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Review Article

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Recent Development of a Fluorescent Probe for In Vivo Detection of Hydrogen Peroxide

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Abstract : Hydrogen peroxide (H_2O_2), an important and distinct member of the ROS (reactive oxygen species) family, is produced by the incomplete reduction of oxygen and also oxidative decomposition process during metabolism. *In vivo* endogenous hydrogen peroxide is associated with many of the diseases including diabetes, cancer, cardiovascular disease, and neurodegenerative disorders. Thus, it is of great significance to track this small molecule, H_2O_2, simply and accurately in *in vivo* biological systems. Recently, various researches have been reported to develop the investigation to detect H_2O_2 in biological systems. Among them, only *in vivo* tracking applications of this molecule are now considered as a potential tool to analyse several diseases. But due to the interference of intrinsic background and dynamic complexity, the *in vivo* tracking of H_2O_2 is a challenging task for the scientists. We have incorporated here in this review some of the interesting research works having rational design strategies which deal with *in vivo* detection of H_2O_2 reported recently. For the first time, Fenton reaction has been utilized to demonstrate as a powerful tool for *in vitro* detection of H_2O_2 reported by Z. Qing, R. Yang's groups. Interesting nanoprobe was designed for specific recognition of H_2O_2 using two-photon microscopy techniques based on target-activated trigger and dual-emission fluorescence modulator developed by S. Yang, R. Yang's groups. Also, fluorescent probes were developed based on the Intramolecular Charge Transfer (ICT) process by Z. Lu, Z. Wang's groups. In addition, near infrared (NIR) fluorescence probes were synthesised based on Cyanine and Aza-BODIPY derivatives by the various groups of researchers for the detection of H_2O_2 and successfully applied to *in vivo* imaging of endogenous H_2O_2. Although, till date, many probes were designed so far for the detection of H_2O_2, real-time probes for rapid response are still expected for *in vivo* researches.

Keywords: Fluorescent probe, Detection, Hydrogen peroxide, *in vivo*, imaging

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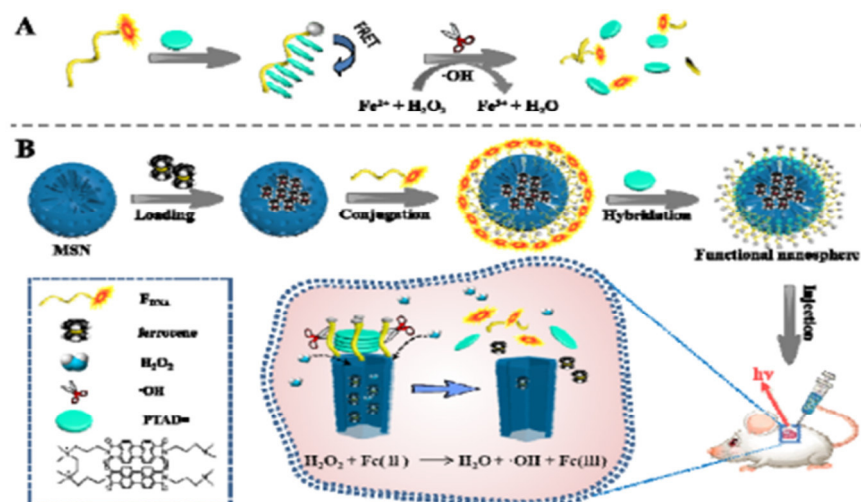
1. INTRODUCTION

Inter cellular production of Reactive oxygen species (ROS) play a significant role in regulating physiological functions.[1-6] ROS being a group of free radicals or non-free radicals, display high reactivity towards biomolecules.[7,8] Hydrogen peroxide (H_2O_2) is an significant member of the ROS family due to its mild reactivity in biological systems.[9-11] H_2O_2 is produced due to the incomplete reduction of oxygen and generated endogenously in a variety of enzyme-catalyzed reactions.[12] It plays a significant role in the various biological progresses such as immune response, host defence, etc.[13,14] However, enhanced concentration of H_2O_2 may damage DNA and RNA structure of organisms that can result many diseases e.g. diabetes,[15] cancer,[16] cardiovascular disease,[17] and neurodegenerative disorders[18] such as Parkinson's disease and Alzheimer disease. Also damage of neurons by H_2O_2 leads to an irreversible process. So, H_2O_2 has been considered as a possible diagnostic tool for several diseases and the ability to monitor H_2O_2 level in vivo is becoming very essential. Therefore, to monitor physiological H_2O_2, development of effective and real time strategies for detection is urgently needed. At present, a greater number of detection methods for H_2O_2 have been reported in literature. Though, most of the reported methods involve complex sample preparation, robust procedures, and disrupting cell and/or tissue structures.[19-21] Among them, only fluorescence-based analytical methods combination with confocal imaging have been considered as an attractive tool for of H_2O_2 detection due to their high selectivity, sensitivity and fast responsiveness. During the last few years, extensive efforts have been made to design and develop fluorescent probes for H_2O_2 detection for in vivo application. The well known Fenton reaction deals with the catalysed reaction of H_2O_2 in presence of low oxidation state transition metal ions e.g. Fe^{2+} to generate hydroxyl radical (·OH). This reaction has been widely applied nowadays as a powerful tool for biochemical analysis. Though the biochemical analysis based on Fenton reaction has shown fast kinetics and excellent sensitivity, it was limited to the applications in cell lysate. Various molecular fluorescent probes were designed and synthesised based on various methods e.g. hydrolysis of sulfonic esters by H_2O_2, diketone to acid group conversation, the reaction of aryl boronates in presence of H_2O_2 to phenols, protein sensors etc. Further, utilizing reaction-based methods to detect H_2O_2 endogenously are now a promising methods coupled with fluorescent response with analyte-associated responsiveness. Among potential fluorophores available in literature, cyanine dye and boron-dipyrromethane derivatives have grown significant attention in the field of near infrared (NIR) molecular probes to be used as sensors. This is because of their high quantum yield, excellent fluorescence behaviour, good photobleaching resistance etc. However, most of the currently developed cyanine dye and BODIPY-based molecular probes still suffer from a lack of ratiometric mode into the near-infrared region, less Stokes shift, water solubility etc. At present in the literature, there have been a greater number of detection methods for H_2O_2 tracking it in vivo. However, the explored detection methods using molecular probes always have some major and/or minor defects. And most of them require complex sample preparation and manipulation procedures, as well as disrupting cell and tissue structures. This review deals with the fluorescence behaviour towards sensing response, designing and structural features, mechanistic study of sensing and in vivo imaging application.

1.1 FLUORESCENT 'TURN-ON' PROBES

Z. Qing, R. Yang and their co-workers have developed, for the first time, a fluorescent approach for in vivo imaging of H_2O_2 via Fenton reaction.[22] For this purpose, they have synthesised a functional nanosphere, Fc@MSN-FDNA/PTAD which is made-up from mesoporous silica nanoparticle (MSN), a Fenton reagent of ferrocene (Fc), 6-carboxylrhodamine (ROX)-labeled single stranded DNA (FDNA), and a cationic perylene derivative (PTAD). The synthetic route of the functional nanosphere and the design mechanism were shown in Scheme 1. The pore entrances of MSN was locked by ferrocene and exterior of it was covalently immobilized with FDNA. Here, PTAD can simultaneously acts as both the gatekeeper of MSN and efficient ROX quencher. Movement of H_2O_2 into the nanosphere triggered the reaction with ferrocene to generate hydroxyl radical (·OH). This type of Fenton reaction cleaves FDNA and separate ROX from PTAD, and thus ROX fluorescence lighting up. This method meets the requirement for real applications because of the high specificity for H_2O_2 towards fast kinetics of Fenton reaction. The fluorescence imaging application of Fc@MSN-FDNA/PTAD to detect in vivo exogenous and endogenous H_2O_2 in mice was demonstrated in Figure 1. The detection limit of this method was evaluated as low as 2.4 nM under physiological condition.

Scheme 1
Schematic illustrations for *in vivo* imaging of H_2O_2 in living system *via* Fenton reaction. Reprinted with permission from ref. 22, Copyright ACS.
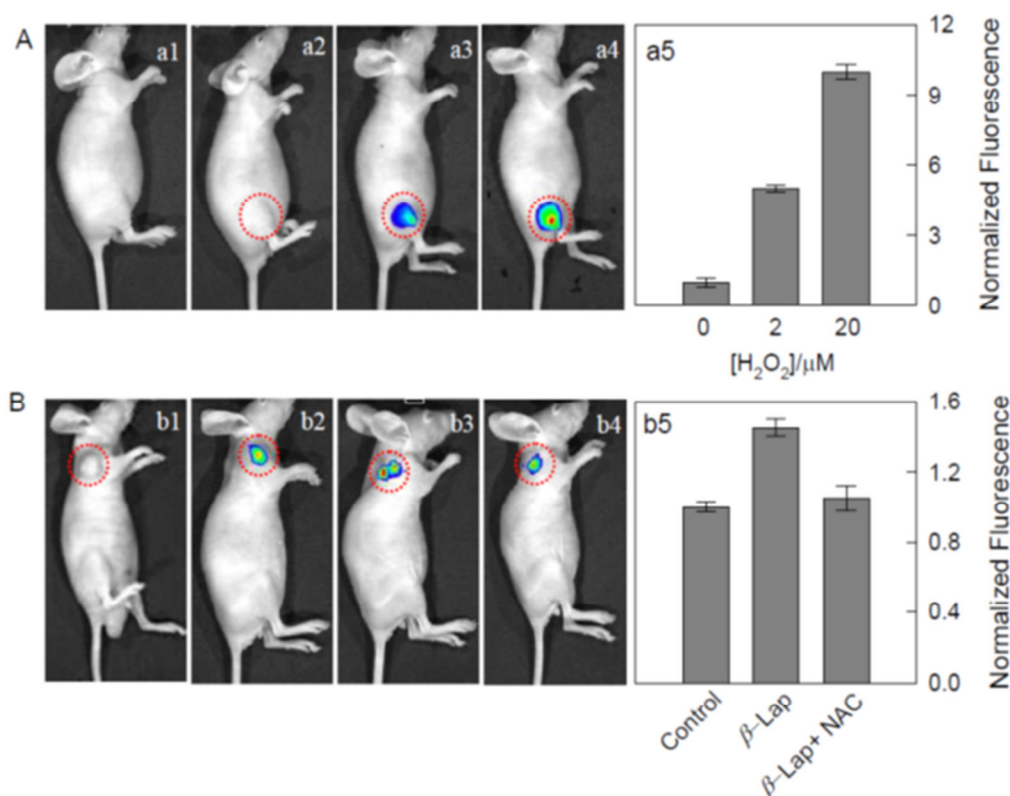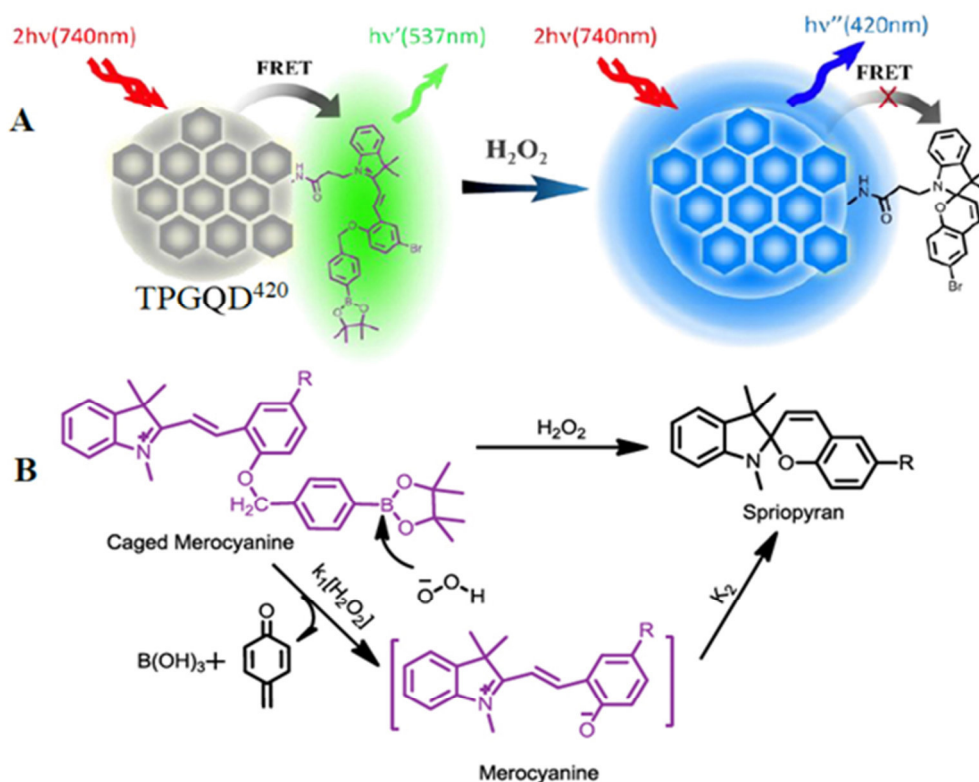
Fig 1. Exogenous and endogenous imaging (*in vivo*) of H$_2$O$_2$ with the nanosphere.
(A) images of the legs of mice treated with fc@msn-fdna/ptad (1.0 mg/ml) and exogenous h$_2$o$_2$: a1) saline; a2) only fc@msn-fdna/ptad (control); a3), fc@msn-fdna/ptad + 2.0 μm h$_2$o$_2$; a4), fc@msn-fdna/ptad+20.0 μm h$_2$o$_2$, and a5), total fluorescence intensity integrated for 1.0 h after injection with the mixture of fc@msn-fdna/ptad and h$_2$o$_2$. (b) representative images of hela-xenograft tumor models treated with β-lapachone (2.0 μm) and nac (1.0 mm), following fc@msn-fdna/ptad (1.0 mg/ml): b1), saline; b2) only fc@msn-fdna/ptad (control); b3), β-lapachone+fc@msn-fdna/ptad; b4), β-lapachone+nac+fc@msn-fdna/ptad, and b5), total fluorescence intensity, integrated for 1.0 h after injection with β-lapachone ± nac and the nanosphere in sequence. Reprinted with permission from ref. 22, copyright acs.

S. Yang, R. Yang and co-researchers have established Graphene Quantum Dots (GQDs) based two-photon fluorescence method for *in vivo* imaging of hydrogen peroxide.[23] This GQDs were fabricated with a boronate ester-functionalized merocyanine (BMC) fluorophore as both target-activated trigger and the dual-emission fluorescence modulator. They introduced a series of boronate ester-functionalized merocyanine (BMC) fluorophores, which can selectively react with H$_2$O$_2$ which modify the maximal absorption as well as the emission wavelengths. Now the final probe, TPGQD^{420}-BMC, was synthesised by covalently attaching the BMC fluorophore on TPGQD^{420} surface, in which the blue colour luminescence of TPGQD^{420} was switched off *via* FRET, showing the green-colour fluorescence of BMC (Scheme 2A). In

the presence of H$_2$O$_2$, the cage structure is removed due to the selective reaction of BMC with H$_2$O$_2$ (Scheme 2B), producing the closed spiropyran derivative. This conversion inhibits the operating FRET process and blue-colour luminescence of TPGQD^{420} is now restored. To enhance biocompatibility of TPGQD^{420}-BMC3 to visualize changes of H$_2$O$_2$ levels in cellular environments, the surface of TPGQD^{420}-BMC3 was modified with PEG chains to generate TPGQD^{420}-BMC3@PEG. The probe is capable to detect endogenous H$_2$O$_2$ in mice and displays flawlessly *in vivo* ratiometric bioimaging of H$_2$O$_2$ (Figure 2). This system showed an interesting very low detection limit of 0.05 μM.

Scheme 2
A) Schematic illustration of target-activated modulation of dual-color and two-photon luminescence of TPGQD420 *via* FRET process. B) Mechanism of H₂O₂-induced tautomerism from open merocyanine to closed spiropyran. Reprinted with permission from ref. 23, Copyright ACS.
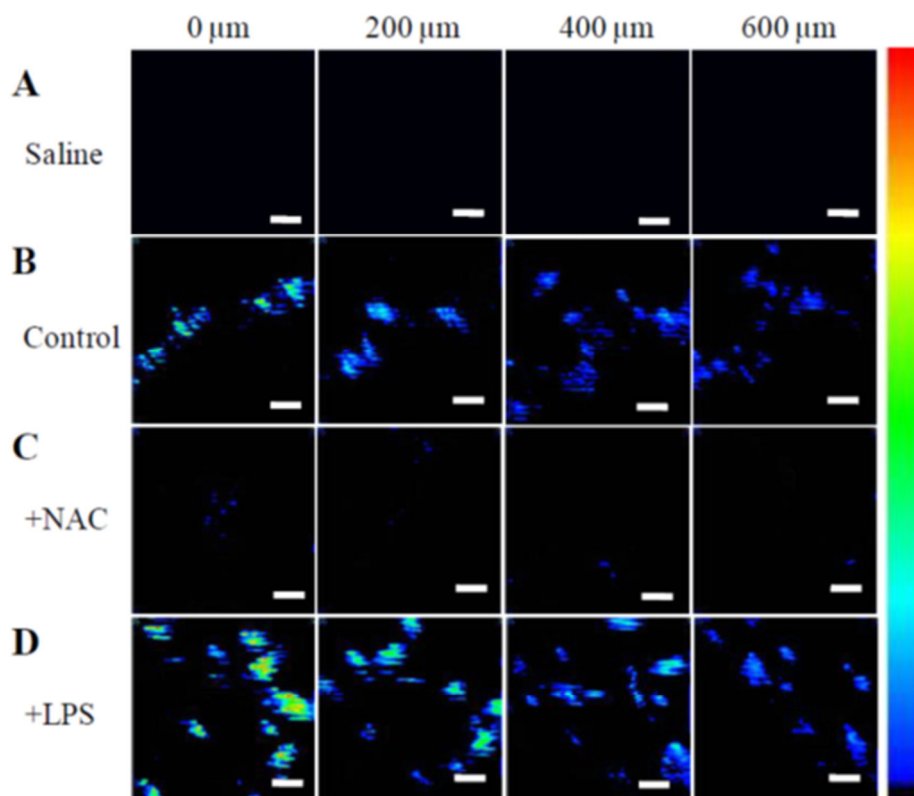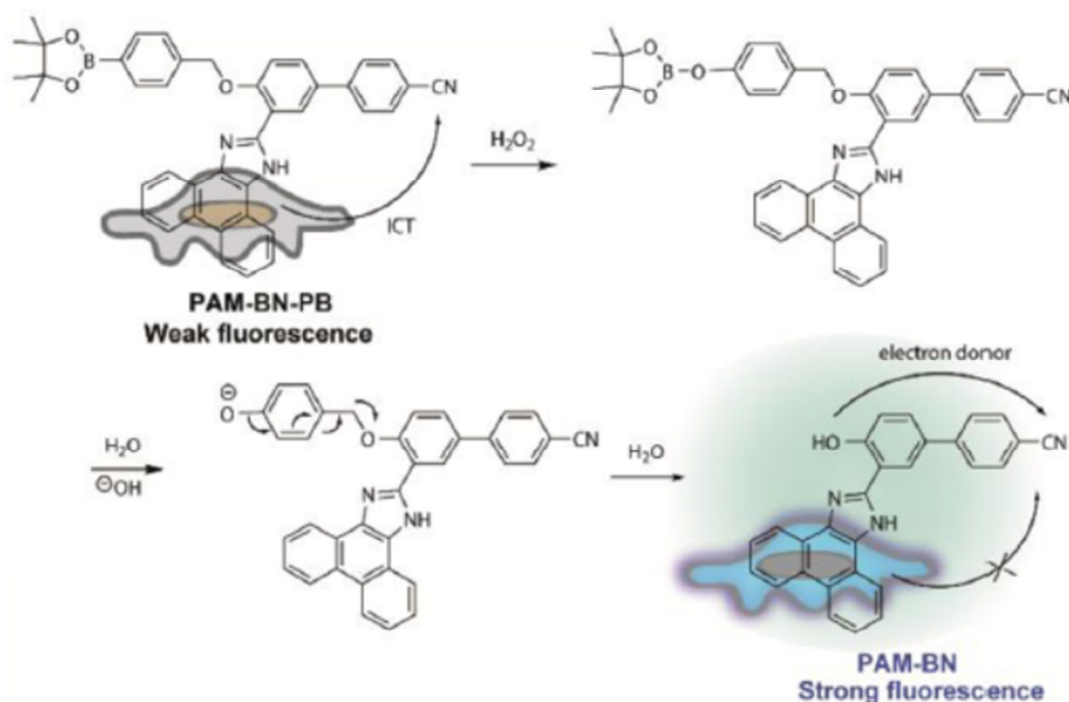

Fig 2. *In vivo* ratiometric TPM images (F_{blue}/F_{green}) of endogenous H₂O₂ levels in mice.
As a control, mice untreated (A, only saline) or unstimulated (B, with TPGQD420-BMC3@PEG, 100 µg/mL) or inhibited (C, treatment with NAC (1.2×10^{-2} M) and then TPGQD420-BMC3@PEG (100 µg/mL)) were imaged. Finally, the LPS (1.0 µg/mL) stimulated mice injected with the nanoprobe (100 µg/mL) (D) were also investigated. The images displayed in pseudo colour were obtained from different depths of the peritoneal cavity by signals process of two channels (400-450 nm, 500-550 nm) upon excitation at 740 nm. Scale bar = 200 µm. Reprinted with permission from ref. 23, Copyright ACS.

Zhengliang Lu, Xuefei Wang, Zhuo Wang and co-researchers have reported a fluorescent probe for the detection of hydrogen peroxide based on the modulation of intramolecular charge transfer for *in vivo* imaging applications.[24] They constructed a three-component fluorescent probe, PAM-BN-PB, using phenanthroimidazol, benzonitrile, and phenyl boronate for the detection purpose. Benzonitrile (BN), being an electron-withdrawing group, is covalently connected with phenanthroimidazole (PAM) to quench the fluorescence due to the possible ICT process operated from PAM to BN (Scheme 3). Then phenylboronate

(PB) has been attached with this system as a recognition unit for H_2O_2. Now, upon the addition of H_2O_2, phenylboronate is converted to the corresponding phenolic derivative, and finally it disrupts the ICT process between PAM and BN because of its good electron donating property. As a result fluorescence recovery was observed and the fluorescence of PAM-BN-PB was changed from "off" to "on" by H_2O_2 (Scheme 3). The probe was also applied to detect H_2O_2 for *in vivo* imaging in mice. In the animal experiment, PAM-BN-PB can image H_2O_2 in the peritoneal cavity of mice (Figure 3).

Scheme 3
Detection Mechanism of PAM-BN-PB upon addition of H_2O_2. Reprinted with permission from ref. 24, Copyright ACS.
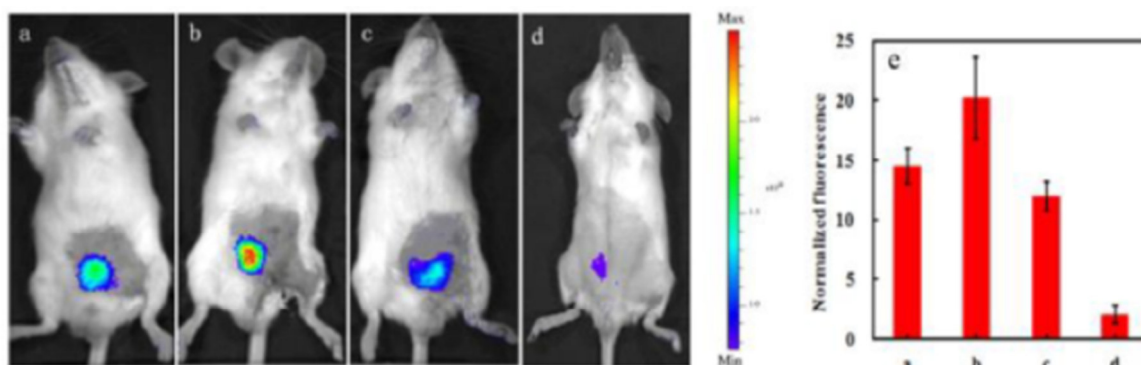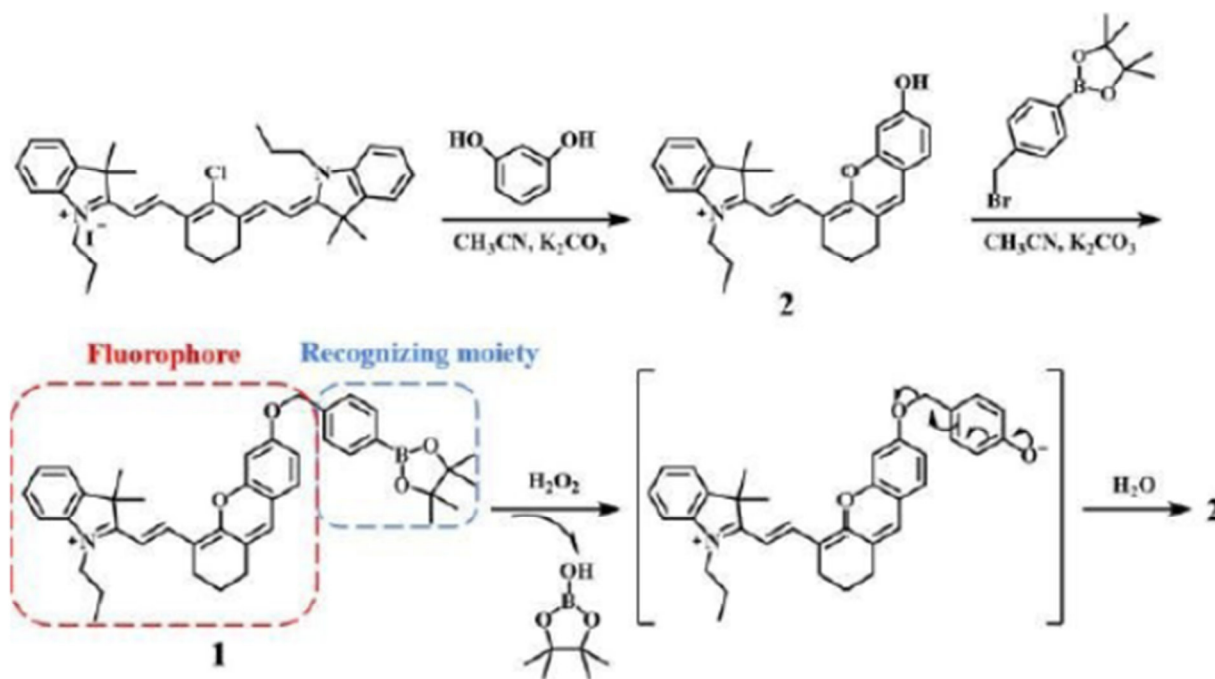

Fig 3. *In vivo* fluorescence imaging: (a) Only PAM-BN-PB was injected as the negative control. (b) Mice were injected with rotenone first, followed by injection of PAM-BN-PB after 1 h. (c) Rotenone and NAC were injected into the mice in turn and then PAM-BN-PB was injected into the same region. (d) Mice were injected with NAC first, followed by injection of PAM-BN-PB after 1 h. (e) Relative fluorescence intensities of a-d. Reprinted with permission from ref. 24, Copyright ACS.

Z. Li, C. Zhang and co-workers recently reported a near-infrared fluorescence probe for the detection of hydrogen peroxide in living systems.[25] They have designed and synthesised a fluorescent probe, 1, (*E*)-3,3-dimethyl-1-propyl-2-(2-(6-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)-2,3-dihydro-1*H*-xanthen-4-yl)vinyl)-3*H*-indol-1-

ium for endogenous H_2O_2 detection. In the probe construction procedure, the decomposed product of the unstable precursor of IR-780 has been used as a fluorophore that shows high stability and near-infrared fluorescence emission feature. On the other hand, 4-(bromomethyl) benzeneboronic acid pinacol ester, a selective recognition

moiety of H_2O_2, was covalently attached with this fluorophore. Upon the addition of H_2O_2 boronic acid pinacol ester was decomposed to give the unstable corresponding phenolic derivative which again transformed to the fluorophore 2 by the expulsion of p-quinomethane molecule

(Scheme 4). Moreover, probe I was successfully introduced to detect endogenous H_2O_2 in mice (Figure 4). The limit of detection was evaluated as 0.14 µM by the 3σ/S model.

Scheme 4
Synthesis of probe I and its reaction with H_2O_2. Reprinted with permission from ref. 25, Copyright RSC.
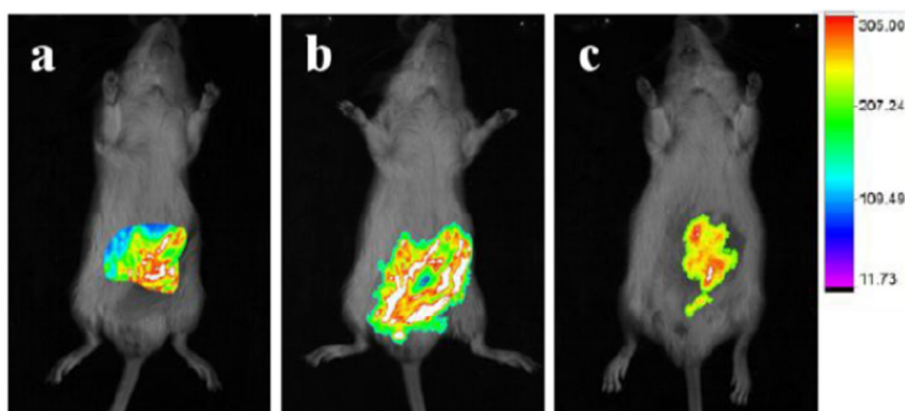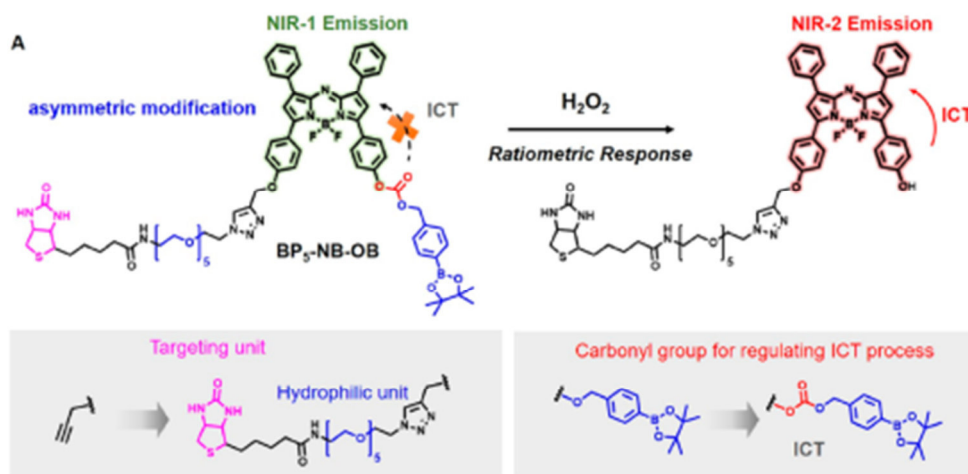

Fig 4. Representative fluorescence images of BALB/c mice (pseudocolor). (a) The mouse received an injection of probe I (200 µM, 100 µL) into the i.p. cavity as the control. (b) The mouse was pre-treated with rotenone (2.5 mM, 100 µL) and followed by injection of probe I (200 µM, 100 µL) at the same region after I h. (c) The mouse was successively treated with rotenone (2.5 mM,100 µL) for I h, NAC (20 mM, 100 µL) for I h, and probe I (200 µM, 100 µL) at the same position. Reprinted with permission from ref. 25, Copyright RSC.

Z. Guo and co-researchers have demonstrated a rational design strategy to construct asymmetric aza-boron-dipyrromethane derivative BODIPY-based ratiometric fluorescent probes for *in vivo* tracking H_2O_2.[26] In this prob (BP5-NB-OB) design strategies, the authors introduced carbonyl group as ICT blocker to control absorption and emission spectra shift, the pinacol borate as the H_2O_2 specific recognition moiety, and the hydrophilic PEG-biotin segment

to improve solubility. Moreover, this probe possesses several important characteristics: (i) ratiometric absorption and NIR emission responses, (ii) excellent biocompatibility and (iii) *in vivo* monitoring of endogenous H_2O_2. Upon the reaction of H_2O_2 the pinacol boronic acid ester is hydrolysed to form a free phenol derivative (Scheme 5). Interestingly, the probe BP5-NB-OB was successfully introduced for tracking endogenous H_2O_2 in mice model (Figure 5).

Scheme 5
Chemical structures and proposed sensing mechanism for H_2O_2.
Reprinted with permission from ref. 24, Copyright ACS.

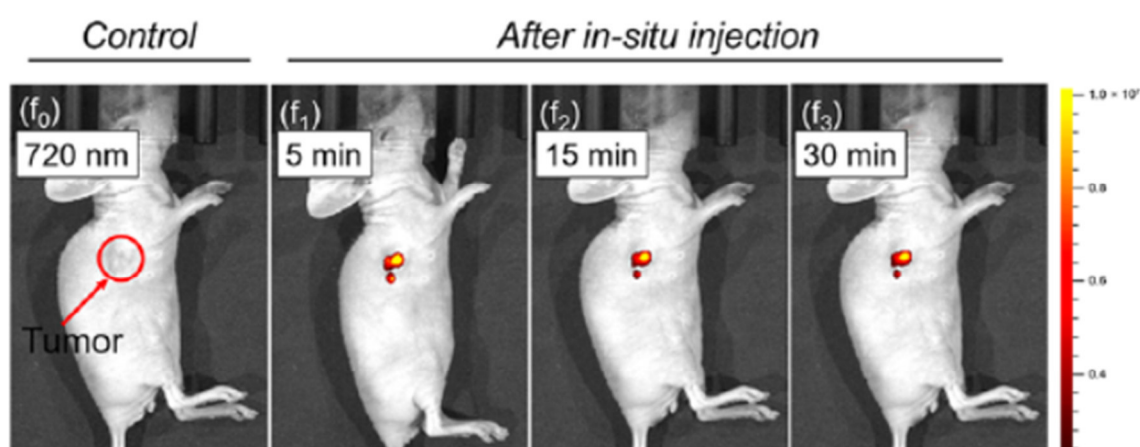**Fig 5. (f_0−f_3)** *In vivo* imaging of A549 xenograft mice after the intratumoral injection of BP5-NB-OB (0.2 mg/kg) (0-30 min). The tumor site is circled in red. Reprinted with permission from ref. 24, Copyright ACS.

2. CONCLUSION

The methods discussed here in this review provide examples of the strategic designing aspects of recently reported fluorescent probes for the detection of H_2O_2 in a selective way. Many of these methods were introduced arylboronic acid pinacol ester as a hydrogen peroxide selective recognition site. This type of ester undergoes an intramolecular elimination reaction upon treatment with H_2O_2 to form the corresponding phenolic compounds with enhanced fluorescence property. One of the above examples were based on Fenton reaction on functional nanospheres. The research works mentioned here in this review were successfully introduced their probes for selective H_2O_2 tracking using *in vivo* application in mice. Although, it is still necessary to develop novel fluorescent probes with high sensitivity, and quick response for real-time detection of H_2O_2 for *in vivo* practical applications.

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3. ACKNOWLEDGEMENTS

4. AUTHORS CONTRIBUTION STATEMENT

Mr. SK Saha gathered data conceptually and Mr. UC Saha analysed the data and inputs the designing of the manuscript. Both the authors discussed the methodology, results and contributed equally to the final manuscript.

5. CONFLICT OF INTEREST

Conflict of interest declared none.

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