Supplementary Information

Boosting abscopal effect of radiotherapy: a smart antigen-capturing radiosensitizer to eradicate metastatic breast tumor

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EXPERIMENTAL SECTION

Materials and reagents. Tetraethyl orthosilicate (TEOS), 1-(3-diaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Alfa Aesar Chemical Ltd. (Tianjin, China); (3-aminopropyl)-triethoxysilane (APTES) and 4-Formylbenzoic acid were purchased from Heowns Biochemical Technology Co., Ltd.; Poly(ethylene glycol) methyl ether (mPEG2k) was purchased from Energy Chemical (Shanghai, China); mal-PEG12-COOH was purchased from Peng Sheng Biotechnology Co., Ltd. (Shanghai, China); Tetrabutyl titanate was purchased from Sinopharm Chemical Reagent Co., Ltd. 4-amino-N-(3-chloro-4-fluorophenyl)-N'-hydroxy-1,2,5-oxadiazole-3-carboximidamide (IDOi, IDO5L) was purchased from Medchemexpress (MCE), USA. 2′, 7′-dichlorofluorescein diacetates (DCFH-DA) was obtained from Beyotime (Nantong, China). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company. Primary antibody for caspase-3 and FITC-labeled secondary antibody were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). Confocal dish was purchased from Cellvis, Mountain View, CA. Annexin V-FITC Apoptosis Detection Kit were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). 4T1 cells and 4T1-Luc cells was purchased from Shanghai AOLU Biological Technology Co. Ltd, China.

Instruments. The transmission electron microscopy (TEM) was carried out on a JEM-2100 electron microscope. Fluorescence spectra were obtained with FLS-980 Edinburgh. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi, China) with a combined glass-calomel electrode. Confocal fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscopy (Leica Germany) with an objective lens (×20). The crystal structure of the samples was determined by powder X-ray diffraction (PXRD) patterns (Bruker D8, Germany). Radiotherapy was carried out on a medical linear accelerator (Varian 23 EX, America) at a power of 6 MV with dose rate of 400 cGy/min and the source skin distance = 100 cm.

Synthesis the hollow mesoporous TiO_2 ($HTiO_2$). Colloidal silica templates were prepared through a modified Stöber method. Tetraethyl orthosilicate (TEOS, 99%) was mixed with the de-ionized water, ethanol and an aqueous solution of ammonia. After stirring for 4 h, the silica particles were separated by centrifugation, washed with ethanol, and then re-dispersed in ethanol. The above silica solution was well dispersed in a mixture of hydroxypropyl cellulose, ethanol, and de-ionized water.

After stirring for 30 min, tetrabutyl titanate in ethanol was injected into the mixture using a syringe pump. After injection, the temperature was increased to 85 °C at 900 rpm stirring under refluxing conditions for 90 min. The precipitate was isolated by centrifugation, washed with ethanol, and re-dispersed in water to give SiO_2@TiO_2 core-shell composites. To obtain hollow shells by removal of the SiO_2 core, an aqueous NaOH solution was added to the solution. After etching, the TiO_2 shells were isolated by centrifugation, washed with de-ionized water and ethanol, dried under vacuum, dispersed in de-ionized water. The resulting precipitates were isolated by centrifugation, washed with de-ionized water and ethanol, dried under vacuum, and calcined at the desired temperature for 2 h in air to obtain hollow TiO_2 shells.

Synthesis of $HTiO_2$-NH_2. The $HTiO_2$ prepared above was dissolved in 40 mL of anhydrous ethanol, and then 600 μL of water and 120 μL of ammonia were added. After mixing, 200 μL of APTES was added to the above solution. After stirring overnight at room temperature, the mixture was centrifuged (10,000 rpm, 10 min), and the precipitate was washed twice with ethanol and water. Finally, $HTiO_2$-NH_2 was dispersed in 10 mL PBS (pH = 7.4, 0.01 M). The amino groups in $HTiO_2$-NH_2 were quantified by TGA.

Synthesis of aldehyde group-modified mPEG. The aldehyde group-modified mPEG (mPEG-CHO) and were prepared according to a simple one-step method. Briefly, mPEG2k, 4-carboxybenzaldehyde, DMAP and EDC·HCl were dissolved in dichloromethane (DCM) and stirred for 48 h. After the reaction was completed, the solution was concentrated by a rotary evaporator, and washed with saturated NaCl solution for five times and 5% NaCl solution for three times. Subsequently, the organic layer was collected and further dehydrate with anhydrous magnesium sulfate. After filtration, the filtrate was concentrated and deposited twice with excess diethyl ether. The product was dried and further dialyzed with deionized water for 3 d.

Synthesis of IDOi@$HTiO_2$-mal-mPEG. 1) COOH-PEG_{12}-maleimide (COOH-PEG-mal) was modified on the surface of $HTiO_2$-NH_2 through the amide-forming reaction to obtain $HTiO_2$-mal NPs. Firstly, COOH-PEG-mal, NHS and EDC were mixed to activate carboxyl groups for half an hour. Secondly, $HTiO_2$-NH_2 was added into the mixed solution for 24 h under gentle stirring. Finally, the product was collected though centrifugation and wash with PBS buffer for three times. 2) The $HTiO_2$-mal was mixed with IDOi in ethanol to obtain IDOi@$HTiO_2$-mal. The mixture was stirred at least 24 h to attain maximum loading. 3) IDOi@$HTiO_2$-mal was mixed with mPEG2k-

CHO under gentle stirring for 30 min. The product IDOi@$HTiO_2$-mal-mPEG were obtained by centrifugation and washed three times with PBS buffer.

The optimization of COOH-PEG-mal and mPEG-CHO. $HTiO_2$-mal-mPEG were prepared according to the above-mentioned method at mole ratio of 0:1, 0.5:1, 1:1, 2:1 and 3:1 for mPEG-CHO: COOH-PEG-mal, respectively. The NPs were treated in PBS (pH 7.4 or pH 5.6) at 37 °C under gentle stirring. The solution was centrifuged and washed with water for two times. Then, these NPs were incubated in BSA solution for 1 h, respectively. The protein adsorption capacity of different NPs was investigated using a bicinchoninic acid analysis.

Preparation of maleimide-liposome (mal-liposome). For comparison, mal-liposomes were prepared by mixing a chloroform solution of DPPC, cholesterol, DSPE-PEG-NH_2 and DSPE-PEG-mal under vigorous stirring overnight. Afterwards, the chloroform was evaporated, and the obtained NPs were dispersed in aqueous solution. Then, NPs was mixed with mPEG-CHO under gentle stirring for 20 min and the final product of mal-liposome were obtained by centrifugation and washed.

Cell culture. The 4T1 cells used in the experiments were treated with 1640 medium containing 10% fetal bovine serum and 1% 100 U/mL penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Anaerobic culture conditions were 5% CO_2, 1% O_2, and 94% N_2 at 37 °C

DNA damage evaluation. DNA damage was detected using the γ-H2AX Phosphorylation Assay Kit. Briefly, the cells were first incubated with different nanoparticles for 12 h in 5% CO_2 at 37 °C. After irradiation with 4 Gy X-rays, the cells were further incubated for 4 h. Next, the cells were fixed with 4% paraformaldehyde for 20 min and were eventually stained with FITC-conjugated anti-phospho-histone γ-H2AX (Ser139). Finally, an imaging flow cytometer (Amnis Corporation) was applied to record the cell images with an excitation of 488 nm for FITC. IDEAS® image analysis software (Amnis) was used to analyze the images.

Caspase 3 activation. Briefly, 4T1 cells were incubated with different nanoparticles for 12 h. Then, the cells were washed thrice with 1640 medium and then subjected to the X-ray for 4 Gy. After further incubation for 12 h, the cells were fixed with paraformaldehyde (4%) for 10 min, and then treated with primary antibody anti-caspase 3, enhanced secondary antibody for 1 h and 40 min at room temperature, respectively. At last, the cells were washed with PBS before CLSM experiment.

Apoptosis analysis *in vitro*. 4T1 cells seeded in 6-well plates (5×10^4 cells/well) were treated with different condition for 12 h. After irradiated with X-ray for 4 Gy and then incubated for another 24 h. Treated cells were harvested, washed twice with ice-cold PBS, stained with Alexa Fluor 488-Annexin V and propidium iodide (PI) for 15 min at room temperature in the dark, and then analyzed by flow cytometry.

Immunogenic Cell Death. 4T1 cells were cultured in a 6-well plate overnight and incubated with different condition for 12 h. After irradiated with X-ray for 4 Gy and then incubated for another 24 h. After washed with PBS buffer and fixed with 4% paraformaldehyde, the cells incubated with AlexaFluor 488-CRT with 1:100 dilution for 2 h, stained with DAPI, and observed by CLSM.

***In vitro* antigen capturing.** The tumor antigens-secreted by tumor cells were quantified using a bicinchoninic acid analysis. 4T1 cells were incubated with various nanoparticles for 12 h and irradiated with 4 Gy X-ray radiation. Subsequently, the cell media was replaced with 1640 media without fetal bovine serum for incubation another 48 h. And then, the supernatant was obtained though centrifugation and the concentration were detected using UV-vis spectra. Preparing tumor antigens from irradiated 4T1 cells. Different NPs were incubated with antigen-containing supernatants, respectively. After incubation, NPs were centrifugated and the supernatant solution were collected. The protein content was quantified using a bicinchoninic acid analysis. The protein uptake by the different NPs was calculated using subtraction. In detail, the protein concentration in the supernatant before or after AC-NP capture were quantified using bicinchoninic acid analysis. And then, the protein concentration uptake by the NPs was obtained using subtraction according to the standard linear calibration curve.

Mice culture. All animal experiments were conducted and agreed with the Principles of Laboratory Animal Care (People's Republic of China). BalB/C mice (4-6 weeks old, female, ~20 g) were fed with normal conditions of 12 h light and dark cycles and given access to food and water ad libitum.

***In vivo* fluorescence imaging of IR780 labeled $HTiO_2$-mal-IR780-mPEG.** IR780 was modified on the surface of $HTiO_2$-NH_2-mal through amide-forming reaction. In detail, IR780, NHS and EDC were added into PBS buffer to activate carboxyl groups for 30 min. And then, $HTiO_2$-NH_2-mal was mixed with the above solution. After 24 h, the $HTiO_2$-mal-IR780 was obtained by centrifugation and wash with PBS buffer for three times. Then, $HTiO_2$-mal-IR780-mPEG were prepared according

to the above-mentioned method. Then 4T1 tumor-bearing BALB/c mice were intravenously injected with $HTiO_2$-mal-IR780-mPEG for *in vivo* fluorescence imaging using IVIS Lumina III imaging system (Perkinelmer, USA).

Bilateral 4T1 tumor model. A bilateral model of 4T1 tumors on BALB/c mice were employed to investigate the abscopal effect of the radiosensitizer. BALB/c mice were subcutaneously (s.c.) injected with 1×10^6 4T1 cells into the right flank (primary tumors). After three days, 1×10^6 4T1 cells were s.c. injected into the left flank (abscopal tumors), respectively. Mice with tumors were divided into seven groups (n=6): (Ⅰ) PBS; (Ⅱ) IDOi@$HTiO_2$-mal-mPEG; (Ⅲ) PBS (+); (Ⅵ) mal-liposome (+); (Ⅴ) $HTiO_2$-mPEG (+); (Ⅵ) $HTiO_2$-mal-mPEG (+); (Ⅶ) IDOi@$HTiO_2$-mal-mPEG (+). NPs were injected intravenously with the dose of 40 mg/kg according to the amount of $HTiO_2$ at day 1 and day 3. Only the primary tumor was treated with 4 Gy of X-ray at day 1 and day 3. The tumor volume was monitored for 20 days and calculated by formula: V=L×W×W/2 (L, the longest dimension; W, the shortest dimension). The survival rates were calculated for 50 days. The lung metastasis was observed at day 24 after treatment and collected the lungs.

***In vivo* immunotherapeutic mechanism study.** To determine the populations of mature DCs after RT, tumor-draining lymph nodes were collected at 5 days after irradiation. Single cells were obtained by passing the lymph-node through a 70-μm pore-sized cell strainer in the presence of red blood cell lysis buffer. For evaluation of the mature DC population in the lymph nodes, the cells were stained with anti-CD11c-FITC, anti-CD80-PE, or anti-CD86-APC (BioLegend). To determine cytotoxic T lymphocytes, abscopal tumors were collected at 5 days after irradiation. Single cells were collected according to the above-mentioned method and then stained with anti-CD3-FITC and anti-CD8-APC (BioLegend). After washed with PBS for three times, the stained cells were analyzed by flow cytometry.

The spontaneous metastatic orthotopic 4T1 murine breast cancer model. 4T1 orthotopic murine breast tumor was established by injecting of Luc-4T1cells into the breast pad of each mouse. Three days after inoculation, 3×10^4 Luc-4T1 cells were additionally injected *via* the tail vein to develop lung metastasis. Mice with tumors were divided into seven groups (n=10): (Ⅰ) PBS; (Ⅱ) IDOi@$HTiO_2$-mal-mPEG; (Ⅲ) PBS (+); (Ⅵ) mal-liposome (+); (Ⅴ) $HTiO_2$-mPEG (+); (Ⅵ) $HTiO_2$-mal-mPEG (+); (Ⅶ) IDOi@$HTiO_2$-mal-mPEG (+). NPs were injected intravenously with the dose of 40 mg/kg according to the amount of $HTiO_2$ at day 1 and day 3. Only the orthotopic

murine breast tumor was treated with 4 Gy of X-ray at day 1 and day 3. The metastasis was monitored using bioluminescence IVIS imaging on days 10. The *in vivo* distribution of luciferase-expressing Luc-4T1 cancer cells were visualized *via* intraperitoneal injection of D-luciferin (Gold Biotechnology, MO, USA). To assess lung metastasis, the mice were sacrificed on day 10. Lung tissues collected on day 10 were embedded and cryo-sectioned for H&E staining. The survival rates were calculated for 30 days.

SUPPORTING FIGURES

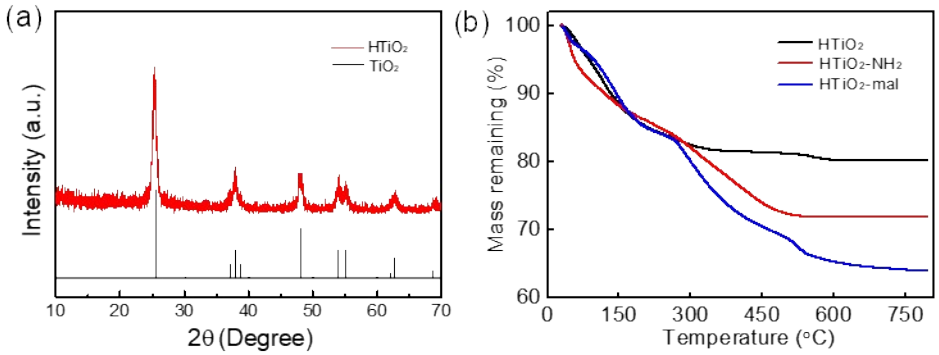

Figure S1. The powder X-ray diffraction (PXRD) patterns of $HTiO_2$ (a). TGA analysis of $HTiO_2$, $HTiO_2$ -NH_2 and $HTiO_2$-mal (b).

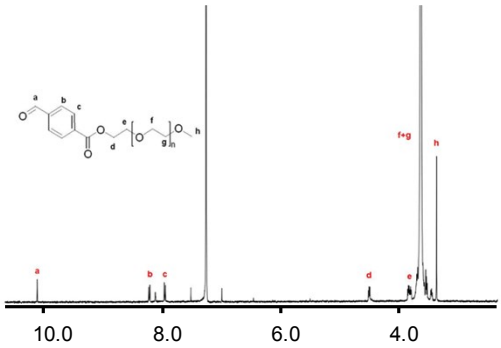

Figure S2. 1H NMR spectra of aldehyde group-modified mPEG.

Protein capture (mg)	mPEG:mal 0:1	mPEG:mal 0.5:1	mPEG:mal 1:1	mPEG:mal 2:1	mPEG:mal 3:1
$HTiO_2$-mal-mPEG (pH 7.4)	0.65±0.02	0.51±0.01	0.31±0.03	0.28±0.02	0.30±0.01
$HTiO_2$-mal (pH 5.6)	0.68±0.03	0.70±0.02	0.70±0.02	0.69±0.02	0.71±0.01

Figure S3. The optimization of COOH-PEG-mal and mPEG-CHO by detecting the protein adsorption capacity.

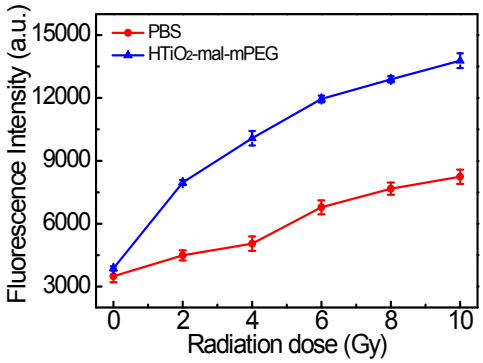

Figure S4. The generation of ROS measured by the fluorescence intensity of DCFH after X-ray irradiation with different treatment.

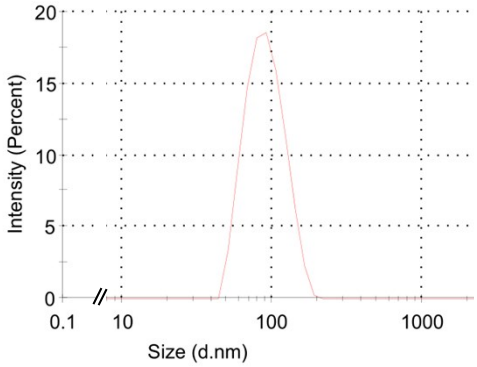

Figure S5. The DLS data of mal-liposome.

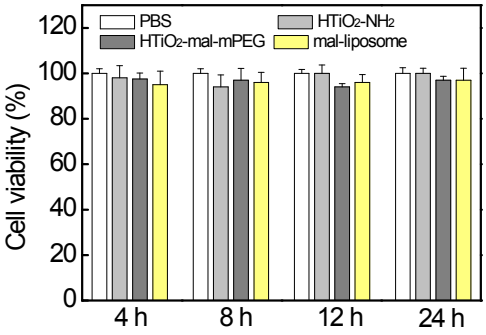

Figure S6. MTT assay of 4T1 cells with different treatments.

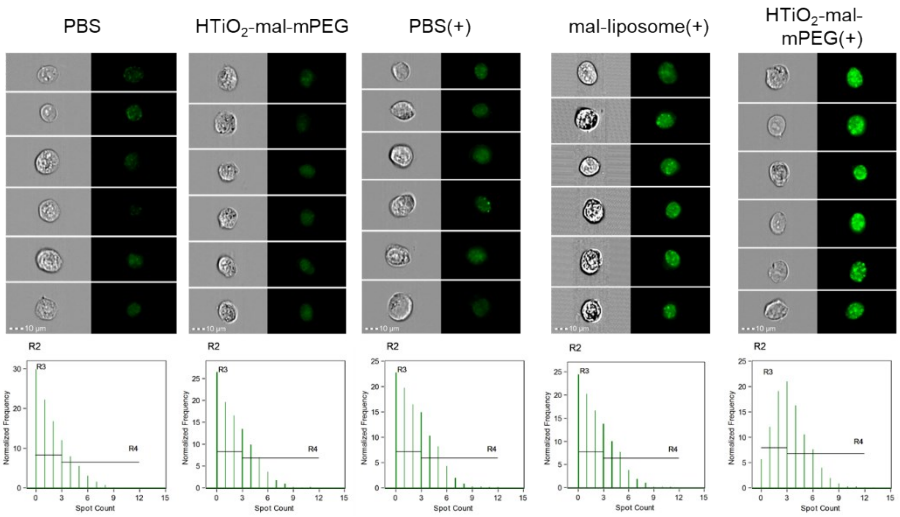

Figure S7. Verification of DNA double-strand breaks in cells via γ-H2AX immunofluorescence staining using imaging flow cytometry under different condition and the dose of X-ray was 4 Gy.

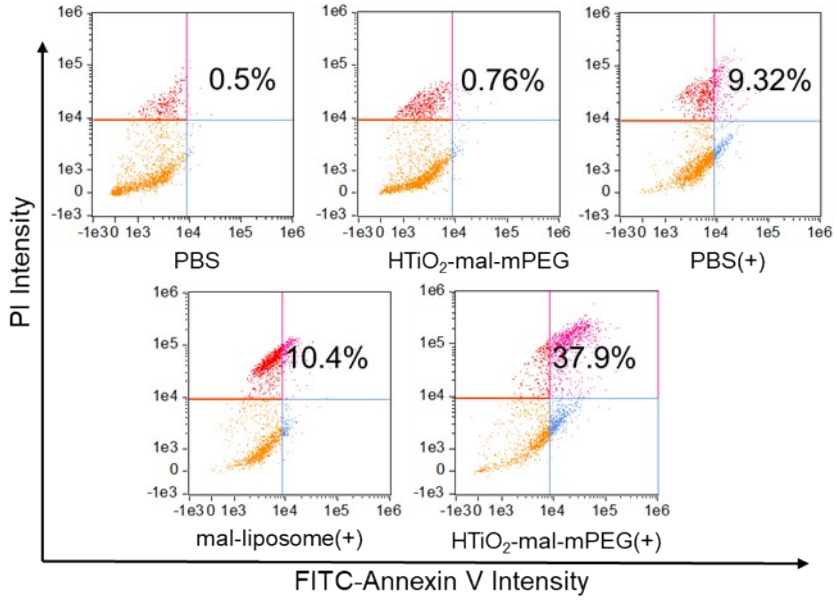

Figure S8. Flow cytometry analysis of apoptosis with Annexin V/PI staining of 4T1 cells.

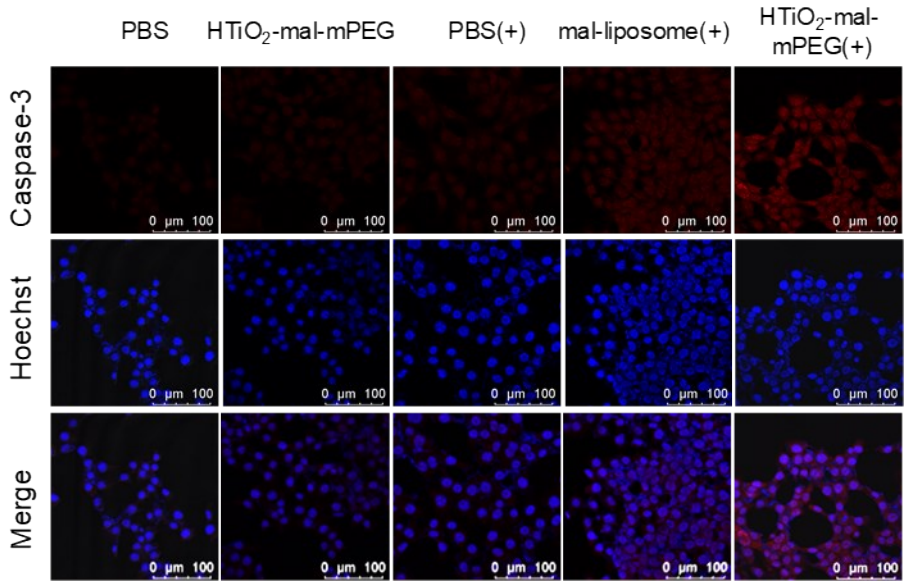

Figure S9. Immunofluorescent staining images of caspase-3 with different treatments for 12 h and the dose of X-ray was 4 Gy.

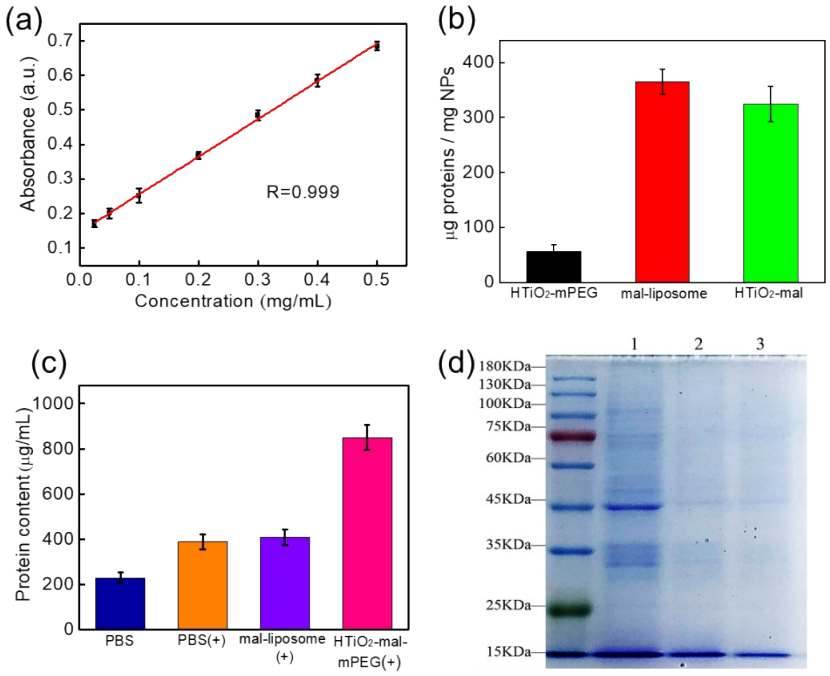

Figure S10. (a) Standard linear calibration curve of protein using a bicinchoninic acid analysis. (b) The quantification of total protein capturing by the NPs. (c) The quantification of tumor antigens-secreted by tumor cells with different treatment. (d) Western blotting analysis for 4T1 cell-specific antigen markers. 1: cancer lysate; 2: mal-liposome-antigen; 3: $HTiO_2$-mal -antigen.

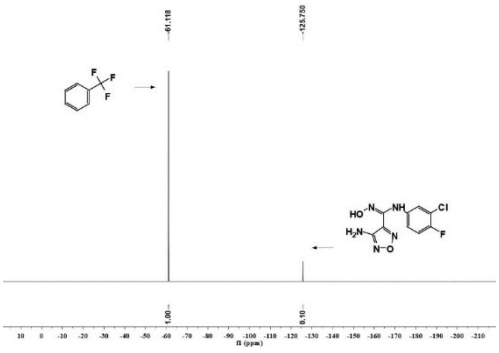

Figure S11. ^{19}F NMR spectrum of IDOi to quantify the loading efficiency.

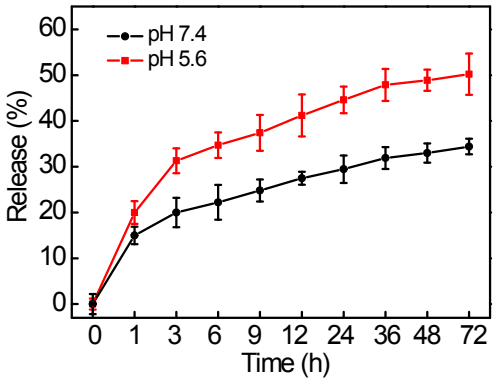

Figure S12. Accumulated leakage of IDOi in radiosensitizer at different pH buffer solution monitored by UV-vis.

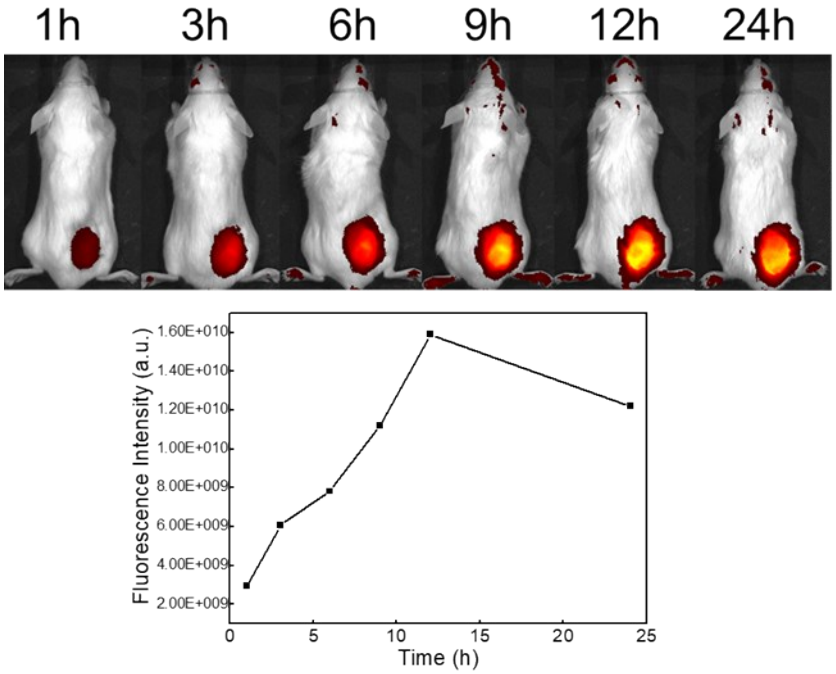

Figure S13. Fluorescence imaging *in vivo* of the 4T1-tumor bearing mice post-injection of the radiosensitizer.

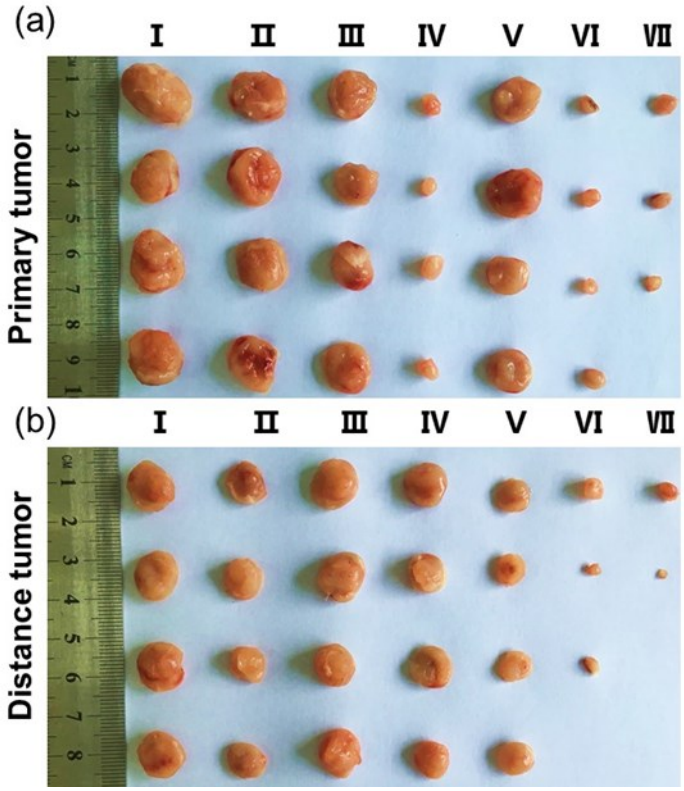

Figure S14. The pictures of (a) primary tumors and (b) distant tumors in 4T1 tumor-bearing mice after different treatments. Ⅰ: PBS; Ⅱ: IDOi@$HTiO_2$-mal-mPEG: Ⅲ: PBS(+); Ⅳ: $HTiO_2$-mPEG(+); Ⅴ: mal-liposome(+): Ⅵ: $HTiO_2$-mal-mPEG(+); Ⅶ: IDOi@$HTiO_2$-mal-mPEG(+).

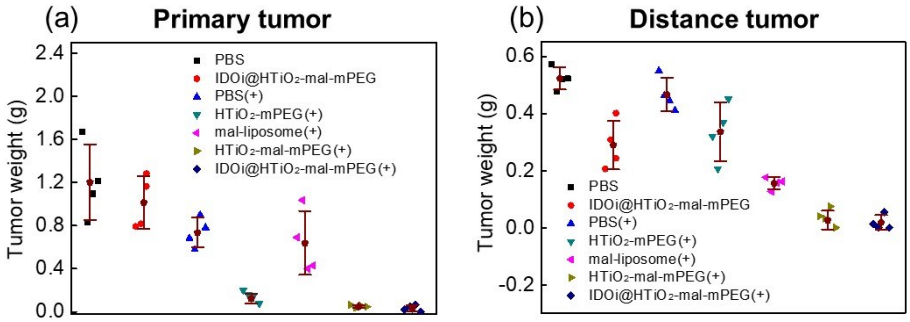

Figure S15. Tumor weight of primary tumors (a) and distant tumors (b) in 4T1 tumor-bearing mice after different treatments.

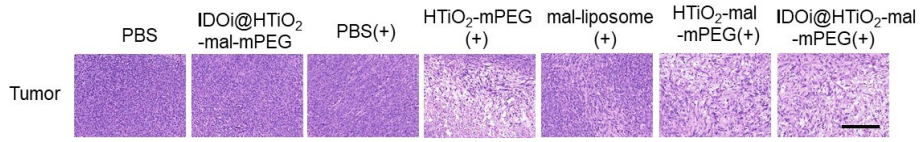

Figure S16. H&E staining of the tumor. H&E staining tissue slides was carried out after mice were treated differently 12 h. Scale bar is 200 μm.

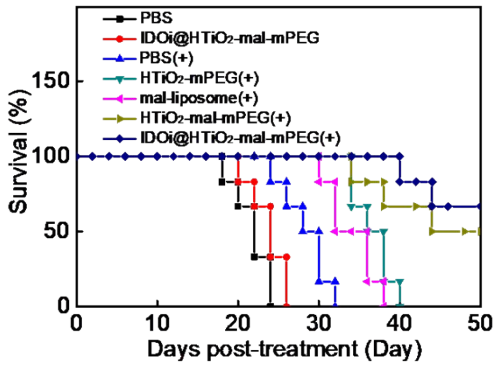

Figure S17. Survival rates for each group after receiving various treatments. (n=6)

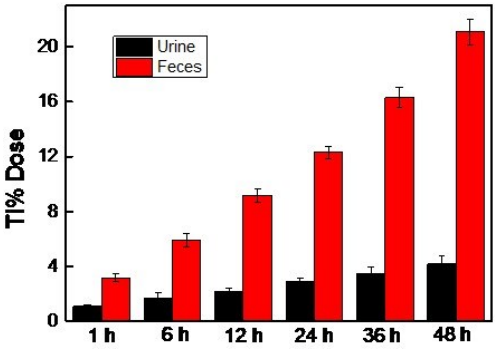

Figure S18. Accumulated Ti amount in urine or feces of mice at various time points after injection.

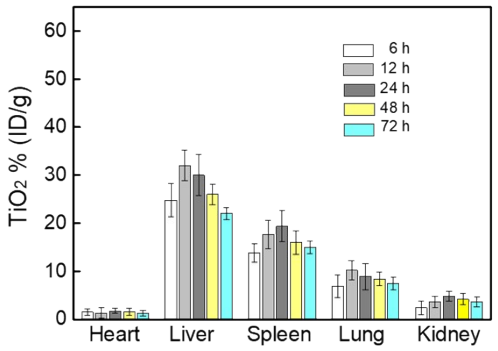

Figure S19. Biodistribution of Ti (% ID of Ti per gram of tissues) in main organs after intravenous injection.

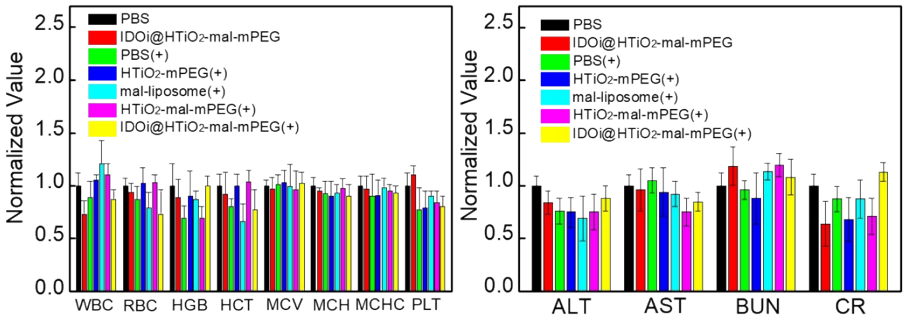

Figure S20. Blood routine tests and blood biochemical tests of mice with various groups.

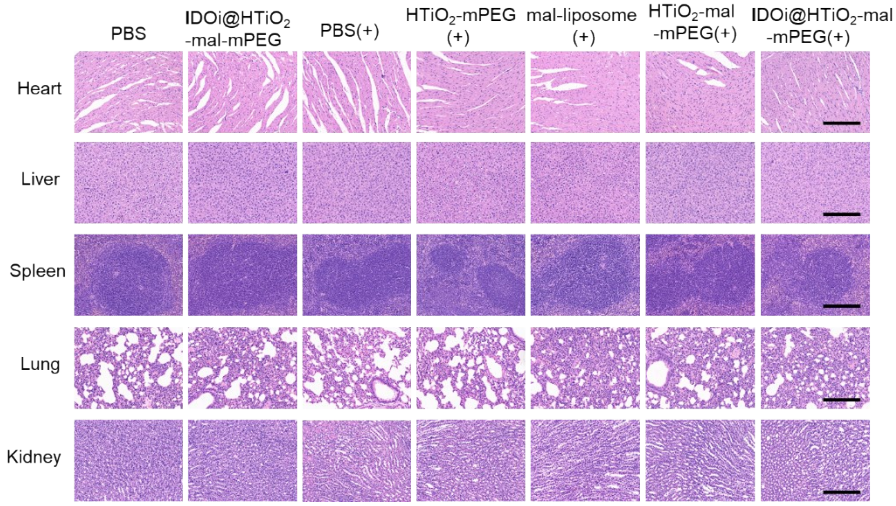

Figure S21. H&E staining of the five major organs (heart, liver, spleen, lung and kidney). The mice were treated differently and H&E staining of tissue slides was carried out at 24 h post-treatment. Scale bar is 200 μm.

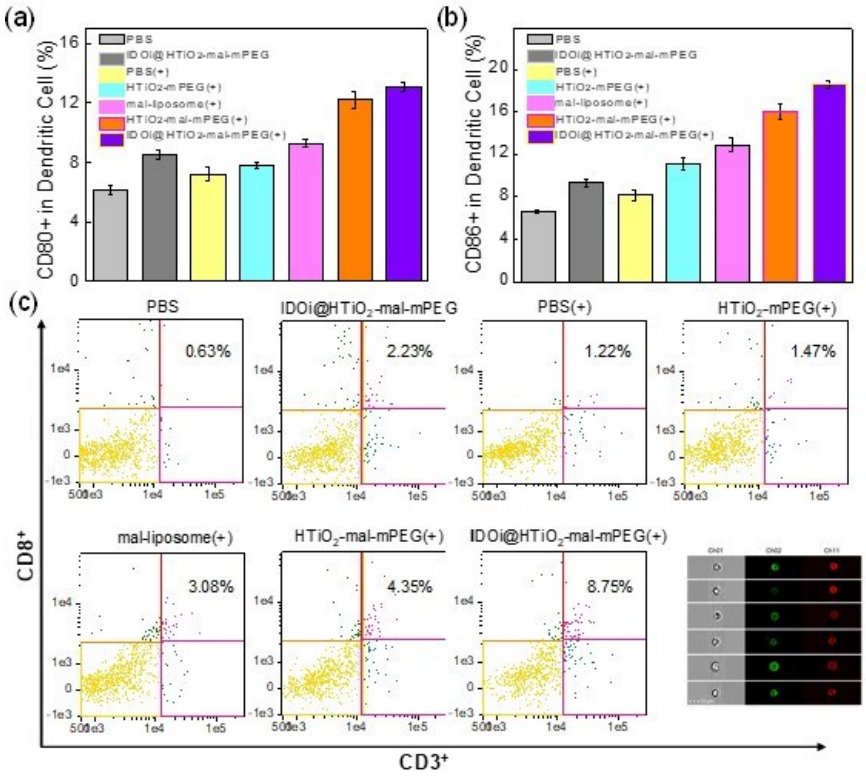

Figure S22. The quantization analysis of flow cytometry for $CD80^+$ (a) and $CD86^+$ (b) expression on lymph nodes treated with various groups. Flow cytometric analyses of the populations of $CD8^+$ ($CD3^+$ $CD8^+$ as the marker) T cells in distant tumor of mice treated by various condition.

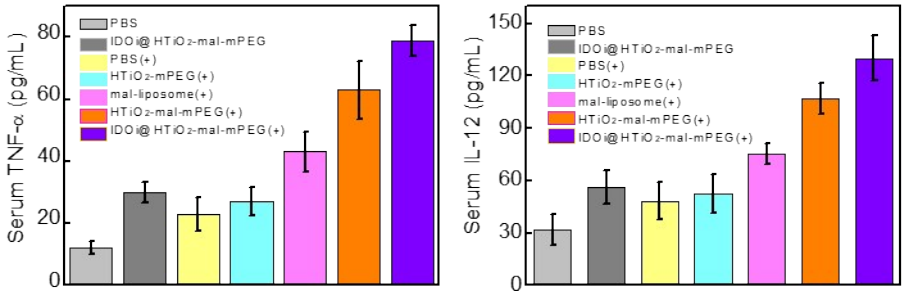

Figure S23. The secretion level of TNF-α (a) and IL-12 (b) in serum obtained from mice with different treatment.

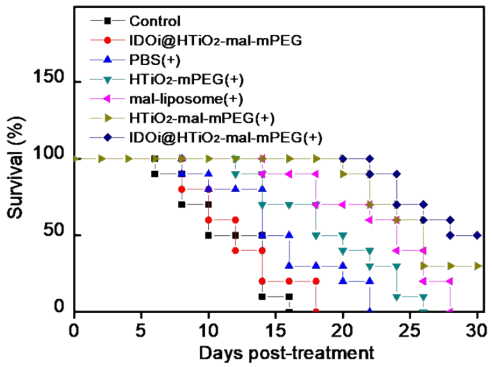

Figure S24. Survival rates for each group after receiving various treatments. (n=10)