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A unique nanogel-based platform for enhanced dual mode tumor MR/CT imaging

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Part of experimental section

Materials

AG (Mw = 12~58 kD, 350-550 mPa.s of viscosity (1%, 20 ^{o}C)) and poly(vinyl alchol) (PVA, 88% alcoholysis degree, Mw = 20-30 kD) were purchased from ACROS Organics (Geel, Belgium). N-Hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC·HCl) were from TCI (Tokyo, Japan). Polyethyleneimine (PEI, Mw = 25 kD) and Diethylenetriaminepentaacetic acid dianhydride (DTPA) were from Sigma-Aldrich (St. Louis, MO). Methoxyl PEG maleimide (*m*PEG-MAL, Mw = 2 kD) was supplied by Shanghai Yanyi Biotechnology Corporation (Shanghai, China). Dioctyl sodium sulfosuccinate (Aerosol OT, AOT) and $HAuCl_4 \cdot 4H_2O$ were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dichloromethane (DCM), acetic anhydride, triethylamine, and all other chemicals and solvents were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Shanghai, China). Annexin V-FITC/PI kit was bought form 7Sea Pharmatech Co., Ltd. (Shanghai, China). HeLa cells (a human cervical carcinoma cell line) were provided by Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM, GIBCO, Invitrogen, Carlsbad, CA), fetal bovine serum (FBS, GIBCO), penicillin-streptomycin (HyClone, Thermo Scientific, Logan, UT) and trypsin 0.25% solution (HyClone) were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Water used in all experiments with a typical resistivity higher than 18.2 MΩ.cm was purified by a laboratory water purification system (Cascada I, PALL, Beijing, China). Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 5,000 and 50, 000 were acquired from Fisher (Pittsburg, PA).

Synthesis of PEI-Au-Gd NPs

PEGylated polyethylenimine-entrapped gold nanoparticles loaded with gadolinium (PEI-Au-Gd NPs) were synthesized according to protocols reported in our previous work.[1] Firstly, DTPA (7.15 mg,

0.02 mmol, dissolved in 5 mL water) were mixed with $Gd(NO_3)_3 \cdot 6H_2O$ water solution (9.02 mg, 0.02 mmol, dissolved in 5 mL water) under continuous stirring for 24 h to obtain the DTPA-Gd complexes. Then, *m*PEG-MAL (120 mg, 0.06 mmol, dissolved in 10 mL water) and PEI (50 mg, 0.002 mmol, dissolved in 20 mL water) were mixed under continuous stirring for 24 h. Later, the reaction mixture was extensively dialyzed against water (2 L, three times per day) using a cellulose dialysis membrane with an MWCO of 5,000 for 3 days to remove the redundant reactants, followed by lyophilization to obtain the PEI-*m*PEG powder. After that, a certain amount of $HAuCl_4 \cdot 4H_2O$ water solution with 200 molar equiv. of PEI was dropped into the aqueous solution of the PEI-*m*PEG under vigorous stirring for 30 min, and next the icy water solution of $NaBH_4$ with five-fold molar excess to $HAuCl_4 \cdot 4H_2O$ was quickly added to the above mixture, followed by stirring for 3 h to obtain the raw product of PEI-*m*PEG@Au NPs. Note that the modification of *m*PEG onto the surface of PEI enables much more effective entrapment of Au within the PEI interior for better CT imaging applications.[2] Therefore, we used *m*PEG-PEI instead of PEI to entrap Au NPs in this study. Later on, the DTPA-Gd complexes with 20 molar equiv. of PEI were dropped into the above reaction mixture solution under vigorous stirring for 12 h. DTPA-Gd complexes were conjugated with PEI-*m*PEG@Au NPs througth the chemical reaction between the anhydride groups of DTPA and the surface amine groups of PEI. Then the mixture was extensively dialyzed against water for 3 days using the same procedure described above, followed by lyophilization to obtain the product of PEI-*m*PEG-DTPA(Gd)@Au NPs (for short, PEI-Au-Gd NPs). Part of the PEI-Au-Gd NPs was acetylated to neutralize the remaining PEI surface amines to obtain PEI.Ac-Au-Gd NPs according to the literature[3] for further use.

Characterization techniques

The final Gd and Au contents in the AG/PEI-Au-Gd NGs were measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Leeman Prodigy, Hudson, NH). Zeta potential and dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano ZS system (ZEN3600, Malvern, Worcestershire, UK) equipped with a standard He-Ne laser (λ = 633 nm) to

analyze the surface potential and hydrodynamic size of the NGs. To evaluate the colloidal stability of different AG/PEI-Au-Gd NGs, the hydrodynamic sizes of the NGs were also measured after they were stored at different time periods. UV-vis spectra of PEI-Au-Gd NPs and the AG/PEI-Au-Gd NGs with different AG/PEI-Au-Gd mass ratios were collected using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Boston, MA). Transmission electron microscopy (TEM) images were captured with the LibraTM 120 transmission electron microscope (Zeiss, Oberkochen, Germany) to analyze the size and morphology of the synthesized AG/PEI-Au-Gd NGs. The NG suspension ([Au] = 0.1 mM, 5 μL in water) was deposited onto carbon-coated copper grid and air dried before measurements. The composition of PEI-Au-Gd NPs and AG/PEI-Au-Gd NGs was determined using energy-dispersive X-ray (EDX) spectroscopy (OXFORD INCA ENERGY 350, Oxford Instruments, Abingdon, UK) attached to the TEM instrument. Thermal gravimetric analyses (TGA) of different samples were performed on a TG 209 F1 thermogravimetric analyzer (NETZSCH, Selb/Bavaria, Germany) from room temperature to 800 ^{o}C at a heating rate of 10 ^{o}C/min under nitrogen atmosphere. Fourier transform infrared (FTIR) spectra of AG, PEI-Au-Gd NPs, and AG/PEI-Au-Gd NGs were collected on a Nexus 670 spectrometer (Thermo Nicolet Corporation, Madison, WI) and each sample was mixed KBr crystals and grounded to form KBr pellets before measurements. The crystal structure of the AG/PEI-Au-Gd NGs was identified by X-ray diffraction (XRD) using a D/max 2550 PC X-ray diffractometer (Rigaku Cop., Tokyo, Japan) with Cu K_{α} radiation (λ = 0.154056 nm) at 40 kV and 200 mA and a 2θ scan range of 20-90^{o}.

Detection of the leakage of Gd(III) ions from the hybrid NGs

The stability of hybrid NGs was evaluated by determining the leakage of Gd(III) ions from the AG/PEI-Au-Gd NGs. In brief, the synthesized AG/PEI-Au-Gd NGs were dispersed into PBS ([Gd] = 0.2 mM) and stored at 37 ^{o}C for 1, 3, and 7 days. At each time point, the NGs suspension was centrifuged (15000 rpm, 10 min) to acquire the supernatants. The Gd contents in the supernatants were measured by ICP-OES. Finally, the relative Gd contents (% of original) in the supernatants were

calculated to evaluate the Gd leakage.

T_1 MR relaxometry

T_1 MR relaxometry of the developed AG/PEI-Au-Gd NGs was performed on an NMR analyzing and imaging instrument (0.5 T, room temperature, NMI20, Niumag, Shanghai, China). The samples were prepared by stepwise dilution with water to get different Gd concentrations (0.05, 0.1, 0.2, 0.3, and 0.4 mM, respectively) before measurements. The parameters were set as follows: IR sequence, point resolution of 156 mm × 156 mm, section thickness of 0.8 mm, and excitation number of 1. Meanwhile, T_1 MR relaxometry of PEI.Ac-Au-Gd NPs and clinical contrast agent (DTPA-Gd, Magnevist®) was also measured under the same Gd concentrations and instrumental settings. Additionally, the T_1 MR images of the above three materials were acquired using a clinical MR system (3.0 T, MAGNETOM VERIO, SIEMENS Medical Systems, Erlangen, Germany). The parameters were set as follows: TE = 11 ms, TR = 650 ms, FOV = 116 × 116, and 224 × 320 matrix.

Nuclear magnetic relaxation dispersion (NMRD) experiments

T_1 of the AG/PEI-Au-Gd NGs (Gd = 0.5 mM) was measured using a SMARtracer™ (STELAR, Mede, Italy) Fast Field Cycling NMR relaxometer in the magnetic field range of 2.4×10^{-4} to 0.25 T (corresponding to 0.01 to 10 MHz). Samples were placed in 10-mm diameter glass tubes, and temperature (25 °C) was regulated by gas flow. For each field strength, sixteen experiments of four scans were used for the T_1 determination and each value was automatically acquired with an absolute uncertainty of ± 1%.

X-ray attenuation property

To study the X-ray attenuation property, the AG/PEI-Au-Gd NGs, PEI.Ac-Au-Gd NPs, and Omnipaque (a conventional iodinated CT contrast agent) with different Au or iodine (I) concentrations (0.005, 0.01, 0.015, 0.02 and 0.03 M, respectively) were prepared in 0.5-mL Eppendorf tubes and the tubes were placed in a home-made scanning holder. Subsequently, CT scanning was performed using a dual-source SOMATOM Definition Flash CT system (Siemens, Erlangen, Germany) with 140 kV, and

a slice thickness of 0.6 mm. Later on, the evaluation of the X-ray attenuation intensity was carried out by a standard display program. Contrast enhancement was determined in Hounsfield units (HU) for each sample with different Au or I concentrations.

Cytotoxicity and cellular uptake assays

For cytotoxicity evaluation, HeLa cells were cultivated and passaged with DMEM containing FBS (10%) and penicillin-streptomycin (1%) in a Thermo Scientific cell incubator (Waltham, MA) at 37 ^{o}C and 5% CO_2. When the cells were ready, the 96-well plates were seeded with HeLa cells at a density of 1 × 10^4 cell/well for overnight. The next day, the adherent HeLa cells were incubated with 200 μL of fresh DMEM containing AG/PEI-Au-Gd NGs at various Au concentrations (20, 40, 60, 80, and 100 μM, respectively) for 24 h. After that, the cells were rinsed three times with PBS, and then incubated with 100 μL of DMEM without FBS but supplemented with 10% CCK-8 for 2 h. Subsequently, a Multiskan MK3ELISA reader (Thermo scientific, Logan, UT) was employed to measure the absorbance of each well at 450 nm. The control group was the HeLa cells treated with PBS, and for each sample 5 parallel wells were analyzed to give a mean value and standard deviation.

The cell morphology after treatment with NGs was also observed by a Leica DM IL LED inverted phase contrast microscope (Wetzlar, Germany) with a magnification of 100 × for each sample to further assess the cytotoxicity of the AG/PEI-Au-Gd NGs. Additionally, HeLa cells treated with the AG/PEI-Au-Gd NGs with different concentrations for 24 h were stained with Annexin V-FITC/PI based on the manufacturer's instructions, and subjected to flow cytometric assay using a Becton Dickinson FACSCalibur flow cytometer (East Rutherford, NJ) to evaluate the cytocompatibility of the NGs.

The uptake of the AG/PEI-Au-Gd NGs by HeLa cells was evaluated by ICP-OES. PEI.Ac-Au-Gd NPs were also tested for comparison. Briefly, HeLa cells in DMEM were seeded in 6-well plates at a density of 2 × 10^6 cells/well. After the cell adherence during overnight incubation, the cells were incubated with fresh DMEM containing the AG/PEI-Au-Gd NGs or PEI.Ac-Au-Gd NPs ([Au] = 0, 50,

and 100 μM, respectively) for 4 h at 37 °C and 5% CO_2. Thereafter, the cells were washed with PBS for 3 times, digested by trypsinization, centrifuged (1000 rpm, 5 min), and resuspended in PBS for cell counting by a handheld cell counter (Scepter™ 2.0, MerckMillipore, Bedford, MA). Then the remaining cells were collected by centrifugation (1000 rpm, 5 min) and lysed using 0.5 mL *aqua regia* solution (nitric acid/hydrochloric acid, v/v = 1:3) for one day. After diluting the samples with PBS, the cellular Au uptake was quantified by ICP-OES.

MR and CT imaging of cancer cells *in vitro*

HeLa cells were treated with the AG/PEI-Au-Gd NGs according to a previously reported method[4] and then imaged by MR and CT *in vitro*. Briefly, HeLa cells (8×10^5) seeded in T25 culture flasks and incubated overnight were treated with fresh DMEM containing AG/PEI-Au-Gd NGs ([Au] = 0, 50, and 100 μM, respectively). After 4 h incubation, the cells were washed with PBS for 3 times, digested with trypsinization, centrifuged (1000 rpm, 5 min), and resuspended into 0.2 mL of PBS containing 0.5% agarose. For MR imaging, a clinical MR system mentioned above was employed. The parameters were set as follows: TE = 9 ms, TR = 2000 ms, FOV = 212 × 230, and 222 × 320 matrix. For CT imaging, the cell suspensions were scanned using a dual-source SOMATOM Definition Flash CT system, operating at 80 kV and a slice thickness of 0.6 mm. The MR signal intensities and CT values were acquired on the workstations using the software supplied by the instrument manufacturers. Each sample was analyzed for at least three times for statistical assessments.

***In vivo* biodistribution and histological examinations**

The HeLa tumor-bearing nude mice were subjected to *in vivo* biodistribution analysis to evaluate the metabolic pathway of the AG/PEI-Au-Gd NGs. After intravenous injection of the AG/PEI-Au-Gd NGs ([Au] = 20 mM, 100 μL in PBS solution), the HeLa tumor-bearing mice were executed at different time points postinjection. Then we extracted and weighted the main organs including heart, liver, spleen, lung, kidney, and tumor of the mice. These organs and tumors were cut into small pieces, treated by an aqua regia solution (2 mL, hydrochloric acid/nitric acid, v/v = 3:1) for two days, and

diluted with water (2 mL). Finally, the Au content in different organs and tumors was quantified by ICP-OES measurements.

Hematoxylin and eosin (H&E) staining of main organs were performed to evaluate the toxicity of the developed AG/PEI-Au-Gd NGs. Healthy male Kunming mice (20-30 g, Shanghai Slac Laboratory Animal Center) were intravenously administered with the AG/PEI-Au-Gd NGs ([Au] = 20 mM, 100 μL in PBS solution). PBS (100 μL) was also intravenously injected to be used as control. After 7 days and 30 days, the mice were anesthetized, and the liver, lung, spleen, heart, and kidney were harvested, washed with PBS, and immediately fixed with 4% paraformaldehyde (10 mL). After 48 h fixation, the organs were processed, sectioned, and hematoxylin and eosin (H&E) stained for microscopic observation according to standard protocols reported in the literature.[5] The histological images of these organ sections were acquired by an inverted phase contrast microscope mentioned above with a magnification of 200 × for each sample.

Statistical analysis

Statistical analysis of the experimental data was performed *via* standard one-way ANOVA (analysis of variance) statistical method. In all cases, a value of 0.05 was selected to indicate the statistical significance, and the data were marked with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Zeta potentials and hydrodynamic sizes of PEI-Au-Gd NPs and AG/PEI-Au-Gd NGs ([Au] = 0.1 mM) with different AG/PEI-Au-Gd mass ratios. Data are provided as mean ± SD (n = 3).

Sample	Zeta potential (mV)	Hydrodynamic size (nm)	Polydispersity index (PDI)
PEI-Au-Gd NPs	18.8 ± 1.0	90.2 ± 2.9	0.32 ± 0.26
NGs (1:0.5)	-24.4 ± 0.2	148.3 ± 2.3	0.27 ± 0.18
NGs (1:1)	-16.4 ± 0.5	167.3 ± 4.9	0.14 ± 0.09
NGs (1:2)	-9.6 ± 0.2	202.7 ± 1.6	0.25 ± 0.11

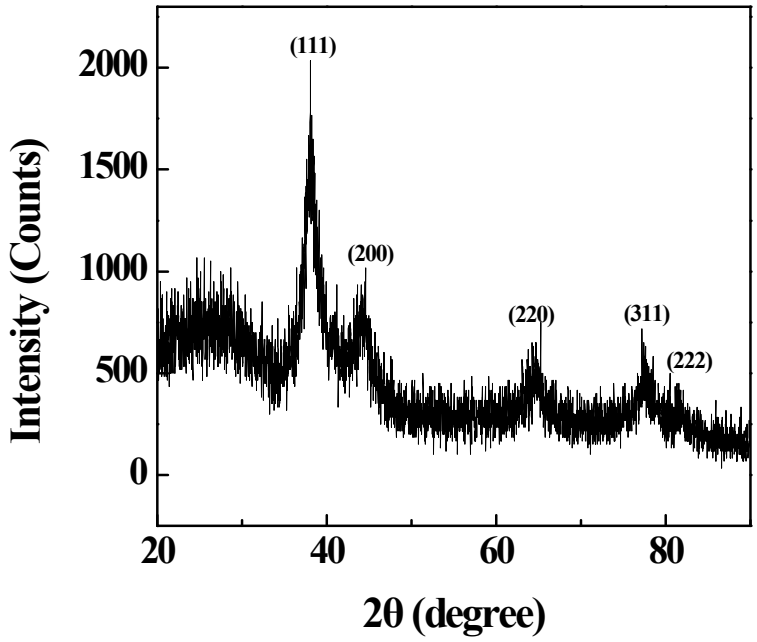

Figure S1. XRD pattern of the AG/PEI-Au-Gd NGs.

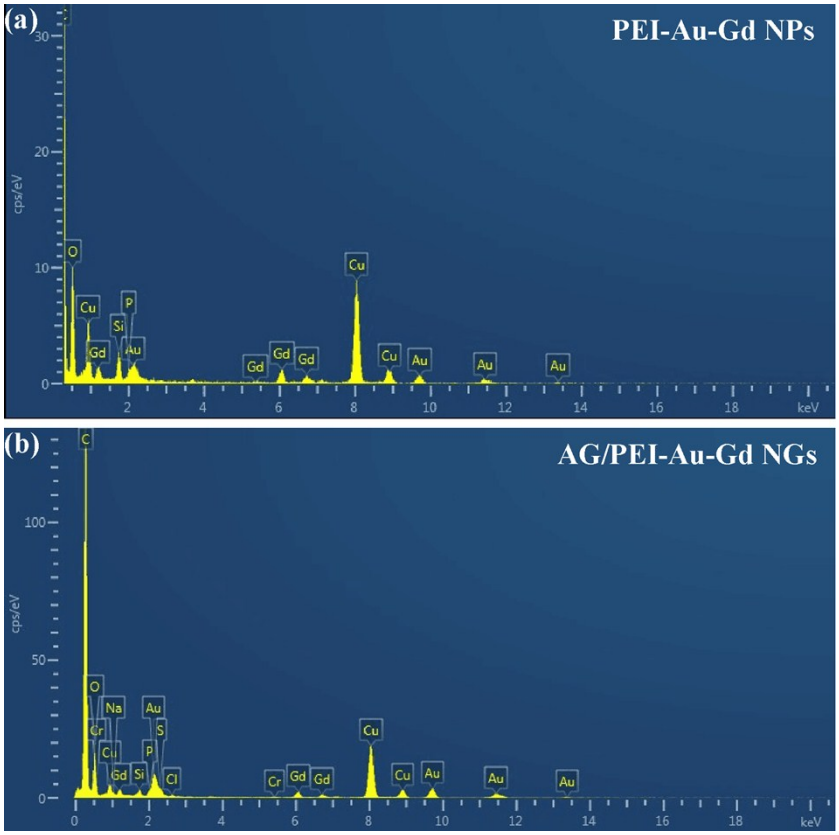

Figure S2. EDX spectra of PEI-Au-Gd NPs (a) and AG/PEI-Au-Gd NGs (b).

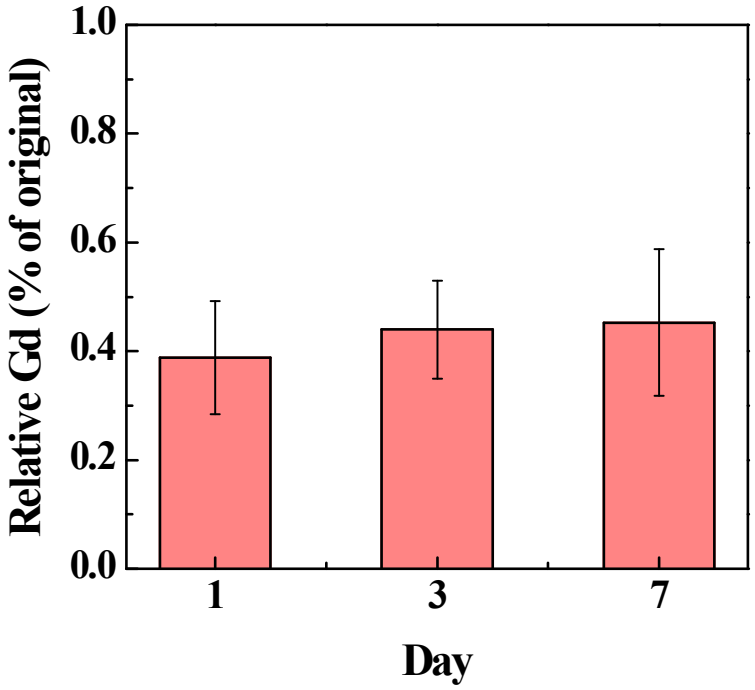

Figure S3. The relative Gd contents (% of original) in the supernatants of AG/PEI-Au-Gd NGs (Gd = 0.2 mM) after stored at 37 ^{o}C for 1, 3, and 7 days, respectively, followed by centrifugation.

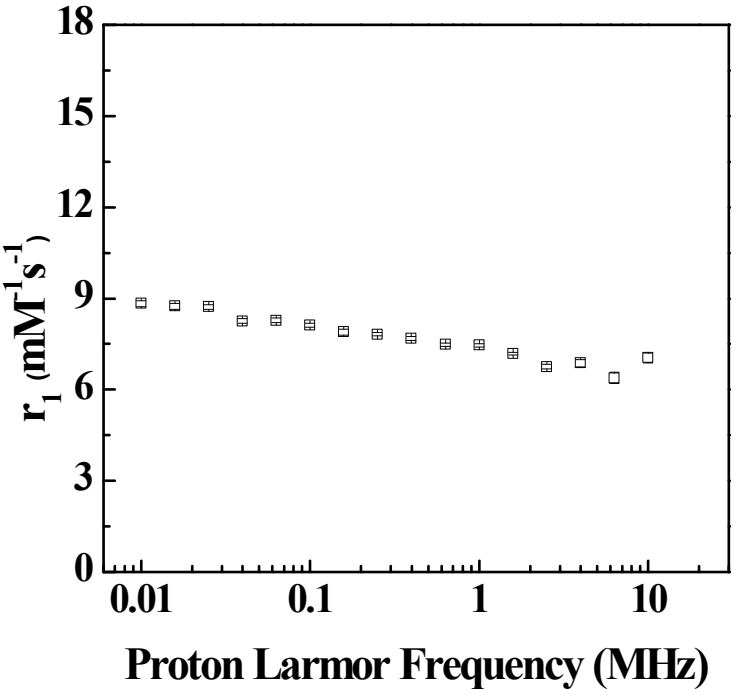

Figure S4. The NMRD profile of the AG/PEI-Au-Gd NGs (Gd = 0.5 mM) at 25 ^{o}C under different magnetic fields.

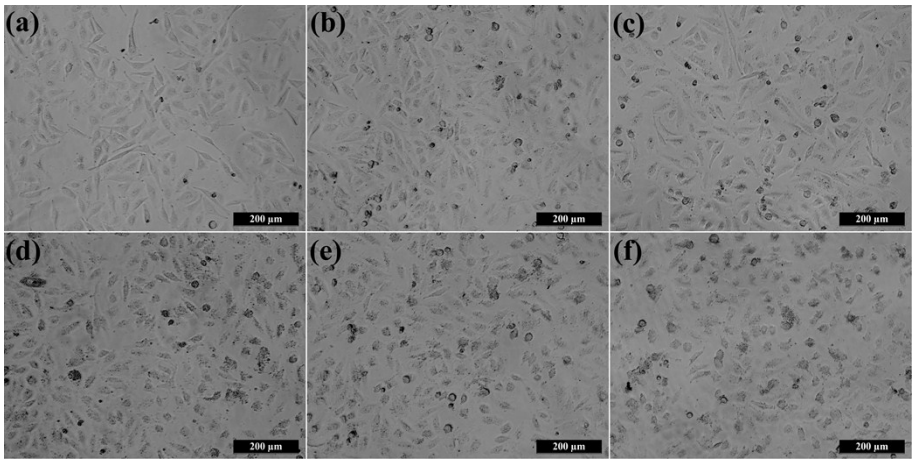

Figure S5. Phase contrast microscopic images of HeLa cells treated with PBS (a) and AG/PEI-Au-Gd NGs at the Au concentrations of 20 μM (b), 40 μM (c), 60 μM (d), 80 μM (e), and 100 μM (f), respectively for 24 h.

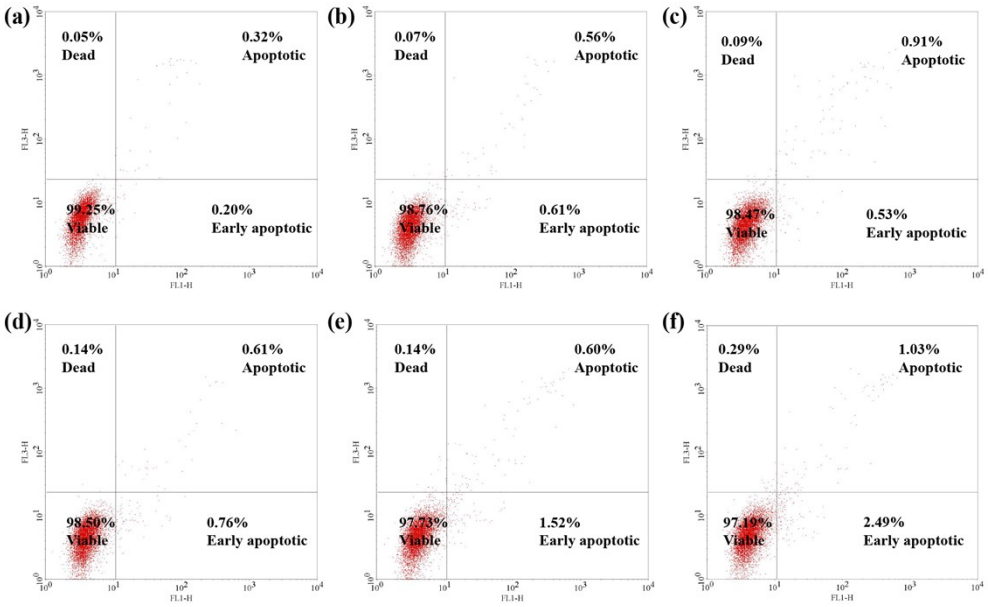

Figure S6. Flow cytometric analysis of HeLa cell apoptosis after the cells were treated with PBS (a) and AG/PEI-Au-Gd NGs at the Au concentrations of 20 μM (b), 40 μM (c), 60 μM (d), 80 μM (e), and 100 μM (f), respectively for 24 h.

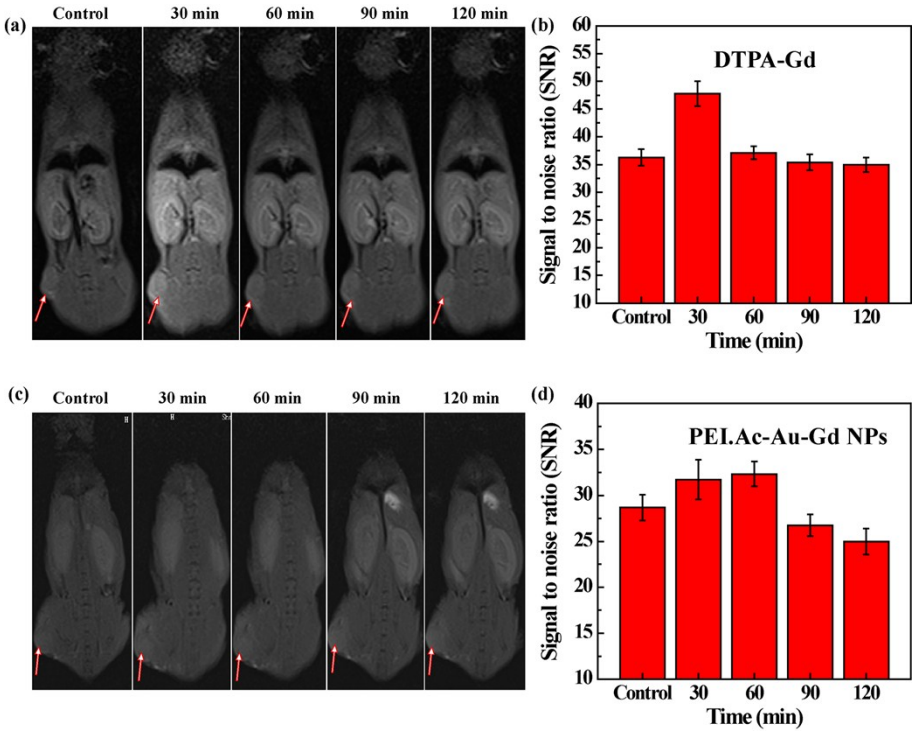

Figure S7. *In vivo* T_1-weighted MR images (a, c) and tumor MR SNR (b, d) of xenografted HeLa tumor-bearing mice before and at different time points post-intravenous injection of the clinical DTPA-Gd (a, b) or PEI.Ac-Au-Gd NPs (c, d). Arrows indicate the tumor regions.

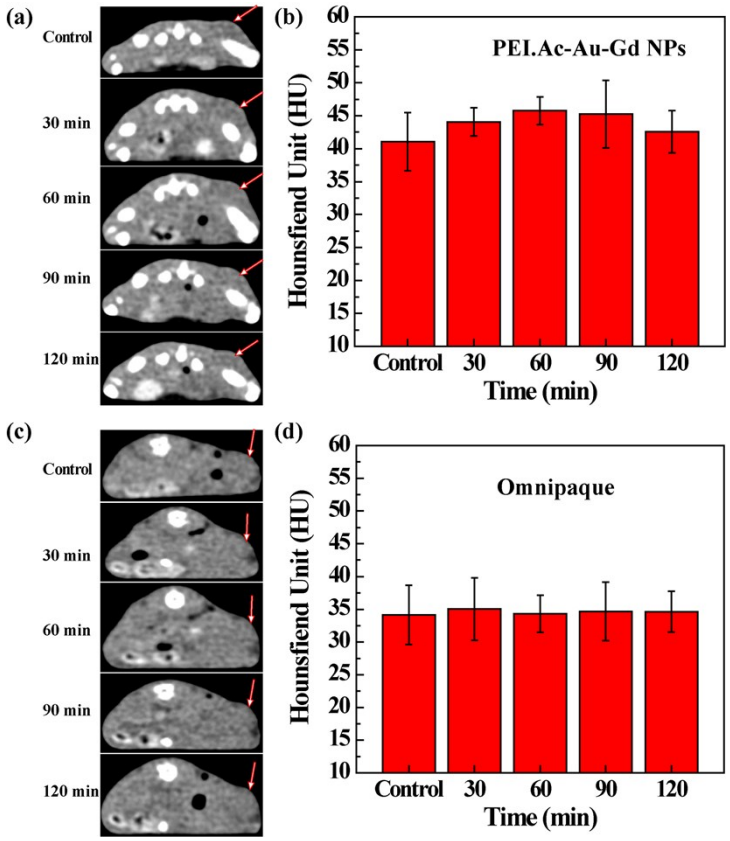

Figure S8. *In vivo* CT images (a, c) and CT values (b, d) of xenografted HeLa tumor-bearing mice before and at different time points post-intravenous injection of the PEI.Ac-Au-Gd NPs (a, b) or clinical Omnipaque (c, d). Arrows indicate the tumor regions.

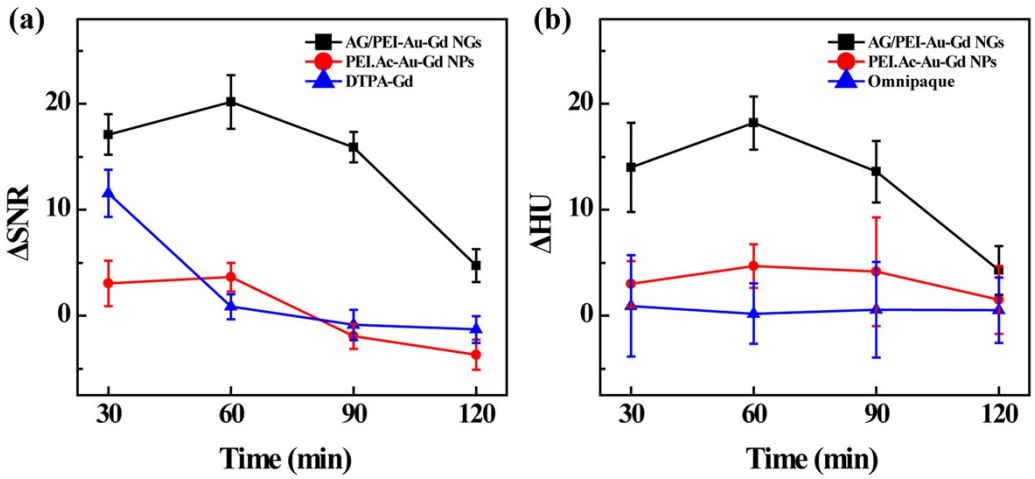

Figure S9. Quantitative analyses of MR SNR changes (a) and CT value changes (b) in tumors at different time points post-intravenous injection of different materials.

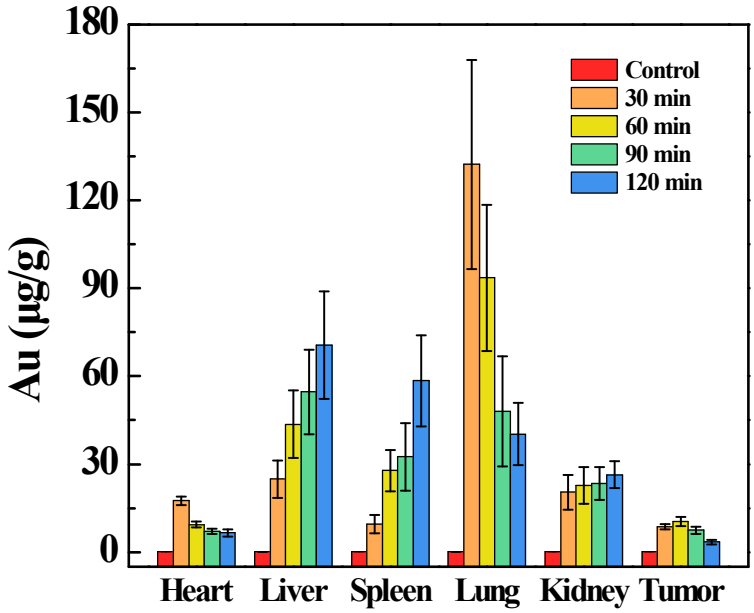

Figure S10. Biodistribution of Au in the major organs of the nude mice including the heart, liver, spleen, lung, and kidney, as well as tumor at 30, 60, 90 and 120 min post-intravenous injection of the AG/PEI-Au-Gd NGs ([Au] = 20 mM, 100 μL in PBS solution for each mouse). Mice injected with PBS (100 μL) were used as control.

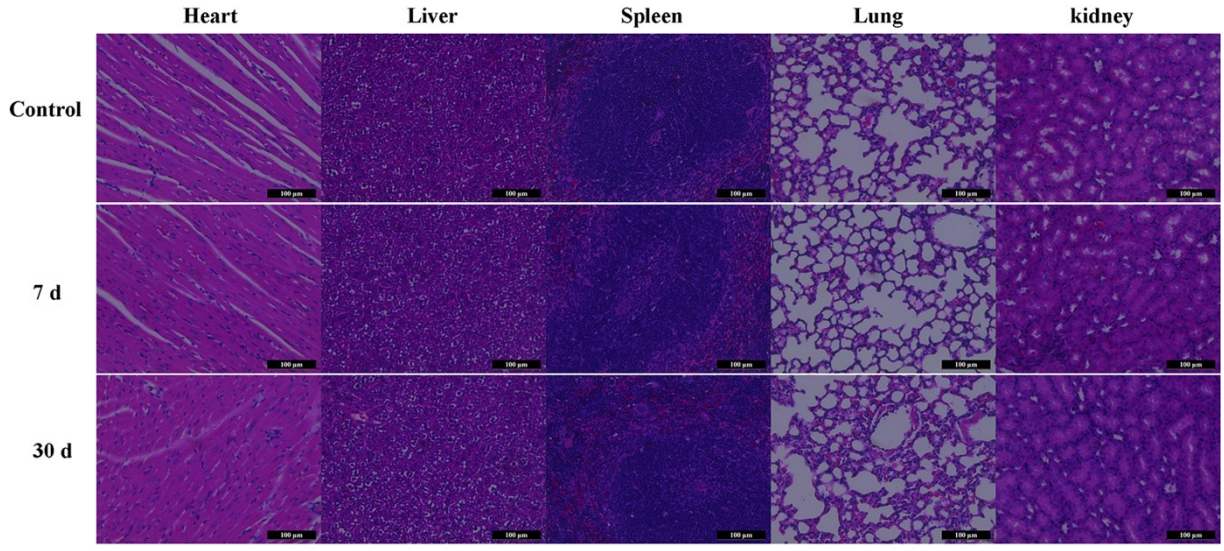

Figure S11. H&E staining of different organs from healthy mice at 7 and 30 days postinjection of the AG/PEI-Au-Gd NGs ([Au] = 20 mM, 100 μL in PBS solution for each mouse). Mice injected with PBS (100 μL for each mouse) were used as control.

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