

Electronic Supplementary Information (ESI)

Targeted tumor SPECT/CT dual mode imaging using multifunctional RGD-modified low generation dendrimer-entrapped gold nanoparticles†

Xiaoying Xu,^{a, 1} Lingzhou Zhao,^{b, 1} Xin Li,^a Peng Wang,^a Jinhua Zhao,^{*b} Xiangyang Shi,^{*a,c}
Mingwu Shen^{*a}

^a State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, People's Republic of China

^b Department of Nuclear Medicine, Shanghai General Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200080, People's Republic of China

^c CQM-Centro de Química da Madeira, Universidade da Madeira, Campus da Penteada, 9000-390 Funchal, Portugal

* To whom correspondence should be addressed. E-mail: zhaojinhua1963@126.com (J. Zhao), xshi@dhu.edu.cn (X. Shi) and mwshen@dhu.edu.cn (M. Shen).

¹ Authors contributed equally to this work.

Experimental section

Materials

2,2'-(7-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (NOTA-NHS ester) was purchased from CheMatech (Dijon, France). N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and sodium borohydride (NaBH_4) were from J&K Chemical Ltd. (Shanghai, China). Ethylenediamine core amine-terminated generation 2 poly(amidoamine) (PAMAM) dendrimers (G_2NH_2) were acquired from Dendritech (Midland, MI). Thiolated cyclic arginine-glycine-aspartic (Arg-Gly-Asp, RGD) peptide ($M_w = 690.93$) was purchased from GenicBio (Shanghai, China). $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$ was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). PEG with maleimide group at one end and carboxyl group at the other end (MAL-PEG-COOH, $M_w = 2000$) and PEG monomethyl ether with carboxyl terminal group (*m*PEG-COOH, $M_w = 2000$) were supplied by Shanghai Yanyi Biotechnology Corporation (Shanghai, China). $^{99\text{m}}\text{Tc}$ -pertechnetate was acquired from Shanghai GMS Pharmaceutical Co., Ltd. (Shanghai, China). Stannous chloride (SnCl_2), dimethyl sulfoxide (DMSO) and all other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. C6 glioma cells (a rat C6 glioma cell line) were obtained from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Cell counting kit-8 (CCK-8) was supplied by 7Sea Pharmatech Co., Ltd. (Shanghai, China). Calcein-AM was obtained from KeyGEN BioTECH. Co., Ltd. (Nanjing, China). The water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) and had a

resistivity greater than 18.2 M Ω ·cm. Regenerated cellulose dialysis membranes possessing molecular weight cut-offs (MWCOs) of 1000, 3000, and 8000 were acquired from Shanghai Yuanye Biotechnology Corporation (Shanghai, China).

Synthesis of the $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs

MAL-PEG-COOH (150.0 mg, dissolved in 6 mL DMSO) was mixed with equal molar equiv. RGD peptide (52.4 mg) predissolved in DMSO (3 mL) under stirring for 24 h. Then the mixture was dialyzed against water (9 times, 2 L) for 3 days *via* a cellulose dialysis membrane with an MWCO of 1000, followed by lyophilization to get RGD-PEG-COOH product. To obtain a raw product of G2-NOTA dendrimers, G2.NH₂ (30.0 mg) dispersed in 5 mL DMSO was reacted with 5 molar equiv. NOTA-NHS ester (30.4 mg, dispersed in 4 mL DMSO) under stirring for 24 h. Then the raw product of G2-NOTA dendrimers was mixed with 10 molar equiv. RGD-PEG-COOH (184.2 mg) preactivated *via* EDC (79.5 mg) and NHS (47.8 mg) in 5 mL water, and the reaction was continued for 3 days under magnetic stirring. The mixture was dialyzed against water (9 times, 2 L) for 3 days *via* a cellulose dialysis membrane with an MWCO of 3000. After lyophilization, the G2-NOTA-PEG-RGD dendrimers were acquired and then used as templates to entrap Au NPs through NaBH₄ reduction chemistry according to the literature.¹ The gold salt/dendrimer molar ratio was set at 6:1. For comparison, the non-targeted $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs without RGD modification were also obtained.

^{99m}Tc labeling of the $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs

The $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs (30 mg) and reductant SnCl₂ (10 mg) were co-dissolved in 200 μ L of phosphate buffered saline (PBS), and a sterile ^{99m}Tc-pertechnetate (111 MBq, 0.8 mL) solution was rapidly added into the above solution. The mixture was continuously stirred for 30 min at room temperature. The $\{(Au^0)_6\text{-G2-NOTA}(\text{}^{99m}\text{Tc})\text{-PEG-RGD}\}$ DENPs were purified

using PD-10 desalting columns (GE Pharmacia, GE Inc., Fairfield, CT) according to the literature.² The non-targeted $\{(Au^0)_6\text{-G2-NOTA}^{(99mTc)\text{-}m\text{PEG}}\}$ DENPs were also obtained under the same conditions.

Characterization techniques

¹H NMR spectra were recorded on a Bruker AV400 nuclear magnetic resonance spectrometer (Karlsruhe, Germany). D₂O was used as a solvent to dissolve samples before measurements. Zeta potential and dynamic light scattering (DLS) were carried out using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, U.K.) coupled with a standard 633 nm laser. Leeman Prodigy inductively coupled plasma optical emission spectroscopy (ICP-OES) (Teledyne Leeman Labs, Hudson, NH) was used to measure the Au concentration of the particle suspension. UV-vis spectroscopy was implemented using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Boston, MA). Before the experiments, all samples were dissolved in water. Transmission electron microscopy (TEM) imaging was executed using a JEOL 2010F analytical electron microscope (JEOL, Tokyo, Japan) at an operating voltage of 200 kV. TEM samples were prepared by dropping an aqueous particle suspension (1 mg/mL) onto a carbon-coated copper grid, and the aqueous suspension was air-dried before measurements. CT scans were performed using a GE LightSpeed VCT imaging system (GE Medical Systems, Milwaukee, WI) with 100 kV, 220 mA, and a slice thickness of 1.25 mm according to the literature.² $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs and Omnipaque (300 mg·mL⁻¹, used as control, GE Healthcare, Princeton, NJ) with Au or iodine concentration range of 0.005-0.04 M were placed in 1.5 mL Eppendorf tubes. The radioactivity was measured by a CRC-15R radioisotope dose calibrator (Capintec, Inc., Ramsey, NJ).

Radiochemical purity and radiostability evaluation

The radiochemical purity of the $\{(Au^0)_6\text{-G2-NOTA}^{(99mTc)\text{-}PEG\text{-RGD}\}$ DENPs was measured

by instant thin layer chromatography (ITLC) using silica gel-coated fiber glass sheets (Macherey-Nagel, GmbH & Co. KG, Düren, Germany). Saline was utilized as the mobile phase, and the sheets were analyzed with a thin layer chromatogram scanner (Bioscan Inc., Tucson, AZ). Radiostability of the $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-PEG-RGD}\}$ DENPs (200 μL , 1 $\text{mg}\cdot\text{mL}^{-1}$ in PBS) was assessed by checking the radiochemical purity at different time points (1, 6, and 12 h) at room temperature *via* ITLC.

***In vitro* cytotoxicity and cellular uptake assays**

C6 glioma cells were regularly cultured and passaged in DMEM supplemented with 10% FBS, 100 $\text{U}\cdot\text{mL}^{-1}$ penicillin, and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin at 37 °C and 5% CO_2 .

To assess the cytotoxicity of the $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ and $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs, C6 glioma cells were seeded into a 96-well culture plate at a density of 1×10^4 cells per well with 200 μL DMEM for 24 h. Then the medium was substituted with fresh medium containing $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ or $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs with different Au concentrations (200, 500, 1000, 2000, 4000 nM, respectively), followed by incubation for 24 h (PBS was used as control). Then the cells were rinsed with PBS for 3 times and incubated with DMEM containing 10% CCK-8 solution (100 μL) for each well of cells. The cells were then cultured for an additional 4 h. The absorbance of each well was recorded using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA) at 450 nm. For each sample, mean and standard deviation (SD) of six parallel wells were recorded.

To further confirm the cytocompatibility of the $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ and $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs, C6 glioma cells were treated with the particles at various Au concentrations (20, 500, 1000, 2000, 4000 nM, respectively) for 24 h. The cells were rinsed three times with PBS and added with 100 μL DMEM containing 0.125% calcein-AM for each well. After staining for 15

min, the cells were rinsed three times with PBS and observed by Zeiss Axio Vert. A1 inverted fluorescence microscope (Carl Zeiss, Jena, Germany) with a magnification of 100× for each sample.

ICP-OES analysis was performed to quantify the specific cellular uptake of the $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs in C6 glioma cells. Briefly, 2×10^5 cells were seeded into each well of a 12-well culture plate for 24 h to bring the cells to confluence. After that, the medium was replaced with fresh medium containing $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ or $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs with different Au concentrations (1000 and 4000 nM), and the cells were incubated for 4 h (PBS was used as control). Next, the cells were rinsed three times with PBS, trypsinized, and centrifuged to remove the supernatant. Thereafter, all cells were digested by *aqua regia* (nitric acid/hydrochloric acid, v/v = 1: 3) for 10 min, and the solution was diluted for subsequent ICP-OES measurements of Au concentration. The cellular uptake of the $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ or $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs was quantified *via* the determination of Au concentration. For the free RGD blocking experiments, C6 glioma cells were co-incubated with 2 μM RGD for 3 h and then the cells were subjected to the same treatments as above to assess the cellular uptake.

***In vitro* CT and SPECT imaging of C6 glioma cells**

C6 glioma cells were seeded into a cell culture flask at a density of 2×10^6 cells with 2 mL DMEM for 24 h to bring the cells to confluence. Subsequently the medium was replaced with fresh medium containing PBS, $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ or $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs with different Au concentrations (1000 and 4000 nM) and the cells were incubated for another 4 h. After being rinsed 3 times with PBS, the cells were trypsinized, centrifuged, and resuspended in 0.5 mL PBS in 1.5 mL Eppendorf tubes. The cell suspensions were scanned using a GE LightSpeed VCT imaging system (GE Medical Systems, Milwaukee, WI) with 100 kV, 220 mA, and a slice thickness of 1.25 mm.

For SPECT imaging, C6 glioma cells were cultured in the same way as described above, followed by incubation with fresh medium containing $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-PEG-RGD\}$ or $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-mPEG\}$ DENPs with different radio dose levels (37.25, 72.5, 125, 150, 300 and 600 $\mu Ci \cdot mL^{-1}$, respectively) for 4 h. Thereafter, the cells were treated under the above conditions and SPECT scanned according to our previous work.³

SPECT/CT imaging of a xenografted tumor model *in vivo*

BALB/c nude mice were purchased from the Shanghai SLAC Laboratory Animal Center (Shanghai, China). To establish the xenografted tumor model, male 4-6-week-old BALB/c nude mice (15-20 g) were subcutaneously injected with 5×10^6 C6 glioma cells per mouse in the right forelegs. When the tumor nodules reached a volume of 0.6-1.0 cm^3 at about 4 weeks postinjection, the mice were anesthetized with 4% isoflurane in oxygen by mask, and then the $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-PEG-RGD\}$ or $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-mPEG\}$ DENPs were intravenously injected into each nude mouse *via* the tail vein ($[Au] = 0.1 M$, $[^{99m}Tc] = 140 MBq \cdot mL^{-1}$, in 150 μL PBS). Subsequently, the nude mouse was placed in an anesthetic chamber confined in a gantry and maintained with 2% isoflurane. SPECT/CT scanning of the mice was carried out using a Nano SPECT/CT PLUS small animal imaging tomographic γ -camera (Bioscan Inc., Washington, DC) with a tube voltage of 80 kV, tube current of 450 μA , and slice thickness of 45 μm .

Biodistribution

To assess the biodistribution of the $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-PEG-RGD\}$ or $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-mPEG\}$ DENPs, the tumor-bearing BALB/c nude mice were intravenously injected with the particles ($[Au] = 0.1 M$, in 150 μL PBS). The mice were euthanized at 24 h postinjection and the major organs including the heart, liver, spleen, lung, kidney and tumor were extracted and weighed. The organs were cut into small pieces and digested by *aqua regia* for 2 days before ICP-

OES quantification of the Au element. For comparison, tumor-bearing mice injected with PBS (150 μ L) were used as control.

Histological examinations

Healthy BALB/c nude mice were intravenously injected with PBS containing $\{(Au^0)_6$ -G2-NOTA(^{99m}Tc)-PEG-RGD $\}$ or $\{(Au^0)_6$ -G2-NOTA(^{99m}Tc)-*m*PEG $\}$ DENPs ($[Au] = 0.1$ M, in 150 μ L PBS) through the tail vein (PBS was used as control). After 2 weeks treatment, the mice were anesthetized and sacrificed, and their liver, lung, spleen, heart, and kidney were harvested. Then these organs were fixed, embedded, sectioned, and stained with H&E according to protocols reported in the literature.⁴ Finally, the organ sections were observed under a Leica DM IL LED inverted phase contrast microscope (Wetzlar, Germany) at a magnification of 100 \times for each sample.

Statistical analysis

One-way ANOVA statistical analysis was performed to evaluate the significance of the experimental data. A value of 0.05 was selected as the significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Zeta potentials and hydrodynamic sizes of the $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ and $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs. Data are provided as mean \pm SD (n = 3).

Samples	Zeta potential	Hydrodynamic size	Polydispersity index (PDI)
	(mV)	(nm)	
$\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$	11.4 \pm 1.15	127.1 \pm 5.41	0.485 \pm 0.039
$\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$	9.5 \pm 0.87	139.7 \pm 2.65	0.367 \pm 0.041

Table S2. Radiochemical purity of $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-}m\text{PEG}\}$ DENPs and $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-PEG-RGD}\}$ DENPs exposed to PBS at room temperature for different time periods (0, 1, 6 and 12 h).

Sample	Radiochemical purity (%)			
	0 h	1 h	6 h	12 h
$\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-}m\text{PEG}\}$ DENPs	100	99.970	99.162	99.082
$\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-PEG-RGD}\}$ DENPs	100	100	99.080	98.909

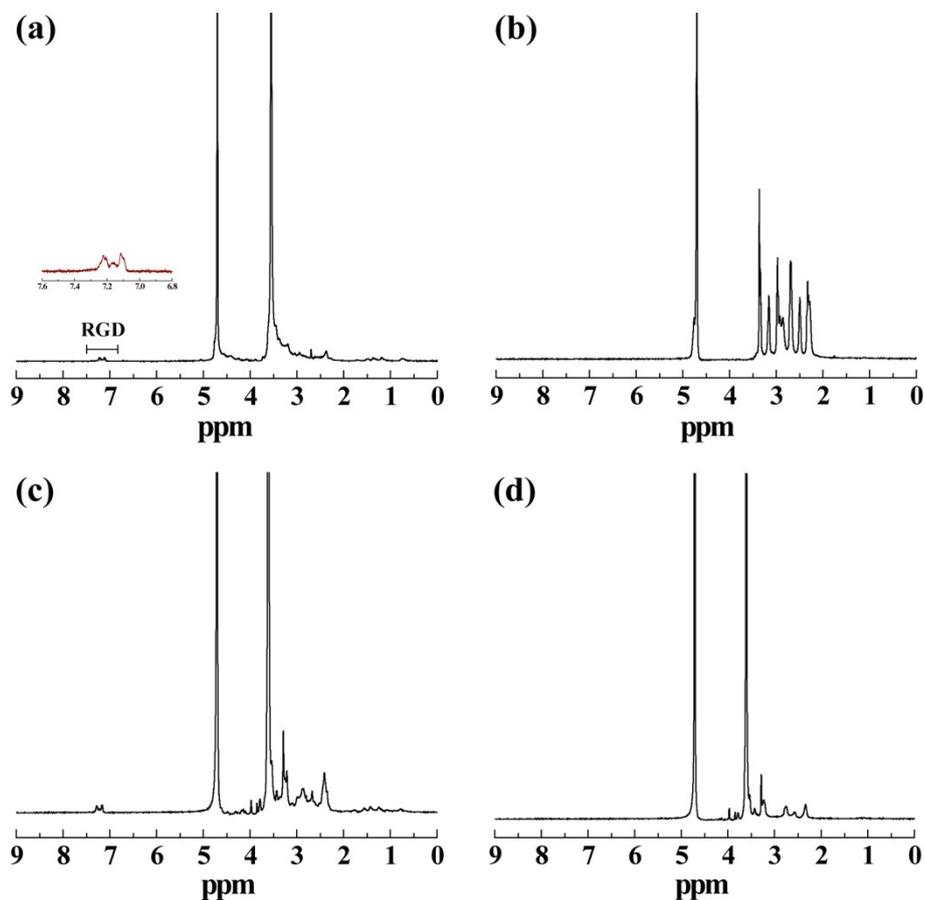


Figure S1. ¹H NMR spectra of RGD-PEG-COOH (a), G2-NOTA (b), G2-NOTA-PEG-RGD (c) and G2-NOTA-*m*PEG (d).

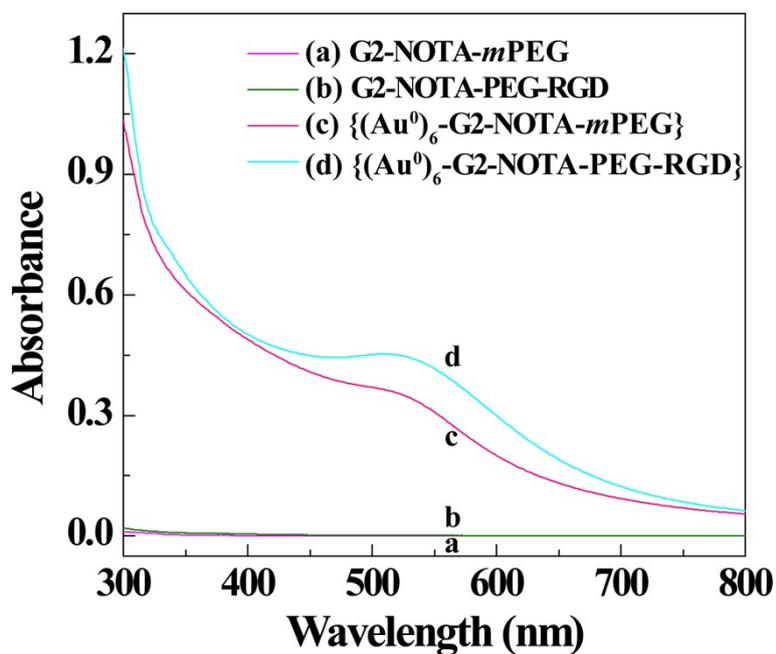


Figure S2. UV-vis spectra of G2-NOTA-*m*PEG (a), G2-NOTA-PEG-RGD (b), {(Au⁰)₆-G2-NOTA-*m*PEG} DENPs (c) and {(Au⁰)₆-G2-NOTA-PEG-RGD} DENPs (d).

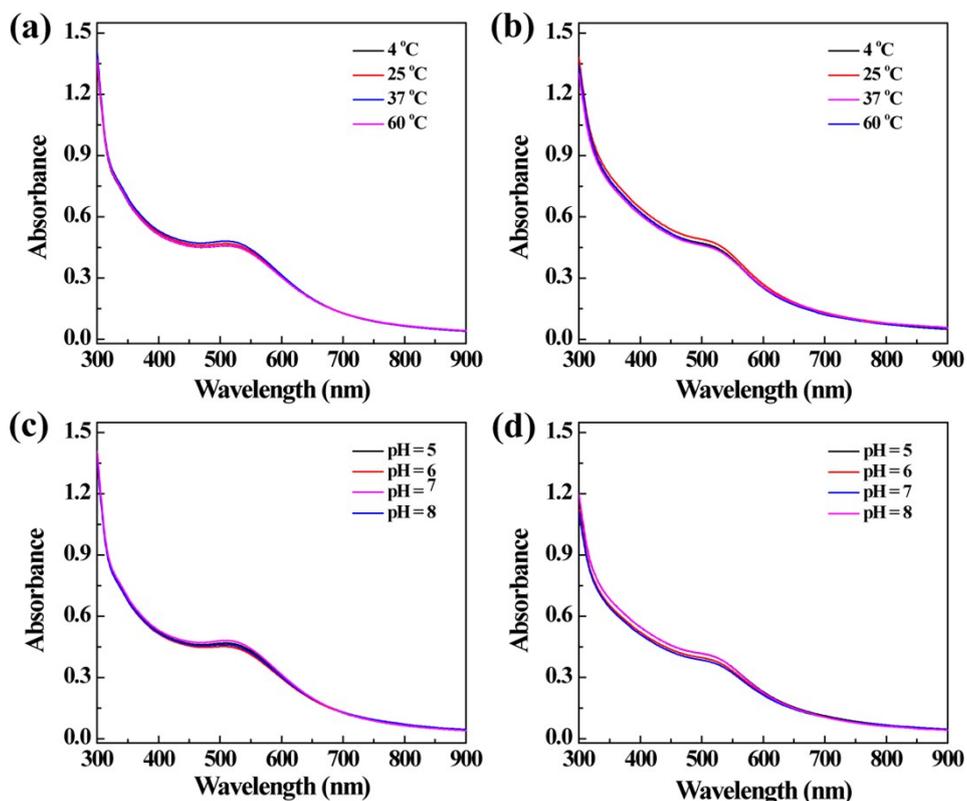


Figure S3. UV-vis spectra of $\{(\text{Au}^0)_6\text{-G2-NOTA-PEG-RGD}\}$ (a, c) and $\{(\text{Au}^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs (b, d) under different temperature (4, 25, 37 and 60 °C) and pH (pH = 5, 6, 7 and 8) conditions.

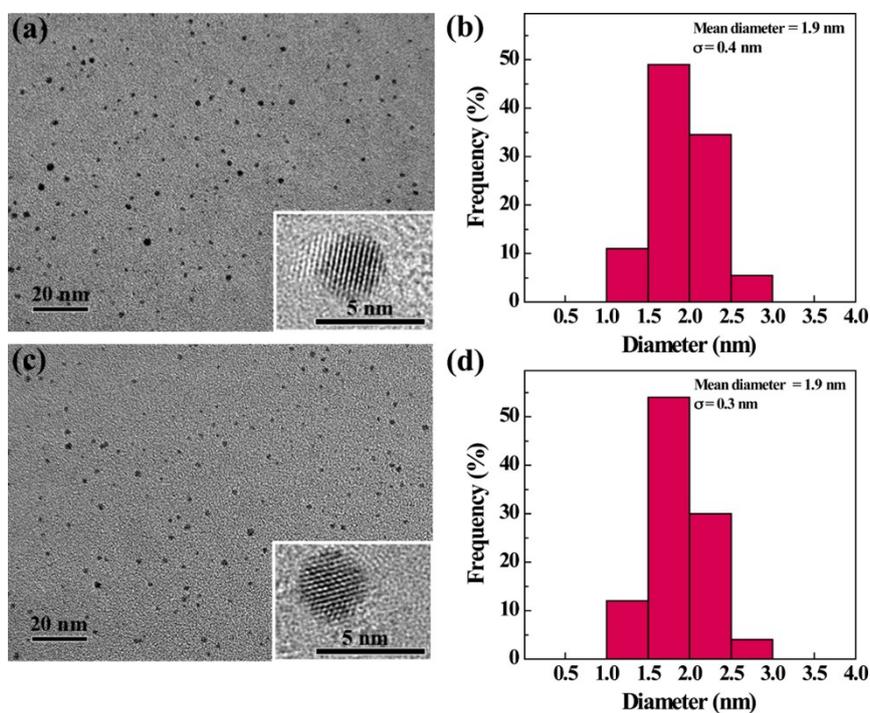


Figure S4. TEM images and size distribution histograms of the $\{(\text{Au}^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ (a, b) and $\{(\text{Au}^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs (c, d), respectively.

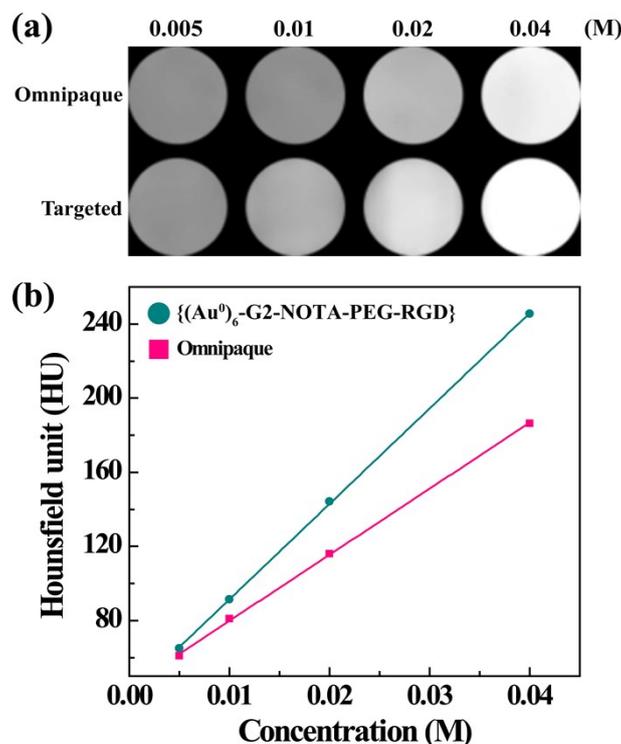


Figure S5. CT images (a) and the quantitative CT values (b) of the $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs and Omnipaque at the Au or I concentration of 0.005, 0.01, 0.02 and 0.04 M, respectively.

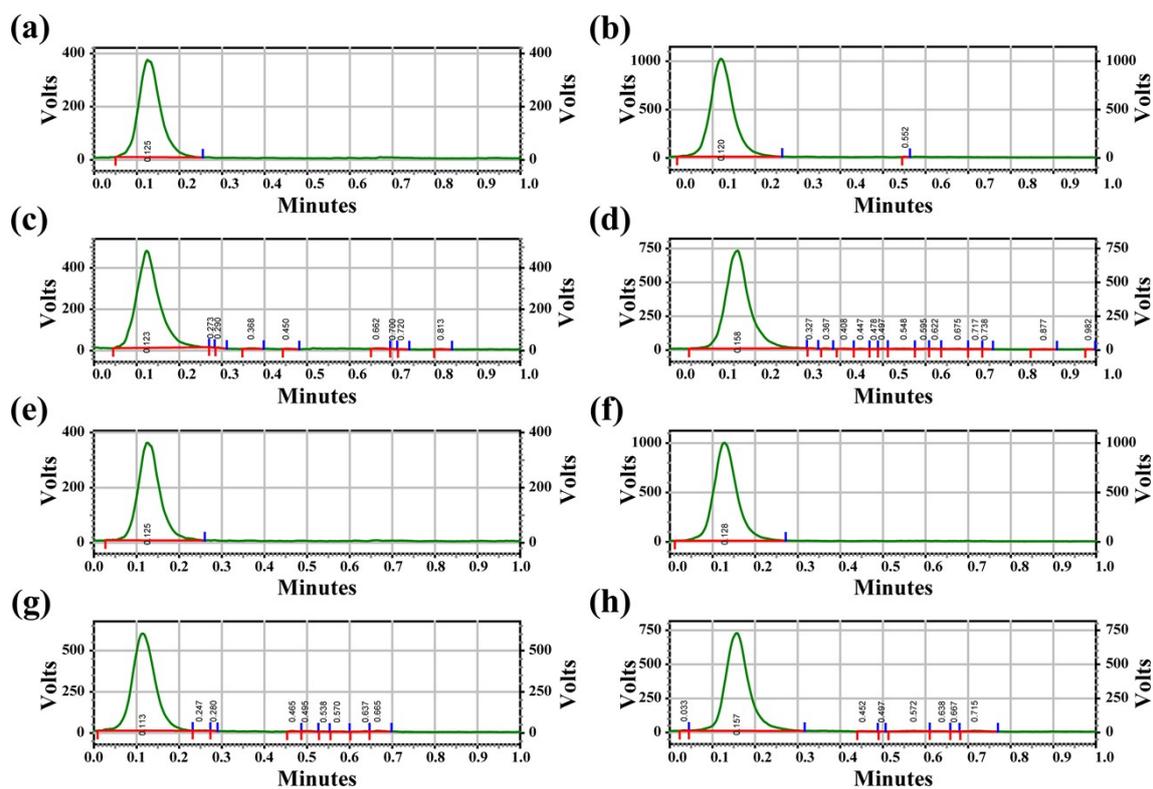


Figure S6. Radiochemical purity of the $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-}m\text{PEG}\}$ (a-d) and $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-PEG-RGD}\}$ (e-h) DENPs dispersed in PBS at 0 h (a, e), 1 h (b, f), 6 h (c, g) and 12 h (d, h), respectively at room temperature.

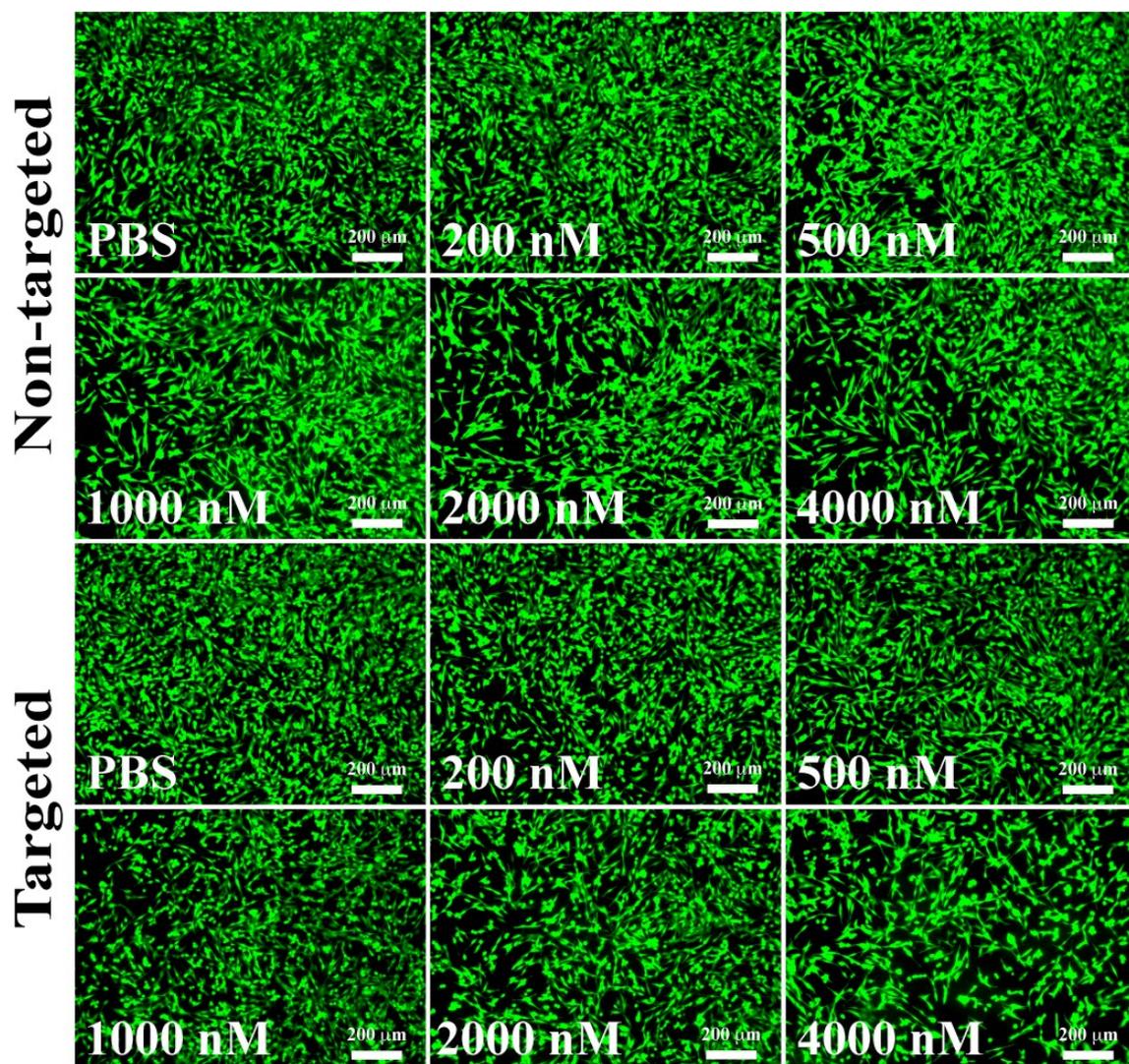


Figure S7. Fluorescence microscopic images of C6 glioma cells stained with calcein-AM after treatment with the $\{(Au^0)_6\text{-G2-NOTA-}m\text{PEG}\}$ DENPs or $\{(Au^0)_6\text{-G2-NOTA-PEG-RGD}\}$ DENPs for 24 h at the Au concentration of 0, 200, 500, 1000, 2000 and 4000 nM, respectively.

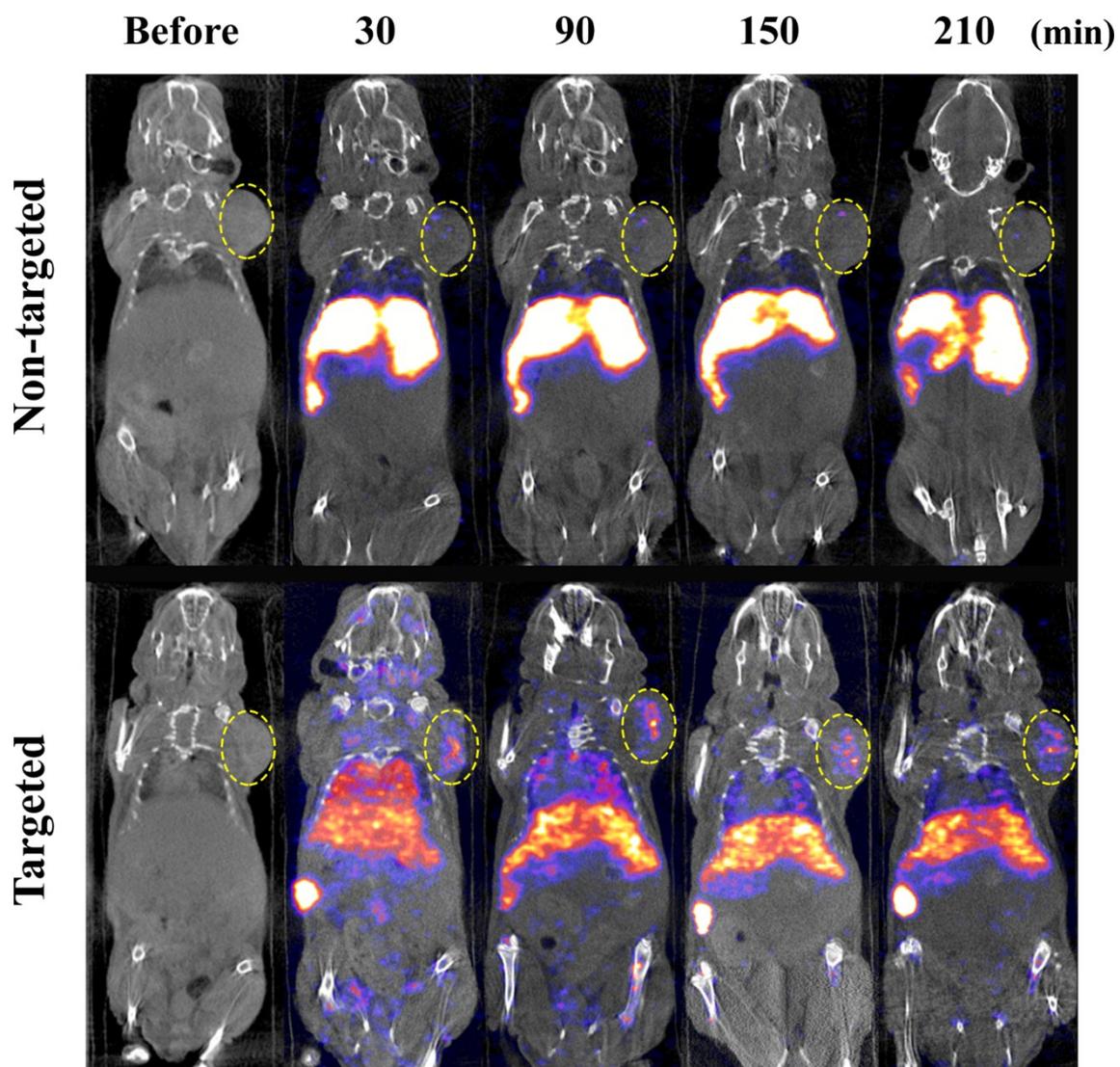


Figure S8. The cross-sectional SPECT/CT images of the C6 glioma tumor before and after intravenous injection of the $\{(Au^0)_6\text{-G2-NOTA}({}^{99m}\text{Tc})\text{-}m\text{PEG}\}$ or $\{(Au^0)_6\text{-G2-NOTA}({}^{99m}\text{Tc})\text{-PEG-RGD}\}$ DENPs ($[Au] = 0.1 \text{ M}$, $[{}^{99m}\text{Tc}] = 140 \text{ MBq}\cdot\text{mL}^{-1}$, in $150 \mu\text{L}$ PBS). The yellow circle points to the tumor site.

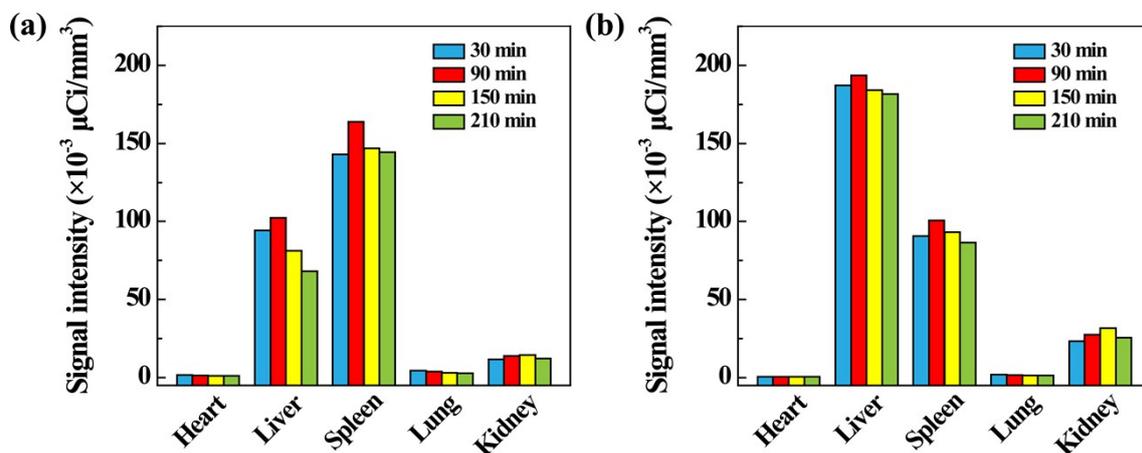


Figure S9. Quantitative SPECT signal intensity of the major organs of the mice including the heart, liver, spleen, lung and kidney at different time points post intravenous injection of the $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-PEG-RGD}\}$ or $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-mPEG}\}$ DENPs ($[Au] = 0.1 \text{ M}$, $[^{99m}\text{Tc}] = 140 \text{ MBq}\cdot\text{mL}^{-1}$, in $150 \mu\text{L}$ PBS).

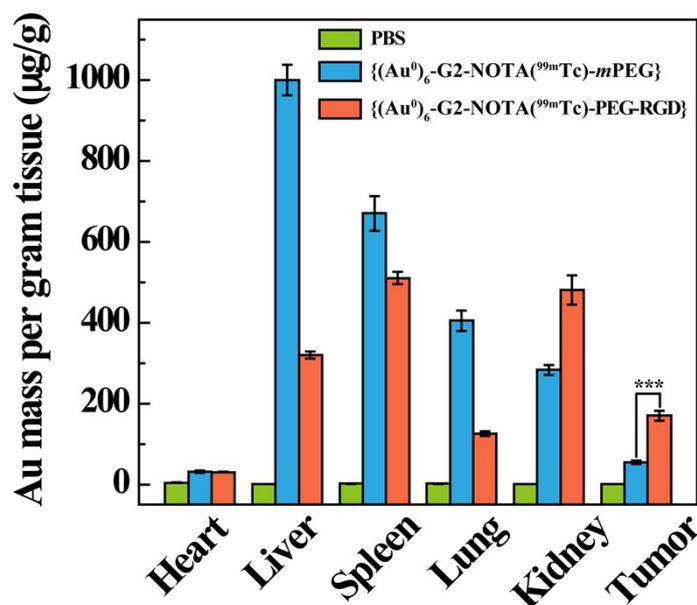


Figure S10. Biodistribution of Au element in the tumor and the major organs of the mice including the heart, liver, spleen, lung and kidney. The data were recorded from the whole organ of tumor-bearing mice taken at 24 h postinjection of the $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-mPEG}\}$ or $\{(Au^0)_6\text{-G2-NOTA}^{(99m)\text{Tc}}\text{-PEG-RGD}\}$ DENPs ($[Au] = 0.1 \text{ M}$, $[^{99m}\text{Tc}] = 140 \text{ MBq}\cdot\text{mL}^{-1}$, in $150 \mu\text{L}$ PBS for each mouse, $n = 3$).

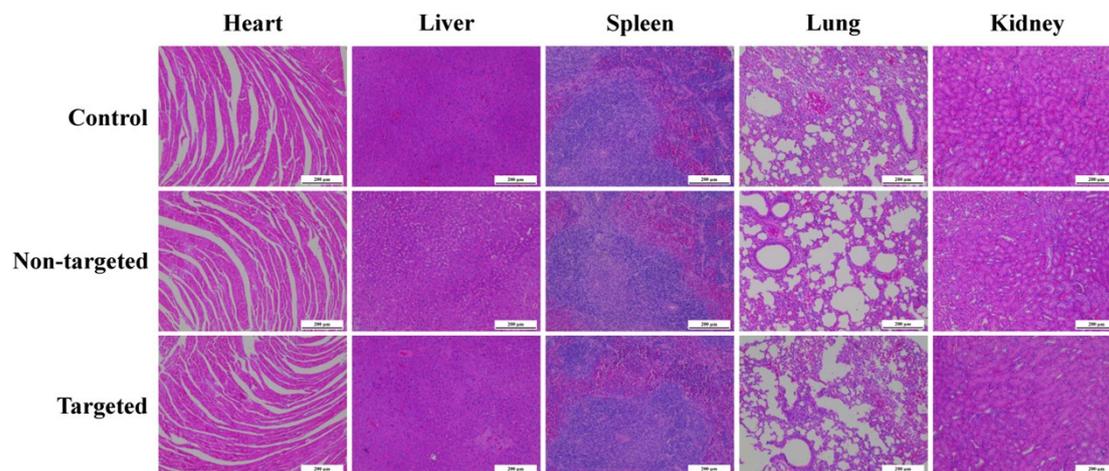


Figure S11. Histological changes in the heart, liver, spleen, lung and kidney of the mice at 2 weeks postinjection of the $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-mPEG\}$ DENPs or $\{(Au^0)_6-G2-NOTA(^{99m}Tc)-PEG-RGD\}$ DENPs ($[Au] = 0.1 \text{ M}$, $[^{99m}Tc] = 140 \text{ MBq}\cdot\text{mL}^{-1}$, in $150 \text{ }\mu\text{L}$ PBS for each mouse). Mice injected with PBS ($150 \text{ }\mu\text{L}$) were used as control. The organ sections were H&E stained and observed under Leica DM IL LED inverted phase contrast microscope at a magnification of $100 \times$ for each sample (the scale bar in each panel indicates $200 \text{ }\mu\text{m}$).

References

- 1 S. Wen, K. Li, H. Cai, Q. Chen, M. Shen, Y. Huang, C. Peng, W. Hou, M. Zhu, G. Zhang and X. Shi, *Biomaterials*, 2013, **34**, 1570.
- 2 S. Wen, L. Zhao, Q. Zhao, D. Li, C. Liu, Z. Yu, M. Shen, J. Majoral, S. Mignani, J. Zhao and X. Shi, *J. Mater. Chem. B*, 2017, **5**, 3810.
- 3 X. Li, Z. Xiong, X. Xu, Y. Luo, C. Peng, M. Shen and X. Shi, *ACS Appl. Mater. Interfaces*, 2016, **8**, 19883.
- 4 B. Zhou, Z. Xiong, J. Zhu, M. Shen, G. Tang, C. Peng and X. Shi, *Nanomedicine*, 2016, **11**, 1639.