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Communication

Design of a novel mitochondria targetable turn-on fluorescence probe for hydrogen peroxide and its two-photon bioimaging applications

Kangnan Wang[a,1], Wen Ma[b,1], Yuchun Xu[b], Xin Liu[a], Gui Chen[b], Meng Yu[b], Qiling Pan[a], Chaobo Huang[c], Xiaochuan Li[d], Qingchun Mu[d,*], Yongbing Sun[e,*], Zhiqiang Yu[b,*]

[a] *The First People's Hospital of Shunde, Southern Medical University, Foshan 528300, China*
[b] *School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*
[c] *Joint Laboratory of Advanced Biomedical Technology, College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, China*
[d] *The People's Hospital of Gaozhou, Gaozhou 525200, China*
[e] *Division of Pharmaceutics, National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China*

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ABSTRACT

Considering that hydrogen peroxide (H_2O_2) plays significant roles in oxidative stress, the cellular signal transduction and essential biological process regulation, the detection and imaging of H_2O_2 in living systems undertakes critical responsibility. Herein, we have developed a novel two-photon fluorescence turn on probe, named as Pyp-B for mitochondria H_2O_2 detection in living systems. Selectivity studies show that probe Pyp-B exhibit highly sensitive response toward H_2O_2 than other reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as biologically relevant species. The fluorescence colocalization studies demonstrate that the probe can localize in the mitochondria solely. Furthermore, as a bio-compatibility molecule, the highly selective and sensitive of fluorescence probe Pyp-B have been confirmed by its cell imaging application of H_2O_2 in living A549 cells and zebrafishes under the physiological conditions.

As a class of indicators of oxidative stress, reactive oxygen species (ROS) exhibit high reactivity to biomolecules and play vital roles in both of physiological and pathological processes [1,2]. As a critical member of ROS family, hydrogen peroxide (H_2O_2) has been testified as a small molecule messenger in signal transduction of various transduction events [3–6]. H_2O_2 also plays important role in the processes of cell growth and metabolism, the production of living material and energy, and so on [7–9]. It is also known that excessive production of H_2O_2 can impair tissues even living systems, leading to aging and functional decline of living systems, which is connected with diverse human diseases, such as inflammation, cancer, malignant tumor, cardiovascular disease, diabetes [10–15]. Therefore, the development of sensitive, selective detection methods for monitoring of H_2O_2 in living cells, especially in the particular subcellular organelles such as mitochondria, is of great significance.

So far, fluorescence sensing and imaging methodologies based upon small molecular probes have been recognized as one of the most used ways for monitoring biological substances in living systems, attributing to its distinctive superiority such as high sensitivity, high spatial resolution, non-invasive visibility and simplicity of sample preparation characters [16–20]. To date, a number of fluorescence probes have been developed for H_2O_2 detecting and imaging, based on the unique reaction between H_2O_2 and *p*-phenyl boronic acid ester moiety [21–24]. Some of the well performance fluorescence probes can be brought to the certain organelles, such as mitochondria, providing the versatile platform to trace the location, content in living systems [25,26]. However, to our best knowledge, most of the H_2O_2 detecting probes use one-photon mode (OPM), which can only excited at short wavelengths. Therefore, subjecting to superficial imaging depth, the OPM considerably limiting their application in the imaging of living systems. As a contrast, two-photon mode (TPM) probes are more available in the bioimaging applications owing to its incomparable merits, such as reduced background emission, lower photo-toxicity and photo-bleaching, increased detection sensitivity [27–31]. However, only a few TPM fluorescence probes have been designed and applied to monitor H_2O_2 [32–35]. Therefore, the

* Corresponding authors.
E-mail addresses: muqcns@163.com (Q. Mu), 20101008@jxutcm.edu.cn (Y. Sun), yuzq@smu.edu.cn (Z. Yu).
[1] These authors contributed equally to this work.

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design of highly selective and sensitive TPM fluorescence probes, as well as the mitochondrial-targeted fluorescence probes for H_2O_2 is still of significant goal.

Recently, the quaternized pyridine unit has been testified to locate mitochondria solely [36,37]. Based on this principle, a serious of quaternized pyridine-based fluorescence probes have been designed for the targeted detection of biological substrates in mitochondrial location [38–40]. Inspired by these reports, herein, a novel turn on two-photon fluorescence probe Pyp-B (Scheme 1) has been designed for mitochondria H_2O_2 detection.

In this novel design, a methyl-4-pyridyl ketone conjugated pyrene formaldehyde (Pyp) group has been introduced as the fluorophore owing to its outstanding photophysical character. A H_2O_2-sensitive *p*-phenyl boronic acid ester moiety has been selected as the reactive site based on the high reactivity of H_2O_2 to boronic acid pinacol ester group, which connected with pyridine, thereby forming a quaternized pyridine moiety to improve the feasibility of its mitochondrial targeting [41–43]. Expectably, Pyp-B expresses weak fluorescence at the maximum emission wavelength at 465 nm due to the photoinduced electron transfer (PET) effect. Under physiological conditions, H_2O_2 induces the oxidation of the *p*-phenyl boronic acid ester moiety of probe Pyp-B based on the 1,6-rearrangement elimination reaction mechanism to release fluorophore Pyp. As results, a turn on fluorescence response is discovered, the signal intensity of 465 nm is obviously enhanced and a new emission wavelength appeared at 625 nm, thereby leading to highly sensitive monitoring of H_2O_2. It is fairly attractive that such extraordinarily low background interference could provide high sensitivity for the monitoring of H_2O_2. Moreover, the probe exhibit highly selectivity response toward H_2O_2 than other ROS and reactive nitrogen species (RNS) as well as biologically relevant species. Most absorbingly, this probe presented outstanding mitochondria-targeted features and has been successfully used to the imaging of mitochondrial H_2O_2 in living cells and zebrafishes.

The synthesis and characterizations (^{1}H NMR and ^{13}C NMR) of Pyp and Pyp-B are described in Scheme 1 and Figs. S1-S4 (Supporting information). The results revealed the right structure and high purity of both compounds. With Pyp-B in hand, the photophysical properties of Pyp and Pyp-B were first studied in dimethyl sulfoxide (DMSO) (Fig. S5 in Supporting information). The maximum two-photon action cross-section value ($\Phi_{\delta max}$, in which Φ is the fluorescence quantum yield and δ is the two-photon absorption cross section) of Pyp-B at 800 nm were determined to be 116.5 GM (Fig. S6 in Supporting information). Upon excitation at 405 nm, all the compounds exhibited red fluorescence. The spectroscopic evaluation of probe Pyp-B (10 μmol/L) and its response to H_2O_2 was performed in the PBS/DMSO (99/1, v/v, 20 μmol/L, pH 7.4) system at the room temperature (25 °C). As can be seen from Fig. 1A, the probe Pyp-B exhibit a maximum absorbance at 470 nm. After the probe Pyp-B with H_2O_2, the absorbance peak at 470 nm reduces gradually, while a new absorbance peak at 437 nm appear. Meanwhile, after the reaction between Pyp-B and 6 equiv. H_2O_2 for 4 min approximately, the absorbance intensity at 437 nm essentially achieves a maximum value (Fig. 1B). The rapid response is attractive to targeted monitor H_2O_2 in complex bionic systems. As shown in Fig. 1C, the probe Pyp-B, has a very weak emission with quantum yield Φ_{fl} = 0.072. However, the fluorescence peaks at 465 nm and 625 nm increases quickly with the increasing of H_2O_2 and an excellent linearity response in the equivalent range of (0–4 equiv.) with the limit of detection (LOD) of 0.021 μmol/L ($3\sigma/k$, in which σ means the standard deviation of the blank test, n = 10, and k means the slope of the linear equation) (Fig. 1D).

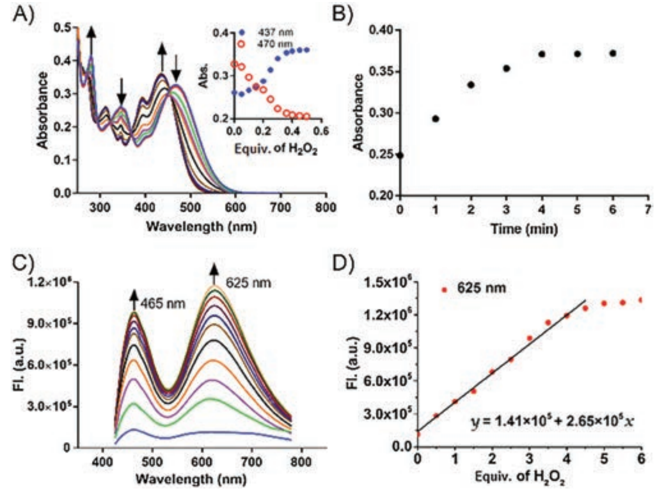
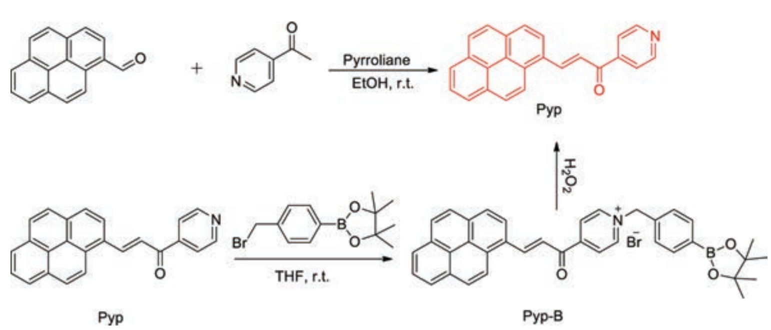


Fig. 1. (A) UV-vis absorption spectra of Pyp-B upon H_2O_2 titration in PBS/DMSO (99/1, v/v, pH 7.4). Inset: absorption intensity changes of 470 nm and 437 depending on the concentration of H_2O_2, respectively. (B) The changes of absorption intensity at 437 nm depending on time. (C) Fluorescence emission spectra of Pyp-B upon H_2O_2 titration in PBS/DMSO (99/1, v/v, pH 7.4). (D) Linear plot of the emission intensity against the equivalent of H_2O_2.

The mitochondria normally sustain a weak alkaline pH (∼7.8) under physiological conditions [44]. To achieve the monitoring of H_2O_2 in mitochondria, the optical response of Pyp-B to different pH in PBS were tested. As results in Fig. S7 (Supporting information), the fluorescence intensity of Pyp-B shows no remarkable difference, while pH value changed from 4.5 to 10, indicate that Pyp-B is pH insensitive. The pH-dominated reaction feature between probe Pyp-B and H_2O_2 is attributed to the phenyl boronic acid ester moiety can react with H_2O_2 only under the mild basic conditions [39]. Notably, the fluorescence emission of this probe remains stable with the changes of pH. In order to verify the selectivity of Pyp-B toward H_2O_2, Pyp-B was incubated with the derives biologically relevant species, ROS and RNS were further



Scheme 1. Synthesis of probe Pyp-B and its recognition to H_2O_2.

determined in PBS/DMSO (99/1, v/v, pH 7.4) system. As can be seen from Fig. S7, only reacted with H_2O_2, a favorable fluorescence enhancement was obviously observed, while the ROS, RNS, and other biological molecules show almost invisible changes.

To understand the mechanism of the turn on fluorescence character of Pyp-B toward H_2O_2, the density functional theory (DFT) calculation was performed with the Gaussian 09 program on the basis of B3LYP/6-31G methodology. As shown in Fig. S8 (Supporting information), due to the high energy gap between LUMO (−2.461 eV) and HOMO (−5.601 eV), Pyp-B only shows very weak fluorescence at 465 nm. With the release of fluorophore Pyp, after Pyp-B react with H_2O_2, the energy level of the LUMO (−5.878 eV) is comparable to that of the HOMO (−7.606 eV), thereby leading to the PET mechanism was relaxed. As result, the fluorescence intensity is obviously enhanced. The reaction between Pyp-B and H_2O_2 was also investigated through high resolution mass spectrometry (HRMS). The peak of Pyp-B ($[C_{37}O_3NBH_{33}]^+$) at *m/z* 550.2602 is found (Fig. S9A in Supporting information). After reacted with H_2O_2, a new peak at *m/z* 334.1267 ($[C_{24}NOH_{15}]^+$) is obviously observed (Fig. S9B in Supporting information), which matches the HRMS of Pyp (Fig. S9C in Supporting information, *m/z* 334.1248). These results further indicate the process of H_2O_2 induced the oxidation of the *p*-phenyl boronic acid ester moiety of probe Pyp-B on basis of the 1,6-rearrangement elimination reaction mechanism to release fluorophore Pyp.

As shown in Fig. S10 (Supporting information), Pyp-B has a very low cytotoxicity to living cells even over 80% cells still remain viable at the concentration of 100 μmol/L after 24 h incubation in A549 and HeLa cells, which indicated Pyp-B has low cytotoxicity

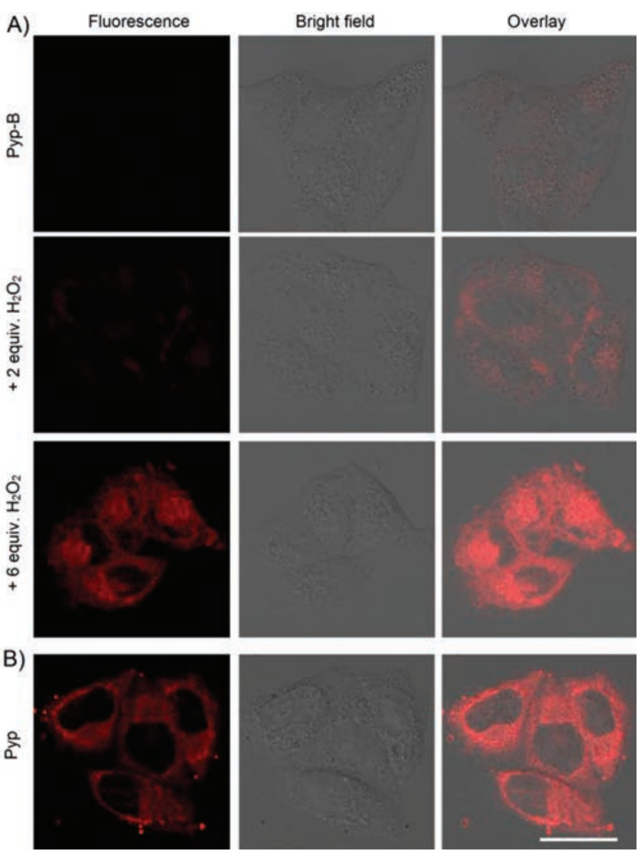


Fig. 2. Two-photon fluorescence imaging of A549 cells incubated with Pyp-B (10 μmol/L) before and after the addition of diverse concentrations H_2O_2 (A), and fluorophore Pyp (10 μmol/L) (B), respectively. λ_{ex} = 800 nm, λ_{em} = 620 ± 20 nm. Scale bar: 20 μm.

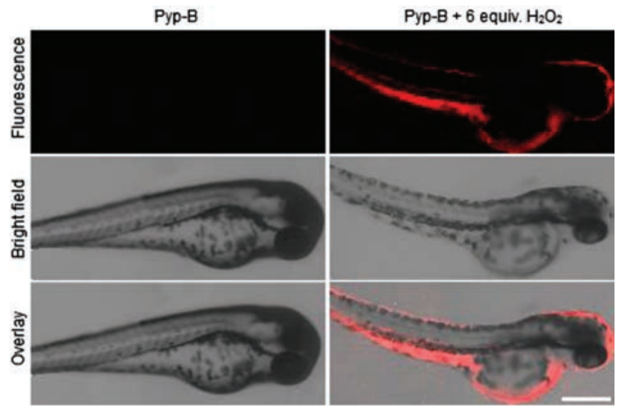


Fig. 3. Two-photon fluorescence imaging of zebrafish (3-day old) with 10 μmol/L probe Pyp-B before and after the addition of 10 equiv. H_2O_2, at 37 °C for 30 min. λ_{ex} = 800 nm, λ_{em} = 620 ± 20 nm. Scale bar: 50 μm.

and excellent biocompatibility. Due to the good two-photon absorption cross section of Pyp-B and Pyp, their two-photon imaging is also well presented (Fig. S11 in Supporting information). After the A549 cells incubating with Pyp-B, there is almost no fluorescence observed (Fig. 2A), while, with the treatment of H_2O_2 in Pyp-B-loaded cells for 30 min, a striking red fluorescence is emerged. The imaging results were consistent with the spectral data in Fig. 1C and the results indicated that Pyp-B has ability to monitor the changes of H_2O_2 in living cells. The fluorescence of Pyp in A549 cells is similar to that after incubation with Pyp-B + 6 equiv. H_2O_2, which confirmed the original hypothesis (Fig. 2B). The fluorescence of Pyp-B and Pyp are well merged with that of mitochondrial tracker deep red (MTDR), with Pearson's colocalization coefficients (PCCs) of 0.82 and 0.93, respectively. (Fig. S12 in Supporting information). The results demonstrated that Pyp-B exhibit favorable localization specificity of cellular mitochondria in living systems, and can detect the H_2O_2 in mitochondrial.

The similar phenomenon of detection of H_2O_2 was also observed in zebrafish. As shown in Fig. 3, after culturing with Pyp-B (10 μmol/L) at 37 °C for 30 min, the two-photon fluorescence have not been observed in zebrafish. Expectably, with the treatment of H_2O_2 in Pyp-B-loaded zebrafish for 30 min, a favorable red fluorescence was clearly presented. As illustration, the imaging of Pyp-B-loaded zebrafish with 6.0 equiv. H_2O_2 is shown in Fig. 3. These results powerfully indicated that Pyp-B has a favorable bio-compatibility and is a potential fluorescence sensor platform for H_2O_2 in living organism.

In summary, a novel mitochondria-targeted turn on two-photon fluorescence Pyp-B for monitoring H_2O_2 has been designed and synthesized. Pyp-B shows high selectivity toward H_2O_2 than other biologically relevant species. Attributed to a serious of highly favorable characters, Pyp-B has been successfully applied for tracing and imaging of H_2O_2 in A549 cells and zebrafish under the physiological conditions. This novel mitochondria-targeted fluorescence probe exhibits powerful potential in practical applications of H_2O_2-mediated biological researches.

Declaration of competing interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cclet.2020.08.039.

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