Part I. Creation of stain for CALAA-01

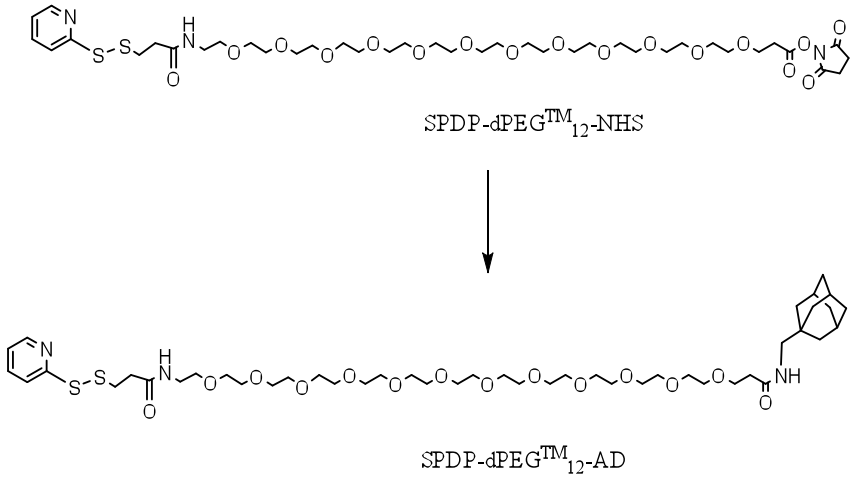
Materials

Methoxy-PEG-thiol (MW 1000 g/mol) was purchased from Laysan Bio (Arab, AL). SPDP-dPEG$^{TM}_{12}$-NHS (MW 912.08 g/mol) ester was purchased from Quanta Biodesign (Powell, OH). 5 nm spherical gold nanoparticles were obtained from Nanopartz (Salt Lake City, UT).

Methods

Preparation of SPDP-dPEG$^{TM}_{12}$-AD (see Scheme SI 1)

To a solution of SPDP-dPEG$^{TM}_{12}$-NHS (100 mg, 0.11 mmol) in 3 ml of anhydrous dichloromethane was added adamantanemethylamine (0.11 mmol, 21 µl). The solution was stirred for 16 h at room temperature, after which the solvent was removed and the product dried under vacuuo. (MALDI-TOF) $[M+Na]^+$ 984.08, $[M+K]^+$ 1000.16



Scheme SI 1: Synthesis of SPDP-dPEG$^{TM}_{12}$-AD

PEGylation of 5 nm gold nanoparticles with SPDP-dPEG$^{TM}_{12}$-AD (Au-PEG-AD)

To a pre-sonicated 1 ml solution of 5 nm gold nanoparticles (10^{13} particles ml^{-1}) was added mPEG-thiol (14 µg, 14 nmol) and SPDP-dPEG$^{TM}_{12}$-AD (16 µg, 14 nmol). The solution was vortexed for 30 min and diluted to 4 ml with deionized water. The resulting 4 ml solution was diafiltered twice with a 10 kDa membrane (Millipore) and finally resuspended to 1 ml.

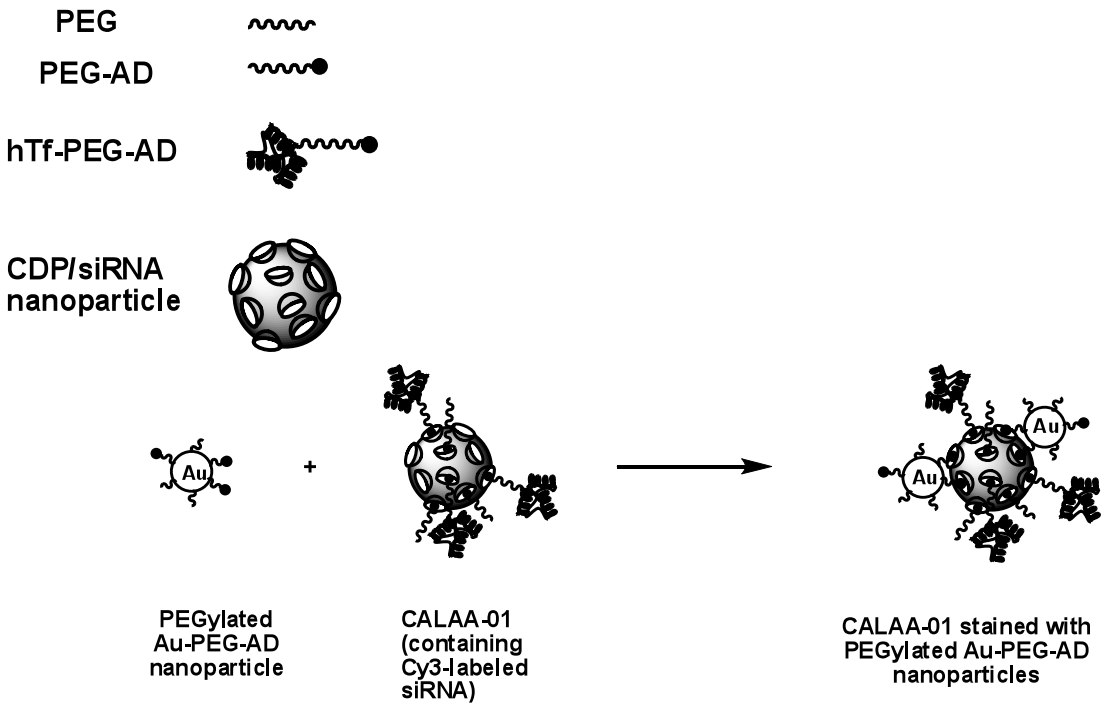
PEGylation of 5 nm gold nanoparticles with methoxy-PEG-thiol (Au-PEG)

To a pre-sonicated 1 ml solution of 5 nm gold nanoparticles (10^{13} particles ml^{-1}) was added methoxy-PEG-thiol (28 µg, 28 nmol). The solution was vortexed for 30 min and diluted to 4 ml with deionized water. The resulting 4 ml solution was diafiltered twice with a 10 kDa membrane (Millipore) and finally resuspended to 1 ml.

Part II: Principle of staining

A schematic representation of the interactions between the Au-PEG-AD particles and CALAA-01 is shown in Schematic SI 2.

Key



Scheme SI 2: Representation of the staining process of CALAA-01 by Au-PEG-AD particles.

Part III: In vitro validation of binding of Au-PEG-AD to the siRNA-containing nanoparticles

A sandwich ELISA assay was developed to test for the binding of the gold stain (Au-PEG-AD) onto the siRNA-containing nanoparticles.

Materials

- Coating buffer: 10 μg ml^{-1} mouse IgG against human transferrin (Bethyl Laboratories, Montgomery, TX) in 50 mM carbonate-bicarbonate buffer (pH = 9.6)
- Wash buffer: 50 mM Tris, 140 mM NaCl, 0.05 % Tween 20, pH = 8
- Blocking buffer: 50 mM Tris, 140 mM NaCl, 1 % bovine serum albumin (BSA), pH = 8
- Targeted particle solution: Transferrin-targeted particles containing siRNA (0.5 mol % AD-PEG-Tf) in 150 mM phosphate buffered saline (PBS)
- Staining solution: Au-PEG-AD or Au-PEG particles in 150 mM PBS

Method

The entire ELISA procedure was conducted at room temperature with mild shaking on a plate shaker. Into each well of a 96-well ELISA plate was coated with 0.1 ml of coating buffer that contains the primary antibody, IgG against human transferrin for 1 h. Three rinses with 0.1 ml of wash buffer followed to remove unbound antibodies. Next, blocking with BSA by adding 0.2 ml of blocking buffer into each well took place for 1 hour, followed by three brief rinses with 0.1 ml of wash buffer. After that, each well was added with 0.1 ml of either targeted particle solution or 150 mM PBS as a negative control and incubated for 2 h. After five rinses with 0.1 ml of wash buffer to remove unbound targeted particles, each well was loaded with 0.1 ml of staining solution containing either Au-PEG-AD or Au-PEG at different concentrations and incubated for 2 h. Upon five rinses with 0.1 ml of wash buffer to remove unbound targeted particles, each well was loaded with 0.1 ml of 150 mM PBS for absorbance measurement at 520 nm (surface plasmonic peak for 5 nm Au-PEG particles) using a Safire^2 Microplate Reader (Tecan, Salzburg, Austria). Reported values are mean absorbance from duplicates of experiments.

Results

The binding of Au-PEG-AD to the siRNA-containing nanoparticles relies upon the interaction between ADs and the cyclodextrin (CD) in a dose dependent fashion.

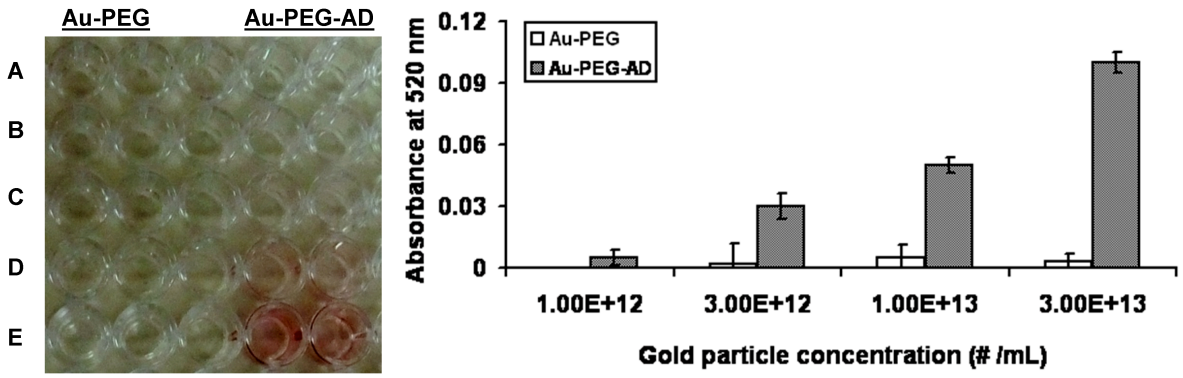


Figure S1: (A) The ELISA plate captures the red color due to binding of Au-PEG-AD onto targeted siRNA particles via specific CD-AD interactions. Legend: - A: no targeted nanoparticles + gold particles (10^{12} # ml^{-1}); B: targeted nanoparticles + gold particles (10^{12} # ml^{-1}); C: targeted nanoparticles + gold particles ($3x10^{12}$ # ml^{-1}); D: targeted nanoparticles + gold particles (10^{13} # ml^{-1}); E: targeted nanoparticles + gold particles ($3x10^{13}$ # ml^{-1}). (B) This graph demonstrates a dose-dependent binding of Au-PEG-AD onto siRNA-containing, CDP targeted nanoparticles (rows B to E in Figure S1A). As the negative control, the wells not loaded with any targeted nanoparticles do not show detectable gold absorbance (row A in Figure S1A).

Part IV: In vivo validation of binding of Au-PEG-AD to siRNA-containing nanoparticles in murine models

The use of imaging techniques serves to ascertain whether Au-PEG-AD can recognize intravenously injected targeted, siRNA-containing nanoparticles in a mouse tumor tissue.

Methods

Tumor Formation and Systemic Delivery of siRNA-containing Nanoparticles

All animal experiments were performed with sterile techniques and complied with the NIH Guidelines for Animal Care and as approved by the Caltech Institutional Animal Care and Use Committee. Neuro2A (mouse neuroblastoma) cells were cultured in complete growth medium (DMEM supplemented with 10 % fetal bovine serum (FBS), 100 units ml^{-1} penicillin, and 100 units ml^{-1} streptomycin). In the right hind flank, four immunodeficient (NOD.CB17-Prkdcscid/J) mice (The Jackson Laboratory, Bar Harbor, ME) received subcutaneous implantation of N2A cells (at 10^6 cells per mouse per 0.1 ml of DMEM). Before injection, tumors reached 100-200 mm^3 in size, as determined by caliper measurements ($0.5 \times l \times w^2$). Two mice received intravenous administration of siRNA-containing, cyclodextrin-based, targeted nanoparticles at a dose level of 10 mg siRNA kg^{-1} animal via the tail-vein. Formulated in 0.1 ml of 5 % glucose in water (D5W), the injected dose contained targeted nanoparticles carrying Cy3-labeled siRNA (20% of the total siRNA in the nanoparticles). As controls, two other mice received intravenous injections of 0.1 ml of D5W. Animal sacrifice by CO_2 overdose took place 24 h after injection, followed by tumor extraction and immersion fixation in 4 % paraformaldehyde (PFA).

Transmission Electron Microscopy

PFA-fixed blocks (~ 1 mm^3 in volume) of mouse tumor samples received fixation with 2.5 % glutaraldehyde (in 100 mM sodium cacodylate, pH = 7.4) for 2 h, post-stain by 1 % OsO_4 at 4 °C for 2 h, and 0.9 % OsO_4 and 3 % $K_3Fe(CN)_6$ at 4 °C for 2 h. Subsequent dehydration with an ethanol gradient and propylene oxide enabled the embedding tissue blocks polymerized from Epon 812 resins (EMS; Electron Microscopy Sciences, Hatfield, PA). 80 nm thick sections were deposited on carbon and formvar-coated, 200-mesh, nickel grids (EMS) and later stained with 3 % uranyl acetate (SPI Supplies, West Chester, PA) and Reynolds lead citrate for visualization under a 120 kV BioTwin CM120 TEM (Philips). All electron micrographs are from randomly chosen facets of the tissue block.

Results

Transmission electron microscopy confirms the existence of siRNA containing, targeted nanoparticles inside a mouse subcutaneous tumor.

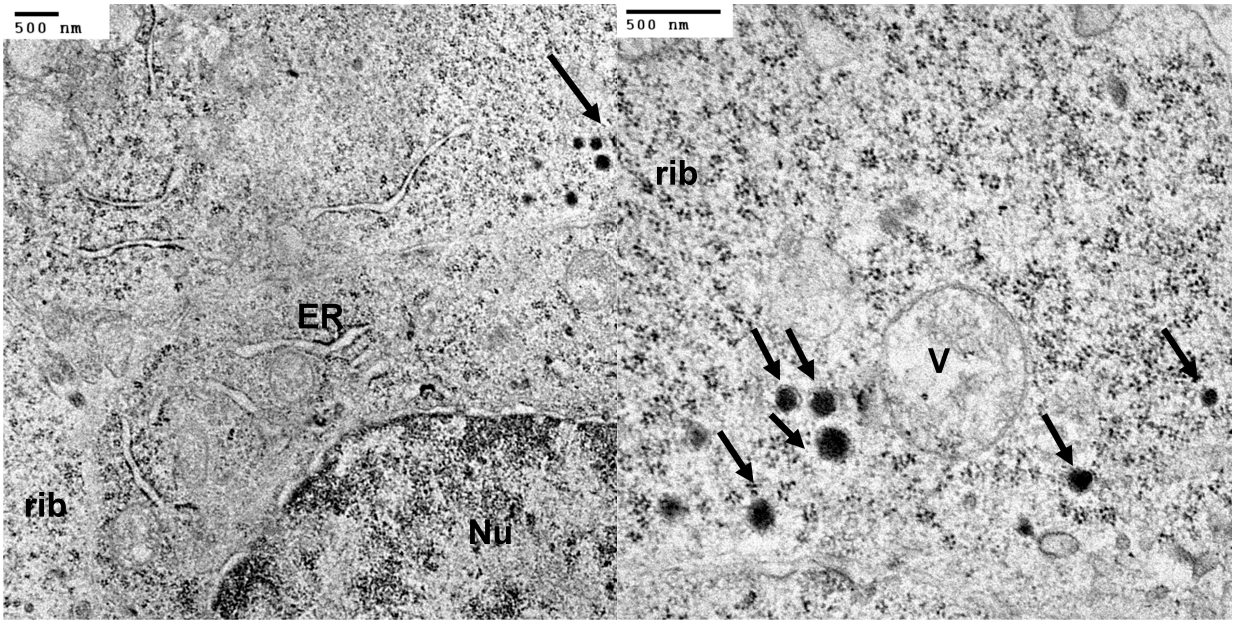


Figure S2: Transmission electron micrographs showed intracellular localization of siRNA-containing, cyclodextrin-based, targeted nanoparticles (dark round objects – the siRNA within the nanoparticle is stained by the presence of the uranyl ions that bind to the nucleic acid) inside N2A cells of the tumor subcutaneously implanted in mice (same tumor tissue used for confocal fluorescence imaging in Figure 1). (Left) The proximity of targeted nanoparticles to the nucleus shows their intracellular localization. (Right) Expanded view of nanoparticles shown in left panel (Scale bar = 500 nm). Solid arrows point to the nanoparticles. Labeling is as follows, Nu: nucleus, rib: ribosome, ER: endoplasmic reticulum, V: vesicle.

Upon the confirmation of the presence of targeted particles inside the tumor following their systemic injection, confocal microscopy can validate the utility of Au-PEG-AD as a stain for the targeted nanoparticles in the same tumor tissues extracted from the same mice.

Method

Fluorescence Microscopy with Au-PEG-AD

After immersion fixation in 4 % PFA in PBS for 3 d, ~2 cm^3 mouse tumor tissue blocks passed through an increasing sucrose gradient up to 30 % sucrose, and were later embedded in 9 % gelatin in PBS. The gradual freezing of gelatin tissue blocks to -80 °C allowed the generation of 10 µm-thick cryosections. After brief rinsing with PBS to remove any surface gelatin as well as fixation with acetone at -20 °C to permeabilize the cell membrane, tissue sections underwent staining of PEGylated, adamantane-modified gold nanoparticles (Au-PEG-AD) in the dark for 2 h. Rinsing with PBS removed any non-specifically bound gold particles, before the mounting of Au-PEG-AD stained tumor sections with 16.7 % (w/v) Mowiol 4-88 and 33 % (v/v) glycerol in PBS. A Zeiss LSM 510 confocal scanning microscope served to reveal the *in vivo* tumor biodistribution of the Cy3-siRNA containing targeted nanoparticles (excitation: 555 nm; emission: 570 nm), whose presence was further confirmed by its colocalization with Au-PEG-AD (excitation: 488 nm; emission: 507 nm).

Results

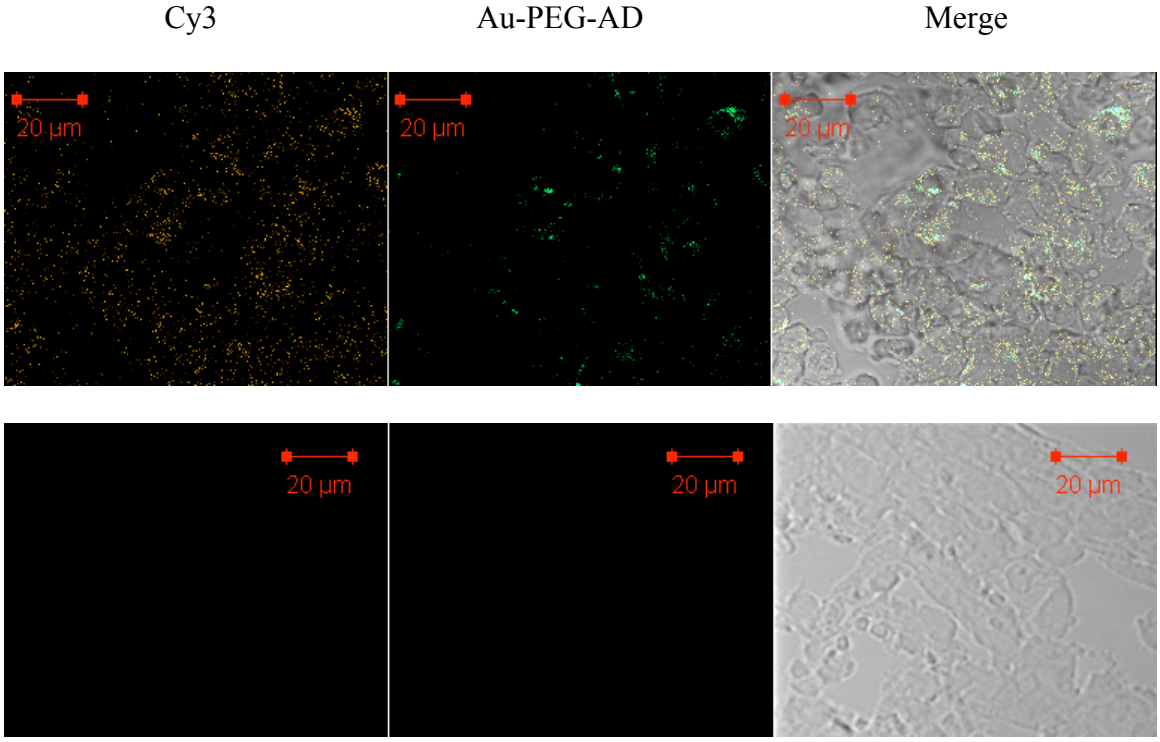


Figure S3: (Top) Tumor tissue collected from mice receiving intravenous (tail vein) injections of Cy3-siRNA containing, targeted nanoparticles. Au-PEG-AD particles (green: middle; emission: 507 nm) specifically bind to the Cy3-siRNA (orange: left; emission: 570 nm) containing, cyclodextrin-based targeted nanoparticles, whose localization was strictly intracellular, as seen from the merged bright-field image (right). (Bottom) As a negative control, tumor tissues were collected from mice that received intravenous injections of D5W. Au-PEG-AD particles failed to stain the tumor tissue in the absence of targeted nanoparticles. (Scale bar = 20 µm)

Part V: Staining of biopsy samples with Au-PEG-AD (Patient Pre-treatment samples)

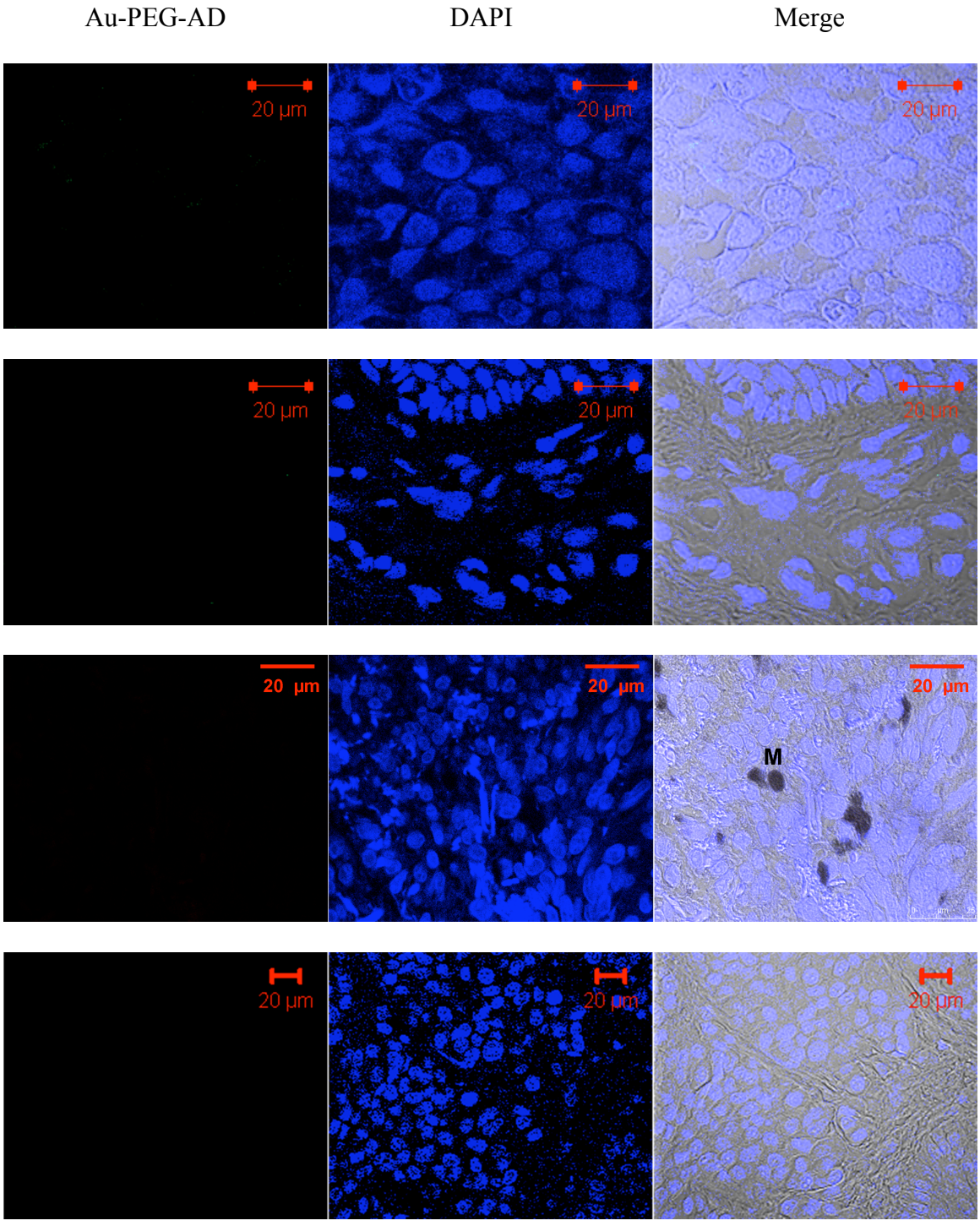


Figure S4: Au-PEG-AD staining of CALAA-01 nanoparticles in tumor biopsy samples using Au-PEG-AD and DAPI nuclear counter-stain. All pre-treatment samples, including A_{pre} (top row), B_{pre} (second row), $C1_{pre}$ (third row), and $C2_{pre}$ (bottom row), reveal undetectable gold

staining. (Scale bar = 20 microns) Legend: M = melanophage (black objects inside the melanoma tissue).

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