Supplemental Information:

A Novel Combination Treatment for Melanoma: FLASH Radiotherapy and Immunotherapy Delivered by a Radiopaque and Radiation Responsive Hydrogel

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1. Supplementary Materials and Methods

1.1. Materials. Gold (III) chloride trihydrate (99.9%), sodium borohydride powder (98.0%), poly(bis(4-carboxyphenoxy)phosphazene) disodium salt (PCPP, 1 MDa), L-glutathione reduced (GSH), selenocystamine dihydrochloride and hexamethylenediamine were purchased from Sigma-Aldrich (St.Louis, MI). Imiquimod (IMQ) was purchased from Selleck Chemicals (Houston, TX). B16-F10 cell line was purchased from ATCC (Manassas, VA). Cells were cultured in Dubecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (10000 units/mL) from Life Technologies Invitrogen (Grand Island, NY). Mouse TNF alpha and IL-6 ELISA kits were purchased from Abcam (Cambridge, UK). C57BL/6J mice were obtained by The Jackson Laboratory (Bar Harbor, ME). Milli-Q deionized (DI) water (18.2 MΩ cm) was used throughout the experiments.

1.2. Synthesis of GSH-Coated AuNP. Ultrasmall GSH-coated AuNP were synthesized by a slight modification of the Turkevich method that our group has previously reported.[1] Briefly, a 2 mL freshly prepared sodium borohydride solution (5 mg mL^{-1}) was slowly added dropwise to 100 mL of DI water containing 16 mg of gold chloride under stirring (350 rpm). The color of the mixture changed from pale-yellow to black, then to wine-red after adding sodium borohydride. After 20 minutes stirring at room temperature, 1 mL of capping ligand GSH (5 mg mL^{-1}) was prepared in DI water and was quickly added to the mixture. The resulting mixture was incubated at 4 °C overnight. The nanoparticle solution was purified by centrifugation in 10 kDa molecular weight cut off (MWCO) filtration tubes (Sartorius Stedim Biotech, Germany). The tubes were spun at 4000 rpm for 5 minutes and rinsed twice with DI water. After the last wash, the nanoparticles were concentrated to a final volume of 1 mL and filtered through a 0.22 μm filter and stored at 4 °C for use in subsequent experiments. The final nanoparticle solution was suspended in PBS for future studies. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was performed to determine the final concentration of the stock AuNP solution.

1.3. Hydrogel Characterization

1.3.1. Electron Microscopies. For TEM, hydrogels were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde. The embedded samples were then cut into sections in 60 nm thickness and mounted onto Formvar carbon-coated copper grids with 200 mesh (Electron Microscopy Sciences, Hatfield, PA). TEM micrographs were acquired using a JEOL 1010 microscopy (JEOL, Tokyo, Japan) with an acceleration voltage of 80 kV. SEM was done as previously reported.[2]

1.3.2. ICP-OES. The elemental gold concentrations in the hydrogels were determined using ICP-OES (Spectro Genesis, Germany). 10 μL of hydrogel stock were placed in 15 mL conical tubes. The hydrogels were dissolved in 1 mL of aqua regia (200 μL of nitric acid, 600 μL of HCl and 200 μL of DI water) followed by dilution with 9 mL of DI water. The final gold concentrations were determined by multiplying the concentrations obtained by the ICP-OES by their dilution factor.

1.3.3. Energy Dispersive X-ray Spectroscopy (EDX). 10 μL of diluted hydrogel sample was dried onto Formvar carbon-coated copper grids with 200 mesh. The samples were then imaged using a FEI Quanta 600 field emission gun scanning electron microscope (FEI, Hillsboro, OR) equipped with EDX detectors at 15 kV.

1.3.4. X-ray Powder Diffraction (XRD). The XRD patterns of dried hydrogel samples were recorded using a GiegerFlex D/Max-B X-ray diffractometer (Rigaku, Tokyo, Japan) at 45 kV and 30 mA with a monochromatized Cu Ka radiation wavelength of 1.5406 Å. A scan range of 20-60° and scan rate of 2° per minute were used.

1.3.5. Fourier Transform Infrared (FT-IR) Spectroscopy. The infrared spectra of hydrogel samples were collected using a JASCO FT/IR-480 Plus spectrophotometer (JASCO, Maryland, USA). The samples were prepared by grinding 5 μL diluted selenocystamine solution (5 mg mL^{-1}), PCPP solution (1 mg mL^{-1}), or hydrogel samples with 100 mg of dried potassium bromide (KBr) powder and pressing them into compact pellets.

1.3.6. Rheology. The hydrogel samples were vortexed for 30 seconds and sonicated for 30 minutes in an ultrasound water bath. Then, the hydrogels were loaded onto the Peltier plate on a rheometer (AR 2000 EX, TA Instruments, New Castle, DE). A parallel plate geometry with a gap size of 100 μm was used. The following measuring conditions were used for the measurements: frequency sweep (0.001-500% strain, 10 Hz), continuous flow (shear rates from 0 to 50 s^{-1} over 2 minutes 30 seconds), and cyclic strain (low = 0.2% strain, high = 500% strain, 10 Hz). The measurements were carried out at 25 °C.

1.3.7. Hydrogel Irradiation. The photon irradiation was performed on an X-RAD 320ix irradiator (PXi, North Brandford, CT) operated at 320 kV and 12.5 mA. An aluminum filter of 2 mm was used to deliver the radiation dosage at a rate of 2.65 Gy/min. The proton irradiation was performed using an IBA Proteus One C230 Cyclotron (Louvain-LA-Neuve, Belgium) and was used to deliver a 230 MeV proton beam at either a conventional dose rate (0.5 Gy/s) or FLASH ultrafast dose rate (95-103 Gy/s). A field size of 26 mm in diameter was used at the point of irradiation. Gafchromic EBT3 film was used to verify the flatness and shape relative dose profiles. NIST-traceable calibrated Advanced Markus Chamber was used to confirm the output dose prior to every experiment.

1.4. AuNP and IMQ Release.

1.4.1. Effect of Radiation on AuNP and IMQ Release. Release studies were performed to determine the percentage cumulative release of AuNP and IMQ drug over time. Hydrogels loaded with AuNP ([Au] = 0.2 mg mL^{-1}) and drug ([IMQ] = 0.2 mg mL^{-1}) were prepared as described above. Then, hydrogels were suspended into 1 mL of freshly prepared release media comprising of PBS and 10% of FBS in micro-centrifuge tubes. The hydrogels were centrifuged at 4,000 rpm for 8 minutes. Samples were prepared in triplicate for each radiation dose treatment and irradiated with the desired radiation dose (e.g., 15, 30, 45 and 60 Gy) using a radiotherapy at conventional dose rate (CONV-RT) or FLASH-RT. Following that, samples were incubated at 37 °C in a water bath for the overall experiment time. At the desired time points (1, 4, 7 days post-irradiation), samples were vortexed and centrifuged at 4,000 rpm for 8 minutes.

The supernatants were gently collected for quantification of the released AuNP by ICP-OES and the released IMQ by UV-vis spectroscopy. 1 mL of freshly prepared release media was added to each sample, and the samples were incubated at 37 °C in a water bath until the next study time point.

1.4.2. Effect of Oxygen and Radical Oxygen Species (ROS) Scavenging on AuNP Release. The effect of radiation on the AuNP hydrogel in conditions preventing ROS formation was investigated by performing release studies in degassed medium or in the presence of ROS scavengers. AuNP hydrogels were prepared as described above. To degas the release media, a continuous nitrogen flow was added to freshly prepared medium comprising of PBS and 10% of FBS in a single neck flask for 30 minutes. To scavenge ROS production, 1 mg of ascorbic acid was added per 1 mL prepared release media. The hydrogels were then suspended into 1 mL of degassed or ascorbic acid supplemented medium. The hydrogels were centrifuged at 4,000 rpm for 8 minutes and irradiated with FLASH-RT at 0 Gy (mock irradiation) or 60 Gy. Following the irradiation, samples were incubated at 37 °C in a water bath. The supernatants were gently collected for quantification of the released AuNP at desired time points (1, 3, 7 days post-irradiation).

1.5. CT Phantom Imaging. The inherent X-ray contrast generation of hydrogels and degraded hydrogels upon hydrolysis and FLASH-RT were examined. Samples were scanned using a MILabs microCT (MILabs, Utrecht, The Netherlands) system before and after FLASH-RT. In brief, hydrogels with gold concentrations from 0 to 10 mg mL^{-1} were prepared in 200 μL micro-centrifuge tubes in triplicates. Samples received 60 Gy FLASH-RT and were incubated at 37 °C in a water bath for 7 days. On day 7, hydrogels were vortexed and reformed by centrifuging at 4,000 rpm for 8 minutes. The supernatants were discarded, and the hydrogels were resuspended in DI water. Samples were then scanned with MILabs microCT at a tube voltage of 55 kV and a tube current of 190 μA with exposure time of 75 ms. Slices of 100 μm thickness with an increment of 0.1 mm were reconstructed using the algorithm provided by the manufacturer. Image analyses were performed using OsirixX (v.3.7.1 64-bit software). A circular ROI was

drawn on the coronal view of each tube. The CT attenuation value for each sample tube at each concentration was recorded as the average of three samples of that same concentration.

1.6. Cell Culture

1.6.1. Biocompatibility of Non-Drug Loaded AuNP Hydrogels. Cells were seeded in 35 mm dishes with 20 mm bottom wells at a density of 8,000 cells per well. Meanwhile, the AuNP hydrogel ([Au] = 8 mg mL^{-1}) was incubated in cell medium at 37 °C for 24 hours. The supernatants were collected as described above and used to treat the cells for 8 hours. Once the treatment was done, the LIVE/DEAD assay (Invitrogen Life Technologies, Grand Island, NY) was performed. In brief, the cells were rinsed with DPBS twice, and 400 μL of a solution containing 0.025% live cell dye calcein-AM and 0.1% dead cell dye ethidium-1 homodimer in DPBS was added to the cells. Cells were incubated with this dye solution for 20 minutes. The cells were then imaged with a Nikon Eclipse Ti-U fluorescence microscope with FITC (ex: 495, em: 519 nm) and Texas Red (ex: 595, em: 613 nm) filters to image live and dead cells, respectively. Four different areas of each well were imaged. The number of live and dead cells was measured using a custom MATLAB (Mathworks, Natick, MA) code. The percentage viability was determined by calculating the ratio of live to dead cells normalized to control.

1.6.2. Effect of Free IMQ Treatment. An MTS assay was performed to assess the cytotoxicity of the free IMQ on B16-F10 cells. Cells were seeded in three 96-well plates at a density of 5,000 cells per well and incubated at 37 °C and 5% CO_2 for 24 hours. Following the incubation period, IMQ was prepared in DMSO at concentrations of 0 (control), 20, 50, 100 and 200 ug mL^{-1}. Cells were treated for 24, 48 or 72 hours. Once the treatment was done for each group, the cells were washed with DPBS and incubated with the MTS assay solution (Promega, Madison, WI) comprised of 20 uL of stock MTS solution and 100 uL of cell medium. After the 2-hour incubation period, a microplate reader (Synergy H1, BioTek, VT) was used to record the absorbance at 490 nm. The cell viability relative to control (%) for each concentration and treatment duration was determined.

1.7. *Ex Vivo* Studies.

1.7.1. Biodistribution. At 18 days post-treatment, mice were euthanized with CO_2, followed by cervical dislocation to analyze the biodistribution of released AuNP. Blood samples, and major organs (i.e., heart, lungs, liver, spleen, and kidneys) from each AuNP hydrogel-treated mouse were harvested and digested by 1 mL of nitric acid overnight at 75 °C followed by 2 more hours of 250 μL of HCl at 75 °C. The samples were then diluted to a final volume of 10 mL with DI water. The concentrations of gold in the digested samples were measured by ICP-OES.

1.7.2. Histology. The organs of interests and tumors from the following groups: 1) DPBS control, 3) AuNP-gel + FLASH-RT and 5) AuNP-IMQ-gel + FLASH-RT, were harvested. The organs and tumors were rinsed with chilled DPBS and then cut into 5-6 mm thick pieces. Samples were immediately transferred to 10% buffered formalin solution and stored at 4 °C overnight for fixation. The samples were dehydrated, embedded, sectioned, and stained with hematoxylin end eosin (H&E) by the Children's Hospital of Philadelphia Pathology (CHOP) core. The slides were analyzed with a slide scanner Aperio CS-O (Leica Biosystems).

2. Supplementary Results

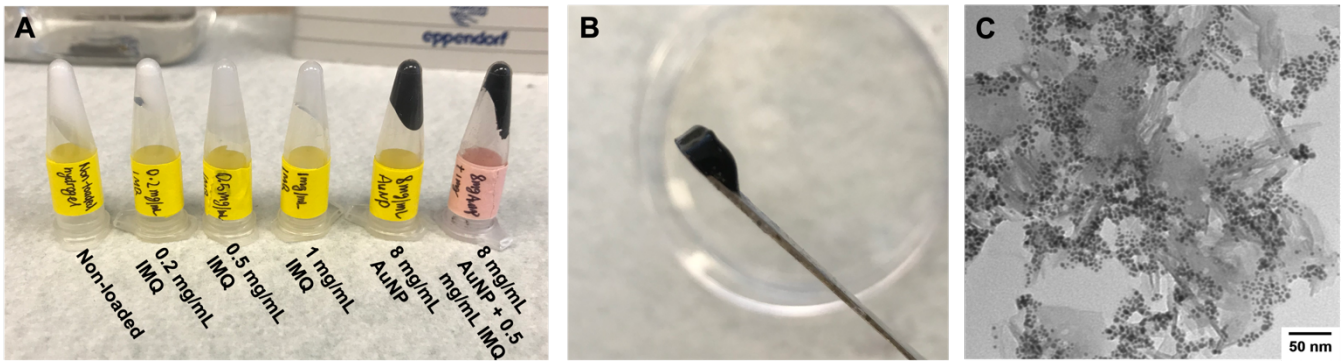


Figure S1. (A) Photographs of hydrogels nonloaded, loaded with IMQ in different concentrations, loaded with AuNP, or loaded with both AuNP and IMQ. **(B)** Photograph of IMQ-AuNP hydrogel on a spatula. **(C)** Representative TEM micrograph of AuNP-IMQ hydrogel in dry state. Scale bar = 50 nm.

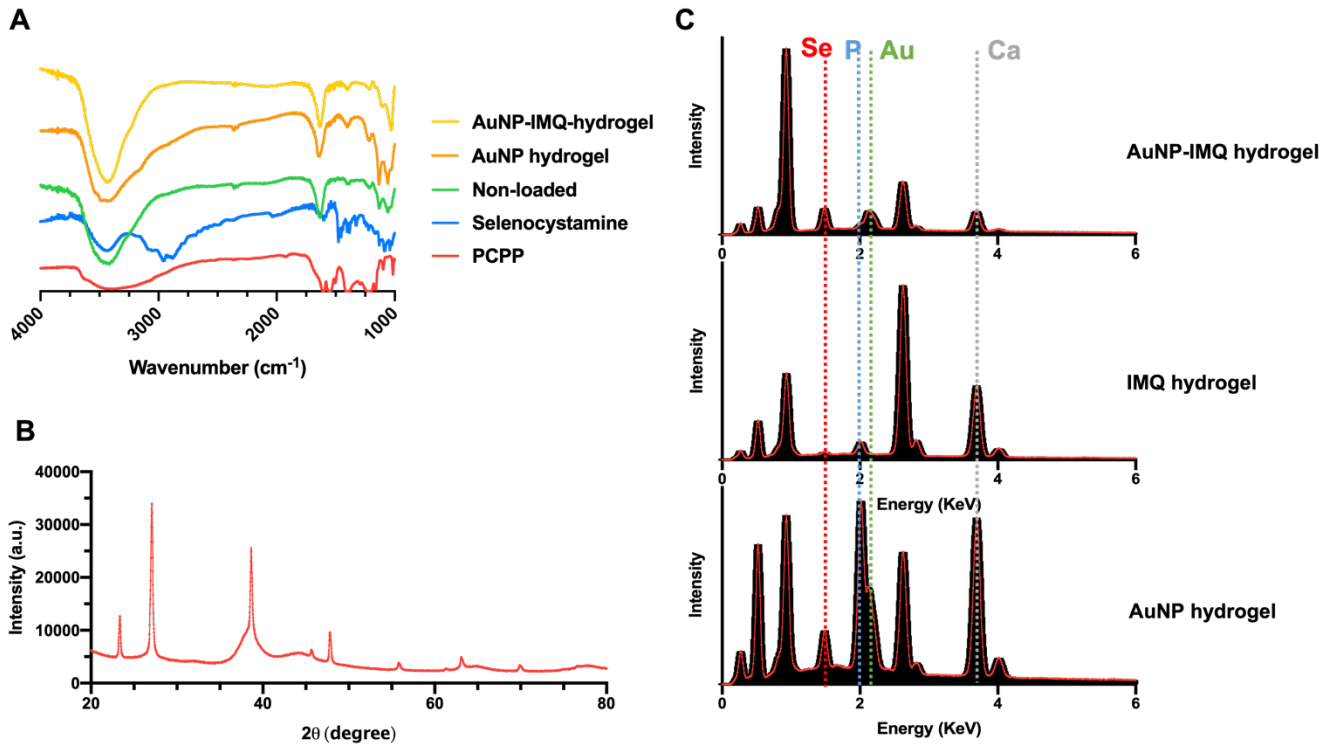


Figure S2. Characterization of AuNP-IMQ hydrogel. **(A)** FT-IR spectra of AuNP-IMQ loaded, AuNP loaded and non-loaded hydrogels in comparison to free selenocystamine and PCPP. **(B)** XRD spectrum of AuNP-IMQ hydrogel. **(C)** EDX spectra of AuNP-IMQ, IMQ, and AuNP hydrogels.

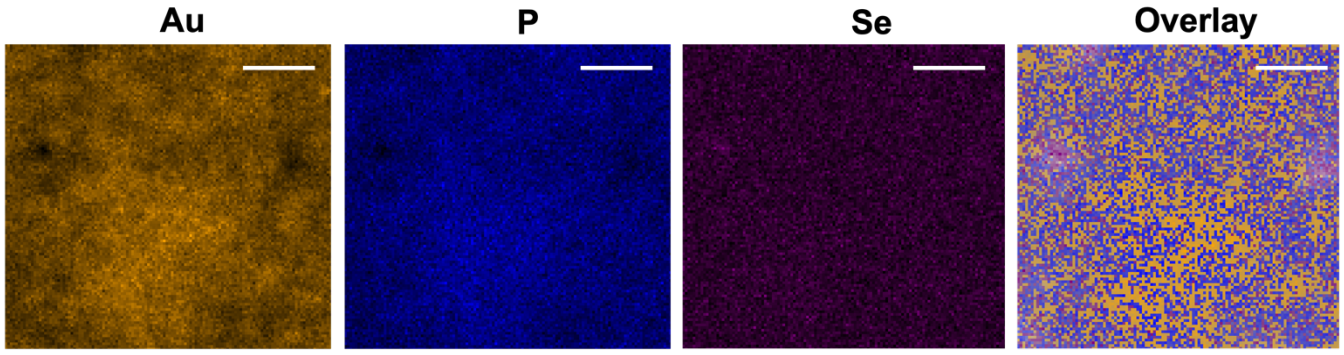
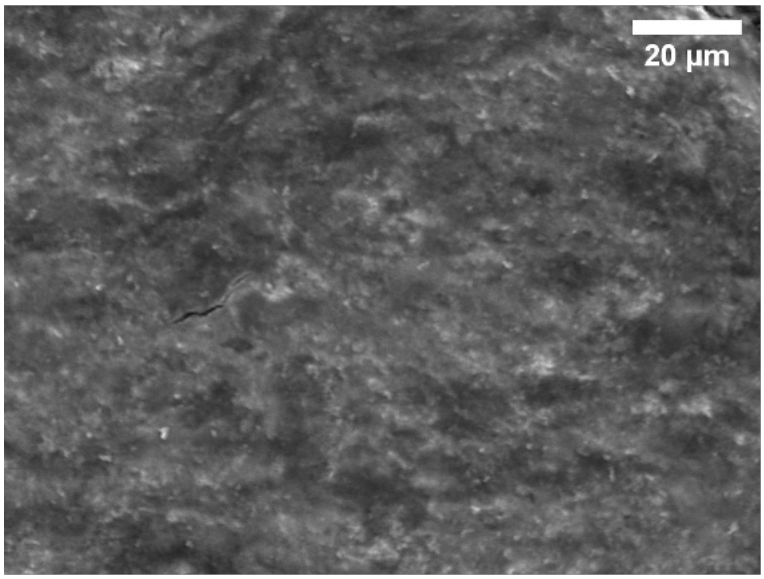


Figure S3. Elemental mapping of AuNP-IMQ hydrogels. All scale bars are 20 μm.



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192 **Figure S4**. Scanning electron microscopy of the hydrogel.

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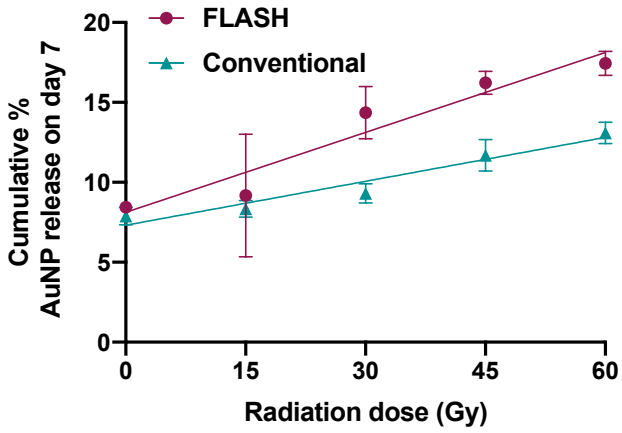

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195 **Figure S5.** Quantification of the released gold from the hydrogel irradiated with FLASH-RT or CONV-RT

196 at a range of radiation doses.

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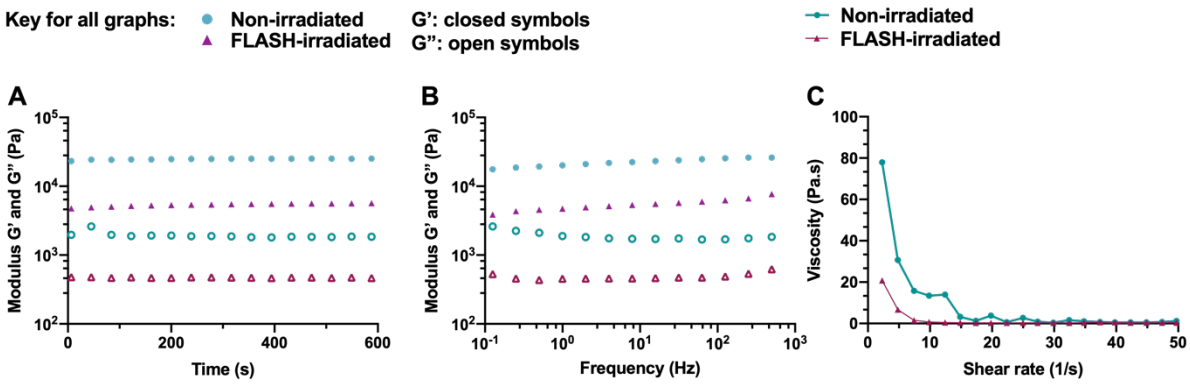

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Figure S6. Rheological characterization of AuNP-IMQ-gel. Non-irradiated or FLASH-irradiated hydrogels characterized using oscillatory shear rheometry, including: storage (G', closed symbols) and loss (G'', open symbols) moduli **A)** over time at 0.2% strain and 10 Hz, **B)** across a range of frequencies at 0.2% strain and **C)** viscosity with increasing shear rate.

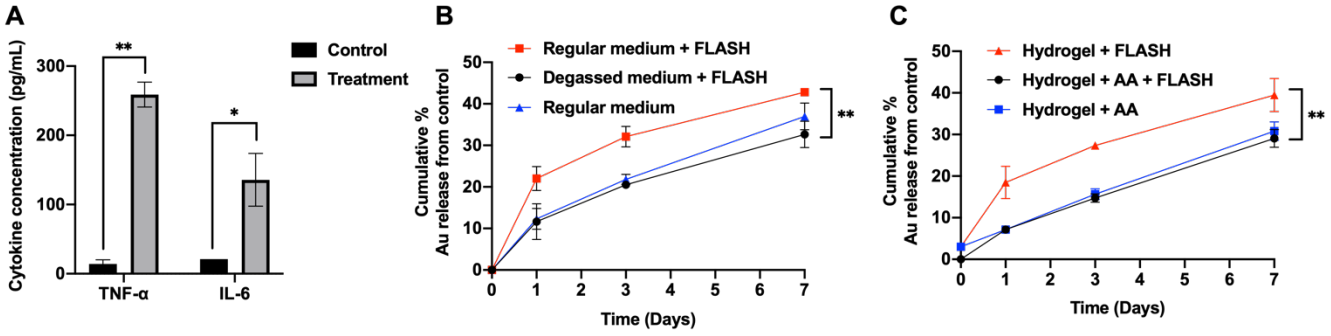

Figure S7. A) ELISA quantification of cytokine secretion of TNF-α and IL-6 in the splenocyte supernatants with treatment of FLASH-RT triggered IMQ release. Quantification of cumulative gold release from the hydrogel after FLASH-RT with either 0 Gy or 60 Gy in **B)** a regular or degassed medium and **C)** a regular or ROS scavenging medium.

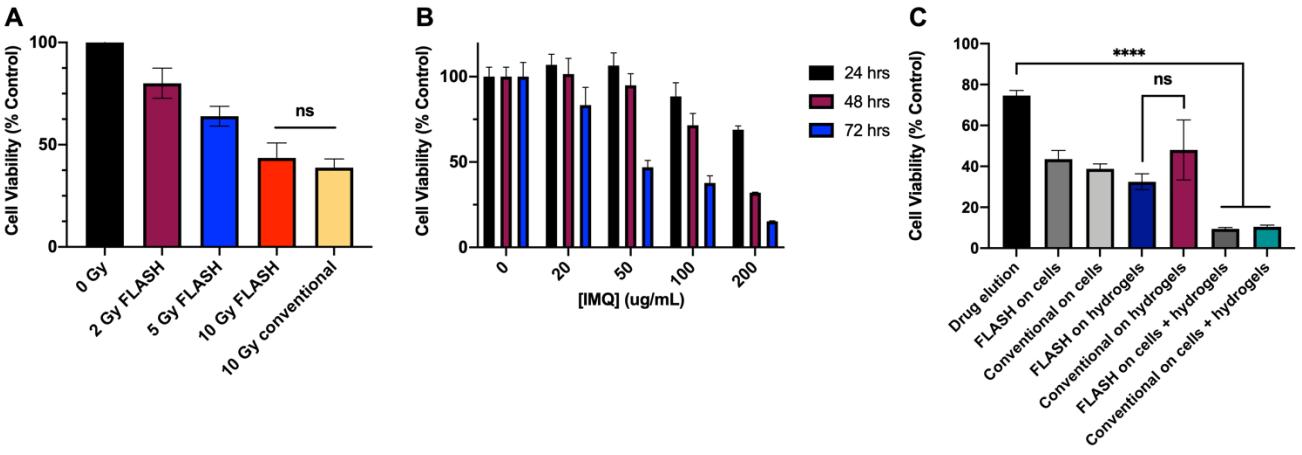

Figure S8. Viability of B16-F10 cells **A)** at 48 hours after receiving radiotherapies in a range of radiation doses. **B)** at 24, 48 or 72 hours after receiving free IMQ treatment in a range of concentrations. **C)** at 48 hours after receiving different treatments.

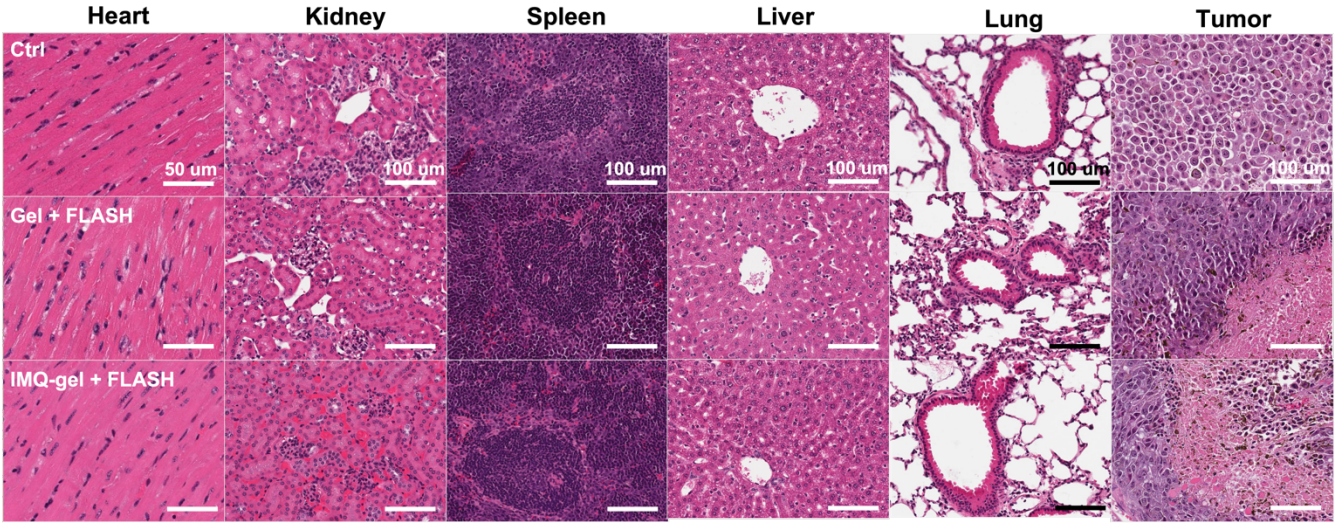

Figure S9. Representative micrographs of tissue from major organs and tumors, after H&E staining, of mice injected with DPBS (top row), treated with AuNP-gel + FLASH-RT (middle row), and AuNP-IMQ-gel + FLASH-RT (bottom row).

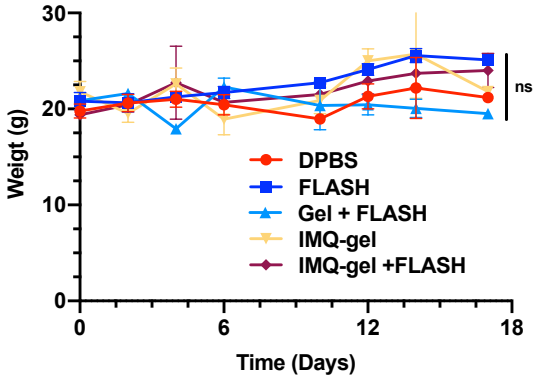

Figure S10. Body weight of control mice (injected with DPBS) and treated mice over 18 days.

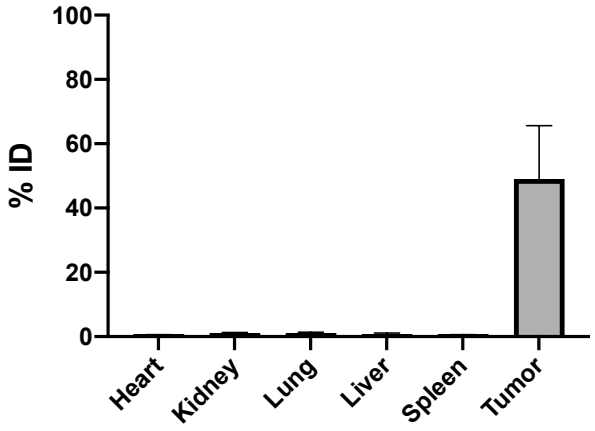

Figure S11. Biodistribution of AuNP.

References

(1) Cheheltani, R.; Ezzibdeh, R. M.; Chhour, P.; Pulaparthi, K.; Kim, J.; Jurcova, M.; Hsu, J. C.; Blundell, C.; Litt, H. I.; Ferrari, V. A.; et al. Tunable, biodegradable gold nanoparticles as contrast agents for computed tomography and photoacoustic imaging. *Biomaterials* **2016**, *102*, 87-97. DOI: https://doi.org/10.1016/j.biomaterials.2016.06.015.

(2) Bouche, M.; Dong, Y. C.; Sheikh, S.; Taing, K.; Saxena, D.; Hsu, J. C.; Chen, M. H.; Salinas, R. D.; Song, H.; Burdick, J. A.; et al. A novel treatment for glioblastoma delivered by a radiation responsive and radiopaque hydrogel. *ACS Biomater. Sci. Eng.* **2021**, *7* (7), 3209-3320.