Supporting Information

Polyoxomolybdate (POM) Nanoclusters with Radiosensitizing and Scintillating Properties for Low Dose X-ray Inducible Radiation-Radiodynamic Therapy

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Experimental section

Synthesis of POMo nanoparticles. 5 mmol of Phosphomolybdic acid was dispersed in 2 mL of DI water followed by addition of 2 mL NaOH (2 M). 0.5 mL (0.1 mmol) of Sodium borohydride ($NaBH_4$) was used to reduce Mo (VI) to Mo (V) Phosphomolybdic acid followed by stirring at 50 ^oC for 4h under dark field. The obtained blue colored suspension was purified by adding absolute ethanol followed by centrifugation at 10000 rpm for 10 minutes. The product was freeze dried to obtain polyoxomolybdate nanoclusters (POMo NCs).

Preparation of hollow structured chitosan nanoparticles. Commercially available chitosan was dispersed in 1% acetic acid solution and stirred for 1 day at 80 ^oC. The obtained pale yellowish solution was evaporated completely, obtaining hollow structured chitosan nanoparticles.

Loading of POMo nanoclusters on chitosan (CS). In order to prepare POMo NCs loaded chitosan (CS) nanohybrid (POMo@CS), 2 mL (4 mg/mL) of POMo NCs were added to 2 mL (10 mg/mL) of CS followed by stirring for 24h under dark field. The unbound POMo nanoclusters were removed by filtration using 3500 MW cut-off filter membrane for 24 h.

Rose Bengal (RB) loading. Different concentrations of RB (50, 100, 200 and 500 μg/mL) were added into 2 mL (1 mg/mL of Mo) of POMo@CS solution and then stirred at room temperature for 24 h under dark condition. The free RB was removed by filtration using 3500 MW cut-off filter membrane for 24 h.

Extracellular detection of singlet oxygen under X-ray irradiation. In this experiments, 100 μM of SOSG was added into DI water or aqueous solution of RB or water dispersed POMo NCs or POMo@CS-RB at RB and Mo concentrations of 50 and 100 μg/mL, respectively. The whole experiment was carried out in 96 microplate reader followed by X-ray irradiation (2 Gy). After X-ray treatment, the fluorescence intensity of SOSG was measured at 530 nm upon excitation at 488 nm.

Extracellular detection of reactive oxygen species (ROS) under X-ray irradiation.100 μM of p-aminophenyl fluorescein (APF) was added into DI water or aqueous solution of RB or water dispersed POMo or POMo@CS-RB at RB and Mo concentrations of 50 and 100 μg/mL, respectively. The whole experiment was carried out in 96 microplate reader followed by X-ray irradiation (2 Gy). After X-ray treatment, the fluorescence intensity of APF was measured at 515 nm upon excitation at 490 nm.

Cell viability study by MTT assay and cellular uptake study. The 4T1 cell lines were originally obtained from American Type Culture Collection (ATCC). Cells were grown in normal RPMI-1640 culture medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells pre-seeded into 96 well cell culture plates at 2×10^5 cells/well were incubated with different concentrations of RB, PEGylated POMo@CS and POMo@CS-RB nanoformulations for 24 h, and the standard (MTT) assay was carried out to determine the cell viabilities. The each well plate was treated with 10 μL of freshly prepared MTT solution (5 mg mL^{-1} in PBS buffer of pH 7.4) and incubated for 4 h. The supernatant was carefully removed leaving behind the violet formazon crystals in the plate. The crystals dissolved in dimethyl sulfoxide (DMSO) were recorded at 450 nm using a microplate reader.

The cellular uptake of PEGylated POMo@CS-RB nanoformulation was examined on 4T1 cells. The cells were placed in six-well plates at 1×10^6 cells per well. After adhesion, the nanoformulation were added into the wells at a concentration of 50 μg/mL and cultured for 24 h. After washing three times with PBS (pH 7.4), cells were fixed by 75% ethanol and stained with 4,6-diamidino-2-phenylindole (DAPI) before imaging by confocal laser scanning microscope (CLSM, FV1200, Olympus, Japan).

***In vitro* singlet oxygen generation.** SOSG reagent (sigma) was used to detect singlet oxygen. 4T1 cells were seeded on cover slides in 35 mm tissue culture dishes overnight. PBS, RB, PEGylated POMo@CS orPOMo@CS-RB were added into the cells at RB and Mo concentration of 50 and 100 μg/mL, respectively, and then incubated for 24 h followed by X-ray irradiation (2 Gy). The cells were washed with PBS three times. The 50 μM of SOSG was added in each well and incubated for 30 min. The slides were washed with PBS and imaged by CLSM. The corresponded fluorescence intensity of SOSG was measured by flow cytometry.

γ-H_2AX assay for DNA damage study. 4T1 cells were cultured in 35 mm tissue culture dishes overnight and then incubated with PBS, RB, PEGylated POMo@CS or POMo@CS-RB at RB and Mo concentration of 50 and 100 μg/mL for 24 h followed by X-ray irradiation (2 Gy). Cells were stained with γ-H_2AX assay kits and DAPI for CLSM.

Live/dead cell co-staining assays. The therapeutic effects of POMo@CS-RB were further evaluated by Live/dead cell co-staining assays using Calcein-AM and propidium iodide (PI). The cultured cells were incubated with different nanomaterials for 6 h and then irradiated by X-ray (2 Gy). After 12 h incubation, cells were washed with PBS followed by co-staining with Calcein-AM (25 nM) and PI (5 μM) solution in PBS for 30 min. Finally, the cells were washed with PBS and imaged by CLSM.

Tumor model. Healthy female Balb/c mice were purchased from Nanjing Peng Sheng Biological Technology C, Ltd. All animal procedures were performed in accordance with the guidelines for care and use of laboratory animals of Soochow University and experiments were approved by the animal ethics committee of Soochow University laboratory animal center. The 4T1 tumors were generated by subcutaneous injection of 2×10^6 cells in ~ 40 μL PBS into the right shoulder of each mouse. When tumor volumes were reached at about 100 mm^3, the mice were used for treatment.

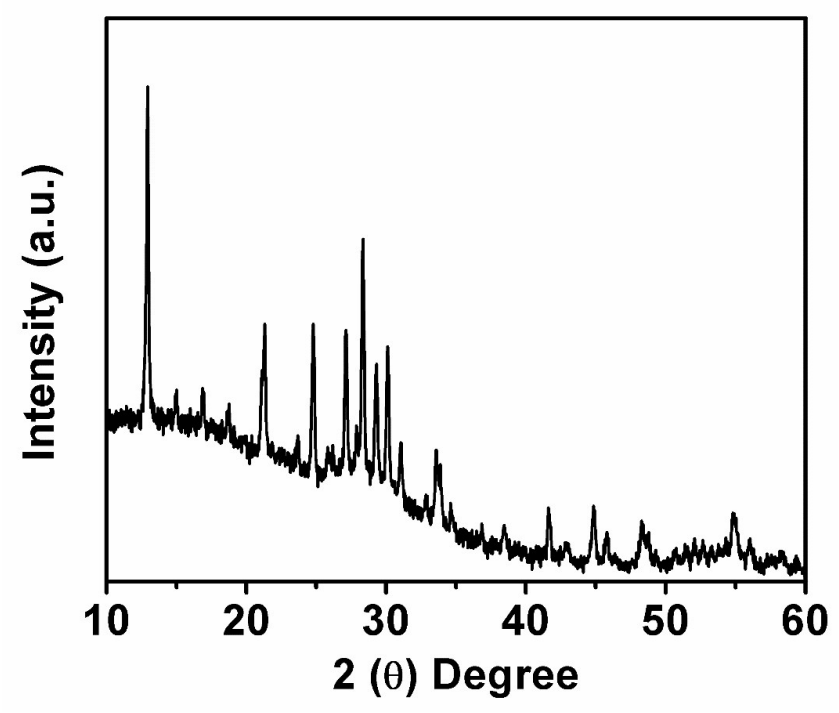
In vivo photoacoustic (PA) imaging. 4T1 tumor-xenograft mice were intravenously injected with PEGylated POMo@CS (25 mg of Mo Kg^{-1}) or PBS. The PA images of mice were captured by small animal PA imaging system at wavelength between 750 -900 nm. The PA imaging was recorded at different time points (1, 2, 4, 8 and 24 h) post injection.

Measurement of blood circulation. Normal Balb female mice (n=3) were intravenously injected with PEGylated POMo@CS (25 mg of Mo Kg^{-1}). At different time points (5 min, 30 min, 1, 2, 6, 12 and 24 h), 10 μL of blood sample was syringed out from the tail vein of the mice followed by dissolving in nitric acid. The concentration of Mo was quantitatively measured by ICP-AES analysis.

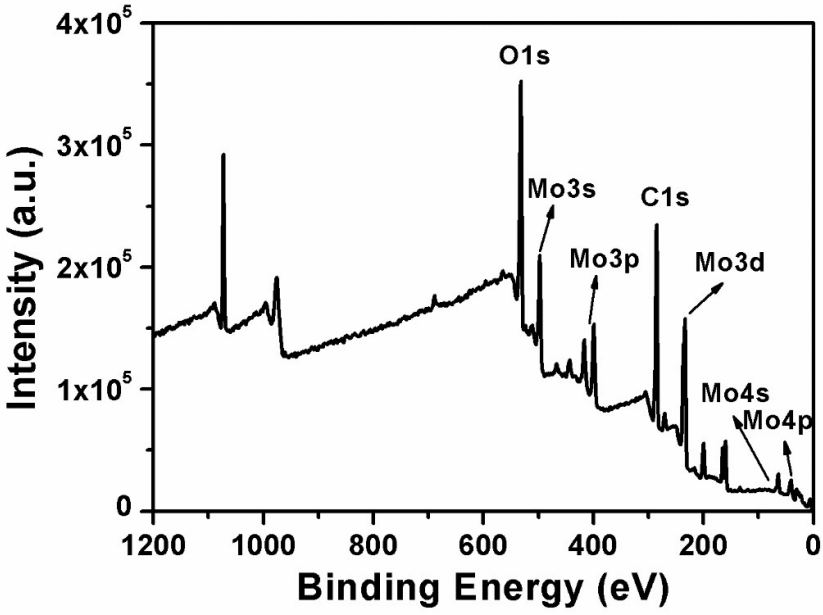
Biodistribution. For biodistribution study, the mice bearing 4T1 tumors treated with PEGylated POMo@CS-RB (25 mg of Mo Kg^{-1}) were sacrificed at 1st and 24th h. The tumors and the major tissues including heart, liver, lungs, kidney and spleen were collected and digested with 3 mL of aqua regia. The concentrations of Mo accumulated in the different organs were quantitatively measured by ICP-AES analysis.

***In vivo* X-RRDT by POMo@CS-RB.** 25 mice bearing 4T1 were subjectively divided into five groups with five mice in each group. Each group of mice was intravenously injected with PBS, POMo (25 mg of Mo Kg^{-1}), RB (12.5 mg of RB Kg^{-1}) and POMo-RB (two groups, 25 mg of Mo Kg^{-1} and 12.5 mg of RB Kg^{-1}). The X-ray beam was produced by a biological irradiator with 160 KeV (Rad Source RS2000 Pro Biological Irradiator). All the treated mice were subjected to irradiate by X-ray at 2 Gy except one group treated by POMo@CS-RB. After various treatments, the tumor length (L) and width (W) were measured by a digital caliper every two days interval. The tumor volume was calculated according to the following formula: Tumor volume (V) = $L \times W^2/2$.

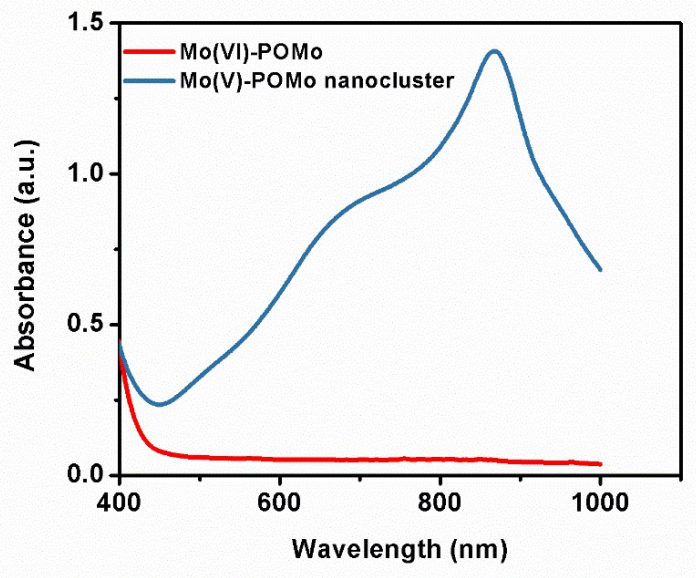
Histological Examination. PBS, POMo (25 mg of Mo Kg^{-1}), RB (12.5 mg of RB Kg^{-1}) and POMo-RB (two groups, 25 mg of Mo Kg^{-1} and 12.5 mg of RB Kg^{-1}) treated mice were sacrificed after 2 days of intravenous injection. Tumors and different major organs, kidney, liver and spleen were collected, sectioned, and stained with hematoxylin and eosin (H&E).



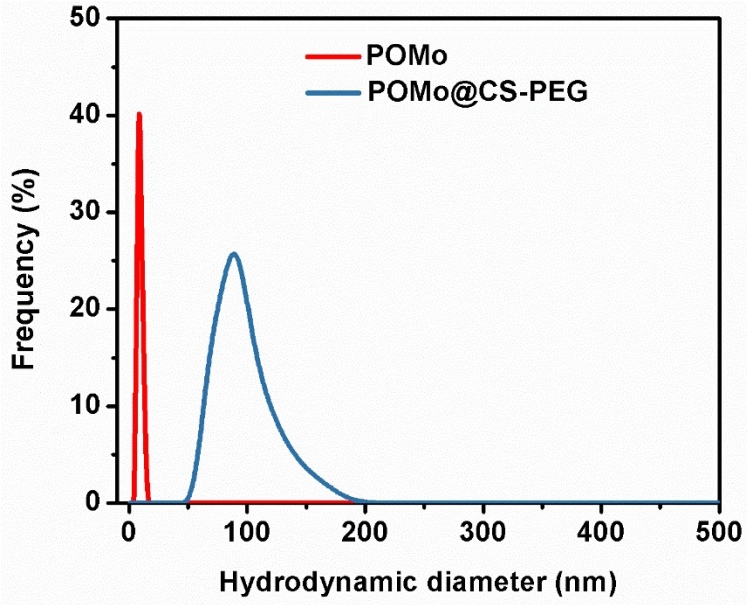
Supporting Information Figure S1. X-ray powder diffraction (XRD) patterns of POMo NCs.



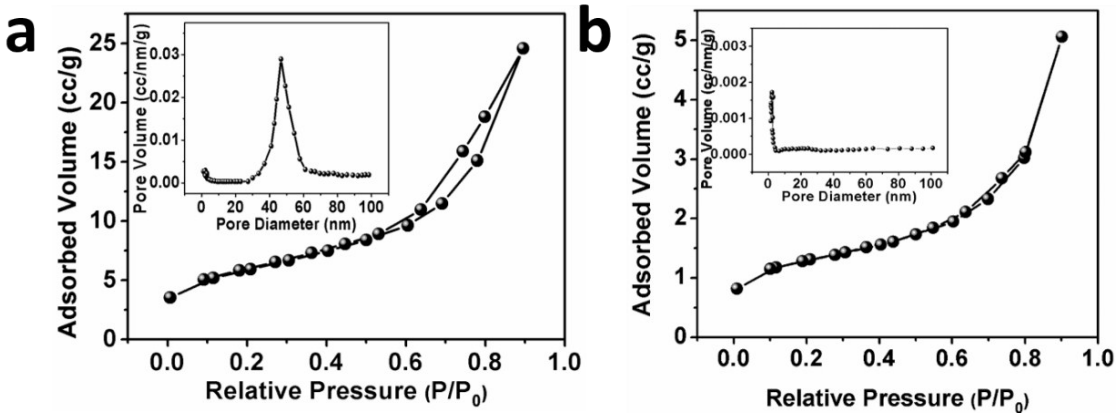
Supporting Information Figure S2. X-ray photoelectron spectroscopy (XPS) measurement of POMo NCs.



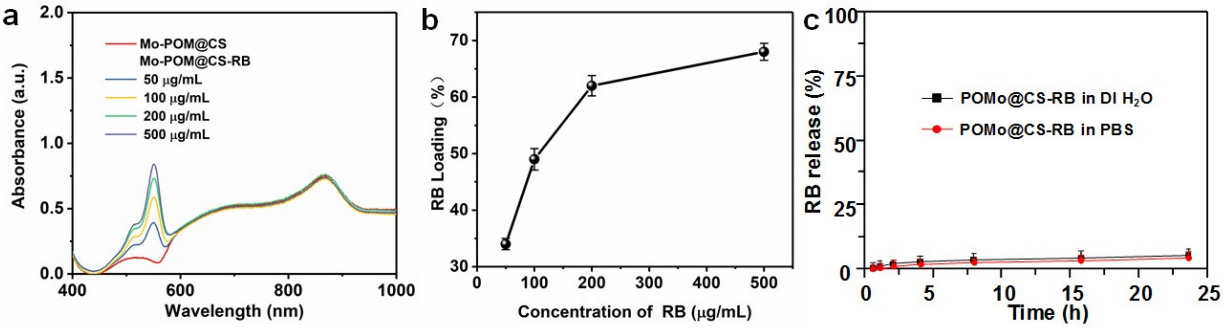
Supporting Information Figure S3. UV-Visible absorbance spectra of Phosphomoybdic (Mo-VI) acid and POMo (V/VI) nanocluster



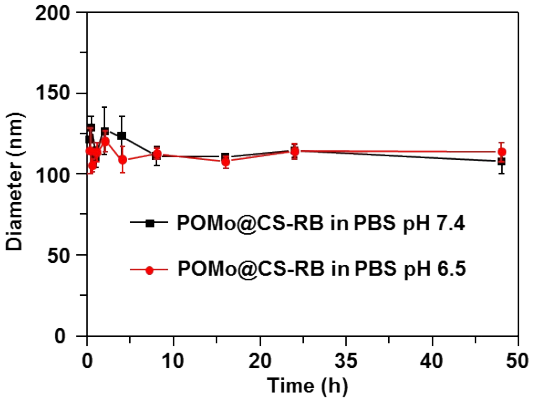
Supporting Information Figure S4. Dynamic light scattering (DLS) of POMo NCs and PEGylated POMo@CS.



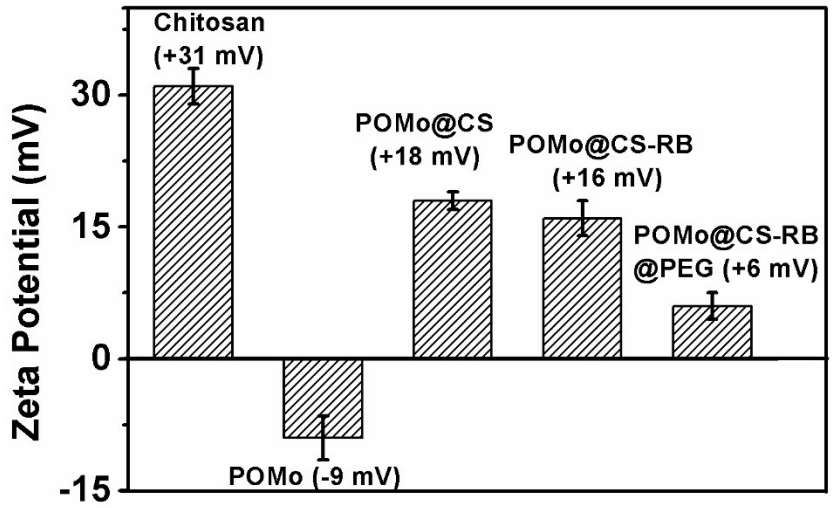
Supporting Information Figure S5. N2 adsorption−desorption isotherm of (a) hollow chitosan and (b) chitosan. Inset shows corresponding BJH desorption, dV/dD, pore volume vs pore diameter curves.



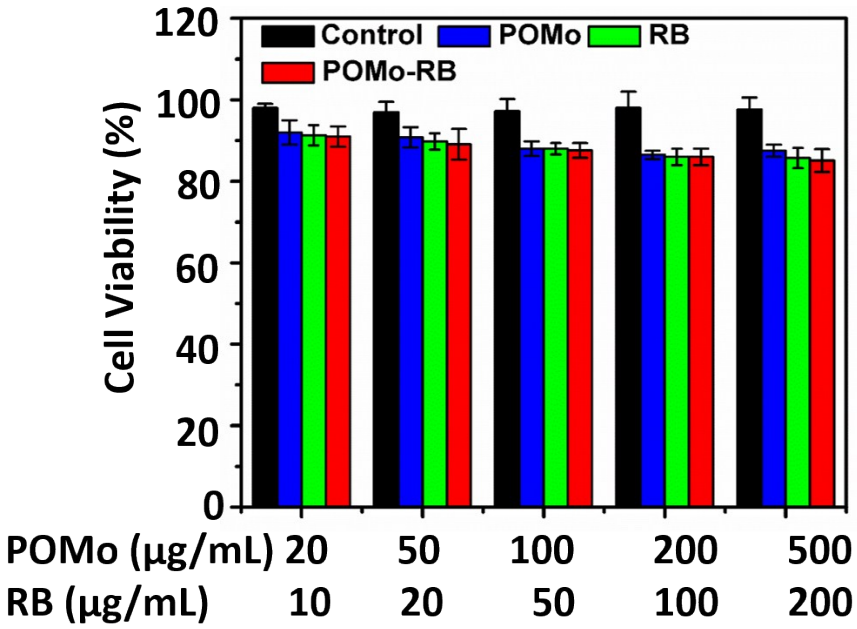
Supporting Information Figure S6. (a) UV-Visible absorbance spectra for RB loading on chitosan nanoparticles. (b) RB loading efficiencies at different RB concentrations. (c) RB release from the POMo@CS-RB.



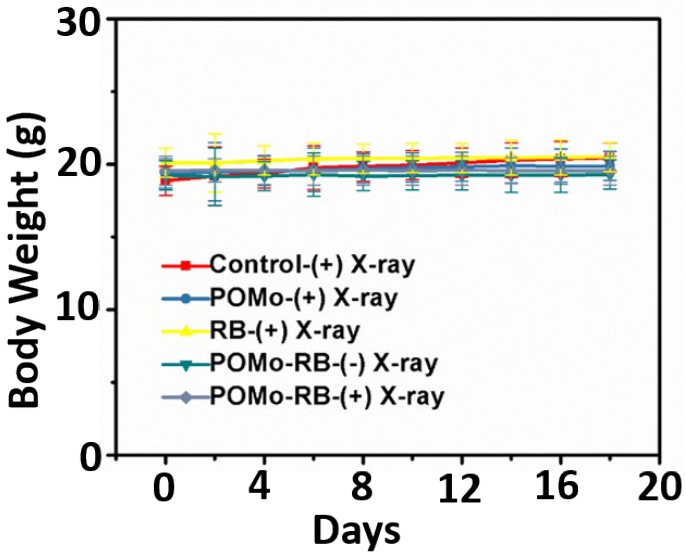
Supporting information Figure S7. The stability of the POMo@CS-RB nanoformualtion in different pH (pH 7.4 and 6.5) solution.



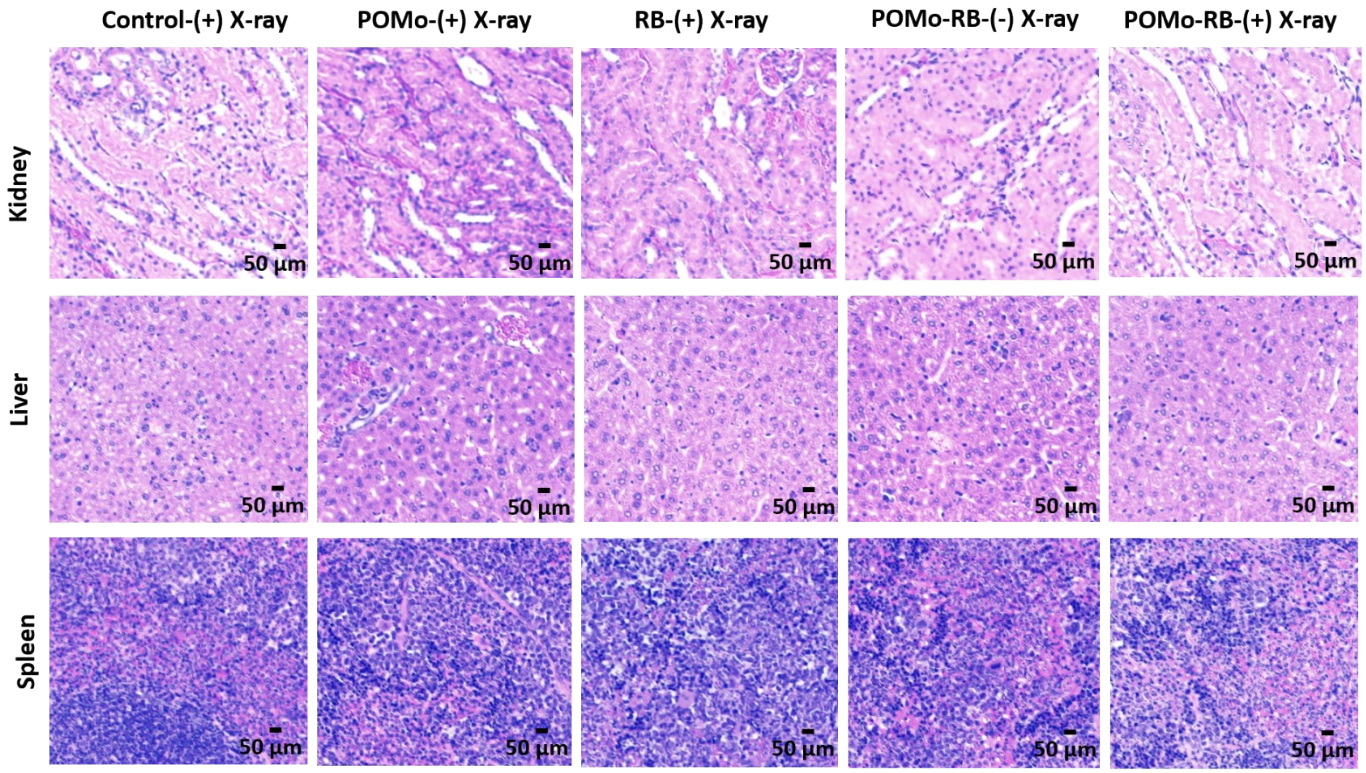
Supporting Information Figure S8. Zeta potential measurements of different nanomaterials.



Supporting Information Figure S9. The cytotoxicity of PBS, POM, RB and POM-RB at different concentration.



Supporting Information Figure S10. Body weight of mice with different treatment.



Supporting Information Figure S11. H&E staining of major organs (liver, spleen and kidney) of mice with different treatment.