

Supporting Information

An Ir-Ho Bimetallic Complex-Mediated Low-dose Radiotherapy/Radiodynamic Therapy *In Vivo*

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Methods

Materials. 2-phenylquionline (pq), iridium trichloride hydrate and holmium (III) acetate were purchased from J&K Scientific. The other reagents were obtained from TiTan. NaN₃ and 9,10-Dimethylantracene (DMA) were bought from Energy Chemical. SOSG was bought from Thermo Fisher. [(pq)₂Ir(H₂dcbpy)]Cl (Irpq) and [Ir₄Ho₂(pq)₈(dcbpy)₄(OAc)₂] (Ir₄Ho₂) were synthesized according to the previous literature^{1,2}.

Characterization. PXRD was measured on an ADVANCE X-ray powder diffractometer (Bruker D8). TEM was measured on a JEOL JEM-2100 TEM. X-ray excited optical luminescence (XEOL) was measured on FLS980 system. A mini-X-ray tube (Amptek Inc.) was used as the X-ray source for experiments. The phosphorescence lifetime was performed on an Edinburgh FLS980 system excited by a 358 nm laser. The quantum yield was measured by Hamamatsu (C13534). The measurement of the hydrodynamic diameter and surface potential of nanoparticles were carried out on Malvern Zetasizer Nano ZS. Flow cytometry (Beckman CytoFLEX FCM, USA) was used to carry out related experiments. X-RAD 225 (Precision.) was used as the X-ray source for experiments in living cells and mice model.

Measurement of the X-ray excited optical luminescence (XEOL) Irpq, and Ir₄Ho₂ were put in 1 mL centrifuge tube, and then irradiated by X-ray (0.05 Gy/h). The luminescence spectra were measured on Edinburgh FLS980 system.

Preparation of Ir₄Ho₂@liposome NPs. DPPC (21 mg, 0.029 mmol), cholesterol (4 mg, 0.010 mmol), and DSPE-mPEG_{2k} (5 mg, 0.0025 mmol) with a molar ratio of 6: 4: 0.5 was dissolved in chloroform and then evaporated to form the lipid membrane. Afterwards, the prepared lipid membrane was hydrated with Ir₄Ho₂ solution (2.5 mg, 0.625 mol) at 45 °C under stirring for 30 min, and then crush with a cell pulverizer for 30 min at 30% power followed by passing through a 450 nm filter twice and a 200 nm filter three times.

The detection of ·OH in an aqueous solution of Ir₄Ho₂ and Ir₄Ho₂@liposome NPs
The quartz dish containing 3 mL solution of MB (0.25 mg/L) and Ir₄Ho₂ (1 mg/mL

based on Ir), Irpq (0.8 mg/mL based on Ir) and H₂O was irradiated under X-ray (0.25 Gy), respectively. The intensity of absorption of MB at 664 nm was measured.

The quartz dish containing MB concentration as above and Ir₄Ho₂@liposome (100 µg/mL based on Ir) was irradiated under X-ray (0, 2, 4, 6, and 8 Gy, respectively). The intensity of absorption of MB at 664 nm was measured.

The detection of ¹O₂ in an aqueous solution of Ir₄Ho₂ and Ir₄Ho₂@liposome NPs

The ¹O₂ generation of Ir₄Ho₂ (1 mg/mL) was monitored by detecting the change of fluorescence intensity of DMA excited by 360 nm. For control, Irpq and NaN₃ inhibition group were analyzed. The solution was irradiated by X-ray (0.25 Gy, 30 min), the fluorescence intensity was measured on Edinburgh FLS980 system. ABDA was used to detect the ¹O₂ generation of Ir₄Ho₂@liposome NPs (100 µg/mL), and the solvent was irradiated by X-ray (0, 2, 4, 6, and 8 Gy, respectively). The same operation was done in the control group and NaN₃ inhibition group. The fluorescence intensity was measured on Edinburgh FS5 system.

The measurement of the characteristic X-ray energy spectrum of Ir₄Ho₂.

Holmium (III) acetate, Irpq and Ir₄Ho₂ mixed with ^{99m}Tc (approximately 0.025 µCi/µL, 1 µL) in 1 mL centrifuge tube were measured by a high-purity gamma-ray spectrometer for testing. For control, the characteristic X-ray of ^{99m}Tc was also measured.

MTT assay. After 4T1 and HUVE cells were incubated with Ir₄Ho₂@liposome NPs with different concentrations for 12 and 24 h, respectively, the cytotoxicity was evaluated by the standard MTT assay. After 4T1 cells were incubated with Ir₄Ho₂@liposome NPs (0, 5, 10, 20, 50, and 100 µg/mL, respectively) for 24 h, and then washed, cells were irradiated with X-ray (2 Gy). The cell viability was measured by the standard MTT assay after another incubation for 48 h.

The uptake of Ir₄Ho₂@liposome NPs. After 4T1 cells were incubated with Ir₄Ho₂@liposome NPs with different concentrations for 4 h, and then washed, the fluorescence intensity was collected by a flow cytometer (Beckman CytoFLEX FCM, USA) excited by 365 nm. The obtained data is processed by FlowJo V10.4 software.

In vitro clonogenic assay. After the incubation with Ir₄Ho₂@liposome NPs (20 µg/mL) for 4 h, 4T1 cells were washed and irradiated with different doses of X-ray (0, 2, 4, 6, and 8 Gy, respectively). Afterwards, the cells were cultured for 7-10 days until visible colonies were formed. After cells were washed with PBS, 4T1 cells were fixed with 4% paraformaldehyde, washed, and stained with crystal violet dye. Finally, the colonies were counted. The colony data were fitted by the following equation: $SF = \exp(-\alpha D - \beta D^2)$, which were processed using matlab R2018a software.

¹O₂ assessment in cancer cells. After 4T1 cells were cultured for 12 h, 1 mL of SOSG (20 µM) was added to each well for 4 h, then cells were irradiated by X-ray (2 Gy) and incubated for further 2 h. Finally, the fluorescence was collected on a flow cytometer (Beckman CytoFLEX FCM) excited by 365 nm. The experimental data were processed using FlowJo V10.4 software. The ¹O₂ generation in 4T1 cells was also detected on the confocal laser scanning microscopy with a similar procedure. The quantitative analysis was performed by image J.

In vitro ·OH generation and DNA double-strand breaks with γ-H2AX assay. After 4T1 cells were incubated with Ir₄Ho₂@liposome NPs (20 µM) for 4 h and followed by an X-ray irradiation (2 Gy), cells were stained immediately with the γ-H2AX for confocal laser scanning microscopy. The quantitative analysis was performed by image J.

In vivo antitumor therapy of radiotherapy. Animal care and handling procedures have been approved by the guidelines of the regional animal experiment ethics committee. All mouse experiments conducted were approved by Shanghai SLAC Laboratory Animal Co., Ltd. The BALB/c mice bearing 4T1 tumor with the tumor volume of ~70 mm³, mice were divided into 4 groups: (i) the control group, (ii) Ir₄Ho₂@liposome group, (iii) X-ray group, and (iv) X-ray + Ir₄Ho₂@liposome group. Mice in group (ii) and (iv) were intratumorally injected with Ir₄Ho₂@liposome NPs (12 mg/kg). Group (iii) and (iv) were irradiated by X-ray (6 Gy). The in vivo antitumor effect was determined by tumor volume. We recorded the body weight and tumor

size every day. After the radiotherapy, the tumor was excised from the mice for the further H&E, TUNEL and HO-1 staining.

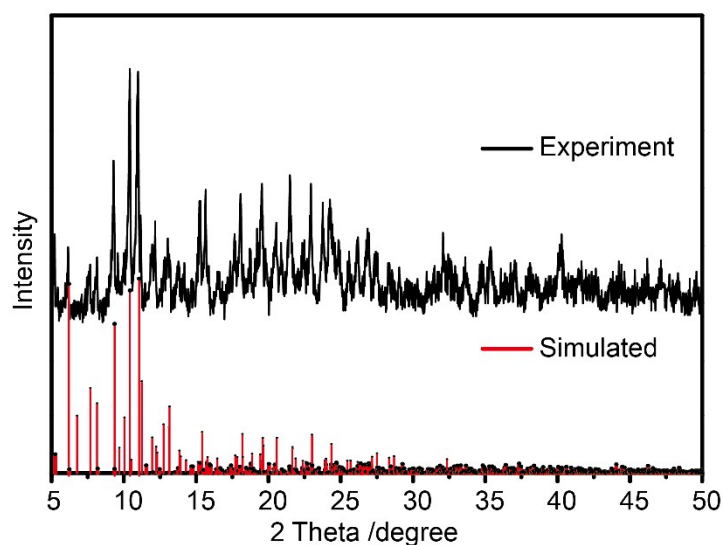


Figure S1. The simulated and experimental PXRD patterns for $[\text{Ir}_4\text{Ho}_2(\text{pq})_8(\text{H}_2\text{dcbpy})_4(\text{OAc})_2]$ (Ir_4Ho_2).

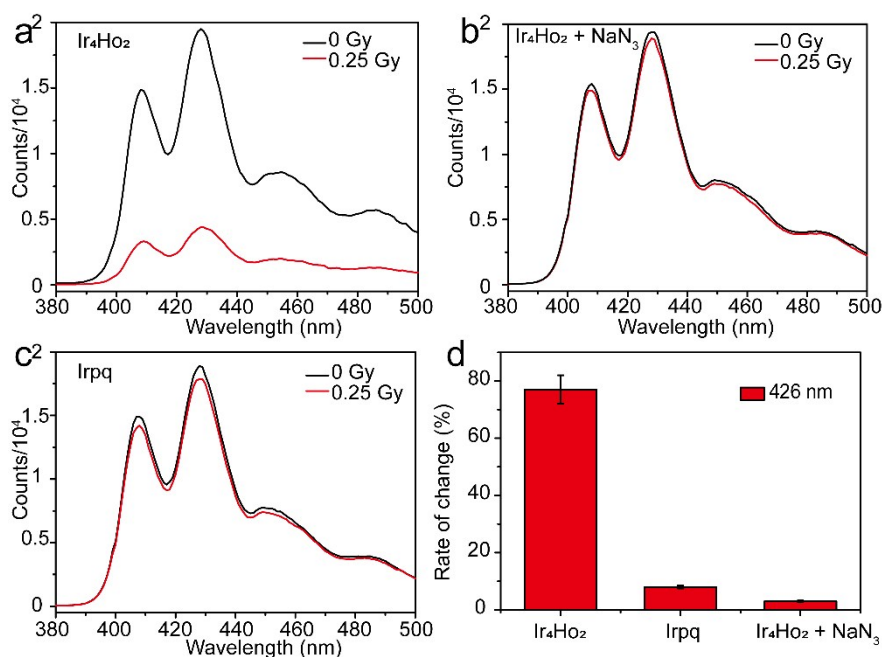


Figure S2. The fluorescence spectra of DMA in the presence of (a) Ir_4Ho_2 (1 mM) , (b) Irpq (1 mM) and (c) Ir_4Ho_2 (1 mM)+ NaN_3 irradiated by X-ray for 30 min, (d) the relative fluorescence intensity of DMA in the presence of Ir_4Ho_2 (1 mM) with NaN_3 irradiated by X-ray for 30 min.

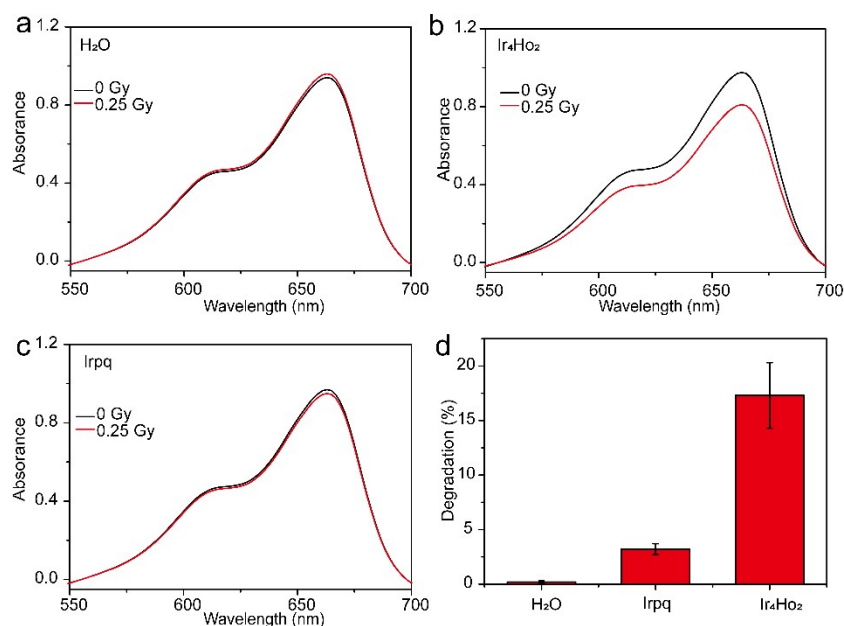


Figure S3. The changes of the absorbance spectra of methylene blue in the presence of (a) H₂O, (b) Ir₄Ho₂ (1 mM) and (c) Irpq (1 mM) irradiated by X-ray (50kV, 70 μA) for 30 min. (d) The degradation of methylene blue in the presence of H₂O, Ir₄Ho₂ (1 mM) and Irpq (1 mM).

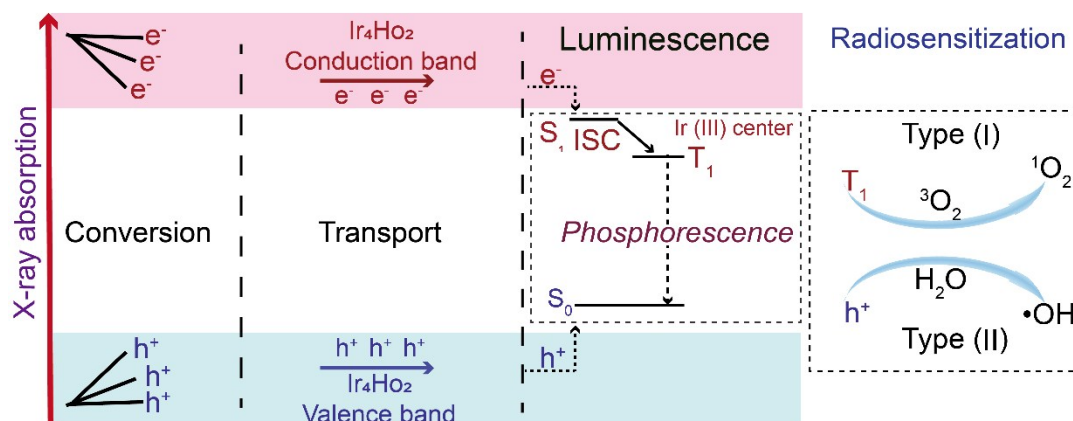


Figure S4. Schematic of the proposed mechanism of Ir₄Ho₂ upon X-ray irradiation

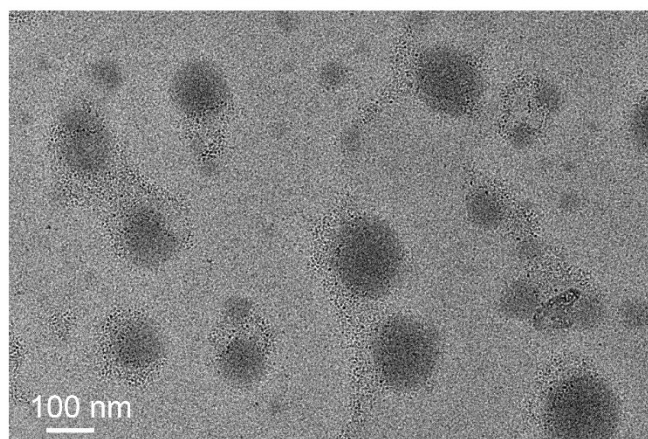


Figure S5. A typical TEM image of Ir₄Ho₂@liposome NPs.

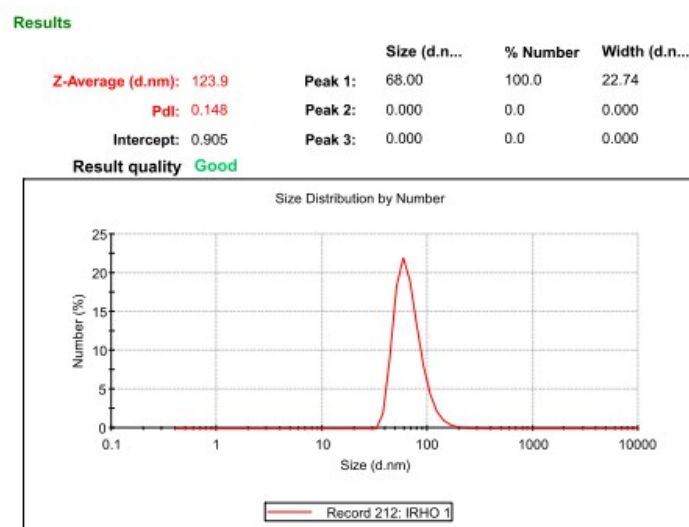


Figure S6. The hydrodynamic diameter of Ir₄Ho₂@liposome NPs

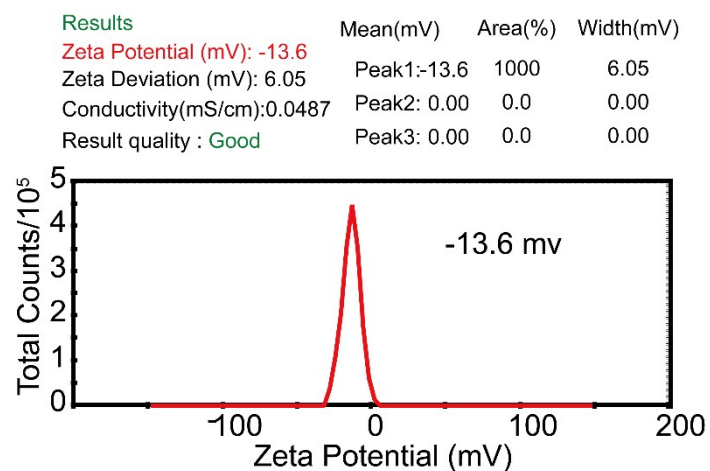


Figure S7. The stability of Ir₄Ho₂@liposome NPs in H₂O, PBS (7.4), and normal saline for 7 days.

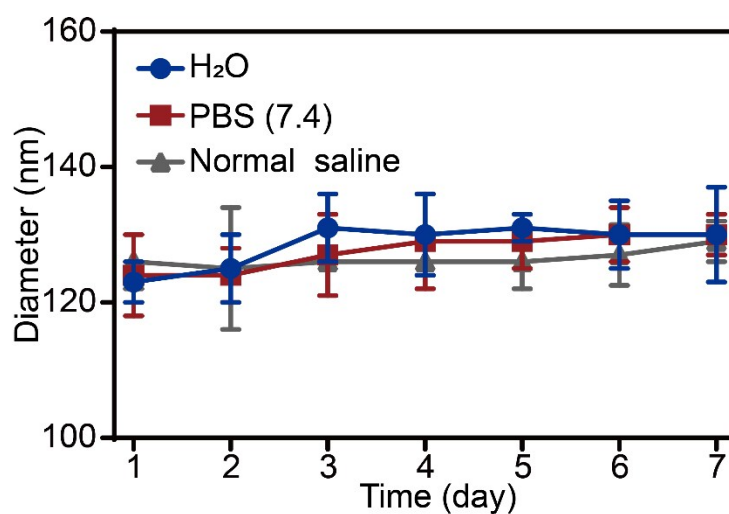


Figure S8. The zeta potential of Ir₄Ho₂ @liposome NPs

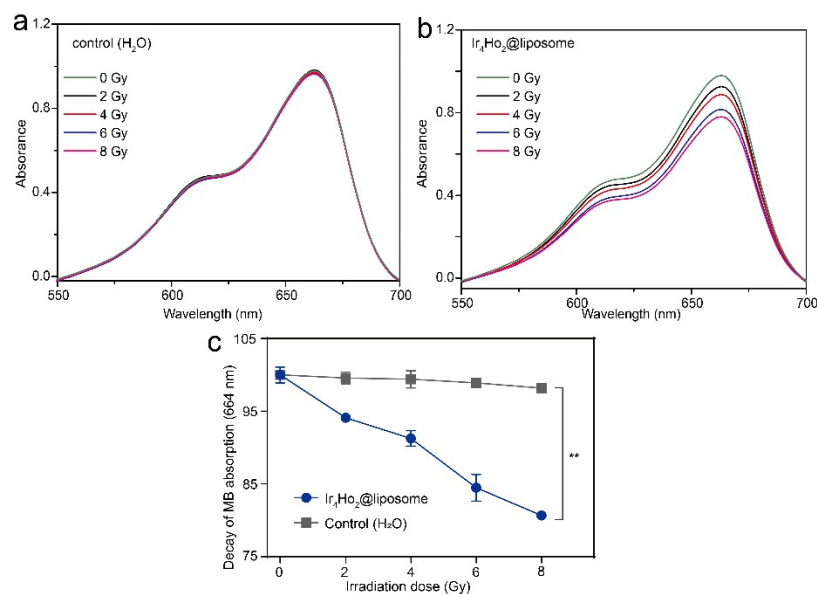


Figure S9. The changes of the absorbance spectra of methylene blue in the presence of H₂O (a), Ir₄Ho₂ @liposome NPs (100 $\mu\text{g mL}^{-1}$ based on Ir) (b) irradiated by X-ray. (c) The decay in absorption of MB located at 664 nm in the presence of Ir₄Ho₂@liposome NPs (100 $\mu\text{g mL}^{-1}$) and H₂O under the same conditions. (*p < 0.05, **p < 0.001, ***p < 0.0001).

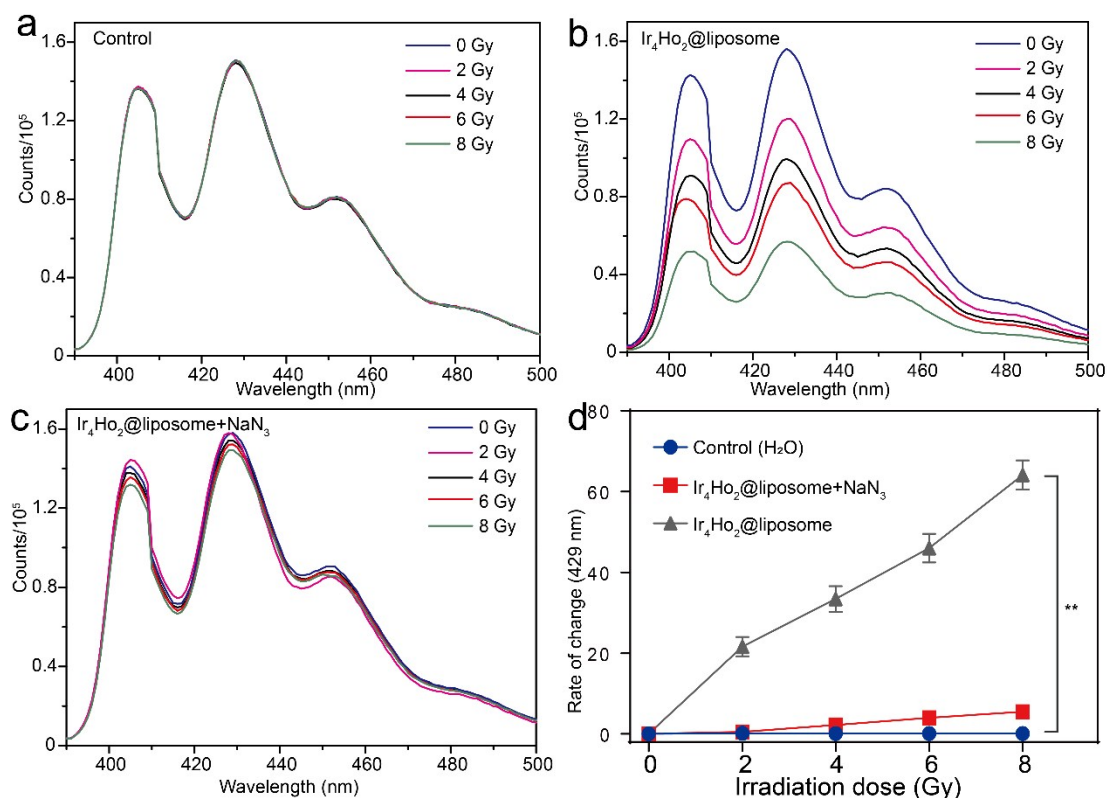


Figure S10. The changes of fluorescence spectra of ABDA in the presence of H₂O (a), Ir₄Ho₂ @liposome NPs (b), and Ir₄Ho₂ @liposome NPs+NaN₃ (c) irradiated by X-ray. The concentration was 100 $\mu\text{g mL}^{-1}$ based on Ir. (d) The change in the fluorescence of ABDA centered at 429 nm in the presence of Ir₄Ho₂@liposome NPs

(100 $\mu\text{g mL}^{-1}$), Ir_4Ho_2 @liposome NPs (100 $\mu\text{g mL}^{-1}$) + NaN_3 , and H_2O under the same conditions. (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

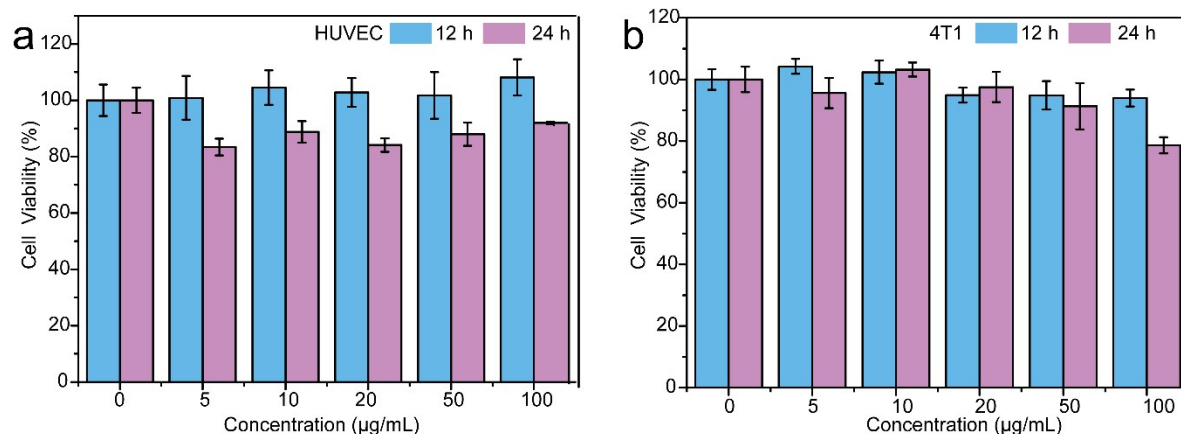


Figure S11. The viability of (a) HUVE and (b) 4T1 cells incubated with Ir_4Ho_2 @liposome NPs for 12 and 24 h, respectively.

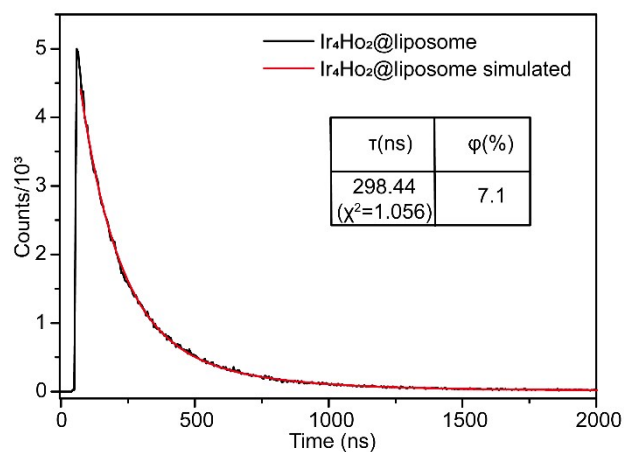


Figure S12. The fluorescence lifetime of Ir_4Ho_2 @liposome NPs excited by 358 nm laser.

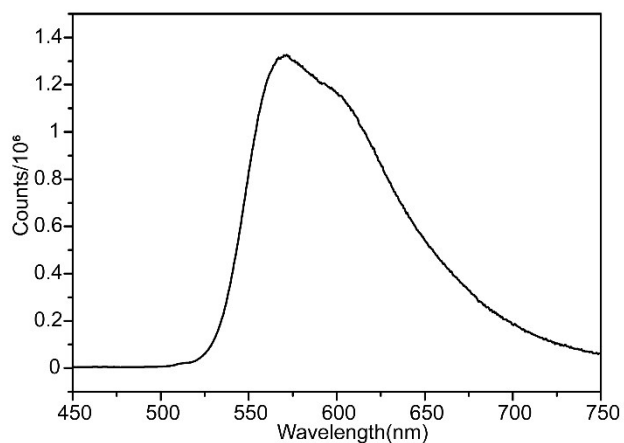


Figure S13. The emission spectrum of Ir_4Ho_2 @liposome NPs excited by 358 nm.

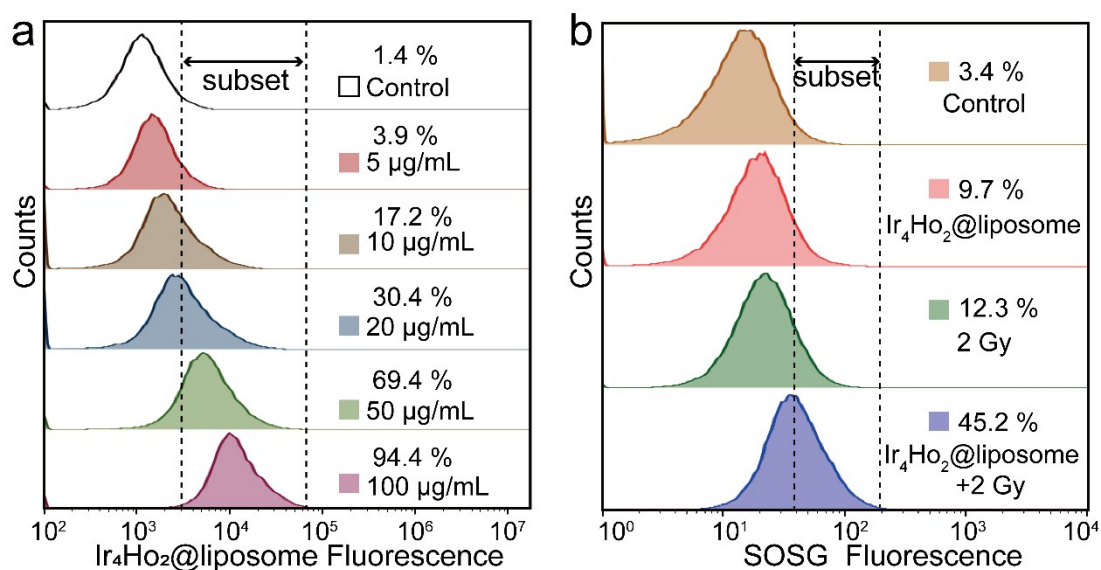


Figure S14. (a) The cellular uptake of Ir_4Ho_2 @liposome NPs measured by flow cytometry after 4T1 cells were incubated with 0, 5, 10, 20, 50, and 100 $\mu\text{g mL}^{-1}$ Ir_4Ho_2 @liposome NPs for 4 h. The detection of singlet oxygen generated by Ir_4Ho_2 @liposome NPs by flow cytometry (b)

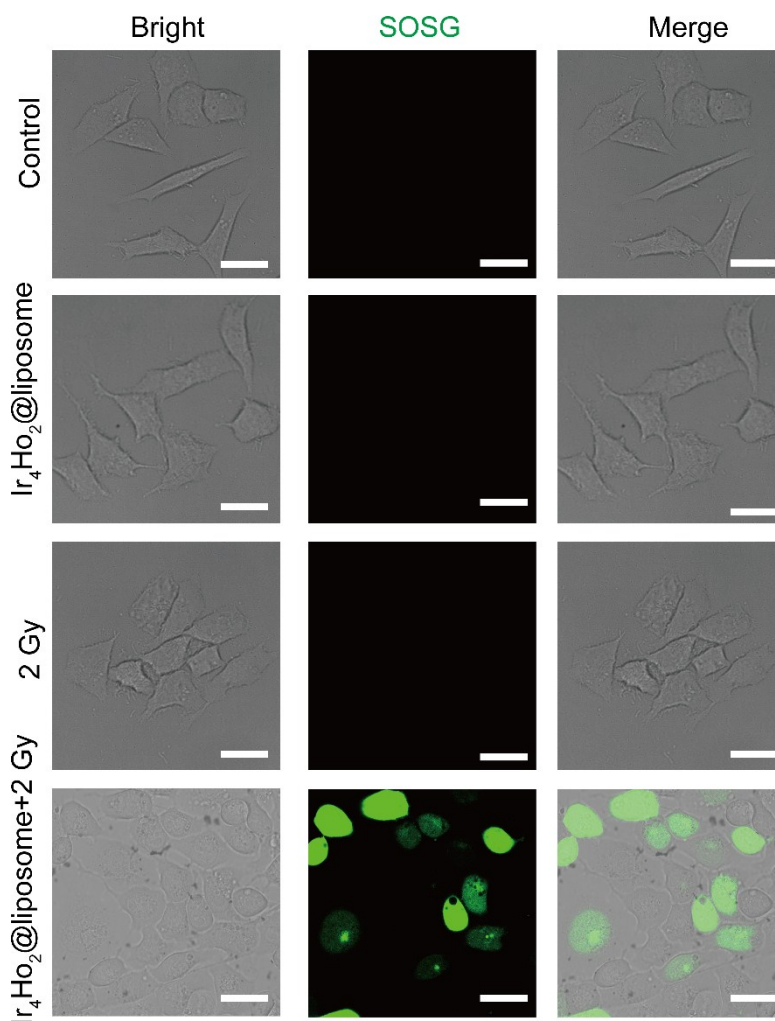


Figure S15. The confocal fluorescence imaging of 4T1 cells stained by SOSG after different treatments.

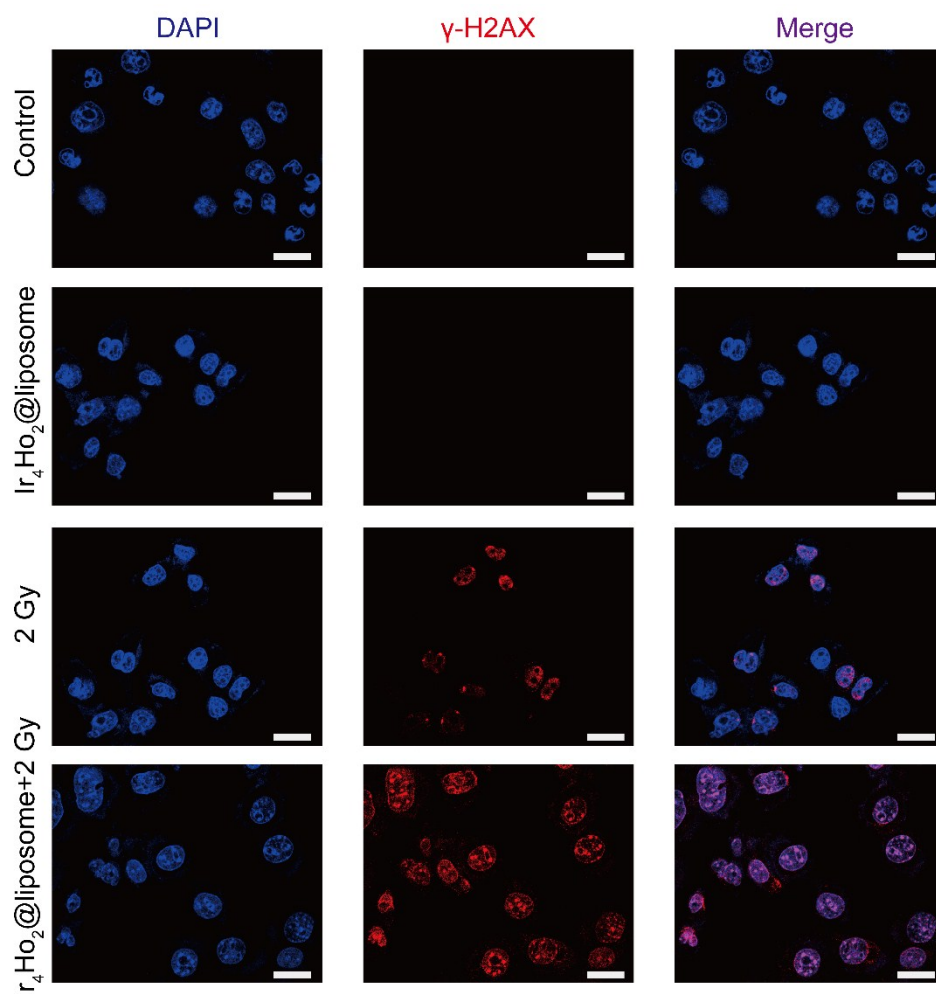


Figure S16. The confocal fluorescence imaging of 4T1 cells stained by γ -H2AX after different treatments.

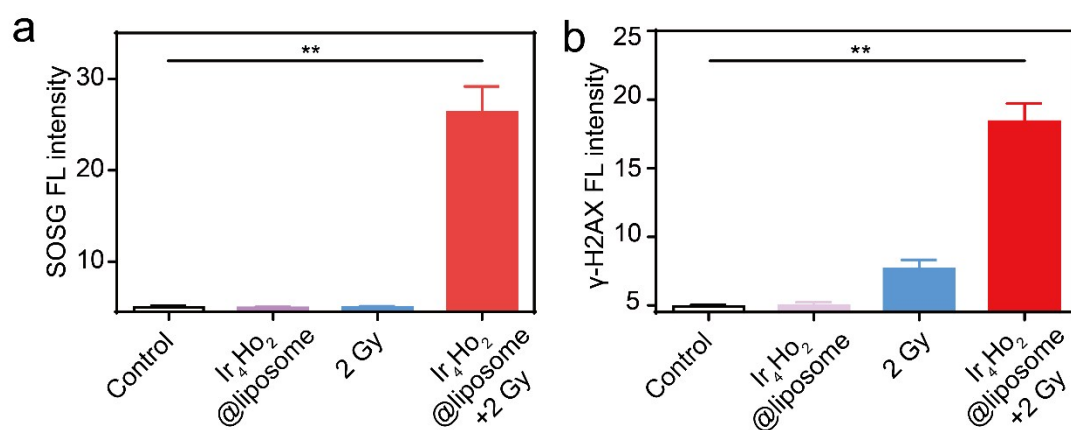


Figure S17. The relative fluorescence intensities of SOSG (a) and γ -H2AX (b) measured by confocal laser scanning microscopy after various treatments.

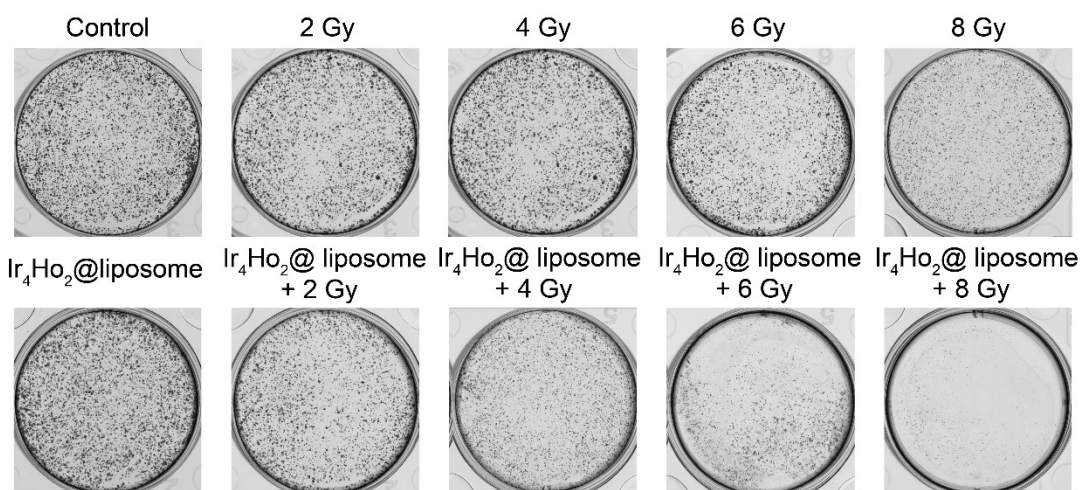


Figure S18. Representative images of colony formation of 4T1 cells after incubation with $\text{Ir}_4\text{Ho}_2\text{@liposome}$ NPs ($20 \mu\text{g mL}^{-1}$) under X-ray irradiation (0, 2, 4, 6, 8 Gy).

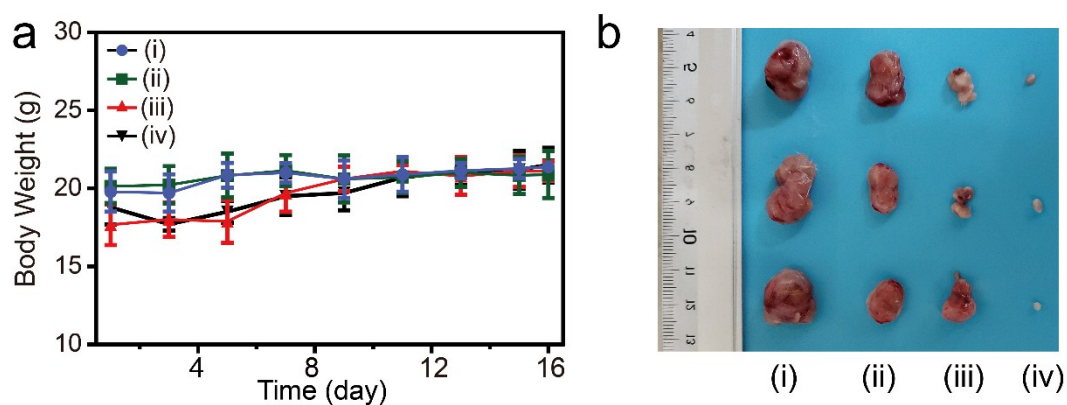


Figure S19. body weights (d), and tumor photos (e) of different groups of mice during 16 days of treatment (n=4)

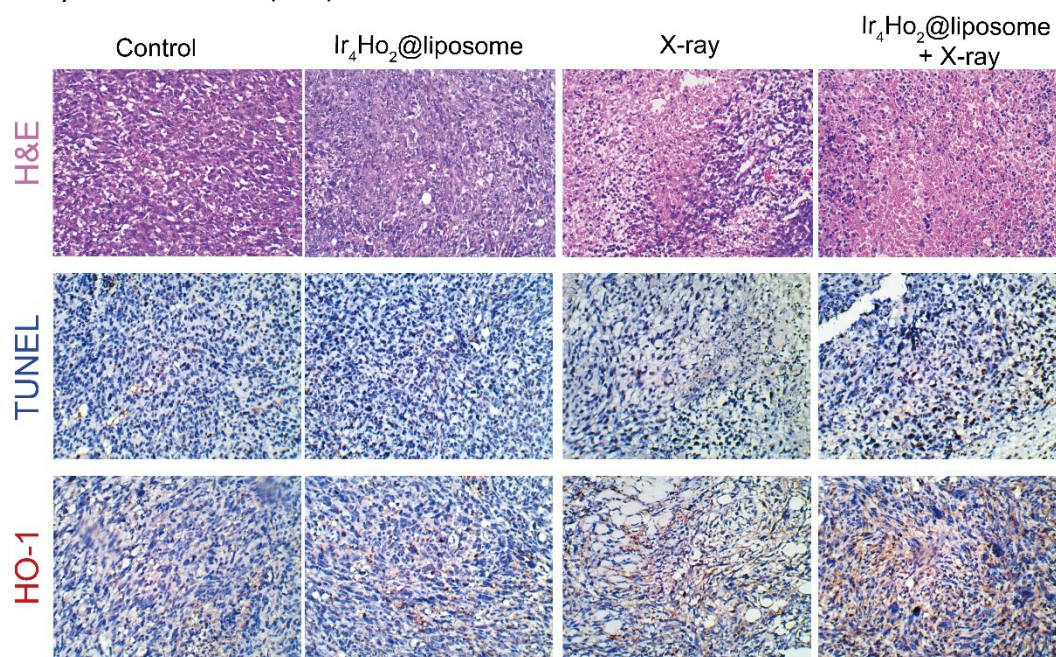


Figure S20. The pathological analysis of tumor tissues stained with H&E, TUNEL, and HO-1, respectively, 200× magnifications.

Table S1. Fitting results of the clonogenic assay

Data	α	β	R^2
Control	0.01638	0.01996	0.998
Ir ₄ Ho ₂ @liposome	0.1837	0.3256	0.994

References:

1. Xue, F.; Lu, Y.; Zhou, Z.; Shi, M.; Yan, Y.; Yang, H.; Yang, S. *Organometallics* **2015**, *34*, 73.
2. Cui, L.; Wang, C.; Zhang, H.; Ma, Y.; Deng, G.; Zhou, Z.; Lin, J.; Yang, H.; Yang, S. *Chem. Commun.* **2019**, *55*, 9120.