

Supporting Information

A Donor- π -acceptor Fluorescent Probe for Hydrogen Peroxide Monitoring Reveals Oxidative Imbalance and Mitochondrial Damage Caused by Cd/Hg Exposure

Jiangkun Tan^{a,d}, Congzheng Zhu^e, Liangwei Zhang^{a,d*}, Yunqing Wang^a, Xiaoyan Wang^e, Ping Liu^b, Li Zhang^b, Qian Yang^b, Jinmao You^b, Lingxin Chen^{a,b,c*}

^aShandong Key Laboratory of Coastal Zone Environmental Processes and Ecological Security, Coastal Zone Ecological Environment Monitoring Technology and Equipment Shandong Engineering Research Center, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

^bCollege of Chemistry and Chemical Engineering, Institute for Advanced Study, Shaoxing University, Shaoxing 312000, China

^cLaboratory for Marine Biology and Biotechnology, Qingdao Marine Science and Technology Center, Qingdao 266237, China

^dUniversity of Chinese Academy of Sciences, Beijing 100049, China

^eSchool of Pharmacy, Shandong Medical and Pharmaceutical University, Yantai 264003 China

* Corresponding authors.

E-mail: liangweizhang@yic.ac.cn (L. Zhang); lxchen@yic.ac.cn (L. Chen)

1 General Methods

1.1 Reagents, Materials and instruments

All reagents were analytically pure and purchased from commercial supplies without further purification. Mass spectra was tested by Liquid Chromatography Ion Trap Mass Spectrometer (LCQ Fleet, Thermo). ¹H NMR and ¹³C NMR spectra were recorded on Bruker AVIII 500 spectrometer. Absorption spectra were collected on an UV-Visible spectrophotometer (Thermo, Evolution 220) with 1.0-cm quartz cell. Fluorescence spectra were obtained by a HITACHI F-4700 spectro fluorometer with Xenon lamp. The fluorescence imaging of cells and zebrafish were acquired on an Olympus laser scanning confocal microscope (Fluo View FV1000, Japan). The flow cytometry analysis of cells were used BD Biosciences FACS Aria (USA).

1.2 Spectral titration analysis

TQ-H₂O₂ (10 mM) was prepared in DMSO, and then was diluted to testing concentration (10 μM) with PBS buffer (0.1% DMSO, pH 7.4). UV-vis and fluorescent experiments were performed by titrating aliquots of H₂O₂. Other analytes including HNO, OCl⁻, ONOO⁻, NO₂⁻, Cys, Hcy, GSH, Na⁺, K⁺, CO₃²⁻, HSO₃⁻, SO₃²⁻, O₂⁻ also were explored in specificity experiment. The spectral analysis was equilibrated at 37 °C.

1.3 Cell culture

HepG2 and RAW 264.7 cells were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). And all cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, USA) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin Liquid (Solarbio, China). The cells were seeded on Petri-dishes and cultured under in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. To execute different experimental operations, the cells were seeded at the same density into the glass bottom culture dish which diameter was 15 mm, (80000 cells/ culture dish). Then, the cells were cultured under the same conditions for 24 h before executed different experimental operations. For incubation of probe, all the groups were also carried out same treatment, the cells were treated with 10 μM TQ-H₂O₂ for 40 min before imaging. The quantification of fluorescence imaging were obtained by Image J software. And the average fluorescence intensities of parallel experimental groups were quantified as mean fluorescence intensity of cells images.

1.4 Cytotoxicity

Cytotoxicity induced by the probe was examined through CCK-8 kit in RAW 264.7 and HepG2 cells. The cells were cultured and seeded into 96-well plates for 24 h adherent growth. Next, different concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80 μM) of the probe were respectively incubated cells for another 24 h. After that, 10 μL commercial CCK-8 reagent was added into every well for 1 h staining at 37 °C, and then measured absorbance of the cell samples at 450 nm via microplate reader. Cell viabilities were calculated according to the instructions of CCK-8 kit. Cell viability (%) [(As-Ab) / (Ac-Ab)] 100%, As represents the absorbance of the experimental groups which included CCK-8 and probe, Ac represents the absorbance of the control group which cells in 96-well without probe treatment, and Ab represents the absorbance of blank group.

1.5 Cell and zebrafish imaging

The HepG2 and RAW264.7 cells were pretreated with 1 μg/mL Lipopolysaccharide (LPS) for 6 h, 0.2 μg/mL phorbol-12-myristate-13-acetate (PMA, 1 h) and N-acetylcysteine (NAC, 1 mM, 1 h), exogenous H₂O₂ (100 μM, 30 min) as follow Fig. 2A, B. For Cd²⁺/Hg²⁺ stress cell models, single Cd²⁺/Hg²⁺ (20, 40, 60 μM) was respectively pretreated in HepG2 cells for 30 min. The multiple Cd²⁺/Hg²⁺ condition were divided into 3 groups (Cd²⁺+Hg²⁺, 10+10 μM, 20+20 μM, 30+30 μM) for 30 min. Single Cd²⁺/Hg²⁺ (0.5, 1, 2 μM) was respectively pretreated in HepG2 cells for 24 h under low dose stress. The multiple Hg²⁺/Cd²⁺ condition were divided into 7 groups (Cd²⁺+Hg²⁺, 0.75+0.25 μM, 0.25+0.75 μM, 1.5+0.5 μM, 0.5+1.5 μM, 0.25+0.25 μM, 0.75+0.75 μM, 1+1 μM) for 24 h. Ethylene diamine tetraacetic acid (EDTA) was utilized to remove excessive heavy metals. Imaging analysis of H₂O₂ at different time (1, 2, 3, 4, 5, 6, 9 h) by TQ-H₂O₂ after removing high dose Cd²⁺/Hg²⁺ exposure was listed in Fig. 3E, F. Imaging analysis of H₂O₂ at different time (1, 3, 6, 12, 18, 24 h) by TQ-H₂O₂ after removing low dose Cd²⁺/Hg²⁺ exposure was listed in Fig. 4H, I.

High dose stress: 10–70 μM $\text{Cd}^{2+}/\text{Hg}^{2+}$ were utilized to incubate HepG2 cells for 30 min and then TQ- H_2O_2 (10 μM) for 40 min. Low dose stress: 0.25–2.5 μM $\text{Cd}^{2+}/\text{Hg}^{2+}$ were utilized to incubate HepG2 cells for 24 h.

For zebrafish, incubation of LPS (1 $\mu\text{g}/\text{mL}$, 12 h), NAC (1 mM, 1 h) and H_2O_2 (200 μM , 30 min) for triggering H_2O_2 variation, different dose of single $\text{Hg}^{2+}/\text{Cd}^{2+}$ (1, 2, 3 μM) was respectively pretreated for 24 h under low dose stress. The multiple $\text{Hg}^{2+}/\text{Cd}^{2+}$ condition were divided into 7 groups ($\text{Cd}^{2+}+\text{Hg}^{2+}$, 1.5+0.5 μM , 0.5+1.5 μM , 2.25+0.75 μM , 0.75+2.25 μM , 0.5+0.5 μM , 1+1 μM , 1.5+1.5 μM) for 24 h. The zebrafish were respectively stained 20 μM TQ- H_2O_2 for 40 min.

1.6 Flow Cytometry Analysis (FCA)

The cells were cultured to 2.0×10^5 cells/well in 6-well plates, and then treated with different experiment condition. The cells were suspended with PBS after afresh washing before flow cytometry analysis. The samples were detected by BD Biosciences FACS Aria, and the data were analyzed by Software of Flowing.

1.6.1 Apoptosis Experiments

Annexin V/7-AAD Apoptosis Detection Kit was purchased from BD Biosciences (#559763). Apoptosis experiments were tested by Annexin V/7-AAD Apoptosis Detection Kit. HepG2 cells were inoculated into six well plates (2.0×10^5 cells/well) and cultured. Washing the cells with the PBS for three times and then harvest the cells through digested them into centrifuge tubes. The cells were suspended in 100 μL binding buffer, 5 μL 7-AAD and Annexin V-FITC were added into the above solution, respectively. The cells were kept in dark place at 25 $^\circ\text{C}$ for 15 min. Before analyzed by flow cytometry, cells were suspended with 400 μL binding buffer. The samples were detected by BD Biosciences FACS Aria, and the data were analyzed by Software of Flowing.

1.6.2 Mitochondrial membrane potential Experiments

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine (JC-1) commercial dye was purchased from Thermo Fisher Scientific (#T3168). Mitochondrial membrane potential ($\Delta\Psi_m$) were measured by JC-1 in BD Biosciences FACS Aria. The cells were first washed by PBS for three times and then incubated with the JC-1 dye (10 $\mu\text{g}/\text{mL}$) for 20 min at 25 $^\circ\text{C}$ in dark place. Samples were washed and suspended with PBS for further testing in BD Biosciences FACS Aria. The test parameters were executed as the instruction. When the $\Delta\Psi_m$ is high, JC-1 would accumulate in the mitochondrial matrix to form polymers which can produce red fluorescence ($\lambda_{\text{ex}}=585$ nm, $\lambda_{\text{em}}=590$ nm). Instead, when the $\Delta\Psi_m$ is low, JC-1 can only exist in the endochylema as a monomer and produce green fluorescence ($\lambda_{\text{ex}}=515$ nm, $\lambda_{\text{em}}=529$ nm). The decrease of $\Delta\Psi_m$ can be easily detected by the transition of JC-1 from red fluorescence to green fluorescence. The relative ratio of red and green fluorescence is often used to measure the degree of mitochondria damage.

1.7 Procedures for ROS generation and testing

HOCl: Aqueous NaOCl was diluted with PBS buffer (pH = 7.4). The concentration of HOCl was determined from the absorption at 292 nm ($\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$). The probe's response was recorded immediately after the completion of preparation.

ONOO $^-$: A mixture of sodium nitrite (NaNO_2 , 0.6 M) and hydrogen peroxide (H_2O_2 , 0.7 M) was acidified with hydrochloric acid (HCl, 0.6 M), and sodium hydroxide (NaOH, 3 M) was added within 1-2 s to make the solution alkaline at 0 $^\circ\text{C}$. The remaining H_2O_2 was removed by passing the mixture over a MnO_2 . The concentration of ONOO $^-$ was determined by measuring the absorption of the solution at 302 nm. The extinction coefficient of the ONOO $^-$ solution in 0.1 M NaOH is $1670 \text{ M}^{-1} \text{ cm}^{-1}$, at 302 nm. The prepared solution was stored at -20 $^\circ\text{C}$.

H $_2$ O $_2$: Stock solution of H_2O_2 was prepared by diluting 30% solution with deionized water and the exact concentration was established by UV-vis measurements based on the absorption at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$).

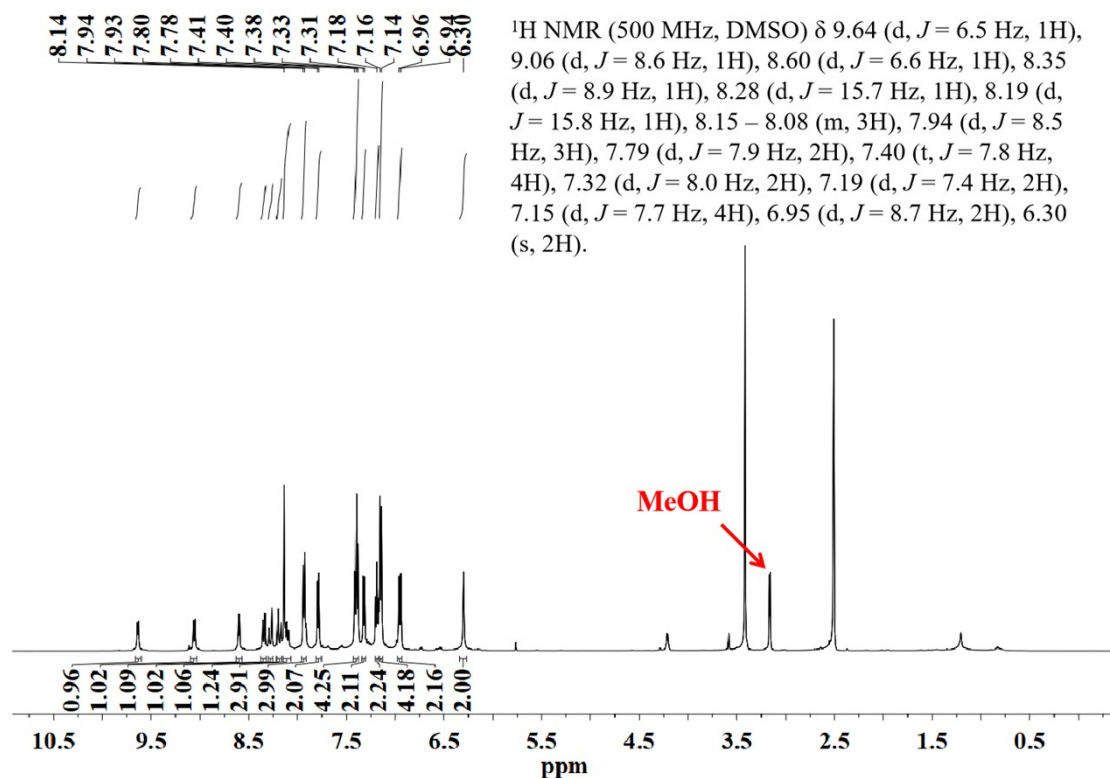


Figure S1. ¹H NMR of the probe TQ-H₂O₂ dissolved in DMSO-*d*₆.

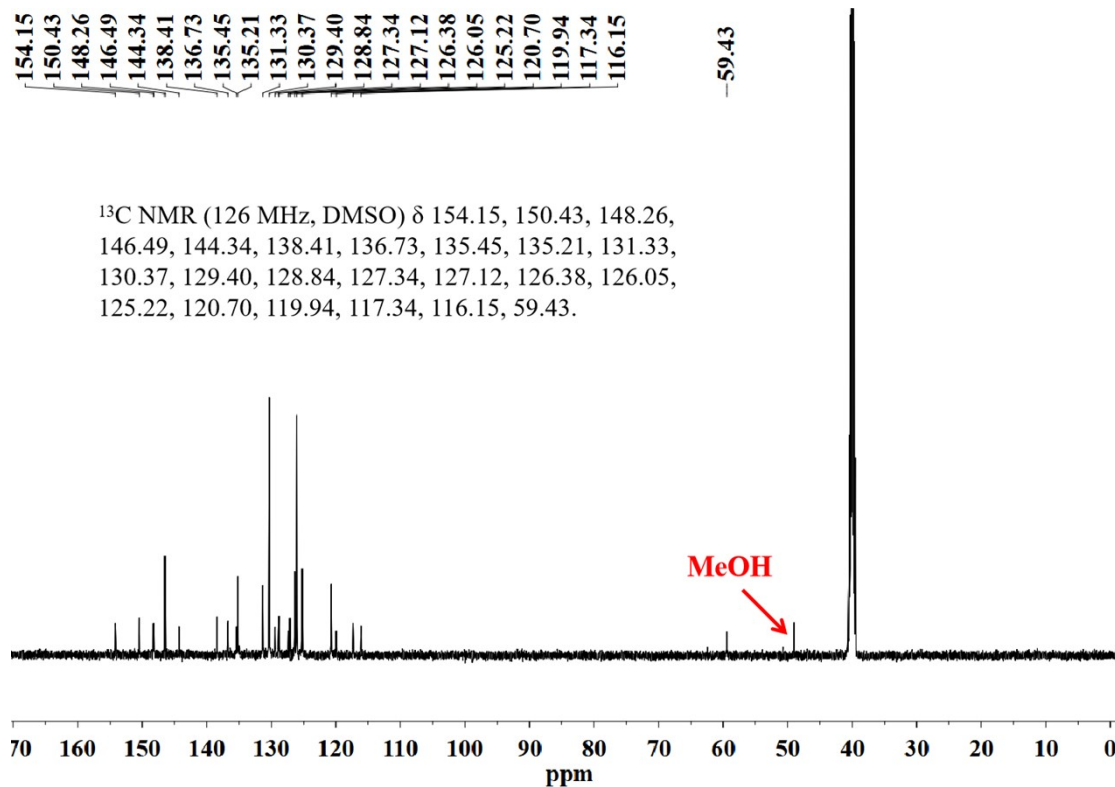


Figure S2. ¹³C NMR of the probe TQ-H₂O₂ dissolved in DMSO-*d*₆.

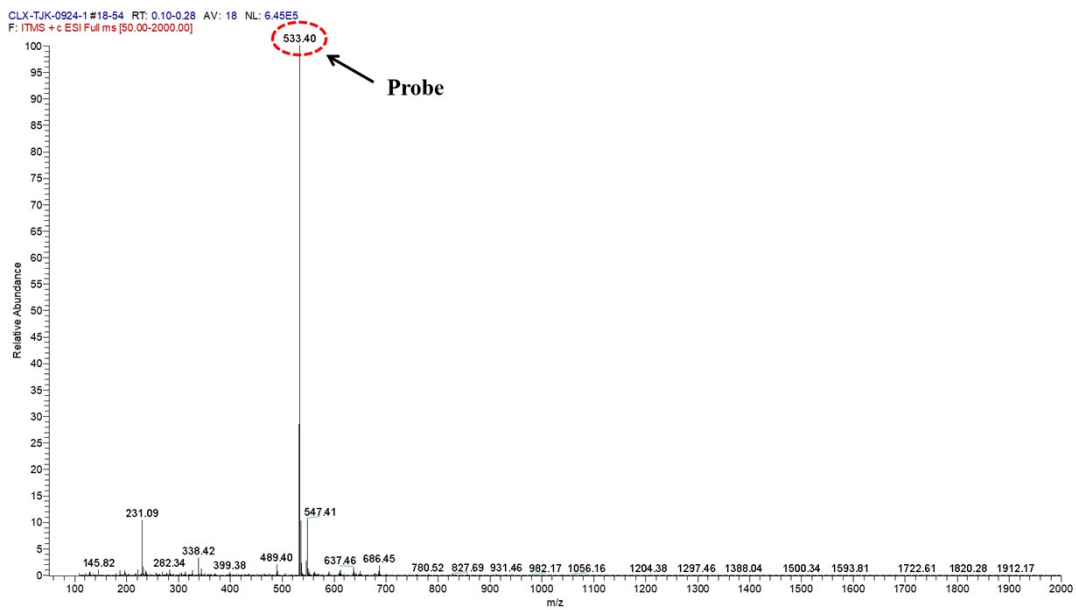


Figure S3. MS spectrum of TQ-H₂O₂.

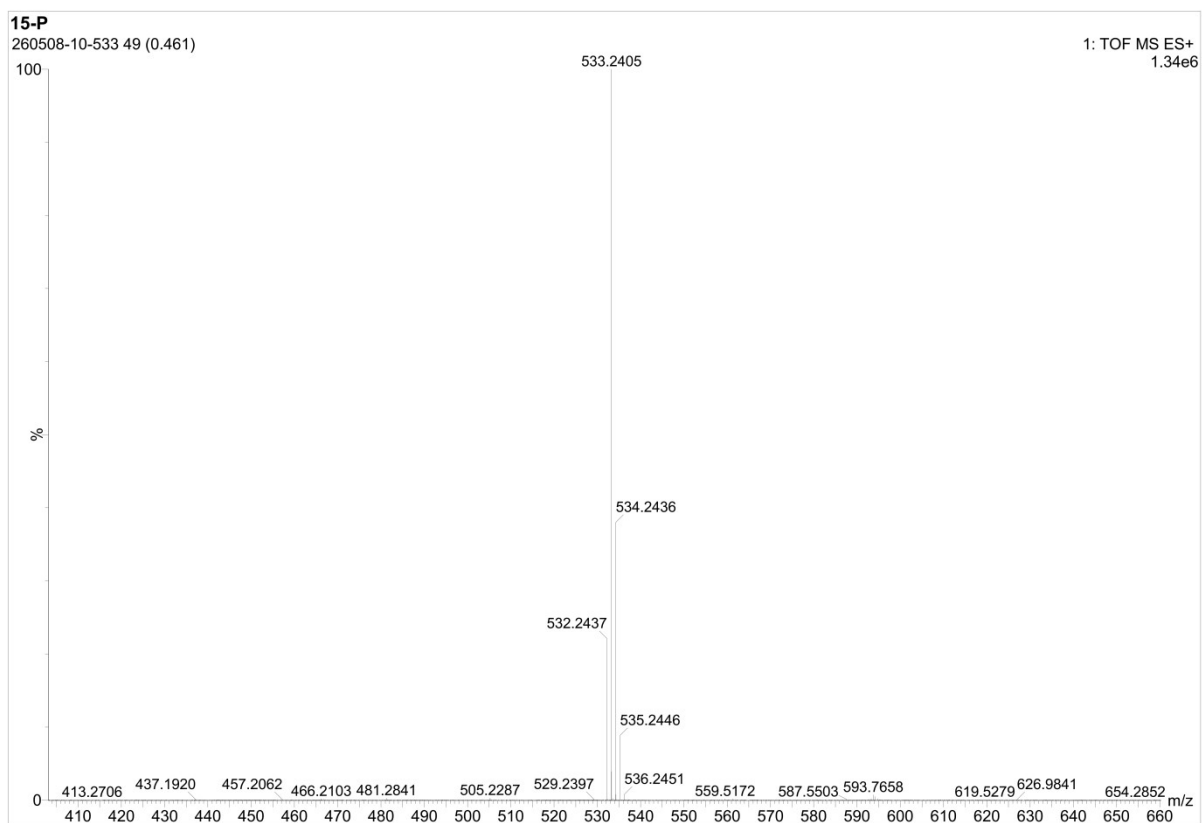


Figure S4. HRMS spectrum of TQ-H₂O₂.

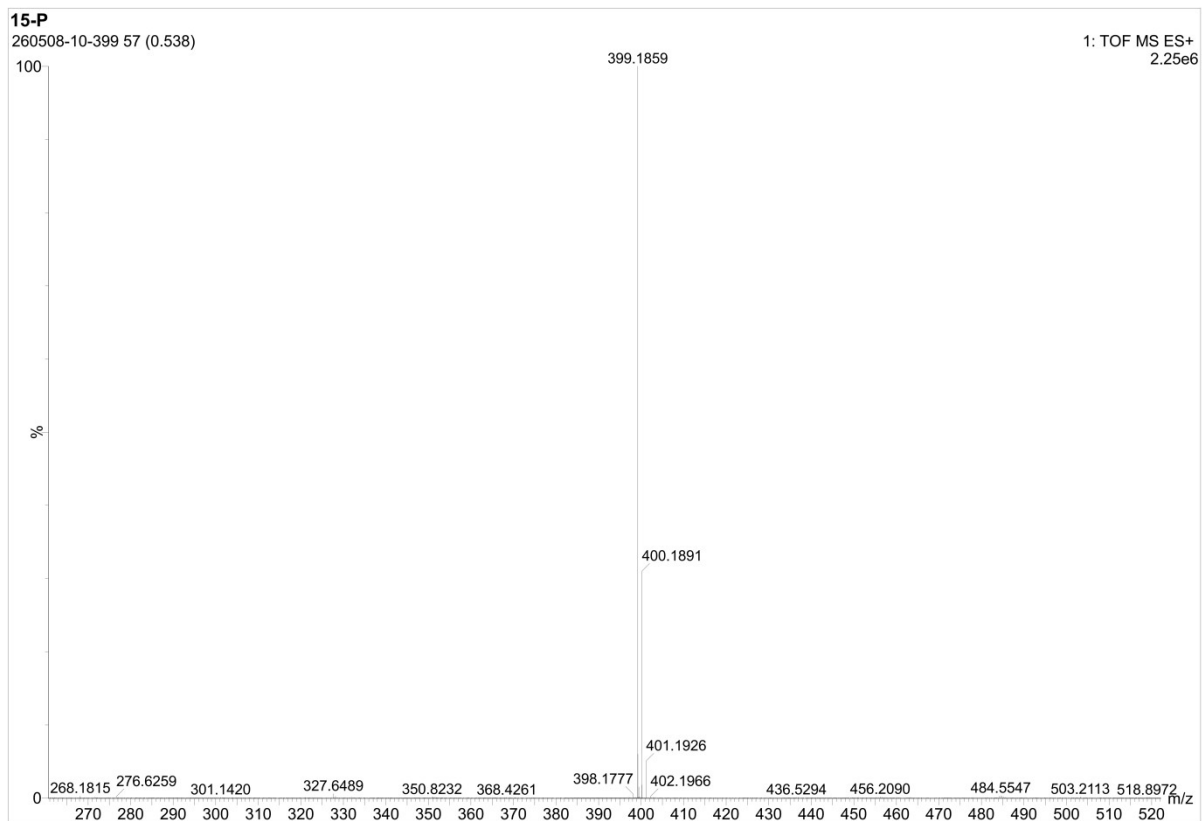


Figure S5. HRMS spectrum of TQ.

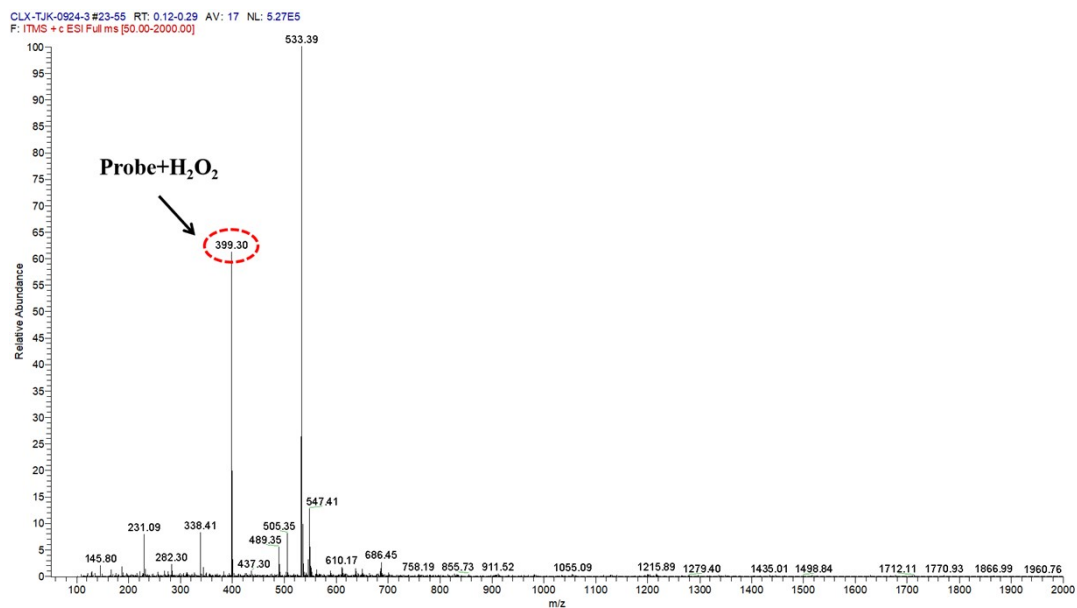


Figure S6. MS spectrum of TQ-H₂O₂ after addition of H₂O₂.

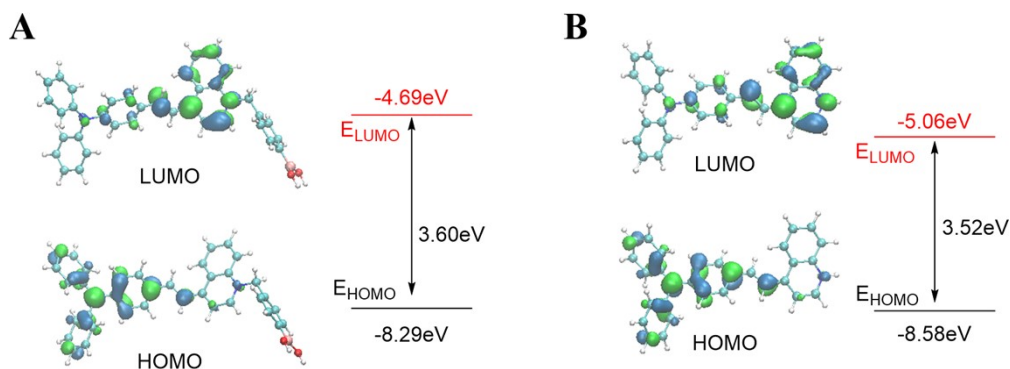


Figure S7. DFT calculations of TQ-H₂O₂ (A) and TQ (B).

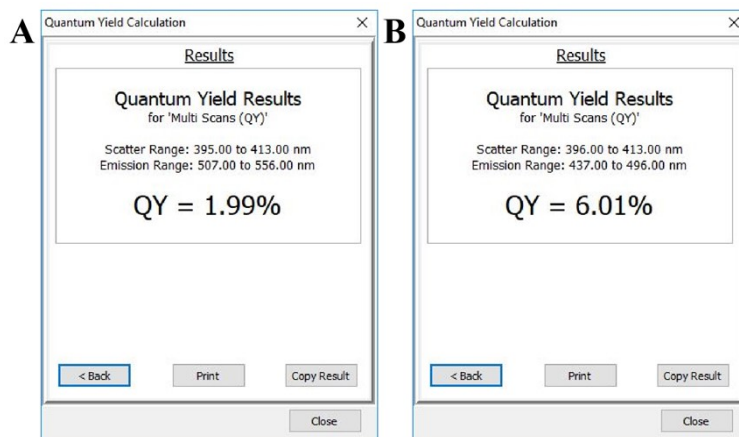


Figure S8. Fluorescent quantum yields (Φ_f) of probe TQ-H₂O₂ without or with H₂O₂ (80 μ M).

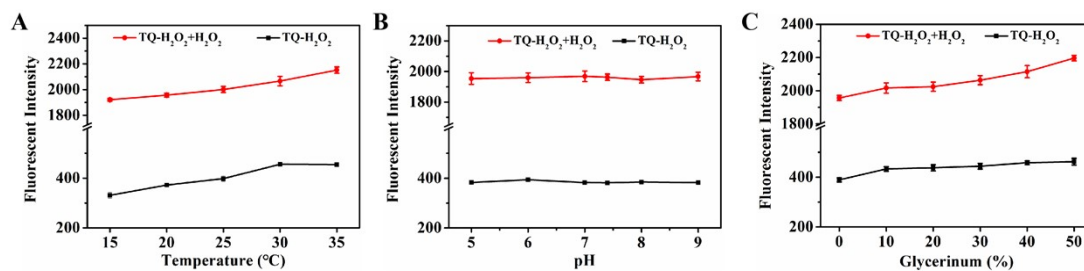


Figure S9. Fluorescence response spectra of TQ-H₂O₂ towards H₂O₂ (80 μ M) under different temperature (A), pH (B) and viscosity (C) with disparate concentration proportion of glycerinum in H₂O.

Table S2. The photophysical characterization of TQ-H₂O₂ and TQ.

	TQ-H ₂ O ₂	TQ
λ_{Abs} (nm)	525	405
ϵ (10 ⁴ L•mol ⁻¹ •cm ⁻¹)	2.14	1.88
λ_{ex} (nm)	525	405
λ_{em} (nm)	738	524
Quantum yield (%)	1.99	6.01
Stokes shift	213	119

4 Cytotoxic assays

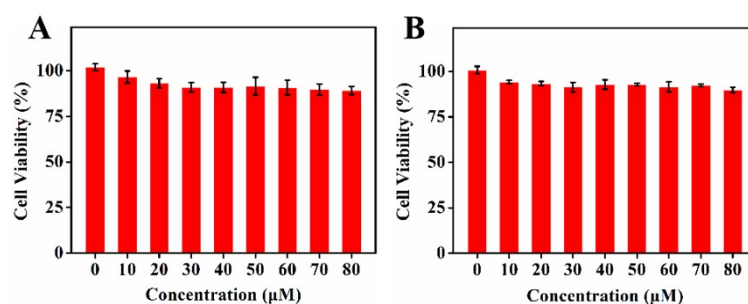


Figure S10. Cytotoxicity assay of the probe in HepG2 (A) and RAW264.7 (B) cells. The cells were respectively treated with different concentrations of the probe (0, 10, 20, 30, 40, 50, 60, 70, 80 µM) for 24 h. The experiments were repeated three times and the data are shown as mean (\pm S.D.).

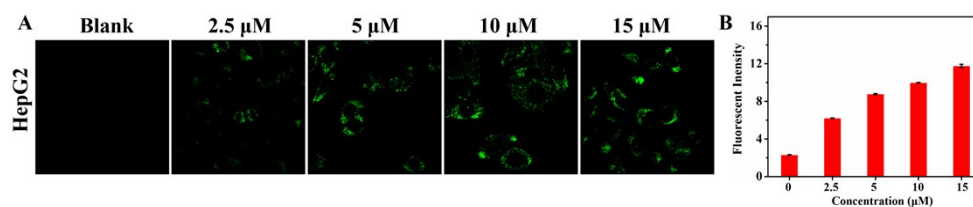


Figure S11. Imaging analysis of different concentration of TQ-H₂O₂ (A). (B) fluorescent intensity (n=3) of (A) was quantified by Image J.

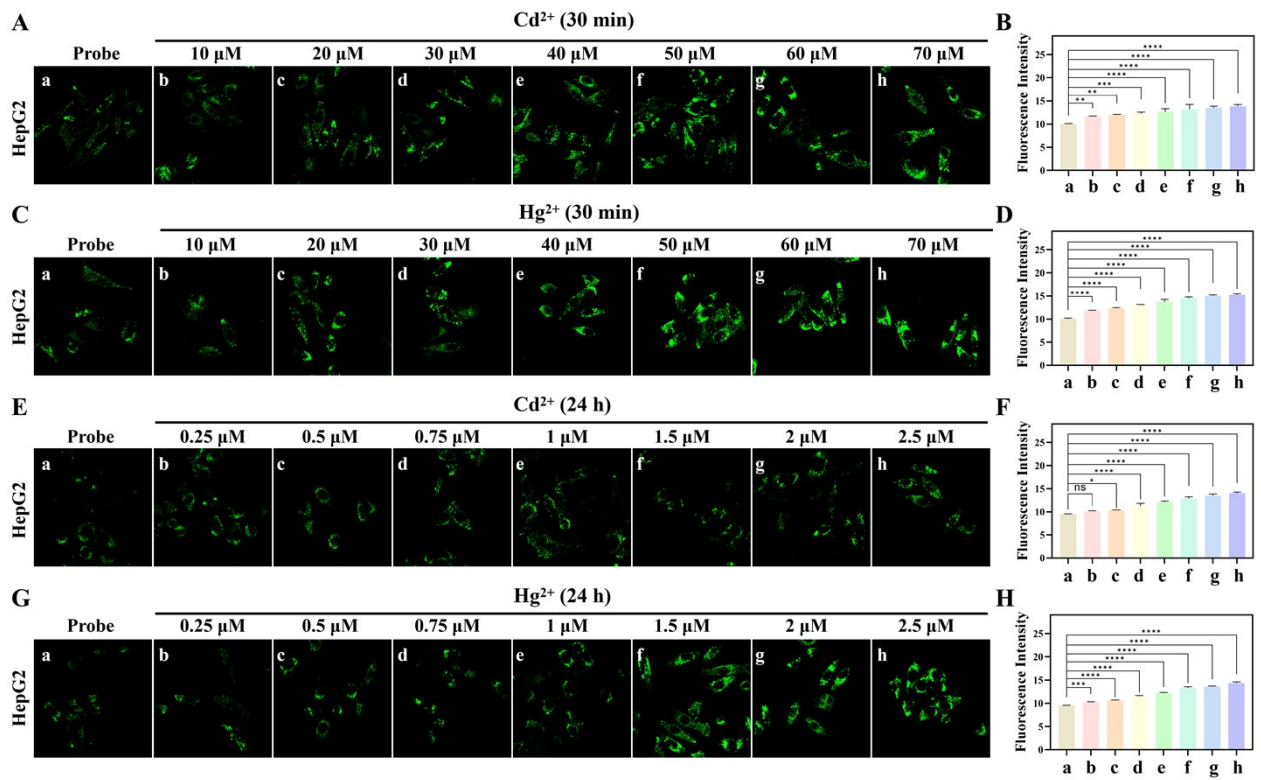


Figure S12. High dose stress: the imaging analysis of Cd^{2+} (A) and Hg^{2+} (C). b-h. 10–70 μM of Cd^{2+} and Hg^{2+} for 30 min; Low dose stress: the imaging analysis of Cd^{2+} (E) and Hg^{2+} (G). b-h. 0.25–2.5 μM of Cd^{2+} and Hg^{2+} for 24 h; (B, D, F, H) fluorescent intensity (n=3) of (A, C, E, G) were quantified by Image J. ns: no significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent S. D. (n = 3).

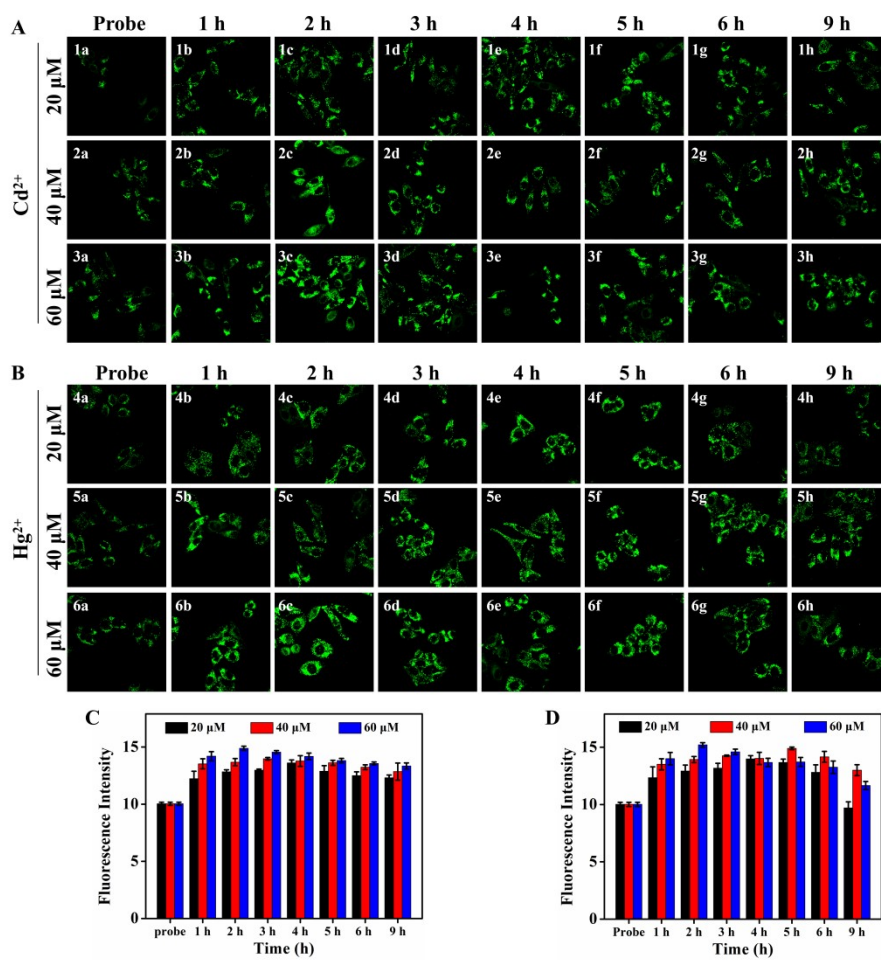


Figure S13. (A, B) Imaging analysis of H₂O₂ at different time by TQ-H₂O₂ after removing Cd²⁺/Hg²⁺ (20-60 μM) exposure. (C, D) Fluorescent intensity (n=3) ratio of (A, B) were quantified.

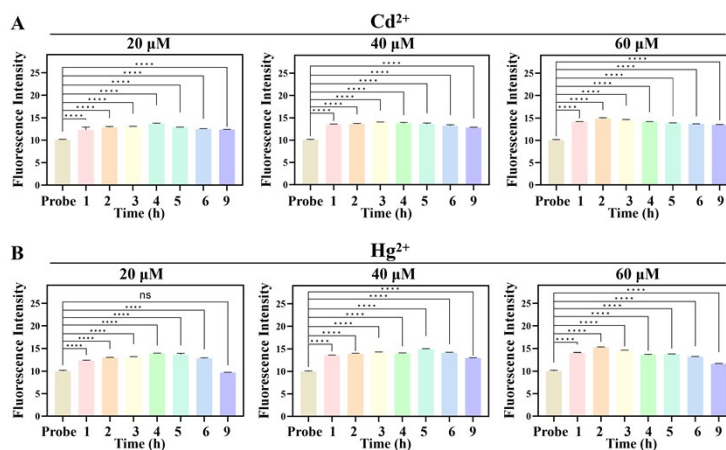


Figure S14. The detailed contrast of imaging analysis of H₂O₂ at different time after removing Cd²⁺/Hg²⁺ acute exposure. ns: no significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent S. D. (n = 3).

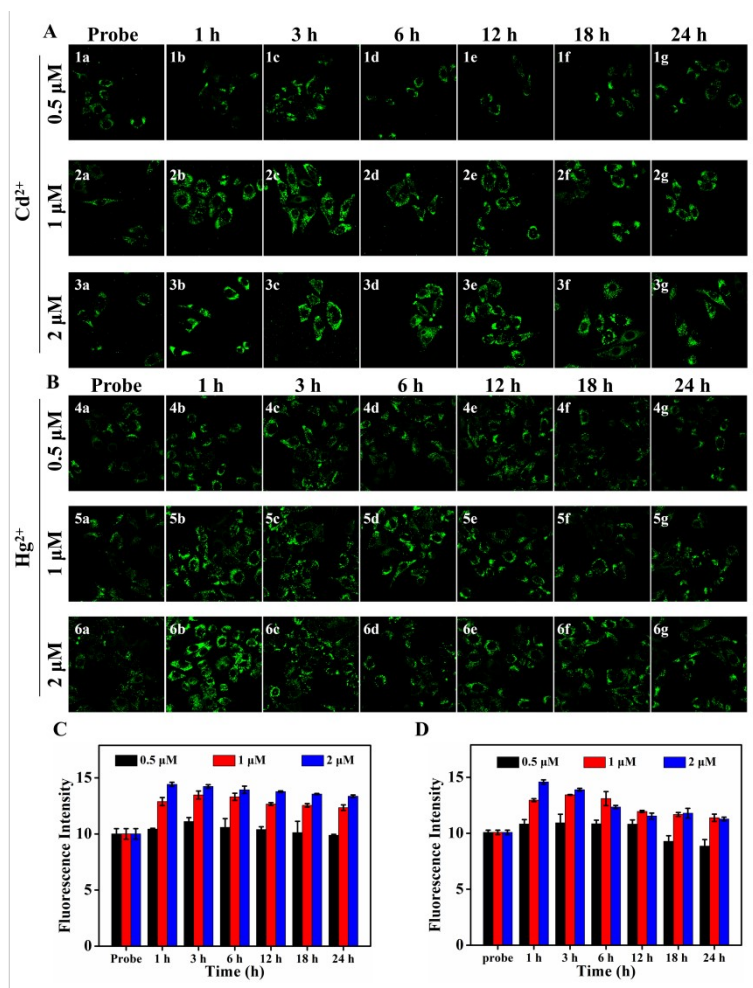


Figure S15. (A, B) Imaging analysis of H₂O₂ at different time by TQ-H₂O₂ after removing Cd²⁺/Hg²⁺ (0.5-2 μM) exposure. (C, D) Fluorescent intensity (n=3) ratio of (A, B) were quantified.

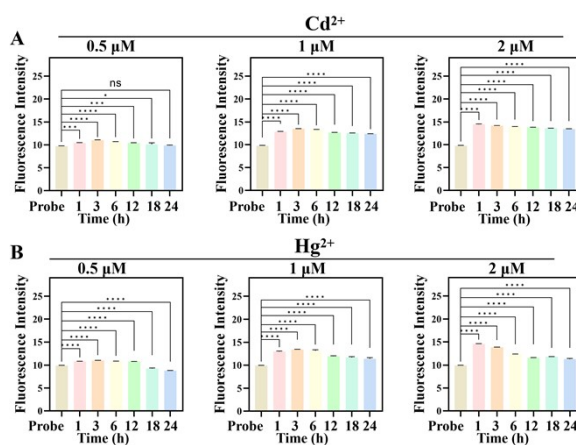


Figure S16. The detailed contrast of imaging analysis of H₂O₂ at different time after removing Cd²⁺/Hg²⁺ chronic exposure. ns: no significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Error bars represent S. D. (n = 3).

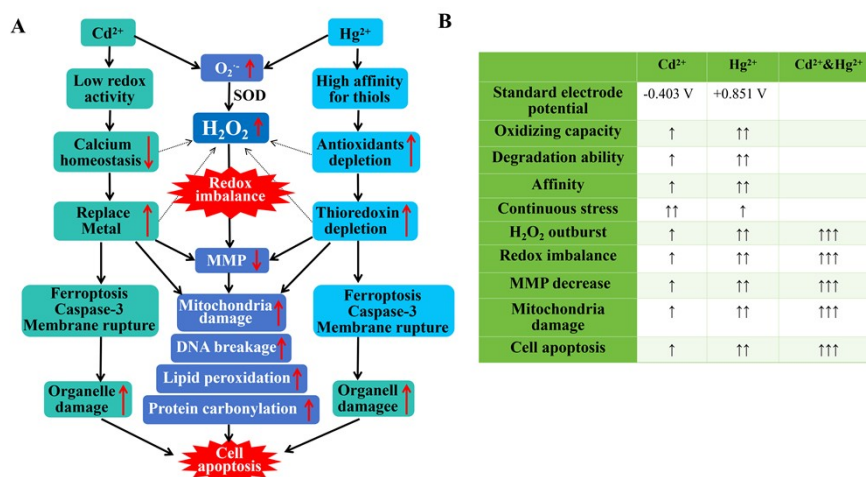


Figure S17. (A) Proposed mechanism diagram of H₂O₂ fluctuation under Cd²⁺/Hg²⁺ single and co-exposure stress. (B) The contrast of physiological activity under different stress (Cd²⁺, Hg²⁺, Cd²⁺&Hg²⁺).

References

- [1] Y. Tian, S. Liu, W. Cao, P. Wu, Z. Chen, H. Xiong, H₂O₂-activated NIR-II fluorescent probe with a large Stokes Shift for high-contrast imaging in drug-induced liver injury mice, *Anal. Chem.* 94 (2022) 11321, <https://doi.org/10.1021/acs.analchem.2c02052>.
- [2] P. Niu, J. Liu, F. Xu, L. Yang, Y. Li, A. Sun, L. Wei, X. Liu, X. Song, Dual-ratiometric fluorescent probe for H₂O₂ and HClO in living cells and zebrafish and application in alcoholic liver injury monitoring, *ACS Applied Bio. Materials* 5 (2022) 1683, <https://doi.org/10.1021/acsabm.2c00058>.
- [3] W.L. Jiang, W.-X. Wang, J. Liu, Y. Li, C.-Y. Li, A novel hepatocyte-targeting ratiometric fluorescent probe for imaging hydrogen peroxide in zebrafish, *Sens. Actuators B Chem.* 313 (2020) 128054, <https://doi.org/10.1016/j.snb.2020.128054>.
- [4] L. Xu, Y. Zhang, L. Zhao, H. Han, S. Zhang, Y. Huang, X. Wang, D. Song, P. Ma, P. Ren, Y. Sun, A neoteric dual-signal colorimetric fluorescent probe for detecting endogenous/exogenous hydrogen peroxide in cells and monitoring drug-induced hepatotoxicity, *Talanta* 233 (2021) 122578, <https://doi.org/10.1016/j.talanta.2021.122578>.
- [5] L. Zhang, C. Yan, Y. Zhang, D. Ma, J. Huang, Z. Zhao, Y. Tao, C. Liu, J. Li, W.-H. Zhu, Z. Guo, Activatable BODIPY-chromene NIR-II probes with small spectral crosstalk enable high-contrast in vivo bioimaging, *Chem. Commun.* 59 (2023) 8388, <https://doi.org/10.1039/d3cc01742g>.