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A mitochondrial-targetable dual functional near-infrared fluorescent probe to monitor pH and H₂O₂ in living cells and mice[†]

 Xueyuan Bi,^a Yingying Wang,^a Dandan Wang,^a Liming Liu,^a Wen Zhu,^a Junjie Zhang^b and Xiaoming Zha^{ID *a}

A lower pH level and high hydrogen peroxide (H₂O₂) concentration in mitochondria is closely associated with a variety of diseases including cancer and inflammation. Thus, determination of changes in the level of acidic pH and H₂O₂ is of great importance and could provide new insights into the key functions under both physiological and pathological conditions. Herein, we present a novel mitochondria-targetable probe **NIR-pH-H₂O₂**, as the first near infrared (NIR) fluorescent small molecule, to monitor changes of endogenous pH ($pK_a = 6.17$) and H₂O₂ with high sensitivity, good compatibility and low cytotoxicity. Furthermore, it was successfully employed to monitor pH and H₂O₂ in a mouse acute inflammation model. These results demonstrate that **NIR-pH-H₂O₂** is a novel bifunctional mitochondrial-targeted NIR probe to sense acidic pH and H₂O₂ *in vitro* and *in vivo*, indicating its huge potential for the diagnosis of pH and H₂O₂-related diseases.

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Introduction

Intracellular pH is an important parameter in mediating many physiological processes such as cell metabolism, proliferation, apoptosis, ion transport and homeostasis and so on.¹⁻⁴ However, abnormal intracellular pH is associated with cellular dysfunctions such as cancer and neurodegenerative disorders.^{5,6} Hydrogen peroxide (H₂O₂), an important member of reactive oxygen species (ROS) mediates signal cascades involved in redox homeostasis.⁷ Substantial evidence has showed that excessive H₂O₂ is relevant to serious diseases including cardiovascular disorders, neurodegenerative diseases, cancer and diabetes.⁸⁻¹³ Wang *et al.* monitored low pH and high H₂O₂ in cancer cells, using dual responsive supramolecular polymer by the host-guest interactions of β -CD-hydrazone-DOX and PEG-Fc.¹⁴ Colon cancer cells were extremely sensitive to pH reduction, which naturally cause metabolic stress in the colon lumen, as well as H₂O₂ and ionizing radiation.¹⁵ A trend for reduced pH and increased H₂O₂ in lower airway inflammation and chronic obstructive pulmonary disease was observed.^{16,17} Mitochondria are cellular powerhouses that produce energy through the respiratory chain.¹⁸ Abnormal levels of pH and H₂O₂ in the mitochondria are associated with various diseases

like cancer, Alzheimer's disease and cardiovascular diseases.¹⁹⁻²² Thus, determination of mitochondrial changes in pH and level of H₂O₂ is of great importance and could provide new insights into the key functions of mitochondria under both physiological and pathological conditions.

Recently, Zhou *et al.* developed a type of two-photon dyes with D- π -A- π -D structure, which possesses near-infrared (NIR) properties, large two-photon absorption cross-section ~ 160 G and high fluorescence quantum yield ~ 0.15 .²³ The effect of pH on this type of dyes had been studied, pH changes had significant effect on this kind of dyes fluorescence intensity, speculating that in a strong base condition the oxygen-bridge bond could be cut off and in acidic medium recovered easily. To the best of our knowledge, we firstly propose that oxygen-bridge group could be appropriate for the development of acidic pH fluorescent probes. In fluorescent detection field, boric acid group has widely been used as the recognition site toward H₂O₂.²⁴⁻²⁶ The chemo-selective deprotection of boric acid to phenols provides a useful reaction-based method which detects H₂O₂ over other ROS.²⁷ Additionally, phenylboronic acid ($pK_a = 8.70$) with relatively weak Lewis acidity is more stability to different pH.²⁸ Moreover, carbonate as the self-immolative system was applied as a linker of probe which can release CO₂ through a 1,6-benzyl elimination.^{29,30}

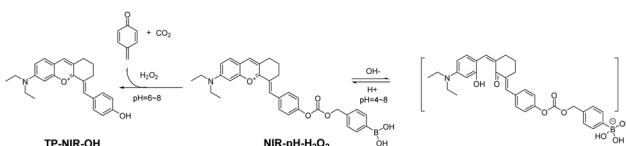
In order to study the biological function or harmful effects of pH and H₂O₂ in mitochondria, we present the first mitochondria-immobilized NIR bifunctional fluorescent probe **NIR-pH-H₂O₂** for monitoring pH and H₂O₂ (Table S1[†]). As illustrated in Scheme 1, **NIR-pH-H₂O₂** consists of **TP-NIR-OH** fluorophore, phenylboronic acid and carbonate linker. The **TP**-

^aSchool of Engineering, China Pharmaceutical University, 639 Longmian Avenue, Nanjing 211198, China

^bSchool of Pharmacy, China Pharmaceutical University, 639 Longmian Avenue, Nanjing 211198, China. E-mail: xmzha@cpu.edu.cn

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Scheme 1 Proposed reaction mechanism of **NIR-pH-H₂O₂** towards pH and H₂O₂.

NIR-OH fluorophore was predicted to display a NIR fluorescence “off-on” signal response to acidic pH and its positively charged was expected to display mitochondria-specific staining capability. Phenylboronic acid moiety was oxidized by H₂O₂ to release CO₂ through a 1,6-benzyl elimination, resulting in the recovery of fluorescence. It was successfully applied for image of pH and H₂O₂ in mitochondria of live cells and monitoring inflammation in living mice. The present results strongly demonstrated that **NIR-pH-H₂O₂** would serve as an excellent bifunctional fluorescent probe to monitor mitochondrial pH and H₂O₂ fluctuations in living biological samples.

Experimental section

Materials and instruments

Absorption spectra measurements were performed on a Shimadzu UV-vis spectrophotometer, UV-2550. Fluorescent spectra measurements were performed on a Thermo Scientific Lumina fluorescence spectrophotometer. The pH was carried out by a PB 10 digital pH meter. ¹H NMR (400 MHz) and ¹³C NMR (125 MHz) spectra were measured on a Bruker ACF-300 spectrometer (Bruker Corp., Billerica, MA, USA) using TMS as internal reference. Mass spectra was carried on an Agilent 1100 Series (Agilent Technologies, Santa Clara, CA, USA) LC/MSD high performance ion trap mass spectrometer and a Mariner ESI-TOF spectrometer. All starting materials and reagents were purchased from commercial sources and used without further purification. Deionized water was prepared through a water purification system. Fluorescent microscopy imaging was carried out by a confocal laser scanning microscope (CLSM, LSM700, Zeiss, Germany). Fluorescent images of mice were taken by an IVIS Spectrum.

General procedure for fluorometric analysis

A stock solution of **NIR-pH-H₂O₂** (1 mM) was prepared in DMSO. Stock solutions of H₂O₂ and other various analytes were prepared with distilled water freshly. A series of standard pH buffer solutions with different values were prepared by the standard procedures. Before absorption and fluorescence spectral measurements, all the resulting solutions were shaken well and incubated for 60 min at room temperature, all fluorescence measurements were executed with the excitation wavelength of 560 nm and the emission wavelength of 680 nm unless other noted.

The *pK_a* values of **NIR-pH-H₂O₂**

The *pK_a* values of **NIR-pH-H₂O₂** were calculated using the Henderson–Hasselbalch equation: $\log[(I_{\max} - I)/(I - I_{\min})] = \text{pH} - pK_a$. Where *I* was the observed fluorescence intensity, *I_{max}* and *I_{min}* were the corresponding maximum and minimum fluorescence intensity, respectively.³¹

Cytotoxicity assay and fluorescence imaging toward pH and H₂O₂ in living cells

The cytotoxic effect of **NIR-pH-H₂O₂** was assessed by the MTT assay. After cells attachment, MCF-7 and Hela cell lines pre-treated in medium with **NIR-pH-H₂O₂** at different concentrations. 200 μ L DMSO was added to each well and shaken for 20 min. The absorbance was measured at 490 nm with a microplate reader (Tecan US, Inc. USA). Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{optical density value (treatment group)}}{\text{optical density value (control group)}} \times 100\%.$$

Before imaging, cells were passed and seeded in glass dishes. For labeling, the cells were incubated with 10 μ M of **NIR-pH-H₂O₂** for the specific incubation time. Then, cells were washed three times with PBS (phosphate buffered saline). Cell images were then recorded using a confocal laser scanning microscope (CLSM, LSM700, Zeiss, Germany) and processed using the ZEN imaging software. Other information is available in the figure captions.

Animal models and *in vivo* animal studies

Athymic nude mice were purchased from Qinglongshan Animal Breeding Farm (Nanjing, China) for *in vivo* imaging studies. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of China Pharmaceutical University and the experiments were approved by the Animal Ethics Committee of China Pharmaceutical University.

Before animal imaging, nude mice were given an intraperitoneal injection of lipopolysaccharide (2 mg mL⁻¹) in 100 μ L PBS for group A. **NIR-pH-H₂O₂** (250 μ M) in 200 μ L buffered solution was injected in the same way for group B. Lipopolysaccharide (2 mg mL⁻¹) in 100 μ L PBS buffer solution was injected intraperitoneally firstly. After 12 h, **NIR-pH-H₂O₂** (250 μ M) in 200 μ L of buffered solution was injected at the same region for group C. All fluorescence images were acquired with auto exposure (IVIS Spectrum) after incubation for 30 min by using the NIR fluorescence imaging system (excitation wavelength = 560 nm, emission wavelength = 680 nm).³²

Synthesis of **NIR-pH-H₂O₂**

The probe **NIR-pH-H₂O₂** was synthesized by the procedure shown in Fig. S1.[†] TP-NIR-OH and **B1** was synthesized according to the reported methods.^{23,30}



TP-NIR-OH (62 mg, 0.2 mmol), compound **B1** (80 mg, 0.2 mmol) and K_2CO_3 (65 mg, 0.6 mmol) were dissolved in 5 mL anhydrous DMF under nitrogen. The mixture was allowed to stir at room temperature for 2 h. The solvent was removed under reduced pressure. Then the residue was purified on a silica gel column with CH_2Cl_2 to CH_2Cl_2 /methanol (v/v, 200/1 to 15/1) as eluent to afford **NIR-pH-H₂O₂** as black solid (30 mg, 21% yield). 1H NMR (500 MHz, $DMSO-d_6$) δ 8.55 (s, 1H), 8.06 (s, 2H), 8.04 (s, 1H), 7.90 (d, J = 9.5 Hz, 1H), 7.79 (d, J = 7.7 Hz, 2H), 7.67 (d, J = 8.6 Hz, 2H), 7.48–7.46 (m, 1H), 7.38–7.36 (m, 4H), 7.30 (d, J = 1.7 Hz, 1H), 5.25 (s, 2H), 3.68 (m, 4H), 2.90–2.83 (m, 4H), 1.81 (m, 2H), 1.21 (t, J = 6.8 Hz, 6H). ^{13}C NMR (125 MHz, $DMSO-d_6$) δ 161.81, 159.16, 156.67, 153.13, 151.66, 149.34, 137.02, 134.82, 134.61, 133.65, 132.67, 132.45, 129.25, 127.66, 123.94, 122.15, 120.02, 119.37, 95.98, 70.51, 70.27, 55.40, 46.21, 40.52, 40.36, 40.19, 40.02, 39.86, 39.69, 39.52, 31.76, 30.88, 29.49, 27.19, 26.99, 22.57, 21.59, 14.42, 12.89. HRMS (ESI): calcd for $C_{32}H_{33}BNO_6^+ [M]^+$ m/z 538.2395, found 538.2416. Purity: 98.5% (HPLC).

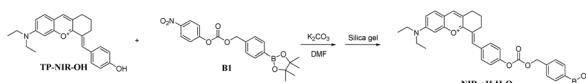
Results and discussion

UV-vis and fluorescence responses to pH

Scheme 2 shows the synthetic route adopted for the preparation of **NIR-pH-H₂O₂**, whose structure was further characterized by 1H NMR, ^{13}C NMR and HRMS (Fig. S8–S10†). The fluorescence behaviors of **NIR-pH-H₂O₂** (10 μ M) towards pH were obtained containing 6% CH_3CN (v/v). As shown in Fig. 1, a significantly large stokes shift of 120 nm was noticed of **NIR-pH-H₂O₂** with an absorption maximum (λ_{abs}) of 560 nm (Fig. 1A) and an emission maximum (λ_{em}) of 680 nm (Fig. 1B). Both intensity of absorption at 560 nm and fluorescence at 680 nm were dramatically reduced as the value of pH (2.96–9.08) gradually increased (Fig. 1C). Moreover, there is an excellent linear relationship of pH over the range of 4.93 to 7.47 (Fig. 1C inset, R^2 = 0.9937). By the nonlinear regression analysis, the pK_a value was calculated to be 6.17, indicating that **NIR-pH-H₂O₂** is capable of assessing acidic media. Additionally, the probe shows satisfactory reversibility between pH 4.0 and pH 8.0 (Fig. 1D).

UV-vis and fluorescence responses to H₂O₂

To investigate the interaction between H₂O₂ and **NIR-pH-H₂O₂**, UV titration was performed (Fig. 2A). The main absorption peak at 560 nm obviously increased with H₂O₂ (200 μ M). Subsequently, the fluorescence of **NIR-pH-H₂O₂** were explored (Fig. 2B). The free probe exhibited a weak fluorescence emission at 680 nm in PBS buffer. However, its fluorescence immediately increased in the presence of H₂O₂ (200 μ M). This result was consistent with its UV absorption spectrum to H₂O₂. As expected, **NIR-pH-H₂O₂** is weak-fluorescent (Fig. 2B) in NIR area.



Scheme 2 Synthesis route for **NIR-pH-H₂O₂**.

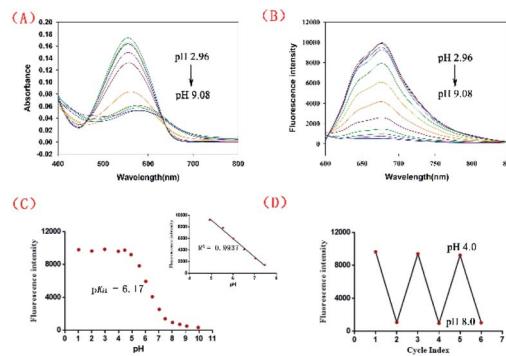


Fig. 1 (A) Absorption and (B) fluorescence emission spectra of **NIR-pH-H₂O₂** (10 μ M) in the phosphate buffer (6% CH_3CN) with various pH values. (C) Plot of I_{680} vs. pH in the range of 1.02 to 9.94. Inset: the linear fitting curve of $I_{680\text{nm}}$ vs. pH from 4.93 to 7.47. (D) Reversible fluorescence changes of **NIR-pH-H₂O₂** between pH 4.0 and 8.0 with λ_{ex} at 560 nm.

The introduction of H₂O₂ could specifically convert the phenylboronic acid unit into an electron-donor-OH group with CO₂ released through a 1,6-benzyl elimination, which resulted a significant fluorescence enhancement at 680 nm. A fluorescence titration of **NIR-pH-H₂O₂** was carried out to further investigate the quantitative relationship between **NIR-pH-H₂O₂** and H₂O₂. With the increasing of H₂O₂, its fluorescence intensity at 680 nm gradually increased (Fig. 2C and D). When the concentration increased to 130 μ M, the fluorescence almost kept stable. In addition, the fluorescence intensity of **NIR-pH-H₂O₂** showed an excellent linear relationship with the H₂O₂ concentration (R^2 = 0.9968 Fig. 2D inset). The detection limit (utilizing the $3\sigma/k$ method) for H₂O₂ was determined 2.097 μ M, which was enough for direct image of intracellular H₂O₂ as its concentrations are usually in sub-micromolar range.²⁵ To ensure whether **NIR-pH-H₂O₂** could be applied for real-time detection, the kinetic experiments towards H₂O₂ were explored (Fig. S1†). It was displayed that the detection process balanced within 30 minutes, after adding H₂O₂ to the probe

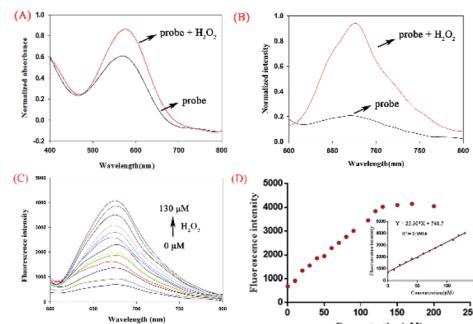


Fig. 2 Absorption (A) and fluorescence (B) spectra of **NIR-pH-H₂O₂** (10 μ M) in PBS buffer (6% CH_3CN). (C) Fluorescence spectra of **NIR-pH-H₂O₂** (10 μ M) in the presence of various amounts of H₂O₂ (0–130 μ M) in PBS buffer. (D) The curve was plotted with fluorescence intensity vs. H₂O₂ concentration (0–200 μ M) inset: linear relationship between fluorescence intensity of **NIR-pH-H₂O₂** and H₂O₂ concentration (0–130 μ M) at 680 nm (λ_{ex} = 560 nm).



solution and a small quantity of enhancement was observed within the next 20 minutes.

Sensitivity detection

Subsequently, the fluorescence specificity of **NIR-pH-H₂O₂** was examined over various other species. As shown in Fig. 3, the fluorescence intensity at 680 nm of H⁺ and H₂O₂ are stronger than the other tested analytes in the phosphate buffer (6% CH₃CN) indicating that **NIR-pH-H₂O₂** was sensitive to protonic acid (pH = 4) and H₂O₂. These unique properties enable it to be feasible to accurately measure intracellular pH and H₂O₂ with high selectivity.

The response between **NIR-pH-H₂O₂** and H₂O₂ at different pH

It was of great significance to know the response between **NIR-pH-H₂O₂** and H₂O₂ at different pH levels. The fluorescence intensity of **NIR-pH-H₂O₂** and **NIR-pH-H₂O₂ + H₂O₂** was then measured in PBS buffer from pH 1 to 10, respectively (Fig. S2†). It was found that fluorescence intensity of **NIR-pH-H₂O₂** can increase upon the addition of H₂O₂ in the pH (1–10) range and the most obvious increase toward H₂O₂ could be observed from pH 6.0 to 8.0, indicating it can be applied in most living organisms (the physiological environment pH = 7.4).

Mechanism studies

The phenylboronic acid group in **NIR-pH-H₂O₂** was the reaction site with H₂O₂ (Scheme 1). The starting material **TP-NIR-OH** was produced through a 1,6-benzyl elimination. The result of the electrospray ionization mass spectrometry (ESI-MS) confirmed the mechanism of this reaction. Addition of H₂O₂ in the **NIR-pH-H₂O₂** solution resulted in a new peak at 360.19578 which correspond to **TP-NIR-OH** [M + H⁺] (Fig. S12†). The same results were also shown in the HPLC analysis (Fig. S13†). Thus, the results were in line with the proposed mechanism.

Cytotoxicity and cell mitochondria-targeting specificity of **NIR-pH-H₂O₂**

Before intracellular experiments, a standard MTT assay of MCF-7 and HeLa cell lines were carried out to evaluate the

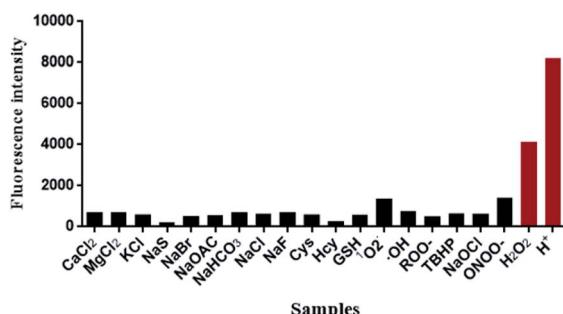


Fig. 3 Fluorescence response of **NIR-pH-H₂O₂** (10 μ M) upon addition of various analytes (200 μ M: CaCl₂; MgCl₂; KCl; NaS; NaBr; NaOAC; NaHCO₃; NaCl; NaF; Cys; Hcy; GSH; O₂⁻; ·OH; ROO⁻; TBHP; NaOCl; ONOO⁻; H₂O₂ and H⁺ (pH = 4) in PBS buffer (6% CH₃CN)).

biocompatibility of **NIR-pH-H₂O₂**. When the probe concentration reached 50 μ M, **NIR-pH-H₂O₂** displayed negligible cell toxicity since nearly 80% of MCF-7 cells (Fig. S3A†) and 85% of HeLa cells (Fig. S3B†) survived and could be applied to biological cells. To confirm the presumed mitochondria-targeting specificity of **NIR-pH-H₂O₂**, the colocalization experiments were performed in MCF-7 cells using Mito Tracker Green (a commercial mitochondria-targeting dye). As expected, the fluorescence image produced using **NIR-pH-H₂O₂** (Fig. 4A) overlaps with that obtained using Mito Tracker Green (Fig. 4B), the fluorescence distribution of **NIR-pH-H₂O₂** overlaps well with that of Mito Green (Pearson's coefficient, 0.90; Fig. S4, parts D and E†). In summary, these data proved that **NIR-pH-H₂O₂** was a good mitochondria-targeted fluorescent probe.

Cell imaging of **NIR-pH-H₂O₂** for pH and H₂O₂

To explore capacity of **NIR-pH-H₂O₂** for living cell fluorescence imaging of pH changes, MCF-7 cells and HeLa cells were evaluated using the confocal fluorescence imaging. The fluorescence intensity of **NIR-pH-H₂O₂** in MCF-7 cells (Fig. 5A) and HeLa cells (Fig. S4A†) gradually decreased with increasing pH over the range of pH 5.08 to 7.84, indicated that **NIR-pH-H₂O₂** could be applied for the detection of pH in living cells. We then employed **NIR-pH-H₂O₂** to determine the pH change in mitochondria with a rapamycin model.³² Rapamycin, a widely used macrolide antibiotic, induces mitophagy in a variety of cell types and acidification of mitochondria has been observed during the mitophagy.^{33–35} MCF-7 cells and HeLa cells were treated with **NIR-pH-H₂O₂** and rapamycin for 1 h then fluorescence images were recorded. As expected, the merged images of the rapamycin-treated MCF-7 cells (Fig. 5B) and HeLa cells (Fig. S4B†) exhibit more obvious colour change, which is ascribed to the rapamycin-induced generation of mitophagy and decrease of the mitochondrial pH.

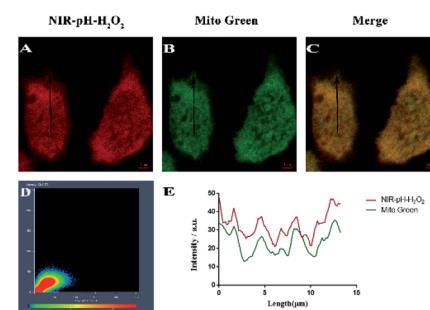


Fig. 4 Colocalization experiments involving **NIR-pH-H₂O₂** and Mito Tracker Green (MTG) in MCF-7 cells. The cells were incubated with **NIR-pH-H₂O₂** (10 μ M) and MTG (5 μ M) for 30 min at 37 °C, followed by treatment with 200 μ M H₂O₂ for 30 min at 37 °C. Images for **NIR-pH-H₂O₂** (A) and MTG (B) were then recorded using excitation wavelengths of 555 nm and 485 nm, and band-path emission filters at 680 nm and 514 nm, respectively. Panels (C) shows a merged image of (A) and (B). (D) Intensity scatter plot of channels 1 and 2. (E) Intensity correlation plot of **NIR-pH-H₂O₂** and MTG (Pearson's coefficient 0.90). Scale bar: 2 μ m.



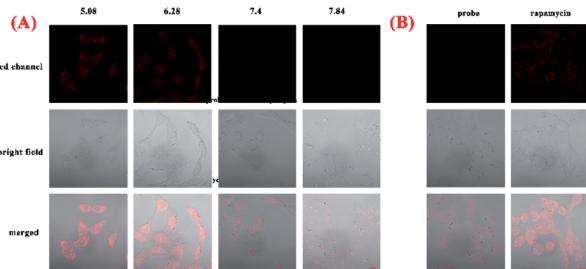


Fig. 5 (A) Fluorescent microscopic images of MCF-7 cells with **NIR-pH-H₂O₂**. Incubated cells with 10 μ L of **NIR-pH-H₂O₂** (10 μ M) under the pH 5.08, 6.28, 7.4, 7.84 and (B) rapamycin (10 μ M) of the culture medium for 30 min and observed under the microscope ($\lambda_{\text{ex}} = 555$ nm, $\lambda_{\text{em}} = 680$ nm for the red channel). Scale bar: 10 μ m.

Next, we investigated the fluorescence imaging of **NIR-pH-H₂O₂** for H₂O₂ in MCF-7 (Fig. 6a–d) cells and Hela cells (Fig. S5a–d†). After incubation with probe **NIR-pH-H₂O₂** for 30 min at 37 °C, almost no fluorescence could be observed for non-H₂O₂. However, fluorescence intensities became increased after treated with increased exogenous H₂O₂. The results were consistent with our previous results of fluorescence spectra, showing that **NIR-pH-H₂O₂** could be used for H₂O₂ detection in living cells. Next, application of **NIR-pH-H₂O₂** to visualize endogenous H₂O₂ in MCF-7 cells and Hela cells by confocal fluorescence microscopy. To induce acute endogenous H₂O₂ production, the nonspecific protein kinase C activator phorbol myristate acetate (PMA; 200 ng mL⁻¹) was co-incubated with **NIR-pH-H₂O₂** for 30 min, followed by confocal imaging.^{36,37} Fluorescence intensity of **NIR-pH-H₂O₂** sharply enhanced due to PMA treatment triggered an oxidative burst in for MCF-7 cells (Fig. 6e) and Hela cells (Fig. S5e†) compared with the control group. These results indicate the capability of **NIR-pH-H₂O₂** in determining the endogenous changes of H₂O₂ in living cells. Dynamic fluorescence imaging for **NIR-pH-H₂O₂** was further conducted. After incubated with exogenous H₂O₂ and probe **NIR-pH-H₂O₂** in MCF-7 (Fig. S6†) and Hela cell lines (Fig. S7†), red fluorescence was enhanced gradually after incubated for

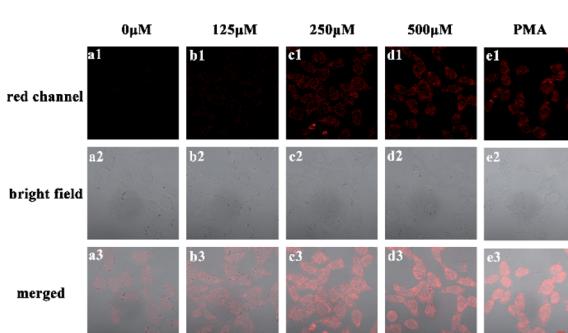


Fig. 6 Fluorescence images of **NIR-pH-H₂O₂** (10 μ M) with various concentrations of H₂O₂ solution and PMA ($\lambda_{\text{ex}} = 555$ nm, $\lambda_{\text{em}} = 680$ nm for the red channel). The MCF-7 was treated with **NIR-pH-H₂O₂** and different concentration of H₂O₂ and PMA for 30 min. Scale bar: 10 μ m.

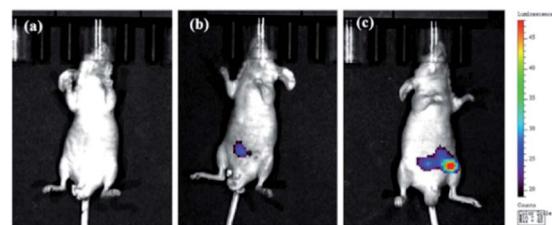


Fig. 7 *In vivo* fluorescence images in the peritoneal cavity of mice injected with **NIR-pH-H₂O₂** during LPS-induced inflammatory response. (a) Only LPS was injected for control. (b) Saline was injected in the peritoneal cavity of mouse, followed by injection of **NIR-pH-H₂O₂** (50 μ M). (c) LPS was injected into the peritoneal cavity of the mouse, followed by injection with **NIR-pH-H₂O₂** (50 μ M). The mice were imaged with an excitation filter at 560 nm and emission filter at 680 nm.

0 min, 10 min, 20 min and 30 min. The results were consistent with our previous results of fluorescence spectra.

Fluorescence imaging of **NIR-pH-H₂O₂** in mice

Having achieved successful cell imaging with **NIR-pH-H₂O₂**, a further exploratory effort was made to determine whether it would be competent of fluorescence imaging *in vivo* and reflect occurrence of biological processes induced by exogenous stimulation. Since abdominal inflammation induced by lipopolysaccharide (LPS) could cause changes of pH and over-production of hydrogen peroxide,^{38–40} we investigated the application of **NIR-pH-H₂O₂** to visualize fluorescence changes in an abdominal inflammation mouse model induced by LPS. As shown in Fig. 7b, the mouse treated with saline and **NIR-pH-H₂O₂** exhibited low fluorescence intensity. However, the fluorescence intensity in the mouse treated with LPS and **NIR-pH-H₂O₂** changed evidently (Fig. 7c), which was attributed to that the inflammatory response could reduce the local pH and increased the level of H₂O₂. As the control, the mouse treated with LPS but no **NIR-pH-H₂O₂** injection showed no fluorescence (Fig. 7a). Therefore, these results demonstrate that **NIR-pH-H₂O₂** is a prominent fluorescent probe for imaging inflammation *in vivo*.

Conclusions

In summary, we firstly reported a novel “off-on” dual functional NIR fluorescent probe **NIR-pH-H₂O₂** to monitor pH and H₂O₂ *in vitro* and *in vivo*. The cellular assay confirmed that **NIR-pH-H₂O₂** is co-localized in mitochondria with low cytotoxicity. Furthermore, the probe shows excellent pH sensitivity, good response to H₂O₂, and successful imaging of pH and H₂O₂ in mice inflammation model induced by LPS. Mitochondria acidification is seen during the mitophagy. Abnormal levels of mitophagy and H₂O₂ are associated with various pathological conditions such as cancer, inflammation and neurodegenerative diseases. On the basis of these results, we envision that **NIR-pH-H₂O₂** could be employed to understand the roles of reduced pH and increased H₂O₂ in pathological conditions and further



for the pH and H_2O_2 related diseases diagnosis and real-time monitoring.

Conflicts of interest

There are no conflicts to declare.

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