Supplementary Information

A near-infrared fluorescent probe for evaluating endogenous hydrogen peroxide during ischemia/reperfusion injury

Runfeng Xu\textsuperscript{a,b}, Yue Wang\textsuperscript{b,d}, Huiyan You\textsuperscript{a*}, Liangwei Zhang\textsuperscript{b*}, Yunqing Wang\textsuperscript{b}, Lingxin Chen\textsuperscript{b,c*}

\textsuperscript{a} School of Environment and Chemical Engineering, Dalian University, Dalian 116622, China. E-mail: dlyhy@dicp.ac.cn (H You)
\textsuperscript{b} CAS Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China. E-mail: liangweizhang@yic.ac.cn (L Zhang); lxchen@yic.ac.cn (L Chen)
\textsuperscript{c} College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu 273165, China.
\textsuperscript{d} University of Chinese Academy of Sciences, Beijing 100049, China.

Contents:
1. Materials and instruments
2. General methods
3. Reaction kinetics of Cy-ArB towards \( \text{H}_2\text{O}_2 \)
4. Effect of pH values to Cy-ArB and Cy-NH
5. Effect of temperature to Cy-ArB and Cy-NH
6. Photo-stability of Cy-ArB and Cy-NH
7. MTT assays for Cy-ArB
8. Colocalization and detection in cells
9. LC-MS and \(^1\text{H}\) NMR of Cy-ArB
1. Materials and instruments

**Materials:** All the reagents were obtained from Aladdin (USA), unless indicated otherwise. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich, and the nucleic acid stain (Hoechst 33342) was purchased from Invitrogen Corporation. DMEM (Dulbecco’s Modified Eagle Medium, #SH30022.01) was purchased from HyClone, and DMEM (no Glucose, #90113-500) was purchased from Solarbio. Fetal Bovine Serum (FBS, #C0252) was purchased from Beyotime. J-aggregate-forming lipophilic cation 5,5’6,6’-tetrachloro-1,1’,3,3’-tetaethylbenzimidazolylcarbo-cyanine iodide (JC-1: #T3168) was purchased from Thermo Fisher Scientific. Annexin V/7-AAD Apoptosis Detection Kit was purchased from BD Biosciences (#559763). All other chemicals were from commercial sources. The solution of the probe Cy-ArB (1 mM) was dissolved in dimethyl sulfoxide (DMSO) as stock solution and maintained in refrigerator at 4 °C. Ultrapure water was used throughout.

**Instruments:** High-resolution mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). $^1$H NMR spectra was obtained on a Bruker spectrometer. Absorption spectra were measured by Lambda 35 UV-visible spectrophotometer (PerkinElmer). Fluorescence spectra were collected by FluoroMax-4 Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. The fluorescence images of cells were taken on a LTE confocal laser scanning microscope (Olympus FV1000 confocal laser-scanning microscope) with an objective lens ($\times$ 60). Flow cytometry data were obtained on BD Biosciences FACSria. Ultrathin sections were cut using Leica EM UC7. BALB / c mice fluorescence images were imaged by Bruker In-vivo Imaging System.

2. General methods

**Spectral experiments:** Entire experimental processes were carried out at 37 °C, ultrapure water was used in all the photophysical characterization experiments. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mM) was purged with N$_2$ for 5 min. Cy-ArB (0.10 mL, 1.0 mM) was added to a 10.0-mL color comparison tube before used. Absorption spectra were obtained with 1.0-cm glass cells. Fluorescence emission spectra were collected by a Xenon lamp and 1.0-cm quartz cells. The fluorescence intensity were measured at $\lambda_{ex/em} = 605/720$ - 770 nm and at $\lambda_{ex/em} = 780/790$ - 820 nm, respectively. After diluted to 10 μM with 10 mM HEPES buffers, H$_2$O$_2$ was added. The mixtures were equilibrated 60 min before measurement.

**Cell culture:** Human liver hepatocellular carcinoma cell line (HepG2) was purchased from the Culture Collection Committee of the Chinese Academy of Sciences (Shanghai, China). HepG2 were incubated in DMEM supplemented with 10% fetal bovine serum (FBS). The cultures were maintained at 37 °C in a 95% humidified atmosphere with 5% CO$_2$.
**Flow cytometry analysis:** The cells were cultured at $2.0 \times 10^5$ cells/well in 6-well plates, and then treated with probes as described in the paper. After harvest, cells were washed three times and suspended in fresh complete medium and analyzed by flow cytometry.

**Fluorescent imaging of mice in vivo:** All biological experimentations were ensure strict observance of the National Guidelines for the Care and Use of Laboratory Animals. And experimental protocols were approved by the Institutional Animal Care and Use Committee in Binzhou Medical University, Yantai, China. BALB/c mice were anesthetized with isoflurane before fluorescence imaging using a Bruker In-vivo Imaging System.

3. Reaction kinetics of Cy-ArB towards H$_2$O$_2$

Fig. S1. Time dependent fluorescent of probe Cy-ArB (10 µM) towards H$_2$O$_2$ (200 µM) during 0-100 min, H$_2$O$_2$ was added at the reaction time of 20 min in 10 mM HEPES buffer (pH 7.4). The reactions were measured during 0-100 min at 37°C. a) $F_{806\text{nm}}$: $\lambda_{ex} = 780$ nm, $\lambda_{em} = 806$ nm; b) $F_{758\text{nm}}$: $\lambda_{ex} = 605$ nm, $\lambda_{em} = 758$ nm.

4. Effect of pH values to Cy-ArB and Cy-NH

The experiments were performed in 10 mM HEPES solution, and the concentrations of compounds were 10 µM. As shown in Fig. S2, Cy-ArB and Cy-NH were stable under different pH range from 4.0 to 8.0. On the basis, our probe Cy-ArB was stable under different pH for H$_2$O$_2$ detection.

Fig. S2. Effect of pH values. a) The fluorescence emission of Cy-ArB (10 µM) under different pH. b) The fluorescence emission of Cy-NH (10 µM) under different pH. pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5,
7.0, 7.4, 7.8, 8.0 (10 mM HEPES buffer). Cy-ArB: $\lambda_{\text{ex}} = 780$ nm, $\lambda_{\text{em}} = 806$ nm; Cy-NH: $\lambda_{\text{ex}} = 605$ nm, $\lambda_{\text{em}} = 758$ nm.

5. Effect of temperature to Cy-ArB and Cy-NH

The probe was diluted to 10 µM, and maintained in different temperature (10 °C, 12 °C, 14 °C, 16 °C, 18 °C, 20 °C, 22 °C, 24 °C, 26 °C, 28 °C, 30 °C, 32 °C, 34 °C, 36 °C, 38 °C, 40 °C) for 2 hours. Then the spectral properties of Cy-ArB and Cy-NH were detected. As shown in Fig. S3, our probe was stable under different temperature conditions for H$_2$O$_2$ detection.

![Fig. S3](image)

Fig. S3. Effect of temperature fluctuations on probe stability. a) The fluorescence emission changes of Cy-ArB (10 µM) under different temperature. b) The fluorescence emission changes of Cy-NH (10 µM) under different temperature. Temperature: 10 °C, 12 °C, 14 °C, 16 °C, 18 °C, 20 °C, 22 °C, 24 °C, 26 °C, 28 °C, 30 °C, 32 °C, 34 °C, 36 °C, 38 °C, 40 °C. Cy-ArB: $\lambda_{\text{ex}} = 780$ nm, $\lambda_{\text{em}} = 806$ nm; Cy-NH: $\lambda_{\text{ex}} = 605$ nm, $\lambda_{\text{em}} = 758$ nm.

6. Photo-stability of Cy-ArB and Cy-NH.

The probe was diluted to 10 µM. As shown in Fig. S4, the fluorescent intensity of Cy-ArB and Cy-NH were recorded after the stock solution exposed in the air for 0-7 days. As plotted in Fig. S4, the probe could be stabilized in air. In brief, our probe was stable enough to meet different experimental conditions.

![Fig. S4](image)

Fig. S4. The photo-stability of the probe. a) The fluorescence emission changes of Cy-ArB (10 µM) under different time points. b) The fluorescence emission changes of Cy-NH (10 µM) under different time points. Time points: 0 d, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d. Cy-ArB: $\lambda_{\text{ex}} = 780$ nm, $\lambda_{\text{em}} = 806$ nm; Cy-NH: $\lambda_{\text{ex}} = 605$ nm, $\lambda_{\text{em}} = 758$ nm.
7. MTT Assay for Cy-ArB

To access the potential toxicity of Cy-ArB, MTT assays were carried out. HepG2 cells (10^6 cells/mL) were planted into 96-well microtiter plates in DMEM with 10% fetal bovine serum (FBS). Plates were maintained at 37°C in a 5% CO₂/95% air incubator for 24 h. Then the cells were incubated for 24 h at 37 °C in a 5% CO₂/95% air in dark upon different concentrations probe of 0-100 μM respectively. MTT solution (5.0 mg/mL, PBS) was added to each well. After 4 h, the remaining MTT solution was removed, and 200 μL of DMSO was added to each well, shaking 10 min to dissolve the formazan crystals at room temperature. Absorbance was measured in a TECAN infinite M200pro microplate reader.

Fig. S5. MTT assay of HepG2 cells treated with different concentrations of Cy-ArB (0 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, 100 μM). The experiments were repeated three times and the data were shown as mean (± S.D.).

8. Colocalization and detection in Cells

Fig. S6. Colocalization and detection in cells. a) Green channel. b) Blue channel. c) Bright-field image.
9. LC-MS and $^1$H NMR of Cy-ArB

Fig. S7. LC-MS spectra of Cy-ArB.

Fig. S8. The $^1$H NMR spectra of Cy-ArB in CDCl$_3$-$d_1$. 