Electronic Supplementary Information

Targeted CT/MR dual mode imaging of human hepatocellular carcinoma using lactobionic acid-modified polyethyleneimine-entrapped gold nanoparticles†

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Experimental Section:

Materials

Branched PEI (Mw = 25000), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO). Methoxy-poly (ethylene glycol)-carboxylic acid (*m*PEG-COOH) and amino-poly (ethylene glycol)-carboxylic acid (NH_2-PEG-COOH) with Mw 2000 were supplied from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). 2,2',2''-(10-(2-(2,5-Dioxopyrrolidin-1-yloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid (DOTA-NHS) was purchased by CheMatech (Dijon, France). Sodium borohydride, LA, acetic anhydride, triethylamine, and all the other chemicals and solvents were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HepG2 cells (Hepatocellular Carcinoma cell line) were supplied by Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS) was obtained from HyClone Lab., Inc. (Logan, UT). Dulbecco's modified eagle's medium (DMEM), penicillin, and streptomycin were from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Cellulose dialysis membranes (molecular weight cut-off, MWCO = 14,000 or 2,000) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China).

Synthesis of LA-PEG-COOH

LA-PEG-COOH was synthesized according to the literature.[1] In brief, LA (53.7 mg, 20 mL in water) was activated by 57.2 mg EDC under vigorous stirring. Three hours later, the activated LA was added to the water solution of NH_2-PEG-COOH (200 mg, 40 mL) and kept stirring for 3 days. Then, the reaction mixture was dialyzed to remove the excess reactants and by-products to get the product LA-PEG-COOH *via* lyophilization.

Synthesis of Gd-Au PENPs-LA

DOTA-NHS (20 mg, 5 mL in water) was added to a water solution of PEI (50.0 mg, 15 mL) with vigorous stirring to form the raw product of PEI-DOTA. After 24 h, EDC-activated LA-PEG-COOH (117.7 mg, 5 mL in water) was added to the above solution and the mixture was kept stirring for 48 h

to form the raw product of PEI-DOTA-PEG-LA. The formed PEI-DOTA-PEG-LA was used as a template to form Au NPs according to the literature.[2, 3] In brief, 5.5 mL of $HAuCl_4$ (24.8 mg/mL, in water) was added to the above solution of PEI-DOTA-PEG-LA with Au salt/PEI molar ratio of 350:1 under stirring. After 30 min, an icy cold $NaBH_4$ solution (75.7 mg, 3 mL in water) was quickly added to the above solution under stirring. One hour later, $Gd(NO_3)_3$ solution (30.9 mg, 2 mL in water) was added under stirring for 24 h. Then, the remaining amine groups were acetylated according to the literature,[2, 3] followed by dialysis against phosphate buffered saline (PBS, 4L, 3 time) and water (4L, 3 times) for 2 days using a dialysis membrane with an MWCO of 14,000 and lyophilization. The synthesized product was named as {Au350-PEI.NHAc-DOTA(Gd)-(PEG-LA)} NPs (Gd-Au PENPs-LA). For comparison, the non-targeted material of {Au350-PEI.NHAc-DOTA(Gd)-mPEG} NPs (Gd-Au PENPs) and the intermediate products of {Au350-PEI.NHAc-DOTA-(PEG-LA)} NPs (Au PENPs-LA) without Gd and the PEI.NHAc-DOTA(Gd)-(PEG-LA) (PEI-Gd-LA) without Au NPs were also prepared under the same experimental conditions. For the synthesis of the non-targeted product, mPEG-COOH was used to modify the periphery of PEI.

Characterization Techniques

^1H NMR spectra were recorded by a Bruker DRX 400 nuclear magnetic resonance spectrometer. Samples were dissolved in D_2O before measurements. UV-vis spectra were collected on a Perkin Elmer Lambda 25 UV-vis spectrophotometer (Waltham, MA). All samples were dissolved in water before the analysis. Transmission electron microscopy (TEM) was performed using a JEOL 2010F analytical electron microscope (JEOL, Japan) at 200 kV. A typical TEM sample was prepared by depositing a sample solution (1 mg/mL, in water) onto carbon-coated copper grid, and the sample was air dried before measurements. Zeta potential and dynamic light scattering (DLS) measurements were conducted with a Malvern Zetasizer Nano ZS system (Worcestershire, UK). The Au and Gd composition of the formed materials were measured by Leeman Prodigy inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Hudson, NH). *In vitro* CT phantom studies were performed using a GE LightSpeed VCT imaging system (GE Medical Systems, Milwaukee, WI) with 100 kV, 80 mA, and a slice thickness of 0.625 mm. Solutions of the Gd-Au PENPs-LA or Au PENPs-LA (0.2 mL)

with different Au concentrations were prepared. The contrast enhancement in hounsfield units (HU) for each sample with different Au concentrations was quantified. T_1-weighted MR phantom studies of the Gd-Au PENPs-LA were performed by a 0.5 T NMI20-Analyst NMR Analyzing and Imaging System (Niumag Corporation, Shanghai, China). Gd-Au PENPs-LA were dissolved in water with the Gd concentrations in the range of 0.325-2.6 mM. T_1 relaxation times were measured via an Inversion Recovery sequence, and the instrumental parameters were set as follows: P90 (us) = 14.00, P180 (us) = 28.00, TD = 1024, SW (KHz) = 100, TW (ms) = 25000, RG1 (db) = 20, DRG1 = 3, NS = 2, DR = 1, NTI = 30. The r_1 relaxivity was calculated by linear fitting of the inverse T_1 relaxation time as a function of the Gd concentration.

Cytotoxicity and Cellular Uptake Assays

The cytotoxicity of Gd-Au PENPs-LA was tested by MTT cell viability assay according to the literature.[4] In brief, HepG2 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. After overnight culture to bring the cells to confluence, the medium was replaced with fresh medium containing Gd-Au PENPs-LA with a final Au concentration ranging from 0 to 50 μM, and the cells were incubated for 24 h. Then, MTT (5 mg/mL, 20 μL) was added to each well and the cells were incubated for another 4 h. After that, the medium was removed, followed by addition of DMSO (200 μL) to dissolve the formazan crystals. Finally, the absorbance of each well at 570 nm was recorded by a Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA). The results were shown as mean ± standard deviation for the triplicate wells for each sample.

The specific uptake of the Gd-Au PENPs-LA by HepG2 cells was assessed *via* quantification of Au in cells by ICP-AES according to the literature.[5, 6] Approximately 5×10^5 HepG2 cells were plated in each well of a 24-well plate and cultured overnight to bring the cells to confluence. Then, the medium was replaced with fresh medium containing Gd-Au PENPs or Gd-Au PENPs-LA ([Au] = 0 and 25 μM) and the cells were incubated for another 3 h. After counting the cell number in the cell suspensions, the cells were centrifuged, digested by aqua regia solution overnight, and diluted. ICP-AES was performed to determine the Au content in the cell samples.

In Vitro Targeted CT/MR Imaging of Cancer Cells

Approximately 4×10^6 HepG2 cells were incubated in the medium containing Gd-Au PENPs or Gd-Au PENPs-LA with different Au concentrations ([Au] = 0, 5, 10, 25, and 50 μM, respectively) for 3 h. After that, the cells were washed, trypsinized, centrifuged, and resuspended in 0.1 mL phosphate buffered saline (PBS). For CT imaging, the cell suspensions were scanned using the same CT imaging system described above. The T_1-weighted MR imaging was performed using a spin echo acquisition in a 0.5 T system described above and the MR signal intensity was measured carefully to calculate the relative signal-to-noise ratio (SNR).

In Vivo Targeted Dual Mode CT/MR Imaging of a Xenograft Tumor Model

Male 5-week-old BALB/c nude mice (Shanghai SLAC Animal Center, Shanghai, China) were subcutaneously injected with 1×10^6 cells for each mouse in the right side of their oxter. After the tumor nodules reached a volume of 0.3-0.5 cm^3, the mice were anesthetized, intravenously injected with Gd-Au PENPs-LA ([Au] = 0.1 M, in 150 μL PBS). For comparison, Gd-Au PENPs with the same dose were also injected. For CT imaging, the scans were carried out before and at 1, 2, 4, and 24 h postinjection using the CT imaging system described above for *in vitro* imaging and the CT values (HU) of tumor site were carefully measured.

In terms of MR imaging, the scans were also performed before and at 1, 2, 4, and 24 h postinjection using a 1.5 T Signa HDxt superconducting clinical GE Medical magnetic resonance system (Milwaukee, WI). And Two-dimensional spin-echo MR images were recorded with a 2 mm slice thickness, a 6 × 6 cm FOV, TR/TE 2000/81.9 ms, and a 256 × 160 matrix.

In Vivo Biodistribution and Histological Examinations

The *in vivo* biodistribution of the Gd-Au PENPs-LA in the tumor-bearing mice at different time points postinjection of the particles was assessed according to the literature protocols.[5] The tumor bearing BALB/c nude mice (22-24 g) were first anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/ kg). After intravenous injection of Gd-Au PENPs-LA ([Au] = 0.1 M, in 150 μL PBS) at different time points (2, 12, 24, 48, and 96 h, respectively), the mice were euthanized.

Then, the blood, tumor and major organs (heart, liver, spleen, lung and kidney) were carefully extracted and weighed. After digested in aqua regia solution overnight, the content of Au in the blood, tumor and different organs was measured by ICP-AES.

Similarly, the organ toxicity of the material was assessed by hematoxylin and eosin (H&E) staining of the major organ of healthy mice at one month postinjection of the particles according to protocols reported in the literature.[7] Briefly, healthy Balb/c mice (25 g) intravenously administrated with Gd-Au PENPs-LA ([Au] = 0.1 M, in 150 μL PBS) were selected as the test group, while those administrated with 150 μL saline were set as control group. One month later, the Balb/c mice were euthanized and their major organs were harvested. The obtained organs were first fixed in 10% formalin, embedded in paraffin, and then sectioned to have a thickness of 4 μm and stained with H&E. Finally, these organ sections were observed by a Leica DM IL LED inverted phase contrast microscope.

Statistical Analysis

Statistical analysis was carried out *via* standard ANOVA statistical method using Origin 8.0 software according to our previous work.[8] 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.

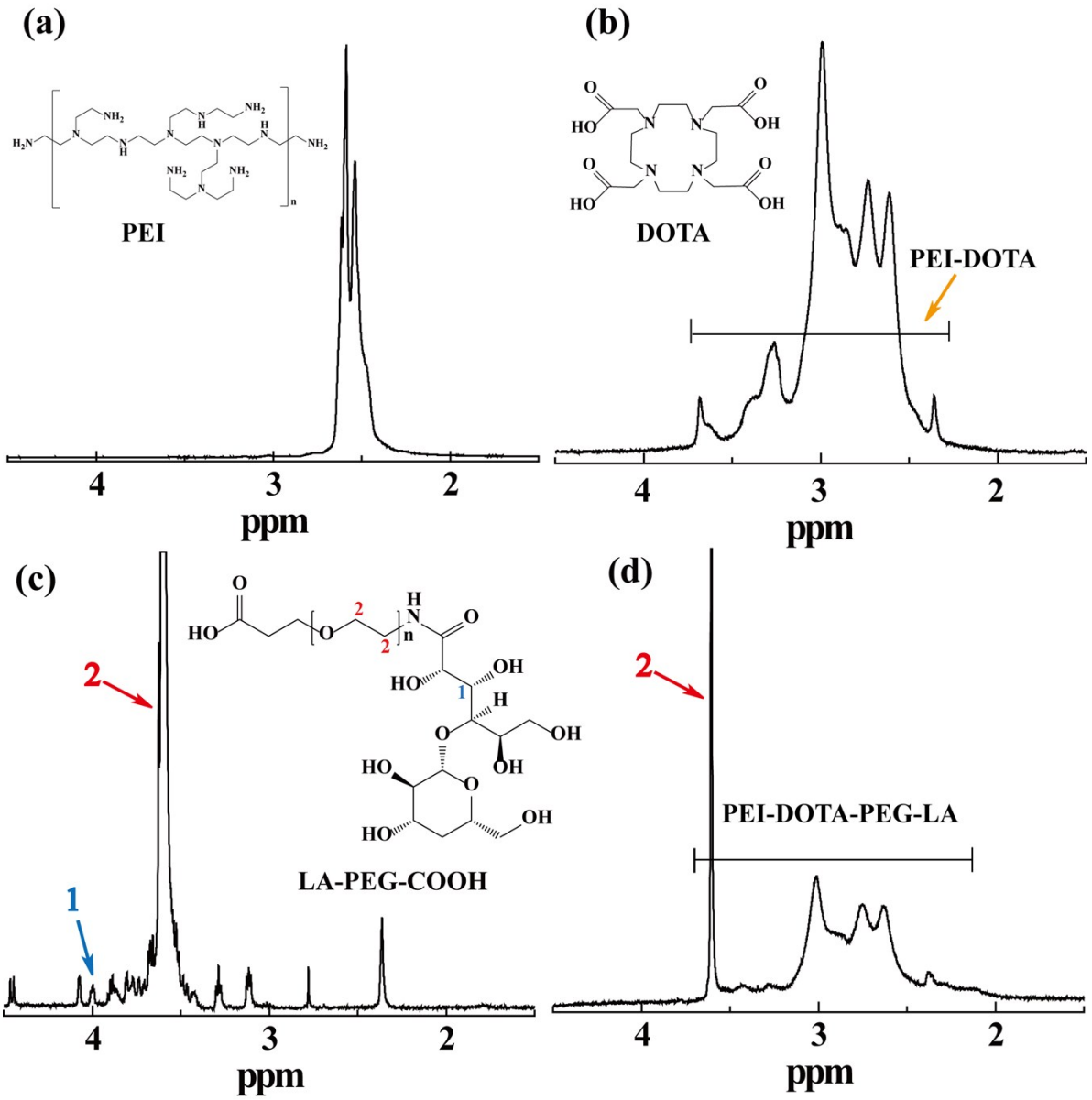

Figure S1. ^{1}H NMR spectra of PEI (a), PEI-DOTA (b), LA-PEG-COOH (c), and PEI-DOTA-PEG-LA (d) dissolved in D_2O.

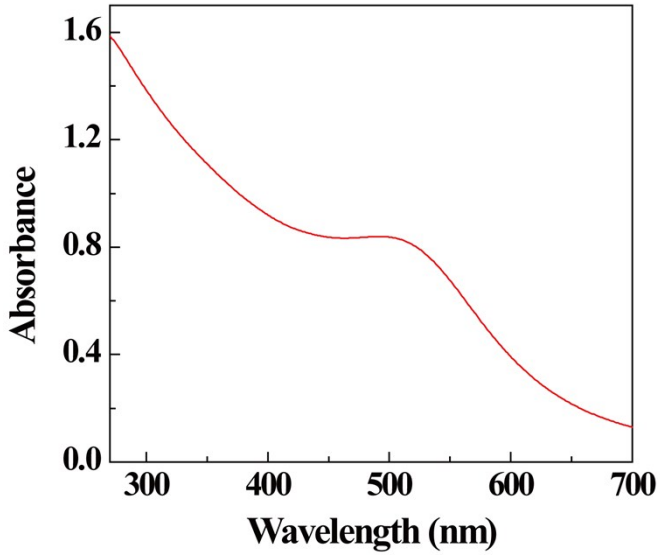

Figure S2. UV-Vis spectrum of Gd-Au PENPs-LA dispersed in water.

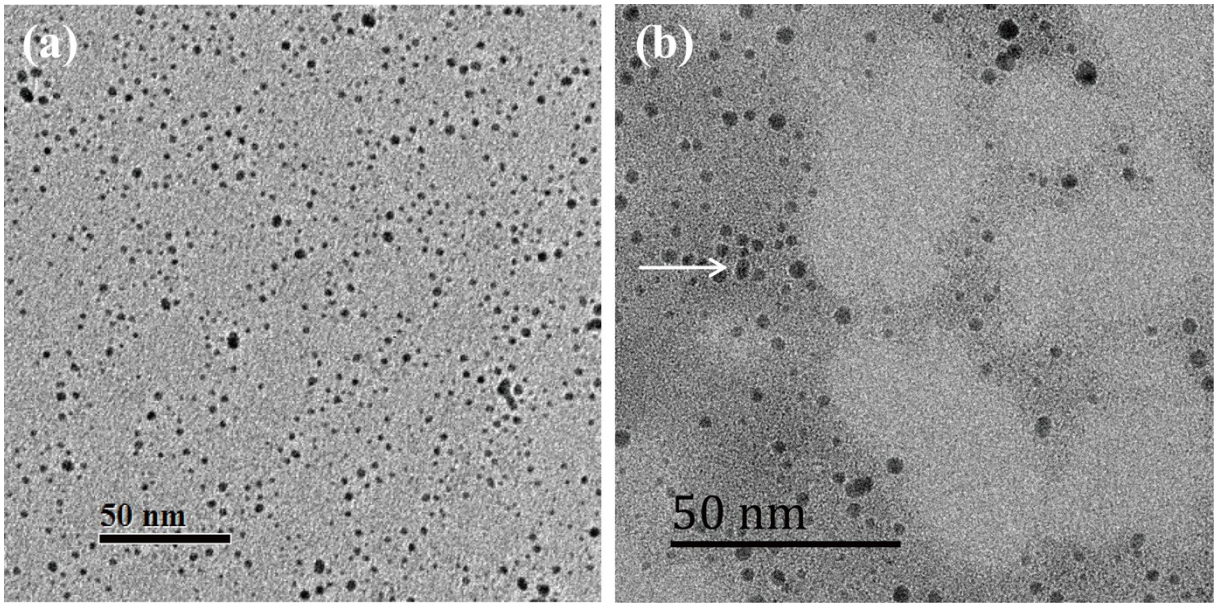

Figure S3. TEM image of the Gd-Au PENPs-LA (a) and Gd-Au PENPs-LA negatively stained with phosphotungstic acid (b).

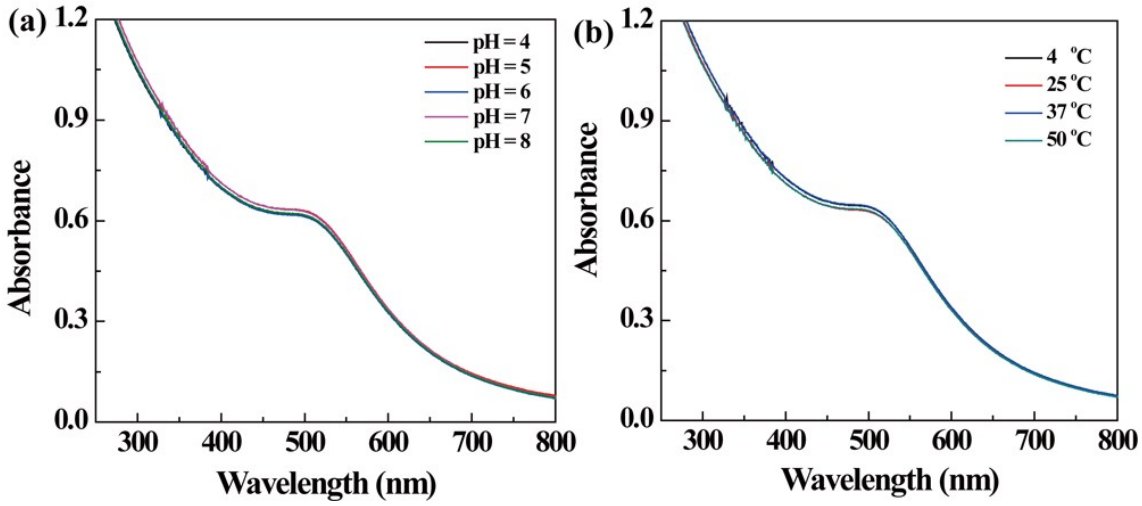

Figure S4. UV-vis spectra of Gd-Au DENPs under different pH (a) and temperature (b) conditions.

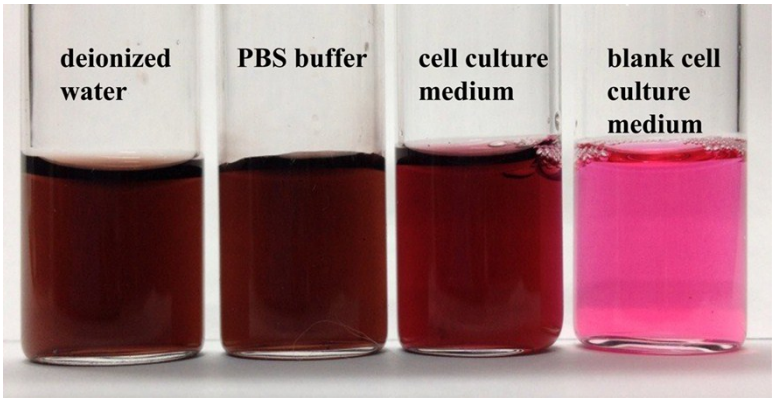

Figure S5. Photos of Gd-Au PENPs-LA dispersed in water, PBS, and cell culture medium with a concentration of 0.2 mg/mL and the blank cell culture medium.

References:

1 F. Fu, Y. Wu, J. Zhu, S. Wen, M. Shen and X. Shi, *ACS Appl. Mater. Interfaces*, 2014, **6**, 16416-16425.

2 B. Zhou, J. Yang, C. Peng, J. Zhu, Y. Tang, X. Zhu, M. Shen, G. Zhang and X. Shi, *Colloids Surf., B*, 2016, **140**, 489-496.

3 B. Zhou, L. Zheng, C. Peng, D. Li, J. Li, S. Wen, M. Shen, G. Zhang and X. Shi, *ACS Appl. Mater. Interfaces*, 2014, **6**, 17190-17199.

4 B. Zhou, Z. Xiong, J. Zhu, M. Shen, G. Tang, C. Peng and X. Shi, *Nanomedicine*, 2016, **11**, 1639-1652.

5 Q. Chen, K. Li, S. Wen, H. Liu, C. Peng, H. Cai, M. Shen, G. Zhang and X. Shi, *Biomaterials*, 2013, **34**, 5200-5209.

6 Q. Chen, H. Wang, H. Liu, S. Wen, C. Peng, M. Shen, G. Zhang and X. Shi, *Anal. Chem.*, 2015, **87**, 3949-3956.

7 J. Zhu, L. Zhao, Y. Cheng, Z. Xiong, Y. Tang, M. Shen, J. Zhao and X. Shi, *Nanoscale*, 2015, **7**, 18169-18178.

8 J. Li, L. Zheng, H. Cai, W. Sun, M. Shen, G. Zhang and X. Shi, *ACS Appl. Mater. Interfaces*, 2013, **5**, 10357-10366.