. Water used throughout all experiments was purified using a Millipore system to a resistivity of 18.2 MΩ cm (Millipore, USA). Column chromatography was conducted on silica gel (200 – 300 mesh) and thin layer chromatography (TCL) was performed using silica gel 60 F254 (Qingdao Ocean Chemicals, Qingdao, China). Electrospray ionization (ESI) mass spectra were acquired on an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). NMR spectra were recorded on a Bruker DRX-400 spectrometer and a Bruker DRX-500 spectrometer using TMS as an internal standard (at 400/500 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR). All chemical shifts are reported in the standard δ notation of parts per million. The pH values were measured with a Mettler-Toledo Delta 320 pH meter. Absorption spectra were obtained on a Shimadzu 2450 UV – visible Spectrophotometer (Tokyo, Japen) with a 1 cm quartz cuvette. The one-photon excited fluorescence spectra were conducted on a Fluoromax-4NIR spectrofluorometer (HORIBA JobinYvon, Edison, NJ) at room temperature with a 1 cm quartz cuvette. For the dramatic time-dependent fluorescence response of probe **1**, fluorescence spectra were conducted on a Fluoromax-4 spectrofluorometer (HORIBA JobinYvon, Edison, NJ) with a temperature controller set at 25 °C. Both the excitation and emission bandwidths were set at 5.0 nm. TP fluorescence images of RAW 264.7 cells (mouse macrophage cells) and mouse liver slices were obtained by using an Olympus FV1000-MPE multi-photon laser scanning confocal microscope (Japan) with 720 nm as the excitation wavelength.

2.2. Synthesis of compound 2

First, 2-aminothiophenol (1.4 g, 11 mmol) was dissolved in ethanol (5 mL). This solution was then dropped into a solution of 6-hydroxy-2-naphthaldehyde (1.7 g, 10 mmol) and p-toluenesulfonic acid monohydrate (1.90 g, 10 mmol) in ethanol (35 mL). The mixture was refluxed for 12 h (Scheme 2). After the solution was concentrated *in vacuo*, the product was purified by column chromatography (petroleum ether/ethyl acetate = 6:1)to give compound **2** as a yellow solid (2.1 g, 78%). ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 10.13 (s, 1H), 8.54 (s, 1H), 8.14-8.13 (m, 1H), 8.08 (d, J = 8.7 Hz, 1H), 8.04 (d, J = 8.1 Hz, 1H), 7.99 (d, J = 8.6 Hz, 1H), 7.84 (d, J = 8.6 Hz, 1H), 7.55-7.52 (m, 1H), 7.46-7.43 (m, 1H), 7.21 (s, 1H), 7.19-7.17(m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm): 167.70, 157.14, 153.68, 136.14, 134.33, 130.71, 127.34, 127.29, 127.25, 127.09, 126.57, 125.27, 124.16, 122.59, 122.25, 119.77, 108.91. MS (EI) m/z, found: 277.0 (M^+); calculated: 277.0 (M^+).

2.3. Synthesis of probe 1

A solution of compound **2** (280 mg, 1.00 mmol) was dissolved in anhydrous pyridine (5 mL) and dry dichloromethane (DCM, 5 mL) at −78 °C. Trifluoromethanesulfonic anhydride (340 μL, 2.00 mmol) was added to the solution dropwise under nitrogen atmosphere (Scheme 2). The mixture was kept stirring at −78 °C for 20 min and then at room temperature for another 25 min. Next, $NaHCO_3$ (aq) was added to the solution to quench the reaction at room temperature. The reaction solution was diluted with 100 mL ethyl acetate and then washed three times with HCl (150 mL, 1 M) and water (100 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The targeted compound **1** (probe **1**) was achieved as an ivory-white solid by silica gel column chromatography purification (petroleum ether/ethyl acetate = 1:1, v/v). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.83 (s, 1H), 8.41-8.39 (d, J = 16 Hz, 1H), 8.37-8.34 (dd, J_1 = 4 Hz, J_2 = 4 Hz, 1H), 8.27-8.25 (m, 2H), 8.22-8.20 (d, J = 8 Hz, 1H), 8.14-8.12 (d, J = 8 Hz, 1H), 7.74-7.71 (dd, J_1 = 4 Hz, J_2 = 4 Hz, 1H), 7.62-7.58 (t, J = 8 Hz, 1H), 7.53-7.50 (t, J = 6 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm): 166.88, 153.79, 147.99, 134.93, 134.49, 132.37, 132.28, 131.94, 131.89, 129.59, 127.45, 127.06, 126.06, 125.87, 123.27, 122.70, 121.07, 120.13, 119.76, 119.69, 116.94, 55.19. MS (EI) m/z, found: 410.0 (M^+); calculated: 409.4 (M^+).

2.4. Spectral measurements

Due to the poor water solubility of probe **1,** an appropriate amount of the compound was dissolved in DMSO solvent to form a 1 mM stock solution. KO_2 powder was dissolved in dry dimethylsulfoxide (DMSO) solvent by adding a suitable amount of 18-crown-6 to increase the solubility. By using a UV – visible spectrophotometer and the extinction coefficient constant (21.1 mM^{-1} cm^{-1}) of ferrocytochrome c at 550 nm, the concentration of generated O_2^- could be calculated after recording the reduction of ferricytochrome c spectrophotometrically.

For the fluorescence response of probe **1** toward O_2^-, probe **1** stock solution (1 mM, 2 μL) and O_2^- at a certain concentration (2 μL) were diluted to 200 μL with PBS buffer (10 mM, pH 7.4) in a test tube. As a result, the detection system was PBS (10 mM, pH 7.4) buffered DMSO (2%, v/v) solution. The resulting mixture was kept at room temperature for 5 min and then the fluorescence intensity