Supporting Information

In Situ Oxygen Generation by Low-Toxicity Ruthenium Electrocatalyst for Multimodal Radiotherapy Sensitization

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1 Material

1.1 Cell lines and reagents

Human cervical cancer cells (HeLa), cisplatin-resistant human cervical cancer cells (R-HeLa), human cervical immortalized squamous cells (Ect1/E6E7), and human renal tubular epithelial cells (HK-2) used in the experiments were purchased from ATCC; pyridine, nitric acid and hydrochloric acid were purchased from Guangzhou Chemical Reagent Factory; 30% acrylamide/methylene bis-acrylamide, 1.5M Tris-HCl (30% acrylamide/methacrylamide, 1.5M Tris-HCl(pH8.8), 1.0M Tris-HCl(pH6.8), TEMED, Tris Base, Tween 20(TW 20), PVP anti-fluorescence quenching sealing solution, 4% paraformaldehyde, trypsin, phosphate buffer solution (PBS) were purchased from Guangzhou Jiebes Biologicals; Ruthenium(III) chloride hydrate was purchased from Aladdin Reagent Co. DMEM medium was purchased from Thermo Fisher Scientific. Fetal bovine serum (FBS), tetrazolium blue (MTT), Ac-DEVD-AMC (caspase 3), dihydroethidium (DHE), hydroxyphenyl fluorescein (HPF), Ac-IETD-AFC (caspase 8), Ac-LEHD-AFC (caspase 9) were purchased from Jabil Biotech. Ac-IETD-AFC (caspase 8), Ac-LEHD-AFC (caspase 9) were purchased from Sigma; Whole Protein Extraction Kit and 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA) were bought from Beijing Solarbio Science & Technology Co., Ltd. Annexin V-FITC Apoptosis Detection Kit was purchased from Guangzhou Qiyun Biotechnology Co Ltd; saline was purchased from Beijing Wokai Biotechnology Co Ltd.

1.2 Instruments and equipment

The complexes were characterised by 1290LC-6545 QTOF MS high-resolution liquid-mass spectrometer, Cary5000 UV-visible spectrophotometer; Hitachi H-7650 transmission electron microscope (Hitachi, Japan); the cell photographs and absorbance measurements were carried out by ELX800/Cytation 5 multifunctional fluorescence enzyme labelling instrument (Bio-Tek, USA); cell collection was performed by CytoFLEXS flow cytometer, EVOS FL Auto fluorescence microscope. Tek, USA); cell acquisition was performed by CytoFLEXS flow cytometer and EVOS FL Auto fluorescence microscope.

2. Method

2.1 Synthesis of complexes

Ru (bbp)Cl_3： 2,6-Bis(2-benzimidazolyl)pyridine (0.311 g, 1 mmol) (bbp) and RuCl3-3H2O (0.2615 g, 1 mmol) were dissolved in 30 ml of 95% ethanol. The mixture was then stirred at 80 °C under nitrogen for 3 hours, collected and washed with ethanol and ether.

Ru(bbp)$(Py)_2Cl$： Ru(bbp) Cl_3 is dissolved in 10 ml of pyridine, stirred and refluxed overnight at 130 °C under nitrogen. Purification by column chromatography with methanol/dichloromethane. Yield:：40.1%. ^{1}H NMR (300 MHz, DMSO-*d*6) δ 14.96 (s, 1H), 8.63 (d, *J* = 7.5 Hz, 2H), 8.49 (d, *J* = 7.7 Hz, 2H), 8.03 (dd, *J* = 21.4, 6.7 Hz, 5H), 7.78 (d, *J* = 7.5 Hz, 2H), 7.52 (p, *J* = 7.1 Hz, 6H), 6.98 (t, *J* = 6.6 Hz, 4H)。

2.2 Cell culture and cell activity tests

HeLa, HK2, ECT/E6E7 cells were purchased from ATCC, and all cell lines were cultured in DMEM medium containing penicillin (100 U/mL), streptomycin (50 U/mL), and fetal bovine serum (10%) in a carbon dioxide incubator at 37 ℃. Hela, R-Hela, Ect1/E6E7 and HK-2 cells were inoculated into 96-well plates at a density of 1.5 × 10^5 cells/mL and cultured in complete medium for 24 hours. Cell viability was determined by MTT at 570 nm after treatment with Ru(bbp)(Py) $_2$Cl for 72 hours.

For the evaluation of the sensitization activity of radiotherapy, Hela cells at a density of 1.5 × 10^5 cells/ml were inoculated into 12-well plates overnight, and then incubated with Ru(bbp)(Py) $_2$Cl for 6 h. The cells were treated with different doses of X-rays, different voltages, and different times of electrostatic treatment. Sensitization was assessed by MTT assay.

2.3 Redox properties of Ru(bbp)$(Py)_2Cl$ determined by cyclic voltammetry

In this experiment, the redox properties of Ru(bbp)(Py) $_2$Cl were evaluated by cyclic voltammetry in acetonitrile using tetrabutylammonium chloride (TBAMC) as the electrolyte solution. A three-electrode system was used, with a saturated calomel electrode as the reference electrode, a glassy carbon electrode as the working electrode and a platinum electrode as the counter electrode.

2.4 Haemolysis experiment

Different concentrations of Ru(bbp)$(Py)_2Cl$ were incubated with human erythrocytes for different times. Erythrocytes treated with Triton-X were used as positive control. PBS-treated erythrocytes were used as the negative control. The morphology of the cultured RBCs was observed microscopically and the absorbance of the supernatant was measured at 540 nm to calculate the hemolysis rate.

$$Hemolysis\ rate(\%T) = \frac{A\ Sample - A\ Negtive\ control}{A\ Positiv\ control - A\ Negative\ control} * 100\%$$

2.5 Clone formation experiment

HeLa cells were inoculated in 6-well plates at a density of 2×10^3 cells/ml overnight, then treated with Ru(bbp)$(Py)_2Cl$ (2.5 and 5 μM) for 6 h. The cells were then treated with X-rays (2 Gy) and electrocatalysis (3 V for 3 min) and cultured in an incubator for a fortnight. The cells were washed with PBS and fixed with 4.0% paraformaldehyde for 20 min, then washed with PBS and snap-dried, stained with 0.5% crystal violet for 20 min and photographed. Evaluation of electrocatalytic sensitization radiotherapy using the number of tumor cell communities.

2.6 Measurement of reactive oxygen species (ROS) generation

HeLa cells were inoculated in 96-well plates at a density of 2×10^6 cells/ml overnight, treated with Ru(bbp)$(Py)_2Cl$ (2.5 and 5 μM) for 6 h, incubated with DCFH-DA/DHE (10 μM) for 30 min at 37 °C, and treated with X-rays (2 Gy) and electrocatalytic treatment (3 V, 3 min) before being processed with a fluorescence microplate reader (ELX800, Bio-Tek, USA, excitation and emission wavelengths set at 480 nm and 525 nm for DCFH-DA, 300 nm and 610 nm for DCFH-DA, respectively). The effect on intracellular ROS was assessed by fluorescence intensity using a fluorescence microplate reader (ELX800, Bio-Tek, USA, excitation and emission wavelengths were set at 480 nm and 525 nm for DCFH-DA, and 300 nm and 610 nm for DHE, respectively).

2.7 Morphological changes in the mitochondria

Mitotracker and DAPI staining were used to monitor morphological changes in mitochondria. HeLa cells were cultured at a density of 2×10^4 cells/mL in 2 cm dishes

overnight, and the cells were treated with Ru(bbp)(Py) $_2$Cl (2.5 and 5 μM), X-rays (4 Gy), and electrocatalytic treatment (2 V, 3 min) for different times before and after the labelling of mitochondria and nuclei with Mitotracker (100 nM) and Hoechst (1 mg/mL), respectively. Mitochondria and nuclei were labelled with Mitotracker (100 nM) and Hoechst (1 mg/mL), respectively, to observe the effects of different treatments on the mitochondria of Hela cells.

2.8 Cell cycle and apoptosis analysis

HeLa cells were pre-cultured in 6 cm dishes for 24 h. Cells were treated with Ru(bbp)(Py) $_2$Cl (2.5 and 5 μM), X-rays (2 Gy) and electrocatalytic treatment (3 V, 3 min) for 48 h. Cells and supernatants were collected, washed with PBS and fixed with pre-cooled 70% ethanol at -20 °C overnight. Cells were processed according to the instructions of the Cycle and Apoptosis Kit and analyzed by flow cytometry.

2.9 Caspase activity assay

HeLa cells (1×10^6 cells/ml) were pre-cultured in 10 cm dishes for 24 h. After treatment of the cells with ruthenium complexes (2.5 and 5 μM), X-rays (2 Gy), and electrocatalytic treatment (3 V for 3 min) for 48 h, the cells and the supernatant were collected, and 100 μL of lysates were added to collect proteins. Then 100 μg of collected proteins and 5 μL of enzyme substrates (Ac-DEVD-AMC for Caspase-3, Ac-IETD-AMC for Caspase-8, Ac-LEHD-AMC for Caspase-9) were added to each well and the solution was replenished to 200 μL with PBS. After incubation at 37 °C for 2 h, the Caspase activity of HeLa cells induced by the conjugate combined with radiotherapy was determined using a fluorescent microplate reader (ELX800, Bio-Tek, USA, excitation and emission wavelengths set at 380 nm and 460 nm, respectively).

2.10 Determination of in vivo anti-tumor capacity

This study was conducted in strict accordance with the National Guidelines for the Breeding and Use of Laboratory Animals and the Ethical Review Guidelines for Laboratory Animals and Animal Welfare (GB/T 35892-2018), and approved by the Ethics Committee for Animal Experimentation of Jinan University (Approval No.: IACUC-20190425-12; IACUC-20200918-04). Female C57BL/6J and Babl/C-nude were purchased from Beijing Viton Lihua Laboratory Animal Technology Co. Briefly,

B16/HeLa cells suspended in 100 μL of serum-free medium were injected subcutaneously into the right abdominal cavity of each mouse to form tumors. After the tumour volume was increased to approximately 35 cubic millimetres, the mice were randomly divided into eight groups. The first group was injected with 100 μl of saline as control; the second group was injected with 100 μL of ruthenium complex (2.5 mg/kg); the third group was electrocatalytic (3 V, 3 min); the fourth group was complex and electrocatalytic; the fifth group was X-ray (2 Gy); the sixth group was complex and X-ray; the seventh group was electrocatalytic and X-ray; and the eighth group was complex, electrocatalytic and X-ray. Group VIII consisted of co-ordination, electrocatalysis and X-rays. The body weight and tumor volume of the mice were measured every two days. At the end of the experiment, the heart, liver, spleen, lung, kidney and tumor of each group were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned. Tumour and organ sections were stained with H&E and observed with a light microscope.

2.11 Statistical analysis

In this study, the experiment was repeated at least three times and all data are expressed as mean ± standard deviation. Multiple group comparisons and statistical analyses were performed using the SPSS program version 25 (IBM Armonk NY) and one-way analysis of variance ANOVA.

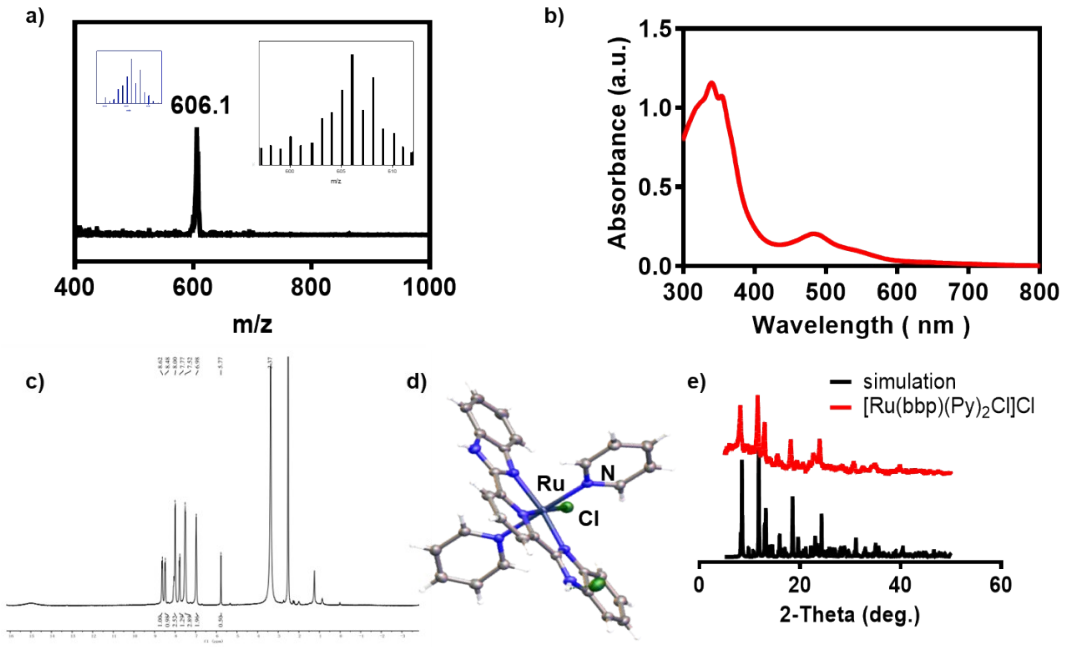

Figure S1 Characterization of Ru (bbp)(py)$_2$Cl). a) ESI-MS spectra of Ru(bbp(py)$_2$Cl; b) UV-Vis of Ru(bbp)(py)$_2$Cl in PBS; c) ^{1}H NMR spectra of the Ru(bbp)(py)$_2$Cl in DMSO-d6; d**)** Single crystal structure of Ru(bbp)(py)$_2$Cl; e**)** XRD and simulation curves of Ru(bbp)(py)$_2$Cl .

Table S1 Crystal data and structure refinement for Ru (bbp) (py)$_2$Cl.

Identification code	Ru (bbp) (py)$_2$Cl
Empirical formula	$C_{11}H_{10}NORuCl_{0.09}$
Formula weight	276.49
Temperature/K	149.99 (10)
Crystal system	monoclinic
Space group	$P2_1/c$
a/Å	10.79963 (14)
b/Å	13.41812 (14)
c/Å	18.7188 (3)
α/°	90
β/°	104.8431 (14)
γ/°	90
Volume/Å^3	2622.04 (6)
Z	11
ρ_{calc}g/cm^3	1.926
μ/mm^{-1}	13.232
F (000)	1502.0

Crystal size/mm^3	0.24 × 0.22 × 0.2
Radiation	Mo Kα (λ = 1.54184)
2Θ range for data collection/°	8.204 to 148.748
Index ranges	-13 ≤ h ≤ 9, -16 ≤ k ≤ 16, -15 ≤ l ≤ 23
Reflections collected	25277
Independent reflections	5237 [R_{int} = 0.0565, R_{sigma} = 0.0305]
Data/restraints/parameters	5237/0/352
Goodness-of-fit on F^2	1.035
Final R indexes [I>=2σ (I)]	R_1 = 0.0464, wR_2 = 0.1254
Final R indexes [all data]	R_1 = 0.0479, wR_2 = 0.1264
Largest diff. peak/hole / e Å^{-3}	1.30/-1.06

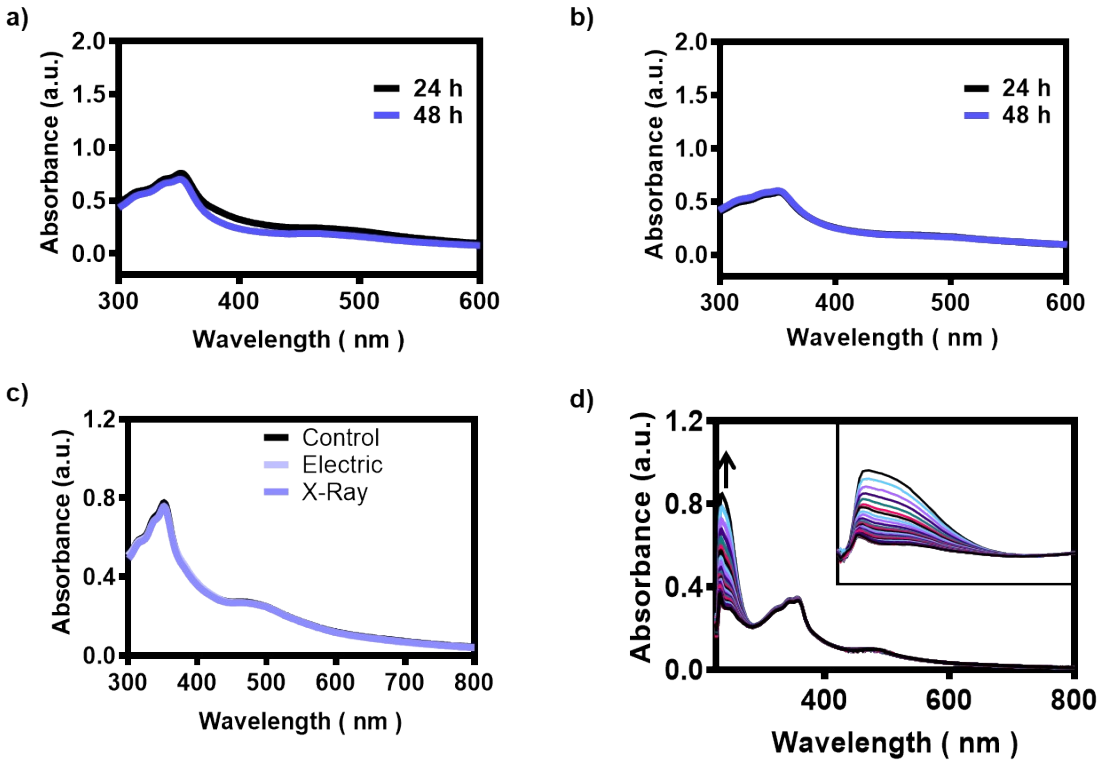

Figure S2 Stability testing of Ru(bbp)(py)$_2$Cl. a) UV spectra of Ru(bbp)(Py)$_2$Cl (10 μM) in PBS solution at different time points; b) UV spectra of Ru(bbp)(Py)$_2$Cl (10 μM) in DMEM solution at different time points; c) UV-visible spectral changes of Ru(bbp)(Py)$_2$Cl (10 μM) in DMEM after treatment with X-ray (2 Gy) and Electiec (3 V, 3 min); d) UV absorption spectra of Ru(bbp)(Py)$_2$Cl (10 μM) in aqueous solution treated electrically (3 V) at different time points.

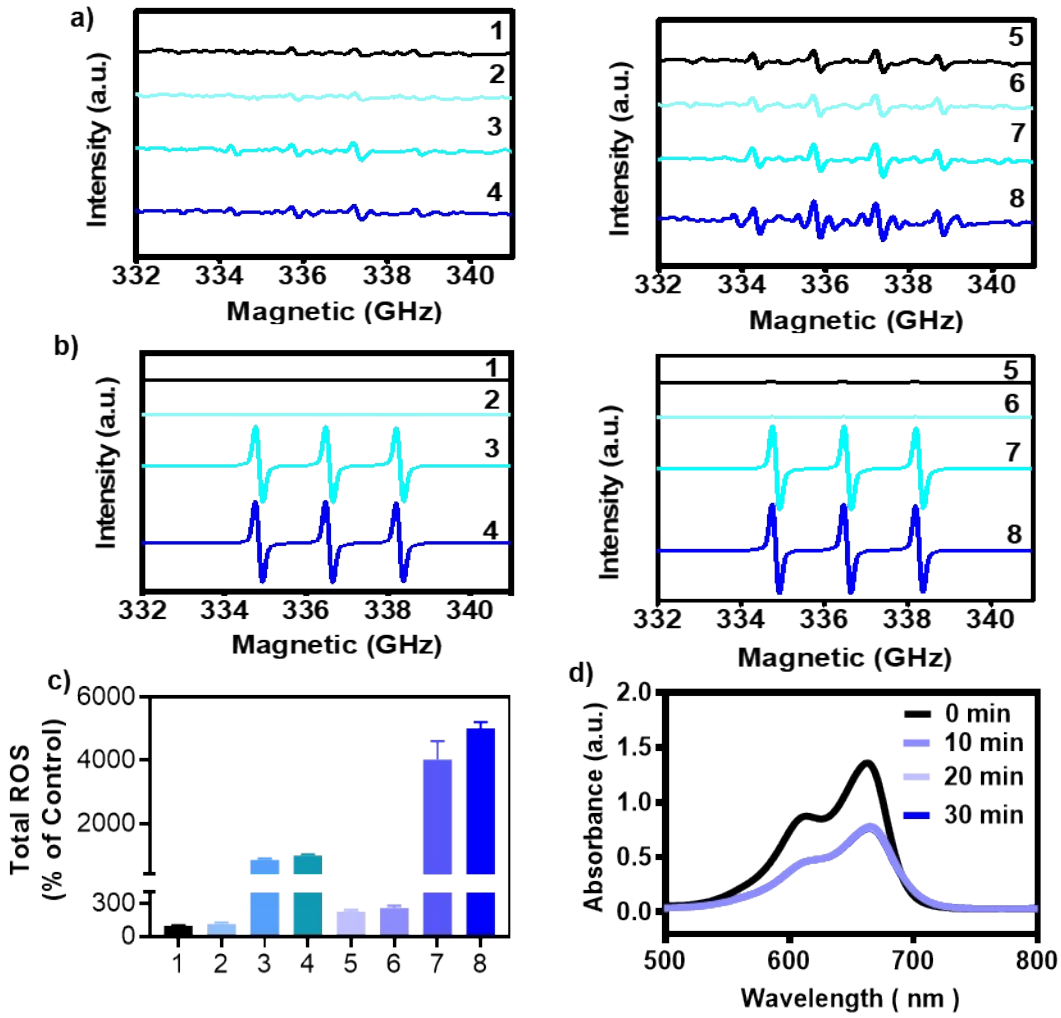

Figure S3 Ru(bbp)(py)$_2$Cl electrocatalytic oxygen production. a) EPR assay DMPO free radical capture reagent captures free radical production from different treatment groups; b) EPR assay TEMP free radical capture reagent captures free radical production in different treatment groups; c) Total reactive oxygen species produced by treatments of different treatment groups; d) Effect of different time points of the final treatment group treatments on the ultraviolet absorption peak of methylene blue. (1:Control; 2:Ru(bbp)(Py)$_2$Cl; 3:Electrocatalytic; 4:Electrocatalytic + Ru(bbp)(Py)$_2$Cl; 5:X-Ray; 6:X-Ray + Ru(bbp)(Py)$_2$Cl; 7:X-Ray + Electrocatalytic; 8: X-Ray + Electrocatalytic + Ru(bbp)(Py)$_2$Cl)

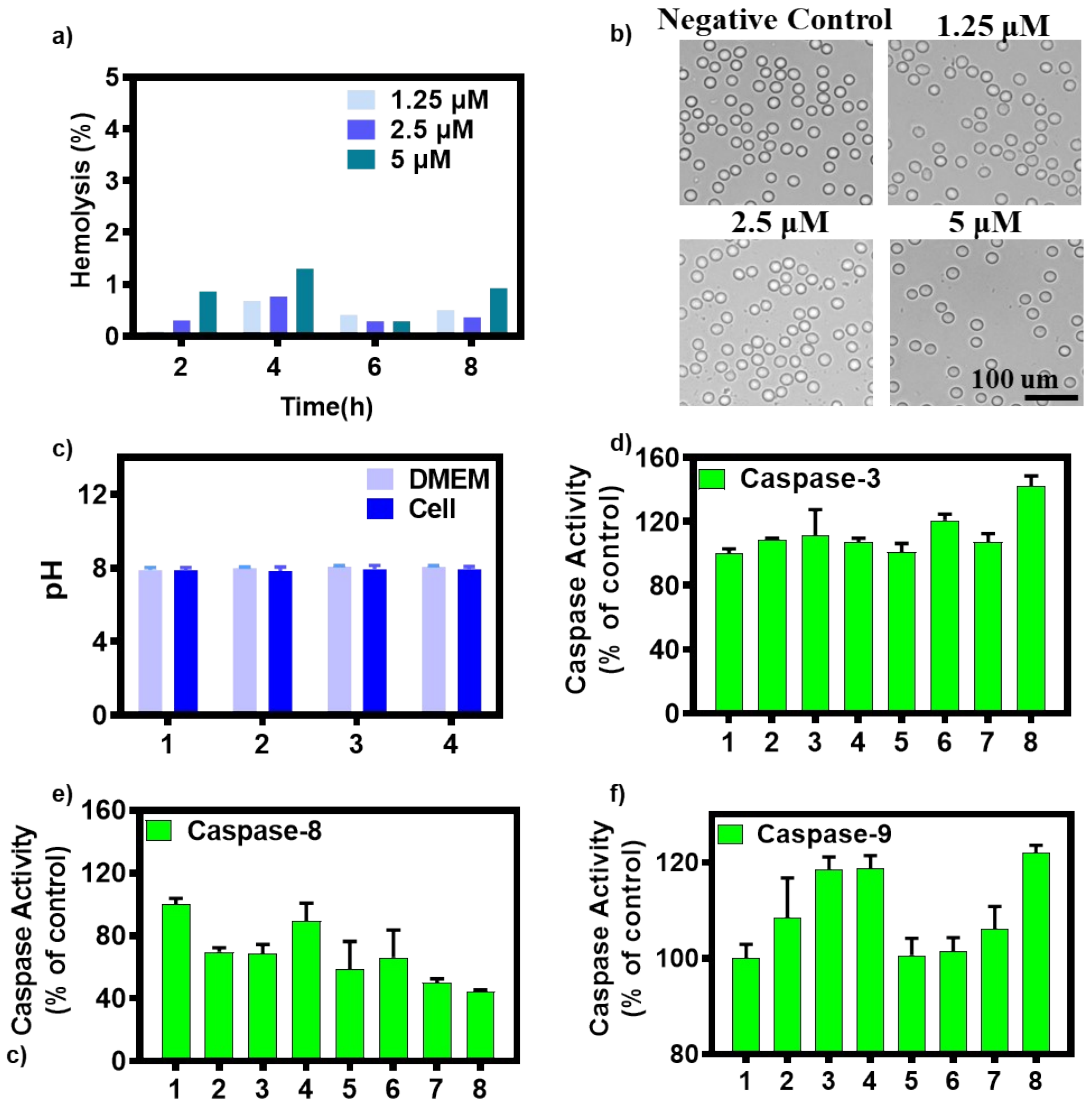

Figure S4 Ru(bbp)(py)$_2$Cl biosafety and Caspase testing. a) Hemolysis of Ru(bbp)(Py)$_2$Cl at different time points of incubation with erythrocytes; b) Pictures of erythrocytes after treatment with Ru(bbp)(Py)$_2$Cl; c) Effect of different treatments on the pH of the cells and the culture medium. d) Effect of different treatments on Caspase 3 in HeLa cells; e) Effect of different treatments on Caspase 8 in HeLa cells; f) Effect of different treatments on Caspase 9 in HeLa cells. (1:Control; 2:Ru(bbp)(Py)$_2$Cl; 3:Electrocatalytic; 4:Electrocatalytic + Ru(bbp)(Py)$_2$Cl; 5:X-Ray; 6:X-Ray + Ru(bbp)(Py)$_2$Cl; 7:X-Ray + Electrocatalytic; 8: X-Ray + Electrocatalytic + Ru(bbp)(Py)$_2$Cl).

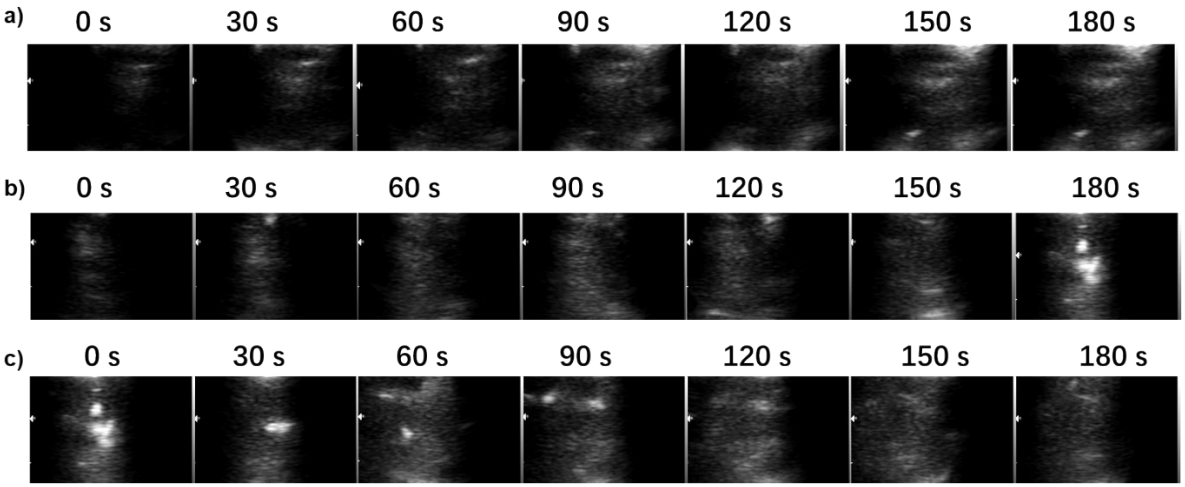

Figure S5 Measurement of electrode spacing. a) Oxygen signals from ultrasound at different times using ultrasound in situ with electrodes spaced 0.5 cm apart; b) Oxygen signals from ultrasound at different times using ultrasound in situ with electrodes spaced 1.0 cm apart; c) Oxygen signals from ultrasound at different time points after 3 minutes of ultrasound at 1.0 cm intervals using B-ultrasound in situ detection electrodes.

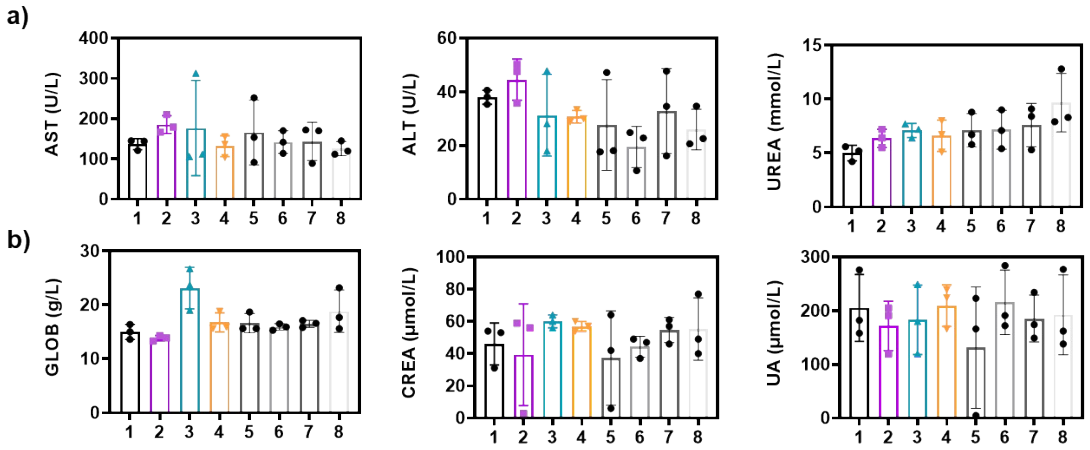

Figure S6 Measurement of liver and kidney indicators in mice. a) Hematologic analysis of liver function indices in different treatment groups after 14 days of treatment; b) Hematologic analysis of renal function indices in different treatment groups after 14 days of treatment. (1: Control; 2:Ru(bbp)$(Py)_2Cl$; 3:Electrocatalytic; 4:Electrocatalytic + Ru(bbp)$(Py)_2Cl$; 5:X-Ray; 6:X-Ray + Ru(bbp)$(Py)_2Cl$; 7:X-Ray + Electrocatalytic; 8:X-Ray+Electrocatalytic+Ru(bbp)$(Py)_2Cl$)

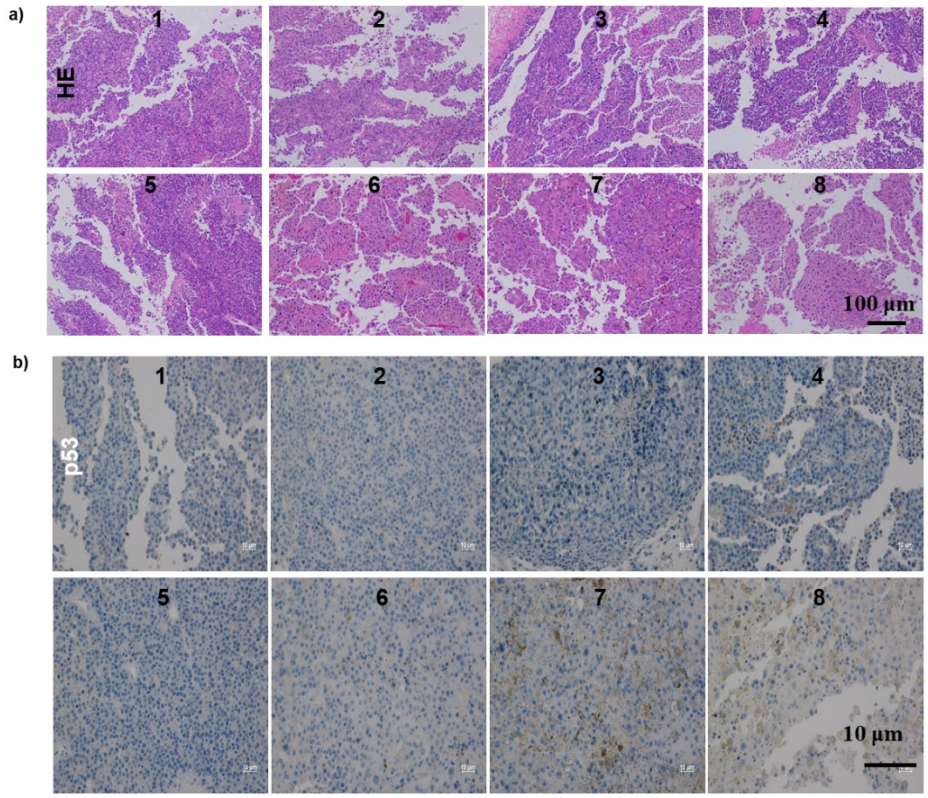

Figure S7 HE and p53 expression sections of mouse tumors. a**)** HE staining in tumor tissue after 14 days of in situ electrocatalytic oxygen-sensitizing radiotherapy treatment; b**)** Tumor p53 in tumor tissue after 14 days of *in situ* electrocatalytic oxygen-sensitizing radiotherapy treatment. (1: Control; 2:Ru(bbp)$(Py)_2Cl$; 3:Electrocatalytic; 4:Electrocatalytic + Ru(bbp)$(Py)_2Cl$; 5:X-Ray; 6:X-Ray + Ru(bbp)$(Py)_2Cl$; 7:X-Ray + Electrocatalytic; 8: X-Ray + Electrocatalytic + Ru(bbp)$(Py)_2Cl$).