Supporting Information

Gene silencing by gold-nanoshell-mediated delivery and laser-triggered release of antisense oligonucleotide and siRNA

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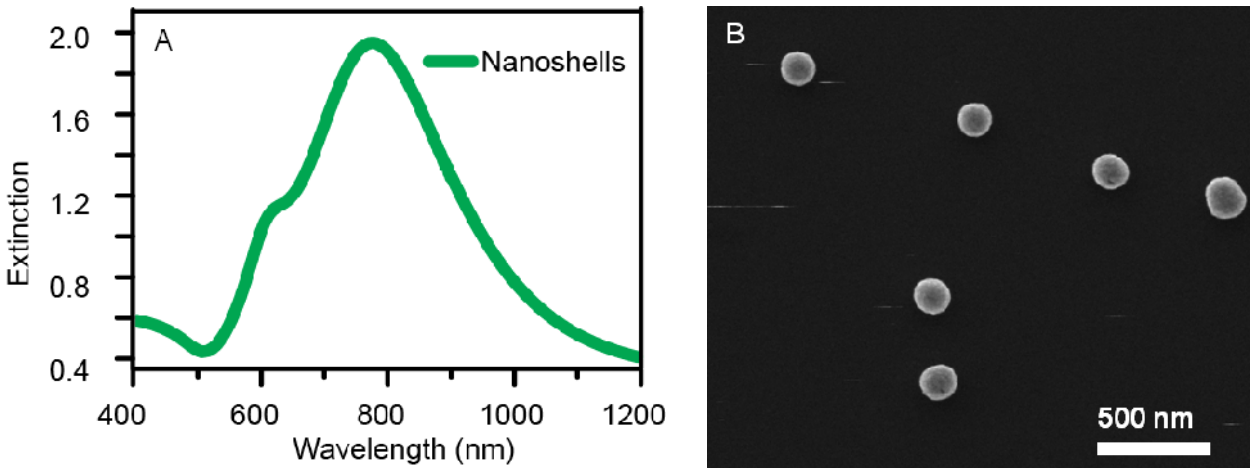

Fig. S1. Characterization of Silica Core-Au shell Nanoshells. (A) Extinction spectrum of an aqueous solution of [r1, r2] = [60, 82] nm gold nanoshells (λ_{peak}=800 nm). (B) Scanning Electron Microscope (SEM) image of gold nanoshells.

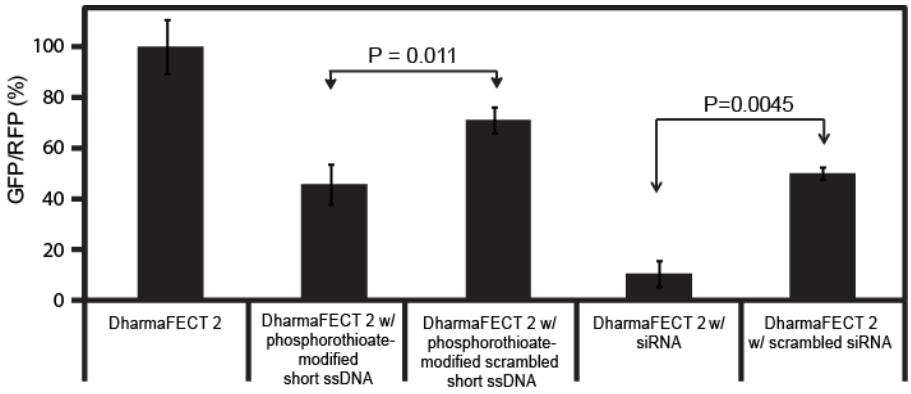

Fig. S2. GFP/RFP (%) of H1299-GFP/RFP cell line treated with DharmaFECT 2 transfection reagent, DharmaFECT 2 with phosphorothioate-modified short ssDNA (Table 1 in manuscript), DharmaFECT 2 with phosphorothioate-modified scrambled short ssDNA, DharmaFECT 2 with siRNA (Table 1 in manuscript) and DharmaFect 2 with scrambled siRNA.

Phosphorothioate-modified scrambled short ssDNA sequence	5' – C*G*T*G*T*G*A*G*T*G*T*G*C*T*G*T*G* T*G*T*G-3'
Scrambled siRNA sense strand	5' – ACAUAUCACCGAUACUCCAGA – 3'
Scrambled siRNA antisense strand	5' – UCUGGAGUAUCGGUGAUAUGU -3'

Table S1. Scrambled sequences of ssDNA and siRNA used in Fig. S2.

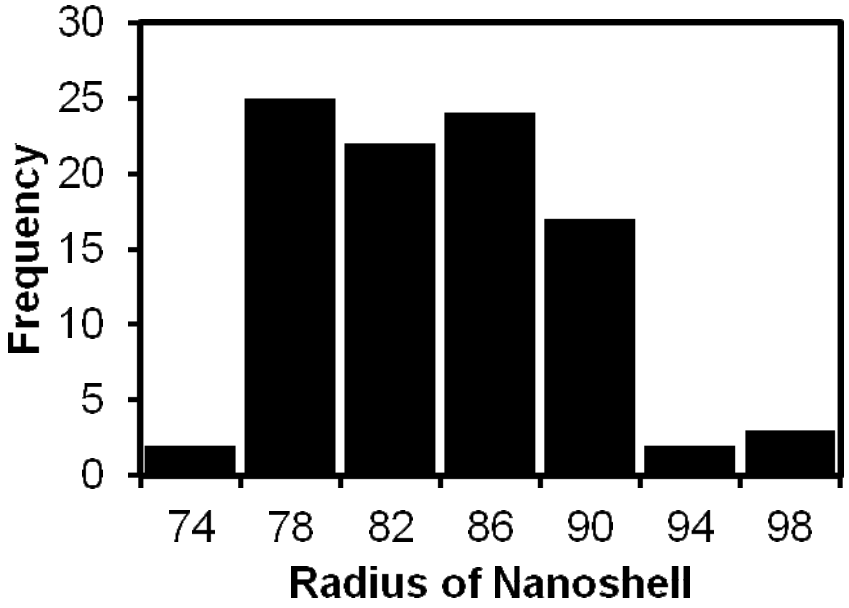

Fig. S3. Histogram of nanoshell outer radius (r_2) determined by measuring over 100 nanoshells by scanning electron microscope images.

Materials and Methods

Nanoshells Synthesis:

Au nanoshells were synthesized according to previously published method.(1) The dimensions of the silica core and the Au shell ([r1, r2] = [60, 82] nm) were chosen such that the peak plasmon resonance in aqueous suspension was 800 nm, corresponding to the laser excitation wavelength used in this experiment.

Polylysine Attachment:

Polylysine peptide was custom synthesized (Biomatik USA, LLC) and consists of the following sequence: Cysteine(C)- Tyrosine(Y)-Serine(S)-Lysine$(K)_{50}$ (CYS-K_{50}). The thiol group on the Cysteine attaches to the Au nanoshell via Au-thiol bond. The tyrosine and serine are spacer amino acids. The concentration of nanoshells is determine by the following equation which is derived from beer's law:

$$\frac{\#\,\text{Nanoshells}}{\text{mL}} = \frac{2.303 x A_{peak}}{\sigma_{ext} x b}$$

where A_{peak}=experimental Absorption at the peak plasmon resonance, σ_{ext} = theoretical extinction cross section taken from mie theory(A free Mie theory simulator is available at http://www.nanocomposix.com/support/tools), and b = path length of the cuvette. The cysteine (C)-Tyrosine (Y)-Serine (S)-Lysine(K)50 (PLL) peptide was custom synthesized (Biomatik USA, LLC)Thepeptidewasreceivedasalyophilizedpowderandresuspended inMilli-Qwatertoa concentrationof500µM. Oncetheconcentrationofnanoshellsisknown,thisPLLpeptideis addedtothesolutionofnanoshellsin100,000molarexcessofPLL. Thissolutionwasallowed toincubatefor24hoursonarockeratroomtemperature.TheexcessPLLwasremovedviatwo

centrifugationcycles(350rcffor15minutes)andresuspendedinMilli-Qwater. ζ-potential measurementsconfirmedPLLattachment(Table3).

ssDNA or siRNA Loading:

AntisensessDNAandsiRNAwerecustomsynthesizedbyIntegratedDNATechnologies, IDTandQiagen,respectively. ssDNAandsiRNAwasresuspendedinTEbufferandnuclease-freewater,respectively,toafinalconcentrationof~100μM.An50,000molarexcessofssDNA orsiRNAwasaddedtotheAunanoshell/PLLsuspensionpreviouslypreparedandallowedto incubatefor24hoursonarockeratroomtemperature. Thenanoshellsolutionwasplacedinthe refrigeratorat4°Candallowedtogentlysettledownfor48hours.Afterwards,thesupernatant waspipettedoffandthepelletwasresuspendedinMilli-Qwater.(Note:Forthefluorescein-tagged-ssDNAreleaseexperiments,resuspensioninTEbuffer(IDT,pH=7.5)isnecessaryto ensurethatthepHisconstantsincetheemissionpropertiesoffluoresceinarepHdependent.)

Thermal Treatment:

To ensure accurate comparison between thermal treatment and light-triggered release each sample prepared was divided in half. Half was used for the thermal treatment and half was used for laser treatment. For both thermal and laser treatment, ssDNA sequences had a fluorescein modification on the 5' end of the DNA sequence. The NS-PLL with ssDNA electrostatically attached (NS-PLL-ssDNA) sample was placed in a water bath and heated slowly (~1°C/minute) while stirring. The slow heating and stirring ensures that the NS-PLL-ssDNA sample is in thermal equilibrium. The solution temperature is monitored by a thermocouple. As the solution temperature rises, aliquots were taken and centrifuged immediately, which separates the released DNA from the nanoshells. The centrifuge speed should be as slow as possible to

ensure minimal removal of ssDNA, which is still attached to the NS-PLL. The fluorescence intensity of the supernatant was measured by a fluorescence spectrophotometer and then converted to DNA concentration using a standard curve of fluorescence intensity versus DNA concentration.

Laser Treatment:

An 800 nm CW Diomed 15 Plus Laser was fibercoupled so that the end of the fibercouple was placed above the sample (2W, spot diameter = 1 cm). The solution was stirred during laser illumination and the temperature was monitored with a thermocouple. The aliquots were treated identically as the thermal treatment described above.

Loading Capacity of Fluorescently-tagged ssDNA on the NS-PLL vector:

Two aliquots of 500 uL of NS-PLL-ssDNA were pipettes into two separate 1.5mL eppindorf tubes. In one tube, 500 uL of TE buffer was added and in the other tube 500 uL of 12mM mercaptoethanol (diluted in TE buffer) was added. Both tubes were covered in aluminum foil and were gently mixed on a rocker plate for 24 hours. Mercaptoethanol displaces the polylysine and ssDNA attached to the nanoshell.(2) After 24 hours, both samples were centrifuged to remove the nanoshells, then the supernatants were measured in a fluorolog (excitation wavelength: 495 nm, Emission wavelengths: 505-540 nm). The difference in fluorolog intensity between samples was used to calculate the difference in DNA concentration by using a standard curve of DNA concentration to fluorescence intensity. The DNA concentration was then divided by the nanoshell concentration, calculated by UV-VIS.

Cell culture. The H1299 and H1299-GFP/RFP lung cancer cell lines were incubated at 5% CO_2, 37°C with RPMI 1640 media with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic solution. 0.25% Trypsin-EDTA was used for cell passaging.

NS-PLL cellular uptake. The H1299-GFP/RFP lung cancer cells were seeded in 4-well chamber slides at 5000 cells/well 24 hours prior to incubation with NS-PLL delivery vectors. The nanoshells were added to the serum containing media in a NS:cell ratio of 5,000:1 and allowed to incubate for 2 hours. Then, the cell culture medium was aspirated off, the cells were washed twice with 1x phosphate buffered saline, the cells were fixed with 4% paraformaldehyde, and stained with Alexa Fluor 555 according to manufacturer instructions. The walls of the chamber slide were removed, and the cells were mounted using Vectashield mounting medium for fluorescence (Vector Laboratories) and 22x50 mm rectangular cover glass (n=1.5) Dark-field and fluorescence images were taken on a Cytoviva microscope equipped with dual-mode fluorescence module and a x-cite light source. For quantification of nanoshells uptaken in cells, ICP-MS measurements were performed. After a 12 hour incubation, the media was aspirated off, the cells were washed twice with PBS, the I_2/KI etchant procedure was perfomed (see methods, supporting information), live cells were sorted and counted using flow cytometry, digested with aqua regia, and then the gold content was measured with the ICP-MS (see methods, supporting information).

Fluorescence images for light-triggered release. NS-PLL-ssDNA delivery vectors were incubated with cells in an identical manner to NS-PLL uptake. The ssDNA is fluorescently tagged with Alexa Fluor 488 (Integrated DNA technologies, IDT) After two hours, the media was aspirated off, the cells were washed twice with 1x phosphate buffered saline, fresh media

was added. The laser treatment group under went a laser treatment (2.5 W/cm^2, 2 minutes). Immediately afterwards, the media was aspirated off, fixed, and stained in an identical procedure as the NS-PLL cellular uptake experiments and imaged with a Cytoviva microscope.

Laser treatment for GFP downregulation. H1299-GFP/RFP cells were plated in a 12-well plate at a density of 95,000 cells/well 12 hours before incubation with NS-PLL, NS-PLL-ssDNA, and NS-PLL-siRNA delivery vectors. These NS-PLL delivery vectors were incubated with cells in an identical manner as the NS-PLL cell uptake experiments. After incubation the cell culture media was aspirated of, the cells were washed with PBS, trypsinized, and resupsended in cell culture media. Half of each cell suspension was irradiated with an 800 nm CW laser for 2 minutes at 2.5 W/cm^2 and the other half did not undergo laser irradiation. The cells are then plated back onto a fresh 24 well plate. Green Fluorescence Protein (GFP) and Red Fluorescence Protein (RFP) were measured at specific time points using a fluorescence plate reader.

GFP/RFP measurements. Green Fluorescence Protein (GFP) and Red Fluorescence Protein (RFP) were measured using a Fluorescence plate reader (Biotek FLx800). For GFP the excitation filter was 485/20 nm and emission filter was 530/25 nm. For RFP the excitation filter was 530/25 and emission filter was 590/35 nm. GFP and RFP measurements were take prior to incubation (0 hours), after incubation and prior to laser treatment (12 hours), and after laser treatment (15, 18, 24, and 36 hours).

Instrumentation. Extinction spectra were obtained using a Cary 5000 UV/vis/NIR spectrophotometer. Fluorescence emission of fluorescently tagged ssDNA was obtained using Jobin Yvon Fluoromax 3. Biotek FLx800 was used for microplate fluorescence measurements.

Perkin Elmer ELAN9000 Inductively Couples Mass Spectrometer. Flow cytometry was done on a BD FACS Aria II.

Inductively-Coupled Mass Spectrometry Measurements. The I_2:KI etchant was made by mixing Iodine (I2, Aldrich) and potassium iodide (KI, Aldrich) to deionized water with a molar ratioof 1:6. The major role of KI is to increase the solubility of I2 in water.Cells were initially seeded in 6-well plates at a density of 400,000 cells/well 24 hours prior to incubation with nanoshell-dsDNA-DAPI complexes. The nanoshells were added to the serum containing media in a NS:Cell ratio of 5,000:1 and allowed to incubate for 12 hours. Then media was aspirated off. The sample was washed with PBS three times, and 1 mL of the I_2/KI etchant (0.17 mM of I_2) was added to the well. After etching at room temperature for 5 min, the solution was removed, the culture plate was washed with 3 times with 2 mL PBS. 500 uL 0.25% Trypsin-EDTA was added. After visual detachment of cells, the trypsin was deactivated by adding 500 uL serum-contating media. This suspension was then analyzed by flow cytometry for viable healthly cells using Propidium Iodide (PI). PI only enters cells apoptotic and necrotic cells which have damaged membranes, binds to the DNA in the nucleus and fluoresces red. PI is excluded from healthy cells. 100,000 cells viable cells (cells that did not fluoresce red) were sorted into a 20-ml sciltian glass vial. Concentrated Aqua regia was added to each glass vial and allowed to sit overnight. (Aqua regia is made of 3:1 of Hydrochloric acid (Sigma Aldrich, 37 wt. % in H_2O, 99.999% trace metals basis):Nitric Acid(Sigma, 70%, ≥99.999% trace metals basis). WARNING: Aqua regia is dangerous and should be used in a chemical hood because it produces toxic nitrosyl chloride and chlorine gases. Do not cap the aqua regia because it produces gases and will explode if capped. The next day, the aqua regia was boiled off by using a hot plate

inside of a chemical hood. Each vial was then resuspended in 1% Aqua regia and filtered with 0.2 μm filters (PALL Acrodisc 32 mm). Gold content was measured via ICP-MS in ppb and then converted to gAu.

Calculation for Number of nanoshells uptaken:

Prior to PLL attachment, the silica core radius and gold shell radii were measured from particle size statistics obtained from SEM images of over 100 silica core particles and 100 Au nanoshells (Figure S3, supporting information). The size of nanoshells (NS) = $[r_1, r_2]$ nm. The Volume of the Au shell per NS was then calculated: Volume of Au shell/NS= $(4/3)\pi(r_2^3-r_1^3)$ cm^3). The grams of Au/NS was then calculated: grams of Au/NS ($\frac{gAu}{NS}$) =Volume of Au shell/NS*(Density of Au ($19.3g/cm^3$)), Flow cytometry gives a specific number of cells (# cells). Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) was used to quantify grams of gold (gAu). The number of nanoshells per cell was then calculated: (#NS/cell)= $\frac{gAu}{\frac{gAu}{NS} \bullet \#\,cells}$

XTT assay

After the laser treatment the cells were allowed to grow for 12 hours, and then the XTT assay (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt) was performed according to XTT kit instructions (ATCC).

1. Oldenburg SJ, Averitt RD, Westcott SL, & Halas NJ (1998) Nanoengineering of optical resonances. Chem Phys Lett 288(2-4):243-247.
2. Demers LM, et al. (2000) A fluorescence-based method for determining the surface coverage and hybridization efficiency of thiol-capped oligonucleotides bound to gold thin films and nanoparticles. Analytical Chemistry 72(22):5535-5541.