Gold Nanocrystal Labeling Allows Low Density Lipoprotein Imaging From The Subcellular To Macroscopic Level

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Supporting figures

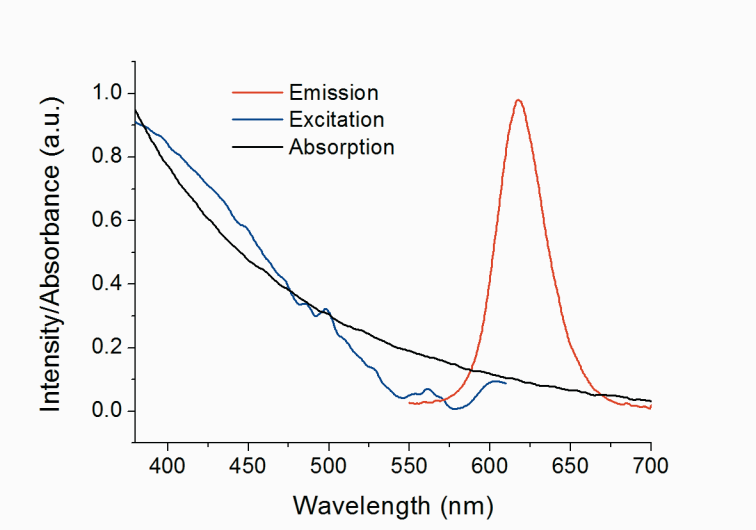

Figure S1. Optical properties of the quantum dots used. Absorption and emission spectra of the quantum dots. AU = arbitrary units. Absorbance and emission both normalized to the peak value recorded.

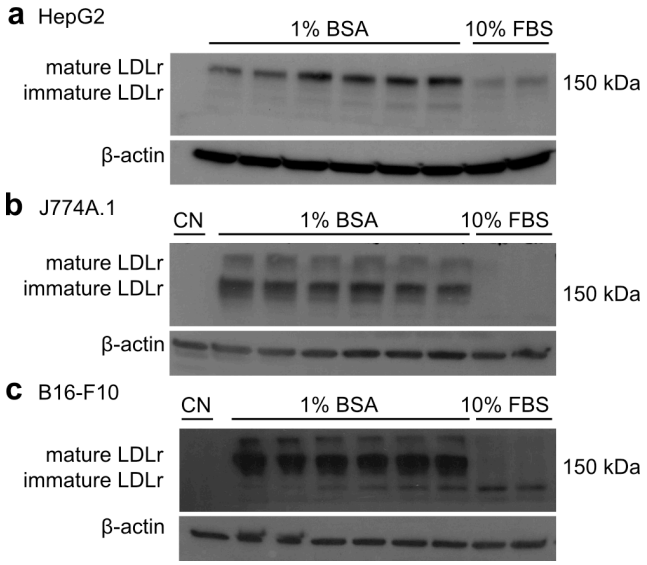

Figure S2. LDL receptor upregulation. Western blots of **a.** HepG2, **b.** J774A.1, and **c.** B16-F10 cells treated with either 1% BSA or 10% FBS. Cells treated with 1% BSA showed upregulation of LDLr (mature LDLr bands) whereas cells treated with 10% FBS did not. CN: negative control.

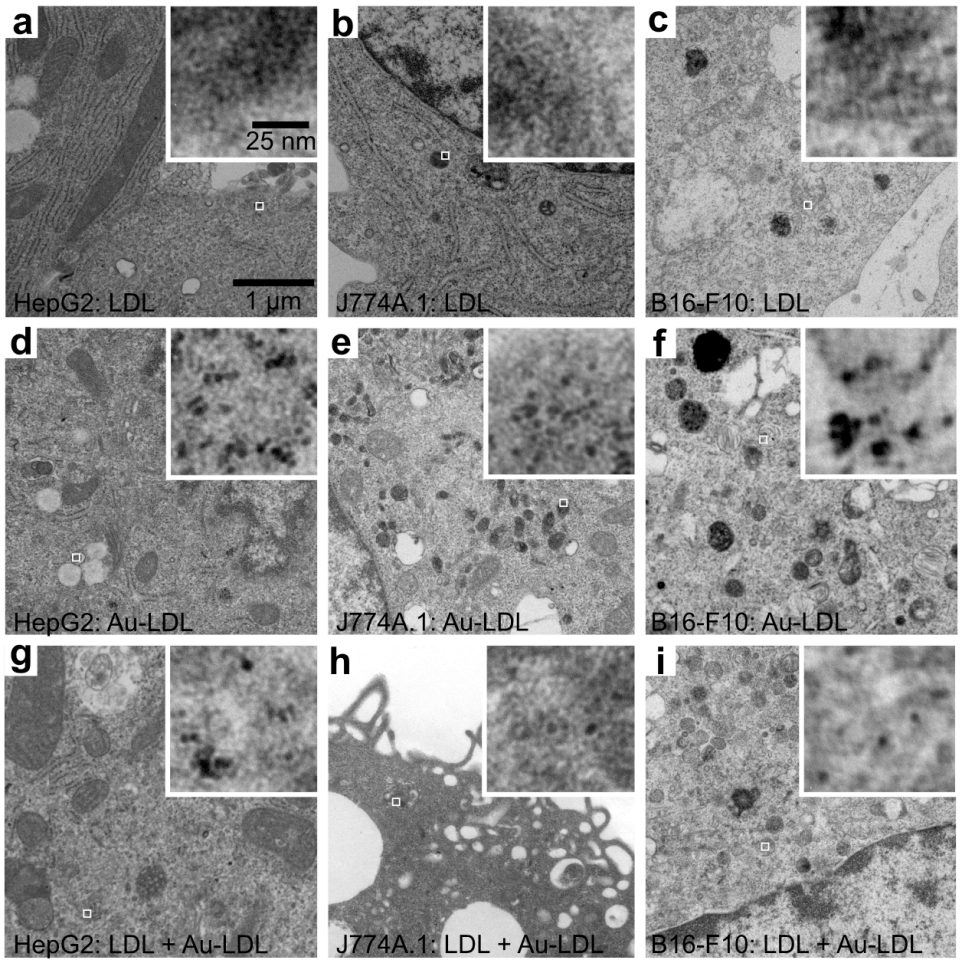

Figure S3. ***In vitro*** **competition inhibition experiment. a-c.** TEM micrographs of control (LDL only) in HepG2, J774A.1, and B16-F10 cells. **d-f.** Au-LDL incubation in HepG2, J774A.1, and B16F10 cells. **g-i.** Competition inhibition (excess LDL + Au-LDL) in HepG2, J774A.1, and B16F10 cells. Scale bars are the same for all micrographs.

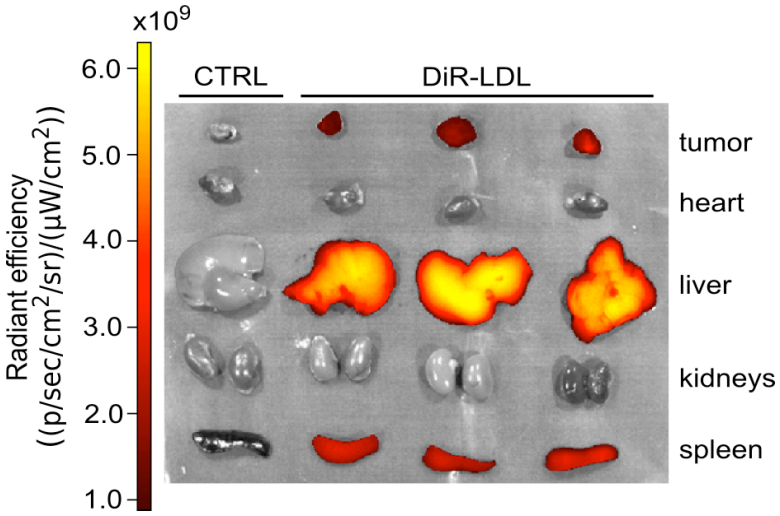

Figure S4. Fluorescence image of the organs of mice injected with DiR-LDL, compared with the organs of the uninjected mice.

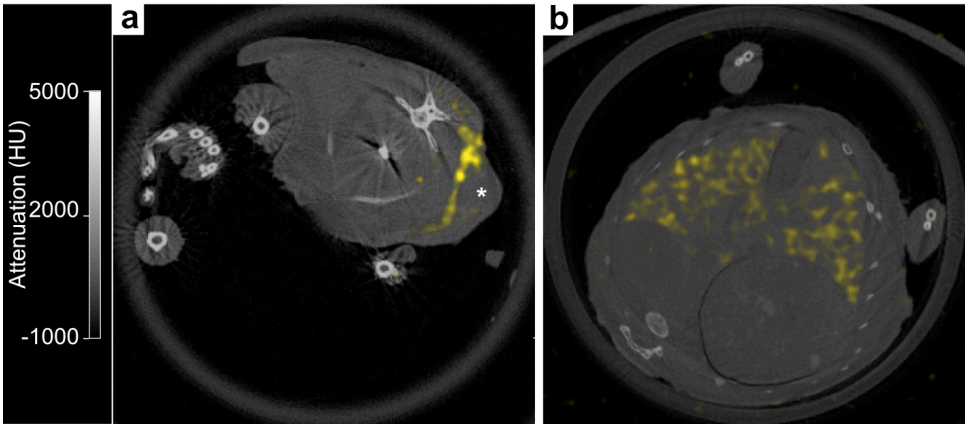

Figure S5. Spectral CT images of mice injected with Au-LDL. a. Image acquired at the level of the tumor in Figure 7a. * indicates the B16-F10 tumor. Gold accumulated at the rim of the tumor. **b.** Image acquired at the level of the liver in Figure 7b. Accumulation in the liver, we expect, is likely due to normal LDL liver uptake.

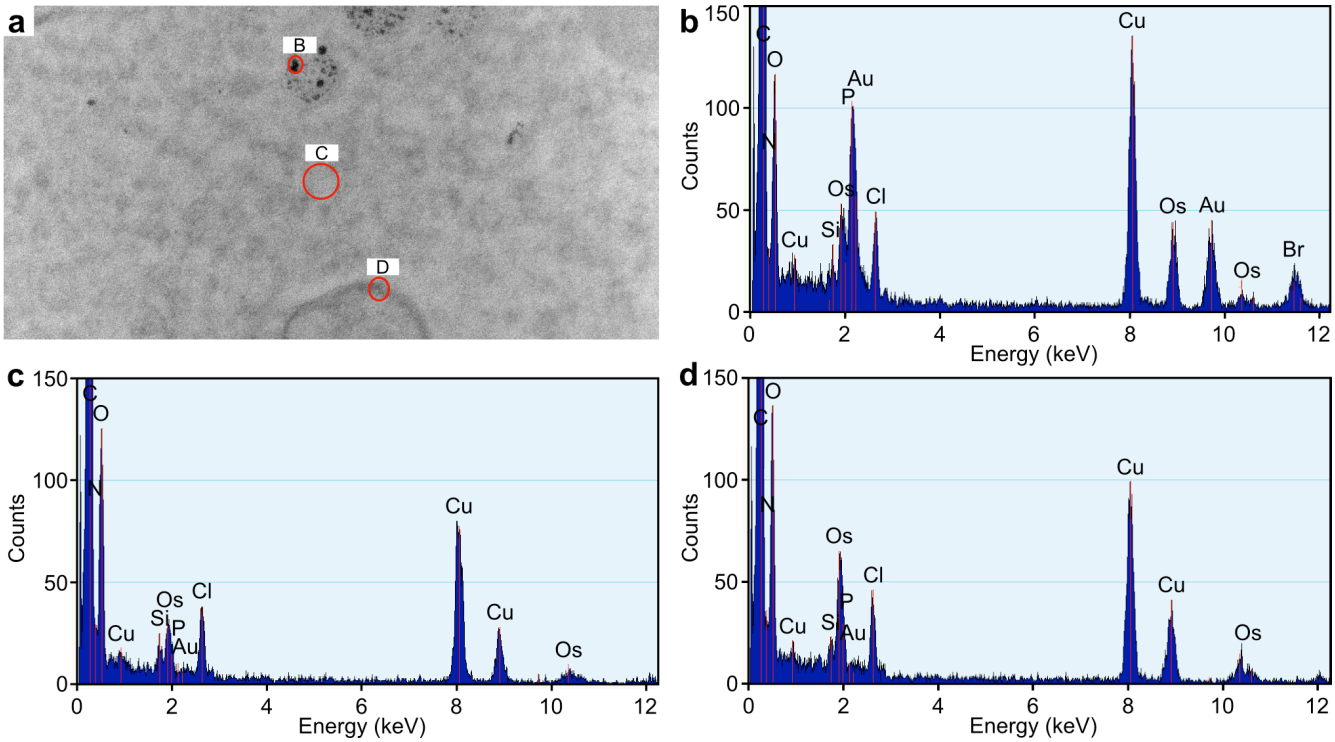

Figure S6. **EDX analysis of B16-F10 tumor tissue of Au-LDL mouse. a.** TEM micrograph of the EDX analysis sites (B-D). **b-d.** EDX graphs of sites b-d confirming the presence of gold (Au) in the dark spheres.

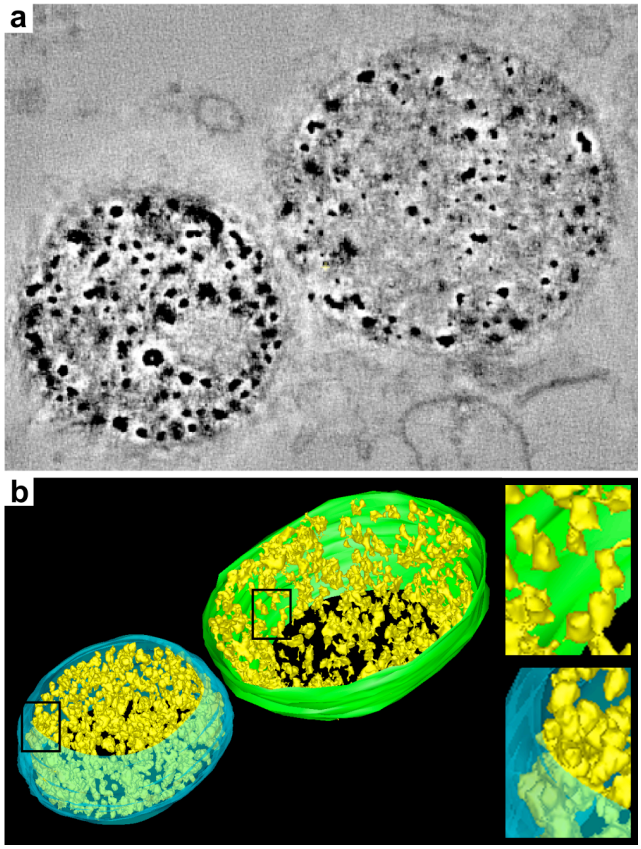

Figure S7. Electron tomography of gold loaded vesicles in tumor tissue. a. TEM image of two vesicles loaded with gold particles. **b.** 3D rendering shows that these vesicles are extensively loaded with gold nanocrystals. Magnifications are shown of both vesicles containing gold.

Materials

Myristoyl hydroxy phosphatidylcholine (MHPC), dimyristoyl phosphoethanolamine (DMPE), distearoylphosphatidylethanolamine polyethylene glycol (DSPE-PEG), and distearoylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids, Inc, Alabaster, AL. The Cy5.5 NHS ester was purchased from GE Healthcare Life Sciences, Piscataway NJ 08854. Antibodies anti CD31, anti CD11b, and anti Gr-1 for FACS were purchased from eBioscience (11-0311-81, 17-0112-81, and 45-5931-80 respectively). Cis-9-octadene-1-thiol and collegenase # C5138-1G were purchased from Sigma Aldrich. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Roswell Park Memorial Institute – 1640 (RPMI-1640) medium and penicillin/streptomycin (p/s) were purchased from Invitrogen, Carlsbad, CA. Hepatoma cells (HepG2), Murine macrophages (J774A.1), and melanoma cells (B16-F10) were purchased from ATCC, Manassas, VA. NCR/NU mice were obtained from NCI Frederick.

Methods

Synthesis

Low density lipoprotein isolation

Low density lipoprotein (LDL) was isolated from human plasma by sequential ultracentrifugation as described previously.[1] Briefly, 800 mL plasma was equally divided between four 250 mL tubes and spun for 30 min at 26,000 *g*. The top white layer containing chylomicrons was discarded, and the remaining divided between twelve 60 mL tubes. 5 mL deionized water (diH_2O) was layered on top of each tube and plasma was spun for 21 h at 137,902 *g*. The top yellowish layer containing very low density lipoproteins was discarded and the remaining plasma collected and stirred well. 35 mg KBr was added, plasma was stirred again, and divided between ten 60 mL tubes. 5 mL 1.25 mg/mL KBr solution was layered on top and tubes were spun for 21 h at 137,902 *g*. The top layer containing LDL was collected, and the density was measured, averaging 1.06 mg/mL.

LDL desalting

The KBr buffer of the LDL, collected after ultracentrifugation, was exchanged for PBS by loading the LDL solution onto a desalting column (HiTrap, GE Healthcare Life Sciences, Piscataway NJ 08854), and flushing with PBS using a syringe pump with a flow rate of 900 µL/min. LDL was collected, the ApoB100 concentration in the LDL solution determined using a modified Lowry protein assay (see 'Characterization' section), and stored at 4 °C.

Cy5.5 conjugation to lipid

Cy5.5 NHS ester was conjugated to DMPE and MHPC as described previously.[2] In short, 5 mg Cy5.5 NHS ester was dissolved in 200 µL dimethyl sulfoxide (DMSO) and 800 µL $CHCl_3$, while 8.4 mg DMPE was dissolved in 350 µL CH_3OH, and 1.4 mL $CHCl_3$. 10 µL triethylamine was mixed with 72 µL $CHCl_3$ and 18 µL DMSO. Subsequently, the dissolved DMPE and Cy5.5 NHS ester, 10 µL triethylamine solution, and 1 mL CH_3OH were mixed. This solution was covered in aluminum foil, to protect the Cy5.5 ester against light, and stirred for at least 8 h at 4 °C. 7 mg MHPC was dissolved in 1 mL 20:1 $CHCl_3$:CH_3OH and dissolved in the reaction solution. The solvents were evaporated to make a lipid film, which was hydrated to form micelles using 4 mL diH_2O, and washed 5 times with PBS using a Vivaspin 10,000 molecular weight cut off (MWCO) tube. The Cy5.5-DMPE in diH_2O was dried under vacuum for three days, redispersed in a 4:1 $CHCl_3$:CH_3OH solvent mixture at 1.24 mg Cy5.5/mL, and stored in the dark at -20 °C.

Characterization

Gold concentration determination of Au-LDL

Samples were scanned using a clinical 256-slice Brilliance iCT scanner (Philips Medical Systems Nederland B.V., The Netherlands). The same scanner was used in all CT experiments. For

gold concentration, samples were scanned with an X-ray tube voltage of 140 kV, a current of 250 mA, slice thickness of 0.67 mm, increment of 0.3 mm, field of view of 200 nm, 768 x 768 matrix and reconstructed using a 'Y-sharp' algorithm. Gold concentrations of samples were determined from these images *via* the following method: attenuations in Hounsfield Units (HU) of sample, PBS background, and air were measured by drawing regions of interest (ROIs), using the scanner's own software, and the Au concentration in the sample was calculated using the following formula:
[Au] (mg/mL) = $((HU_{SAMPLE}*1000/(HU_{PBS}-HU_{AIR}))-HU_{PBS})/29.7$, which had been previously determined from a standard curve. CT attenuation performance of Au-LDL was determined from a phantom in a triplicate dilution sequence containing the following dilutions from the stock concentration (35.74 mg Au/mL): 1, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1. These vials were scanned in water 21 cm in height and 24 cm in width to mimic conditions *in vivo* and their attenuations were measured as described above. The samples were scanned with an X-ray tube voltage of 120 kV, a current of 300 mA, slice thickness of 0.9 mm, increment of 0.45 mm, field of view of 250 mm, 512 x 512 matrix and reconstructed using a 'smooth' algorithm.

Protein concentration determination of LDL and Au-LDL

The ApoB100 concentration in Au-LDL was determined using a modified Lowry[3] protein assay kit (Thermo Scientific, Waltham, MA). A protein standard was made from 2 mg bovine serum albumin (BSA) stock in the following concentrations: 0, 0.001, 0.005, 0.025, 0.125, 0.25, 0.5, 0.75, 1, and 1.5 mg/mL. 40 µL of the standard as well as of the sample diluted 1:20 and 1:40 (n=3 in each case), were pipetted into a clear flat bottom 96-wells plate. 200 µL Lowry reagent was added and incubated in the dark for 10 min. Subsequently, 20 µL Folin-Ciocalteu (FC) reagent was added and incubated in the dark for 30 min. Absorbance was measured at 750 nm using a plate reader. The intrinsic Au absorbance, which interferes with the protein absorbance, was measured by adding 220 µL PBS (the combined volume of the Lowry and FC reagents) and reading the absorbance. The total concentration

was calculated by subtracting the Au absorbance from the total sample absorbance and comparing to the standard curve.

Gel electrophoresis of LDL and Au-LDL

Protein content of LDL and Au-LDL was compared and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained overnight with Coomassie Blue staining solution (0.025% Coomassie Brilliant Blue R250, 40% CH_3OH, 7% acetic acid). The next day, samples were destained in two steps for 30 min using 40% CH_3OH/7% acetic acid and 1 h using 7% acetic acid/5% CH_3OH.

Western blot of LDL and Au-LDL

Western blotting was performed to isolate and compare the ApoB100 content of LDL and Au-LDL. Sample buffer (10% w/v SDS, 10 mM dithiothreitol, 20% v/v glycerol, 0.2 M Tris (pH 6.8), and 0.05% bromophenol blue), 1 L running buffer (25 mM, 200 mM glycine, and 0.1% w/v SDS), 7% acrylamide running gel solution (15.3 mL diH_2O, 7.5 mL 1.5 M Tris-HCl (pH 8.8), 150 µL 20% w/v SDS, 6.9 mL 30%/1% w/v acrylamide/bis-acrylamide, 150 µL 10% w/v ammonium persulfate, and 20 µL tetramethylethylenediaminde (TEMED)) and a 4% acrylamide stacking gel solution (3.075 mL diH_2O, 1.25 mL 0.5 M Tris-HCl (pH 6.8), 25 µL 20% w/v SDS, 670 µL 30%/1% w/v acrylamide/bis-acrylamide, 25 µL 10% w/v ammonium persulfate, and 5 µL TEMED) were prepared.

LDL and Au-LDL samples were diluted in sample buffer and the samples were incubated in a 37 °C water bath for 30 min to denature the proteins. 20 µL of the sample (containing 10 ng LDL or Au-LDL by protein content) or molecular marker (High Weight Molecular Marker kit, GE Lifesciences, Piscataway, NJ) were added in triplicates to each well of the gel. The gel was run for 2 h at 60 V.

After SDS-PAGE, the samples were tank electroblotted. The tank transfer to a nitrocellulose membrane (Amersham Hybond ECL, GE Lifesciences, Piscataway, NJ) was run at 400 mA for 2 h.

The effectiveness of the transfer was confirmed using Ponceau S staining solution (Thermo Scientific, Waltham, MA). Proteins were blocked by incubating the membrane for 1 h at room temperature (RT) with 10% non-fat dry milk in Tris-buffered saline with Tween 20 (1X TBS-T). Subsequently, the membrane was washed five times 5 min with 1X TBS-T at RT, and the membrane was probed using a 1:1000 dilution of anti-apolipoprotein B primary antibody (ab7616, Abcam Inc., Cambridge, MA) in 5% non-fat dry milk at 4 °C for 12 h. The membrane was washed again five times 5 min with 1X TBS-T, and incubated with 1:12,000 dilution of peroxidase anti-goat IgG secondary antibody (Vector Laboratories Inc., Burlingame, CA) in 5% milk at RT for 1 h. The membrane was developed using Amersham Hyperfilm ECL radiography film and the ECL technique as described by the Amersham ECL Prime Detection Kit (GE Lifesciences, Piscataway, NJ).

Phosphate concentration determination of LDL and Au-LDL

Phosphorus content of LDL and Au-LDL was measured using Rouser's phosphorus assay.[4] A phosphate standard was made of 0 – 160 µL from a 500 mM standard phosphate solution. Samples should have a phosphate concentration between 10 and 80 nmol. PBS (control) LDL and Au-LDL samples were tested in 1:100, 1:100, and 1:10 dilutions respectively in triplicates in glass tubes. The tubes were heated in a 155 °C block heater for approximately 45 min until the samples were dry. Samples were taken from the block heater, 300 µL perchloric acid was added to each tube and a glass marble was placed on top of the tube. Samples were placed back in the block heater for approximately 1 h, until the solutions were clear. The tubes were then cooled, 1 mL diH_2O, 1 mL 1.25% hexa-ammoniummolybdate solution, and 0.4 mL freshly made 5% ascorbic acid were added. Subsequently, marbles were replaced, the tubes vortexed and placed in boiling water for 5 min. The tubes were cooled in an ice bath to RT. Absorbance was determined at 797 nm cuvettes and the phosphate concentration deduced from the standard curve.

Negative stain transmission electron microscopy

Samples and grids were prepared *via* the method described by Forte and Nordhausen.[5] In detail, the buffer of 5 µL of concentrated Au-LDL was replaced by transmission electron microscopy (TEM) buffer (0.125 M $CH_3CO_2NH_4$ 2.6 mM $(NH_4)_2CO_3$ 0.26 mM tetrasodium EDTA at pH 7.4) by washing it twice using a Vivaspin 10,000 MWCO tube. Samples were diluted with TEM buffer until it was slightly pink, 7.5 µL 2 % phosphotungstic acid was mixed with 7.5 µL sample and a drop of 10 µL was placed on a 100 mesh Formvar coated nickel grid (Electron Microscopy Sciences) for 30 sec. Excess fluid was removed using filter paper and the grids were left to dry. Grids were examined using a Hitachi 7650 TEM coupled to a Scientific Instruments and Applications (SIA) digital camera controlled by Maxim CCD software at 80 kV and at 50,000 x magnification. Success of labeling could be observed and the diameters of the LDL nanoparticles were measured using the software's scale bar.

Oxidation determination of LDL and Au-LDL

Oxidation levels of LDL and Au-LDL samples were measured to determine whether the sonication and gradient centrifugation procedures oxidize the Au-LDL nanoparticles. An enzyme-linked immunosorbent assay (ELISA) was used. LDL and Au-LDL samples were diluted 600 times as described by the OxiSelect Human Oxidized LDL ELISA kit (Cell Biolabs Inc., San Diego, CA). Oxidation levels were calculated using the kit's standard curve and oxidized content of LDL and Au-LDL were analyzed in relation to their ApoB100 protein content.

Fluorescence imaging

To confirm fluorophore inclusion and retained fluorescence activity, samples of DiR-LDL and Cy5.5-Au-LDL were imaged with a fluorescence imaging system (IVIS, Xenogen, Alameda, CA). In the

case of DiR-LDL 745 nm excitation and 820 nm emission was used. For Cy5.5-Au-LDL, 675 nm excitation and 720 nm emission was used.

In vitro experiments

Cell cultures

In vitro incubations were performed in three different cell types; HepG2, J774A.1, and B16-F10. All cells were cultured in DMEM, substituted with 10% FBS and 1% p/s in culture flasks. For passaging, HepG2 and B16-F10 cells were treated with trypsin (10 and 2 min respectively), and J774A.1 cells were scraped. HepG2 and J774A.1 were typically passaged in a 1:6 ratio, whereas the fast growing B16-F10 in a 1:10 or 1:20 ratio.

Western blot of cells

To determine LDLr upregulation in medium with 1% BSA instead of 10% FBS, the same Western blot procedure was used as described above in the 'Characterization' section. Briefly, cell lysates of B16-F10 and J774A.1 cells incubated in DMEM medium containing either 10% FBS (no upregulation) or 1% BSA (upregulation) were obtained. For LDLr detection, as primary antibody rabbit polyclonal anti-human LDLr (Fitzgerald, Cat. No 20R-LR002) in a 1:100 dilution and as secondary antibody ECL anti-rabbit IgG horseradish peroxidase linked whole antibody from sheep (GE Healthcare) were used. For β-actin detection as primary antibody mouse monoclonal anti-β-actin (Sigma-Aldrich, Cat. No A2228) in a 1:15,000 dilution and as secondary antibody ECL anti-mouse IgG horseradish peroxidase linked whole antibody from sheep (GE Healthcare) were used.

Competition inhibition assay for transmission electron microscopy

Competition inhibition assays were based on a previous described method.[6] Briefly, cells were

cultured as described above, and 80% confluent cells were passaged into 6-wells plates in a 1:1 dilution. After at least 12 h to allow the cells to adhere and normalize, the media was changed to DMEM with 1% BSA, p/s, but no FBS, to upregulate the LDL receptors. 20 h later, the cells were washed and incubated with 1 mL incubation medium with the following conditions: 1) control – containing native LDL 2) competition inhibition – containing Au-LDL with a five fold excess of LDL 3) test – containing Au-LDL. For TEM, LDL with a concentration of 100 µg ApoB100 /mL was used, for fluorescence 20 µg ApoB100/mL and for CT 500 µg ApoB100/mL. Incubation times differed for CT (24 h) and TEM and fluorescence (3 h). Subsequently, the incubation was stopped by removing the medium containing the nanoparticles, and washing twice with PBS. Cells were detached from the bottom of the well using either trypsin or the scraping method (depending on the cell type), and spun down in a 1.5 mL centrifuge tube for 5 min at 1,000 rpm in an Eppendorf Minispin Plus. Trypsin was carefully removed, and the pellet was resuspended in 100 µL 2.5% glutaraldehyde for fixation, and left to settle. Cell pellets were processed with the standard procedure with osmium tetraoxide, embedded in epoxy resin blocks, cut to 60 nm sections with a microtome and grids were post stained with 4% uranyl acetate and lead citrate. The same electron microscope and imaging conditions were used as described in the 'Characterization' section.

Competition inhibition assay for fluorescence microscopy

Au-LDL competition inhibition assays for fluorescence microscopy were performed using a similar method as for TEM, however, cells were grown on a glass cover slip in the 6-wells plate. After washing with PBS, and fixation in 4% paraformaldehyde, the cover slips were mounted on a glass slide with a drop of mounting medium containing DAPI, sealed with transparent nail polish, and kept in the dark. Fluorescence microscopy was performed on a Leica DM6000 microscope controlled by InVivo software (Media Cybernetics, Inc.) and using a Leica DFC350 FX camera. DAPI and Rhodamine filters were used.

CT of cell pellets

The same CT scanner was used as described in the 'Characterization' section. Cell pellets were scanned with an X-ray tube voltage of 140 kV, a current of 250 mA, slice thickness of 0.67 mm, increment of 0.3 mm, field of view of 200 mm, 768 x 768 matrix and reconstructed using a 'smooth' algorithm. Attenuations of the cell pellets prepared for TEM were measured using OsiriX v.3.7.1 64-bit software (Geneva, Switzerland; www.osirix-viewer.com).

In vivo assessments

Mouse model

All experiments were approved by the Institutional Animal Care and Use Committee. *In vivo* studies were performed in ~7 weeks old female NCR/NU mice (NCI Frederick). B16-F10 melanoma cells were used for *in vivo* studies of Au-LDL due to their high levels of LDLr expression and fast growth.[7-9] 100 µL of DMEM containing ~ 1 million B16-F10 cells were injected in the right flank of the mice. Tumor growth was monitored and when the tumors reached the desired size of 4 mm^3 nanoparticles were injected. For the experiments with DiR-LDL, an alternative LDLr-over expressing tumor cell line, Lewis lung carcinoma, was used, with the same protocol, except 2 million cells were injected per mouse.

Nanoparticle injections

Mice were divided into four groups by size, with Group A having the smallest tumors and Group D having the largest tumors. These mice were randomly divided in three groups, where each group was composed of one mouse each of Groups A-D. The following treatments were given to the groups: 1) control – no injections (n=4), 2) Au-LDL injection (n=4), 3) Au-NE injection (n=3). The

nanoparticles were each injected intravenous in the tail at a 250 mg Au/kg doses and the nanoparticles were allowed to circulate for 24 h before imaging and sacrifice.

IVIS of mice

Fluorescence in the mice was measured using a Xenogen IVIS Spectrum (Alameda, CA). Mice were anesthetized using isoflurane, with a 4% induction dose. Then, mice were positioned in the IVIS with isoflurane administered at 1.5% *via* a nose cone. Tissue was excited at 745 nm and the emission spectra recorded from 820 nm.

CT imaging of mice

The same CT scanner was used as described in the 'Characterization' section. Mice were anesthetized by a 100 mg/kg ketamine/10 mg/kg xylazine injection in the left flank. All mice and a control 50 mL Falcon tube containing PBS were scanned in a single acquisition using an X-ray tube voltage of 120 kV, a current of 100 mA, slice thickness of 0.8 mm, increment of 0.4 mm, field of view of 350 mm, 1024x1024 matrix and reconstructed using a 'smooth' algorithm. Data were analyzed using OsiriX v.3.7.1 64-bit software (Geneva, Switzerland; www.osirix-viewer.com).

Statistical analysis of attenuations

A two tailed t-test was calculated of Au-LDL and Au-NE mice versus LDL (control) mice using Python's Scipy (www.scipy.org).

Animal sacrifice and tissue collection

Two mice each from the Au-LDL, Au-NE and control groups were anesthetized with isoflurane and were sacrificed using perfusion, during which at least 30 mL PBS was perfused through the heart. Tumors were removed and half of the tissue was collected in RPMI-1640 medium for fluorescence

activated cell sorting (FACS) analysis and a quarter was trimmed and placed in 2.5% glutaraldehyde for TEM preparation. The remaining two Au-LDL, two control, and one Au-NE mice were sacrificed with CO_2, snap-frozen with liquid N_2 and were subsequently scanned with spectral CT.

FACS of tumor tissue

Tumor tissue was cut in small pieces and placed in 1 mL fresh cold RPMI-1640 medium in a 15 mL Falcon tube. Collagenase (Sigma-Aldrich, Cat# C5138-1G) was added to the tube to reach a final concentration of 10 U/mL and the tissue was incubated for 45 min at 37 °C. Released cells were harvested and passed through a 70 µm cell strainer (Fischer Scientific) to remove excessive extra cellular matrix. Cells were spun at 400 *g* for 5 min and resuspended in 2 mL PBS+BSA (0.1% w/v). Cells were transferred to a 96-wells round bottom plate (100 µL/well). To each well 0.2 µL FC blocker was added and the plate was incubated on ice for 15 min. Antibody cocktails (anti CD31 11-0311-81, anti CD11b 17-0112-81, and anti Gr-1 45-5931-80) were prepared and incubated with cells for an additional 30 min on ice. Cells were further stained with DAPI and subjected to flow cytometric analysis (LSRII, BD Biosciences). Endothelial cells were identified as CD31+CD11b- Gr-1- and macrophages as CD11b+ Gr-1+. Tumor cells were identified by size and SSC anti CD31.

TEM and energy-dispersive X-ray spectroscopy of tumor tissue

Tumor tissue was fixed and post stained as described above. Sections were examined in the Hitachi 7650 TEM, and gold existence was shown using the unstained sections. Energy-dispersive X-ray spectroscopy (EDX) was performed on the same samples using a Tecnai 20 FEG.

Electron tomography of Au-LDL tumor tissue

A detailed description of the 3D electron tomography procedures is given in Geerts *et al.*[10] In short, thick sections (250–300 nm) on grids were placed in a Fischione Model 2040 rotation holder

(Fischione Instruments Inc., Export, PA). Double tilt series were acquired in 1 degree intervals from -60 to +60 degrees on various regions of interest using a Tecnai-20 microscope (FEI Company, Eindhoven, The Netherlands) and Xplore3D software (FEI Company, Eindhoven, The Netherlands). The thus acquired tilt series were aligned and tomograms were reconstructed using the IMOD software package.[11] Orthogonal single-axes tomograms were subsequently merged into one tomogram. Tomogram processing was then performed using the aforementioned IMOD software.

Spectral CT

The spectral CT scanner used in this study has been described in detail elsewhere.[12] In brief, the scanner's single slice photon counting detector (Gamma Medica-Ideas, Northridge, Ca., USA) comprises 3 mm thick CdTe direct conversion material. A KEVEX PXS10-65 W micro-focus tube (Thermo Scientific, Scotts Valley, CA), serves as the X-ray source. The x-ray tube exit window is made of beryllium. A 2 mm thick aluminum filter was used during scanning. Spectral CT scanning was performed at 50 μA and 130 kV using six energy bins (*i.e.* 25-33, 33-50, 50-81, 81-91, 91-110 and 110-130 keV). Mice were imaged using a magnification of 6, resulting in 100 μm in-plane resolution. Slice thickness was also 100 μm. The photon-counting raw data from the six energy bins were pre-processed to correct for detector imperfections (as explained in Schlomka *et al.* 2008[12]). This data was subsequently decomposed into material equivalents using a maximum likelihood approach (as per the method given in Roessl and Proksa 2007[13]). The photo-Compton decomposition basis of Alvarez and Macovski[14] was extended by the contrast material, *i.e.* gold.

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