# Silica-Coated Gold Nanorods with Hydrophobic Modification Show Both Enhanced Two-Photon Fluorescence and Ultrasound Drug Release

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## Materials and Methods.

Chloroauric acid, silver nitrate, CTAB, TEOS, sodium oleate, sodium hydroxide and ascorbic acid were all purchased from Sigma Aldrich. The PTX was purchased from MedChemExpress. The DPPC and DSPE-PEG-2k-methoxy were purchased from Avanti Polar Lipids. The DiO was purchased from Life Technologies. The dodecyltrichlorosilane was purchased from TCI America. Isopropanol and sodium borohydride were purchased from Fisher Chemical. Methanol, hexane, and 37% hydrochloric acid were purchased from Macron Fine Chemicals. Prior to usage, water (dd-$H_2O$) was deionized using Milli-Q Advantage A-10 water purification system (Millipore, USA). TEM imaging was performed on a FEI Technai T12 with 120kV laB6 filament.

Synthesis of gold nanorods (AuNR). The gold nanorods were created in a seed nucleation method similar to a procedure reported by Ye *et al*[42] and Mueller *et al*.[28] First, the growth solution was created by dissolving 0.7 g of cetrimonium bromide (CTAB) and 0.1234 g of sodium oleate in 25 mL of water and heating the solution to 30℃. 2.4 mL of 4 mM aqueous silver nitrate was added to the solutions and left undisturbed in the dark for 15 min. 25 mL of 1 mM chloroauric acid was then added to the solution and stirred at 200 rpm for 1.5 h. While the growth solution was mixing, the seed solution was created by mixing 1 mL of 0.5 mM chloroauric acid and 1 mL of 0.2 M CTAB. 200 µL of 0.6 mM sodium borohydride dissolved in ice cold water was then added to the seed solution, which was mixed vigorously for 2 min and then left undisturbed for 30 min. While the seed solution was aging, 187.5 µL of 37% hydrochloric acid was added to the growth solution and mixed at 100 rpm for 15 min. Finally, 125 µL of 0.064 M ascorbic acid was added to the solution, which was mixed vigorously for 30 s at RT. 40 µL of the seed solution was added to the growth solution and the solution was aged at 30℃ for 12 h. The mixture was then centrifuged at 6700*g* for 20 min, then washed twice with water to decrease the CTAB concentration in solution.

Synthesis of silica-coated gold nanorods (MSiO2-AuNR). The silica coating of the AuNR followed modified Stöber method.[28,51] To briefly summarize, the silica coating involved creating

1 mL solution of 1.8mM solution of CTAB and 1 nM of AuNR in water. Afterwards, the solution was mixed overnight. 10 μL of 0.1 M NaOH was added to the solution and mixed for 30 min. Next 48 μL of 5% v/v of tetraethylorthosilicate was added to the solution in four 12 μL doses, each being added in 30 min intervals. The reaction was then left to moderately stir overnight. The particles were then centrifuged at 6700 g for 15 min and redispersed in 1 mL of methanol.

Synthesis of hydrophobically-modified silica-coated gold nanorods (HMSiO2-AuNR). The MSiO2-AuNRs were modified with hydrophobic groups following a biphasic reaction scheme.[28] The hydrophobic modification by concentrating 10 mL of surfactant extracted via centrifugation at 6700*g* for 15 min and then redispersing the rods in 1 mL of methanol. 10 μL of 37% hydrochloric acid was then added to the 1 mL of concentrated, surfactant-extracted MSiO2-AuNR. 5 mL of hexane was then added to the solution, and 500 μL of dodecyltrichlorosilane was added dropwise to the solution. The solution was then mixed at 900 rpm for 2 h and sealed with Parafilm. The hexane layer was then extracted from the biphasic mixture and centrifuged at 6700*g* for 15 min. The solution was centrifuge washed once with hexane and thrice with chloroform. Finally, the solution was redispersed in 2.1 mL of chloroform to achieve an OD of ~6.8.

Addition of lipid monolayer to hydrophobically-modified silica-coated gold nanorods (PL-HMSiO2-AuNR). This lipid monolayer addition protocol was adapted from a procedure reported by Kattack et. al.[52] In the lipid monolayer addition, 600 μL of the concentrated HMSiO2-AuNRs dispersed in chloroform (see previous step) were mixed with 135 μL of 10 mg/mL DPPC and 18 μL of DSPE-PEG-2k-methoxy in chloroform. 6 mL water was then added to the mixture, and the entire reaction vessel was heated to 45℃ and set to mix at 1500 rpm with a cylindrical stir bar for 2 h. The final mixture was then centrifuged at 6700*g* for 15 min and centrifuge washed twice with water, then redispersed in 1 mL water.

Multiphoton microscopy imaging of PL-HMSiO2-AuNR and MSiO2-AuNR. Both the motion and the intensity of individual nanorods were tracked by multiphoton microscopy using a Mai Tai eHP DS femtosecond pulsed laser with a pulse width of 70 fs and 80 MHz repetition rate. The samples were imaged using a 25x NA1.05; FV30-AC25W truResolution water immersion objective with a galvometer, recording two-minute length movies at a frame rate of 1.8 fps in a home-built flow cell. The brightness of each rod was analyzed utilizing a MATLAB (Mathworks) script, where the fluorescence intensity of the nanoparticles above the background fluorescence

background was integrated in a histogram format to calculate the intensity of nanorod signal in each frame.

General HIFU setup. The setup for applying HIFU to samples has been reported elsewhere and is repeated here.[14] For HIFU exposure, a spherically focused, single-element, HIFU transducer (Sonic Concepts H101, 64.0 mm Active Diameter by 63.2 mm Radius of Curvature) equipped with a coupling cone (Sonic Concepts C101) was used. When in operation, both the HIFU transducer and any samples were completely submerged in a water bath, and the HIFU cone was filled with degassed water. The HIFU transducer was connected to a 30 MHz Function/Arbitrary Waveform Generator (Agilent Technologies) via an AG Series Amplifier (T&C Power Conversion, Inc.), the amplifier operating at 100% output. For calibration, a capsule hydrophone (HGL-0400, Onda Corp.) was used to estimate the peak negative and peak positive pressures, pulse duration, and focal dimensions of the HIFU pulses, as described elsewhere.[14]

For cavitation detection experiments, a 20 MHz single element immersion transducer (Olympus Corp.) was aligned orthogonal to the focal zone of the transducer. This transducer was connected to a 5072PR Pulser/Receiver (Olympus Corp.) operating with +40dB gain in receive-only (passive detection) mode. A EF513 6.7 MHz high pass filter (ThorLabs, Inc.), connected between output of the Pulser/Receiver and a Tektronix TDS2012C oscilloscope, was used to eliminate any signal contributions from the HIFU. The external trigger of the oscilloscope was connected to a Function/Arbitrary Waveform Generator (Agilent Technologies) so that each waveform triggered an oscilloscope reading.

For ultrasound imaging, the setup was the same as above, except in place of the 20 MHz transducer, a Seqouia Acuson 512 scanner was used with a 4V1 (Acuson) transducer. Images were acquired in cadence pulse sequence mode at 1.5 MHz.

Measurement of acoustic cavitation threshold. PL-HMSiO2-AuNR and MSiO2-AuNR samples were first matched to an optical density of 2.6 at 800 nm. The PL-HMSiO2-AuNR and the MSiO2-AuNR were each bath sonicated for 5 min. Each suspension was loaded into the bulb of an inverted 3 mL Fisherbrand plastic pipette and allowed to equilibrate for 5 min. The sample was then placed onto a focusing cone which was placed on a 1.1 MHz HIFU transducer submerged in a water bath according to the general procedure described above. The particles were then irradiated for 30 s at the following input peak-to-peak voltages from the function generator: 0, 200, 400, 500, 600, 700, 800, 900, and 1000 mV. The frequency was 1.1 MHz, burst period was 100

ms, and cycle count was 12. The signal was read using a 20 MHz transducer and recorded by a TDS 2012C oscilloscope. The signal was then quantified by squaring the voltage and integrating in the time domain from 40-70 μs. Imaging was performed as described in the general HIFU setup.

Loading of DiO into PL-HMSiO2-AuNRs. This protocol was very similar to the lipid monolayer addition, however DiO was added during the lipid addition step, so that 600 μL of the concentrated HMSiO2-AuNRs dispersed in chloroform were mixed with 135 μL of 10 mg/mL DPPC, 18 μL of DSPE-PEG-2k-methoxy, and 100 μL of 1 mg/mL of DiO in chloroform. The remainder of the procedure was the same.

Following loading, the amount of encapsulated DiO was calculated using a calibration curve of DiO in chloroform and DiO encapsulated in liposomes. It was assumed that the liposomes were purely Rayleigh scatterers, and a $4^{th}$ order polynomial was fitted outside of the DiO absorption region. The fitted polynomial was then subtracted from the UV-Vis spectrum of DiO in liposomes, and a calibration curve was formed. The encapsulation and the loading efficiency were calculated by subtracting the DiO adhered to the stir bar and vial and the DiO in the supernatant from the initial loading amount.

Measurement of DiO release. 100 μL DiO loaded PL-HMSiO2-AuNR in PBS with an optical density of 2.6 at 800 nm was prepared and transferred to the bulb of an inverted 3 mL Falcon plastic pipette. The sample was subjected to HIFU with the following parameters: frequency 1.1 MHz, burst period 100 ms, cycle count 12, input peak-to-peak voltage 1000 mV. Samples were insonated for either 0, 2.5, 5, or 10 min. Immediately after insonation was finished, 50 μL of insonated sample was then placed in the plate reader and the fluorescence was measured, with a 480 nm excitation wavelength and emission reading from 520-700 nm. The relative fluorescence decrease was determined by comparing to the "0 min" sample.

Loading of paclitaxel (PTX) into PL-HMSiO2-AuNRs. This protocol was very similar to the lipid monolayer addition, however DiO was added during the lipid addition step, so that 600 μL of the concentrated HMSiO2-AuNRs dispersed in chloroform were mixed with 135 μL of 10 mg/mL DPPC, 18 μL of DSPE-PEG-2k-methoxy, and 200 μL of 1 mg/mL of PTX in chloroform. The remainder of the procedure was the same.

Following loading, PTX encapsulation was determined by performing a calibration curve of PTX in a 1:1 v/v ethanol:acetonitrile solution at 274 nm. To isolate the excess liposomes, the liposomes from the supernatant were lyophilized overnight and redispersed in 1:1 v/v ethanol:

acetonitrile. The scattering caused by the liposomes around 274 nm was accounted by subtracting a linear extrapolation generated from the absorbance from 290-350 nm. The subtracted value at 274 nm was then used to approximate the amount of PTX remaining. The PTX on the stir bar and vial were heated at 120°C overnight to remove of any excess water, and then dissolved in the 1:1 v/v ethanol:acetonitrile. The calculated PTX mass was then subtracted from the initial added mass to determine the mass loaded in the particles.

Measurement of PTX release. 300 μL of 0.57 nM PL-HMSiO2-AuNR loaded with PTX in PBS was prepared and transferred to the bulb of an inverted 3 mL Falcon plastic pipette. The pipette was added to the standard ultrasound setup and insonated for 2.5 min at the following conditions: frequency 1.1 MHz, burst period 100 ms, cycle count 12, input peak-to-peak voltage 1000 mV. The liquid portion was deposited to a new pipette and shaken in an incubator for 1 h at 37℃. The sample was then added to a new pipette and this procedure was repeated for 1, 2, 4, 6, 12, 24, 36, 48, and 72 h. After each time point, 150 μL of butanol was added to the now-empty Falcon plastic pipette and allowed to sit for 2.5 min for PTX dissolution. The amount of PTX released at each time point was calculated using UV-Vis spectroscopy, comparing at 227 nm to a calibration curve of PTX in butanol. As this procedure gives release at specific intervals, cumulative release was calculated by summing the calculated concentrations from t=0 to each time point (e.g., for cumulative release at 6 h: 1 h + 2 h + 4 h + 6 h).

Measurement of cell toxicity to PTX-loaded PL-HMSiO2-AuNR and HIFU. MDA-MB-468 breast carcinoma cells were grown to confluence at 37℃ in a flask. 60 wells in a 96 well plate was then created with a seeding density of 12,500 cells/well. 36 wells were created for the PTX loaded particles, 12 wells for the 10x concentration of particles, 9 for the positive controls and 3 for the controls with no particles. The cells were then incubated for 24 h. The wells were then washed with PBS two times and then the treatments were deposited. The treatments included positive controls performed in triplicate: 100 μL of 10 μM PTX in Cremophor EL dispersed in cell media, 100 μL 1 μM PTX in Cremophor EL dispersed in cell media, and 100 μL of 0.1 nM PTX in Cremophor EL dispersed in cell media. 100 μL of the PTX loaded particles in cell media were then added with a concentration of 0.57nM, 0.057 nM, and 0.0057 nM of rods dispersed in cell media were added to 36 wells, 12 for each concentration. 100 μL of 0.57 nM particles without drug were then added 12 of the wells. Finally, the last three wells were filled with 100 μL of cell media to act as a control. The well plate was then wrapped in 3 layers of parafilm to minimize

water intruding into the well plate. The particles were then allowed to incubate for ### h to allow for endocytosis of the nanorods into the cells. Afterwards, the particles were then irradiated at a PNP of 0 or 13.94 MPa for 2.5 min for the designated well. The parafilm was then removed from well plate and the particles were incubated with the cells for 24 h. The cells were then with PBS twice and the 100 µL of 0.5 mg/mL of MTT in cell media was then added to the wells. The cells were then incubated for 4 h. The MTT solution was then removed and the MTT crystals were dissolved in 150 µL of isopropyl alcohol. The plate was placed on shaker at room temperature for 2 h, and the absorbance was read using the plate reader at 560 nm. The background was then subtracted by reading the background absorbance at 650 nm. Assuming the control cells are considered 100% viable, the corrected absorbances were scaled to the absorbance of the control wells to determine the cell viability of the different treatments that were performed.

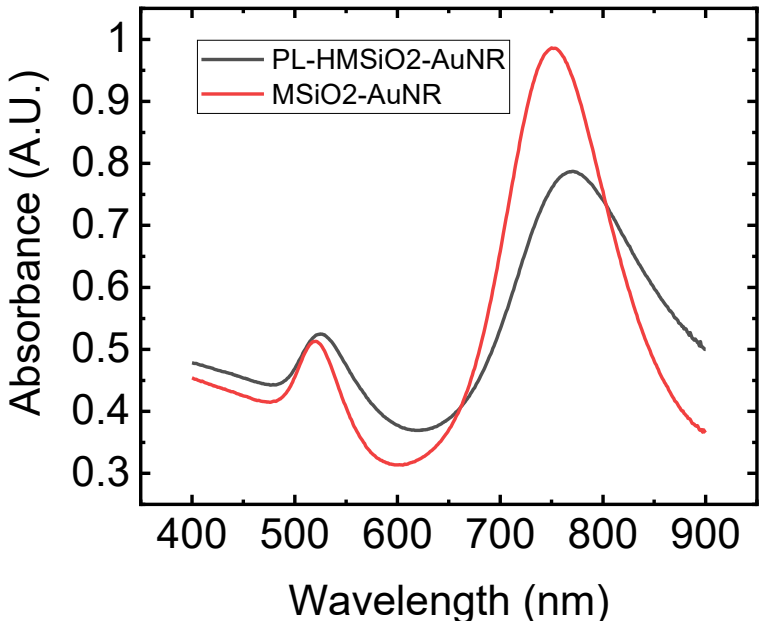


Figure S1. UV-Vis spectra of PL-HMSiO2-AuNR and MSiO2-AuNR with concentrations adjusted to match absorbance at 800 nm.

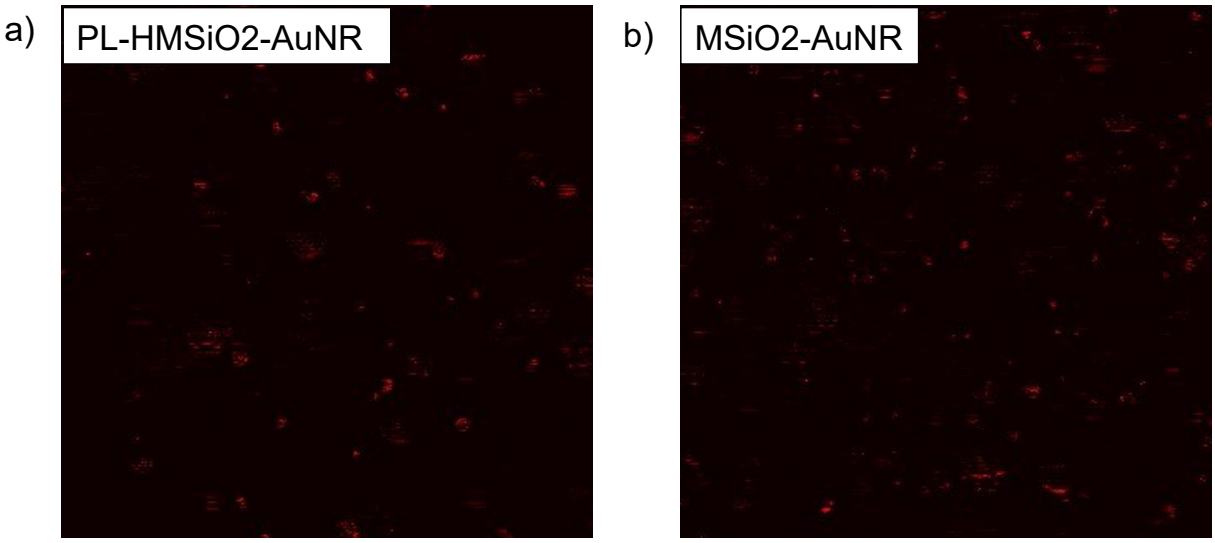


Figure S2. Two-photon luminescence images of (a) PL-HMSiO2-AuNRs and (b) MSiO2-AuNR at 800 nm and 432 mW input laser power.

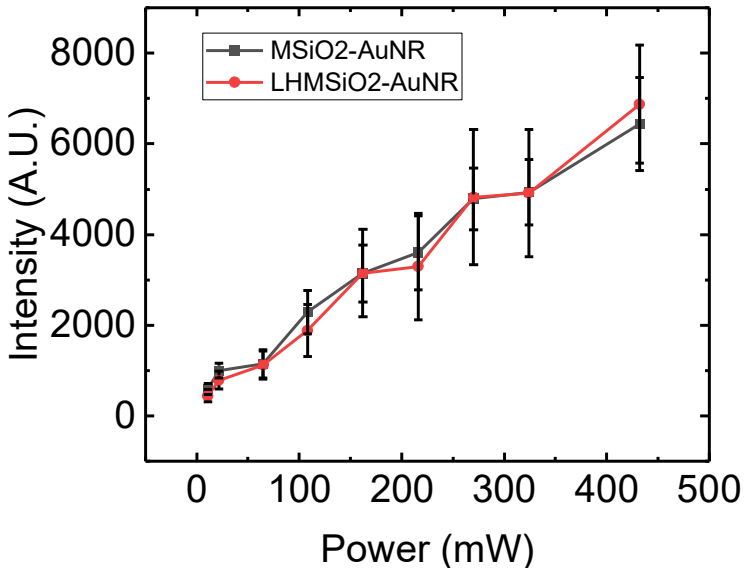


Figure S3. Intensity of two-photon luminescence of PL-HMSiO2-AuNR and the MSiO2-AuNR at 120 s irradiation at different input laser powers.

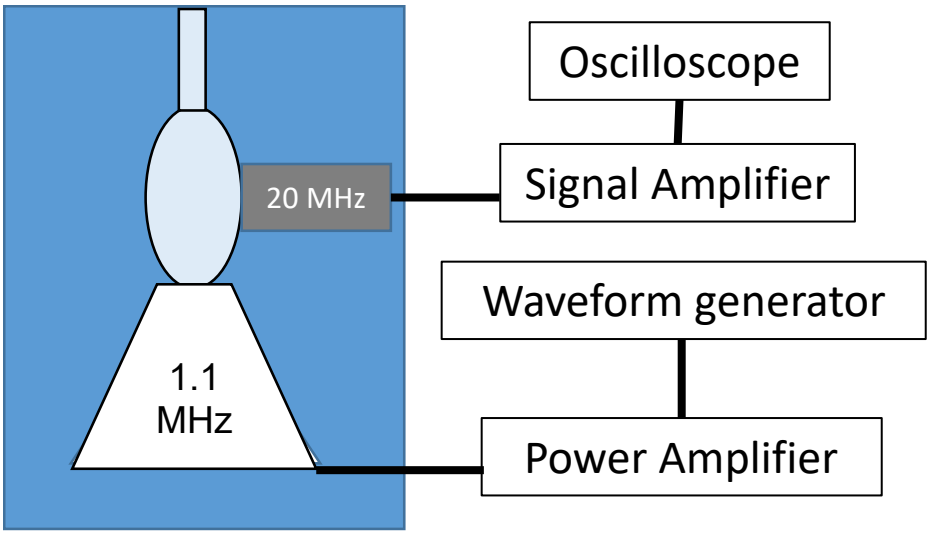


Figure S4. Schematic of HIFU setup for passive cavitation detection.

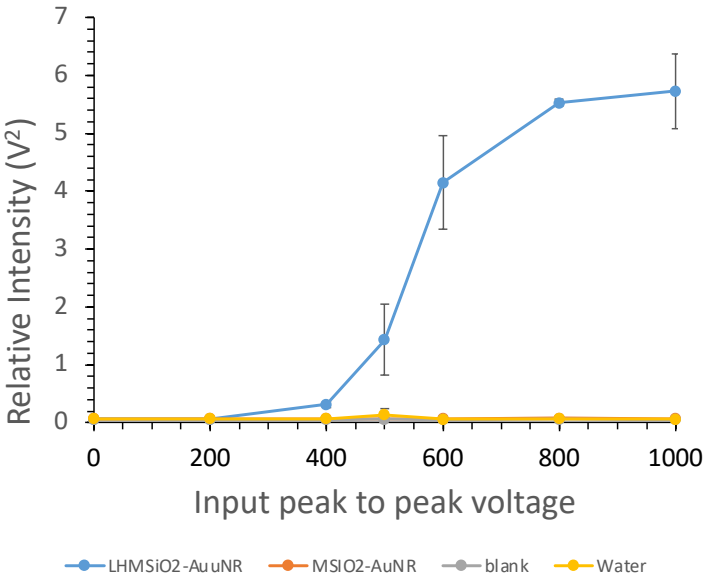


Figure S5. Intensity of received acoustic signal vs. input driving voltage from the PL-HMSiO2-AuNR, MSiO2-AuNR, water, and blank (no sample holder).

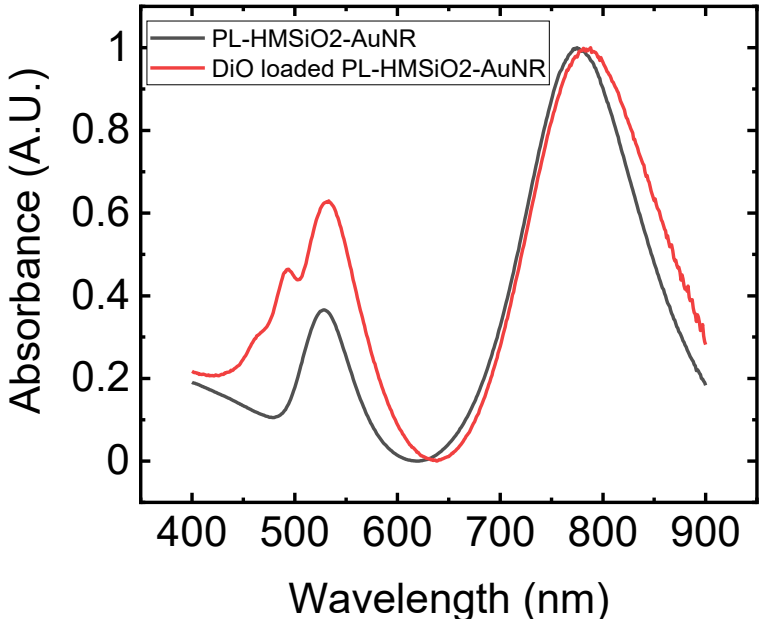


Figure S6. UV-Vis spectra of PL-HMSiO2-AuNR with and without loading of DiO.

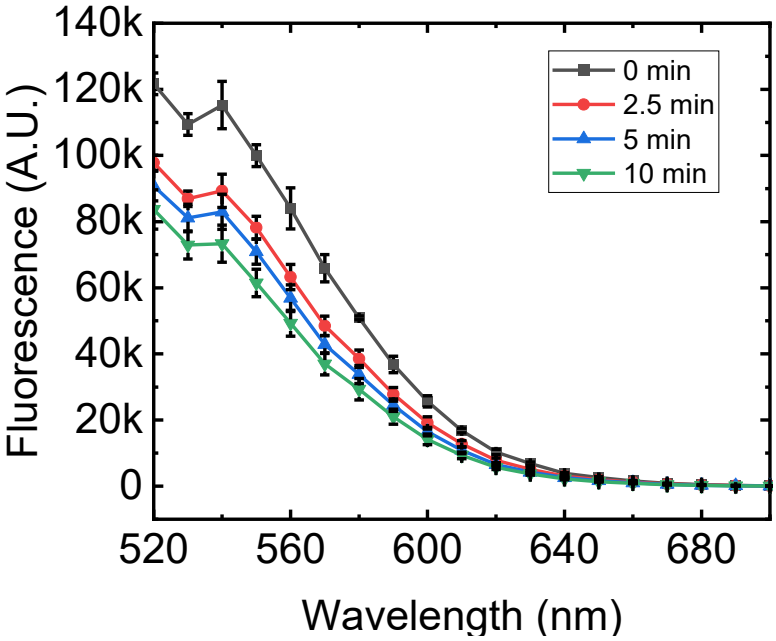


Figure S7. Fluorescence emission spectra of DiO-loaded PL-HMSiO2-AuNRs following 0, 2.5, 5, and 10 min HIFU insonation.

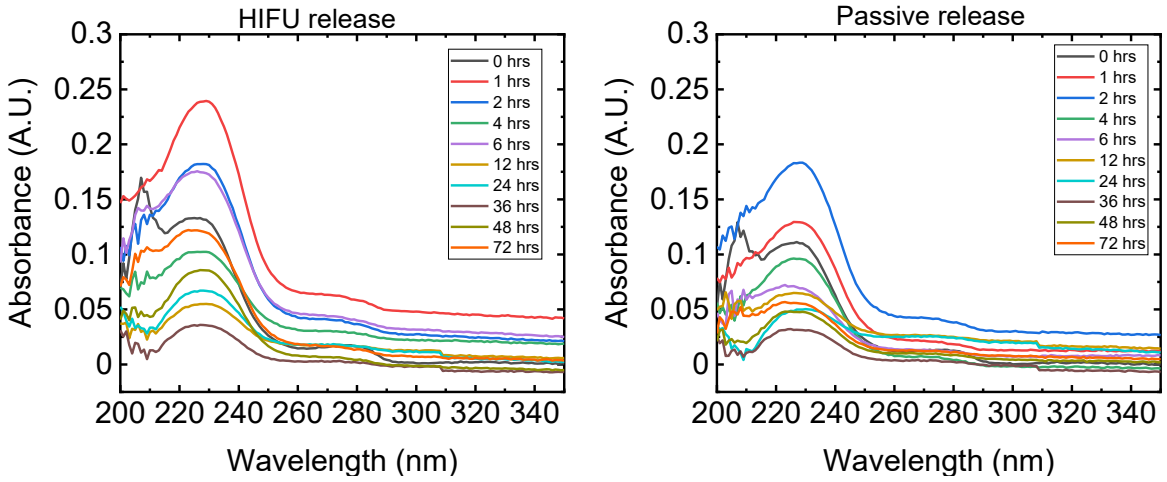


Figure S8. UV-Vis spectra of PTX released from PL-HMSiO2-AuNR at various timepoints with either (a) HIFU applied or (b) HIFU not applied.

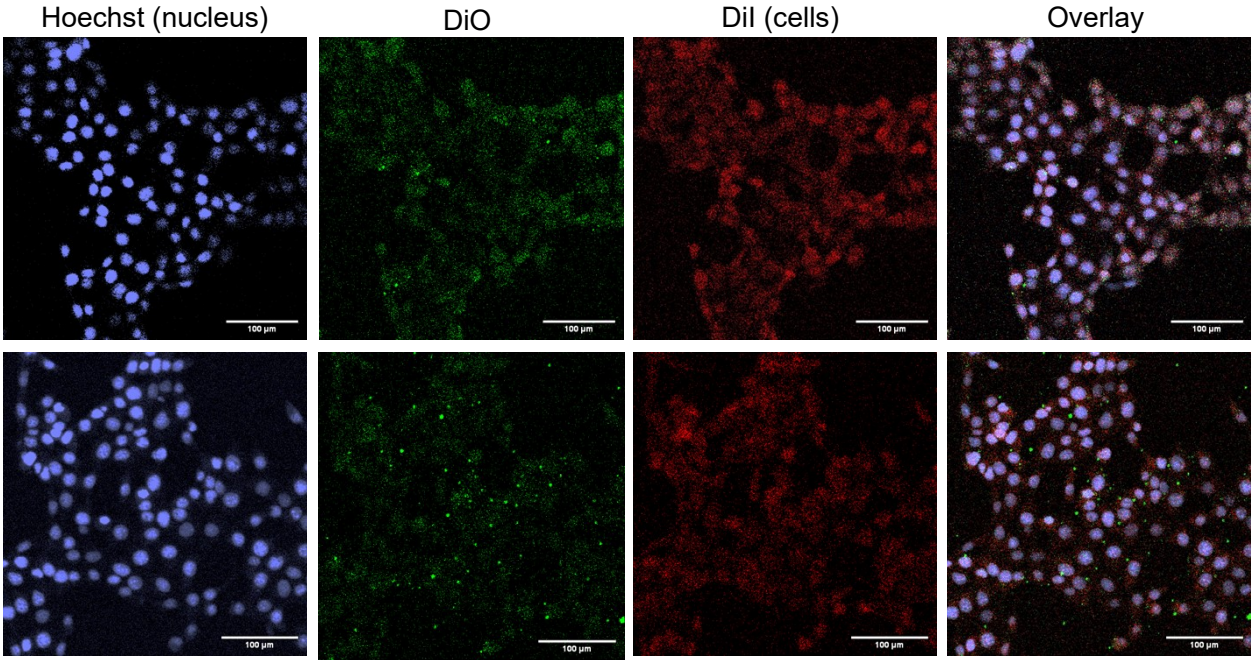


Figure S9. Confocal microscopy images showing MDA-MB-468 cells incubated with PTX-loaded PL-HMSiO2-AuNRs.