Electronic supplementary Information

Multifunctional gold nanocomposites designed for targeted CT/MR/optical trimodal imaging of human non-small cell lung cancer cells

Jingwen Chen,‡[a] Yingqi Sun,‡[a] Qian Chen,‡[b] Le Wang,[c] Suhe Wang,[d] Yun Tang,*[e] Xiangyang Shi*[bf] and Han Wang*[ag]

[a.] *Department of Radiology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, People's Republic of China. Email: 13564112852@163.com*

[b.] *State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, Donghua University, Shanghai 201620, People's Republic of China*

[c.] *Department of Radiology, Yueyang Hospital affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200437, People's Republic of China*

[d.] *Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigen, Ann Arbor, Michigen 48109-5648, USA*

[e.] *Department of Chemistry, iChem (Collaborative Innovation Center of Chemistry for Energy Materials), Fudan University, Shanghai 200433, People's Republic of China. Email: yuntang@fudan.edu.cn*

[f.] *College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, People's Republic of China. Email: xshi@dhu.edu.cn*

[g.] *National Engineering Research Center for Nanotechnology, Shanghai 200241, People's Republic of China. Email: 13564112852@163.com*

‡ These authors equally contributed to this work.

***Corresponding Author**

Han Wang,[a*] Tel: +86 21 37798252, E-mail: 13564112852@163.com, fax: +86 21 37798252;

or Xiangyang Shi,[b,f*] Tel: +86 21 67792656, E-mail: xshi@dhu.edu.cn;

or Yun Tang, [e*] Tel: +86 21 51630325, E-mail: yuntang@fudan.edu.cn

Experimental Section

1. Materials

Ethylenediamine core amine-terminated G5.NH_2 PAMAM dendrimers were purchased from Dendritech (Midland, MI), which with a polydispersity index less than 1.08. 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) were gotten from CheMatech (Dijon, France). PEG monomethyl ether with one end of carboxyl group (mPEG-COOH, Mw = 2,000), a dual functional PEG, which has one end of amine group and the other end of carboxyl group (NH_2-PEG-COOH, Mw = 2,000), and Cyanine5.5 NHS ester (Cy5.5) were obtained from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China). FA, acetic anhydride, triethylamine, sodium hydroxide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), hydrochloric acid and all the other chemicals and solvents were purchased from Aldrich (St. Louis, MO). Water, which used in experiments, was purified by a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with the resistivity higher than 18 MΩ·cm. Cellulose dialysis membranes (MWCO = 14,000 or 1,000) were gotten from Thermo Fisher Scientific (Waltham, MA).

2. Synthesis of Cy5.5-Gd-Au DENPs-FA

The synthesis process of the Cy5.5-Gd-Au DENPs-FA product is illustrated in Figure 1. Firstly, DOTA-NHS (23.85 mg) dissolved in DMSO (10 mL) was dropwise added to a DMSO solution of G5.NH_2 dendrimers (81.47 mg, 20 mL) under vigorous magnetic stirring for 24 h. This led to the formation of the raw product of G5.NH_2-DOTA dendrimers. Then, FA-PEG-COOH was synthesized according to the reported protocol.[31] FA-PEG-COOH (61.04 mg) pre-activated by EDC (48.04 mg in 10 mL DMSO) for 3 h was dropwise added to the above solution of the G5.NH_2-DOTA dendrimers under vigorous magnetic stirring for 3 days. The formed raw product of G5.NH_2-DOTA-(PEG-FA) was further modified with mPEG-COOH (93.96 mg) which was pre-activated by EDC for 3 h. The reaction was performed for 3 days under magnetic stirring to get the raw product of the G5.NH_2-DOTA-(PEG-FA)-mPEG dendrimers. Finally, the raw product of G5.NH_2-DOTA-(PEG-FA)-mPEG was further modified with 3 molar equivalents of Cy5.5 (6.73 mg) under vigorous magnetic stirring for 1 d in the dark to get the raw product of G5.NH_2-DOTA-(PEG-FA)-mPEG-Cy5.5.

The G5.NH_2-DOTA-(PEG-FA)-mPEG-Cy5.5 dendrimers (the above raw product) were then used as templates to synthesize Au NPs. Briefly, an aqueous solution of $HAuCl_4 \cdot 4H_2O$

(200 molar equivalents of the G5 dendrimer, 30 mg/mL, 5.72 mL) was added into the solution of G5.NH_2-DOTA-(PEG-FA)-mPEG-Cy5.5 dendrimers under vigorous stirring. After 30 min, an icy cold aqueous $NaBH_4$ solution (78.98 mg, 10 mL) with 5 times molar excess to the Au salt was added to the above mixture solution under stirring. The reaction mixture changed to be wine red within a few seconds after addition of the $NaBH_4$ solution, indicating the formation of Au NPs. The reaction mixture was stirred for 2 h to complete the reaction. After that, an aqueous $Gd(NO_3)_3$ solution 32.80 mg, 1 mL) with 35 molar equivalents to the G5 dendrimer was dropwise added to the above mixture solution under stirring for 24 h to form the {$(Au^0)_{200}$-G5.NH_2-DOTA(Gd)-(PEG-FA)-mPEG-Cy5.5} DENPs.

A final acetylation step was used to convert the remaining dendrimer terminal amines to acetamide groups. Briefly, triethylamine (160.0 μL) was added to the above solution of the {$(Au^0)_{200}$-G5.NH_2-DOTA(Gd)-(PEG-FA)-mPEG-Cy5.5} DENPs under magnetic stirring for 30 min. Then, acetic anhydride (130.3 μL) was added to the above mixture solution under stirring. The reaction finished after 24 h. The reaction mixture was then removed the excess reactants and by-products by extensively dialyzed against PBS (3 times, 2 L) and water (3 times, 2 L) for 3 days. Finally, the target Cy5.5-Gd-Au DENPs-FA product was obtained by lyophilization.

3. Characterization techniques

^1H NMR spectra were measured by a Bruker DRX 400 NMR spectrometer. UV-Vis spectra were recorded by a Lambda 25 UV-Vis spectrometer (PerkinElmer, Waltham, MI). TEM was performed using an analytical electron microscope (JEOL 2010F, Japan) working at 200 kV. DLS measurements (Zetasizer Nano ZS, Malvern, Worcestershire, UK) were performed with a standard 633 nm laser. ICP-AES (Prodigy, Leeman Labs, Hudson,NH) was used to analyze the composition of Au and Gd in the multifunctional NPs.

4. Cell culture

NCI-H460 human NSCLC cells were cultured using RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin at 37 ℃.in a humid incubator containing 5% CO_2

5. CT/MR/Optical tri-mode imaging of NCI-H460 cells *in vitro*

NCI-H460 cells were incubated with Cy5.5-Gd-Au DENPs-FA for 4 h at a concentration of 0, 0.25, 0.5, 1, and 2 mg/mL, respectively. The 1×10^6 treated cells were washed with PBS, and then were trypsinized, centrifuged and resuspended in 100 uL for the CT and Optical imaging experiments. For the MR imaging experiments, the same quantitative treated cells

were dispersed and fixed in 0.5% agarose for 100 uL. The treated cell suspension in each micro-tube was imaged respectively using: (1) a CT imaging system (SOMATOM Definition Flash, SIEMENS, Germany) with parameters as: tube voltage = 80 kV, tube current = 20 mA, and slice thickness = 0.6 mm; (2) a 3.0 Tesla MR imaging system (Discovery MR750, GE Healthcare, USA) with a wrist receiver coil. T1W images were acquired using an SE/2D sequence with the following parameters: TR = 400 ms, TE= 12.2 ms, NEX=4.00, matrix = 256 × 256, slice thickness = 2 mm, slice space=0.8 mm, and FOV= 12 cm; and (3) an near-infrared (NIR) optical imaging system (SPEXTRAL Ami and Amix manual v1.0, Spectral Instruments Imaging, USA) with a 675 nm excitation light and a 730 nm receiving light. CT values, T1W MR imaging signal intensities, and optical imaging signal intensities were measured using the software supplied by the manufacturers. Each imaging experiment was performed in triplicate.

6. Confirmation of the cellular uptake of nano-probes *in vitro*

The *in vitro* cellular uptake of Cy5.5-Gd-Au DENPs-FA was confirmed by ICP-AES and TEM.

For ICP-AES experiments, NCI-H460 cells were incubated for 24 h at the density of 3×10^5 cells per well in 6-well plates, and were then treated with Cy5.5-Gd-Au DENPs-FA for 4 h at the concentration of 0, 0.25, 0.5, 1, and 2 mg/mL, respectively. The treated cells were washed with PBS, and then typsinized, collected, and finally digested by aqua regia. The amount of Au element in the cells was quantitatively measured by a ICP-AES system (Prodigy, Leeman Labs, Hudson,NH). Each experiment was performed in triplicate.

For TEM imaging, the cells were treated with Cy5.5-Gd-Au DENPs-FA at the concentration of 1 mg/mL for 12 h. After washed with PBS and trypsinized, the treated cells were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h at 4 ℃ and post-fixed with 1% OsO_4 in 0.2 M phosphate buffer (pH 7.2) for 2 h at 4 ℃. After additional washed with PBS, the treated cells were then dehydrated in a series of ethanol solutions at the concentration of 30%, 50%, 70%, 95%, and 100%. The cell samples were finally embedded by Epon 812 (Shell Chemical, UK), followed with polymerization. Embedded cells were then sectioned for the thickness of 75 nm using a ReicharteUltramicrotome. The sectioned slices were mounted onto copper grids, and were then counterstained by uranyl acetate and lead citrate. The samples were imaged by a TEM system (H600, Hitachi, Japan) with the working voltage of 60 kV.

7. Cytotoxicity assay

The cytotoxicity of the Cy5.5-Gd-Au DENPs-FA was evaluated by MTT assay of treated cells. Cells were incubated in a 96 well plate for 4 h with the Cy5.5-Gd-Au DENPs-FA at the concentrations of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2 mg/mL, respectively. The treated cells were washed with PBS and then added with 200 mL fresh RPMI 1640 medium and 20 mL 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, 5 mg/mL in PBS) in each well, and were subsequently incubated for 4 h. Finally, after washed with PBS, the treated cells were added 200 mL DMSO into each well. The absorbance values were read using a microplate reader (Bio-tek) at the wavelength of 490 nm.

8. Establishment of the mouse NSCLC tumor model

The protocol of animal experiments was approved by the institutional committee for animal care, and also in compliance with the policy of the National Ministry of Health. Twenty-four male 4-week-old BALB/C nude mice (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were subcutaneously injected with 1×10^6 NCI-H460 cells per mouse in the right side of their back. Around 3 weeks after injection, the tumors grew to a volume of 1.0±0.15 cm^3. Then, five tumor-bearing mice were used for the *in vivo* targeted CT/MR/Optical tri-mode imaging experiments, eighteen mice were used for the investigation of *in vivo* nano-probes biodistribution, and the last one was used for the tumor pathological confirmation by FRs immunohistochemistry staining.

9. *In vivo* targeted CT/MR/Optical tri-mode imaging of NCI-H460 human NSCLC tumor

Firstly, the mice were intraperitoneally injected the chloral hydrate solution (10 wt%) for anesthesia. The targeted tri-mode imaging procedure was then sequentially performed using the NIR optical, CT, and MR imaging. Images of each modality were sequentially acquired both before and after intravenous injection of Cy5.5-Gd-Au DENPs-FA with a single-dose (a 100-μL PBS suspension containing 15 mg of Cy5.5-Gd-Au DENPs-FA) at time points of 1, 2, 4, and 6 h post-injection. Multi-mode images of the tumor-bearing mice were respectively acquired using: (1) a CT imaging system with 80 kV, 50 mA, and the slice thickness of 0.6 mm; (2) a 3.0 Tesla MR imaging system with a custom-built rodent receiver coil (Chenguang Med Tech, Shanghai, China). T1W images were acquired using an SE/2D sequence with the following parameters: TR= 2000 ms, TE= 81.2 ms, NEX=4.00, matrix= 256 × 160, slice thickness= 2 mm, slice space= 0.8 mm, and FOV= 6 cm; and (3) an optical imaging system

with an excitation light at 675 nm. CT values, MR signal intensities and optical imaging signal intensities of tumors were measured using the software supplied by the manufacturers.

10. Confirmation of the *in vivo* tumor uptake of nano-probes

The *in vivo* tumor uptake of Cy5.5-Gd-Au DENPs-FA was confirmed by silver enhancement staining. In brief, the tumors were cut and then fixed by 10% buffered formalin solution after imaging experiments. After dehydrated and embedded with paraffin, the tumors were sectioned for the thickness of 3 mm, and then were dewaxed and subsequently stained with the silver enhancement kit (Sigma-Aldrich) in accordance with the manufacturer's instruction. Sections were then washed with PBS, counterstained with 1% nuclear fast red, finally dehydrated and mounted for light microscopic observation.

Furthermore, both the FR immunohistochemistry staining and the silver enhancement staining were carried out on the same tumor section for co-localizing of the FRs and the nanoprobes in the NCI-H460 tumor according to the protocol.[31]

11. *In vivo* biodistribution of nano-probes

Fifteen tumor-bearing mice were euthanized after intravenous administration of the Cy5.5-Gd-Au DENPs-FA probes with the same dose (a 100-μL PBS suspension containing 15 mg of Cy5.5-Gd-Au DENPs-FA) as that in the imaging experiments. The tumor and main organs (eg. kidneys, liver, spleen, lung, heart) of each mouse were respectively extracted and then weighed at different time points after the administration of nanoprobes (1, 2, 4, 6, and 24 h, respectively). At each time point, three mice were euthanized to get the organs and tumors. For the negative control, three tumor-bearing mice untreated by Cy5.5-Gd-Au DENPs-FA probes were euthanized to obtain the organs and tumors as well. All organs and tumors were disposed by aqua regia. The amount of Au element in different organs or tumors was measured by ICP-AES.

12. Statistical analysis

All quantitative data was recorded as means ± S.D. The data between two groups was compared by Student's t-test, and the data among multiple groups was analyzed by one-way ANOVA followed by LSD's test. SPSS 17.0 software was used for the statistical analyses. A P value < 0.05 was considered the statistically significant.

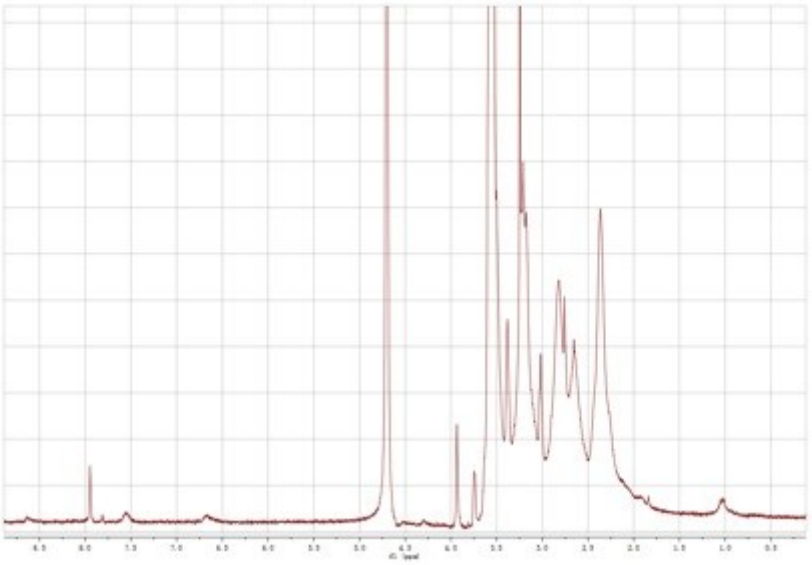

Fig. S1. ^{1}H NMR spectrum of G5.NH_2-DOTA-(PEG-FA)-*m*PEG dendrimer.

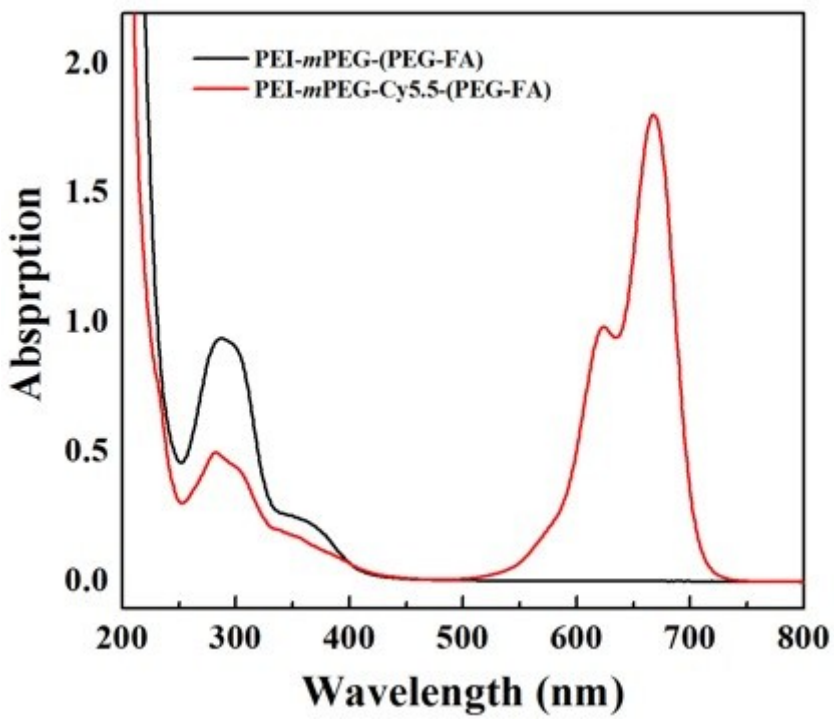

Fig. S2. UV-Vis spectra of G5.NH_2-DOTA-(PEG-FA)-mPEG dendrimer (black line) and G5.NH_2-DOTA-(PEG-FA)-mPEG-Cy5.5 dendrimers (red line) dispersed in water (pH = 6.0) at room temperature (25℃).

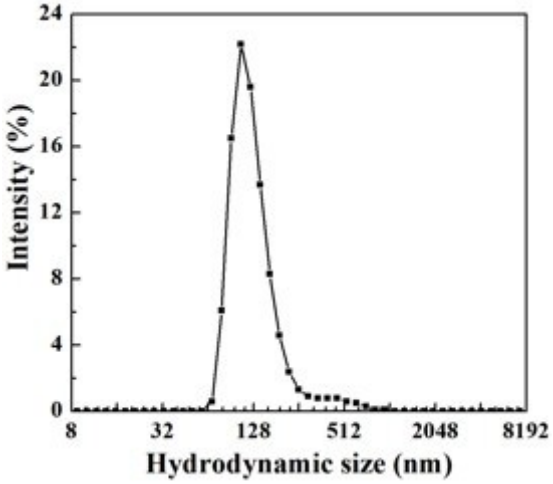

Fig. S3. The hydrodynamic size distribution of the Cy5.5-Gd-Au DENPs-FA probes dispersed in water.

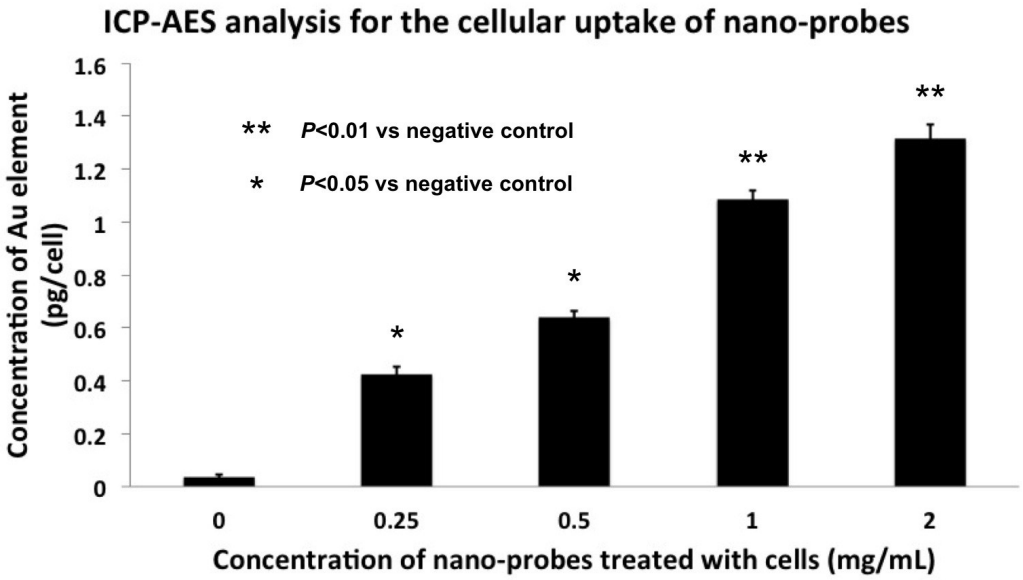

Fig. S4. The Au element uptake in the cells incubated with Cy5.5-Gd-Au DENPs-FA at different concentrations (0, 0.25, 0.5, 1, and 2 mg/mL). * indicates $P < 0.05$ vs negative control (0 mg/mL), ** indicates $P < 0.01$ vs negative control. (n=3).

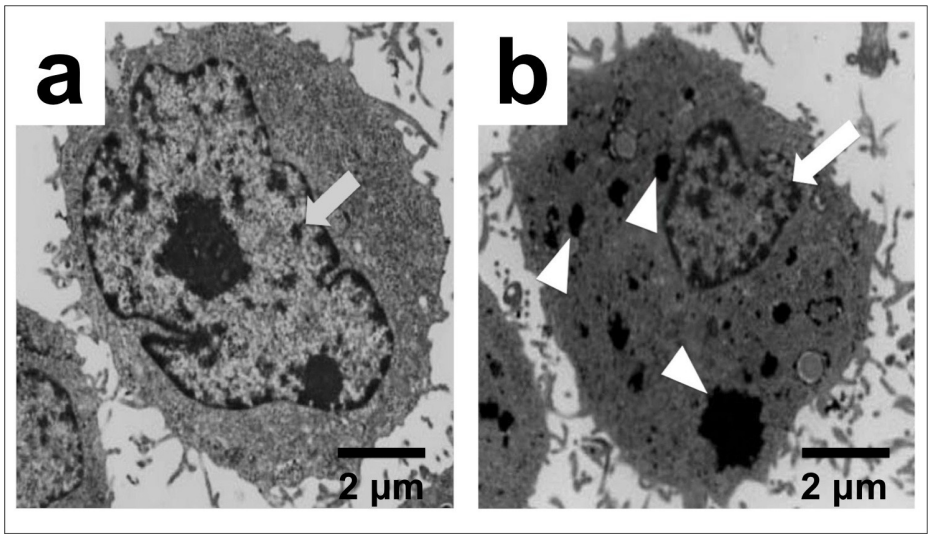

Fig. S5. TEM images of the negative cell (a) and the cell treated with (b) Cy5.5-Gd-Au DENPs-FA at concentration of 1 mg/mL for 12 h. The arrowheads indicate the high electron-staining nanoparticles in the cytoplasm of the treated cells. The white arrows indicate the nucleus of the cells.

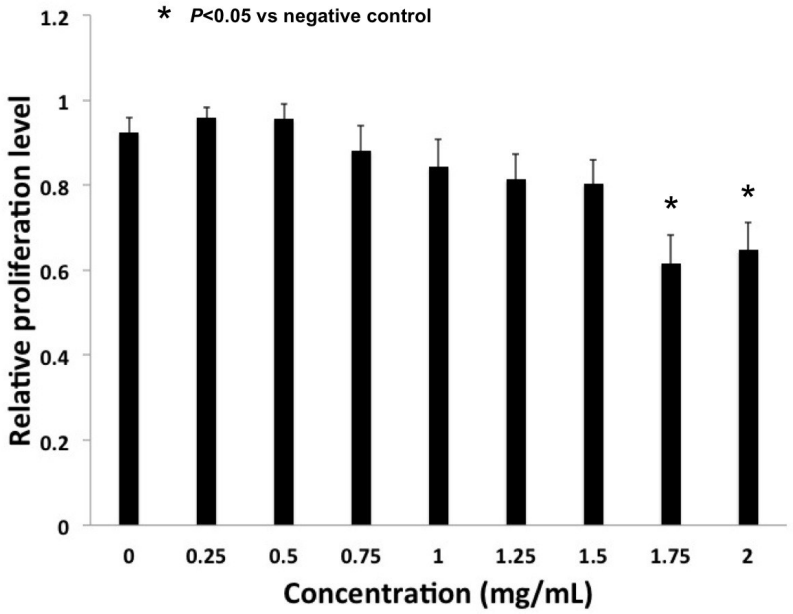

Fig. S6. MTT assay of the viability of SPC-A1 cells treated with Cy5.5-Gd-Au DENPs-FA at different concentrations for 4 h. * indicates $P < 0.05$ vs negative control. (n=5).

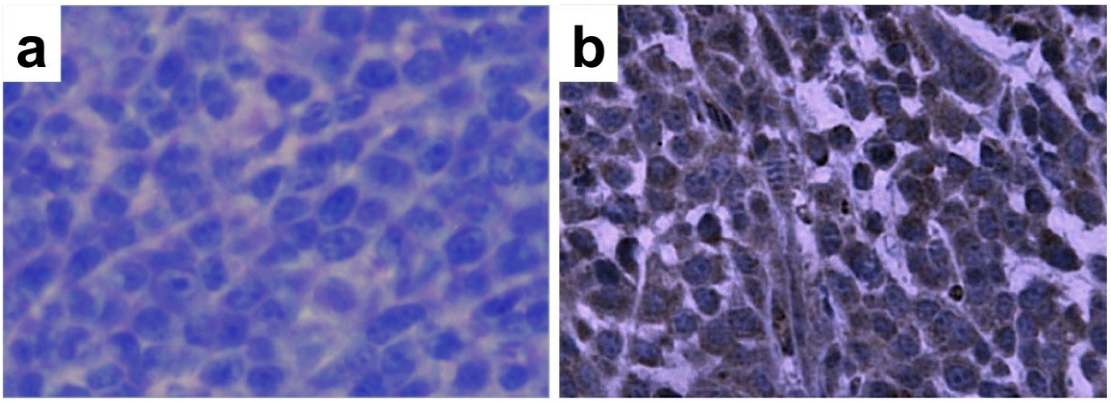

Fig. S7. The optical microscope images of FR immunohistochemistry staining of xenografted NCI-H460 tumor tissue. The FR positive cells show as dark brown staining (b), the negative control staining tumor tissue which do not display the brown sites (a). The magnification for tumor section was set at 400 ×.

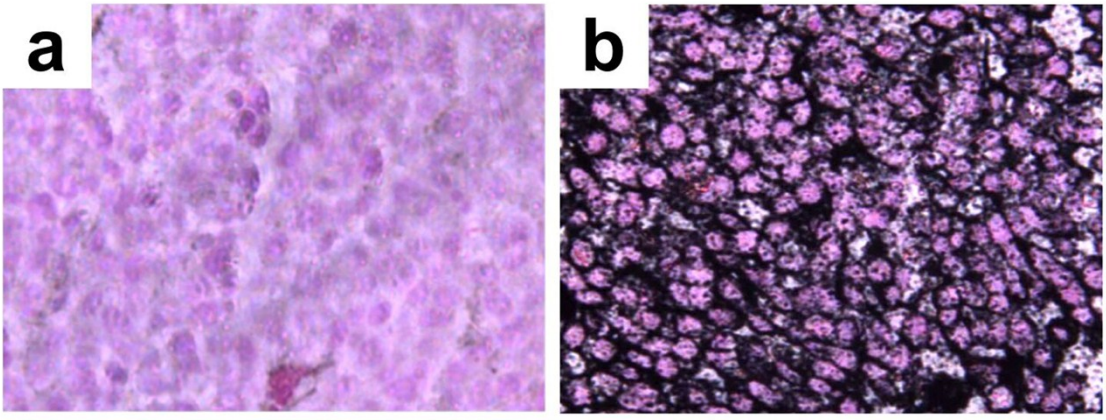

Fig. S8. The silver enhancement staining optical microscope images of the xenografts NCI-H460 tumor tissue: (a) the negative control tumor without treated with Cy5.5-Gd-Au DENPs-FA, (b) xenografts tumor after intravenous injection with Cy5.5-Gd-Au DENPs-FA.

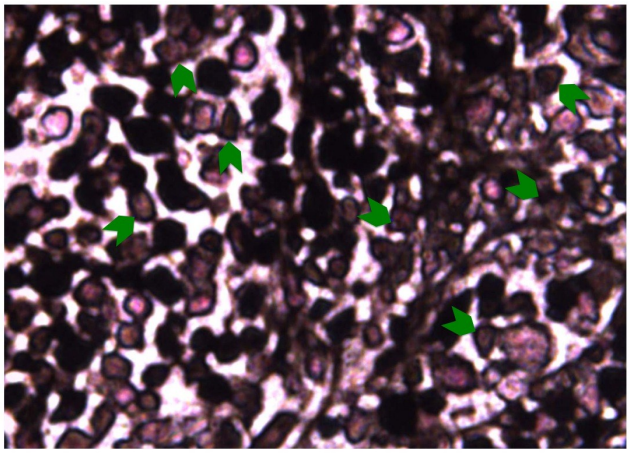

Fig. S9. The optical microscope image of the silver enhancement and the FR immunohistochemistry co-staining of xenografted NCI-H460 tumor tissue after intravenous injection with Cy5.5-Gd-Au DENPs-FA. Green arrowheads show the Cy5.5-Gd-Au DENPs-FA probes as black spots in cells in accordance with the expression of FR as brown staining in the same NCI-H460 cells. The magnification was set at 400 ×.

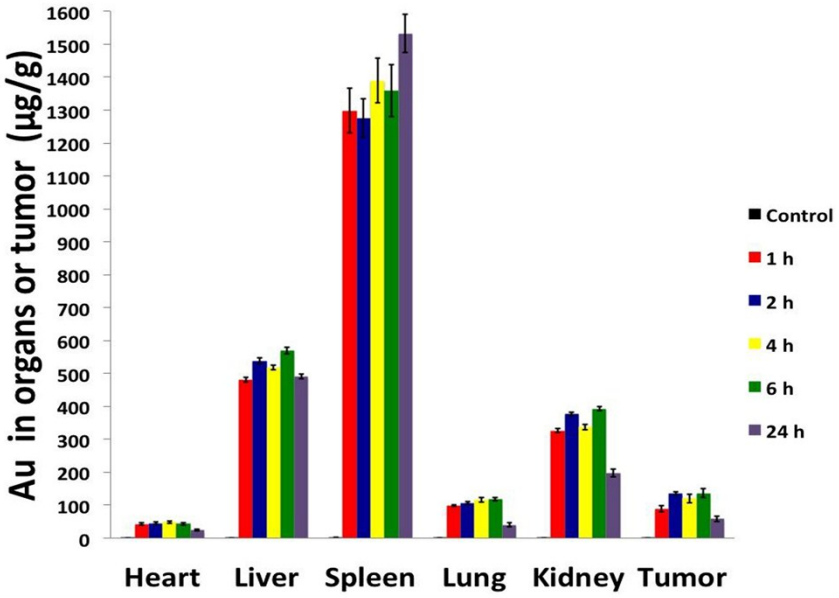

Fig. S10. Biodistribution of Au in the major organs of the mice including heart, liver, spleen, lung, kidney, and tumor. The data was recorded from the whole organ taken at different time points after intravenous injection of Cy5.5-Gd-Au DENPs-FA. (n=3).

Table S1

Biodistribution of Au in the major organs of the mice (n=3)

Time point after injecton of probes	Au in organs or tumor (μg/g)					
	Heart	Liver	Spleen	Lung	Kidney	Tumor
before (control)	0.59±0.33	0.24±0.02	1.39±0.03	0.64±0.02	0.29±0.02	0.05±0.01
1 h	42.73±3.21	480.39±7.83	1298.46±67.37	98.94±2.39	326.20±6.78	88.93±9.19
2 h	45.23±3.56	538.30±9.27	1276.31±59.09	107.87±3.46	376.68±5.88	135.63±3.83
4 h	48.28±3.59	518.94±7.64	1389.80±68.38	115.78±6.62	336.73±7.82	120.24±13.18
6 h	43.22±4.01	569.62±9.65	1359.27±78.83	117.84±5.29	382.77±5.72	136.76±13.29
24 h	23.66±2.56	490.57±6.73	1532.63±57.84	39.78±6.27	198.26±12.36	58.29±8.19