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A two-photon fluorescent probe for exogenous and endogenous superoxide anion imaging *in vitro* and *in vivo*

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ABSTRACT

Herein, we report a novel quinoline derivative-based two-photon fluorescent probe 6-(dimethylamino) quinoline-2-benzothiazoline (**HQ**), which is capable of tracking superoxide anion in organisms with specific "turn-on" fluorescence response based on extension of π-conjugations and moderate ICT process. The probe exhibited favorable photophysical properties, a broad linear range and high photostability. It can specifically detect superoxide anion with a significant fluorescence enhancement and great linearity from 0 to 500 μM in PBS buffer. Furthermore, **HQ** shows low cytotoxicity and excellent photostability toward living cells and organisms, which was able to monitor endogenous superoxide anion fluxes in living cells and *in vivo*. For the first time, endogenous superoxide anion in lung inflammation was visualized successfully by using **HQ** through two-photon microscopy, and the probe **HQ** shows great potential for fast *in-situ* detecting of inflammatory response in live organisms.

1. Introduction

Reactive oxygen species (ROS) are a group of substances derived from oxygen molecule, involving superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and the singlet oxygen ($^1O_2$) (Dickinson and Chang, 2011; D'Autréaux and Toledano, 2007; Sena and Chandel, 2012). Overproduction of ROS results in excessive oxidative stress and disruption of the homeostasis, which is closely linked with the first-line defense of mammalian phagocytes in innate immunity (Nathan and Cunningham-Bussel, 2013; Imlay, 2013, 2008). Furthermore, as a by-product of oxidative energy metabolism, superoxide anion ($O_2^{\bullet -}$) is not only a remarkable precursor of endogenous ROS but also a sink for most of radicals generated intracellularly (Zhang et al., 2016; Laura and MacManus-Spencer, 2005; Brand, 2010). Free radicals produced via numerous physiological processes can regulate various of biological activities like cellular signaling, host defense and inflammation (Bókkon, 2012; Gao et al., 2007). Noteworthily, superoxide anion ($O_2^{\bullet -}$), a reactive oxygen radical, has been proved to play an essential role in inflammatory diseases (Kundu et al., 2009; Shepherd et al., 2007). However, due to the fact that all the reactive oxygen species possess similar chemical properties including widespread compartmental distribution, high reactivity and short half-life, it is difficult to identify and quantify single ROS in cells and tissues (Dickinson and Chang, 2011; Hu et al., 2015). Therefore, methods for recognition and tracking of superoxide anion ($O_2^{\bullet -}$) in living systems for investigating the relation and mechanism between superoxide anion levels and inflammatory diseases should be critically important.

There have been a variety of methods reported for the detection of $O_2^{\bullet -}$, such as electron paramagnetic resonance (EPR), high performance liquid chromatography (HPLC), electrochemical sensors, mass spectrometry (MS), and optical methods (Si et al., 2015; Abbas et al., 2014; Yasui and Sakurai, 2000). Although the aforementioned analytical methods can effectively measure the level of $O_2^{\bullet -}$, the limitation is obvious, *i.e.*, most of these methods are only suitable for detection of extracellular radicals (Chen et al., 2004; Hong et al., 2014). In order to monitor $O_2^{\bullet -}$ in live cells and *in vivo*, there is a growing interest to develop small$^-$molecule based fluorescent probes in view of their high sensitivity, excellent specificity, and nondestructive visibility (Anand et al., 2014, 2015a, 2015b; Sivaraman et al., 2012, 2014a, 2014b, 2014c; Vengaian et al., 2016).

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Till now, some elegant fluorescent probes for $O_2^{\bullet -}$ measurement in living biological samples have been reported (Ma et al., 2009; Manjare et al., 2014; Yu et al., 2015; Zhang et al., 2015; Murale et al., 2013; Maeda et al., 2005). Despite their considerable contributions to $O_2^{\bullet -}$ related studies, the application of fluorescent probes for $O_2^{\bullet -}$ detection and imaging in tissues and live organisms is still limited, mainly because most of the probes are one-photon (OP) excited fluorescent probes, which usually excited at UV–Vis wavelengths (<550 nm) (Anand et al., 2015a, 2015b; Sivaraman et al., 2013; Sivaraman et al., 2014a, 2014b, 2014c; Sunnapu et al., 2016). Compared to one-photon excited fluorescent probes, two-photon (TP) excited fluorescent probes with excitation wavelengths in near infrared region (700–900 nm) have been drawing increasing attention in the past decade (Xu et al., 2007; Robinson et al., 2006). The advantages of two-photon microscopy (TPM) make it a powerful technique for *in vivo* imaging, which can provide minimum photodamage to biological samples, weak autofluorescence and self-absorption, large penetration depth and low photobleaching of fluorophores (Kim and Cho, 2009; Phan and Bullen, 2010; Dong et al., 2009). Nonetheless, TP fluorescent probe for detecting $O_2^{\bullet -}$ that can be applied to evaluate the relation between superoxide anion levels and various pathological processes *in vivo* was rarely reported (W. Zhang et al., 2013; X. Zhang et al., 2013; Li et al., 2013). Moreover, some major problems have yet to be addressed, such as the low selectivity over competing ROSs, long reaction time, and narrow linear response.

In this work, we developed a quinoline derivative-based two-photon fluorescent probe **HQ** for selectively monitoring superoxide anion fluxes in organisms, which shows a specific "turn-on" fluorescence response toward superoxide anion as a result of extended π-conjugations and moderate internal charge transfer (ICT) process demonstrated by theoretical calculations. Fluorescence detection *in vitro* and *in vivo* reveals that **HQ** is a reliable fluorescent probe for specific detection of endogenous superoxide anion with a wide linear range. In addition, **HQ** was utilized to detect the superoxide anion level produced in the process of lung inflammation in model animals for the first time, which suggests that **HQ** may hold great promise in studying roles of superoxide anion in various pathological processes in the future.

2. Materials and method

2.1. Materials and Instrumentation

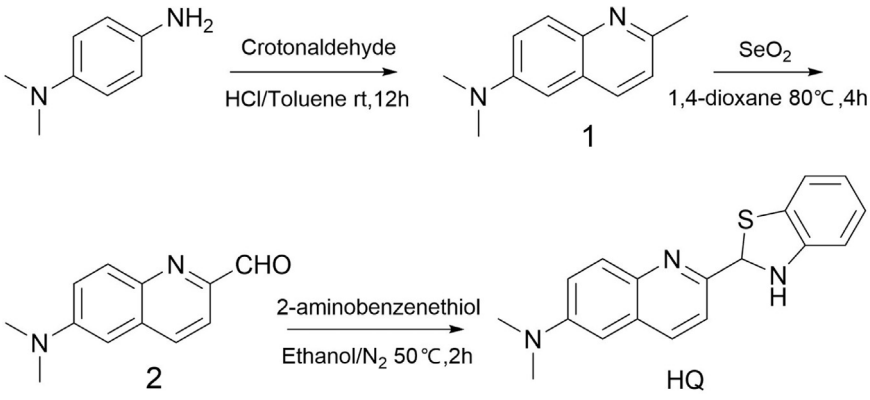
All solvents and reagents were commercial products and used without further purification. All the chemicals used were of analytical grade. Absorption spectra were conducted from an UV–vis spectrophotometer (Shimadzu UV-2550, Japan), and one-photon fluorescence spectra were measured on a fluorescence spectrophotometer (Shimadzu RF-5301 PC, Japan). Two-photon fluorescence spectra were obtained with a DCS200PC photon counting with Omno-λ5008 monochromator (Zolix, China) by exciting with a mode-locked Ti: sapphire femtosecond pulsed laser (Chameleon Ultra I, Coherent, America). One-photon fluorescence images of RAW 264.7 were acquired on a confocal laser scanning microscope (Nikon C1-si TE2000, Japan). Two-photon microscopy of RAW 264.7 cells was collected from a spectral confocal and multiphoton microscope (Carl Zeiss, LSM 780 NLO, Germany). Two-photon microscopy images of zebrafish were performed on a Nikon Ni-E Microscope equipped with multiphoton laser. Two-photon microscope images of inflamed tissues were captured with a multiphoton laser scanning confocal microscope (Carl Zeiss, LSM 710, Germany).

2.2. Synthesis of 6-(dimethylamino)quinoline-2-benzothiazoline (HQ)

Details about preparation of **HQ** were depicted as below (Scheme 1). 6-(*N,N*- dimethylamino)quinaldine (**1**) and 6-(dimethylamino)quinoline-2-carbaldehyde (**2**) were synthesized by the literature route (Petit et al., 2012). Briefly, a mixture of compound **2** (0.1 g, 0.5 mmol) and ethanol (100 ml) was heated at 50 °C. After addition of 2-amino-benzenethiol (0.063 g, 0.5 mmol) the mixture was stirred for 20 min under the nitrogen protection. The reaction process was monitored by TLC. After cooling down to room temperature, the solution was extracted with dichloromethane and the organic layer was washed with water, dried over Na_2SO_4, and the solvent was then removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (dichloromethane) then (Petroleum ether: Ethyl acetate 10:1), and recrystallized in ethanol. **HQ** was obtained as sand-brown solid (0.098 g, 67%). ^{1}H NMR (400 MHz, DMSO$^-d_6$): δ 8.12 (t, J=9.3 Hz, 1H), 7.81 (d, J=9.3 Hz, 1H), 7.63 – 7.55 (m, 1H), 7.46 (dd, J=9.3, 2.8 Hz, 1H), 7.17 (t, J=10.0 Hz, 1H), 7.02 (d, J=7.4 Hz, 1H), 6.96 – 6.87 (m, 2H), 6.70 – 6.58 (m, 2H), 6.48 (d, J=2.7 Hz, 1H), 3.08 (d, J=30.9 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.36, 148.95, 148.34, 140.55, 135.75, 129.63, 129.38, 125.98, 125.00, 121.60, 120.04, 119.24, 118.94, 109.10, 105.18, 70.94, 40.57.

2.3. Theoretical calculation

All the calculations were implemented by the Gaussian 09 program package (Frisch et al., 2009). Geometry of **HQ** and 6-(dimethylamino)quinoline-2-benzothiazole (**4**) were optimized by using the three-parameter hybrid B3LYP density method, and a basis set of 6–311++g (2d, p) (Becke, 1993; Lee et al., 1988). The



Scheme 1. Synthesis route for **HQ**.