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Near-infrared fluorescence probe for in situ detection of superoxide anion and hydrogen polysulfides in mitochondrial oxidative stress

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ABSTRACT: H$_2$S plays important physiological and pathological roles in cardiovascular system and nervous system. But recent evidences imply that hydrogen polysulfides (H$_2$S$_n$) are the actual signaling molecules in cells. Although H$_2$S$_n$ have been demonstrated to be responsible for mediating tumor suppressors, ion channels, and transcription factors, more of their biological effects are still need to be elaborated. On one hand, H$_2$S$_n$ have been suggested to be generated from endogenous H$_2$S upon reaction with reactive oxygen species (ROS). On the other hand, H$_2$S$_n$ derivatives are proposed to be a kind of direct antioxidant against intracellular oxidative stress. This conflicting results should be attributed to the regulation of redox homeostasis between ROS and H$_2$S$_n$.

Superoxide anion (O$_2^-$•−) is undoubtedly the primary ROS existing in mitochondria. We reason that the balance of O$_2^-$•− and H$_2$S$_n$ are pivotal in physiological and pathological processes. Herein, we report two near-infrared fluorescent probes Hcy.Mito and Hcy.Biot for the detection of O$_2^-$•− and H$_2$S$_n$ in cells and in vivo. Hcy-Mito is conceived to be applied in mitochondria, and Hcy-Biot is designed to target tumor tissue. Both of the probes were successfully applied for visualizing exogenous and endogenous O$_2^-$•− and H$_2$S$_n$ in living cells and in tumor mice models. The results demonstrate that H$_2$S$_n$ can be promptly produced by mitochondrial oxidative stress. Flow cytometry assays for apoptosis suggest that H$_2$S$_n$ play critical roles in antioxidant systems.

INTRODUCTION

Reactive oxygen species (ROS) and reactive sulfur species (RSS) are endogenously widespread in cells. These species are involved in a wide range of physiological and pathological processes including cytoprotection, signal transduction, neurodegenerative injury, inflammation, and carcinogenesis. Since the prolonged exposure of ROS will potentially damage organelles, cells have developed several defense mechanisms, which comprise antioxidant enzymes and diametrically targeted elimination pathways. Therefore, the intracellular oxidative stress is interrelated with an imbalance between ROS production and cellular antioxidant capacity. Accumulated evidences suggest that hydrogen sulfide (H$_2$S, a member of RSS) plays essential roles in regulating the intracellular redox status and fundamental signaling processes. However, recently interests are focused on physiological functions of another member of RSS: hydrogen polysulfides (H$_2$S$_n$, n > 1). H$_2$S$_n$ are composed of a combination of polysulfur molecules, which are inextricably balanced by H$_2$S oxygen-dependent catalytic processes in mitochondria. Research results imply that H$_2$S$_n$ behave the properties of antioxidant, cytoprotection, and redox signaling in tissues and organs, and H$_2$S may be the terminal product of H$_2$S$_n$ when function in physical activity. The endogenous H$_2$S can generate H$_2$S$_n$ by reacting with ROS. However, H$_2$S$_n$ derivatives are proposed to be direct antioxidants against ROS in cells. There is no doubt that H$_2$S$_n$ possess their own bio-functions, but the evidence is limited owing to the lack of accurate and sensitive detection methods. We assume that these conflicting findings should be driven by the redox homeostasis between H$_2$S$_n$ and ROS.

Superoxide anion (O$_2^-$•−) is the primary one-electron reduced product from mitochondrial electron transport of respiratory chain. It serves as the major source for other ROS, O$_2^-$•− has long been recognized as a vital cellular signaling molecule involved in numbers of physiological processes from innate immunity to metabolic homeostasis. Nevertheless, excessive amounts of O$_2^-$•− induces damages for biological membranes and tissues. The mitochondria fraction of H$_2$S$_n$ and their derivatives are approximately up to 60%. We hypothesize that the mitochondrial redox state may have closed relationship between H$_2$S$_n$ and O$_2^-$•−.

Owing to their biological functions either in cells or in vivo, the elucidation of combined bioeffects of H$_2$S$_n$ and O$_2^-$•− have become an important area in research. The major obstacle for research is the rapid catabolism properties of both H$_2$S$_n$ and O$_2^-$•−, which will result in the continuous fluctuations of their concentrations. Another challenge for the detection is that it is impossible to immediately separate H$_2$S$_n$ and O$_2^-$•− from biological systems. All these issues make it difficult to direct detection of H$_2$S$_n$ and O$_2^-$•− sensitively and selectively. A few methods have been developed for the detection of H$_2$S$_n$ and O$_2^-$•− including colorimetry, electrochemical analysis, and gas chromatography. However, these technologies often require
post-mortem processing and destruction of tissues or cells. Obviously, these technologies are not suitable for in real-time analysis of endogenous H$_2$S$_n$ and O$_{2}^{-}$. Compared with these biological detection technologies, technology based on fluorescence probes for visualizing physiological and pathophysiological changes in cells has become increasingly indispensable, as this technology enables high sensitivity, superior selectivity, less invasion, more convenience, readily available instruments, as well as simple manipulation. Additionally, near-infrared (NIR) absorption and emission profiles can maximize tissue penetration while minimizing the absorbance of heme in hemoglobin and myoglobin, water, and lipids. Therefore, fluorescent probes that have NIR absorption and emission are preferential candidates for fluorescence imaging in in cells and vivo. To date, the fluorescent probes for O$_{2}^{-}$ and H$_2$S$_n$ separate detection have been elegantly developed. However, the combined-response fluorescent probes for in situ detection of O$_{2}^{-}$ and H$_2$S$_n$ are urgently needed, because the crosstalk process between O$_{2}^{-}$ and H$_2$S$_n$ includes signal transduction, homeostasis regulation, oxidative stress, and antioxidant repair in in living cells. The integration of multi-reaction to multi-species by a single probe can demonstrate the redox homeostasis process more clearly. Moreover, the multiresponse probes can benefit from inaccurate calibration. And they can also avoid potobleaching rates of individual probes, uneven probe loading, nonhomogeneous distribution uncontrollable localization, larger invasive effects, metabolisms, and interference of spectral overlap.

Herein, we conceived two new multiresponse fluorescent probes, Hcy-Mito and Hcy-Biot, for successively detecting the O$_{2}^{-}$ and H$_2$S$_n$ in living cells and in vivo. The two probes could provide high sensitivity and selectivity toward O$_{2}^{-}$ and H$_2$S$_n$. Hcy-Mito was prioritized to image the redox homeostasis process between O$_{2}^{-}$ and H$_2$S$_n$ in mitochondria. The results indicated that the burst of O$_{2}^{-}$ would induce the rapid generation of H$_2$S$_n$ in cells. However, the excessive accumulation of O$_{2}^{-}$ could cause mitochondrial oxidative stress and induce cell apoptosis. Additionally, the application of Hcy-Biot in tumor-bearing mice (murine sarcoma S180) displayed that the probe could locate in cancerous tissue for the detection of O$_{2}^{-}$ and H$_2$S$_n$.

**EXPERIMENTAL SECTION**

**Apoptosis Experiments:** Human umbilical vein epithelial cells (HUVECs) were cultured at 2.0 × 10$^5$ cells/well in 6-well plates, and then treated as described in Figure 3. After harvest, cells were washed and suspended in 400 µL Binding Buffer, and then treated with 5µL Annexin V-FITC and 5 µL Propidium Iodide for 10 min at 25 °C in dark. At last, the cells were analyzed by flow cytometry. Annexin V-FITC and Propidium Iodide were detected by green fluorescent channel and red fluorescent channel, respectively.

**Establishment of the Murine Sarcoma S180 Tumor:** S180 murine sarcoma cells were purchased from Cell Preserve Center (Wuhan University, Wuhan, P.R.China). The culture flasks were covered with S180 murine sarcoma cells, and then we shifted the cells and cell culture medium to centrifuge tubes in super clean bench. The cells were centrifuged at 1000 r/min for 10 min and discarded the supernatant. We adjusted the cell concentration by Hanks solution to cause S180 ascites tumor model. Six to eight-week-old BALB/C mice weighing about 20-25 g each were used. S180 murine sarcoma cells grown for 7 days in the ascites fluid of the mice were injected into an intracutaneous site on the right armpit of about 11-week-old mouse, at a dose of 0.2 mL 3×10$^6$/mL cells per site. The tumor-bearing mice received regular food, and the tumors were allowed to grow for about 8 to 10 days until the tumor diameter reached 8 to 10 mm.

**Fluorescent Imaging of BALB/c Mice:** BALB/c mice were obtained from Binhuzh Medical University. Mice were group-housed on a 12:12 light-dark cycle at 22 °C with free access to food and water. BALB/c mice, 20-25 g, were selected and divided into different groups. Mice were anesthetized prior to injection and during imaging via inhalation of isoflurane. All the BALB/c mice were selected and divided into three groups. In the control group a, the peritoneal cavities of BALB/c mice were injected with 50 µL 100 µM Hcy-Mito solution (DMSO/saline = 1:9, v/v). Group b were given i.p. cavity injection with Hcy-Mito for 20 min, and then injected with phorbol myristate acetate (PMA) 30 min to induce O$_{2}^{-}$ generation (50 nM, 50 µL in 1:99 DMSO/saline v/v). Group c were per-treated as indicated in group b. Subsequently, these mice were given i.p. cavity injection with NaS$_n$ (50 µM, 100 µL in saline) for 30 min as the source of H$_2$S$_n$. Twenty minutes later, fluorescence images were constructed from fluorescence collection (750 nm to 850 nm, $\lambda_{ex} = 730$ nm) and using in vivo imaging system (Bruker). Additionally, we merged the fluorescence image with the corresponding X-ray image to clearly display the reaction site of the mice.

**Fluorescent Imaging of Acute Hepatitis Mice and Sarcoma S180 BALB/c Mice:** Sarcoma S180 BALB/c mice were group-housed on a 12:12 light-dark cycle at 22 °C with free access to food and water. We induced acute liver injury by D-galactosamine (D-GalN)/LPS in mice for the production of O$_{2}^{-}$ and H$_2$S$_n$. The mice in group a were intravenously injectep with D-GalN/LPS (500 mg/kg and 50 µg/kg LPS in saline) for 24 h. Subsequently, the mice were with intravenous injection of Hcy-Biot (1 µM, 50 µL in 1:99 DMSO/saline v/v) and maintained 1 h. The murine sarcoma S180 tumor bearing mice were injected intravenously with Hcy-Biot (1 µM, 50 µL in 1:99 DMSO/saline v/v) for 1 h. Images were taken by Xenogen IVIS Spectrum Pre-clinical In Vivo Imaging System.

**RESULTS AND DISCUSSION**

**Design Strategies for Probes Hcy-Mito and Hcy-Biot.** The synthetic approaches of multiresponse fluorescence probes Hcy-Mito and Hcy-Biot were outlined in Scheme S1. The synthetic details of compounds were shown in the Supporting Information (SI). The multiresponse mechanism for the probes was shown in Scheme 1. A heptamethine cyanine dye with NIR fluorescence emission was chosen as fluorophore. Our desirable probes could be readily available from the versatile fluorophore. The reducibility of N$^+$ site in cyanine platform is selected to detect O$_{2}^{-}$. Studies indicate that the nitro group can be reduced by H$_2$S to produce the corresponding amino group under mild conditions. We hypothesized that the stronger electronophile H$_2$S$_n$ would behave more reducibility than nitro group than H$_2$S. The integration of m-nitrophenol into the fluorescent platform would dramatically quench the fluorescence of fluorophore. The mechanism for this fluorescence quenching was attributed to photoinduced electron transfer (PET) process from the excited fluorophore to a strong electron-withdrawing group (donor-excited PET; d-PET). The negative potential of mitochondrial inner mem-

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brane would predominantly accumulate probes which occupy the positively charged groups. To prevent the leakage of neutral probes from mitochondria, we induced a location group benzyl chloride to immobilize the neutral probe within mitochondria. Finally, we obtained a new probe Hcy-Mito for the in situ detection of O$_2^\bullet^-$ burst inducing H$_2$S$_n$ generation in mitochondria. It is suggested that O$_2^\bullet^-$ and H$_2$S$_n$ also play important roles in cells growth and division. Dysfunction of the balance of O$_2^\bullet^-$ and H$_2$S$_n$ in cells may result in carcinoma. In order to test the behavior of O$_2^\bullet^-$ and H$_2$S$_n$ in carcinoma, we integrated biotin as carcinoma tissues targeting group into the probe Hcy-Biot. Hcy-Mito and Hcy-Biot had the same reaction mechanisms except the targeting functions. The proposed reaction mechanism was illustrated in Scheme 1. Hydrogen abstraction reaction between O$_2^\bullet^-$ and the two probes could recover their conjugated systems and emit low fluorescence. After the nitro groups were reduced with H$_2$, the maximum absorption of Cy.Mito appeared at 770 nm ($\varepsilon_{770\text{ nm}} = 8.9 \times 10^4$ M$^{-1}$ cm$^{-1}$). The probe Cy-Mito exhibited emission spectra centered at 780 nm, which extended across the NIR region (Figure 1a). In order to obtain easy and precise determination of the intracellular O$_2^\bullet^-$, we established a linear relationship between emission intensity ($F_{780\text{ nm}}$) and O$_2^\bullet^-$ concentrations. As shown in Figure 1b, our probe was suitable for O$_2^\bullet^-$ quantitative detection. The regression equation was $F_{780\text{ nm}} = 2.58 \times 10^5 \cdot [O_2^\bullet^-] \cdot \mu$M$^{-1} \cdot 3.75 \times 10^4$, with $r = 0.9964$. The limit of detection was calculated to be 50 nM (3σ/k), and the experimental detection limit was measured to be 0.1 µM.

It was noteworthy that the low fluorescence intensity of Cy-Mito was attributed to $d$-PET process ($\Phi_{\text{Cy-Mito}} = 0.015$). The reducibility of nitro group to amino group with H$_2$S$_n$ was then assessed. H$_2$S$_n$ were derived from H$_2$S and S$_n$ in our tests. After scavenged the redundant O$_2^\bullet^-$ by ascorbic acid, Cy-Mito was continuously treated with increasing concentrations of H$_2$S$_n$ (0 - 50 µM). The fluorescence peak centered at 780 nm strongly increased due to the removal of $d$-PET process ($\Phi_{\text{Cy-Mito}} = 0.10$). To evaluate the ability of Cy-Mito in the determination of H$_2$S$_n$ concentrations, the fluorescence intensity at 780 nm was linear to the H$_2$S$_n$ concentrations under the given range (Figure 1c) revealing the ability of Cy-Mito for quantitative and qualitative detection of H$_2$S$_n$. The regression equation was $F_{780\text{ nm}} = 6.20 \times 10^4 \cdot [H_2S_n] \cdot \mu$M$ + 4.87 \times 10^2$, with $r = 0.9987$. The limit of detection was calculated to be 80 nM (3σ/k), and the experimental detection limit was measured to be 0.2 µM under the experimental conditions. These results indicated that our probe could in situ detect O$_2^\bullet^-$/$H_2S_n$ concentrations, which made our probe a promising candidate for application in cells.

**Figure 1.** Fluorescence emission spectra changes of Hcy-Mito (10 µM) in the presence of various concentrations of O$_2^\bullet^-$ and H$_2$S$_n$. a) Spectra were acquired after 10 min upon addition of 0 - 25 µM O$_2^\bullet^-$ in HEPES (pH 7.4, 10 mM) at 37 °C. After scavenged the redundant O$_2^\bullet^-$ by ascorbic acid, spectra were acquired after 10 min upon addition of 0 - 50 µM H$_2$S$_n$. b) and c) The linear relationship between fluorescence intensities and O$_2^\bullet^-$/$H_2S_n$ concentrations, respectively. All data were obtained at $\lambda_{ex} = 730$ nm, $\lambda_{em} = 780$ nm.

**Imaging of Cells Response to O$_2^\bullet^-$ and H$_2$S$_n$.** Based on the above experiments, it is necessary to verify whether our probe has great potential to detect O$_2^\bullet^-$ and H$_2$S$_n$ in complex biological systems. Firstly, we tested our probe for its ability to respond to exogenous O$_2^\bullet^-$ and H$_2$S$_n$ in mouse macrophage cell line (RAW264.7 cells). The cells in Figure 2a were treated with 1 µM Hcy-Mito for 15 min as control. The cells were washed with Dulbecco’s Modified Eagle Medium (DMEM) three times before imaging. After treated as described in control, RAW264.7 cells in Figure 2b were incubated with 10 µM O$_2^\bullet^-$ for 15 min before imaging. We observed red fluorescence emission inside the cells. When the cells were further treated with 50 µM Na$_2$S$_n$ for 15 min, we obtained stronger fluorescence increase in cells. These results indicated that Hcy-Mito could directly detect exogenous O$_2^\bullet^-$ and H$_2$S$_n$ in living cells. We next sought to check whether our probe could detect changes of endogenous O$_2^\bullet^-$ and H$_2$S$_n$. O$_2^\bullet^-$ burst can be triggered using paraquat in RAW264.7 cells. The cells in Figure 2c were per-stimulated with 50 µM paraquat for 8 h to provide the overproduction of O$_2^\bullet^-$, which results in cell death. After incubated with Hcy-Mito, the cells provided gradual increase in fluorescence indicating the detection of endogenous O$_2^\bullet^-$. Glutathione peroxidase (GPxs) can scavenge ROS by depleting bio-thiol. We supposed that GPxs could scavenger ROS through converting H$_2$S to H$_2$S$_n$. Next group of cells (Figure 2d) were treated as described in Figure 2c, then 50 µM H$_2$S were added to the cells for 30 min. As expected, remarkable increase in the fluorescence intensity was obtained. However, once inhibited the GPx activity by racemic misonidazole, the increase in fluorescence emission were suppressed (Figure 2e). These results...
It is reported that lipopolysaccharide (LPS) can induce cystathionine γ-lyase (CSE) mRNA overexpression in RAW264.7 cells for promoting the initial real-time production rate of H$_2$S. 50 However, LPS can often be employed to quickly stimulate ROS generation in macrophages. 51 We hypothesized that the overexpression of CSE actually triggered by the imbalance of the intracellular redox states. CSE-mediated cysteine metabolism could produce H$_2$S to scavenge the excess ROS. 52 We now verified that the intracellular H$_2$S pool could be perturbed by O$_2^-$ burst. The cells in Figure 2f were incubated with Hcy.Mito. After washed with DMEM, the cells were stimulated with 1 µg/mL LPS for 30 min. The cells would give red fluorescence response to the generation of O$_2^-$. These cells were continuously cultured for more 16 h to prompt endogenous H$_2$S generation. The strong fluorescence emission indicated the high level of endogenous H$_2$S. If the enzyme CSE was inhibited by 100 µM PAG, 52 as shown in Figure 2g, there was no further enhancement of fluorescence intensity, which revealed that CSE contributed to the H$_2$S generation. The cell body regions in the visual field (Figure 2a - g) were selected as the regions of interest (ROI), and the average fluorescence intensity was shown for the direct contrast distinction (Figure S10). All these data enabled our probe direct in situ visualization of endogenous O$_2^-$ and H$_2$S cross-talk in living cells.

**Cytoprotection from Cross-talk between O$_2^-$ and H$_2$S.**

H$_2$S is proposed to be more nucleophilic and superior reducing agent for cellular antioxidant activities. The enzyme CSE is considered to be the main source of H$_2$S. 53 It is suggested that H$_2$S production via CSE is dependent on NADPH oxidase (Nox)-derived H$_2$O$_2$. 54 Whereas, the predominant source of O$_2^-$ is also dependent on the regulation of Nox. As known, O$_2^-$ can be immediately converted to H$_2$O$_2$ by peroxidase. The long-time burst of O$_2^-$ in cells would cause cellular oxidative stress and induce apoptosis. We reasoned that this H$_2$O$_2$-related H$_2$S production should be related to the redox homeostasis between O$_2^-$ and H$_2$S, which should play important role in cytoprotection. We now strove to examine the relationship between O$_2^-$ and H$_2$S in cytoprotection. Human umbilical vein endothelial cells (HUVECs) were employed as test models. As shown in Figure 3, all experimental parallel groups were per-incubated with 1 µM Hcy-Mito for 15 min and washed with DMEM three times before imaging. All the fluorescence responses were further confirmed via flow cytometry assay. Apoptosis induced by cellular oxidative stress was also evaluated. As control, the cells in Figure 3a emitted no fluorescence. The apoptosis rate in the control cells was almost 0.0%. The treatment of cells with VEGF 40 ng/mL for 15 min would trigger O$_2^-$ burst (Figure 3b). 55 As continuously cultured 30 min, cells in Figure 3b emitted strong fluorescence indicating the rising level of H$_2$S. The rate of apoptosis was 11.5%. Provided per-treated the cells with 100 µM PAG for 10 min to inhibit the activity of CSE, the rate of apoptosis increased up to 20.9% (Figure 3c). As an additional control experiment, treatment with 100 µM PAG and 40 µM racemic misonidazole for 10 min prompted the apoptosis rate increased to 26.0% (Figure 3d), as PAG and racemic misonidazole could inhibit the activities of CSE and GPx, respectively. The results demonstrated that the main antioxidant activities in HUVECs were attributed to H$_2$S. It was also implied that oxidative stress damage by the burst of O$_2^-$ could be balanced by the production of H$_2$S. However, the extreme oxidative damage by O$_2^-$ would lead to collapse of antioxidant system. In order to verify excessive O$_2^-$ could induce serious apoptosis, the assays that directly induced apoptosis by different doses of O$_2^-$ were performed. HUVECs cells in Figure 3e were treated with VEGF 40 ng/mL for 15 min, and then incubated with 40 µM O$_2^-$. Cell imaging illustrated the critical collapse of the antioxidant system, because the weak fluorescence emissions were obtained even at the time point 60 min (Figure S12). The apoptosis rate was 40%. The cells in Figure 3f and 5g were incubated with VEGF 40 ng/mL for 15 min, then treated with doses of 60 and 80 µM O$_2^-$, respectively. All the two groups...
cells displayed low fluorescence. The results indicated that the excess O$_2^{-}$ had failed cytoprotective mechanism of H$_2$S$_n$. The apoptosis rates were up to 45.3% and 53.7%, respectively.

**Detection of O$_2^{-}$ and H$_2$S$_n$ in Mitochondria.** Mitochondria are known as the main production source of O$_2^{-}$. Mitochondrial oxidative stress can cause cell injury and apoptosis. Therefore, it is crucial to control mitochondrial redox homeostasis. Mitochondria hold approximately 60% of H$_2$S$_n$ and their derivatives in cells, which implies the antioxidant and cytoprotective properties of H$_2$S$_n$ in mitochondria. We next investigated whether Hcy-Mito could in situ detect O$_2^{-}$ and H$_2$S$_n$ in mitochondria. We employed the multicolor colocalization method to confirm whether Hcy-Mito functioned in mitochondria or not. The costaining dyes were mitochondria tracker MitoTracker® Green FM and DNA marker Hoechst 33342. Respiratory burst in HUVECs was per-induced utilizing 50 µM paraquat for 8 h. After washed with DMEM, cells were loaded with 1 µg/mL Hoechst 33342 for 30 min, 1 µg/mL MitoTracker® Green FM for 15 min and 1 µM Hcy-Mito for 15 min. The spectrally separated images acquired from the three dyes were estimated using Image-Pro Plus software. As shown in Figure S15, the probe could respond to O$_2^{-}$ within 15 min. Although the images of Cy.Mito and MitoTracker® Green FM merged well (Figure S15c), the relatively low fluorescence of Cy.Mito would interfere with the resolution of colocalization. We continued to culture cells until Cy-Mito was reduced and emitted strong fluorescence. As illustrated in Figure 4, the cells were stimulated with 50 µM paraquat for 8 h. Then the costaining assays were performed with 1 µM Hcy-Mito and 1 µg/mL MitoTracker® Green FM. After incubated with the dye for 15 min, the cells were washed with DMEM. The spectrally separated images acquired from the two dyes were constructed at the time point of 30 min. The color-pair intensity correlation analysis for Figure 4a and 6b illustrated high correlated plot between the costaining dyes (Figure 4e). We obtained the Pearson’s coefficient Rr =
Figure 4. Mitochondrial multicolor colocalization in HUVECs with Hcy-Mito and MitoTracker® Green FM. a) HUVECs cells were treated with 50 µM paraquat for 8 h, and then incubated with Hcy-Mito 1 µM for 15 min. Fluorescence imaging of Hcy-Mito at 30 min. b) Fluorescence imaging of MitoTracker® Green FM for 15 min. c) Merged red and green channels. d) Merged red, green channels and bright field. e) Displayed the colocalization-correlation between red and green channels in c); f) Pseudo-color of a) according to fluorescence intensity profiles. g) Merged f) and bright field; Surface plots of a), h) perspective observation. i) Vertical view. j) Merged i) and bright field. Images displayed represent fluorescence emission collected windows: 750 to 800 nm (λ<sub>ex</sub> = 730 nm) for Hcy-Mito; 500 to 580 nm (λ<sub>ex</sub> = 488 nm) for MitoTracker® Green FM. HUVECs were stimulated with paraquat 1 mM for 8 h, then incubated with 1 µM Hcy-Mito and 1 µg/mL MitoTracker® Green FM for 15 min before imaging.

0.93 and the Manders’ coefficients m<sub>1</sub> = 0.95, m<sub>2</sub> = 0.96 implying the preferential distribution of our probe in mitochondria (Figure 4c and 6d). Flow cytometry analysis of mitochondrial isolation for these cells delivered more evidence of our probe targeting in mitochondria (Figure S16). Fluorescence imaging of Figure 4f was additionally presented in pseudo-color according to fluorescence intensity profiles to further identify mitochondrial morphology. Figure 4g was showed as plan view. Figure 4h was perspective observation. And Figure 4i was displayed as vertical view. These imaging analysis convinced that our probe could specifically target in mitochondria for the in situ detection of O<sub>2</sub><sup>-</sup> and H<sub>S</sub><sub>2</sub> level changes successively. The results were of great importance to clarify the physiological relationships between O<sub>2</sub><sup>-</sup> and H<sub>S</sub><sub>2</sub> in cells.

**Visualization of O<sub>2</sub><sup>-</sup> and H<sub>S</sub><sub>2</sub> in Vivo.** NIR fluorescence facilitates bioimaging in vivo because it can avoid interference from cell auto-fluorescence, minimize photo damage, and penetrate tissue deeply. The application of Hcy-Mito for fluorescence imaging in vivo was studied in BALB/c mice. As shown in Figure 5, BALB/c mice were divided into three groups. Mice in Group a were given intraperitoneal (i.p.) cavity injection with Hcy-Mito (1 µM, 50 µL in 1:99 DMSO/saline v/v) for 20 min as control. Group b were given i.p. cavity injection with Hcy-Mito for 20 min, and then injected with phorbol myristate acetate (PMA) 30 min to induce O<sub>2</sub><sup>-</sup> generation (50 nM, 50 µL in 1:99 DMSO/saline v/v). Group c were per-treated as indicated in group b. Subsequently, these mice were given i.p. cavity injection with Na<sub>S</sub><sub>2</sub> (50 µM, 100 µL in saline) 30 min as the source of H<sub>S</sub><sub>2</sub>. The mice were subjected to imaging on an in vivo imaging system (Bruker). As expected, the control group showed almost no fluorescence. Group b displayed an increase fluorescence indicating that Hcy-Mito responded to O<sub>2</sub><sup>-</sup> in mice. The strong fluorescence in group c suggested that our probe had been reduced by H<sub>S</sub><sub>2</sub>. These results confirmed that the NIR excitation and emission of our probe possessed desirable penetration for successive imaging O<sub>2</sub><sup>-</sup> and H<sub>S</sub><sub>2</sub> in vivo.

The imbalance of O<sub>2</sub><sup>-</sup> and H<sub>S</sub><sub>2</sub> in cells may result in carcinoma. We expected that the probe Hcy-Biot with biotin as targeting group could evaluate the levels of O<sub>2</sub><sup>-</sup> and H<sub>S</sub><sub>2</sub> in cancerous tissue. To explore the targeting effects of Hcy-Biot, the in vivo tumor imaging was performed in xenograft BALB/c mice (murine sarcoma S180 tumor). The level of ROS in cancerous tissue is high. However, the low level of O<sub>2</sub><sup>-</sup> in the healthy mice could not light the fluorescence on within short time. We induced acute liver injury by D-galactosamine (D-GalN)/LPS in mice for the production of O<sub>2</sub><sup>-</sup>. As shown in Figure 6, the mice in group a were intravenously injected with D-GalN/LPS (500 mg/kg and 50 µg/kg LPS in saline) for 24 h.

**Figure 5.** Representative NIR fluorescence imaging for visualizing O<sub>2</sub><sup>-</sup> and H<sub>S</sub><sub>2</sub> in BALB/c mice. a) Mice were i.p. cavity injected with Hcy-Mito (1 µM, 50 µL in 1:99 DMSO/saline v/v) for 20 min; b) Mice were loaded with 1 µM Hcy-Mito for 20 min, then injected i.p. with PMA (50 nM, 50 µL in 1:99 DMSO/saline v/v) for 30 min; c) Performed as indicated in group b, then the mice were given i.p. cavity injection with Na<sub>S</sub> (50 µM, 100 µL in saline) for 30 min; d) Mean fluorescence intensity of a), b) and c). Images displayed represent emission intensities collected window: 750 to 850 nm, λ<sub>ex</sub> = 735 nm. d) The total number of photons from the entire peritoneal cavity of the mice was integrated for quantification. Data are presented as means ± SD (n = 5).
Subsequently, the mice were with intravenous injection of Hcy-Biot (1 µM, 50 µL in 1:99 DMSO/saline v/v) and maintained 1 h. The murine sarcoma S180 tumor bearing mice (Figure 6) were injected intravenously with Hcy-Biot (1 µM, 50 µL in 1:99 DMSO/saline v/v) for 1 h. Obviously, the liver and tumor tissue could be very quickly distinguished from the surrounding tissues. Fluorescent signal could be maintained even 24 h after the injection (Figure 6D), which demonstrated the targeting delivery and long retention of Hcy-Biot in vivo. The high fluorescence contrasts in Figure 6A were attributed to the depth of liver and tumor tissue (Figure 6B). The penetration depth of Hcy-Biot was at least 1 cm in vivo. H&E (hematoxylin and eosin) staining were carried out to confirm the liver of acute hepatitis and the model of S180 sarcoma tumor (Figure 6E-6G). These results indicated that the NIR excitation and emission of our probe possessed desirable penetration depth for in vivo imaging. Ex vivo imaging clearly showed that the selective location of Hcy-Biot was at the tumor tissue over other organs including lung, liver, kidney, spleen and brain tissue (Figure 6B and 6C). The results also revealed that high level of O$_{2}^-$ and H$_{2}$S$_{n}$ might result in the deregulated rapid cell division and proliferation in tumor tissue.

**CONCLUSIONS**

In summary, we have successfully developed two near-infrared fluorescent probes Hcy-Mito and Hcy-Biot for the detection of O$_{2}^-$ and H$_{2}$S$_{n}$ in cells and in vivo. As a representative for the examination, Hcy-Mito exhibits outstanding sensitivity and selectivity for endogenous O$_{2}^-$ and H$_{2}$S$_{n}$ detection. The bioassays in RAW264.7 cells and HUVECs have fully demonstrated that H$_{2}$S$_{n}$ can be prompted to be generated by mitochondrial oxidative stress. Flow cytometry assays for apoptosis prove that H$_{2}$S$_{n}$ play important roles in antioxidant systems. H$_{2}$S$_{n}$ can protect cells from mitochondrial oxidative stress by direct scavenging O$_{2}^-$ in vivo. Moreover, fluorescence imaging illustrates that our probes possess desirable penetration depth for imaging O$_{2}^-$ and H$_{2}$S$_{n}$ in vivo. Furthermore, bioassays in RAW264.7 cells and HUVECs have further application of these probes in cells and in vivo will reveal bio-roles of O$_{2}^-$ and H$_{2}$S$_{n}$ in physiological and pathological processes.

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**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website. Experimental detail procedures, synthetic procedures and characterization details, reaction kinetics and selectivity, and additional data. (PDF)

**Notes**

The authors declare no competing financial interest.

**REFERENCES**
