Supporting information

Title: Smart Gold Nanoparticle-Stabilized Ultrasound Microbubbles as Cancer Theranostics

Authors: Young Il Yoon[1ac], Xin Pang[1b], Sungwook Jung[1a], Guofeng Zhang[d], Minsuk Kong[e], Gang Liu[*b], Xiaoyuan Chen[*a]

Affiliations:

[a] Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), Bethesda, Maryland, 20892, USA.

[b] State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics & Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, Xiamen, 361102, China

[c] IT and Medical Research Team, Korea Textile Development Institute (KTDI), 136 Gukchaebosangro, Seo-gu, Daegu, 41842, South Korea.

[d] Trans-NIH Shared Resource on Biomedical Engineering and Physical Science, National Institutes of Health (NIH), Bethesda, Maryland, 20892, USA.

[e] Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20892, USA.

[*] **Corresponding author:** Gang Liu (gangliu.cmitm@xmu.edu.cn); Xiaoyuan Chen (shawn.chen@nih.gov).

[1] These authors contributed equally to this study.

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Materials and Methods

Chemicals: Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium citrate dihydrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$), sodium borohydride ($NaBH_4$) and BSA were purchased from Sigma-Aldrich. 4-(2-(6,8-dimercaptooctanamido) ethylamino)-3-methyl-4-oxobut-2-enoic acid ("pH-responsive ligand") was kindly provided by Dr. Sungwook Jung. All chemicals were used without further purification.

Preparation of SAuMBs: To synthesize AuNPs with 20 nm diameter, 3.125 mL of 20 mM aqueous $HAuCl_4 \cdot 3H_2O$ and 1.875 mL of 100 mM aqueous sodium citrate dihydrate were added to 250 mL of boiling H_2O. The color of the solution changed to red after vigorously stirring for 10 min. To prepare SAuNPs, 60 mL of the AuNP solution was dialyzed twice using H_2O and centrifugal filters (Amicon Ultra, 50 KDa molecular weight cutoff), and then the volume of the AuNP solution was adjusted to 6 mL. The pH-responsive ligand (20.6 mg) and $NaBH_4$ (18.9 mg) were added to 1 mL of H_2O, stirred for 1 h at room temperature, and then poured into 6 mL of the dialyzed AuNP solution and stirred at room temperature overnight.[1] This solution was dialyzed twice using H_2O and centrifugal filters for purification, and the volume of the SAuNP solution was finally adjusted to 6 mL. Phosphate buffered saline (PBS) containing 1 mL of 1 nM SAuNPs was added to 1 mL of BSA solution (2 mg mL^{-1}). To prepare SAuMBs, the SAuNP solution with BSA was vigorously mixed for 2 min at 20,000 rpm using a T18 digital S1 disperser (IKA, USA). The SAuMBs were stored at 4 °C before use.

Characterization of SAuNPs and SAuMBs: UV-vis spectrophotometry (UV-3100PC, VWR, USA), dynamic light scattering (DLS) (SZ-100, Horiba, Japan), and transmission

electron microscopy (TEM) (Tecnai T12, FEI, Netherlands) were used to measure sizes, zeta potentials, and aggregation behaviors of SAuNPs and SAuMBs. To measure the sizes and structures of SAuMBs, an upright light microscope (BX41, Olympus, Japan) and field emission-scanning electron microscope (S-4800, Hitachi, Japan) were used.

Photothermal effect measurement: The pH of 0.25 nM SAuNP solution was adjusted to 5.5 with an acetic acid buffer and then irradiated at 671 nm using a laser intensity of 0.5 W cm^{-2} for 5 min (LRS-0671, Laserglow Technologies, Canada). The laser density was measured by a laser energy meter (Coherent, Santa Clara, USA). Temperature changes in the solution were recorded using an infrared camera (SC300, FLIR Systems, USA).

Cytotoxicity assay: U-87 MG human glioblastoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.[2] The cytotoxicity of SAuNPs and SAuMBs was measured using Alamablue® reagent (ThermoFisher, USA).[3] Cells (2,000 cells per well) were cultured in 96-well plates and incubated overnight. Different concentrations of SAuNPs and SAuMBs were added to each well for 30 min. For laser irradiation groups, the cells were irradiated at 671 nm using a laser intensity of 0.5 W cm^{-2} for 5 min. Afterwards, the cells were incubated for 3 days, and 10 μL of the reagent for every 100 μL of sample was added to each well. Fluorescence signals from each well were measured at 590 nm using a multimode reader (Synergy 2, BioTek, USA). Also, a live/dead cell double staining kit (Sigma-Aldrich, USA) containing calcein-AM and propidium iodide solutions was used to confirm viable and dead cells after laser irradiation according to kit instructions.[4] After samples were prepared, their fluorescent properties

were characterized using a confocal laser scanning microscope (Fluoview FV10i, Olympus) or fluorescence-activated cell sorter (FACSCelesta™, BD Biosciences, USA).

Cellular uptake assay of SAuNPs: To confirm cellular uptake of SAuNPs, an inverted light microscope (IX81, Olympus) and TEM were used. Samples were prepared through simple processes. In chamber slides, U-87 MG cells treated with SAuNPs or SAuMBs were fixed and mounted with 4% paraformaldehyde and then DAPI solution to stain nuclei. To observe nuclei and SAuNPs in cells, fluorescent and dark field modes of the inverted microscope were utilized.

Ultrasound treatment: To induce temporary sonoporation of cell membranes, cells treated with SAuMBs were subjected to ultrasound treatment. A 1-MHz probe-equipped sonoporator (Sonidel, Ltd., Ireland) was used with an intensity of 1 W cm^{-2}, duration of 2 min, and 50% duty cycle.[5] After 30 min, cells on plates and slides were washed to remove excess SAuMBs and SAuNPs which were not cdelivered into the cells and then were incubated further for other experiments.

Ultrasound and PAT: A clinical ultrasound device (iU22, Philips, USA) and VevoLAZR imaging system (Fujifilm VisualSonics, Canada) were used to confirm the effects of SAuMBs as ultrasound and PA contrast agents.[6] A gel phantom made with 1% agarose was set to check the contrast effects. For ultrasound imaging, a 5- to 12-MHz probe was used, and for PA imaging, light generated from a tunable laser (680-700 nm) was irradiated through the transducer.[7]

Animal model preparation: All animal studies were approved by the Animal Management and Ethics Committee of Xiamen University, China and performed in accordance with its rules and guidelines. The U-87 MG cell line was used to create a U-87 MG xenograft

mouse model because preparation methods and techniques of the animal model are well-established in our lab and moreover, as is well known, U-87 MG has been widely used for determining theranostic effects of various agents.[8] The tumor model was established by subcutaneously inoculating 1.0×10^6 U-87 MG cells suspended in Matrigel (1:1) into the right shoulder of mice. Tumor growth was monitored for 1 month.

SAuMB biodistribution analysis: To estimate the biodistribution of SAuNPs and SAuMBs, mice injected with SAuMBs were visualized using a fluorescence imaging device (Maestro EX, Caliper Life Sciences, USA).[9] Before tail vein injection of the SAuMBs (10 nM, 150 μL), mice were anesthetized with an O_2/air mixture containing 2% isoflurane. After administration of SAuMBs and ultrasound treatment using a multifunctional ultrasound instrument (CSD-1B, Southwest University, Chongqing, China) at a central frequency of 1.0 MHz and intensity of 1.0 W cm^{-2}, 50% duty cycle, 5 min, mice were scanned at 1, 3, 6, 12, and 24 h postinjection. For isolated organ imaging, mice were carefully euthanized and heart, liver, spleen, lungs, kidneys, and tumor harvested.

Theranostic effects of SAuMBs on U-87 MG tumor xenografts: Before and directly after (within a minute) injection of SAuMBs into the tail vein, ultrasound scanning was performed (iU22, Philips). Tumor-bearing mice were treated with SAuMBs, followed by ultrasound and laser irradiation to confirm the therapeutic effect. Ultrasound treatment was carried out for 5 min, with a 50% duty cycle and 1 W cm^{-2} intensity. Six hours postinjection, the laser was irradiated for 8 min, at 671 nm, and 0.5 W cm^{-2} intensity. Temperature changes (ΔT) and relative tumor volumes (final/initial volume) were monitored for 8 min and 14 d, respectively. Survival ratios of U-87 MG xenograft mice

were checked for 30 days (interval: 2 days), and tumors were harvested and measured on the fourteenth day.

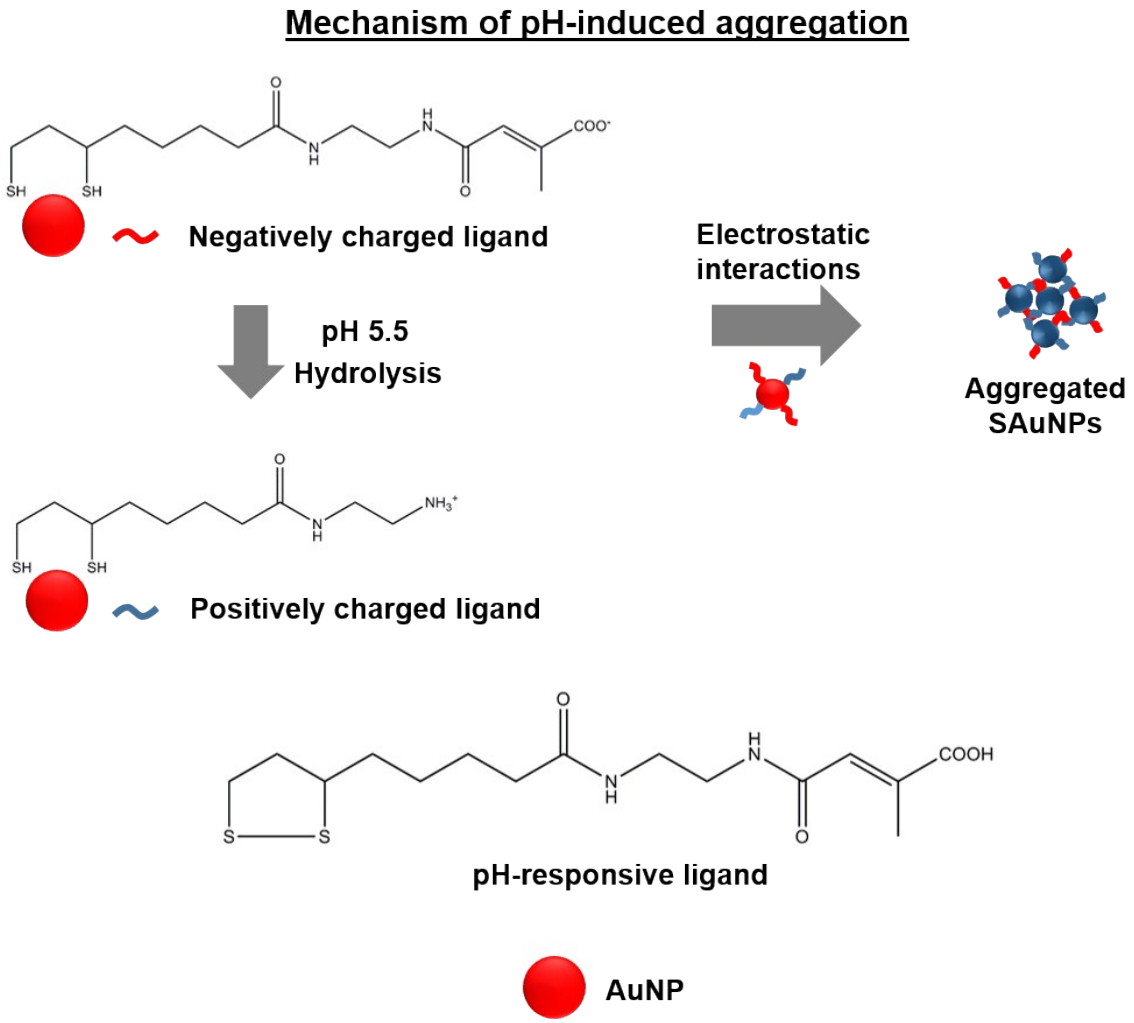

Figure S1. Scheme explaining the aggregation mechanism of smart ligand-bound gold nanoparticles (SAuNPs) depending on pH. Introduction of thiol-terminated ligands called "pH-reponsive" on AuNPs progressed smoothly, and SAuNPs had negative charges owing to COO^- groups on the ligands. A part of the ligand was cleaved via hydrolysis under acidic conditions, and consequently, the surface charge of the SAuNPs became positive. Electrostatic interactions among positively and negatively charged SAuNPs caused their

aggregation. As a result, the size of the SAuNPs increased, and the color of the SAuNP colloid changed from red to blue.

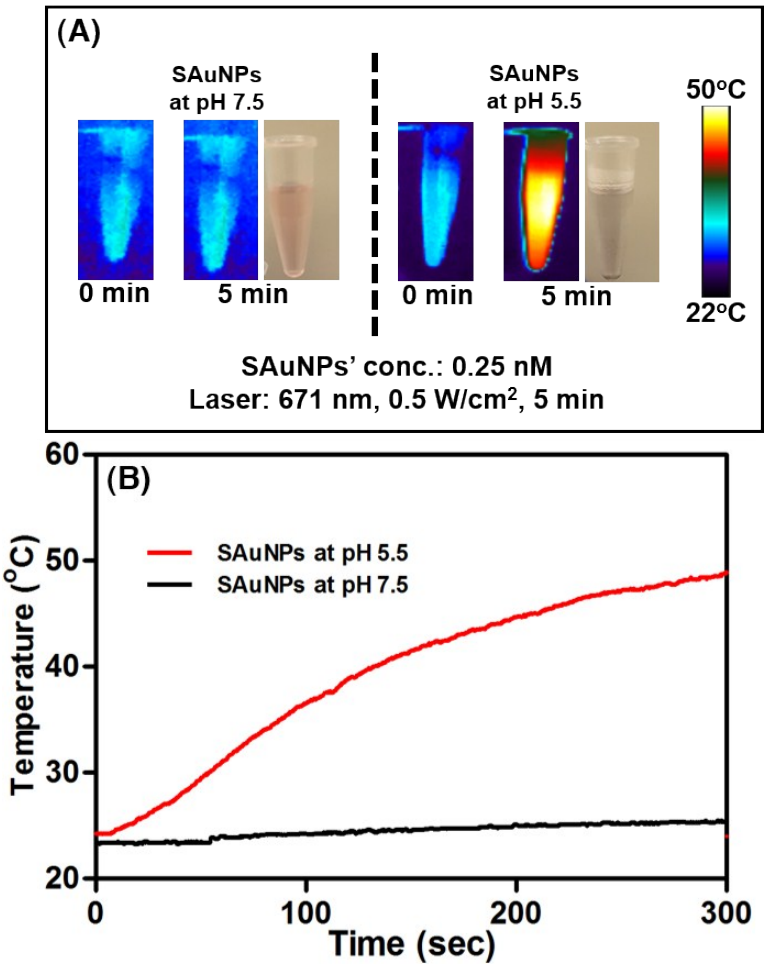

Figure S2. Photothermal effects of SAuNPs. To assay photothermal effects of SAuNPs based on pH, the temperature of SAuNP colloids was measured for 5 min under laser irradiation (671 nm, 0.5 W cm^{-2}). While the temperature of the SAuNP colloid steadily increased to about 50 °C at pH 5.5, there was no change in the colloid temperature at pH 7.5 (A). (B) Detailed temperature changes of the SAuNP colloids. Aggregated SAuNPs at pH 5.5 effectively absorbed the laser energy and released heat.

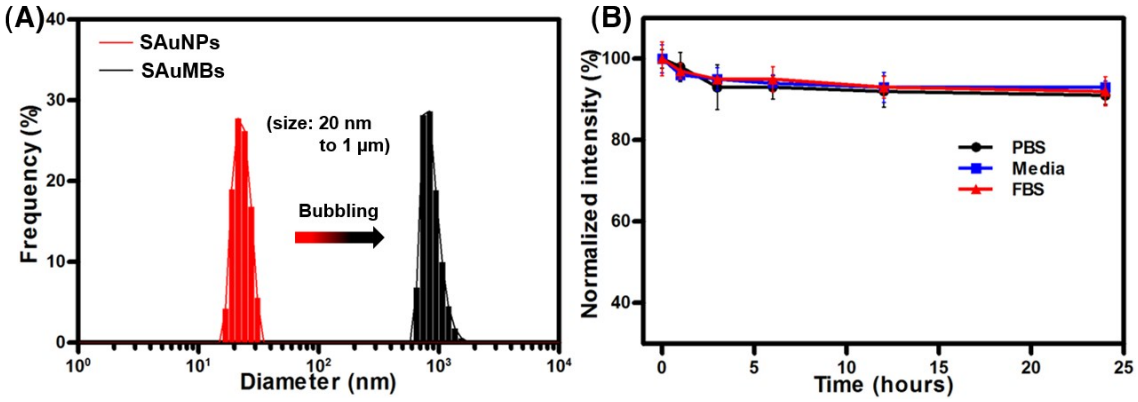

Figure S3. Characterization of SAuMBs after bubbling. Prepared SAuMBs were analyzed by dynamic light scattering (DLS). The size of SAuMBs after bubbling was about 1 μm (A). SAuMBs maintained good stability in various conditions (phosphate buffered saline, culture media, and fetal bovine serum) for 24 h ($N = 3$) (B). The SAuMBs were prepared as expected, and they could be used for further *in vitro* and *in vivo* studies.

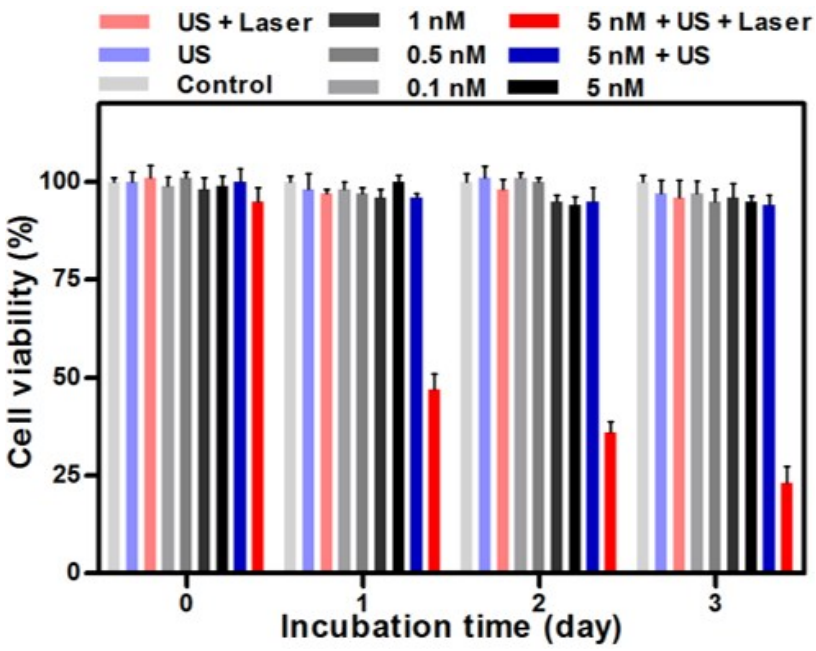

Figure S4. Photothermal therapy (PTT) effects of SAuMBs. To assay cytotoxicity and PTT effects, U-87 MG cancer cells were monitored for 3 d (N = 3). The SAuMBs in the range of 0.1 to 5 nM based on the AuNP were selected. US and laser irradiation were used to deliver SAuNPs into cancer cells and attact the cells respectively. Only when cells were treated with SAuMBs, US, and laser irradiation did cell viability decrease to 25%. Cell viability was barely affected by the ultrasound solely or the ultrasound plus the 671 nm laser without SAuMBs.

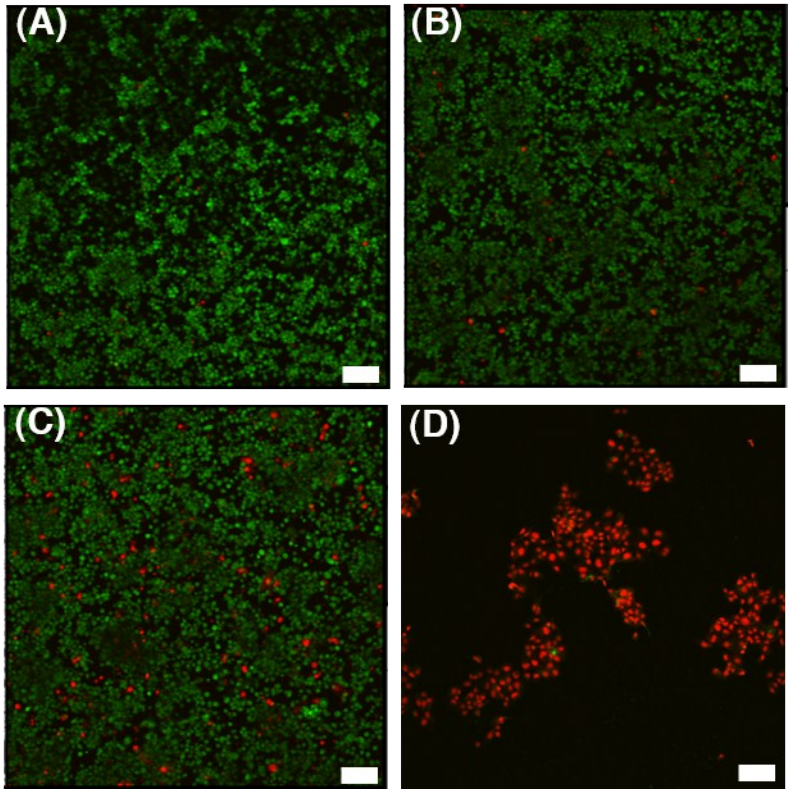

Figure S5. Cell viability assay. The viability of U-87 MG cancer cells treated with SAuNPs or SAuMBs were estimated using a live/dead double-staining kit and confocal laser scanning microscopy. Positive calcein-AM staining (green) indicates live cells, while propidium iodide staining (red) indicates dead cells. Compared with control (a), SAuMB only (b), and SAuNP only (c) treatment groups, and SAuMB administration followed by US treatment (SAuMBs+US) (d) showed the most red staining, in line with results in Figure S4. Scale bar: 100 μm.

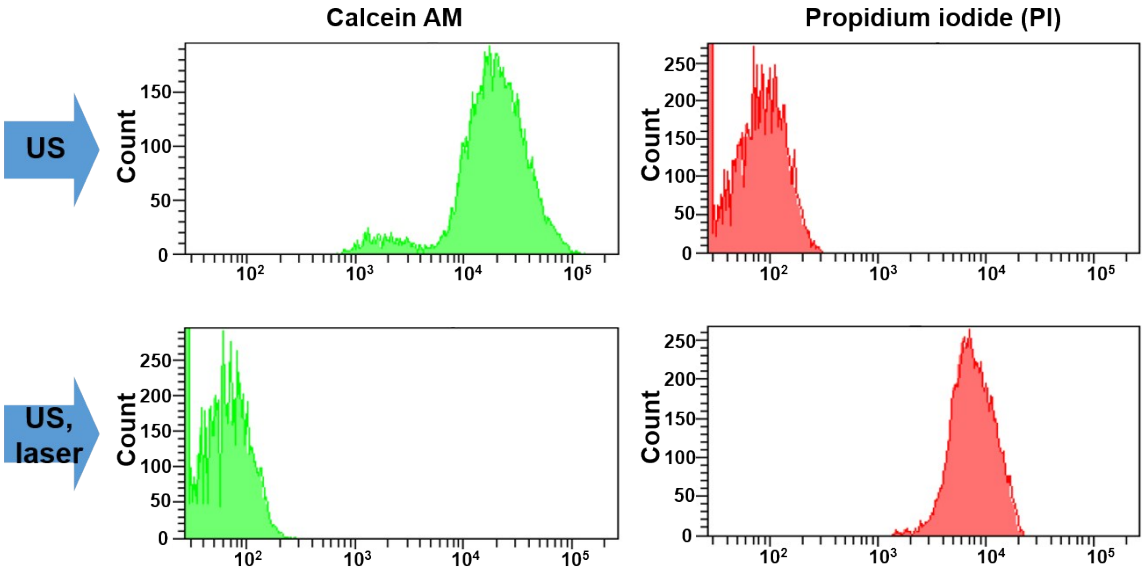

Figure S6. Fluorescence-activated cell sorting. Conditions of U-87 MG cancer cells treated with SAuMBs and US were assayed using a live/dead cell double-staining kit and fluorescence-activated cell sorting. Positive calcein-AM staining (green) of live cells was predominant in the US only group, while propidium iodide staining (red) of dead cells was dominant in the US + laser irradiation group, in line with results in Figure S4 and S5.

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