Supporting Information for

Perivascular targeting gold nanocages significantly enhance photothermal treatment efficacy in solid tumors despite minimal bulk temperature change

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Synthesis of Au nanocages (AuNCs).

The AuNCs were synthesized by galvanic replacement reaction between Ag nanocubes and $HAuCl_4$ as previously described.[1] First, Ag nanocubes were synthesized by the polyol method.[2] Briefly, 50 mL EG was added to a 250-mL round-bottom flask equipped with a stirring bar and placed in an oil bath at 150 °C. After the temperature equilibrated (30~45 min), EG solutions of 0.6 mL of 3 mM NaHS, 5 mL of 3 mM HCl, 12.5 mL of PVP (0.25 g), and 4 mL of 282 mM AgTFA were sequentially added to the reaction flask. Once the LSPR peak reached ~440 nm (~35 min after addition of AgTFA), the reaction was quenched in an ice bath. Upon cooling, the product was collected by adding acetone to the reaction solution at a ratio of 5:1 and centrifuging at 6,000 rcf for 10 min. The resulting pellet was purified twice with H_2O and collected by centrifugation at 20,000 rcf for 10 min, and resuspended in 10 mL of H_2O for future use. To synthesize AuNCs, 10 mL of H_2O was heated to boiling in a 50-mL round-bottom flask equipped with a stirring bar. To the boiling liquid, 1 mL of the Ag nanocubes described above was added and subsequently 1 mM $HAuCl_4$ was added using a syringe pump at a rate of 45 mL/h until the LSPR maximum was at 750 nm. The AuNCs were purified by saturated NaCl solution to remove by-product AgCl, washed 3 times by centrifugation at 20,000 rcf for 10 min, and resuspended in H_2O at a concentration of 5 nM for future use.

Synthesis of PDA coated AuNCs (AuNC@PDA).

The AuNC@PDA were prepared by self polymerization of dopamine on the surface of AuNCs under basic conditions in the presence of O_2.[3] Briefly, 3 mL of 5 nM AuNC aqueous suspension was diluted to 200 mL using Tris-buffered saline (20 mM Tris and 100 mM NaCl, pH = 9) in a 250-mL, 3-neck, round-bottom flask. The reaction flask was briefly flushed with O_2 and placed in a bath sonicator held at 4 °C with ice. Dopamine hydrochloride (0.2 mmol, 36.0 mg) was added to the flask, the vessel was sealed under 1 atm O_2, and the mixture was sonicated throughout the reaction until the LSPR had redshifted ~50 nm (~75 min). After this reaction, the product was collected by centrifugation at 6,000 rcf for 10 min, washed with H_2O twice and recovered by centrifugation at 19,000 rcf for 10 min at 4 $_{o}$C. The AuNC@PDA was resuspended in H_2O at a concentration of 6 nM for characterization and future use.

Cell lines.

Murine breast cancer cells (4T1, ATCC CRL-2539) and murine endothelial cells (2H11, ATCC CRL-2163) were purchased and cultured in DMEM supplemented with 10% FBS and 1%

P/S and passaged biweekly. Tumors were implanted into mice at $1x10^5$ 4T1 cells per mouse in the right hind limb, and allowed to grow for approximately 1 week to a size of 8-10 mm. Ear tumors were inoculated using a 25 µL volume.

High- Resolution Photoacoustic Mapping of Nanoparticles in Mouse Tissues

PA imaging and quantification of nanoparticles *in vivo* in live mouse ear tissues and *ex vivo* in tissue sections was performed using a custom laser scanning PA microscope coupled to Olympus IX81 microscope. A pair of galvo mirrors (6215H, Cambridge Technologies, Lexington, MA) steered 820 nm and 532 nm laser beams across the sample in a XY raster pattern. The laser operated at 10 kHz pulse repetition rate. The field of view was limited to 650 µm by the focal area of the ultrasound transducer (3.5 MHz, model 6528101, 4.5 mm in diameter; Imasonic Inc., Besançon, France). Wide area imaging was performed in a mosaic mode by shifting sample position using a mechanical stage (1.0 mm steps) and stitching individual PA images together in automated mode. The samples were mounted at the bottom of a plastic bath filled with deionized water to provide acoustic coupling. Signals from the transducer were amplified (5662B, Panametrics) and recorded by a PC equipped with a high-speed digitizer (PCI-5124, 12-bit card, 128 MB of memory, National Instruments, Austin, TX). Control over the mirrors and overall synchronization of the system was performed using a digital waveform generator (DG4062, Rigol, Beijun, China). Serial samples were fixed with acetone and stained with silver enhancement kit per the manufacturer's instructions (Ted Pella) and/or FITC-conjugated CD-31 in 5% bovine serum albumin to identify endothelial cells. Fluorescence images were acquired using the cytation 5 and contrast enhanced on the software.

Photothermal Microscopy of Single Cells and Tissue Sections

A custom photothermal microscope (PTM)[4] capable of visualizing single nanoparticles using their light absorption contrast was built on a platform of Olympus IX73 microscope and featured c.w. (continuous wave) pumping laser (532 nm, LaserGlow LLS-0532). Laser intensity was modulated at 500 kHz (sine wave) using electro-optical modulator (EO-AM-NR-C4, Thorlabs, Newton, NJ). Absorption of the laser energy by nanoparticles and subsequent thermal relaxation created a local transient phothothermal element (heated zone) within the sample, which was probed using c.w. 635 nm laser (Thorlabs CLD1010) focused into the same sample location. The probe beam was collected after the sample using a custom condenser featuring 40x objective and PDA-015 photodetector (Thorlabs Inc. Newton, NJ). Changes in the probe beam

intensity were analyzed using a lock-in amplifier (MFLI, Zurich Instruments, Switzerland) connected to a PC for data collection. Fluorescence imaging of the sample has been performed using conventional wide-field fluorescence imaging mode with DAPI and FITC filters (Semrock Inc., Rochester, NY) and Olympus DP80 color camera. PT and fluorescence images were analyzed and merged in ImageJ software (NIH) using a color-coded scale to display PT signal amplitudes.

Inductively coupled plasma mass spectrometery

Laser ablation ICP-MS (LA-ICP-MS) element maps were acquired using a New Wave Research/ESI 193nm excimer laser ablation system coupled with a Thermo Scientific iCapQ quadrupole mass spectrometer. Ablation was conducted on tissue samples harvested 24 h following particle injection, frozen, and sectioned at 20 µm that were then adhered to a glass slide and fixed with acetone. Elemental mapping was performed using a 50 µm x 50 µm laser spot, a repetition rate of 10Hz, a fluence of ~3.5 J/cm^2, a scanning speed of 250 µm/s, and a line spacing of 40 µm. The elements analyzed were: ^{29}Si, ^{31}P, ^{43}Ca, ^{44}Ca, ^{63}Cu, ^{66}Zn, ^{107}Ag, and ^{197}Au. NIST standard glass 612[5] was analyzed prior to and after each sample map. Data reduction was performed using the Iolite Software Program.[6] ^{29}Si was used for calibration.

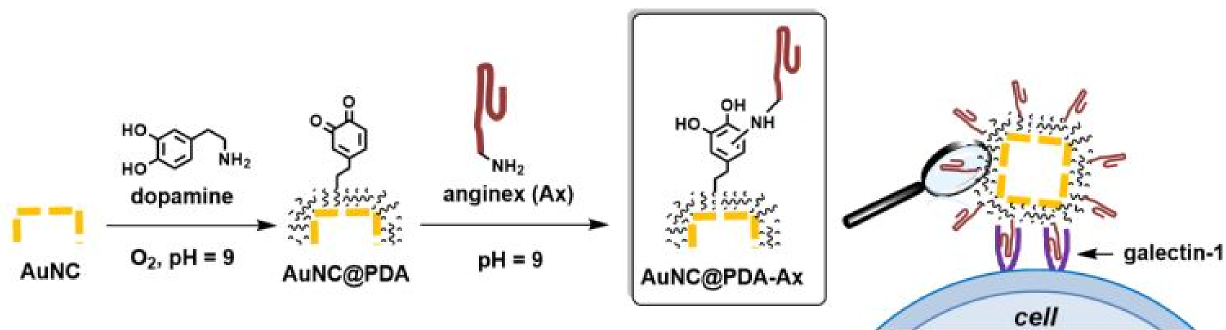

Figure S1. Schematic representation of the synthetic procedure to generate AuNC@PDA-Ax. Reproduced from Ref 3.

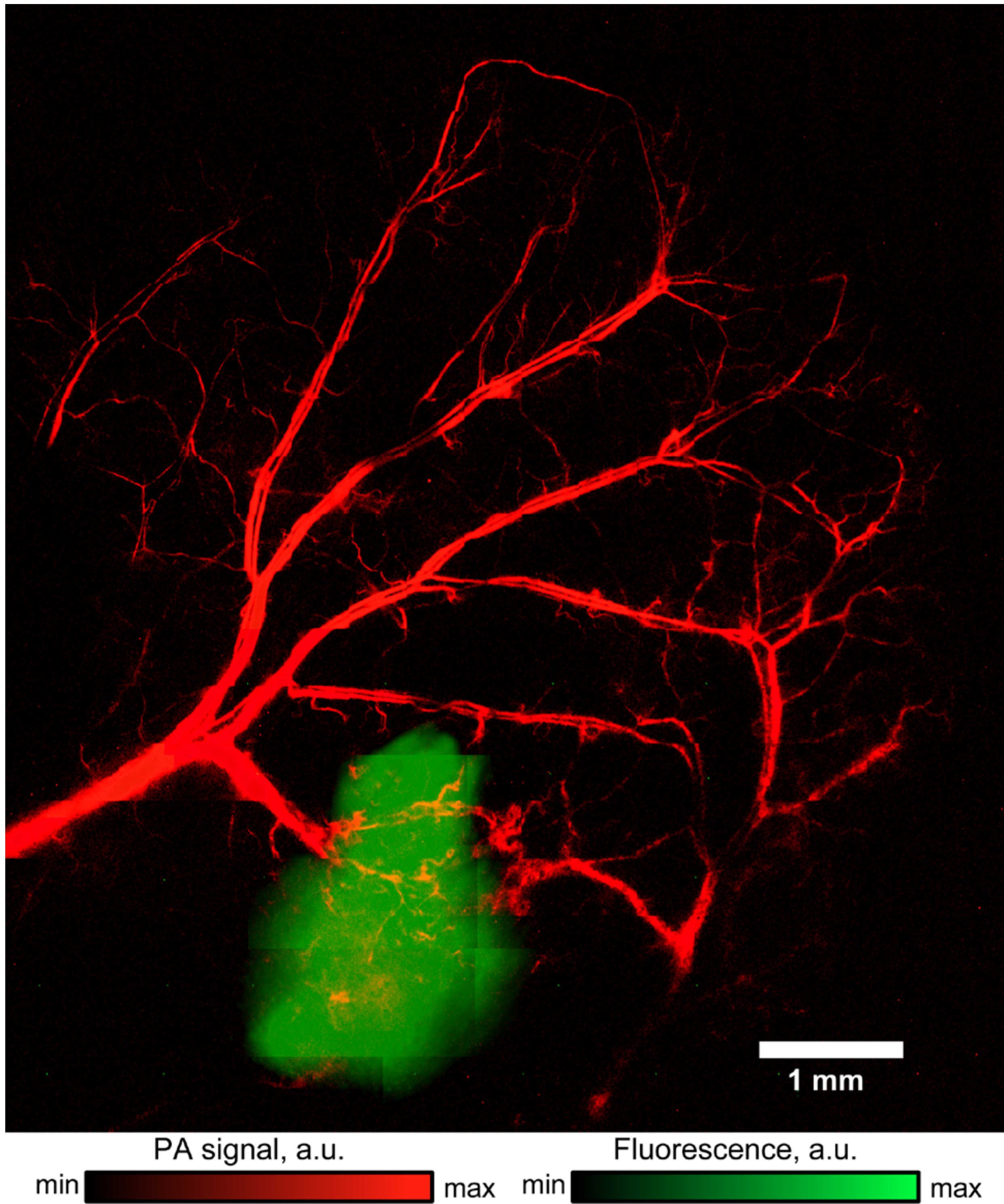

Figure S2. Whole mouse ear containing a GFP-expressing 4T1 tumor imaged via fluorescence and photoacoustic signal to confirm tumor location

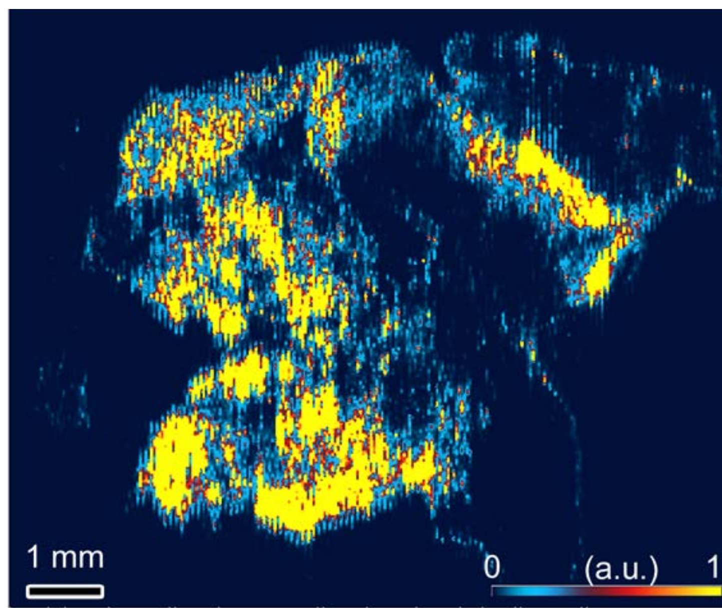

Figure S3. LA-ICP-MS mapping of tumor tissue 24 hours following injection of AuNC@PDA

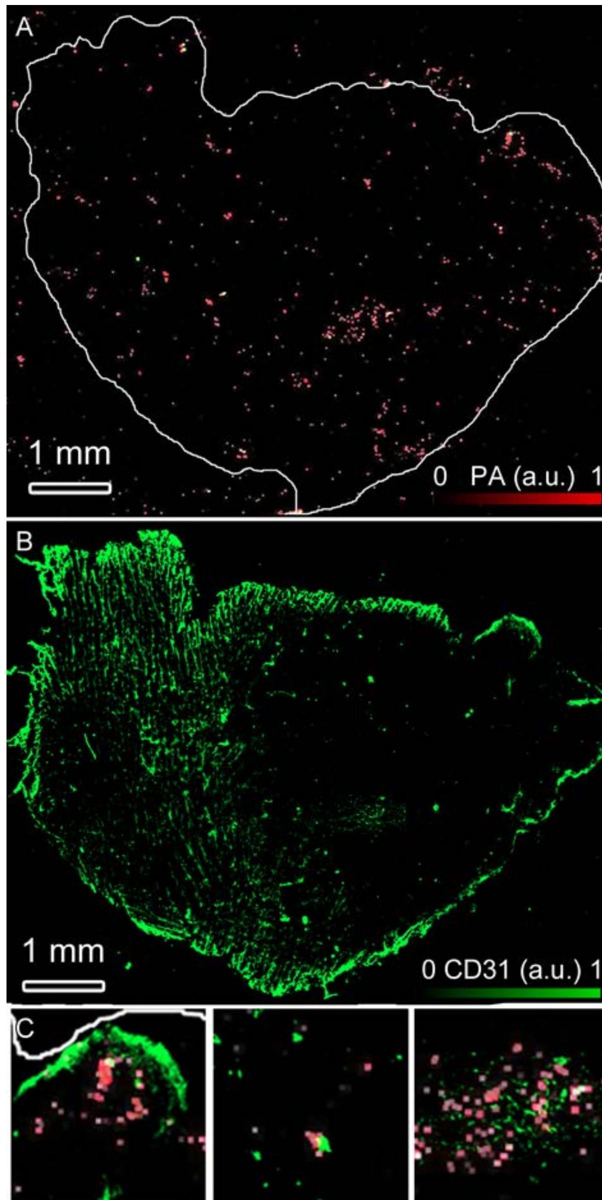

Figure S4. (A) Photoacoustic mapping (as seen in Fig. 6C of the main text) and (B) contrast-enhanced CD-31 staining with a FITC conjugated antibody of serial sections of 4T1 tumor harvested 24 h post injection. (C) Expanded theoretical overlays of several regions of tissue.

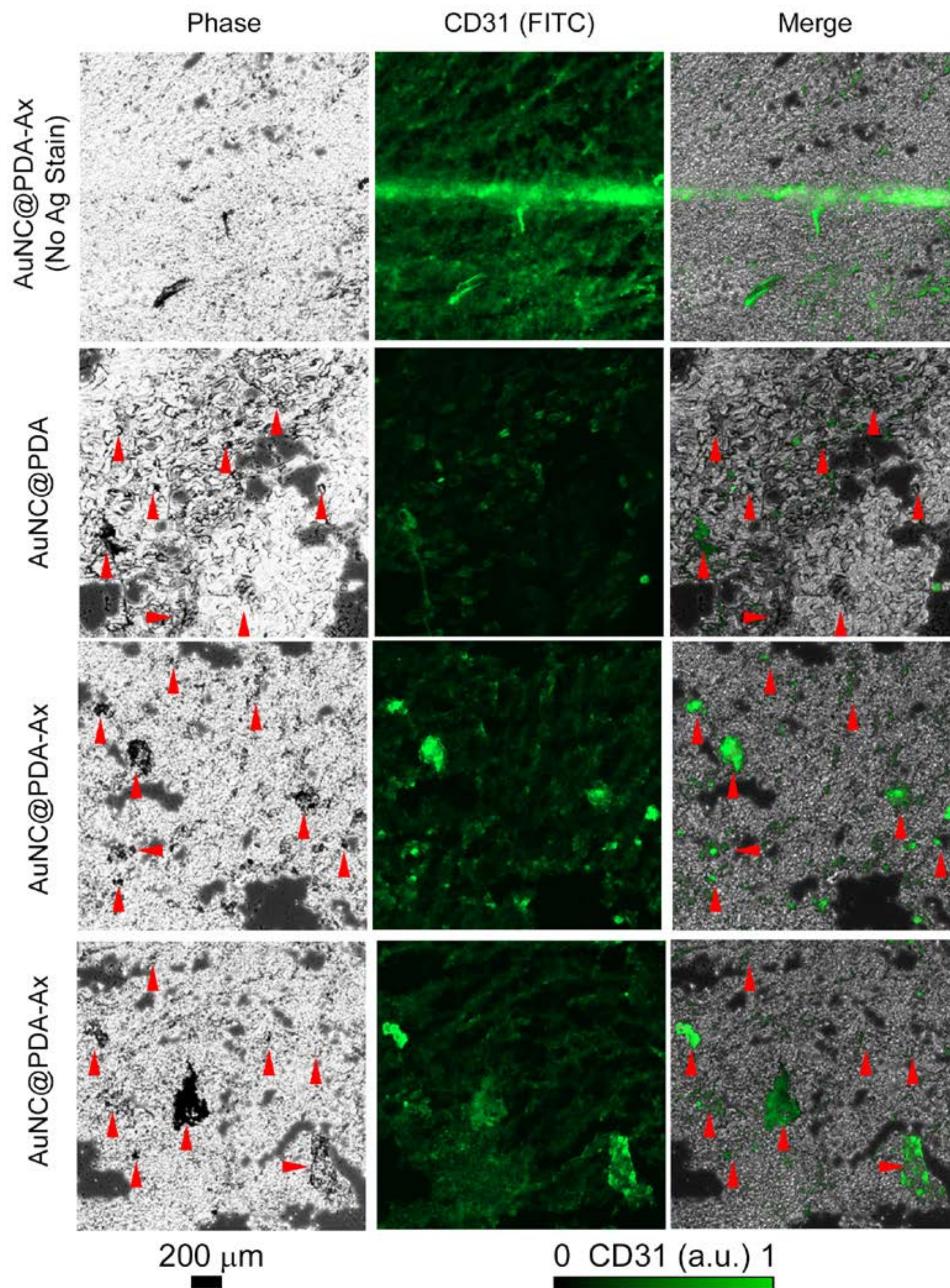

Figure S5. (Left) Phase contrast image following silver stain (center) FITC labeled CD31 and (right) overlay of representative regions of 4T1 tumors 24 h following i.v. injection of AuNC@PDA or AuNC@PDA-Ax. Red arrowheads are used to highlight some regions of silver staining.

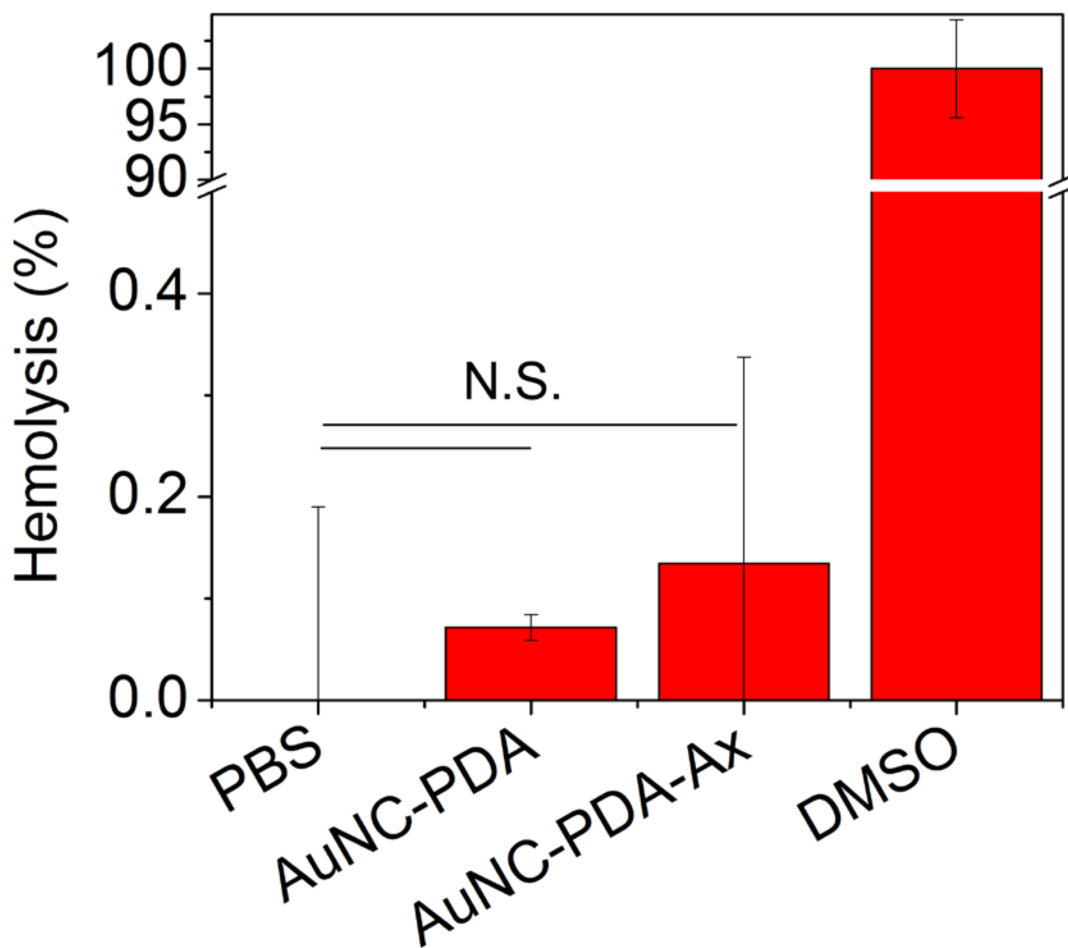

Figure S6. Hemolysis assay using PBS as negative control, and DMSO as a positive control.

References

1. Skrabalak, S. E.; Au, L.; Li, X.; Xia, Y., Facile synthesis of Ag nanocubes and Au nanocages. *Nat Protoc* **2007,** *2* (9), 2182-90.
2. Zhang, Q.; Li, W.; Wen, L.-P.; Chen, J.; Xia, Y., Facile Synthesis of Ag Nanocubes of 30 to 70 nm in Edge Length with CF(3)COOAg as a Precursor. *Chemistry (Weinheim an der Bergstrasse, Germany)* **2010,** *16* (33), 10234-10239.
3. Jenkins, S. V.; Nedosekin, D. A.; Miller, E. K.; Zharov, V. P.; Dings, R. P. M.; Chen, J.; Griffin, R. J., Galectin-1-based tumour-targeting for gold nanostructure-mediated photothermal therapy. *International Journal of Hyperthermia* **2018,** *34* (1), 19-29.
4. Nedosekin, D. A.; Galanzha, E. I.; Dervishi, E.; Biris, A. S.; Zharov, V. P., Super-resolution nonlinear photothermal microscopy. *Small* **2014,** *10* (1), 135-42.
5. Jochum, K.; Weis, U.; Stoll, B.; Kuzmin, D.; Jacob, D.; Stracke, A.; Birbaum, K.; Frick, D.; Günther, D.; Enzweiler, J., *Determination of reference values for NIST SRM 610–617 glasses following ISO Guidelines*. 2011; Vol. 35, p 397-429.
6. Paton, C.; Hellstrom, J.; Paul, B.; Woodhead, J.; Hergt, J., Iolite: Freeware for the visualisation and processing of mass spectrometric data. *Journal of Analytical Atomic Spectrometry* **2011,** *26* (12), 2508-2518.