Supporting Information for

Ultrasound Activated Cyanine-Rhenium(I) Complex for Sonodynamic and Gas Synergistic Therapy

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Contents

Experimental section

Chemicals and reagents

1,1,2-trimethyl-1H-benzo[e]indole, iodoethane, pentacarbonyl chlororhenium(I) ($Re(CO)_5Cl$), 2,2'-bipyridine-4,4'-dicarbaldehyde and PBS solution were purchased from Macklin. 9,10-diphenylanthracene (DPA), 2,2,6,6-tetramethylpiperidine (TEMP), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), methylene blue (MB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Roswell Park Memorial Institute medium (RPMI-1640) and Penicillin-Streptomycin were from HyClone (USA). FBS was purchased from Gibco (USA). Calcein-AM and propidium iodide (PI) were purchased from Biolegend (USA). DAPI was purchased from Thermo Fisher Scientific (USA). 2',7'-dichlorofluorescin diacetate (DCFH-DA) and cell apoptosis detection Annexin V/PI kit were obtained from Life Technologies. Mouse breast cancer (4T1) cell line was purchased from ECACC.

Instruments

NMR spectra were recorded by a Bruker AV-600MHz spectrometer. Positive ion ESI-MS spectra were obtained using an Agilent 6130B single quad coupled to an automated sample delivery system. UV-visible absorption spectra were recorded by a Shimadzu UV-3600PLUS spectrophotometer. The emission spectra were recorded with an Edinburgh FS5 Fluorimeter. ESR spectra were recorded using a Bruker Model A300 ESR spectrometer equipped with a Bruker ER 4122 SHQ resonator. Confocal images were recorded by a Zeiss LSM 880 confocal microscopy. The cell viability assay was done with a Promega microplate reader. DJO-2776 sonicator was used to generate ultrasound during the treatment.

Synthesis of 1-ethyl iodide-2,3,3-trimethylbenz[e]indole

A mixture containing 1,1,2-trimethyl-1H-benzo[e]indole (10.06 g, 0.065 mol) and iodoethane (8.97 g, 0.057 mol) in 50 mL CH_3CN was refluxed under nitrogen atmosphere for 24 h in the dark. Upon cooling to the room temperature, a large amount of gray precipitate was formed which then washed with diethyl ether for

3 times. The gray powder was obtained by recrystallization from ethanol. The yield was approximately 40.5%. ^{1}H NMR (600 MHz, DMSO-d^{6}) δ 8.38 (d, J = 8.3 Hz, 1H), 8.31 (d, J = 8.9 Hz, 1H), 8.23 (d, J = 8.1 Hz, 1H), 8.16 (d, J = 8.9 Hz, 1H), 7.77 (dt, J = 28.3, 7.5 Hz, 2H), 4.63 (d, J = 7.4 Hz, 2H), 2.95 (s, 3H), 1.77 (s, 6H), 1.51 (t, J = 7.3 Hz, 3H).

Synthesis of Bpy-Cy

A mixture containing 1-ethyl iodide-2,3,3-trimethylbenz[e]indole (2 equiv, 0.366 g, 1 mmol) and 3,3'-bipyridine-6,6'-diformaldehyde (1 equiv, 0.106 g, 0.5 mmol) refluxed in 30 mL absolute ethanol for 24 h. After cooling to room temperature, and then filtration gave red brown color precipitate. The precipitate was washed with a mixed solution of petroleum ether and ethyl acetate (v : v = 3 : 1) for 3 times. The red brown power was obtained after drying. Yield: 44.4%. ^{1}H-NMR (600 MHz, DMSO-d^{6}) δ 9.04 (d, J = 36.4 Hz, 4H), 8.69 (d, J = 17.2 Hz, 2H), 8.48 (d, J = 8.2 Hz, 2H), 8.34 (d, J = 11.0 Hz, 4H), 8.23 (dd, J = 16.1, 8.2 Hz, 4H), 8.03 (d, J = 16.2 Hz, 2H), 7.81 (d, J = 28.2 Hz, 4H), 4.95 (s, 4H), 2.06 (s, 12H), 1.57 (s, 6H).

Synthesis of Re-CHO

$Re(CO)_5Cl$ (0.2896 g, 0.8 mmol) and 2,2'-bipyridine-4,4'-dicarbaldehyde (0.169 g, 0.8 mmol) were taken in 20 mL toluene and then the reaction mixture was refluxed under nitrogen for 24 h in the dark. After cooling the reaction mixture to the room temperature, a light red precipitate was obtained which then collected by filtration and washed 3 times with n-hexane and diethyl ether. The yield was approximately 63.7%. ^{1}H-NMR (600 MHz, DMSO-d^{6}) δ 10.24 (s, 2H), 9.34 (d, J = 4.9 Hz, 4H), 8.16 (d, J = 6.9 Hz, 2H). ^{13}C-NMR (126 MHz, DMSO-d^{6}) δ 197.81, 191.54, 156.73, 155.08, 144.47, 126.67, 124.13. ESI-MS (m/z): $C_{15}H_8ClN_2O_5KNa(^{185}Re)$ **[Re-CHO**+Na+K]:579. Elemental Analysis: Anal. Calcd. For $C_{15}H_8ClN_2O_5Re$: C, 34.79; H, 1.56; N, 5.41; Found: C, 34.77; H, 1.58; N, 5.40.

Synthesis of Re-Cy

Re-CHO (0.104 g, 0.2 mmol) and 3-ethyl-1,1,2-trimethyl-1H-benzo[e]indol-3-ium iodide (0.146 g, 0.4 mmol) were taken in 20 mL anhydrous ethanol. The mixture was then refluxed in the dark under nitrogen atmosphere for 24 h. The deep red product, obtained from the reaction, was filtered and collected and was washed with petroleum ether and ethyl acetate (*v* : *v*=3:1) for 3 times. The yield of the solid was approximately 51.2%. ^{1}H-NMR (600 MHz, DMSO-d^{6}) δ 9.59 (s, 2H), 9.36 – 9.22 (m, 3H), 8.66 (d, J = 16.7 Hz, 2H), 8.54 – 8.43 (m, 4H), 8.37 (d, J = 8.1 Hz, 2H), 8.25 (d, J = 11.0 Hz, 5H), 7.85 (d, J = 6.5 Hz, 2H), 7.80 (d, J = 7.2 Hz, 2H), 5.05 (d, J = 5.3 Hz, 4H), 2.10 (s, 12H), 1.62 (d, J = 7.2 Hz, 6H). ^{13}C-NMR (126 MHz, DMSO-d^{6}) δ 182.11, 156.30, 146.35, 145.11, 140.75, 138.68, 134.21, 131.94, 130.66, 130.65, 130.61, 129.21, 128.55, 128.53, 127.15, 124.43, 124.11, 119.66, 114.18, 55.17, 46.25, 45.35, 44.45, 25.30, 15.08. ESI-MS (m/z): $C_{49}H_{44}ClN_4O_3KNa$(185Re) [**Re-Cy**-I-I+K+Na]$^{2+}$: 511.0; [**Re-Cy**-I-I+K+Na]$^{+}$: 1019.7. Elemental Analysis: Anal. Calcd. For $C_{49}H_{47}ClI_2N_4O_3Re$: C, 48.42; H, 3.90; N, 4.61; Found: C, 48.45; H, 3.94; N, 4.58.

Synthesis of COP-1

According to Chang's method, COP-1 was synthesized and characterized by ^{1}H-NMR and ESI-MS.[1] ^{1}H-NMR (600-MHz, DMSO-d^{6}) δ 7.49 (s, 1H), 7.23 (d, J = 7.3 Hz, 1H), 7.01 (d, J = 7.3 Hz, 1H), 6.16 (s, 2H), 4.18 (s, 2H), 2.71 (d, J = 32.9 Hz, 6H), 2.44 (s, 6H), 1.42 (s, 6H). ESI-MS (m/z): $C_{44}H_{52}B_2Cl_2F_4N_6Pd_2$ [COP-1+2H]$^{2+}$: 527.1.

Detection of CO in solution

Gas chromatography method: Release of CO in solution under US irradiation was monitored by gas chromatography. 4 mL DMSO solution containing 4 mg **Re-Cy** or **Re-CHO** was placed in a sealed cuvette and degassed for 5 min, then irradiated with US (0.3 W/cm^{2}, 3 MHz) for 0.5 h. Gas chromatography of the reaction mixture was recorded by quickly collecting the gas from the cuvette. Chromatogram of a gas mixture showing the retention time for CO ~ 19.3 min. As the control, **Re-Cy** or **Re-CHO** in DMSO without US irradiation were also studied as well.

COP-1 method: The CO release under US irradiation was also monitored by the CO fluorescence probe COP-1 (500 μM stock solution in DMSO). In detail, fluorescence of 2 mL phosphate buffer solution (PBS) containing 50 μM **Re-Cy** or **Re-CHO** and 10 μM COP-1 was after various US irradiation durations (0.3 W/cm^2, 3 MHz).

Hemoglobin method: The CO release was also detected by detecting the conversion of hemoglobin (Hb) to carboxyhemoglobin (HbCO) by UV-vis spectroscopy.[2] Hb was dissolved completely in PBS (4.2 μM, 1 mL, pH 7.4) and then reduced by sodium dithionite (SDT, 0.4 mg) in nitrogen atmosphere. 1 μL **Re-Cy** or **Re-CHO** (1 mM) was added to the above solution. After US irradiation the absorption spectra of the solution were collected by UV-vis spectrophotometer. Two strong characteristic absorption bands at 410 nm and 430 nm are assignable to HbCO and Hb, respectively. Which were used to calculate the conversion of Hb-to-HbCO. The Beer-Lambert law was used to calculate the Hb-to-HbCO conversion percentage (x) and the concentration of released CO (C_{CO}) was coordinated with Hb, as indicated by the following functions:

$$C_{CO} = \frac{528.6 \times I_{410\,nm} - 304 \times I_{430\,nm}}{216.5 \times I_{410\,nm} + 442.4 \times I_{430\,nm}} C_{Hb}$$

.

Detection of ROS generation

Total ROS measurement: Fluorescence of 10 μM DCFH-DA and 10 μM **Re-Cy** or **Re-CHO** in H_2O was measured by fluorescence spectrometer after different durations of US (0.3 W/cm^2, 3 MHz) irradiation. Fluorescence intensities of DCFH-DA at 525 nm were monitored to quantify the generation rate of ROS.

$^1O_2$ measurement: The $^1O_2$ generation under US irradiation was measured using the probe 9,10-diphenylanthracene (DPA). In brief, a DMSO:H_2O solution (1:9 v/v) containing 5 μM **Re-Cy** or **Re-CHO** and 2 μg/mL DPA was monitored by UV-vis spectroscopy after different durations of US irradiation (0.3 W/cm^2, 3

MHz) (irradiation interval: 20 min). The absorbance at 378 nm was monitored to quantify the rate of $^1O_2$ generation.

•OH measurement: The absorbance of MB-based band at 664 nm was monitored to quantify the rate of •OH generation. 5 μM **Re-Cy** or **Re-CHO** and 5 μg/mL methylene blue (MB) in H_2O was monitored by UV-vis spectroscopy after different durations of US irradiation (0.3 W/cm^2, 3 MHz).

ESR measurement

ESR measurements were recorded on Electron Paramagnetic Resonance A300, TEMP and DMPO were used to trap $^1O_2$ and $^{\bullet}OH$, respectively. 20 μL TEMP (40 mM) or DMPO (90 mM) was mixed with 80 μL **Re-Cy** or **Re-CHO** (5 mM) and irradiated by US (0.3 W cm^{-2}, 3 MHz, 1 h). As a control, the **Re-Cy** or **Re-CHO** mixed with TEMP or DMPO without US were studied as well.

Cytotoxicity test

Cytotoxicity test was performed by MTT assay.[3] Approximately 5 × 10^3 cells per each well were seeded onto a 96-well plate, followed by 36 h incubation for attachment. Then 4T1 or MCF-10A cells were incubated with various concentrations of **Re-Cy** or **Re-CHO** for 1 h. After incubation, the culture media was replaced with fresh culture media followed by US irradiation (0.3 W/cm^2, 3 MHz) for different time durations (0-20 min). The change in optical density (OD) at 490 nm was monitored using microplate reader (Promega). For *in-vitro* apoptosis imaging, 4T1 cells were incubated with **Re-Cy** or **Re-CHO** (10 μM) for 1 h, then exposed to US irradiation (0.3 W/cm^2, 3 MHz, 15 min). After that, the 4T1 cells were stained with calcein AM (AM, stained live cell) and propidium iodide (PI, stained dead cell) for 30 min. The cell images were acquired using a Zeiss LSM 880 confocal microscope.

Intracellular CO measurement

The intracellular CO release under the US irradiation was monitored by the fluorescence probe of COP-1. Already attached 4T1 cells were treated with the **Re-Cy** or **Re-CHO** (10 μM) for 1 h, then washed with PBS and exposed to US

irradiation (0.3 W/cm^2, 3 MHz, 15 min). After that the cells were incubated with 10 μM of COP-1 for 30 min and Hoechst 33342 for 30 min in the dark. For COP-1 excitation wavelength was set at 488 nm, and emission was collected at 550 ± 50 nm . For Hoechst 33342 excitation wavelength was set at 405 nm, and emission was collected between 475 ± 50 nm.

Intracellular ROS measurement

The intracellular ROS generation by the complexes under US irradiation was studied by fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The 4T1 cells were treated with the **Re-Cy** or **Re-CHO** for 1 h, washed with PBS and then exposed to US irradiation (0.3 W/cm^2, 3 MHz, 15 min). After that the cells were incubated with 10 μM of DCFH-DA for 30 min in the dark. The cells were imaged by confocal microscopy immediately. The excitation wavelength was set as 488 nm, and the fluorescence was collected at 530 ± 20 nm.

Depletion of GSH in solution

The consumption of GSH was measured using 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB). **Re-Cy** or **Re-CHO** (10 μM) was mixed with GSH (200 μM) at room temperature in PBS. After different US irradiation time points, 100 μL of this solution was added to 900 μL PBS and then 4 μL DTNB (10 mg/mL) was added to it. After 5 min incubation of the resultant reaction mixture, the absorbance spectra of the solution were quickly recorded by UV-vis spectroscopy.

Cellular GSH depletion detection

The Re(I) complexes induced GSH depletion in cancer cells was detected by the commercial GSH assay kit. The 4T1 cells (1×10^7) in a 13 cm culture dish were treated by the Re(I) complexes at a dose of 20 μM and 10 μM. The incubation time was 1 h and drugs-free cells were used as the control. Then the harvested cells were suspended in 0.6 mL PBS and sonicated for 10 min at 4 ^{o}C. After that, based on the product protocol, 100 μL disrupted cell solution was mixed with 100 μL reagent 1 in the GSH assay kit. Then the mixture was centrifuged at 3,500 rpm 10 min, followed by mixing the supernatant with detection reagents prior to

reading the absorbance at 412 nm. The total protein content was determined by Bradford protein assay kit.[4] The absolute GSH level in 4T1 cells with reference to unit protein concentration were calculated according to the product protocol.[5]

Western blotting of GPX4

4T1 cells were seeded into 6-well cell culture plate at 10^6/well for 24 h, then the cells were incubated with **Re-Cy** (5 μM) in RMPI 1640 culture medium. After 4 h incubation, the culture media was replaced by fresh medium and then the cells were irradiated by US (3 MHz, 0.3 W/cm^2) for 15 min and re-cultured for 20 h. All cells were collected, the expression of GPX4 (22 kDa) in 4T1 cells were analyzed by western blotting. The protein bands were measured via the ECLTM western blotting detection reagents.

***In-vivo* experiments**

Tumor model: Balb/c female mice (8 weeks) were purchased from Guangdong Experimental Animal Center. Animal care and experiments were performed in compliance with the relevant laws and institutional guidelines from the Animal Research Ethics Committee of Shenzhen University, and the committee has approved our experiments, permit number AEWC-201412003.4T1 cells (2×10^6) suspended in PBS (25 μL) were subcutaneously injected into the back of each female Balb/c mice to construct the tumor model.

***In-vivo* tumor treatment:** 4T1 tumor-bearing mice were randomly divided into four groups (5 mice per group): (1) control group with intra-tumor (i.t.) injection of 25 μL PBS; (2) only US stimulation at the tumor sites for 15 min (3 MHz, 0.3 W/cm^2); (3) i.t. injection of 25 μL **Re-Cy** (500 μM); (4) i.t. injection of 25 μL **Re-Cy** (500 μM), after injection, the tumor sites were treated with US irradiation for 15 min (3 MHz, 0.3 W/cm^2). The tumor sizes were measured and recorded at every two days. Tumor volumes were calculated by the following formula: Volume = 0.5*Width^2*Length. For histology diagnosis, at 24 h after treatment, tumor tissues were collected from different group of mice. The tumor tissue was fixed with 10% formalin, then embedded in paraffin, and then sectioned and

stained with H&E and TUNEL. For ROS staining, the mice were sacrificed and the tumors were collected for frozen sections. The tumor slices were stained with DAPI and DCFH-DA and imaged by a Leica confocal fluorescence microscope.

References

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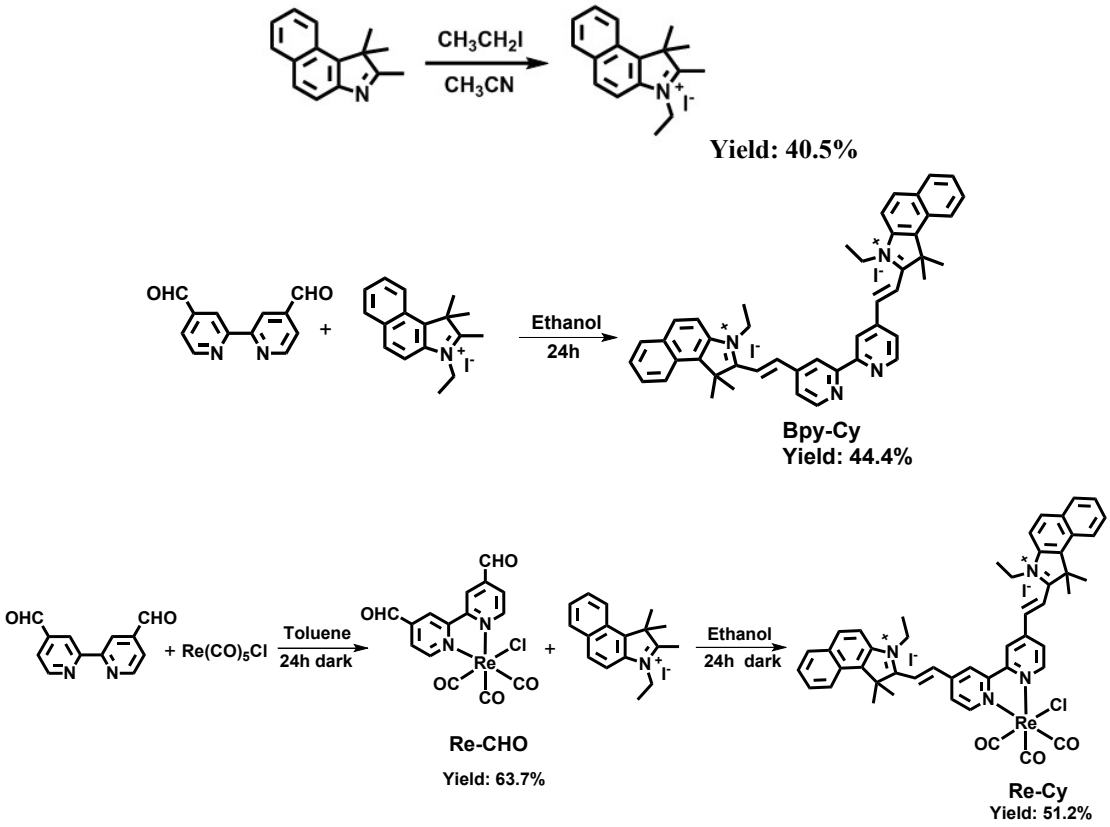
3 M. V. Berridge, P. M. Herst and A. S. Tan, Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction, *Biotechnology annual review*, 2005, **11**, 127-152.

4 A. S. Karim and M. C. Jewett, in *Methods in Enzymology*, ed. N. Scrutton, Academic Press, 2018, vol. 608, pp. 31-57.

5 T. Xu, Y. Ma, Q. Yuan, H. Hu, X. Hu, Z. Qian, J. K. Rolle, Y. Gu and S. Li, Enhanced Ferroptosis by Oxygen-Boosted Phototherapy Based on a 2-in-1 Nanoplatform of Ferrous Hemoglobin for Tumor Synergistic Therapy, *ACS Nano*, 2020, **14**, 3414-3425.

Scheme and Figures



Scheme S1. The synthetic route of the compounds.

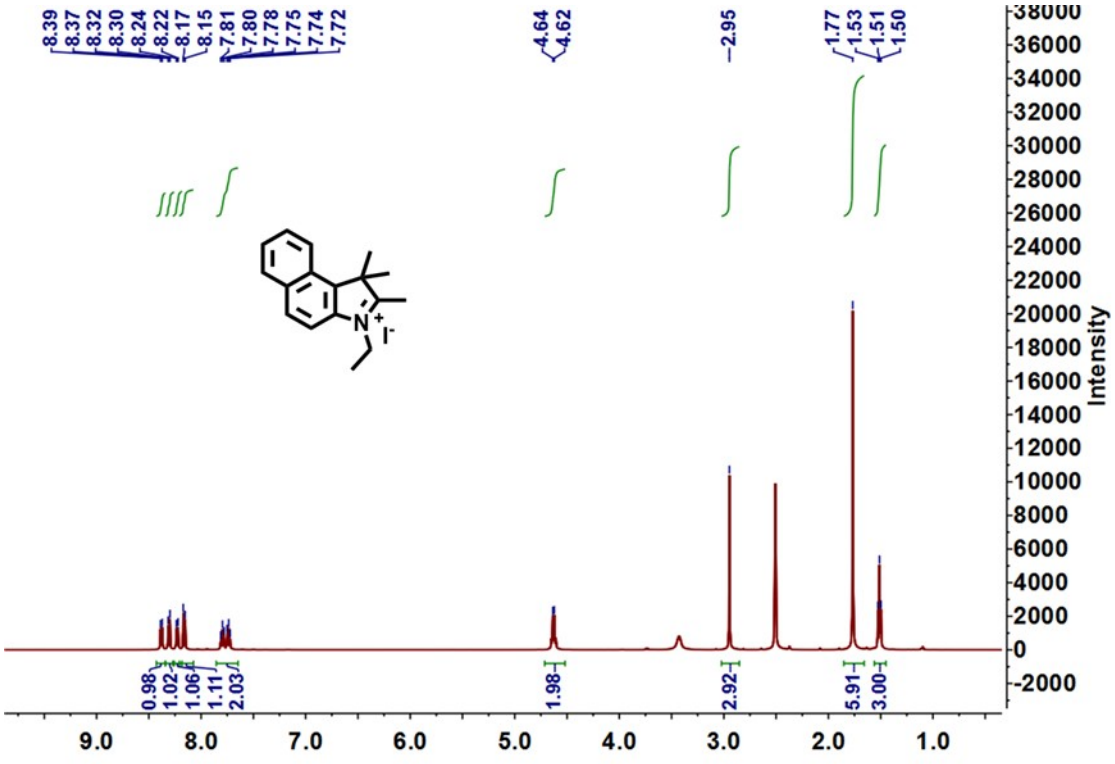


Figure S1. ^{1}H NMR spectrum of 1-ethyl iodide-2,3,3-trimethylbenz[e]indole (600 MHz, DMSO-d_6).

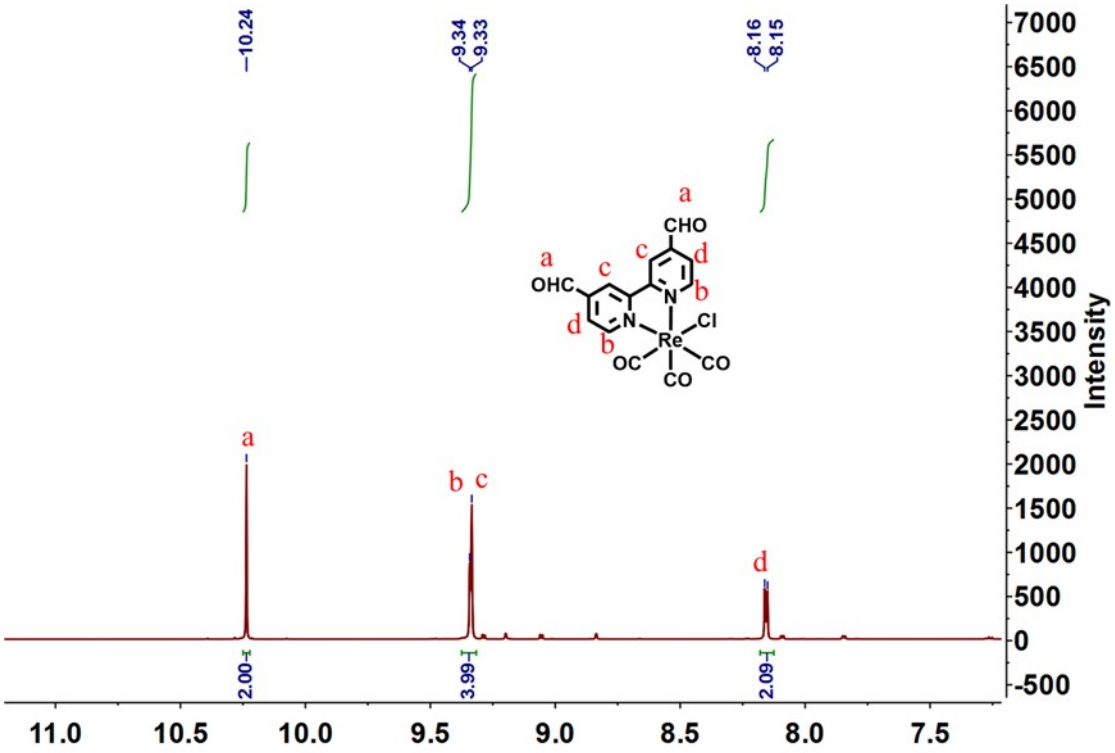


Figure S2. ^{1}H NMR spectrum of **Re-CHO** (600 MHz, DMSO-d_6).

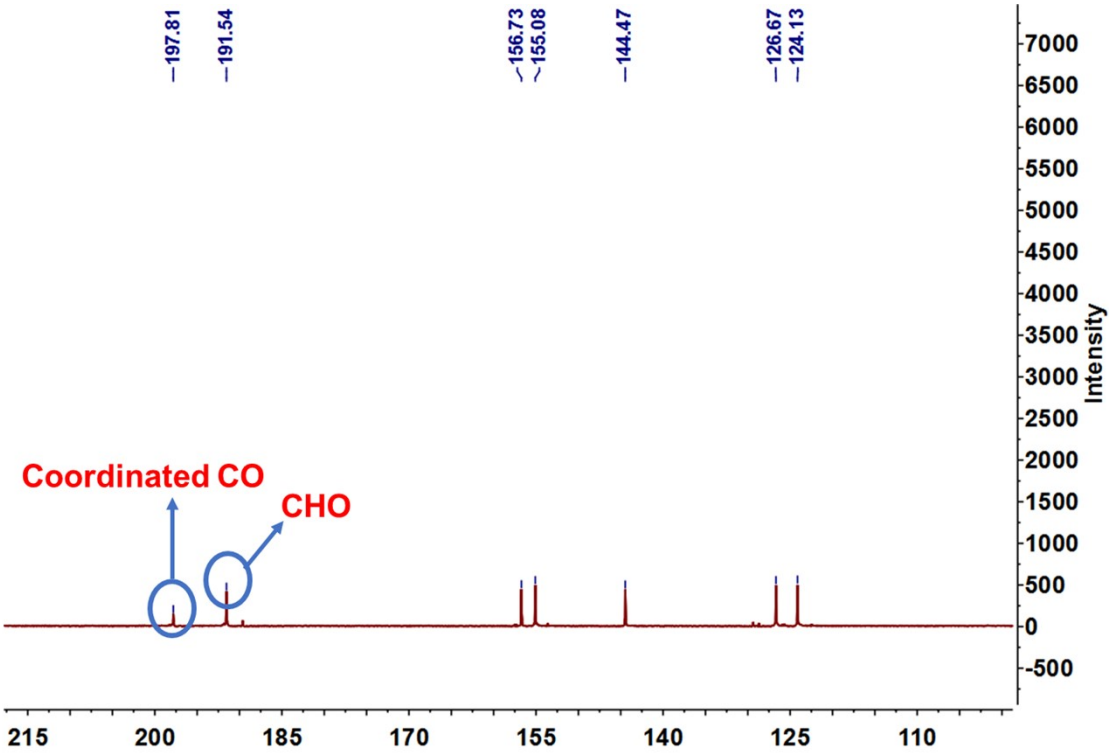


Figure S3. ^{13}C NMR spectrum of **Re-CHO** (125 MHz, DMSO-d_6).

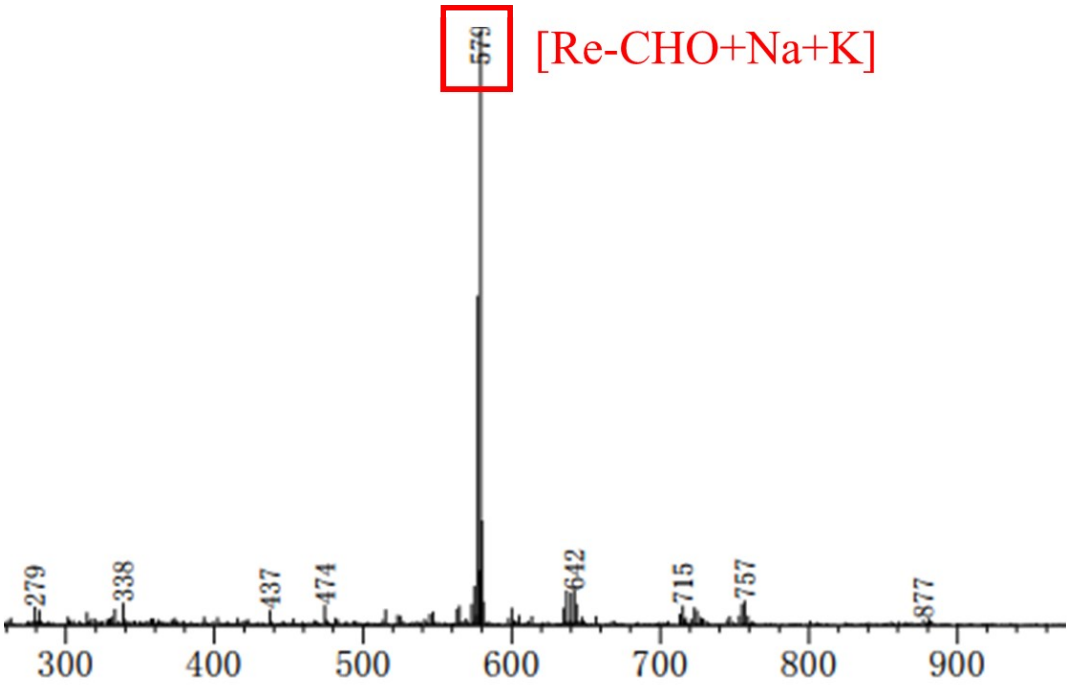


Figure S4. The ESI-MS spectrum of **Re-CHO**.

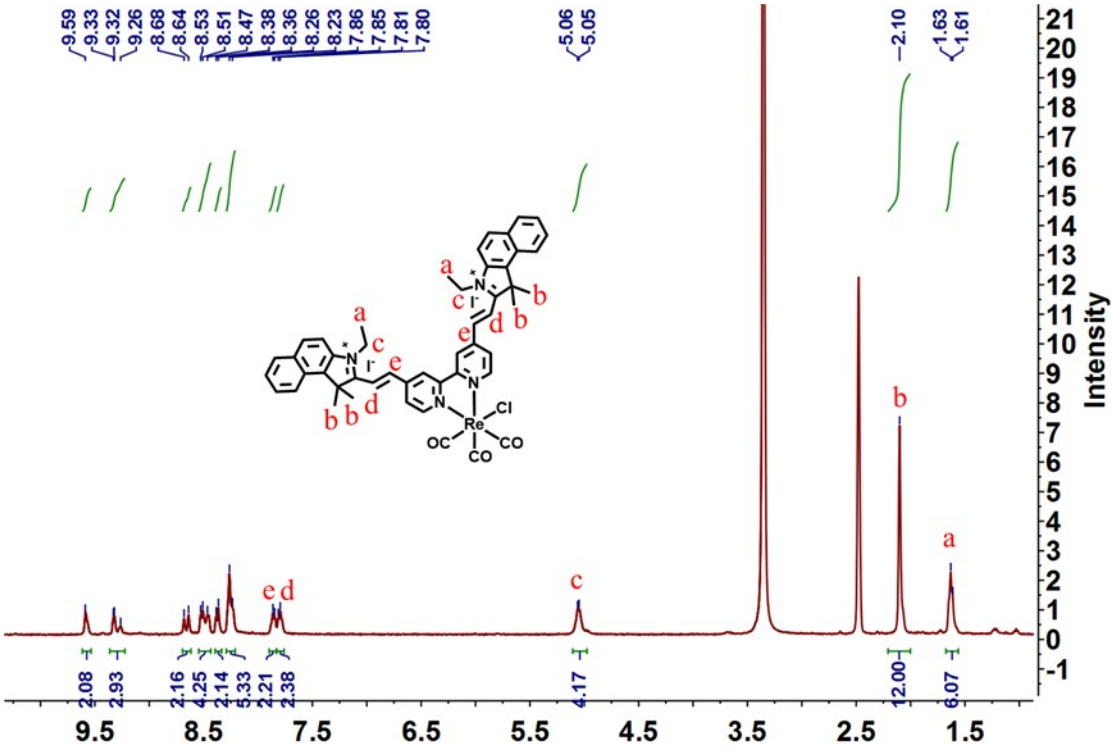


Figure S5. ^{1}H NMR spectrum of **Re-Cy** (600 MHz, DMSO-d_6).

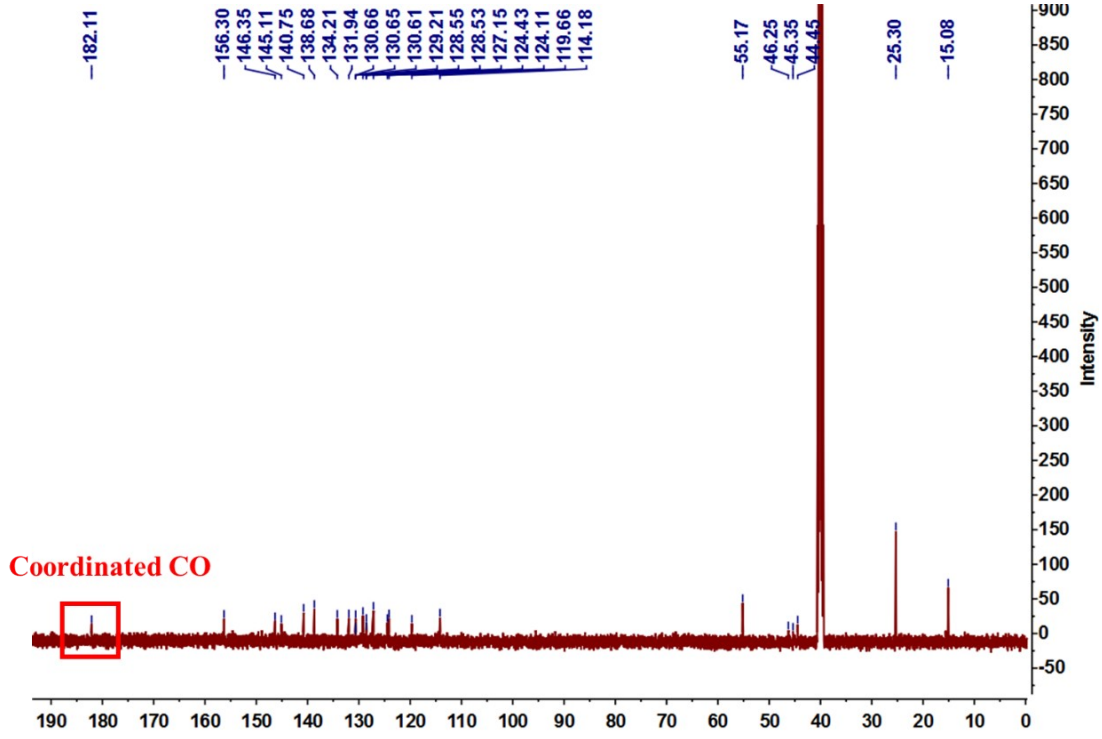


Figure S6. ^{13}C NMR spectrum of **Re-Cy** (125 MHz, DMSO-d_6).

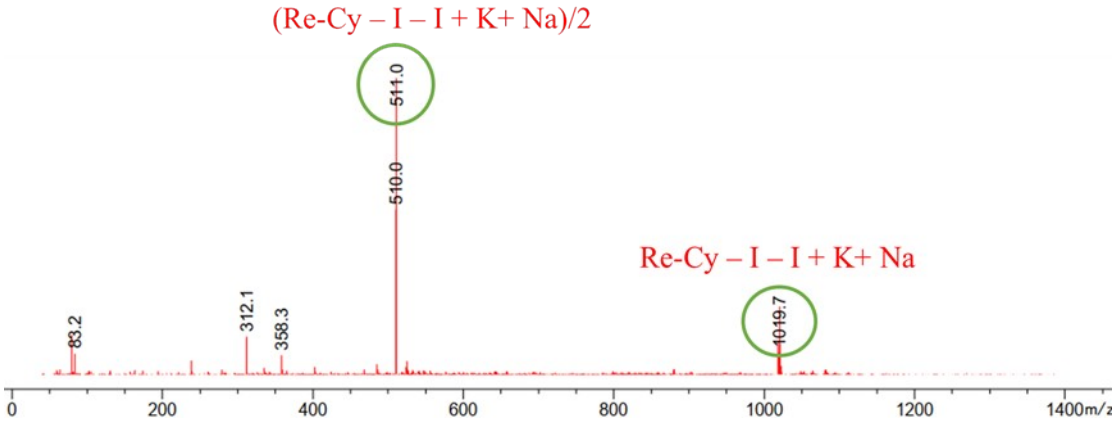


Figure S7. The ESI-MS spectrum of **Re-Cy**.

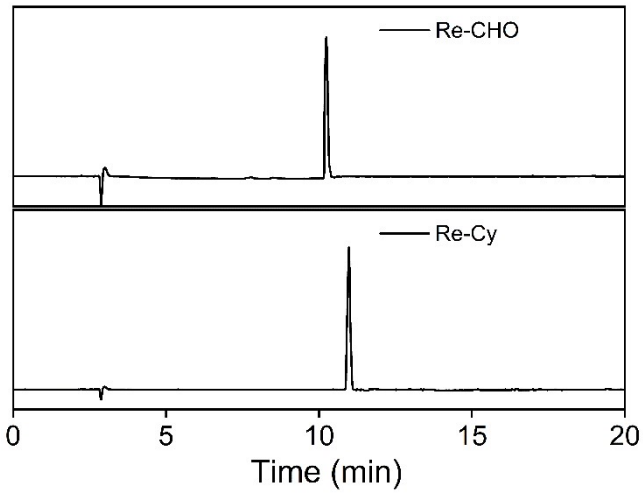


Figure S8. The HPLC spectrums of the **Re-CHO** and **Re-Cy**, the purities of **Re-CHO** and **Re-Cy** were 97.4% and 96.3%, respectively.

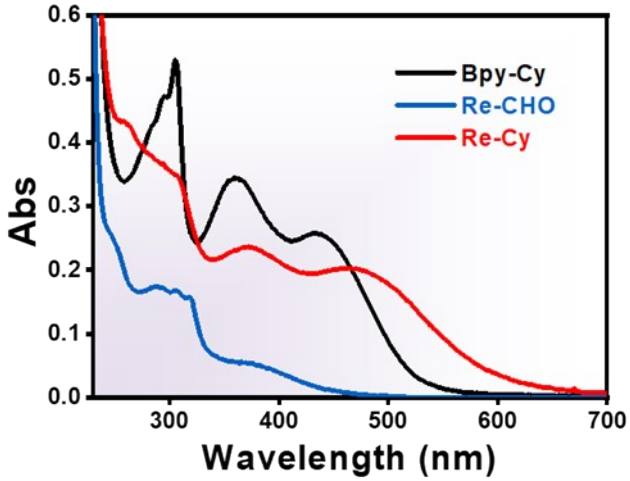


Figure S9. The UV-vis absorption of **Bpy-Cy**, **Re-CHO** and **Re-Cy** in the PBS solution.

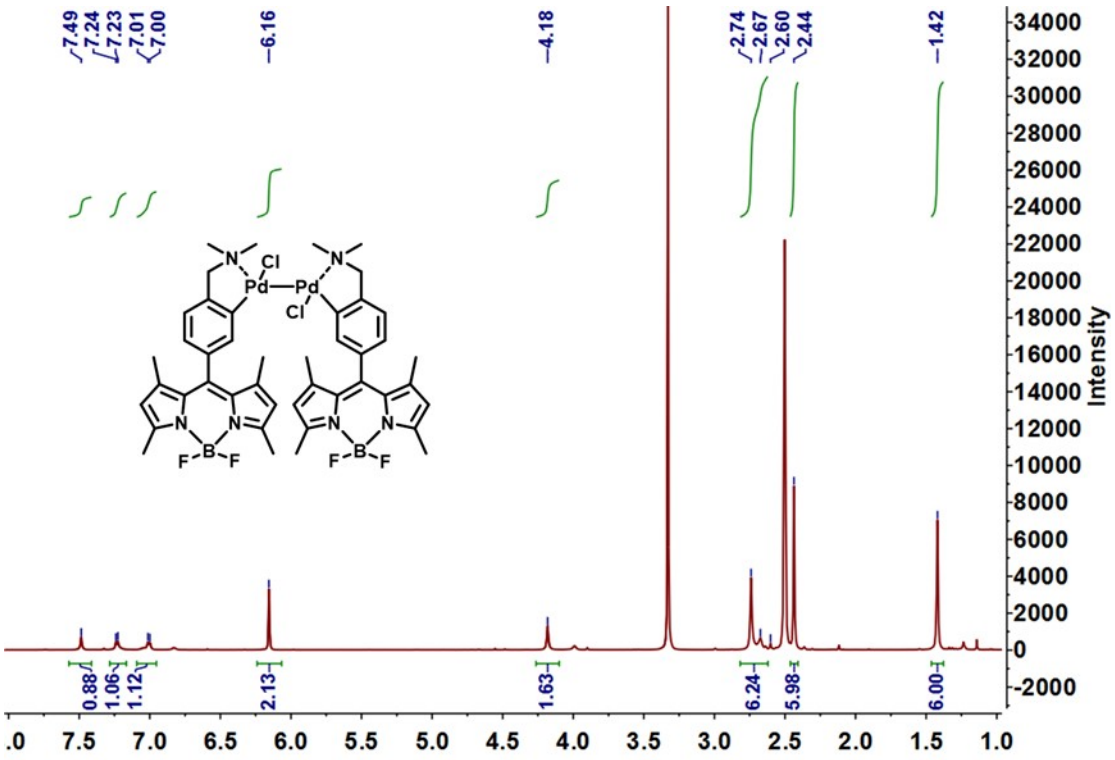


Figure S10. ^{1}H NMR spectrum of **COP-1** (600 MHz, DMSO-d_6).

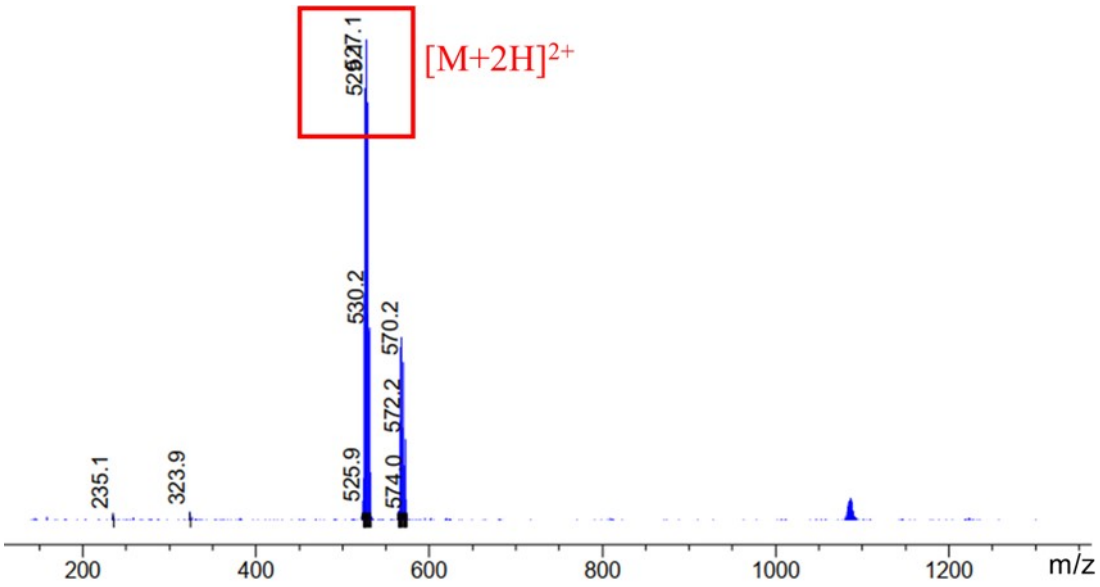


Figure S11. The ESI-MS spectrum of **COP-1**.

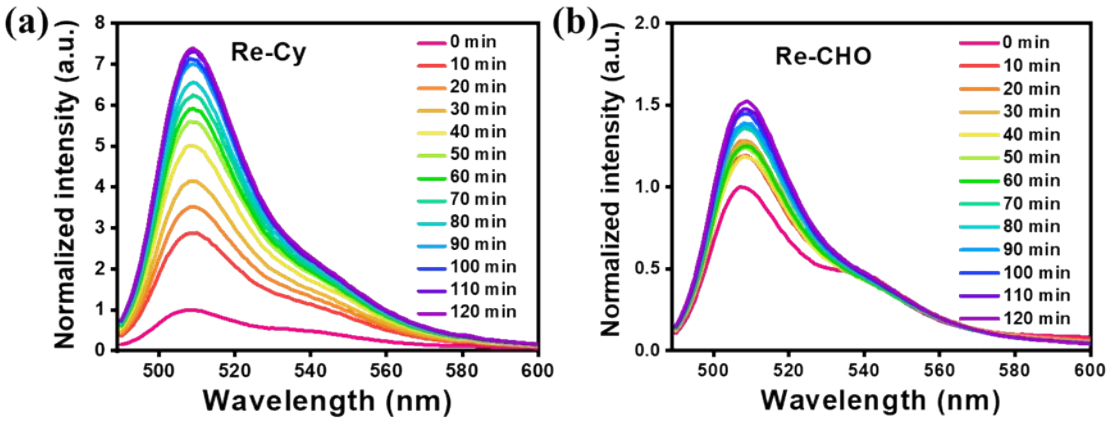


Figure S12. Time-dependent CO released from 50 μM **Re-Cy** (a) and **Re-CHO** (b) was detected by change in the fluorescence intensity of COP-1 (10 μM) under US irradiation (0.3 W cm^{-2}, 3 MHz).

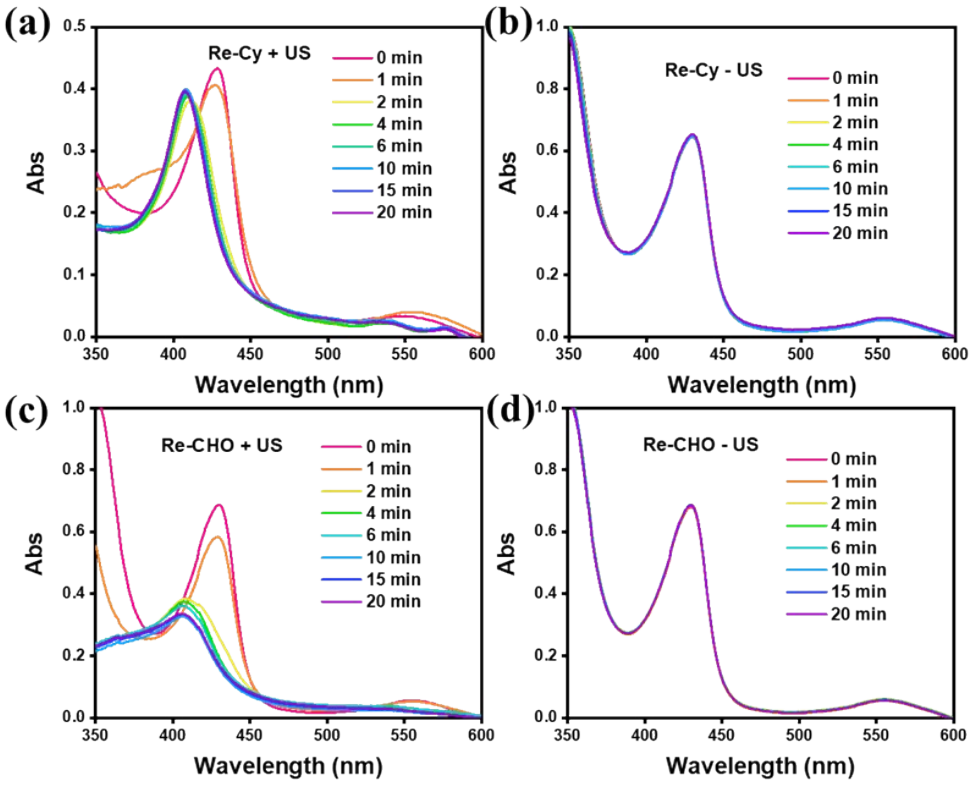


Figure S13. Time-dependent CO released from 1 μM **Re-Cy** or **Re-CHO** was detected by change in the UV-Vis spectra of Hb (4.2 μM) in presence or absence of US irradiation (0.3 W cm^{-2}, 3 MHz).

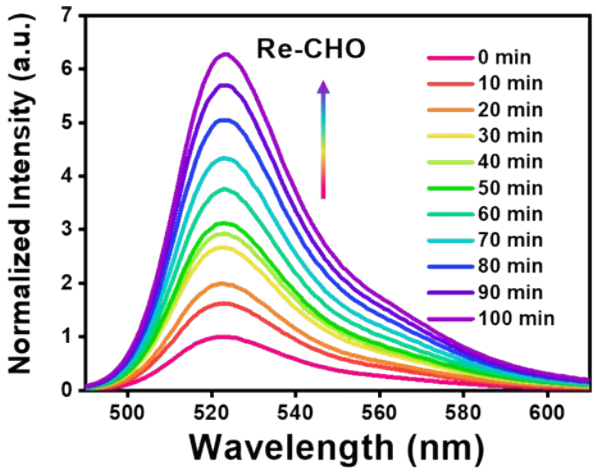


Figure S14. Time-dependent ROS generation by **Re-CHO** (10 μM) detected with the change in the fluorescence intensity of DCFH-DA under US irradiation (0.3 W cm^{-2}, 3 MHz).

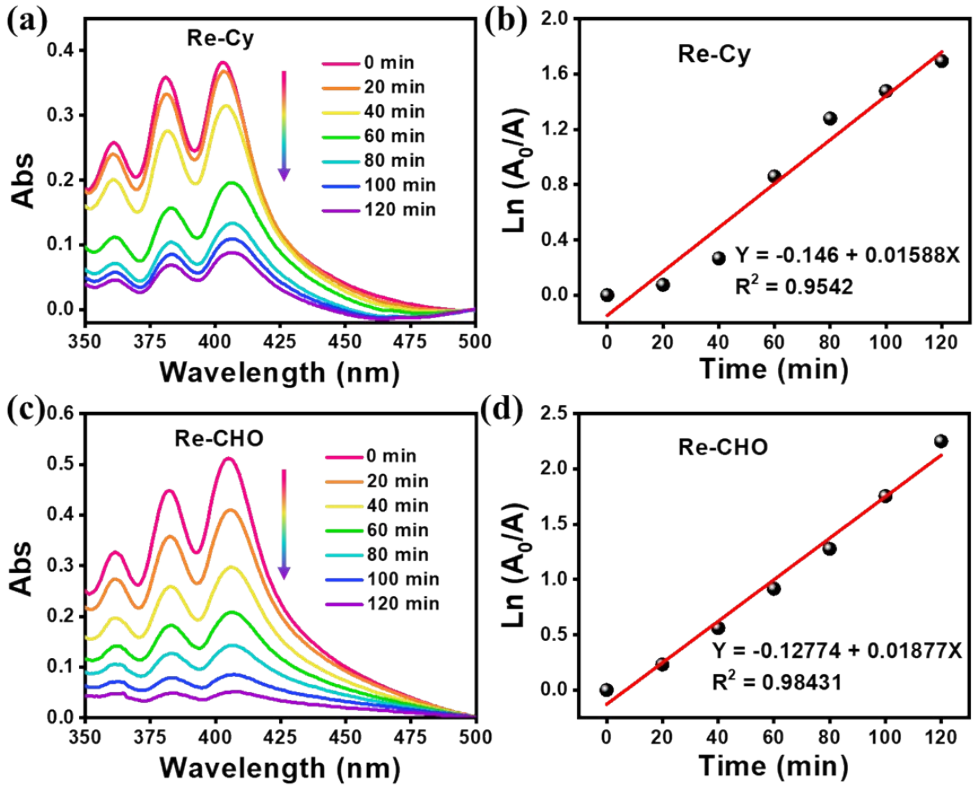


Figure S15. Time-dependent oxidation of DPA indicating $^1O_2$ generation by 10 μM **Re-Cy** (a), and **Re-CHO** (c) under US irradiation (0.3 W cm^{-2}, 3 MHz). Rate constant for DPA decomposition in the presence of **Re-Cy** (b), and **Re-CHO** (d) according to the absorbance of DPA at 378 nm in (a) and (c), respectively.

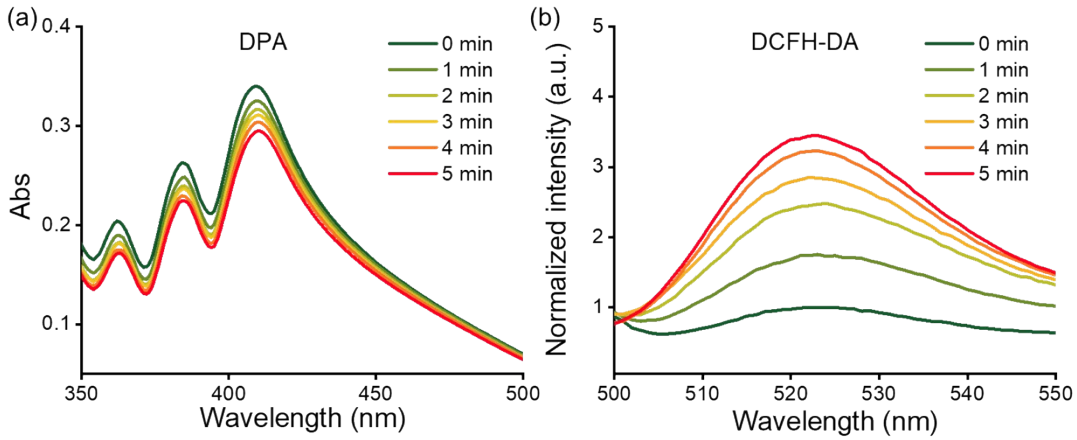


Figure S16. (a) Time-dependent ROS generation by 10 μM **Re-Cy** detected by monitoring the change in the fluorescence intensity of DCFH-DA under US irradiation (0.3 W cm^{-2}, 3 MHz). (b) Time-dependent oxidation of DPA indicating $^1O_2$ generation by 10 μM **Re-Cy** under US irradiation (0.3 W cm^{-2}, 3 MHz).

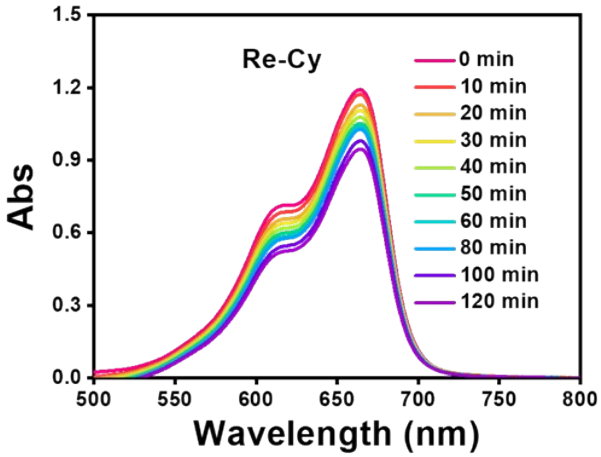


Figure S17. Time dependent insignificant degradation of MB on US irradiation (3.0 MHz, 0.3 W cm^{-2}) indicating that **Re-Cy** can hardly produce any •OH.

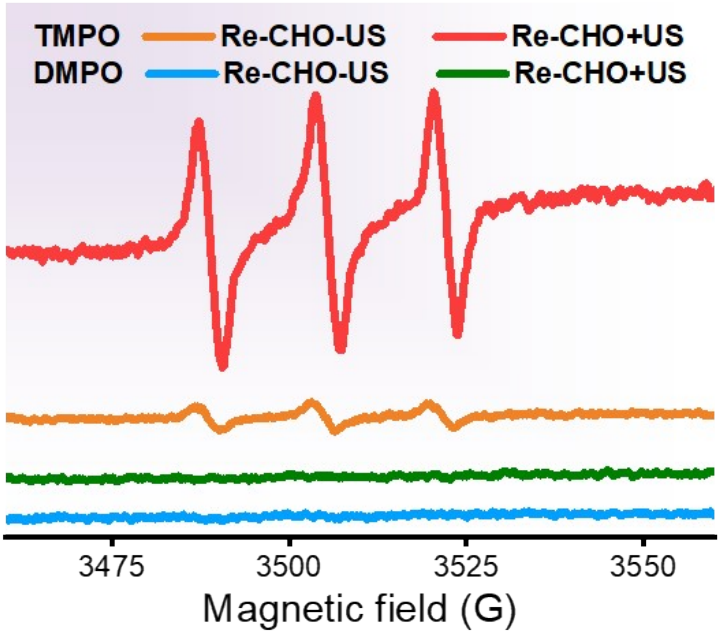


Figure S18. ROS generation by **Re-CHO** under US irradiation detected by ESR studies.

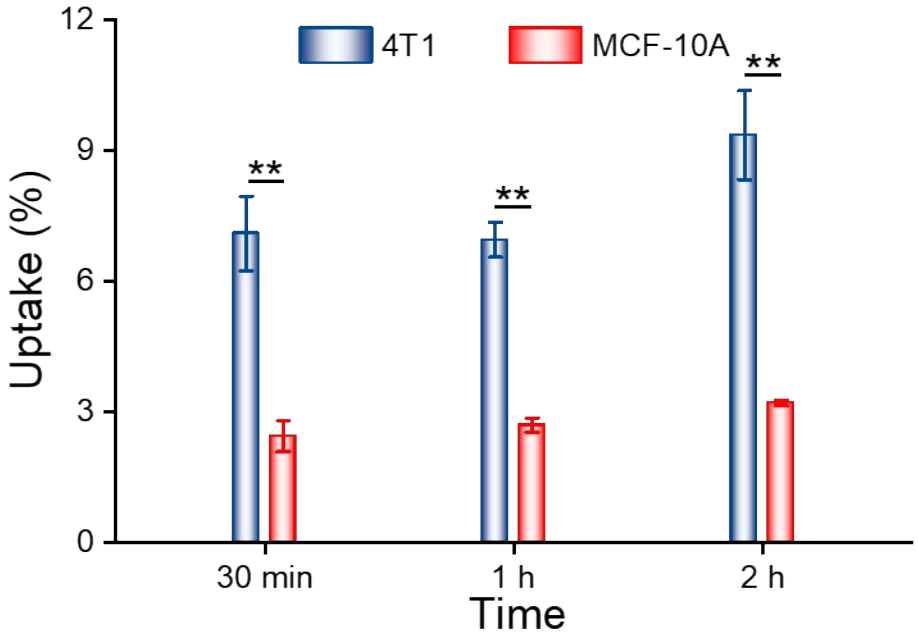


Figure S19. Cell uptake of **Re-Cy** at different incubation time points.

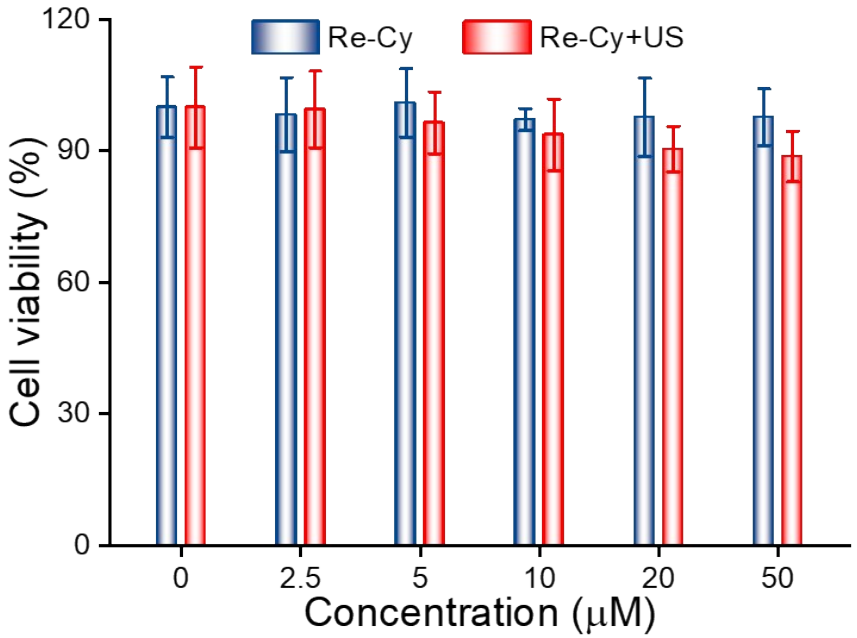


Figure S20. Cell viabilities of MCF-10A cells after incubating with different concentrations of **Re-Cy** in the presence or absence of US irradiation (3 MHz, 0.3 W/cm^2, 15 min).

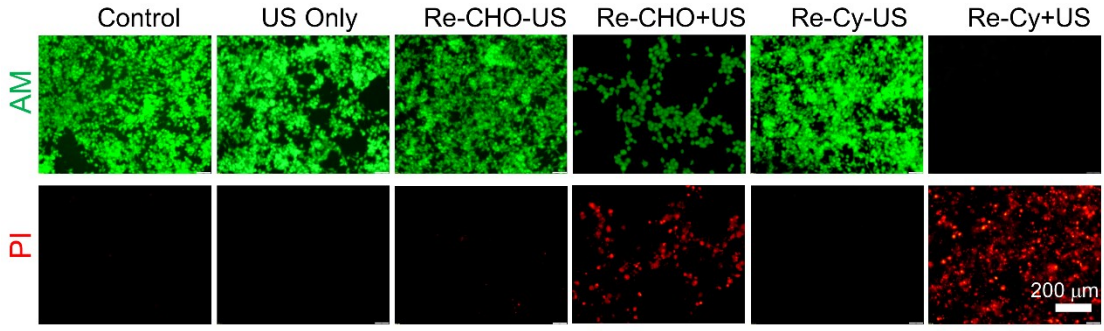


Figure S21. Confocal microscopy images of the living 4T1 cells treated with **Re-CHO** or **Re-Cy** (10 μM, 4 h) and co-stained with Calcein AM (green, live cells) (4 μM, 0.5 h) and propidium iodide (red, dead cells) (6 μM, 0.5 h) after different treatments. The Calcein AM was excited at 460 nm and the emission was collected at 540 ± 30 nm. The PI was excited at 540 nm and the emission was collected at 610 ± 30 nm Scale bar: 200 μm.

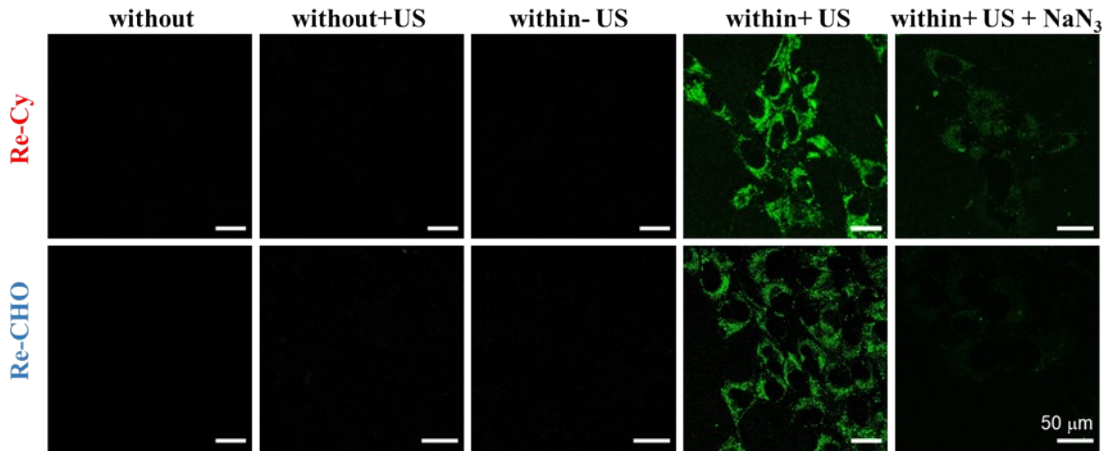


Figure S22. Confocal microscopy images of 4T1 cells treated with **Re-Cy** or **Re-CHO** (10 μM, 1 h) and co-stained with SOSG (2.5 μM, 30 min) in presence or absence of NaN_3 (5 mM, 1 h) under different conditions. The SOSG probe was excited at 488 nm and the emission was collected at 525 ± 30 nm. Scale bars: 50 μm.

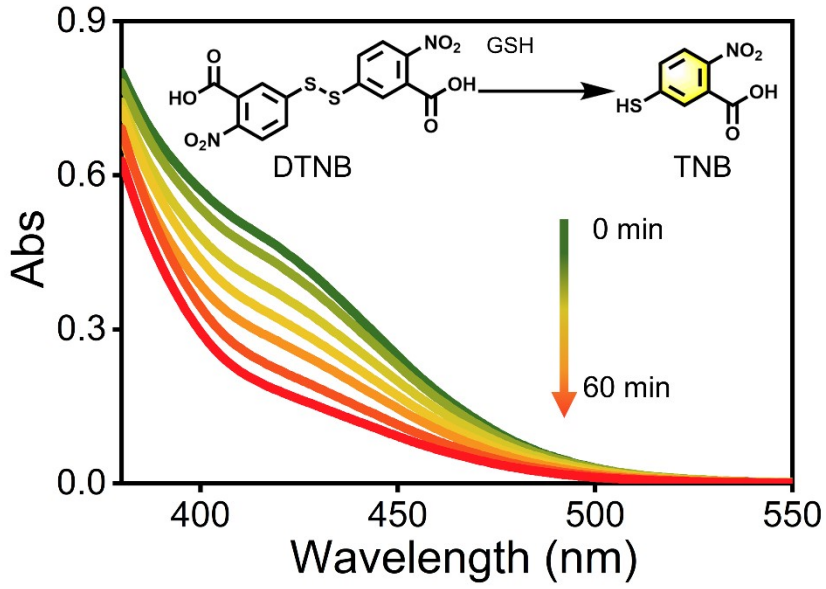


Figure S23. Time-dependent GSH depletion by **Re-Cy** upon US irradiation.

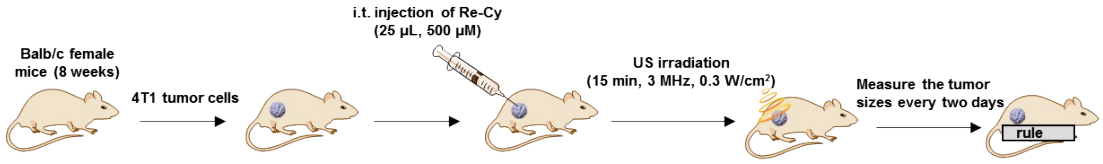


Figure S24. Schematic diagram of the *in-vivo* sonodynamic therapy.

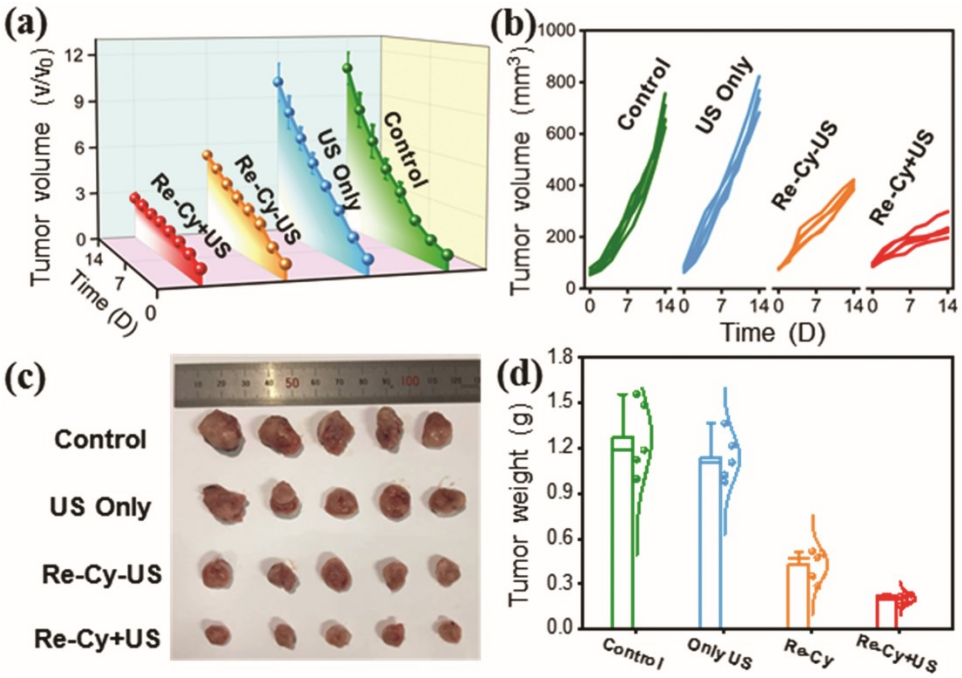


Figure S25. (a) The average and (b) individual tumor growth curves in mice after different treatments. (c) Photos of tumors from mice at day 14 after various treatments. (d) Average tumor weights at day 14 post various treatments.

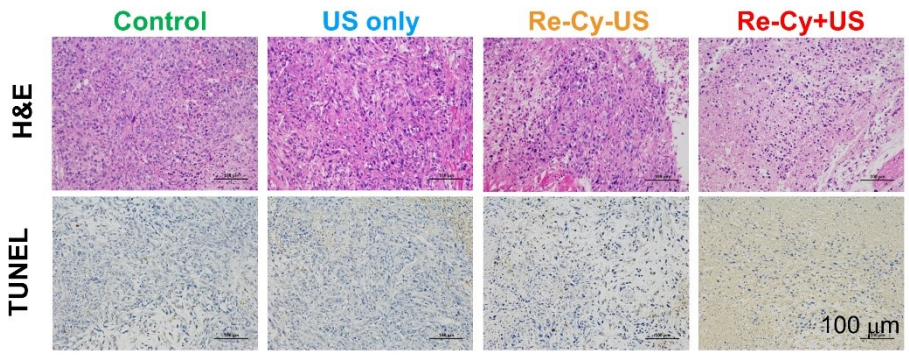


Figure S26. (a) Microscopy photos of H&E and TUNEL stained tumor slices. Tumor tissue were collected from mice at 24 h post various treatments.

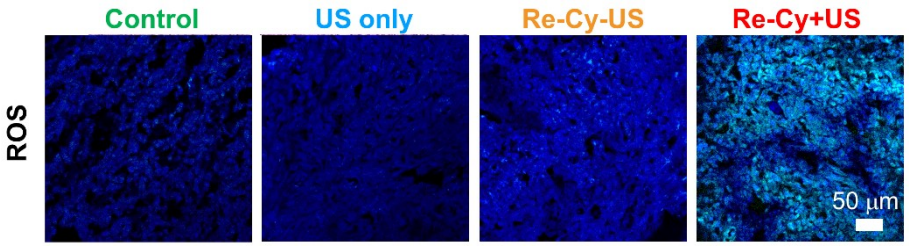


Figure S27. Fluorescence images of DCFH-DA and DAPI co-stained tumor slices collected from mice.