Supporting Information

Core–Shell Nanostars for Multimodal Therapy and Imaging

Mengyuan Li, Lele Li, Changyou Zhan, Daniel S. Kohane*

Laboratory for Biomaterials and Drug Delivery, Department of Anesthesiology, Division of Critical Care Medicine, Children's Hospital Boston, Harvard Medical School, Boston, MA, 02115. Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, 02139. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge MA 02139.

Correspondence author: daniel.kohane@childrens.harvard.edu

Chemicals and Materials. All the chemicals and materials used were commercially available unless otherwise stated and were used without further purification. Gadolinium(III) chloride hexahydrate (GdCl$_3$•6H$_2$O) and gold(III) chloride trihydrate (HAuCl$_3$•3H$_2$O) were purchased from Sigma-Aldrich (St. Louis, MO). Gemcitabine-5’-monophosphate disodium salt were from HDH Pharma (Morrisville, NC).

Preparation of AuNS@CP Nanoparticles. AuNS were synthesized by reducing HAuCl$_3$•3H$_2$O in HEPES buffer to fabricate surfactant-free gold nanoparticles based on a previously reported procedure.[1] Typically, 150 µL of 40 mM HAuCl$_3$•3H$_2$O was added to 30 mL of 140 mM HEPES buffer (pH 7.4). The resonance wavelength of the reaction mixture was monitored using UV-vis-NIR absorption spectroscopy (Tecan Infinite M200 spectrophotometer, Tecan Group, Switzerland). AuNS were collected by ultrafiltration and washed thoroughly with water when the plasmon resonance peak was around 760 nm (indicating the formation of AuNS). The as-synthesized AuNS (600 µL, 1.0 mg/mL in deionized water) were mixed with GMP solution (600 µL, 1.0 mg/mL in deionized water). After stirring overnight, the mixture was added to GdCl$_3$•6H$_2$O solution (1.2 mL, 0.2 mg/mL in HEPES buffer solution (100 mM, pH 7.4)) under stirring. Then, PEG-DiP (100 µL, 4 mg/mL in deionized water) was added and the resulting mixture was incubated at room temperature for 3 h. The AuNS@CP were collected after ultrafiltration. To determine the content of Au and Gd in AuNS@CP, a sample of collected AuNS@CP nanoparticles was dissolved in 2% HNO$_3$ and analyzed by ICP-MS (Sciex Elan 6100, Perkin Elmer, Norwalk, CT). The GMP content of AuNS@CP was calculated by subtracting the amount of GMP in the supernatant (determined by HPLC, Agilent Technologies, Santa Clara, CA ) from the total amount of GMP used for the reaction. TEM images were taken on a JEOL 2100 (JEOL, Peabody, MA) advanced high performance microscope with an accelerating voltage of 200 kV. The sizes and polydispersities of NPs were characterized by particle analyzer (Delsa Nano C, Beckman Counter).

Photothermal Effect of AuNS@CP Nanoparticles. 100 µL of AuNS@CP saline solution (0.5 mM Au in saline) in a 96 well plate was irradiated with an 808 nm laser (GCSLS-05–7W00 fiber-coupled diode laser system, Daheng Science&Technology, China) at different power densities (42, 107 and 205 mW/cm$^2$) for 8 min. The spot size of the laser was adjusted to cover the entire solution surface. The temperature increase of the solution was recorded by an FLIR E50 infrared imaging camera (FLIR Systems, Wilsonville, OR). Saline was irradiated at 205 mW/cm$^2$ as a control. The temperature increase


of 100 uL of AuNS@CP saline solution at different concentrations (Au concentration: 0.0625, 0.125, and 0.25 mM) were also measured at power density of 0.5 W/cm² for 5 min.

**Release of GMP from AuNS@CP.** 1.0 mL of AuNS@CP (2.5 mM Au) in saline was placed in a dialysis tube (Slide-A-Lyzer® MINI, MWCO 1 KDa, Thermo Fisher Scientific Inc, Grand Island, NY). The tube was then immersed in 13.5 mL of saline and incubated at 37°C. At predetermined time intervals, 1.0 mL of the external saline was removed for determination of GMP content by HPLC, and 1.0 mL of fresh saline was added.

**Cell Cytotoxicity Assay.** 4T1 breast cancer cell line was cultured in RPMI 1640 medium (Life Technologies, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. On the day of experiments, cells were washed with pre-warmed PBS and incubated with pre-warmed RPMI 1640 medium contain GMP, AuNS, or AuNS@CP. After 6 h, the cells were irradiated with 808 nm NIR light (0.25 W/cm² or 0.5 W/cm² for 3 min). Cytotoxicity was evaluated by MTS (3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay after 24 or 48 h. MTS assay is a colorimetric method for quantification of viable cells in proliferation and cytotoxicity assay. The MTS tetrazolium compound can be reduced by viable cells and generate a colored formazan dye which can be quantified by measuring the absorbance at 490-500 nm.

**Magnetic Relaxivity Measurement.** A range of concentration of aqueous AuNS@CP (200 μL) were transferred into tubes for longitudinal magnetic relaxivity measurements. T₁-weighted MR images were acquired on a Siemens Magnetom Trio with a 7T magnet field (Erlangen, Germany). The parameters were set as follows: 256 × 256 data matrix; 45 × 45 mm field of view; 10 slices; a slice thickness of 0.5 mm; repetition time (TE) = 8.92 ms, echo time (TR) = 5000 ms, and inversion recovery times (TI) = 400, 800, 1200, 1600, 2000, 2400, 2800, and 3200 ms. The following standard inversion-recovery formula was used to calculate T₁ values of each tube: \[ S(TI) = S_0 \times \left(1 - 2e^{-\frac{TI}{T_1}}\right) \] to fit the T₁ recovery curve, where S(TI) is the signal measured at a certain TI and S₀ is the signal that would be available at full longitudinal magnetization. The resulting mean T₁ values over the region of interest were plotted as 1/T₁ (R₁) vs molar concentration of Gd(III). The molar relaxivity r₁ was calculated from the slope of the plotted line.

**4T1 Tumor Model and in vivo MR Imaging.** Immunodeficient 6-8 week nu/nu nude female mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and maintained under pathogen-free conditions for all animal studies. The study protocol was reviewed and approved by the MIT Committee on Animal Care. For subcutaneous 4T1 tumor models, about 1 × 10⁶ cells /100 µL 4T1 cells in 1:1 (v/v) PBS and Matrigel (BD Biosciences, Franklin Lake, NJ) were injected subcutaneously in the flank. When the tumors reached ~200 mm³, mice were anaesthetized with isoflurane and injected i.v. with AuNS@CP nanoparticles (0.05 mmol/kg Gd(III)). T₁-weighted MR images were acquired on a Siemens Magnetom Trio with a 7T magnet field (Erlangen, Germany) at designated time points after injection. The detailed imaging parameters were set as follows: TR/TE 900/10.17ms, 2 averages; 256 × 256 data matrix; 45 × 45 mm field of view; 10 slices; a slice thickness of 0.5 mm.

**TPL Imaging of Tumor Microenvironment.** TPL tumor imaging was performed on an Olympus FV1000 multiphoton laser scanning confocal microscope. Mice were injected subcutaneously with 1 × 10⁶ cells /100 µL 4T1 cells in 1:1 (v/v) PBS and Matrigel (BD Biosciences, Franklin Lake, NJ) in the flank. When the tumors reached ~200 mm³, mice were injected i.v. with 200 µL AuNS@CP (Au content 1.2 mg/mL, i.e., 12 mg Au/kg body weight). At designated time points after i.v. injection, mice were injected i.v. with
0.1 mL 2.5 wt% Texas Red dextran solution (molecular mass 70 kDa; Life Technologies) to demarcate the vasculature for microscopy. Immediately following injection of Texas Red dextran, mice were anesthetized by isoflurane and the tumor was exposed by a skin incision. The tumor was then immobilized on the microscope stage for TPL imaging, while continuity with the body vasculature was maintained.[2] The collagen matrix in the tumor microenvironment was imaged by second harmonic generation (e.g. in our experiment the excitation wavelength was 810 nm, the emission wavelength was 405 nm).[3]

**Tumor Growth Inhibition Studies.** Mice were injected subcutaneously with \( 1 \times 10^6 \) cells /100 µL 4T1 cells in 1:1 (v/v) PBS and Matrigel (BD Biosciences, Franklin Lake, NJ) in the flank. When the tumors reached ~50 mm³, mice were anaesthetized and injected i.v. with a total volume of 200 µL of one of the following: saline, GMP (1.03 mg/mL, i.e., 10.3 mg GMP/kg body weight), AuNS (Au content at 1.2 mg/mL, i.e., 12 mg Au/kg body weight), a mixture of GMP and AuNS (with GMP concentration at 1.03 mg/mL and Au content at 1.2 mg/mL), or AuNS@CP (with equivalent GMP concentration at 1.03 mg/mL and Au content at 1.2 mg/mL). For the laser-treated groups, the tumor site was irradiated with an NIR laser (808 nm, 0.5 W/cm²) for 3 minutes at 6 hours after i.v. injection. The 6 hour timepoint was selected because MRI data suggested that nanoparticle accumulation had plateaued by 4 h, and began to decrease by 8h (Figure 2); TPL data confirmed that particles were still present in tissues at 6 hours. The thermal images and temperature increase in the irradiated tumor region was monitored by an FLIR E50 infrared imaging camera (FLIR Systems, Wilsonville, OR). Animal body weight and tumor size were measured every 2-3 days. Tumor length and width were measure with calipers, and the tumor volume was calculated using the following equation: tumor volume = length × width × width / 2. Organs (heart, liver, spleen, lung, kidney) of mice were fixed, sectioned, and processed for H&E staining.

**Statistical Analysis.** Data were described with means and standard deviations and compared with unpaired t-tests. All data analyses were performed using Origin 8 software (Northampton, MA).

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**Figure S1.** Molecular Structure of GMP and PEG-DiP.
Figure S2. Size distribution of AuNS by DLS.

Figure S3. FT-IR spectrum of PEGylated AuNS@CP. See text for discussion of spectrum features.
Figure S4. TEM images of the core-shell AuNS@CP nanoparticles with various shell thicknesses. Scale bar: 50 nm. Mass ratio = AuNS:GMP:Gd(III):PEG-DiP. The mass ratios of GMP and Gd(III) – the only two that change – are in bold.

<table>
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<th>Mass ratio</th>
<th>Shell (nm)</th>
<th>High Magnification</th>
<th>Low Magnification</th>
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Figure S5. EDX characterization of the AuNS@CP.
Figure S6. TEM image of the AuNS@CP incubated in human serum buffer (human serum : saline = 1:1, v/v, pH 7.4) for 24 h. Scale bar: 100 nm.

Figure S7. Release profile of GMP from AuNS@CP in saline. Data are means ± SD, N = 4.
Figure S8. Effect of concentration and duration of irradiation (808 nm continuous wave NIR laser, 0.5 W/cm²) on the temperature of a solution of AuNS@CP nanoparticles. Data are means ± SD (N = 4).

Figure S9. TPL imaging of AuNS@CP nanoparticles in the tumor microenvironment 6 hours after injection. The 3D reconstructed image showed AuNS@CP (green) and blood vessels (red; 70 kDa Texas Red dextran) in the tumor microenvironment. AuNS@CP are inside (yellow) and mostly outside (green) of blood vessels.
Figure S10. Cytotoxicity to 4T1 cancer cells of AuNS@CP and equal concentrations of gemcitabine-5’-monophosphate (GMP) after 24 or 48 h of incubation. Data are means ± SD; N = 5.

Figure S11. Cytotoxicity of AuNS to 4T1 cancer cells after 48 h exposure. Data are means ± SD; N = 5.
Figure S12. Cytotoxicity of AuNS, GMP, and AuNS@CP to 4T1 cells without or with irradiation. 4T1 cells were incubated with AuNS, GMP, or AuNS@CP (with an equivalent GMP concentration of 20 μg/mL and Au concentration of 23.4 μg/mL) then irradiated with a 808 nm laser (0, 0.25, or 0.5 W/cm²) for 3 min, 6 h after exposure to treatment groups. After 24 h, cell viability was quantitated by the MTS assay. Data are means ± SD; N = 4.

Figure S13. Cytotoxicity of AuNS@CP to 4T1 cells without or with irradiation. 4T1 cells were incubated with different concentrations of AuNS@CP, then irradiated with a 808 nm laser (0.5 W/cm²) for 3 min, 6 h after exposure to treatment groups. After 24 h, cell viability was quantitated by the MTS assay. Data are means ± SD; N = 4.
Figure S14. Characterization of PEGylated AuNS nanostructures. (A, B) TEM images of PEGylated AuNS. Scale bar: 50 nm. (C) UV–vis–NIR absorbance spectra of PEGylated AuNS.
Figure S15. Tumor cell density counts of hematoxylin and eosin stained sections of tumor collected from mice 3 days after treatment.

Figure S16. Body weight of mice in various treatment groups over time.
Figure S17. Representative hematoxylin and eosin stained sections of organs from animals treated with AuNS@CP nanoparticles (GMP dose: 10.3 mg/kg) followed 6 h later by NIR laser irradiation (0.5 W/cm², 3 min). Tissue samples were harvested 15 days after intravenous injection. Scale bar: 100 µm.

Reference

