# Electronic Supplementary Information

# Dendrimers meet zwitterions: Development of a unique antifouling nanoplatform for enhanced blood pool, lymph node and tumor CT imaging

Zhijuan Xiong,[‡a] Yue Wang,[‡b] Jingyi Zhu,[c] Xin Li,[a] Yao He,[b] Jiao Qu,[b] Mingwu Shen,[a] Jindong Xia,*[b] Xiangyang Shi*[ad]

[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 Radiology, Shanghai Songjiang District Central Hospital, Shanghai 201600, People's Republic of China.

[c] College of Materials Science and Engineering, Donghua University, Shanghai 201620, People's Republic of China.

[d] CQM-Centro de Química da Madeira, Universidade da Madeira, Campus da Penteada, 9000-390 Funchal, Portugal

[‡] These authors equally contributed to this work.

* To whom correspondence should be addressed. E-mail addresses: xshi@dhu.edu.cn (X. Shi) and xiajd_21@163.com (J. Xia)

## Materials and Methods

### Materials

Ethylenediamine core G5 PAMAM dendrimers with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). PEG monomethyl ether with the other end of carboxyl group (*m*PEG-COOH, Mw = 2000) was purchased from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). N-[3-(Dimethylamino)propyl] acrylamide (DMAPA, 98%) was purchased from TCI (Shanghai, China). 2,6-Bis(1,1-dimethylethyl)-4-methylphenol (BHT) and β-propiolactone (98%) were from J&K Scientific (Shanghai, China). Acetic anhydride, triethylamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and all the other chemicals and solvents were purchased from Aldrich (St. Louis, MO) and used as received. Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18 MΩ·cm. Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 1000 or 8000-14000 were acquired from Fisher (Pittsburgh, PA). RAW 264.7 cells (a murine macrophage cell line) and U87MG cells (a human glioblastoma cell line) were from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified eagle medium (DMEM), penicillin, fetal bovine, streptomycin, and fetal bovine serum (FBS) were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Cell counting kit-8 (CCK-8) was purchased from 7Sea Pharmtech Co., Ltd. (Shanghai, China). Male 4- to 6-week-old BALB/c mice, Sprague-Dawley (SD) rats (180-210 g) and female Japanese long-ear white rabbits (1.5 kg) were obtained from Shanghai Slac Laboratory Animal Center (Shanghai, China).

### Synthesis of CBAA monomer

CBAA was first synthesized according to the literature.[1] DMAPA (1.00 g) and β-propiolactone (0.64 g) were co-dissolved in anhydrous acetone (10 mL) and reacted at 0 $^{o}$C for 3 h under $N_2$ atmosphere. The CBAA monomer was obtained by washing the white precipitate with anhydrous acetone and anhydrous ether, followed by being dried in vacuum, and stored at 4 °C. Yield: 80%. $^{1}$H NMR (in $D_2O$): 6.11 (t, 1H, CHH=CH), 6.05 (t, 1H, CHH=CH), 5.62 (t, 1H, CHH=CH), 3.40 (t, 2H, N-$CH_2$-

$CH_2$-COO), 3.20 (m, 4H, NH-$CH_2$-$CH_2$-$CH_2$), 2.91 (s, 6H, N-$(CH_3)_2$), 2.49 (t, 2H, $C_2$-COO), 1.89 (t, 2H, NH-$CH_2$-$CH_2$-$CH_2$).

**Synthesis of dendrimer-based CT contrast agents**

G5 PAMAM dendrimers were modified by CBAA. In brief, G5 PAMAM dendrimer (50.00 mg) dissolved in 10 mL methanol was reacted with different amounts of CBAA (75.00 mg or 200.00 mg) predissolved in an aqueous solution of NaCl (10 mL, 0.138 M) under stirring. BHT (0.10 mg) was then added to inhibit the polymerization of CBAA. The reaction mixture was magnetically stirred for 48 h at room temperature, followed by extensive dialysis against phosphate buffered saline (PBS, 3 times, 4 L) and water (3 times, 4 L) through a 8000-14000 molecular weight cut-off (MWCO) membrane for 3 days, and then lyophilization to get the product of G5.$NH_2$-$CBAA_{20}$ and G5.$NH_2$-$CBAA_{80}$.

For comparison, G5 PAMAM dendrimer was also reacted with *m*PEG-COOH to generate G5.$NH_2$-*m*$PEG_{20}$ according to our previous work.[2] *m*PEG-COOH (76.89 mg, in 10 mL water) with 20 molar equiv. of G5.$NH_2$ PAMAM dendrimer (50.00 mg), was reacted with EDC (36.83 mg, in 5 mL water) and NHS (22.14 mg, in 5 mL water) under vigorous magnetic stirring for 3 h. The activated *m*PEG-COOH was then dropwise added into the G5 PAMAM dendrimer solution (50.00 mg, in 10 mL water) under stirring for 3 days at room temperature. Then the mixture solution was extensively dialyzed against PBS (3 times, 4 L) and water (3 times, 4 L) through an 8000-14000 MWCO membrane for 3 days, and then lyophilized to get the product of G5.$NH_2$-*m*$PEG_{20}$.

G5.$NH_2$-$CBAA_{20}$ (100.00 mg, in 20 mL water), G5.$NH_2$-$CBAA_{80}$ (144.97 mg, in 20 mL water) or G5.$NH_2$-*m*$PEG_{20}$ (215.74 mg, in 20 mL water) was mixed with $HAuCl_4 \cdot 4H_2O$ (134.30 mg, in 4.48 mL water) under vigorous magnetic stirring for 30 min, respectively. Then $NaBH_4$ (61.80 mg, in 5 mL water) was added to each mixture solution under stirring for 3 h at room temperature. Next, triethylamine (303.20 μL) was added to each mixture solution with continuous stirring for 30 min, followed by addition of acetic anhydride (171.70 μL) and the reaction mixture was stirred overnight. At last, each mixture solution was subjected to the same dialysis and lyophilization processes to get

the products of {$(Au^0)_{100}$-G5.NHAc-$CBAA_{20}$}, {$(Au^0)_{100}$-G5.NHAc-$CBAA_{80}$} or {$(Au^0)_{100}$-G5.NHAc-*m*$PEG_{20}$}. In order to track the intracellular uptake of the Au DENPs, G5.$NH_2$ dendrimers were first reacted with fluorescein isothiocyanate (FI) according to our previous work,[3] followed by the same modification of either CBAA or *m*PEG, entrapment of Au NPs, and final acetylation to generate {$(Au^0)_{100}$-G5.NHAc-FI-$CBAA_{20}$}, {$(Au^0)_{100}$-G5.NHAc-FI-$CBAA_{80}$} or {$(Au^0)_{100}$-G5.NHAc-FI-*m*$PEG_{20}$}.

**Characterization techniques**

$^1$H NMR measurements were carried out on a Bruker DRX 500 NMR spectrometer operating at 400 MHz. $D_2O$ was used as a solvent to dissolve all materials before measurements. Zeta-potential and dynamic light scattering (DLS) measurements were performed using a Malvern Zetasizer Nano ZS system (model ZEN3600, Worcestershire, UK) equipped with a standard 633 nm laser. The composition of the Au DENPs was measured by Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH). The size and morphology of the Au DENPs were characterized by transmission electron microscopy (TEM, JEOL 2010F, Tokyo, Japan). UV−vis spectroscopy was carried out using a Lambda 25 UV−vis spectrophotometer (PerkinElmer, Boston, MA). CT phantoms studies of Au DENPs were performed according to our previous work.[4] In brief, aqueous solutions of the Au DENPs (0.1 mL) at different Au concentrations and iohexol 300 (Omnipaque 300 mg/mL, GE Healthcare) at different iodine concentrations (0.1 mL) were prepared in 2.0 mL-Eppendorf tubes and placed in a homemade scanning holder. CT scanning was carried out using a clinical Brilliance 64-slice CT imaging system (Philips Healthcare, Andover, MA) with 120 kV, 200 mA, and a slice thickness of 1.5 mm. Evaluation of X-ray attenuation intensity was carried out by loading the digital CT images in a standard display program and then selecting a uniform round region of interest on the resultant CT image for each sample. The CT contrast enhancement was evaluated in Hounseld units (HU) for each concentration of the Au DENPs or Omnipaque.

**Protein resistance assay**

We used protein resistance assay to investigate the antifouling property of the {$(Au^0)_{100}$-

G5.NHAc-CBAA$_{80}$}, {(Au$^0$)$_{100}$-G5.NHAc-CBAA$_{20}$}, and {(Au$^0$)$_{100}$-G5.NHAc-*m*PEG$_{20}$} DENPs. UV−vis spectroscopy was employed to check the absorbance of bovine serum albumin (BSA, 1 mg/mL, in PBS)/Au DENPs mixture at 278 nm at the time of mixture and after incubation with the Au DENPs at different concentrations (0, 0.125, 0.5 and 2 mg/mL, respectively) at 37 °C for 4 h, followed by centrifugation. After interaction with the Au DENPs, the mixtures were centrifuged and the supernatant was measured. The reduced absorbance at 278 nm for the BSA/Au DENPs mixture was calculated to quantify the antifouling property of the Au DENPs.

**Cytotoxicity assay and cytoskeleton observation**

U87MG cells (a human glioblastoma cell line) and RAW264.7 cells (a murine macrophage cell line) were continuously cultured and passaged in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a 5% $CO_2$ incubator.

Cell Counting Kit-8 (CCK-8) assay was used to test the cytotoxicity of the Au DENPs. In brief, $1\times10^4$ U87MG cells were seeded into each well of a 96-well plate and cultured overnight. Then the medium was replaced with fresh medium containing {(Au$^0$)$_{100}$-G5.NHAc-CBAA$_{20}$}, {(Au$^0$)$_{100}$-G5.NHAc-CBAA$_{80}$} or {(Au$^0$)100-G5.NHAc-*m*PEG$_{20}$} at different Au concentrations (0, 10, 25, 50, 100, 200, and 400 μM, respectively) and the cells were incubated for 24 h. After that, the medium of each well was discarded and the cells were washed with PBS for 3 times. The cells in each well were added with 100 μL medium containing 10 μL CCK-8 and incubated for another 4 h. A Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA) was used to record the absorbance at 450 nm for each well. Mean and standard deviation of the triplicate wells for each sample were reported.

To further study the cytotoxicity of Au DENPs, the cytoskeleton of cells after 24 h incubation with Au DENPs ([Au] = 400 μM) was observed. In brief, cover slips with a diameter of 14 mm were pretreated with 5% HCl, 30% $HNO_3$, and 75% alcohol and then placed in 12-well tissue culture plate. $2\times10^5$ U87MG cells were seeded into each well of a 12-well plate and cultured overnight. Then the medium was replaced with fresh medium containing the {(Au$^0$)$_{100}$-G5.NHAc-CBAA$_{20}$}, {(Au$^0$)$_{100}$-

G5.NHAc-CBAA$_{80}$} or {(Au$^0$)$_{100}$-G5.NHAc-*m*PEG$_{80}$} DENPs with an Au concentration of 400 μM and the cells were incubated for 24 h to allow the cells to attach onto the cover slips. After that, the medium of each well was discarded and the cells were washed with PBS for 3 times. The cells in each well were added with 500 μL 4% paraformaldehyde solution and incubated for 5 min. Next, the cells were washed with PBS for 3 times, followed by addition of 0.1% Triton X-100 (500 μL, in PBS) to each well for 4 min. After that, the cells were washed with PBS for 3 times. Then, a fluorescein isothiocyanate (FI)-Phalloidin solution (500 μL, in methanol) was added into each well and the cells were incubated for 30 min. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei for 7 min at room temperature. Finally, the cells were washed with PBS for 3 times and samples were scanned using a 63× oil immersion objective lens.

**Cellular uptake assays**

To check the cellular uptake of the FI-labeled Au DENPs, RAW264.7 cells were seeded in 12-well cell culture plates at a density of $2\times10^5$ cells per well the day before the experiment. The medium were replaced with fresh DMEM containing the {(Au$^0$)$_{100}$-G5.NHAc-FI-CBAA$_{20}$}, {(Au$^0$)$_{100}$-G5.NHAc-FI-CBAA$_{80}$}, or {(Au$^0$)$_{100}$-G5.NHAc-FI-*m*PEG$_{20}$} DENPs at different particle concentrations (0, 1, 2 and 4 μM, respectively). After 4 h incubation, the cells were trypsinized, resuspended in PBS, and analyzed using a Becton Dickinson FACScan analyzer equipped with a 15 mW, 488 nm, and air-cooled argon ion laser. The fluorescence mission was collected through a 530 nm band-pass filter and acquired in log mode. The FL3-fluorescence of 10 000 cells was measured, and the mean fluorescence of gated viable cells was quantified.

To further study the uptake of {(Au$^0$)$_{100}$-G5.NHAc-FI-CBAA$_{20}$}, {(Au$^0$)$_{100}$-G5.NHAc-FI-CBAA$_{80}$}, or {(Au$^0$)$_{100}$-G5.NHAc-FI-*m*PEG$_{20}$} DENPs by RAW264.7 cells, confocal microscopy (Carl Zeiss LSM 700, Jena, Germany) was used according to our previous report.[5] Briefly, cover slips with a diameter of 14 mm were pretreated and fixed in a 24-well tissue culture plate according procedures described above. RAW264.7 cells were seeded at a density of $8\times10^4$ cells per well within 1 mL fresh medium and cultured at 37 $^o$C and 5% $CO_2$ for 24 h to allow the cells to attach. Then, the

medium was discarded, and replaced with 1 mL fresh medium containing PBS (control), or different Au DENPs (1 and 4 μM, respectively). After 4 h incubation, the cells were washed, fixed, and counterstained with DAPI (1 mg/mL) using a standard procedure as described above. The optical section thickness was set at 5 mm. Samples were scanned using a 63× oil immersion objective lens.

**Pharmacokinetics and CT imaging of blood pool of rats**

Animal experiments were carried out according to protocols approved by the ethical committee of Shanghai General Hospital for animal care and also in accordance with the policy of the National Ministry of Health. Pharmacokinetics was performed using female Spraguee-Dawley rats (200-240 g) according to the protocols described in our previous study.[6] Each rat was intravenously injected with a saline solution containing different Au DENPs (500 μL, [Au] = 0.1 M) through tail vein. The blood samples were collected at different time points postinjection (from 0 to 72 h). The Au concentration in the blood samples was measured by ICP-OES according to our previous report.[7] The half-decay time ($t_{1/2}$) of Au DENPs was analyzed by DAS Software 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

Female Spraguee-Dawley rats (200-240 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). Au DENPs (500 μL, [Au] = 0.1 M) were then tail vein intravenously injected into rats. Then the rats were scanned by a clinical Brilliance 64-slice CT imaging system (Philips Healthcare, Andover, MA) with 80 kV, 450 mA, and a slice thickness of 45 mm.

**CT imaging of lymph node of rabbits**

The female Japanese long-ear white rabbits (1.5 kg) were anesthetized using pentobarbital sodium (40 mg/ kg). Then, Au DENPs (500 μL, [Au] = 0.1 M) were injected intradermally into the right paw of the rabbits (n = 3). CT imaging of lymph node was performed before and at different time points postinjection using the same instrument and parameters as mentioned above.

**CT imaging of a xenografted mouse tumor model**

Male 4- to 6-week-old BALB/c nude mice (n = 6) were used to establish a xenografted U87MG

tumor model (0.1 - 0.3 $cm^3$) according to protocols described in the literature.[8] The mice were intravenously delivered with Au DENPs (150 μL, [Au] = 0.1 M), placed in a scanning holder, and then CT scanned using the same CT imaging system with the same parameters described above.

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

Male 4- to 6-week-old BALB/c nude mice were selected to investigate the *in vivo* biodistribution of the injected Au DENPs ([Au] = 0.1 M, in 150 μL saline). The mice were euthanized at the time point of 0, 2, 8, 12 and 24 h postinjection. The heart, liver, spleen, lung, kidney were extracted and weighed. The organs were cut into 1 - 2 $mm^2$ pieces and incubated in aqua regia solution for 4 h. Au content was determined by a Leeman Prodigy ICP-OES system (Hudson, NH03051, USA).

To evaluate the long-term organ toxicity of Au DENPs *in vivo*, male 4- to 6-week-old BALB/c nude mice were intravenously administered with Au DENPs ([Au] = 0.1 M, in 150 μL saline). Saline (150 μL) was used as control. After one month, the mice were anesthetized, and the liver, lung, spleen, heart, and kidney were harvested, washed, fixed, sectioned, and hematoxylin and eosin (H&E) stained according to standard protocols for optical microscopic observation. [7]

**Statistical analysis**

One way ANOVA statistical analysis was performed to evaluate the significance of the experimental data. 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.** Surface potential and hydrodynamic size of the Au DENPs.

| Sample | Surface potential (mV) | Hydrodynamic size (nm) | Polydispersity index (PDI) |
|---|---|---|---|
| $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{20}\}$ | 5.8±0.5 | 140.0±0.9 | 0.343 |
| $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{80}\}$ | 12.1±0.6 | 138.2±2.7 | 0.510 |
| $\{(Au^0)_{100}$-G5.NHAc-*m*$PEG_{20}\}$ | 2.6±0.5 | 109.2±1.3 | 0.318 |

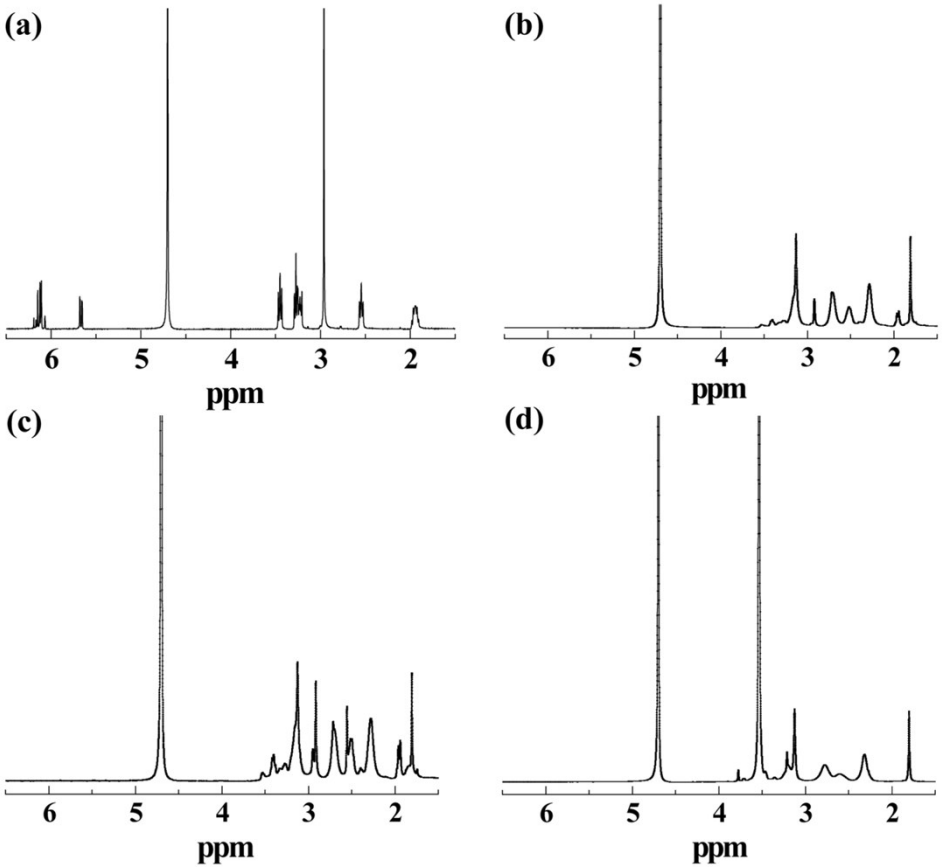


**Fig. S1.** $^1H$ NMR spectra of CBAA (a), $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{20}\}$ (b), $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{80}\}$ (c) and $\{(Au^0)_{100}$-G5.NHAc-*m*$PEG_{20}\}$ (d) in $D_2O$.

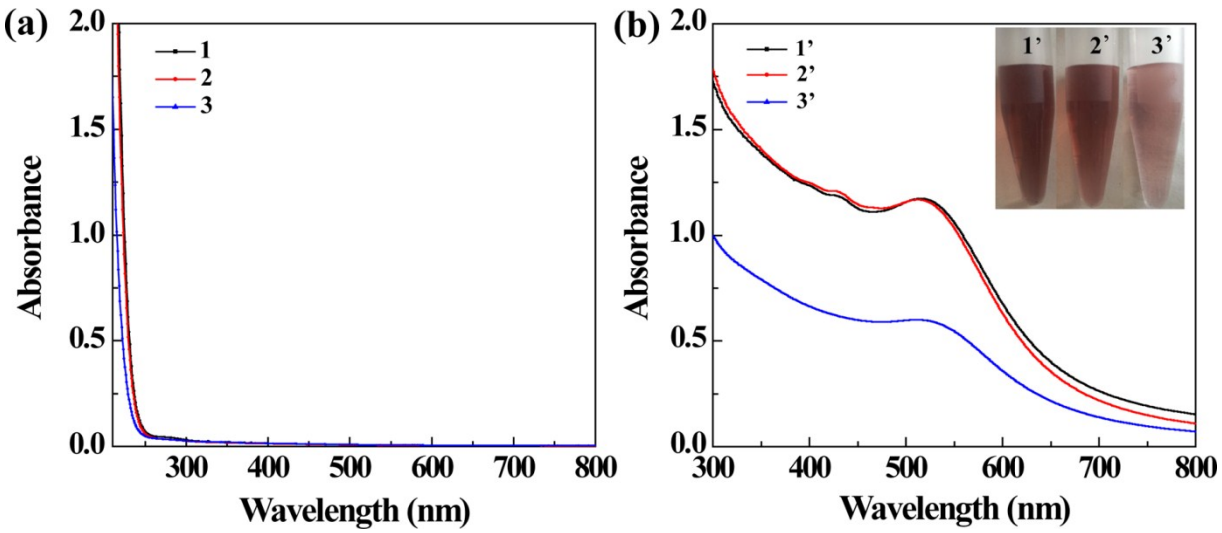


**Fig. S2.** (a) UV-vis spectra of (1) G5-CBAA$_{20}$, (2) G5-CBAA$_{80}$, and (3) G5-*m*PEG$_{20}$ dendrimers. (b) UV-vis spectra of (1') {(Au$^0$)$_{100}$-G5-CBAA$_{20}$}, (2') {(Au$^0$)$_{100}$-G5-CBAA$_{80}$} and (3') {(Au$^0$)$_{100}$-G5-*m*PEG$_{20}$} NPs. Inset of (b) shows the photographs of the respective aqueous suspension of the Au DENPs (0.25 mg/mL).

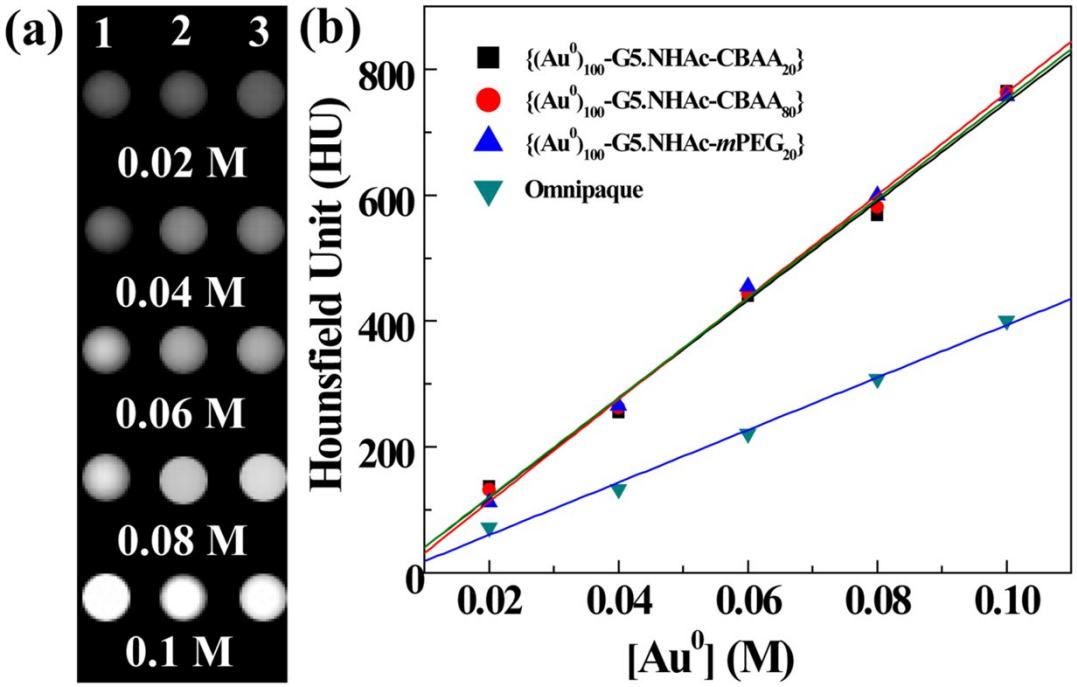


**Fig. S3.** (a) CT images of {(Au$^0$)$_{100}$-G5.NHAc-CBAA$_{20}$} (1), {(Au$^0$)$_{100}$-G5.NHAc-CBAA$_{80}$} (2) and {(Au$^0$)$_{100}$-G5.NHAc-*m*PEG$_{20}$} (3) and (b) X-ray attenuation intensity of Au DENPs and Omnipaque as a function of the molar concentration of the radiodense element (Au or iodine).

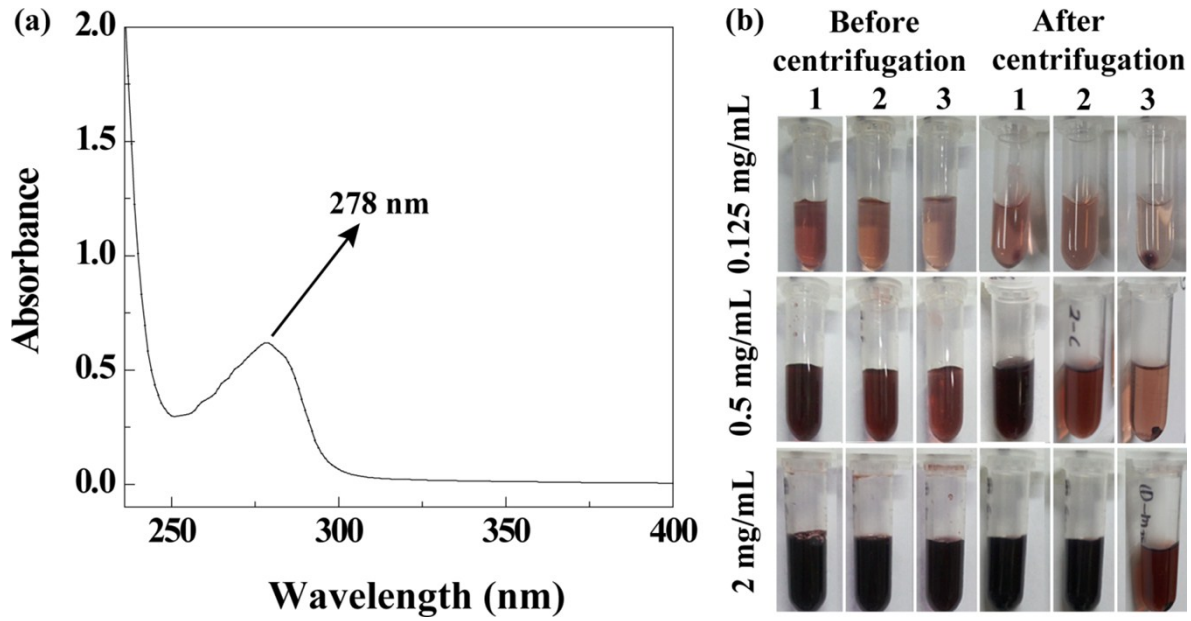


**Fig. S4.** UV-vis spectrum of BSA dispersed in PBS at room temperature (a) and photographs of BSA/Au DENPs mixture after centrifugation (b). BSA (1 mg/mL) was incubated with $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{20}\}$ (1), $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{80}\}$ (2) or $\{(Au^0)_{100}$-G5.NHAc-*m*$PEG_{20}\}$ (3) at different concentrations for 4 h, respectively, followed by centrifugation (8000 rpm, 5 min).

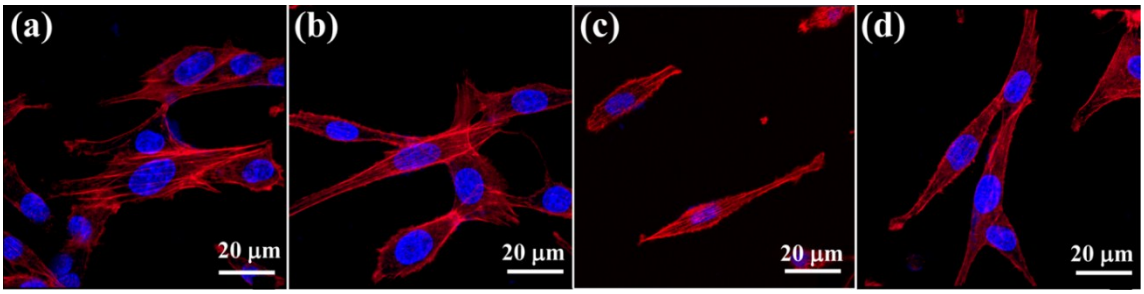


**Fig. S5.** Confocal microscopic images of the cytoskeleton of U87MG cells treated with PBS (a), $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{20}\}$ (b), $\{(Au^0)_{100}$-G5.NHAc-$CBAA_{80}\}$ (c) and $\{(Au^0)_{100}$-G5.NHAc-*m*$PEG_{20}\}$ (d), respectively for 24 h. The Au concentration for all Au DENPs used was 400 μM.

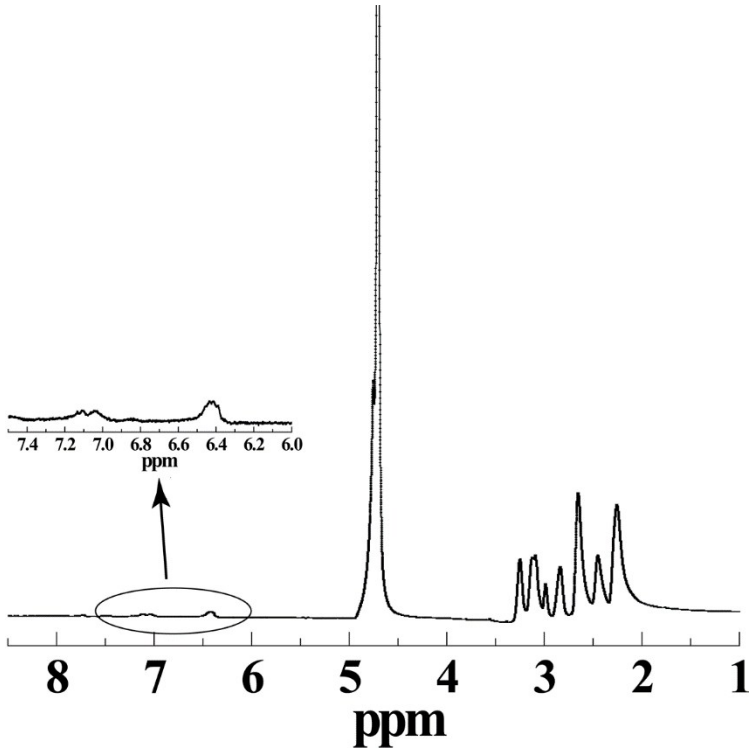


**Fig. S6**. $^{1}$H NMR spectrum of G5.$NH_2$-FI dendrimers.

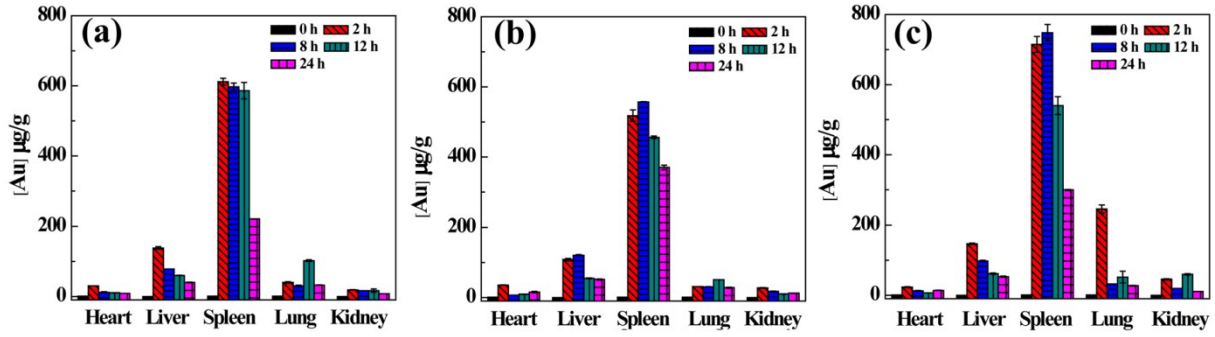


**Fig. S7.** The biodistribution of {$(Au^0)_{100}$-G5.NHAc-$CBAA_{20}$} (a), {$(Au^0)_{100}$-G5.NHAc-$CBAA_{80}$} (b) and {$(Au^0)_{100}$-G5.NHAc-*m*$PEG_{20}$} (c) in different organs of mice. The data were obtained by ICP-OES at 0, 2, 8, 12, and 24 h postinjection, respectively.

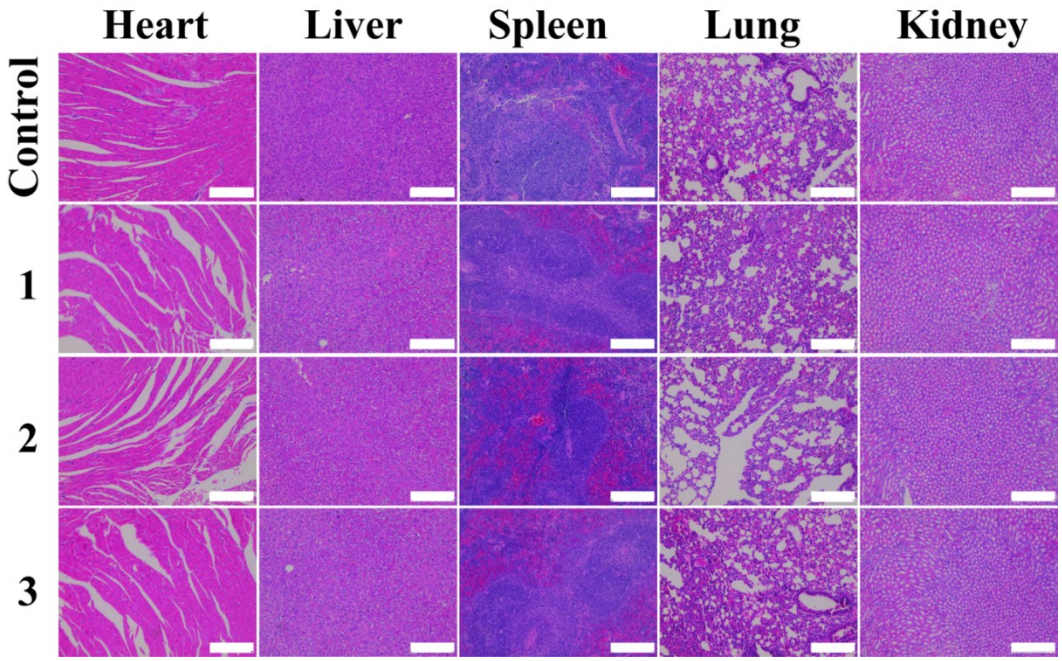


**Fig. S8.** Histological changes in heart, liver, spleen, lung, and kidney of the mice at one month post intravenous injection of $\{(Au^0)_{100}$-G5.NHAc-CBAA$_{20}\}$ (1), $\{(Au^0)_{100}$-G5.NHAc-CBAA$_{80}\}$ (2) or $\{(Au^0)_{100}$-G5.NHAc-*m*PEG$_{20}\}$ (3) ( [Au] = 0.1 M, 150 μL in saline, for each mouse) (n = 3). Mice injected with PBS were used as control. These organ sections were H&E stained and observed under an optical microscope (the scale bar in each panel indicates 200 μm).

## References


1. L. Wang, Z. Wang, G. Ma, W. Lin and S. Chen, *Langmuir*, 2013, **29**, 8914-8921.
2. C. Peng, J. Qin, B. Zhou, Q. Chen, M. Shen, M. Zhu, X. Lu and X. Shi, *Polym. Chem.*, 2013, **4**, 4412-4424.
3. Y. Wang, R. Guo, X. Cao, M. Shen and X. Shi, *Biomaterials*, 2011, **32**, 3322-3329.
4. Y. Cao, Y. He, H. Liu, Y. Luo, M. Shen, J. Xia and X. Shi, *J. Mater. Chem. B*, 2015, **3**, 286-295.
5. J. Zhu, F. Fu, Z. Xiong, M. Shen and X. Shi, *Colloids Surf., B*, 2015, **133**, 36-42.
6. C. Peng, L. Zheng, Q. Chen, M. Shen, R. Guo, H. Wang, X. Cao, G. Zhang and X. Shi, *Biomaterials*, 2012, **33**, 1107-1119.
7. B. Zhou, L. Zheng, C. Peng, D. Lo, J. Li, S. Wen, M. Shen, G. Zhang and X. Shi, *ACS Appl. Mater. Interfaces*, 2014, **6**, 17190-17199.
8. J. Zhu, L. Zheng, S. Wen, Y. Tang, M. Shen, G. Zhang and X. Shi, *Biomaterials*, 2014, **35**, 7635-7646.