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Construction of a lysosome-targetable ratiometric fluorescent probe for H₂O₂ tracing and imaging in living cells and an inflamed model†

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Hydrogen peroxide (H₂O₂), an important reactive oxygen species (ROS) with unique destructive oxidation properties, can be produced in lysosomes to fight off pathogens. Although many fluorescent probes have been developed for the detection and imaging of H₂O₂, the development of a ratiometric fluorescent probe for H₂O₂ detection and imaging in lysosomes and an inflammation model remains rather scarce. Therefore, it is important to develop an efficient tool for monitoring H₂O₂ in inflamed tissues to evaluate the physiological and pathological relationship between inflammation and lysosomal H₂O₂. In this work, a new naphthalimide-based lysosome-targeting fluorescent probe (NPT-H₂O₂) for ratiometric detection and imaging was developed *in vitro* and *in vivo*. The probe exhibited two well-resolved emission peaks separated by 125 nm, rapid response (<40 s), and high selectivity and sensitivity toward H₂O₂, as well as low cytotoxicity *in vitro*. Inspired by prominent features of these results, we further successfully applied NPT-H₂O₂ for H₂O₂ imaging with a dual-channel in living cells, demonstrating that our probe NPT-H₂O₂ was targeted in the lysosomes. Finally, NPT-H₂O₂ was used for H₂O₂ detection in inflamed tissues and achieved satisfactory results. We predict that our probe can be used as a powerful tool to reveal the relationship between physiology and pathology of inflammation and lysosomal H₂O₂.

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Introduction

Reactive oxygen species (ROS), involving hydrogen peroxide (H₂O₂), can be produced through cellular respiration and can regulate the original stability of various physiological processes by varieties of homeostatic mechanisms, and have attracted more and more attention in many intersecting fields such as chemistry and biology.^{1–3} The abnormal production and

accumulation level of ROS were closely associated with many factors, such as inflammation, aging, cell signal transduction pathways disturbance, cancer, *etc.*^{4,5} Among them, H₂O₂ is undoubtedly the most important one, because it acts as a destructive oxidant to resist pathogens or as a fine-tuned second messenger for dynamic cellular signaling pathways. Modern biomedical research showed that H₂O₂ can damage a wide variety of biomacromolecules including DNA, RNA, and proteins,^{6,7} and excessive H₂O₂ could cause cell damage, disorders and even death, thereby causing a series of related diseases, such as Alzheimer's, Parkinson's, Huntington's diseases, and even cancer.^{8–12} In addition, lysosomes are acidic vesicles (pH 4.5–5.5) that contain various enzymes related to physiological and pathological processes,^{13,14} and modern biomedical studies have shown that ROS can permeabilize lysosomal membranes to cause cell death.^{15–17} It is therefore, developing a suitable tool and method for detecting and imaging lysosomal H₂O₂ in biosystems is urgent to clarify its pathophysiology *in vivo* is very meaningful (Scheme 1).

In the near future, organic small-molecule-based fluorescent probes have become an attractive tool and widely used to investigate various biological events in living biological systems due to their merits of non-invasiveness, high spatiotemporal resolution as well as real-time detection/imaging ability.^{18–22}

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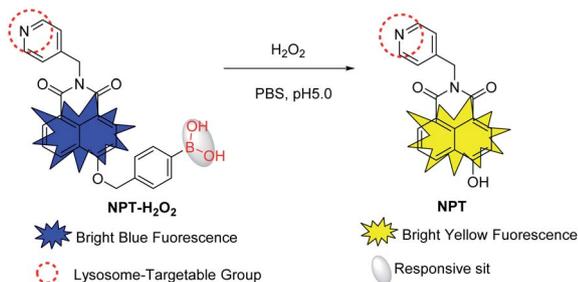
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Scheme 1 Schematic diagram of the response mechanism of the probe NPT-H₂O₂.

Recently, a lot of H₂O₂ fluorescent probes have been successfully developed for the detection and imaging of H₂O₂.^{23–32} However, most of the reported H₂O₂ fluorescent probes were constructed from enhancing or reducing the output signal by a single-channel, which affected by the concentration of probe molecules, environmental factors, instrumental efficiency, *etc.* In contrast, ratiometric fluorescent probes have a built-in correction of the two emission bands, the ratiometric fluorescent probes can eliminate most, if not all, such interference.^{33–37} Moreover, some reported ratiometric H₂O₂ fluorescent probes were affected by low water solubility, poor organelle targeting ability, and small Stokes shift. Some fluorescent probes have a large Stokes shift, which can prevent the self-quenching effect and increase the signal-to-noise ratio. Especially in biological imaging, the crosstalk between the two channels can be minimized to obtain a large signal-to-background ratio. More importantly, the organelle-targeted fluorescent probes can accurately carry out imaging analysis at the organelle level to obtain more accurate results related to the analytes and the physiological and pathological processes. Thus, it is an urgent need to develop an efficient organelle-targeting ratiometric fluorescent probe with a large Stokes shift to improve its precision and sensitivity for concentration of H₂O₂ detecting in biosystems.

Due to the excellent optical properties of donor- π -acceptor (D- π -A)-structured naphthalimide derivatives, various fluorescent probes have been constructed based on them. Inspired by the photophysical properties of naphthalimide, prompting us to construct a new H₂O₂ fluorescent probe based on naphthalimide. Herein, utilizing benzyl boric acid as H₂O₂ reactive moiety (a well-known H₂O₂ responsive moiety), a new ratiometric fluorescent probe NPT-H₂O₂ was constructed for the selective detection of H₂O₂. In the absence of the H₂O₂, it emits a bright blue light, but in the presence of H₂O₂, it emits a bright yellow light, so as to achieve ratiometric detection of H₂O₂. Moreover, because the pyridine group is weakly basic, it can be actively targeting the lysosomes as a lysosomal targeting group. Significantly, NPT-H₂O₂ exhibits excellent sensing performance for H₂O₂: (1) it exhibits a target-regulated ratiometric fluorescence response toward H₂O₂; (2) a large Stokes shift with dual well-resolved channels (425 nm and 550 nm); (3) rapid response time (<1 min) and high selectivity over other analytes. In addition, for fluorescence imaging of H₂O₂ in living cells, and

inflamed tissues, due to an obvious ratio signal between the blue channel and the yellow channel to obtain a large signal-to-background ratio, demonstrating the newly constructed ratiometric probe NPT-H₂O₂ was successfully applied to detect and image H₂O₂ in biological systems.

Experimental section

Materials and apparatus

Materials and equipment are described in the ESI.†

Synthesis of 5 and dye 2 (NPT)

5 and 2 was synthesized according to the previous methods,³⁸ and the synthesis steps were shown in Fig. 1. The detailed operation was as follows: 367 mg (1 mmol) 5, and 326 mg (2 mmol) 4, 417 mg (3 mmol) K₂CO₃, and 20 mL DMF were added to a 100 mL round bottom flask, and then the mixture was stirred at 125 °C overnight. The solvent of DMF was removed by the rotary evaporator under reduced pressure. Following, 20 mL of distilled water was added into the residue, and then the resolution of pH was adjusted to be neutral with hydrochloric acid (pH test paper), a large amount of yellow solid was formed and filtered under reduced pressure. A yellow crude product was obtained by a vacuum drying oven. Finally, the residue was purified by column chromatography with DCM/MeOH = 50 : 1 (v/v) to obtain 222 mg yellow solid. ¹H NMR (400 MHz, d₆-DMSO) δ (ppm): 12.00 (s, 1H), 8.58–8.56 (d, *J* = 8.00 Hz, 1H), 8.50–8.48 (d, *J* = 8.00 Hz, 3H), 8.39–8.37 (d, *J* = 8.00 Hz, 1H), 7.80–7.76 (s, *J* = 8.00 Hz, 1H), 7.33–7.31 (d, *J* = 4.00 Hz, 2H), 7.19–7.17 (d, *J* = 8.00 Hz, 1H), 5.25 (s, 2H); ¹³C NMR (100 MHz, d₆-DMSO) δ (ppm): 164.26, 163.50, 161.13, 149.91, 147.27, 131.97, 129.75, 122.94, 122.04, 112.74, 110.55, 42.46; LC-MS: *m/z*, C₁₈H₁₂N₂O₃, calcd 304.08, found 304.09.

Synthesis of ratiometric fluorescent probe 1 (NPT-H₂O₂)

152 mg (0.5 mmol) 2, 107 mg (0.5 mmol) 3, 139 mg (1 mmol) K₂CO₃ were dissolved in 10 mL DMF, and then the mixture was stirred for 4 h at 50 °C until the reaction was accomplished. The solvent of DMF was removed by the rotary evaporator under reduced pressure. Finally, the residue was purified by column chromatography with DCM/MeOH = 50 : 1 (v/v) to obtain 189 mg yellow solid. ¹H NMR (400 MHz, DMSO-d₆) δ (ppm):

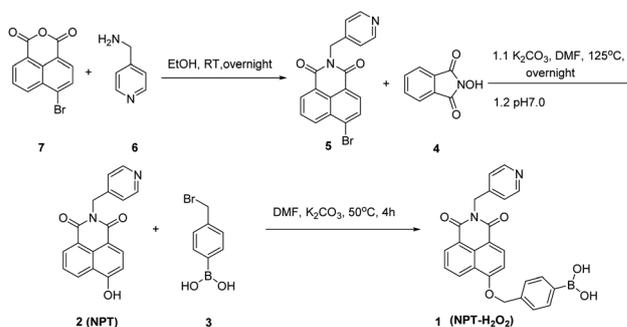


Fig. 1 The synthetic route of NPT-H₂O₂.



9.16–9.14 (d, $J = 8.00$ Hz, 2H), 8.57–8.52 (d, $J = 8.00$ Hz, 1H), 8.52–8.46 (m, 2H), 8.20–8.18 (d, $J = 8.00$ Hz, 2H), 7.89–7.82 (m, 3H), 7.50–7.41 (m, 3H), 5.86 (s, 2H), 5.49 (s, 2H), 5.26 (s, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 163.52, 158.85, 158.37, 144.92, 135.29, 129.07, 128.14, 127.21, 126.52, 123.45, 122.38, 115.28, 108.25, 80.02, 78.53, 63.10, 57.43, 40.58, 35.02, ESI-MS $\text{C}_{25}\text{H}_{19}\text{BN}_2\text{O}_5$, calcd 438.14, found 438.15.

Spectrophotometric measurements

10 mM stock solutions of **NPT-H₂O₂** were obtained by dissolving the right amount of **NPT-H₂O₂** in DMSO, and 10 mM stock solutions of H_2O_2 and other analytes were obtained in 10 mM PBS (pH 4.5). According to the previous references to prepare other ROS,³⁹ and all of them were used freshly. The absorption and fluorescence spectra were measured after the probe and the analytes were incubated for 1 min. The emission range of the fluorescence spectrum was fixed at (400–700) nm, the excitation slit and emission slit widths were fixed at 5 nm and 5 nm, respectively.

Fluorescence imaging of H_2O_2 in living cells

Before fluorescence imaging H_2O_2 in living cells, the MTT method was used to assess the degree of toxicity of the probe **NPT-H₂O₂** to HeLa cells. Fluorescence images of HeLa cells were obtained by an Olympus FV1000 laser scanning confocal microscope (Japan). The fluorescent excitations wavelength were set at 405 and 635 nm, and the fluorescent emissions range were set at (410–460) nm, (500–560) nm, and (640–680) nm, respectively.

Fluorescence imaging of H_2O_2 in inflamed tissues

In order to investigate the probe's imaging of H_2O_2 in the inflammation model, we injured one foot of an adult Kunming mouse, and the other normal foot was used as a control. Following, let the mouse die under the conditions of animal ethics, taken the inflamed tissues of the injured foot and normal tissues of control foot incubated with 5.0 μM probe **NPT-H₂O₂** for 1 h for H_2O_2 images.

Results and discussion

Naphthalimide-based fluorescent probes are widely used due to their D- π -A-structured and their 4-position are easily modified by a responsive group. Therefore, the targeting fluorescent probe is usually constructed by the substitution/modification of the 4-position, and the fluorescent signal change *via* the reaction of the probe with analytes to be regulated or activated. Such a designed structure affords naphthalimide fluorophore has some excellent optical properties. Herein, we used a well-known H_2O_2 responsive group (benzyl boric acid derivatives)⁴⁰ to modify 4-position hydroxyl ($-\text{OH}$), and as well as used a pyridine group with the ability to bind H^+ as a lysosome-targeting group to obtain targeting ratiometric fluorescent probe **NPT-H₂O₂** for effectively detecting H_2O_2 . Because lysosomes are acidic vesicles (pH 4.5–5.5),^{13,14} and $-\text{OH}$ as an electron-donating group has a weak intramolecular charge transfer (ICT) effect, the fluorescence intensity is relatively weak under acidic conditions, and

when $-\text{OH}$ becomes an O^- under alkaline conditions, it has a strong ICT effect and the fluorescence intensity increases. In addition, since the hydrogen proton of the 4-position $-\text{OH}$ of the dye is replaced by the responsive group and becomes an ether bond, the ICT effect is completely ineffective, causing the fluorescence emission of a blue shift. When the probe is in the lysosomal pH range, the boric acid group quickly responds to the H_2O_2 to release the dye **NPT** and restore the weak ICT effect of the $-\text{OH}$, so the ratiometric detection of H_2O_2 can be achieved. According to the synthetic route given in Fig. 1 to synthesize the ratiometric fluorescent probe **NPT-H₂O₂**, and the targeting probe was fully characterized by ^1H NMR, ^{13}C NMR and ESI-MS (see ESI†).

In order to investigate the responsive spectrum properties of the newly designed fluorescence probe **NPT-H₂O₂** toward H_2O_2 under lysosomal physiological pH conditions (normal lysosomal pH 4.0 to 5.5, so, in this work, the pH 5.0 was simulated lysosomal pH value for all experiments). Therefore, the absorption and fluorescence spectra of **NPT-H₂O₂** (5.0 μM) in the absence and in the presence of H_2O_2 in PBS buffer (10 mM, pH 5.0, with 1% DMSO). As shown in Fig. 2a, in the absence H_2O_2 , the newly designed fluorescent probe **NPT-H₂O₂** has a remarkable absorption peak at 375 nm. When in the presence of 60.0 μM H_2O_2 , a significant new absorption peak appeared at 450 nm with a significant red shift 75 nm. At the same time, the response mechanism was also confirmed by mass spectrometry (Fig. S4†). Following, as shown in Fig. 2b–d, the response kinetic of the probe **NPT-H₂O₂** toward H_2O_2 was investigated. The result showed that **NPT-H₂O₂** had a good response kinetic. In the presence of 60.0 μM H_2O_2 , 5.0 μM **NPT-H₂O₂** could quickly react with H_2O_2 during 1 min to reach responsive

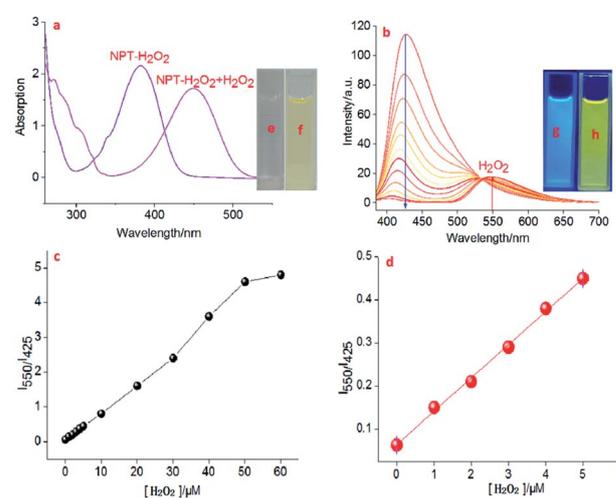


Fig. 2 (a) UV-vis absorption spectra of **NPT-H₂O₂** and **NPT-H₂O₂** + 60.0 μM H_2O_2 ; (b) fluorescence spectra of 5.0 μM **NPT-H₂O₂** after adding different amount of H_2O_2 (0–60.0) μM in 10 mM PBS solution (pH 5.0), $\lambda_{\text{ex}} = 375$ nm; (c) fluorescence ratio (I_{550}/I_{425}) vs. H_2O_2 concentration (0–60.0) μM was plotted the calibration curve; (d) the linear response: plot of fluorescence ratio (I_{550}/I_{425}) vs. the concentration of H_2O_2 (0–5.0) μM ; inset (e)–(h) were **NPT-H₂O₂** before and after 60.0 μM H_2O_2 under the visible light or under 365 nm ultraviolet radiation, respectively.



equilibrium (the result was shown in Fig. S1†). Encouraged by the above experimental results, the newly designed probe **NPT-H₂O₂** of the fluorescence intensity vs. different concentrations of (0–60.0) μM **H₂O₂** were measured in 10 mM PBS (pH 5.0, Fig. 2b), of course, we also carried out the fluorescence response curve with pH = 7.4 (Fig. S3†). With the increase of **H₂O₂** concentration, the emission peak at 425 nm gradually weakened, but a new emission peak was generated at 550 nm and its intensity became stronger and stronger with ~ 5 -fold ratio enhancement (Fig. 2c), and when **H₂O₂** enhance from 0 to 5.0 μM with a good linear response ($I_F = 0.07729 \times [\text{H}_2\text{O}_2] \mu\text{M} + 0.06395$, $R^2 = 0.99878$), according to $3\sigma/\text{slope}$ to obtain the limit of determination (LOD) as low as 12.8 nM (Fig. 2d). Moreover, the fluorescence of the solution changed from bright blue (Fig. 2g) to bright yellow (Fig. 2h) under the excitation of 365 nm UV lamp. Therefore, these results showed **NPT-H₂O₂** has a potential to high sensitive for ratiometric **H₂O₂** detection *in vivo*.

Because selectivity is a very important parameter for a newly designed probe, and high selectivity could eliminate interference from other coexisting biological species *in vivo* to determine whether the developed fluorescent probe could be used in biological systems. Therefore, we further carried out selectivity experiments of **NPT-H₂O₂** with other ROS and other biological species including blank, Gly, Glu, GSH, Cys, Ca^{2+} , Na^+ , K^+ , Fe^{3+} , Mg^{2+} , Zn^{2+} , NO_2^- , SO_3^{2-} , NO, TBO[•], HO[•], *t*-BuOOH, ClO^- , $\text{O}_2^{\cdot-}$, ONOO⁻, and **H₂O₂** (Fig. 3a). Once upon reaction with **H₂O₂**, a large ratio (I_{550}/I_{425}) of fluorescent intensity was enhanced. Although ONOO⁻ with slight interference for probe, the other analytes even exceeds in 10 equivalents caused the changes of the fluorescence intensity were ignored. Therefore, the experimental results showed that, except for **H₂O₂**, the addition of other analytes did not increase the fluorescence ratio value (Fig. 3a, I_{550}/I_{425}) of the newly designed **NPT-H₂O₂**, suggesting **NPT-H₂O₂** to be accurately used in complex biological samples for **H₂O₂** detection.

Additionally, responsiveness of **NPT-H₂O₂** toward **H₂O₂** under different pH (3.0–10.0) values further evaluated, the results as exhibited in Fig. 3b. In the absence of **H₂O₂**, no significant fluorescence intensity changes were caused by the

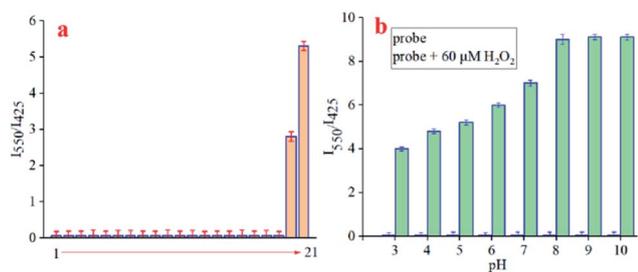


Fig. 3 (a) The photograph of 5.0 μM **NPT-H₂O₂** upon addition of various species in 10 mM PBS buffer (pH 5.0, 1% DMSO): (1 to 21) were blank, Gly, Glu, GSH, Cys, Ca^{2+} , Na^+ , K^+ , Fe^{3+} , Mg^{2+} , Zn^{2+} , NO_2^- , SO_3^{2-} , NO, TBO[•], HO[•], *t*-BuOOH, ClO^- , $\text{O}_2^{\cdot-}$, ONOO⁻, and **H₂O₂**, respectively), the acquisition of photograph 5.0 min after mixing, $\lambda_{\text{ex}} = 375$ nm; (b) The fluorescence ratio (I_{550}/I_{425}) of **NPT-H₂O₂** with different pH values (3.0–10.0) when excited at 375 nm.

pH from 3.0 to 9.0, but when in the presence of **H₂O₂** (60.0 μM), the changes of the fluorescence intensity has changed significantly under different pH values, and the optimal pH response interval >4.0 . All the results further demonstrated that **NPT-H₂O₂** has the potential for **H₂O₂** detecting in the lysosomes.

In order to investigate the potential of the probe **NPT-H₂O₂** for **H₂O₂** detection in living cells model. Before fluorescence imaging, the MTT method was used to evaluate the cytotoxicity of **NPT-H₂O₂** to HeLa cells, and the results exhibited the concentration of probe from 0 to 20.0 μM was almost non-toxic to HeLa cells in Fig. S2,† and the cell viability was maintained at more than 85%. As shown in Fig. 4, when the HeLa cells were only incubated with 5.0 μM **NPT**-enhanced at I_{550}/I_{425} with a very low limit of detection 12.8 nM. In addition, **NPT-H₂O₂** showed low cytotoxicity, lysosome-targeting **H₂O₂** for 30 min, a bright blue fluorescence signal was observed in the blue channel, while displayed only weakly yellow signal in the yellow channel (Fig. 4A(a) and (b)). In contrast, the HeLa cells were pre-treated with 60.0 μM of **H₂O₂** for 30 min, and then continued to add 5.0 μM **NPT-H₂O₂** to co-stain for 30 min. The blue fluorescence signal in the blue channel decreased dramatically while the yellow fluorescence signal in the yellow channel was dramatically increased (Fig. 4B(a) and (b)), suggesting **NPT-H₂O₂** has the ability to **H₂O₂** detection in the living cell. In addition, a colocalization experiment was carried out to further clarify that **NPT-H₂O₂** could detect **H₂O₂** in lysosomes. Specifically, **NPT-H₂O₂** and a commercial lysosome-targeting reagent were co-stained with HeLa cells for 30 min, and then washed 3 times with 10 mM PBS to wash away the excess lysosome-targeting reagent and **NPT-H₂O₂** to carried out colocalization imaging. From the

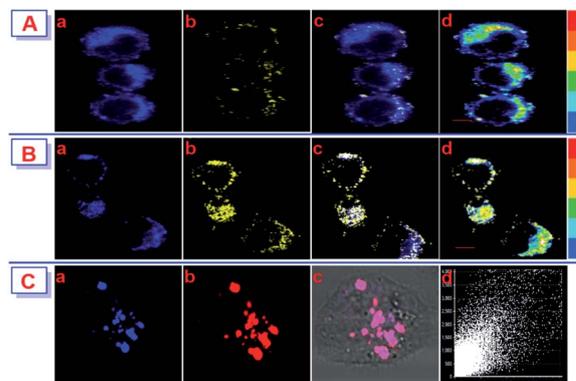


Fig. 4 Fluorescence images in HeLa cells. (A) The HeLa cells incubated with 5.0 μM **NPT-H₂O₂** for 30 min for imaging: (a) the blue channel image, (b) the yellow channel image, (c) overlaid the blue channel (a) and the yellow channel (b) image, (d) the Spec3 image; (B) the HeLa cells pretreated with 60.0 μM **H₂O₂** for 30 min, and then continue to add 5.0 μM **NPT-H₂O₂** to incubate another 30 min for image: (a) the blue channel image, (b) the yellow channel image, (c) overlaid the blue channel (a) and the yellow channel (b) image, (d) the Spec3 image; (C) colocalization imaging: (a) 5.0 μM **NPT-H₂O₂** for the blue channel image, (b) commercial Lyso-Tracker Red for the red channel image, (c) overlaid (a) and (b) image, (d) intensity scatter plot of channels blue and red. $\lambda_{\text{ex}} = 405$ nm or $\lambda_{\text{ex}} = 635$ nm, λ_{em} : (410–460) nm, (500–560) nm, and (650–680) nm, respectively. All images were acquired with a 40 \times oil immersion objective, scale bar: 40 μm .



co-localization imaging results, we can know that **NPT-H₂O₂** located in lysosomes and have a good co-localization correlation coefficient ($pc = 0.85$, Fig. 4C(a)–(d)). All the results indicated **NPT-H₂O₂** can penetrate the cell membrane and can locate the lysosomes for H₂O₂ detecting.

Finally, in order to evaluate the relationship between inflammation and H₂O₂, we constructed a mouse model to clarify the physiology and pathology of H₂O₂ and inflammation. Firstly, under the conditions of animal ethics, injuring the mouse's right hind foot to obtain inflamed tissue, while its left hind foot was kept normal as a control. Secondly, the model mouse was incubated in the incubator for 2 days. Thirdly, 5.0 μM probe solution was injected into the inflammation area of the right hind foot and the left hind foot symmetrical to the inflammation area of the right hind foot, respectively, and then the mouse was placed in an incubator for 1 h. At last, the mice were sacrificed according to the requirements of animal ethics, and taking the inflamed tissue of the right hind foot and the normal tissue of the left hind foot symmetrical to the right hind foot for imaging analysis. The imaging analysis of the tissue was performed with a Zeiss laser scanning confocal microscope (Germany), showing the fluorescence signal of inflamed tissues was significantly enhanced in the yellow channel, while the blue channel was significantly weakened (Fig. 5a and b). In contrast, for the normal tissues, the yellow channel has only a weak fluorescence signal, while the blue channel always has a bright fluorescent signal (Fig. 5a' and b'). Therefore, the experimental results exhibited that the newly designed probe as an important tool could perform H₂O₂ detection in inflamed tissues.

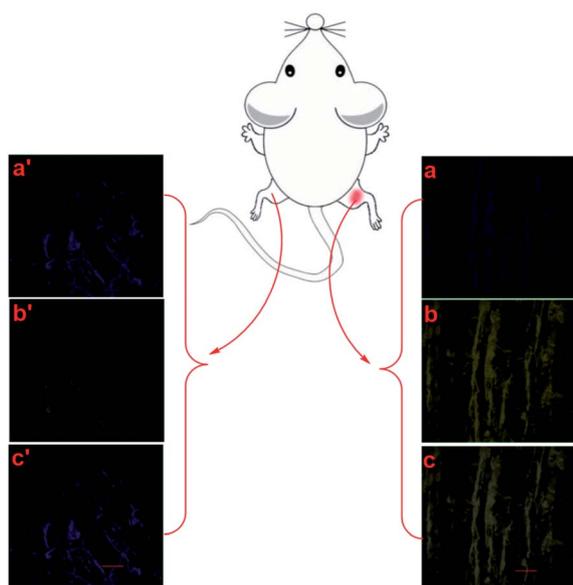


Fig. 5 Confocal images of a frozen inflamed and normal tissue slice from a nude mouse model stained. (a')–(c') Normal tissues injected probe **NPT-H₂O₂** for imaging: (a') blue channel image, (b') yellow channel image, (c') overlay (a') and (b') image; (a)–(c) model of inflamed tissues injected probe **NPT-H₂O₂** for imaging: (a) blue channel image, (b) yellow channel image, (c) overlay (a) and (b) image. $\lambda_{ex} = 405$ nm, λ_{em} : (410–460) nm, and (500–560) nm, respectively. All images were acquired with a 40× oil immersion objective, scale bar: 50 μm.

Conclusions

In summary, in this work, we have designed and synthesized a naphthalimide-based lysosome-targeting ratiometric fluorescent probe **NPT-H₂O₂** with large Stokes shift for H₂O₂ detection in living cells and inflamed tissues. Adopting a well-known H₂O₂ responsive moiety of benzyl boric acid ester to detect H₂O₂. In the absence of H₂O₂ only one fluorescent emission signal at 425 nm, while in the presence of H₂O₂, a new fluorescent emission peak was produced at 550 nm, the two well-resolved fluorescent emission signal separated by 125 nm. Moreover, **NPT-H₂O₂** exhibited a large ratio ability and it was capable of H₂O₂ detection in living cells and inflamed tissues. Therefore, we expect that the ratiometric fluorescent probe **NPT-H₂O₂** as a useful tool to study the physiological pathology related to H₂O₂ and inflammation.

Ethical statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Central South University of Forestry and Technology and approved by the Animal Ethics Committee of Central South University of Forestry and Technology.

Conflicts of interest

There are no conflicts to declare.

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