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# Construction of a lysosome-targetable ratiometric fluorescent probe for H<sub>2</sub>O<sub>2</sub> tracing and imaging in living cells and an inflamed model†

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Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 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 H<sub>2</sub>O<sub>2</sub>, the development of a ratiometric fluorescent probe for H<sub>2</sub>O<sub>2</sub> detection and imaging in lysosomes and an inflammation model remains rather scarce. Therefore, it is important to develop an efficient tool for monitoring H<sub>2</sub>O<sub>2</sub> in inflamed tissues to evaluate the physiological and pathological relationship between inflammation and lysosomal H<sub>2</sub>O<sub>2</sub>. In this work, a new naphthalimide-based lysosome-targeting fluorescent probe (NPT-H<sub>2</sub>O<sub>2</sub>) 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 H<sub>2</sub>O<sub>2</sub>, as well as low cytotoxicity *in vitro*. Inspired by prominent features of these results, we further successfully applied NPT-H<sub>2</sub>O<sub>2</sub> for H<sub>2</sub>O<sub>2</sub> imaging with a dual-channel in living cells, demonstrating that our probe NPT-H<sub>2</sub>O<sub>2</sub> was targeted in the lysosomes. Finally, NPT-H<sub>2</sub>O<sub>2</sub> was used for H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>.

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## Introduction

Reactive oxygen species (ROS), involving hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 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.<sup>1–3</sup> 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.*<sup>4,5</sup> Among them, H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> can damage a wide variety of biomacromolecules including DNA, RNA, and proteins,<sup>6,7</sup> and excessive H<sub>2</sub>O<sub>2</sub> 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.<sup>8–12</sup> In addition, lysosomes are acidic vesicles (pH 4.5–5.5) that contain various enzymes related to physiological and pathological processes,<sup>13,14</sup> and modern biomedical studies have shown that ROS can permeabilize lysosomal membranes to cause cell death.<sup>15–17</sup> It is therefore, developing a suitable tool and method for detecting and imaging lysosomal H<sub>2</sub>O<sub>2</sub> 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.<sup>18–22</sup>

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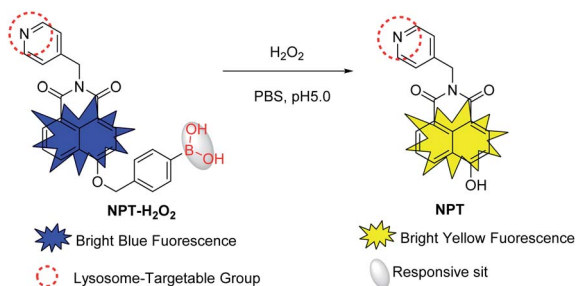
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Scheme 1 Schematic diagram of the response mechanism of the probe NPT- $\text{H}_2\text{O}_2$ .

Recently, a lot of  $\text{H}_2\text{O}_2$  fluorescent probes have been successfully developed for the detection and imaging of  $\text{H}_2\text{O}_2$ .<sup>23–32</sup> However, most of the reported  $\text{H}_2\text{O}_2$  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.<sup>33–37</sup> Moreover, some reported ratiometric  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  detecting in biosystems.

Due to the excellent optical properties of donor- $\pi$ -acceptor (D- $\pi$ -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  $\text{H}_2\text{O}_2$  fluorescent probe based on naphthalimide. Herein, utilizing benzyl boric acid as  $\text{H}_2\text{O}_2$  reactive moiety (a well-known  $\text{H}_2\text{O}_2$  responsive moiety), a new ratiometric fluorescent probe NPT- $\text{H}_2\text{O}_2$  was constructed for the selective detection of  $\text{H}_2\text{O}_2$ . In the absence of the  $\text{H}_2\text{O}_2$ , it emits a bright blue light, but in the presence of  $\text{H}_2\text{O}_2$ , it emits a bright yellow light, so as to achieve ratiometric detection of  $\text{H}_2\text{O}_2$ . Moreover, because the pyridine group is weakly basic, it can be actively targeting the lysosomes as a lysosomal targeting group. Significantly, NPT- $\text{H}_2\text{O}_2$  exhibits excellent sensing performance for  $\text{H}_2\text{O}_2$ : (1) it exhibits a target-regulated ratiometric fluorescence response toward  $\text{H}_2\text{O}_2$ ; (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  $\text{H}_2\text{O}_2$  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- $\text{H}_2\text{O}_2$  was successfully applied to detect and image  $\text{H}_2\text{O}_2$  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,<sup>38</sup> 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)  $\text{K}_2\text{CO}_3$ , 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.  $^1\text{H}$  NMR (400 MHz,  $\text{d}_6$ -DMSO)  $\delta$  (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);  $^{13}\text{C}$  NMR (100 MHz,  $\text{d}_6$ -DMSO)  $\delta$  (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:  $m/z$ ,  $\text{C}_{18}\text{H}_{12}\text{N}_2\text{O}_3$ , calcd 304.08, found 304.09.

### Synthesis of ratiometric fluorescent probe 1 (NPT- $\text{H}_2\text{O}_2$ )

152 mg (0.5 mmol) 2, 107 mg (0.5 mmol) 3, 139 mg (1 mmol)  $\text{K}_2\text{CO}_3$  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.  $^1\text{H}$  NMR (400 MHz, DMSO- $\text{d}_6$ )  $\delta$  (ppm):

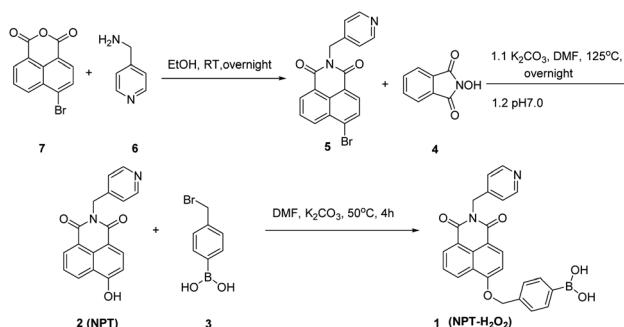


Fig. 1 The synthetic route of NPT- $\text{H}_2\text{O}_2$ .

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);  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$  (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-H<sub>2</sub>O<sub>2</sub>** were obtained by dissolving the right amount of **NPT-H<sub>2</sub>O<sub>2</sub>** in DMSO, and 10 mM stock solutions of  $\text{H}_2\text{O}_2$  and other analytes were obtained in 10 mM PBS (pH 4.5). According to the previous references to prepare other ROS,<sup>39</sup> 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 $\text{H}_2\text{O}_2$ in living cells

Before fluorescence imaging  $\text{H}_2\text{O}_2$  in living cells, the MTT method was used to assess the degree of toxicity of the probe **NPT-H<sub>2</sub>O<sub>2</sub>** 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 $\text{H}_2\text{O}_2$ in inflamed tissues

In order to investigate the probe's imaging of  $\text{H}_2\text{O}_2$  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  $\mu\text{M}$  probe **NPT-H<sub>2</sub>O<sub>2</sub>** for 1 h for  $\text{H}_2\text{O}_2$  images.

## Results and discussion

Naphthalimide-based fluorescent probes are widely used due to their D- $\pi$ -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  $\text{H}_2\text{O}_2$  responsive group (benzyl boric acid derivatives)<sup>40</sup> to modify 4-position hydroxyl ( $-\text{OH}$ ), and as well as used a pyridine group with the ability to bind  $\text{H}^+$  as a lysosome-targeting group to obtain targeting ratiometric fluorescent probe **NPT-H<sub>2</sub>O<sub>2</sub>** for effectively detecting  $\text{H}_2\text{O}_2$ . Because lysosomes are acidic vesicles (pH 4.5–5.5),<sup>13,14</sup> 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  $\text{O}^-$  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  $\text{H}_2\text{O}_2$  to release the dye **NPT** and restore the weak ICT effect of the  $-\text{OH}$ , so the ratiometric detection of  $\text{H}_2\text{O}_2$  can be achieved. According to the synthetic route given in Fig. 1 to synthesize the ratiometric fluorescent probe **NPT-H<sub>2</sub>O<sub>2</sub>**, and the targeting probe was fully characterized by  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and ESI-MS (see ESI†).

In order to investigate the responsive spectrum properties of the newly designed fluorescence probe **NPT-H<sub>2</sub>O<sub>2</sub>** toward  $\text{H}_2\text{O}_2$  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-H<sub>2</sub>O<sub>2</sub>** (5.0  $\mu\text{M}$ ) in the absence and in the presence of  $\text{H}_2\text{O}_2$  in PBS buffer (10 mM, pH 5.0, with 1% DMSO). As shown in Fig. 2a, in the absence  $\text{H}_2\text{O}_2$ , the newly designed fluorescent probe **NPT-H<sub>2</sub>O<sub>2</sub>** has a remarkable absorption peak at 375 nm. When in the presence of 60.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 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-H<sub>2</sub>O<sub>2</sub>** toward  $\text{H}_2\text{O}_2$  was investigated. The result showed that **NPT-H<sub>2</sub>O<sub>2</sub>** had a good response kinetic. In the presence of 60.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 5.0  $\mu\text{M}$  **NPT-H<sub>2</sub>O<sub>2</sub>** could quickly react with  $\text{H}_2\text{O}_2$  during 1 min to reach responsive

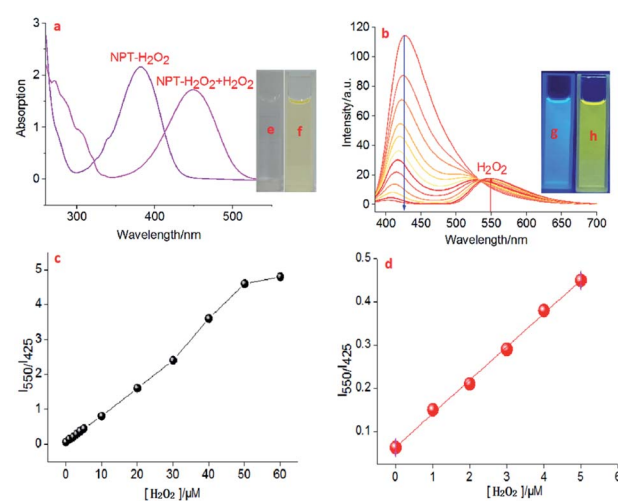
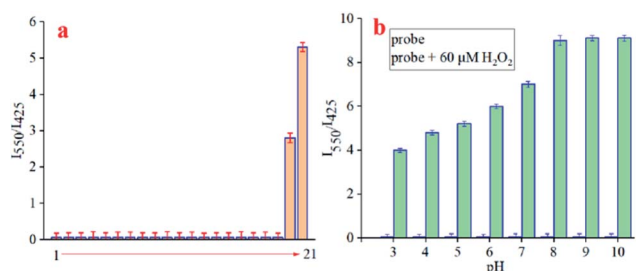


Fig. 2 (a) UV-vis absorption spectra of **NPT-H<sub>2</sub>O<sub>2</sub>** and **NPT-H<sub>2</sub>O<sub>2</sub>** + 60.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ; (b) fluorescence spectra of 5.0  $\mu\text{M}$  **NPT-H<sub>2</sub>O<sub>2</sub>** after adding different amount of  $\text{H}_2\text{O}_2$  (0–60.0)  $\mu\text{M}$  in 10 mM PBS solution (pH 5.0),  $\lambda_{\text{ex}} = 375$  nm; (c) fluorescence ratio ( $I_{550}/I_{425}$ ) vs.  $\text{H}_2\text{O}_2$  concentration (0–60.0)  $\mu\text{M}$  was plotted the calibration curve; (d) the linear response: plot of fluorescence ratio ( $I_{550}/I_{425}$ ) vs. the concentration of  $\text{H}_2\text{O}_2$  (0–5.0)  $\mu\text{M}$ ; inset (e)–(h) were **NPT-H<sub>2</sub>O<sub>2</sub>** before and after 60.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$  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-H<sub>2</sub>O<sub>2</sub>** of the fluorescence intensity vs. different concentrations of (0–60.0)  $\mu\text{M}$  **H<sub>2</sub>O<sub>2</sub>** 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 **H<sub>2</sub>O<sub>2</sub>** 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  $\sim 5$ -fold ratio enhancement (Fig. 2c), and when **H<sub>2</sub>O<sub>2</sub>** enhance from 0 to 5.0  $\mu\text{M}$  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-H<sub>2</sub>O<sub>2</sub>** has a potential to high sensitive for ratiometric **H<sub>2</sub>O<sub>2</sub>** 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-H<sub>2</sub>O<sub>2</sub>** with other ROS and other biological species including blank, Gly, Glu, GSH, Cys,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Fe}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{NO}_2^-$ ,  $\text{SO}_3^{2-}$ , NO, TBO<sup>•</sup>, HO<sup>•</sup>, *t*-BuOOH,  $\text{ClO}^-$ ,  $\text{O}_2^{\cdot-}$ , ONOO<sup>-</sup>, and **H<sub>2</sub>O<sub>2</sub>** (Fig. 3a). Once upon reaction with **H<sub>2</sub>O<sub>2</sub>**, a large ratio ( $I_{550}/I_{425}$ ) of fluorescent intensity was enhanced. Although ONOO<sup>-</sup> 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 **H<sub>2</sub>O<sub>2</sub>**, the addition of other analytes did not increase the fluorescence ratio value (Fig. 3a,  $I_{550}/I_{425}$ ) of the newly designed **NPT-H<sub>2</sub>O<sub>2</sub>**, suggesting **NPT-H<sub>2</sub>O<sub>2</sub>** to be accurately used in complex biological samples for **H<sub>2</sub>O<sub>2</sub>** detection.

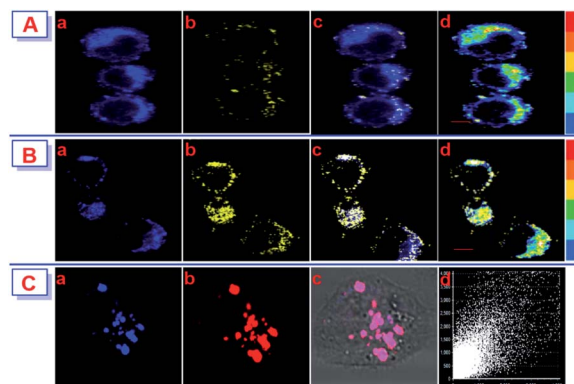
Additionally, responsiveness of **NPT-H<sub>2</sub>O<sub>2</sub>** toward **H<sub>2</sub>O<sub>2</sub>** under different pH (3.0–10.0) values further evaluated, the results as exhibited in Fig. 3b. In the absence of **H<sub>2</sub>O<sub>2</sub>**, no significant fluorescence intensity changes were caused by the



**Fig. 3** (a) The photograph of 5.0  $\mu\text{M}$  **NPT-H<sub>2</sub>O<sub>2</sub>** upon addition of various species in 10 mM PBS buffer (pH 5.0, 1% DMSO): (1 to 21) were blank, Gly, Glu, GSH, Cys,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Fe}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{NO}_2^-$ ,  $\text{SO}_3^{2-}$ , NO, TBO<sup>•</sup>, HO<sup>•</sup>, *t*-BuOOH,  $\text{ClO}^-$ ,  $\text{O}_2^{\cdot-}$ , ONOO<sup>-</sup>, and **H<sub>2</sub>O<sub>2</sub>**, respectively), the acquisition of photograph 5.0 min after mixing,  $\lambda_{\text{ex}} = 375$  nm; (b) The fluorescence ratio ( $F_{550}/F_{425}$ ) of **NPT-H<sub>2</sub>O<sub>2</sub>** 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 **H<sub>2</sub>O<sub>2</sub>** (60.0  $\mu\text{M}$ ), 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-H<sub>2</sub>O<sub>2</sub>** has the potential for **H<sub>2</sub>O<sub>2</sub>** detecting in the lysosomes.

In order to investigate the potential of the probe **NPT-H<sub>2</sub>O<sub>2</sub>** for **H<sub>2</sub>O<sub>2</sub>** detection in living cells model. Before fluorescence imaging, the MTT method was used to evaluate the cytotoxicity of **NPT-H<sub>2</sub>O<sub>2</sub>** to HeLa cells, and the results exhibited the concentration of probe from 0 to 20.0  $\mu\text{M}$  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  $\mu\text{M}$  **NPT**-enhanced at  $I_{550}/I_{425}$  with a very low limit of detection 12.8 nM. In addition, **NPT-H<sub>2</sub>O<sub>2</sub>** showed low cytotoxicity, lysosome-targeting **H<sub>2</sub>O<sub>2</sub>** 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  $\mu\text{M}$  of **H<sub>2</sub>O<sub>2</sub>** for 30 min, and then continued to add 5.0  $\mu\text{M}$  **NPT-H<sub>2</sub>O<sub>2</sub>** 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-H<sub>2</sub>O<sub>2</sub>** has the ability to **H<sub>2</sub>O<sub>2</sub>** detection in the living cell. In addition, a colocalization experiment was carried out to further clarify that **NPT-H<sub>2</sub>O<sub>2</sub>** could detect **H<sub>2</sub>O<sub>2</sub>** in lysosomes. Specifically, **NPT-H<sub>2</sub>O<sub>2</sub>** 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-H<sub>2</sub>O<sub>2</sub>** to carried out colocalization imaging. From the



**Fig. 4** Fluorescence images in HeLa cells. (A) The HeLa cells incubated with 5.0  $\mu\text{M}$  **NPT-H<sub>2</sub>O<sub>2</sub>** 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  $\mu\text{M}$  **H<sub>2</sub>O<sub>2</sub>** for 30 min, and then continue to add 5.0  $\mu\text{M}$  **NPT-H<sub>2</sub>O<sub>2</sub>** 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  $\mu\text{M}$  **NPT-H<sub>2</sub>O<sub>2</sub>** 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,  $\lambda_{\text{em}}$ : (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  $\mu\text{m}$ .



co-localization imaging results, we can know that **NPT-H<sub>2</sub>O<sub>2</sub>** located in lysosomes and have a good co-localization correlation coefficient ( $pc = 0.85$ , Fig. 4C(a)–(d)). All the results indicated **NPT-H<sub>2</sub>O<sub>2</sub>** can penetrate the cell membrane and can locate the lysosomes for H<sub>2</sub>O<sub>2</sub> detecting.

Finally, in order to evaluate the relationship between inflammation and H<sub>2</sub>O<sub>2</sub>, we constructed a mouse model to clarify the physiology and pathology of H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> detection in inflamed tissues.

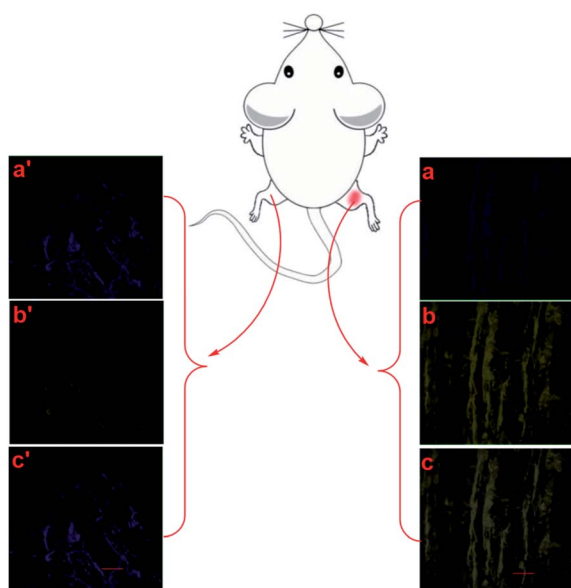


Fig. 5 Confocal images of a frozen inflamed and normal tissue slice from a nude mouse model stained. (a')–(c') Normal tissues injected probe **NPT-H<sub>2</sub>O<sub>2</sub>** for imaging: (a') blue channel image, (b') yellow channel image, (c') overlay (a') and (b') image; (a)–(c) model of inflamed tissues injected probe **NPT-H<sub>2</sub>O<sub>2</sub>** for imaging: (a) blue channel image, (b) yellow channel image, (c) overlay (a) and (b) image.  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}}$ : (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-H<sub>2</sub>O<sub>2</sub>** with large Stokes shift for H<sub>2</sub>O<sub>2</sub> detection in living cells and inflamed tissues. Adopting a well-known H<sub>2</sub>O<sub>2</sub> responsive moiety of benzyl boric acid ester to detect H<sub>2</sub>O<sub>2</sub>. In the absence of H<sub>2</sub>O<sub>2</sub> only one fluorescent emission signal at 425 nm, while in the presence of H<sub>2</sub>O<sub>2</sub>, a new fluorescent emission peak was produced at 550 nm, the two well-resolved fluorescent emission signal separated by 125 nm. Moreover, **NPT-H<sub>2</sub>O<sub>2</sub>** exhibited a large ratio ability and it was capable of H<sub>2</sub>O<sub>2</sub> detection in living cells and inflamed tissues. Therefore, we expect that the ratiometric fluorescent probe **NPT-H<sub>2</sub>O<sub>2</sub>** as a useful tool to study the physiological pathology related to H<sub>2</sub>O<sub>2</sub> 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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