# Treatment of orthotopic U251 human glioblastoma multiforme tumors in NRG mice by convection-enhanced delivery of gold nanoparticles labeled with the β-particle-emitting radionuclide, $^{177}$Lu

## SUPPORTING INFORMATION

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## EXPERIMENTAL SECTION

**Synthesis and *In Vitro* Characterization of $^{177}$Lu-AuNPs.** AuNPs were synthesized by the Turkevitch method [1]. Prior to synthesis, all glassware was thoroughly cleaned using Aqua Regia and rinsed with ample Milli-Q water (EMD Millipore). To synthesize AuNPs, 10 mL of 0.25 mM $HAuCl_4$ (>99.9%, Sigma-Aldrich) aqueous solution was boiled and refluxed in a round bottom flask equipped with a condenser, water bath, and hot plate stirrer (Fisher Scientific) for 15 min. Then 100 µL of 500 mM freshly prepared sodium citrate (>99%, Sigma-Aldrich) aqueous solution was quickly added, and the solution continuously stirred and boiled until the color turned ruby red, indicating formation of AuNPs. Morphology and particle size of the AuNPs were assessed by transmission electron microscopy (TEM). Aqueous solutions of AuNPs were mounted on copper grids and imaged using a Talos L120C Transmission Electron Microscope (ThermoScientific) equipped with a BM-Ceta camera at 120 kV and 45,000 × magnification. The diameters of AuNPs on TEM images were measured using ImageJ. AuNPs were conjugated to a metal-chelating polymer (MCP) for complexing $^{177}$Lu, as described in the main manuscript. Citrate-stabilized AuNPs and MCP-conjugated AuNPs were characterized by UV-visible spectroscopy on an Ultraspec 3100 Pro spectrophotometer (GE Healthcare/Amersham Pharmacia). Particle size distribution and zeta potential were measured by dynamic light scattering (DLS) on a Zetasizer Nano-ZS instrment (Malvern Instruments). The concentration of AuNPs was determined by UV-visible absorbance at 524 nm and the extinction coefficient of AuNPs [2]. The number of AuNPs used in the conjugation reaction was calculated by the volume of AuNPs used and its concentration. The radioactivity bound to AuNPs in the pellet after centrifugation of the conjugation reaction mixture at 15,000 × g for 15 mins was measured using a radioisotope dose calibrator (CRC15R®, Capintec) to estimate the number of $^{177}$Lu-MCP bound to AuNPs based on the known specific

activity of $^{177}$LuMCP and molecular weight of MCP (12,000 Da). The average number of $^{177}$Lu-MCP bound per AuNP was calculated by dividing the number of $^{177}$Lu-MCP bound to AuNPs by the number of AuNPs.

**Clonogenic Survival and DNA Double-Strand Breaks.** The cytotoxicity of $^{177}$Lu-AuNPs *in vitro* on U251-Luc human GBM cells was evaluated by a clonogenic survival assay. Briefly, $1 \times 10^5$ U251-Luc human GBM cells were cultured overnight in 24-well plate. The medium was then removed and the cells treated for 16 h with $^{177}$Lu-AuNPs at increasing specific activity (0.0-2.0 MBq, $4 \times 10^{11}$ AuNPs) in 1 mL of fresh culture medium. The cells were recovered and sufficient cells (250-4,000 cells) were seeded into wells in 6-well dishes containing 3 mL of medium per well to obtain a measureable number of surviving colonies after culturing at 37 $^{o}$C/5% $CO_2$ for 10 d. The colonies were stained with methylene blue (1% in a 1:1 mixture of ethanol and water) and imaged on a ChemiDoc Imaging System (BioRad). Colonies (>50 cells) were counted using ImageJ software and a customized macro [3]. The plating efficiency (PE) was defined as the number of colonies formed divided by the number of cells seeded. The surviving fraction (SF) was calculated by dividing the PE of treated cells by untreated cells. The SF was plotted vs. the activity (MBq) of $^{177}$Lu-AuNPs incubated with the cells and the resulting survival curve was fitted to a linear quadratic model by Prism Ver. 9.2 (GraphPad).

DNA double-strand breaks (DSBs) in U251-Luc cells caused by the β-particles emitted by $^{177}$Lu were identified by confocal immunofluorescence microscopy for phosphorylated histone-2AX (γH2AX) as previously reported [4]. Briefly, cells treated with $^{177}$Lu-AuNPs, unlabeled MCP-conjugated AuNPs or unlabeled MCP were probed with primary anti-phospho-histone H2AX (Ser139, clone JBW301) mouse monoclonal $IgG_1$ (Upstate Biotechnology) then with secondary Alexa Fluor 488 donkey anti-mouse IgG (H+L) (Invitrogen). VECTASHIELD Mounting Medium

with DAPI (Vector Laboratories) was used to counterstain the nuclei blue. Images of γH2AX foci and nuclei were acquired using a confocal microscope (LSM 700) with LSM Zen acquisition software, and using a Plan-Apochromat 63×/1.4 oil DIC M27 objective. At least 30 cells were imaged per slide. Images were processed to derive the mean integrated density of γH2AX foci per unit nucleus area using Volocity (Quorum Technologies Inc., Version 6.5.1). Nuclei and γH2AX foci were identified by setting minimum object size of 50 $\mu m^2$ and 0.01 $\mu m^2$, and low intensity threshold of 100 but no upper bound was set. Foci intersections with nuclei were identified as "true foci" and the total intensity and area was multiplied to obtain the integrated density which was divided by the nucleus area. Results were expressed as fold increase of γH2AX foci, where the density of γH2AX foci of untreated cells was considered baseline.

**Model for Estimation of Radiation Absorbed Doses**

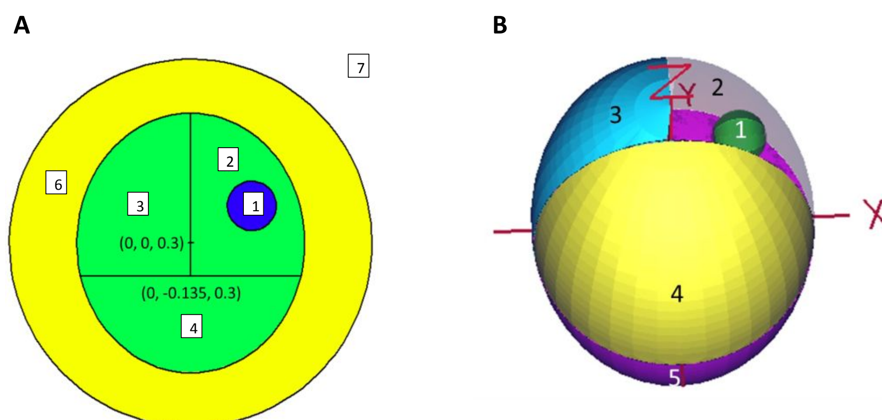


**Figure S1**. Schematic geometry used in the MCNP simulation. (A) x-y plane through the center (0.25, 0.15, 0.30 cm) of a 0.2 cm diameter tumor sphere (blue, labeled 1) in the mouse brain (green) enclosed by a 1.6 cm diameter spherical buffer (yellow, labeled 6) representing the mouse head,

while region 7 is the void space to stop particle transport. (B) 3D dyanamic plotting showing an ellipsoid with principle semi-axis of 0.528, 0.594 and 0.639 cm, respectively, the upper half of which represents the mouse brain connsisting of 4 regions: 1) tumor (labeled 1,dark green), right hemisphere excluding the tumor (labeled 2, transparent grey), non-tumor bearing left hemisphere (labeled 3, cyan) and cerebellum (labeled 4, yellow), and the lower half of which represents the other half of the head excluding the brain (labeled 5, purple). Both regions 6 and 7 indicated in panel A were selected to be hidden in panel B to allow a clear view of regions 1-5. Both A and B were generated using MCNPX version 2.7.E

## RESULTS

**Synthesis and *In Vitro* Characterization of $^{177}$Lu-AuNPs.** TEM revealed that the AuNPs had spherical morphology with a mean diameter of 23 ± 3 nm, range 16-29 nm (**Figure S2A,B**). UV-visible spectra of AuNPs and MCP-conjugated AuNPs showed absorbance peaks at 521 nm and 523 nm, respectively (**Figure S2C**). The absorbance of citrate-stabilized AuNPs aqueous solution was 1.365 at 524 nm. Assuming 100% conversion of $HAuCl_4$ to AuNPs, the concentration of AuNPs was calculated as $4.05 \times 10^{11}$ AuNPs/mL ($6.72 \times 10^{-10}$ M). Thus, the molar absorption coefficient was $2.03 \times 10^{9}$ $M^{-1}cm^{-1}$, which was in good agreement with published molar absorpton coefficients for citrate-stabilized solutions of 21 nm AuNPs ($8.78 \times 10^{8}$ $M^{-1}cm^{-1}$) or 25 nm AuNPs ($2.93 \times 10^{9}$ $M^{-1}cm^{-1}$) [2]. DLS revealed an increase in the z-average diameter and zeta potential after conjugation of MCP to AuNPs (29.2 vs. 19.9 nm, respectively and -17.8 mV vs. -40.0 mV, respectively). Both AuNPs and MCP-conjugated AuNPs showed a narrow size distribution by DLS, with a polydispersity index (PdI) of 0.126 and 0.162, respectively (**Figure S2D**). The labeling efficiency of the MCP with $^{177}$Lu was 92 ± 5 % and the conjugation efficiency of $^{177}$Lu-MCP to

AuNPs was 32 ± 6 %. The average number of MCP conjugated per AuNP was 197 ± 36.

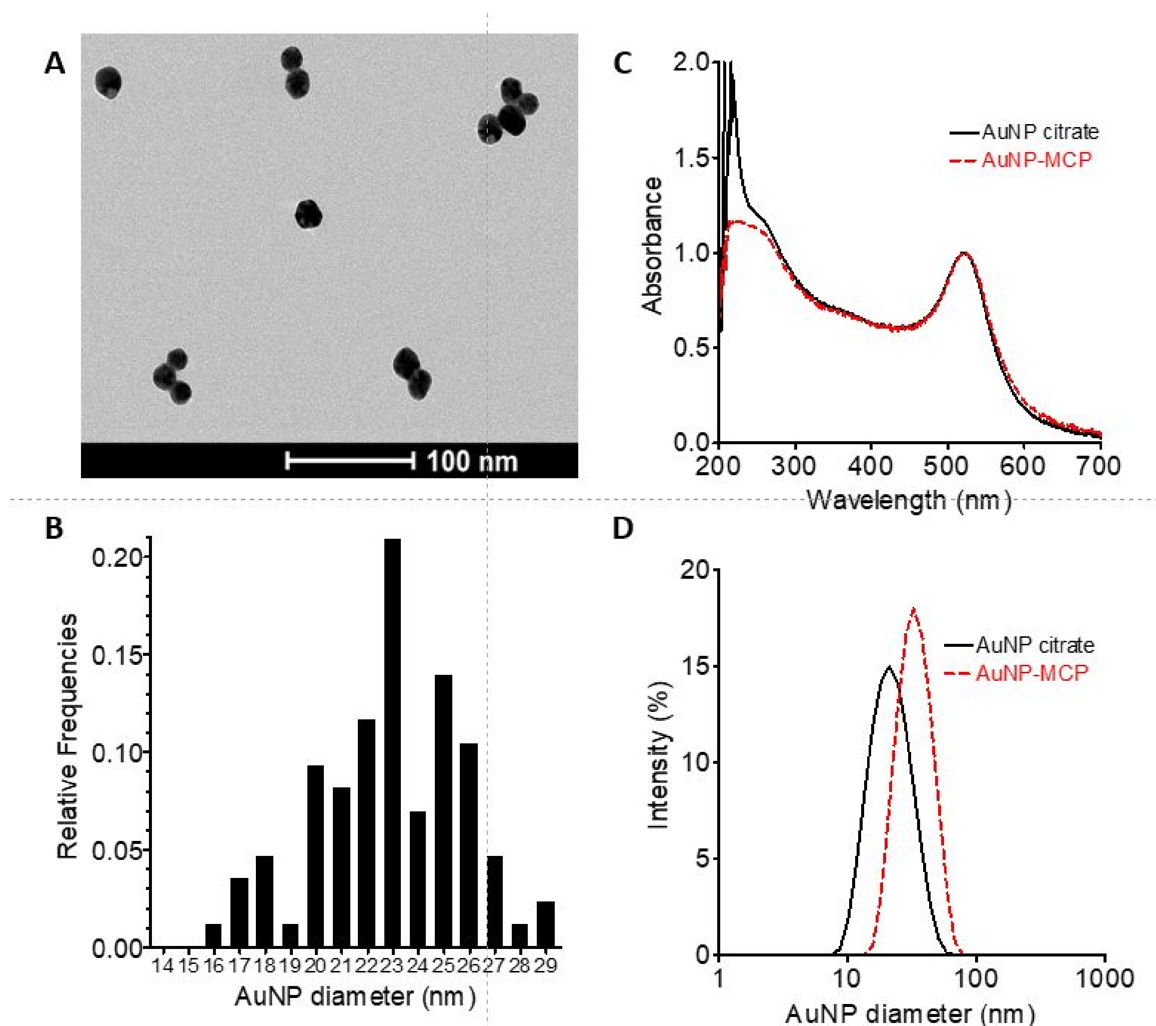


**Figure S2.** (A) TEM images of synthesized AuNPs. (B) Size distribution measured by TEM. (C) UV-visible spectra of AuNPs and AuNPs conjugated to MCP. (D) Size distribution of AuNPs and MCP-conjugated AuNPs measured by DLS.

**Clonogenic Survival and DNA Double-Strand Breaks.** The SF of U251-Luc cells treated with $^{177}$Lu-AuNPs *in vitro* at increasing specific activity (0.0-2.0 MBq, $4 \times 10^{11}$ AuNPs) is shown in

**Figure S3A**. At the highest specific activity, the SF was dramatically reduced to 0.005 ± 0.002. This reduction in SF was correlated with a 14.3-fold significant increase ($P<0.05$) in DNA DSBs in the nucleus of U251-Luc cells measured by immunofluorescence for γH2AX compared to untreated cells (**Figure S3B**). There was no significant increase in γH2AX foci for U251-Luc cells treated with unlabeled MCP or unlabeled AuNPs. Representative immunofluorescence microscopy images probing for γH2AX foci in the nucleus of U251-Luc cells treated with unlabeled MCP, unlabeled AuNPs or $^{177}$Lu-AuNPs or no treatment are shown in **Figure S3C**.

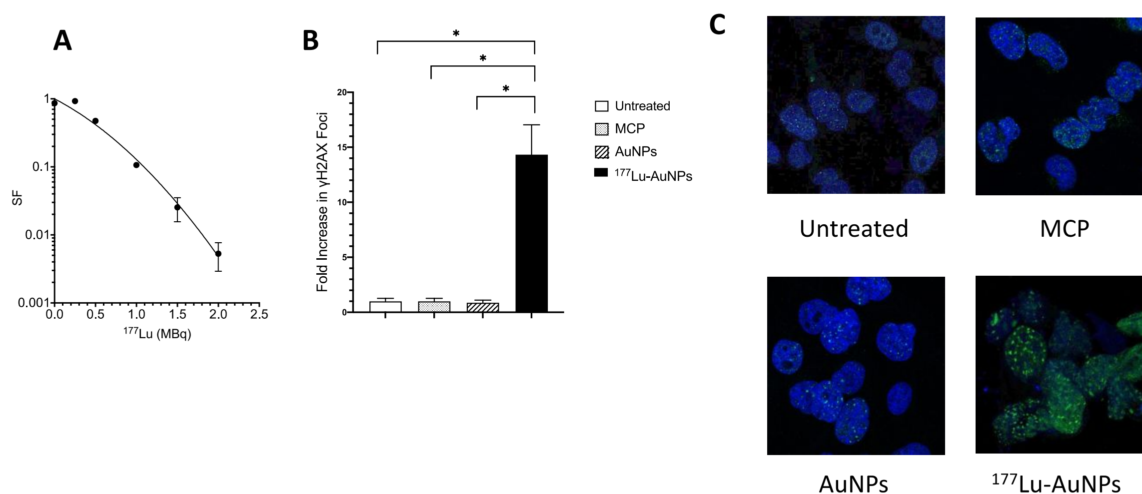


**Figure S3.** (A) Clonogenic survival fraction (SF) of U251-Luc cells treated *in vitro* with increasing specific activity (0.0-2.0 MBq, 4 × $10^{11}$ AuNPs) of $^{177}$Lu-AuNPs. (B) Increase in γH2AX foci in the nucleus of U251-Luc cells treated at the highest specific activity (2.0 MBq, 4 × $10^{11}$ AuNPs) of $^{177}$Lu-AuNPs or with unlabeled MCP (1 μg) or unlabeled AuNPs (4 × $10^{11}$ AuNPs) in 1 mL of medium. Significant differences (P<0.05) are indicated by the asterisks. (C) Representative images of γH2AX foci (green) in the nucleus (blue) of untreated U251-Luc cells or U251-Luc cells treated

with unlabeled MCP (1 μg), unlabeled AuNPs ($4 \times 10^{11}$ AuNPs) or $^{177}$Lu-AuNPs (2.0 MBq, $4 \times 10^{11}$ AuNPs).

**Whole Body SPECT/CT Images**

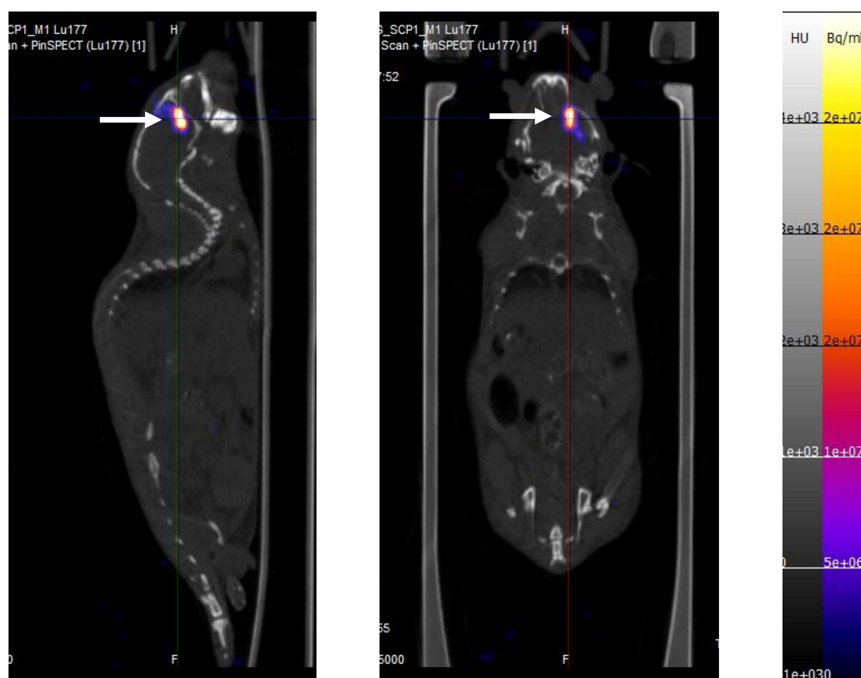


**Figure S4.** Sagittal (left) and coronal (right) SPECT/CT whole body images in a representative NRG mouse with a U251-Luc human GBM tumor at 14 d after CED of 1.0 MBq of $^{177}$Lu-AuNPs ($4 \times 10^{11}$ AuNPs). The image intensity bar is shown at the right. Activity is confined to the site of CED infusion of $^{177}$Lu-AuNPs (arrows) in the brain with no visible redistribution to other regions of the body.

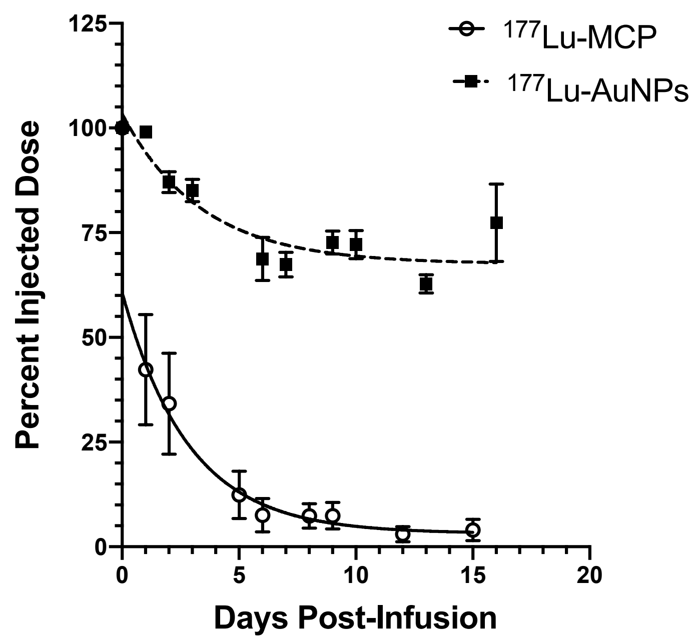


**Figure S5.** Whole body retention of activity in NRG mice with a U251-Luc human GB tumor after CED of 1.0 MBq of $^{177}$Lu-AuNPs (4 × $10^{11}$ AuNPs) or $^{177}$Lu-MCP.

## REFERENCES

(1) Wuithschick, M.; Birnbaum, A.; Witte, S.; Sztucki, M.; Vainio, U.; Pinna, N.; Rademann, K.; Emmerling, F.; Kraehnert, R.; Polte, J. Turkevich in new robes: key questions answered for the most common gold nanoparticle synthesis. *ACS Nano* **2015**, *9*, 7052-7071.

(2) Liu, X. O.; Atwater, M.; Wang, J. H.; Huo, Q. Extinction coefficient of gold nanoparticles with different sizes and different capping ligands. *Colloids and Surfaces B-Biointerfaces* **2007**, *58*, 3-7.

(3) Cai, Z.; Chattopadhyay, N.; Liu, W. J.; Chan, C.; Pignol, J. P.; Reilly, R. M. Optimized digital counting colonies of clonogenic assays using ImageJ software and customized macros: comparison with manual counting. *Int J Radiat Biol* **2011**, *87*, 1135-1146.

(4) Cai, Z.; Vallis, K. A.; Reilly, R. M. Computational analysis of the number, area and density of gamma-H2AX foci in breast cancer cells exposed to $^{111}$In-DTPA-hEGF or gamma-rays using Image-J software. *Int J Radiat Biol* **2009**, *85*, 262-271.