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Short communication

A coumarin-based two-photon probe for hydrogen peroxide

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ABSTRACT

A new fluorescence probe was developed for hydrogen peroxide (H_2O_2) detection based on donor-excited photo induced electron transfer (D-PET) mechanism, together with the benzil as a quenching and recognizing moiety. The benzil could convert to benzoic anhydride via a Baeyer–Villiger type reaction in the presence of H_2O_2, followed by hydrolysis of benzoicanhydride to give benzoic acid, and the fluorophore released. The probe was synthesized by a 6-step procedure starting from 4-(diethylamino) salicylaldehyde. A density functional theory (DFT) calculation was performed to demonstrate that the benzil was a fluorescence quencher. The probe was evaluated in both one-photon and two-photon mode, and it exhibited high selectivity toward H_2O_2 over other reactive oxygen species and high sensitivity with a detection limit of 0.09 μM. Furthermore, the probe was successfully applied to cell imaging of intracellular H_2O_2 levels with one-photon microscopy and two-photon microscopy. The superior properties of the probe made it of great potential use in more chemical and biological researches.

1. Introduction

Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl ($^{\bullet}OH$), singlet oxygen ($^1O_2$), superoxide ($O_2^{-\bullet}$), peroxyl radical ($^{\bullet}ROO$) and hypochlorous acid (HOCl) are endogenously generated from oxygen metabolism in biological systems (Dickinson and Chang, 2011; D'Autreaux and Toledano, 2007; Stadtman, 2006). H_2O_2, a major member of ROS, plays an important role in normal cellular growth and proliferation (Rhee, 2006; Kamata et al., 2005; Finkel and Holbrook, 2000; Veal et al., 2007; Harman, 1981; Stone and Yang, 2006). However, over production of H_2O_2 can cause many diseases, such as inflammatory disease, cardiovascular disease, Alzheimer's disease and cancer (Barnham et al., 2004; Mattson, 2004; Lin and Beal, 2006; Andersen, 2004; Ohshima et al., 2003; Shah and Channon, 2004). Therefore, it is necessary to develop sensitive and selective methods for the detection of H_2O_2 in living cells.

As a useful analytical tool, fluorescent spectroscopy is of broad interest in bioanalysis. In recent years, many fluorescence probes for H_2O_2 have been developed (Li et al., 2013; Chen et al., 2011; Hyman and Franz, 2012; Kim et al., 2010; Lo and Chu, 2003; Chang et al., 2004; Miller et al., 2005; Dickinson et al., 2010; Albers et al., 2008; Du et al., 2008; Setsukinai et al., 2003; Maeda et al., 2004; Lippert et al., 2011; Karton-Lifshin et al., 2011; Zhu et al., 2013; Soh et al., 2005; Xu et al., 2005; Hu et al., 2014; Qian et al., 2012). For example, dichlorodihydrofluorescein derivatives are widely utilized as fluorescent probes for measuring H_2O_2 (Setsukinai et al., 2003). However, they have poor selectivity and undergo autoxidation upon exposure to excitation light. Another reported H_2O_2 probes based on arylsulfonyl (Maeda et al., 2004) and boronate ester (Lippert et al., 2011; Karton-Lifshin et al., 2011; Hyman and Franz, 2012) deprotection mechanism show high selectivity toward H_2O_2 over other ROS, but their response rates are not entirely satisfactory for biological research and clinical needs. Very recently, Negano et al. reported a turn-on H_2O_2 fluorescent probe NBNF based on a Baeyer–Villiger type reaction of the benzil moiety (Abo et al., 2011). As a quencher and specific response group to H_2O_2, the benzil was first applied to design H_2O_2 probe in his work. Moreover, this probe has higher sensitivity and enough selectivity. However, NBZF was evaluated using one-photon excitation (OPE) with relatively short excitation wavelength (490 nm), which limits its further application in tissue imaging. While two-photon excitation (TPE) has attracted much attention in bioanalysis due to its minimum interference from photobleaching and photodamage (Kim and Cho, 2009; Masanta et al., 2012). So it is still significative to extend a new fluorescent probe with TPM using benzil as a recognition moiety of H_2O_2. We selected

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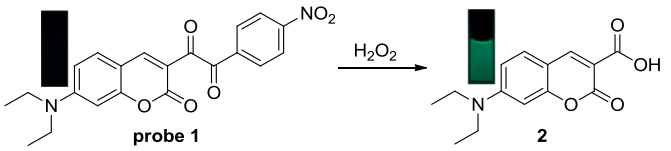

Scheme 1. Mechanism of H_2O_2 sensing by probe **1**.

coumarin as a two-photon fluorophore scaffold, because a coumarin-based fluorophore 6-hydroxycoumarin-4-ylme thanol has been reported to possess TPE properties (Furuta et al., 1999).

Herein, we reported a TPE probe **1** for H_2O_2. The synthesized coumarin derivative **1** was almost non-fluorescent (quantum yield in $H_2O/CH_3CN=8$: 2, $\Phi=0.0202$), indicating that the benzil acted as a fluorescence quencher because of the donor-excited photo induced electron transfer (D-PET) process (Abo et al., 2011). However, compound **1** could convert to **2** in the presence of H_2O_2 (Scheme 1). Owing to the break of benzil's structure, the D-PET process did not occur and the strong fluorescence of coumarin was emitted (quantum yield in $H_2O/CH_3CN=8$: 2, $\Phi=0.0641$).

2. Experimental section

2.1. Materials and instruments

All reagents were purchased from commercial providers and used without further purification. All solvents were used after distillation or dehydration. ^1H NMR and ^{13}C NMR were recorded in $CDCl_3$ at 25 °C on a Bruker DRX400 spectrometer, 400 MHz for ^1H and 100 MHz for ^{13}C, respectively. Mass spectra (ESI) were taken on a Bruker micrOTOF-Q II mass spectrometer. UV–visible spectra were measured using an Analytik Jena SPECORD 50 PLUS. Fluorescence spectra with both one-photon excitation (OPE) and two-photon excitation (TPE) were carried out using the Hitachi F-4500 fluorescence spectrometer and Edinburgh Instrument FLS920, respectively. Conventional fluorescence imaging were performed using a Leica DMI 4000B microscope with DFC420 C camera and a mercury lamp (short ARC) with a BP 450–490 excitation filter and a LP 515 emission filter. Two-photon fluorescence microscopy imaging were captured using Olympus FV1000 laser scanning confocal and multiphoton microscopes (set at wavelength 900 nm and PMT; Gain, 746 V) with 100 × oil immersion objective lens. The signals were collected in a 12 bit unsigned 1024 × 1024 pixels at 8.0 us/pixel sampling speed.

2.2. Absorption and fluorescence spectroscopy

Probe **1** was dissolved in DMF for a stock solution (1 mM). H_2O_2 was from dilution of 30% solution in water. NaClO was from dilution of 10% solution in water. Tert-butyl hydroperoxide was from dilution of 70% solution in water. $^{\bullet}NO$ was produced from sodium nitroferricyanide (SNP). $O_2^{-\bullet}$ was from KO_2 in anhydrous DMSO. $^1O_2$ was produced by mixing H_2O_2 and 100-exceed of NaClO. $^{\bullet}OH$ was produced by Fenton reaction, mixing H_2O_2 and 10-exceed $(NH_4)_2Fe(SO_4)_2$. Test solutions (10 μM) were prepared by displacing 20 μL of the stock solution into a 2-mL mixture of 0.1 M PBS and CH_3CN (8:2, v/v) at pH 7.4.

2.3. Cell culture and cell imaging

MKN-45 gastric cancer cells and SMMC-7721 liver cancer cells were cultured for 48 h in culture media (Dulbecco's Modified Eagle's Medium, High Glucose) with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator which was provided with 5% CO_2 and 95% air. Both of them were cultured in 12-well plate at a density of 104 cells per well in culture media, then incubated with 10 μM probe **1** solution at 37 °C. Before imaging measurement, cells were washed 3 times with phosphate buffered saline (PBS).

3. Results and discussion

3.1. Calculation based on density functional theory (DFT)

In order to understand the effect of the benzil structure on photophysical properties, the calculation based on density functional theory (DFT) for **1** was performed (Fig. 1). According to the comparison between the highest occupied molecular orbitals (HOMO) and the lowest unoccupied molecular orbitals (LUMO), it came to a conclusion that the electron density from the coumarin moiety moved to the benzil moiety upon the excitation. As a result, the fluorescence of coumarin was quenched.

3.2. Synthesis and characterization

The synthesis of the probe **1** began with compound **3** in a straight forward manner by a 6-step procedure. The starting material **3** could convert to **4** according to a routine coumarin formation procedure. The compound **7** was synthesized by using the intermediate **4** as reaction material undergoing bromination, reaction of Sonogashira with trimethylsilyl acetylene (TMSA) and deprotection with K_2CO_3 in CH_3OH. The compound **8** was afforded through the reaction of Sonogashira of **7** with 1-bromo-4-nitrobenzene. At last, the desired target probe **1** was obtained by oxidation reaction of **8** with DMSO catalyzed by $PdCl_2$. The detail characterization data and experimental procedure were shown in the ESI. Scheme 2

3.3. UV–vis absorption and fluorescence spectra of *1* towards H_2O_2

We examined the optical properties of probe **1** in PBS buffer (0.1 M, pH 7.4, 20% CH_3CN). UV–vis spectra of **1** (10 μM) exhibited an absorptions maximum at $\lambda_{abs}=475$ nm. After treatment with 10 equiv. of H_2O_2 at 37 °C, the absorption at 475 nm obviously declined, whereas a new absorption peak was present at 360 nm (Fig. 2a). Accordingly, the emission at 505 nm apparently appeared upon excitation at 380 nm, and the fluorescence intensity increased rapidly. Finally approximately 75-fold fluorescence enhancement after 60 min was observed (Fig. 2b). The sharp increase in the fluorescence intensity could even be perceived by the naked eye under the excitation of ultraviolet lamp (365 nm) (Scheme 1). This result also supported the idea that benzil moiety could be a strong fluorescence quenching. Moreover, this large fluorescence off–on response was extremely desirable for sensitive detection. Furthermore, upon addition of different concentrations of H_2O_2, the fluorescence intensity increased linearly with the concentration of H_2O_2 from 10 μM up to 100 μM (Fig. 2c). Thus, the detection limit (3σ/slope) was calculated to be 0.09 μM (Fig. S1). We then evaluated the ability of probe **1** to H_2O_2 in a two-photon mode. Upon TP excitation at 760 nm, the emission spectra were similar to those obtained in one-photon mode within 60 min (Fig. 2d). Subsequently, the TP absorption cross action spectra of the resulting solution (Fig. S2) exhibited δ_{max} values of 164.7 GM

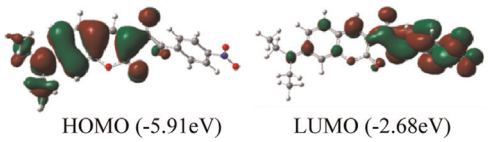

Fig. 1. The HOMO and LUMO orbitals of probe **1**.