

Cite this: *RSC Adv.*, 2019, 9, 39532Received 17th September 2019
Accepted 24th November 2019

DOI: 10.1039/c9ra07517h

rsc.li/rsc-advances

A novel ratiometric fluorescent probe for rapid detection of hydrogen peroxide in living cells†

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In this work, we present a new ratiometric fluorescent probe JNY-1 for rapid and convenient detection of H₂O₂. The probe could selectively and sensitively respond to H₂O₂ within 10 min. In addition, this probe was successfully applied for monitoring and imaging of H₂O₂ in liver cancer HepG2 cells under physiological conditions.

Introduction

Reactive oxygen species (ROS) is a collective term that describes the chemical species that are formed upon incomplete reduction of oxygen and includes the hypochlorous acid/hypochlorite ions (HOCl/OCl⁻), hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and the hydroxyl radical (HO[•]). ROS in the body are generated endogenously from oxygen mainly through the mitochondrial respiration process as well as exogenously by exposure to deleterious conditions including UV light, xenobiotics and infectious agents.¹ ROS are classically known as indicators of oxidative stress, which is a fundamental process that operates in a diverse array of human diseases, including Alzheimer's diseases, cardiovascular disorders, and cancer.^{2–4} Hydrogen peroxide (H₂O₂) is one of the most important ROS. Cellular hydrogen peroxide (H₂O₂) arises primarily as a ubiquitous dismutation product of superoxide. Mounting evidence suggests that H₂O₂ production drives a broad spectrum of cellular processes. For example, when H₂O₂ is generated at low concentrations (<0.7 μM) in a regulated fashion, it functions as a ubiquitous intracellular second messenger, regulating a multitude of physiological and pathological processes in cell.⁵ Under conditions of stress or stimulation by exogenous chemicals, H₂O₂ can be generated aberrantly and can be converted to dramatically more “damaging” ROS such as hypochlorous acid (HOCl) and hydroxyl radical (HO[•]) by myeloperoxidase and Fenton reagents, respectively. The resulting ROS can attack cellular structures or biomolecules such as proteins, liposomes, and DNA, which has been associated with aging, Alzheimer's

disease, and cancer.^{6,7} However, the transient increase of intracellular H₂O₂ may be necessary for mitosis.⁸ Therefore, development of a rapid and highly selective method for monitoring H₂O₂ level change in living cells is of great significance.

However, it is challenging to capture and detect H₂O₂ in living cells. This is mainly due to H₂O₂ in the cells possessing the following characteristics. At first, H₂O₂ can readily move out of cells through free diffusion. So, it's difficult to detect intracellular H₂O₂ *in situ*. Second, H₂O₂ easily reacts with bio-substances to form other ROS. Therefore, in order to accurately detect H₂O₂, the methods require a fast response time. Last but not least, there are many ROS with high reactivity coexisting in the living system which can interfere the detection of H₂O₂. Thus, high selectivity is also expected to be achieved for detection of H₂O₂. Therefore, detection of H₂O₂ with spatial-temporal accuracy is of great significance for mechanistic studies of H₂O₂-related biology.

Fluorescence imaging based on small-molecule fluorescent probes has emerged as an efficient methodology to visualize the spatial and temporal distribution of biomolecules in biological specimens due to its real-time, sensitive, and noninvasive characteristics.^{9–11} Up to now, numerous fluorescent probes for endogenous H₂O₂ detection that have been elaborated,^{12–16} and the detection mechanisms of these H₂O₂ probes are mainly based on boronate oxidation¹⁷ or Baeyer–Villiger-type reactions.¹⁸ To some extent, these probes always have some minor defects. Several boronate-based probes react more slowly with H₂O₂ compared to other reactive species (*e.g.*, peroxynitrite and hypochlorite).^{19–21} Some of them have been used to detect peroxynitrite^{22–24} or benzoyl peroxide²⁵ instead of H₂O₂. On the other hand, the vast majority of these probes are intensity-based turn-on fluorescent probes.²⁶ However, interpretation of the results obtained with turn-on probes could be complex. As the fluorescence intensity is very sensitive to environmental factors such as probe concentration, temperature, medium polarity, and pH and also instrumental set-ups, such intensity-based

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ra07517h



probes are not suitable for quantitative analysis. In this context, ratiometric probes that have an internal correction capability are in great demand. Ratiometric probes provided two separate signals for the probe itself and for its reaction product with the analyte or its analyte bound form, which can normalize the interference. Thus, ratiometric probes with high response rate, excellent selectivity, and good sensitivity should be developed for fulfilling the stringent issues of biological examination.

To address this issue, we herein report a novel ratiometric fluorescent probe **JNY-1** (Scheme 1) for detection of H_2O_2 with high selectivity and sensitivity. In this work, we choose an integration of fluorescein and coumarin fluorophores as the fluorogen because of its' tunable fluorescent properties which could be used for design ratiometric response probes. The pentafluorobenzenesulfonyl group was selected as the reaction unit. As we all known, it is more stable to hydrolysis than ester bond, and the pentafluorobenzene ring enhances the reactivity of the sulfonates toward H_2O_2 , which can contribute to enhance the selectivity and sensitivity of probe towards to H_2O_2 .²⁷ As shown in Fig. 1, in the absent of H_2O_2 , probe **JNY-1** is present in spirocyclic form only with coumarin-like emission. After selectively deprotecting the pentafluorobenzenesulfonyl group by H_2O_2 , probe **JNY-1** is present in its strongly fluorescent quinoid form and the fluorescence shift from 440 nm (coumarin-like emission) to 540 nm (fluorescein-like emission).

Results and discussion

The synthetic route of probe **JNY-1** is shown in Scheme 1. First, an aldehyde-functionalized fluorescein was prepared according to previous reports.^{28,29} The further reaction of fluorescein monoaldehyde with diethyl malonate afforded a coumarin hybrid **1**, which can be treated with 2,3,4,5,6-pentafluorobenzene-1-sulfonyl chloride, eventually resulting in the formation of probe **JNY-1**, which was characterized by ^1H , ^{13}C NMR and mass spectra.

With the probe **JNY-1** in hand, we then tested its spectroscopic properties. The fluorescence and absorption spectra were measured in DMF/phosphate buffer (30 : 70 v/v, 10 mM, pH 7.4) system at room temperature (25 °C). As displayed in Fig. 1b, the probe **JNY-1** itself showed the maximal absorbance centered at 340 nm. With the excitation at 340 nm, the probe **JNY-1** offered fluorescence emission centered at 440 nm, which is belong to coumarin-like emission. Upon the addition of H_2O_2 , the

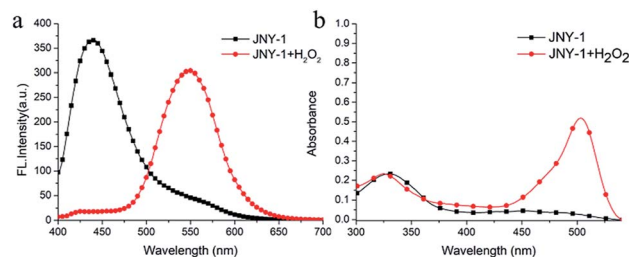


Fig. 1 Fluorescence (a) and UV/Vis absorption (b) spectra of **JNY-1** (10 μM) in the absence and presence of H_2O_2 (100 μM).

reaction of **JNY-1** and H_2O_2 happened and resulted the dissociation of the pentafluorobenzenesulfonyl group from **JNY-1**. Obviously, this detection process is not reversible. Thus, a new absorbance centered at 480 nm emerged. Correspondingly, the fluorescein-like emission centered at 540 nm was observed.

As shown in Fig. 2, the time course of the fluorescent emission of probe **JNY-1** (10 μM) at 540 nm in the presence of H_2O_2 (10 equiv.) was studied. By examining the kinetic data for H_2O_2 , it is apparent that it would take no more than 10 minutes to reach the equilibrium. Generally, it needs 30 min or more to reach the reaction equilibrium between arylboronate based probes and H_2O_2 . **JNY-1** showed faster response to H_2O_2 mainly due to the pentafluorobenzene ring enhances the reactivity of the sulfonates toward H_2O_2 . The rapid response time is favorable for detection and imaging of H_2O_2 in the complex biological systems.

Next, the detailed emission titration experiments of probe **JNY-1** (10 μM) with various concentrations of H_2O_2 were also carried out in the DMF/phosphate buffer (30 : 70 v/v, 10 mM, pH 7.4) system. As shown in Fig. 3, when the probe **JNY-1** was treated with different concentrations of H_2O_2 (0–200 μM), the emission band at 440 nm decreased progressively, while a new emission peak around 540 nm was observed. Importantly, as shown in Fig. 4, the ratio of emission intensities ($I_{540\text{ nm}} : I_{440\text{ nm}}$)

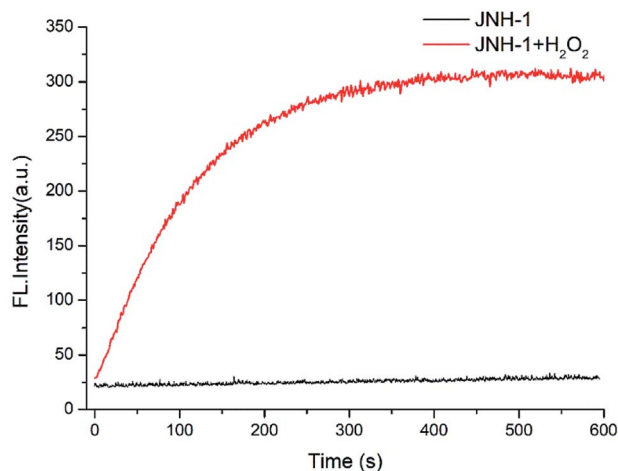
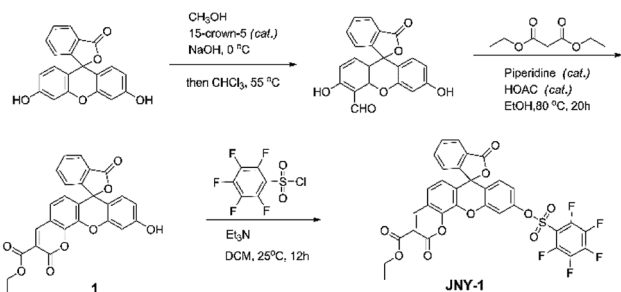


Fig. 2 The time course of the emission intensity of **JNY-1** (10 μM) at 540 nm in the presence and absence of H_2O_2 (100 μM) with $\lambda_{\text{ex}} = 340\text{ nm}$.



Scheme 1 The synthesis route of probe **JNY-1**.



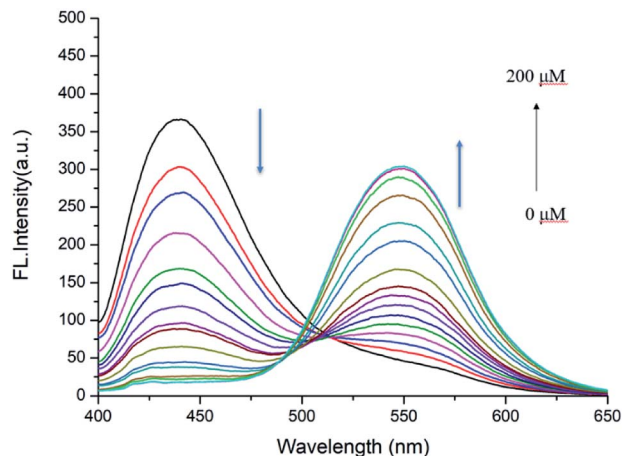


Fig. 3 Fluorescence responses of probe **JNY-1** (10 μM) toward different concentrations of H_2O_2 (0–200 μM) with $\lambda_{\text{ex}} = 380 \text{ nm}$.

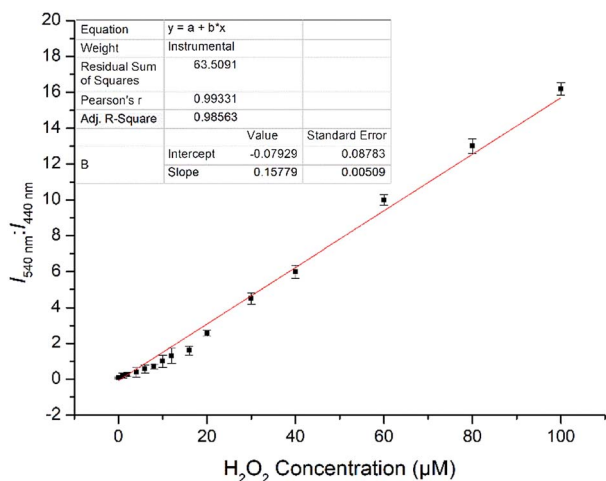


Fig. 4 Fluorescence intensity ratio ($I_{540 \text{ nm}} : I_{440 \text{ nm}}$) of probe **JNY-1** versus H_2O_2 concentration (0–100 μM) with $\lambda_{\text{ex}} = 380 \text{ nm}$, error bars, SD ($n = 3$).

nm) increased by nearly 180-fold and exhibited good linear correlation with the amount of H_2O_2 (0–100 μM , $R^2 = 0.98563$). The detection limit was calculated to be 0.08 μM ($3S/m$, in which S is the standard deviation of blank measurements, $n = 11$, and m is the slope of the linear equation). As shown in Fig. S4,† with addition of H_2O_2 (0–200 μM), the initial absorption peak centered at 340 nm decreased slightly, along with a simultaneous emergence of the red shifted new absorption peaks at 500 nm. Correspondingly, **JNY-1** displayed distinct color changes from colorless to orange, which could be easily observed by the naked eye (Fig. S6†).

To verify the selectivity of **JNY-1** for H_2O_2 , we also measured the ratiometric signals change upon addition of a panel of ROS, reactive nitrogen species (RNS), and the representative biological species. As shown in Fig. 5, only H_2O_2 could trigger a large ratiometric response. However, the ratiometric signals showed no or very minor changes for other species, including $^1\text{O}_2$,

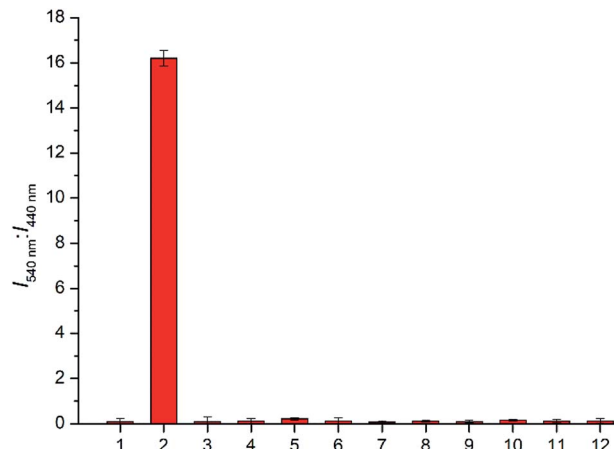


Fig. 5 Fluorescence responses $I_{540 \text{ nm}} : I_{440 \text{ nm}}$ of **JNY-1** (10 μM) with various analytes. (1) Free probe; (2) H_2O_2 ; (3) $^1\text{O}_2$; (4) ONOO^- ; (5) NO ; (6) ClO^- ; (7) *tert*-butyl hydroperoxide (TBHP); (8) $\cdot\text{OH}$; (9) $\text{O}_2^{\cdot-}$; (10) GSH; (11) ascorbic acid and (12) glucose. Each spectrum was recorded at 10 min after the addition, error bars, SD ($n = 3$).

ONOO^- , NO , ClO^- , *tert*-butyl hydroperoxide (TBHP), $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, GSH, ascorbic acid, and glucose. This result indicated that probe **JNY-1** exhibited remarkably high selectivity for H_2O_2 .

To further evaluate the ability of probe **JNY-1** for detection of H_2O_2 in living cells, we conducted the cell imaging experiments. As shown in Fig. 6, the liver cancer HepG2 cells in group A and B were incubated with 10 μM of **JNY-1** at 37 $^\circ\text{C}$ for 15 min before fluorescence imaging. Group A was the control and group B was added 100 μM of H_2O_2 . As described in Fig. 6A, HepG2 cells incubated with 10.0 μM **JNY-1** show fluorescence in blue channel, but there is negligible fluorescence signal through green channel. On the contrary, the probe-loaded cells appear clear cellular profiles with bright green fluorescence when treated with 100 μM H_2O_2 for 10 min, the original fluorescence signal in the blue channel weakened accordingly. These imaging results indicate that **JNY-1** shows pleasurable cell-permeability and could be applied in dual-color fluorescence imaging of intracellular H_2O_2 .

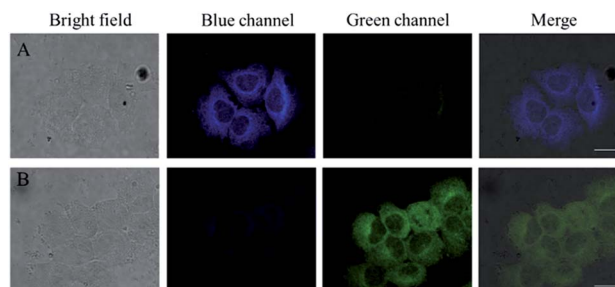


Fig. 6 Confocal fluorescence images of the liver cancer HepG2 cells with probe **JNY-1**. Fluorescence images were obtained before and after incubation for 10 min without (A) or with (B) 100 μM H_2O_2 . The images were collected at 425–465 nm (blue channel) and 520–560 nm (green channel) upon excitation at 405 nm. Scale bar: 20 μm .



Conclusions

In summary, we have described the design and synthesis of a new ratiometric fluorescent probe **JNY-1** for detection of H₂O₂. The probe could selectively and sensitively respond to H₂O₂ within 10 min without interferences of other reactive oxygen species (ROS), reactive nitrogen species (RNS), and biologically relevant species. This probe was successfully applied for monitoring and imaging of H₂O₂ in liver cancer HepG2 cells under physiological conditions. The combination of ratiometric capability, sensitivity, specificity, and rapid response property makes the probe a candidate for various potential applications.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the National Nature Science Foundation of China (No. 81701754 and 81601471), Provincial Natural Science Foundation of Hunan (No. 2019JJ40434) and Scientific Research Project of Hunan Health and Family Planning Commission (No. B20180048).

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