Supporting Information for: 'Nanocrystal core high-density lipoproteins: A multimodal contrast agent platform'

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Methods

Materials

Myristoyl hydroxy phosphatidylcholine (MHPC), gadolinium dimyristoyl phosphoethanolamine diethylenetriamine pentaacetic acid (Gd-DTPA-DMPE), distearoyl phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ammonium salt (PEG-DSPE) and dimyristoyl phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (Rhod-DMPE) were all purchased from Avanti Polar Lipids and used as received. Cell culture supplies were purchased from Invitrogen (Carlsbad, CA).

Synthesis of nanocrystal core HDL

Dodecanethiol coated gold nanoparticles were synthesized by the method of Brust.[1] Quantum dots were synthesized as described by Koole et al.[2] Oleic acid coated iron oxide was purchased from NN Labs (Fayetteville, AR). In an example experiment, 50 mg of MHPC, 50 mg of Gd-DTPA-DMPE and 1 mg of Rhod-DMPE were co-dissolved in 5 ml of a 20:1 chloroform:methanol solvent mixture. This solution was added to 5 ml of chloroform in which 100 mg of dodecanethiol coated gold nanoparticles was dissolved. The resulting lipid-nanoparticle solution was added dropwise to 30 ml of deionized water that was heated to 80 °C, under vigorous stirring. This solution was centrifuged briefly and the sediment (large aggregates or unsuspended nanoparticles) discarded. 3.3 ml of 10x PBS was added to the supernatant to change the buffer to 1X PBS. 40 mg of apoA-I was added and the solution left to incubate at room temperature overnight. This synthesis resulted in a mixture of nanocrystal core HDL and empty-core HDL. These were separated using ultracentrifugation in 30% KBr solution by Havel's lipoprotein separation method.[3] The buffer of nanocrystal core HDL was changed to 1X PBS and the solution concentrated to around 10 mM in Gd or Fe.

Cell incubations

J774A.1 murine macrophage cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin. After the fifth splitting, the cells were put into six wells plates with 2 ml of media in each well. The cells were allowed to grow to 70-80% confluency and the media replaced with fresh, before nanocrystal HDL was added into a well along with relevant control contrast agents at the same concentration into other wells. In the case of Au-HDL, Au-PEG and

Omnipaque, the agents were added so that there was 0.5 mg/ml of Au or I per well, For the QD-HDL and QD-PEG, the agents were added so that there was 0.02 mM Gd in each well. In the case of the iron contrast agents, a concentration of 4 μg Fe/ml was used. No contrast agent was added to the last well. Six such plates were made and one each was allowed to incubate for ½, 1, 2, 4, 7 and 12 hours. The media in the wells was removed and the cells were washed 3 times with PBS. The cells were collected by scraping, subsequently centrifuged to form pellets, dispersed in 4% paraformaldehyde and finally allowed to loosely settle.

CT imaging

CT imaging of phantoms and cell pellets was performed on a Siemens Somatom Emotion 6 at 110 keV and 25 mA. The slice thickness was 1 mm, the field of view 50x50 mm, the matrix size 512x512 and the data was reconstructed using a B30f kernel. Images were analyzed using Osirix v.3.0.1 32-bit (Geneva, Switzerland; www.osirix-viewer.com). Imaging of excised aortas was performed using a microCAT II (ImTek Inc) scanner operating at 60 keV and 0.8 mA. The images were acquired with a 92 x 92 x 92 μm voxel size. The data was converted to DICOM format using (X)MedCon (freeware, xmedcon.sourceforge.net) and analyzed using Osirix.

Fluorescence imaging

Cell pellets incubated with QD-HDL and dilutions of the agents used in this study were imaged using an IVIS Imaging System 200 (Xenogen, Alameda, CA). A continuous external light source of 365 nm and a 610-630 nm emission filter were used. Aortas of

apoE KO mice, injected 24 hours previously with the contrast agents reported in this study, along with the aortas of untreated mice, were imaged using a Maestro^{TM} 500 FL In Vivo Imaging System (Cambridge Research & Instrumentation, Inc, Woburn, MA). A modified DsRed 2 filter set was used where the tissue was excited at 503-555 nm and the spectra recorded from 580 to 800 nm in 10 nm intervals while using a 580 nm longpass emission filter. The data thus recorded were spectrally unmixed using the supplied software by placing ROIs on the autofluorescence on the control and the bright areas on the nanocrystal-HDL treated aortas.

Statistics

Statistical comparisons were carried out using student's T-test, 2-tailed, unequal variance. Differences were considered significant when $p<0.05$.

MR imaging

Our MR system for imaging has recently been extensively described elsewhere.[4] In short, cell pellets and animals that were anesthetized and maintained using isoflurane were scanned using a 9.4 T MRI system supplied by Bruker Instruments. Cell pellets incubated with Au-HDL, Au-PEG, QD-HDL, Qd-PEG or media only were imaged using a spin-echo sequence where the echo time was 10.5 ms, the repetition time 1000 ms, the field of view 3 x 3 cm, the matrix size 192 x 192 and the no. of averages 20. The slice thickness was 0.5 mm and the total scan time 48 minutes. Cell pellets incubated with FeO-HDL, FeO-PEG or media only were imaged using a gradient echo sequence for which the echo time was 50 ms, the repetition time 2000 ms, the field of view 2 x 2 cm,

the matrix size 128 x 128, the slice thickness 1 mm and the number of averages four to yield a total scan time of 13 minutes.

Mice were scanned using the following protocol. The abdominal aorta was identified in a coronal section on a localizing sequence. T1-weighted MRI was performed using a black blood sequence. Twenty-two contiguous 500 μm-thick slices with a microscale in-plane resolution of 101 μm were acquired using a spin echo sequence with a 256 × 256 matrix size. The repetition time (TR) and echo time (TE) for the T1W images were 800 and 8.6 ms, respectively. An inflow saturation band of 3 mm was used with a slice gap of 3 mm for additional luminal flow suppression. Sixteen signal averages were used for a total imaging time of 55 min per scan. A saturation pulse was used to eliminate signal from fat tissue and better delineate boundary of the aortic wall and minimize chemical shift artifacts. After a preinjection, baseline MRI scan, mice were injected, via a tail-vein catheter with a 50 μmol Gd/kg dose of the Au-HDL, Au-PEG, QD-PEG or the QD-HDL. The mice were scanned again at 24 hours post-injection. n=3 apoE-KO mice were injected with each agent. Use of the medical image analysis software package eFilm allowed the MR signal intensity of various tissues from the different time points to be ascertained and the percent change in normalized enhancement ratio (% NER) post-injection to be calculated. Normalization of the MR signal intensity in the aorta wall was performed by rebasing with the signal from a standard that was scanned simultaneously.

Before T2*-weighted imaging was carried out, a short T1 weighted scan (using the same parameters as above but with 4 averages, a slice thickness of 1 mm and 11 slices) was performed on the abdomen of the mouse to identify areas of plaque. Four of

the slices with good plaque were imaged using a T2*-weighted sequence where the TE=4 ms, the TR=10 ms, the slice thickness 1 mm, no. averages=128, the FOV was 1.5 x 1.5 mm, the matrix size 200 x 200, for a total scan time of 6.5 minutes per scan. After such a scanning regime, the mice were injected with a 30 mg/kg dose of the FeO-HDL or FeO-PEG via the tail vein. 24 hrs later the scanning regime was repeated.

Transmission Electron Microscopy

The instrument used was a Hitachi H7650 instrument linked to a SIA (Scientific Instruments and Applications) digital camera controlled by Maxim CCD software. TEM was performed on nanocrystal HDL samples suspended in an ammonium acetate buffer using a 2 % sodium phosphotungstate (pH=7) negative stain as described by Forte and Nordhausen.[5] Cells incubated with nanocrystal HDL were prepared for TEM by fixation in glutaraldehyde, followed by osmium tetraoxide and stained post-sectioning with 4% uranyl acetate and Reynold's lead citrate.[6]

Gel Electrophoresis

The size range of the HDL-like contrast agents were measured by non-denaturing gel electrophoresis (4-20% polyacrylamide, Bio-Rad, Hercules, CA) in 90 mM Tris, 80 mM boric acid, 3 mM sodium azide, 3 mM EDTA buffer, pH 8.3; 80 V for 6 h. Gels were pre-run at 80V for 30 minutes prior to loading samples. Samples were mixed 1:1 (v/v) with a sample buffer containing 20% sucrose and 0.25% bromophenol blue. Prior to staining, the gel was fixed using a glutaraldehyde solution (0.2% glutaraldehyde, 30%

ethanol, 0.2 M sodium acetate) for 30 minutes. Protein bands were visualized by Commassie Blue R-250 staining using standard methods, and particle size (nanometers) was determined by comparison to the migration of the protein standards in a High Molecular Weight Calibration Kit (Amersham Biosciences).

Relaxometry

T1 measurements of solutions were performed on a 60 MHz Bruker Minispec (Bruker Medical BmbH, Ettingen) operating at 40 °C. In order to find the longitudinal and transverse relaxivities of the particles 10, 20, 30, 40 and 50 μl of sample were diluted to 250 ml using 1x PBS and the T1 and T2 was measured for each solution. The longitudinal (r1) and transverse (r2) relaxivities of the samples was then calculated from the slope of the graph of 1/T1 and 1/T2 plotted against the gadolinium concentration.

Particle composition analysis

Gadolinium, gold, iron and cadmium contents were determined by inductively coupled plasma mass spectrometry (Cantest Ltd, Burnaby, Canada). Phosphorous analysis was carried out by the method of Rouser.[7] Protein analysis was performed using the Markwell method.[8]

Calculation of the number of apoA-I molecules per particle

From the TEM derived diameter of the nanocrystal cores and knowledge of the core composition, the average mass of a core was found. Using the relevant metal concentration, the number of particles per ml of solution was found. From the protein

analysis, the number of apoA-I molecules per ml solution was found and comparison of the two values allowed calculation of the number of apoA-I molecules per particle to be calculated.

Confocal microscopy

Imaging was performed on a Zeiss LSM 510 META microscope (Carl Zeiss, Oberkochen, Germany) in an inverted configuration. Objectives used were Plan-Apochromat 20x, Plan-Neofluar 40x (1.3 oil DIC) and Plan-Apochromat 63x (1.4 oil DIC) lenses. Data were captured and analyzed using Zeiss LSM 510 Meta and Image Browser software (Zeiss). Macrophage cells for confocal microscopy were grown on coverslips placed at the bottom of six wells plates. After incubation for 2 hours, the coverslips were washed with PBS and mounted on slides using a media containing DAPI and sealed. Aortas were removed from apoE KO mice at 24 hr post-injection with each type of nanocrystal HDL (i.e. Au-HDL, FeOHDL and QD-HDL). Sections of the aortas were stained with Alexa 647-CD68, a macrophage specific antibody conjugated to a fluorophore, in the case of Au-HDL and FeO-HDL. In the case of QD-HDL aorta sections, macrophage staining was performed using a rat anti-mouse CD 68 primary antibody and Alexa 488 rabbit anti-rat secondary antibody. Lastly, the sections were mounted in DAPI (nuclei specific) containing media. In all cases the signal from the nanocrystal HDL is displayed as red, the signal from the macrophages was displayed as green and DAPI as blue.

Additional Images

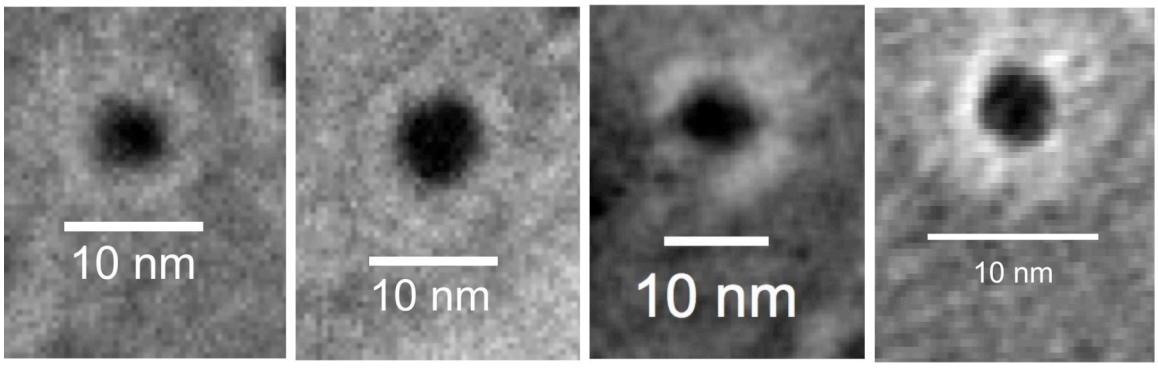

Figure S1 Negative stain TEM images of, from left, Au-HDL, Au-PEG, QD-HDL and QD-PEG.

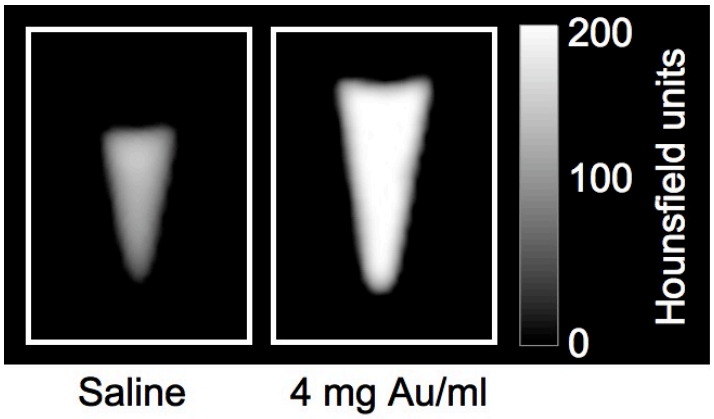

Figure S2 Computed tomography images of saline and 4 mg/ml Au-HDL (as also depicted in Figure 1H in the manuscript) presented with a different window, i.e. 0 to 200 HU. Significant contrast between saline and this low gold concentration can be observed in the 0 to 200 HU window.

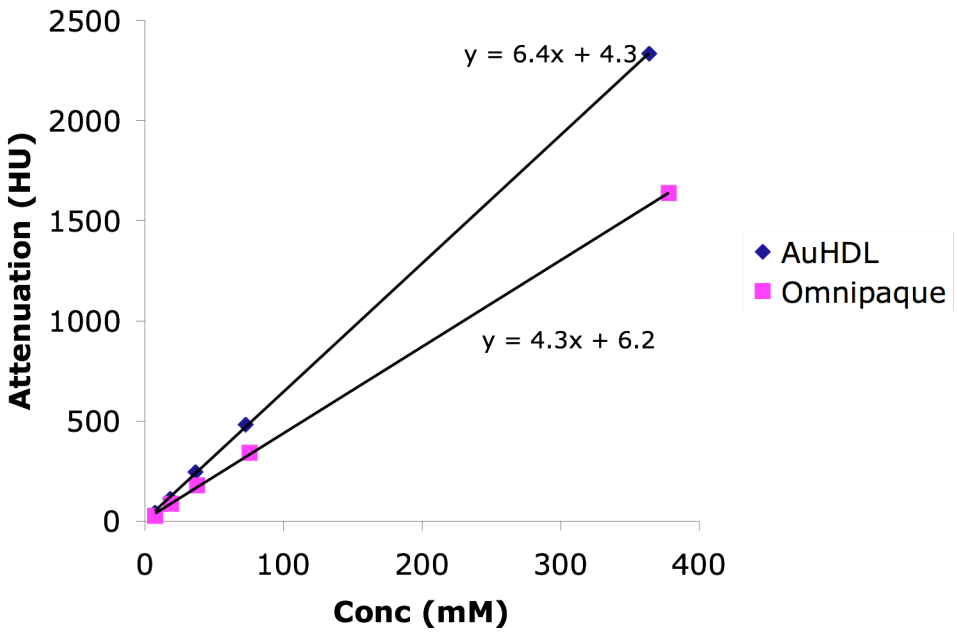

Figure S3 Graph of CT attenuation vs Concentration for Au-HDL and Omnipaque. The gradient of the lines of best fit through the data reveal the attenuation per mM.

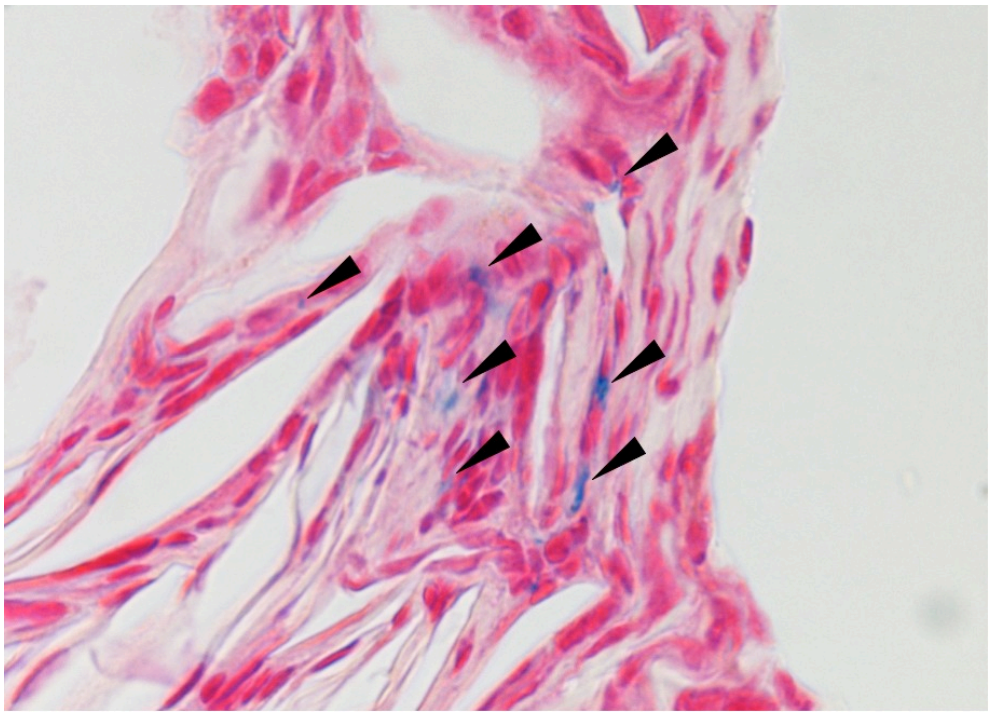

Figure S4 Perl's staining of aortic sections taken from mice injected with FeO-HDL

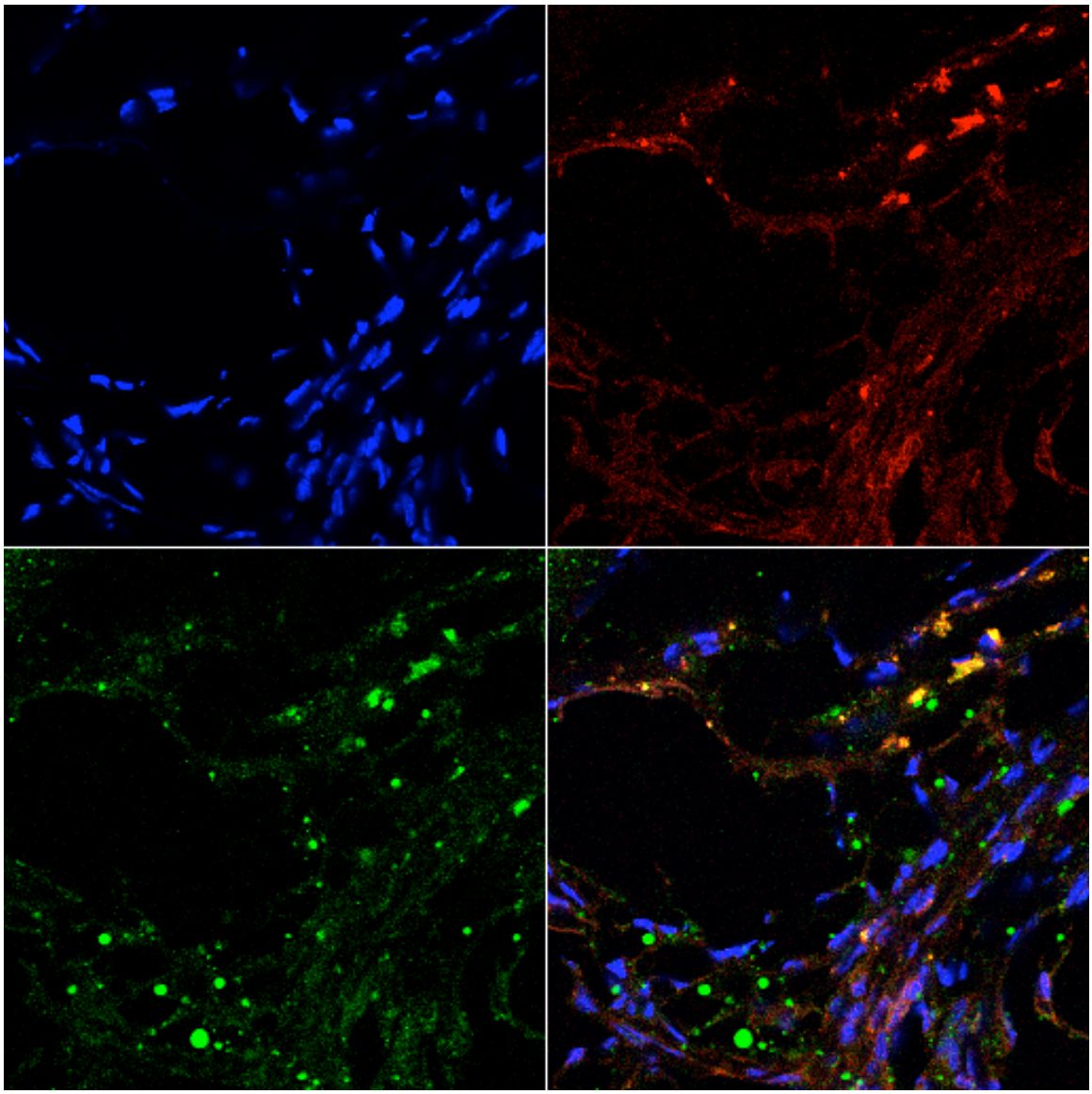

Figure S5 Confocal microscopy image of and aortic section taken from a mouse injected with Au-HDL. Clockwise from bottom left: CD68 macrophage staining, DAPI staining for nuclei, rhodamine from Au-HDL and a merged image.

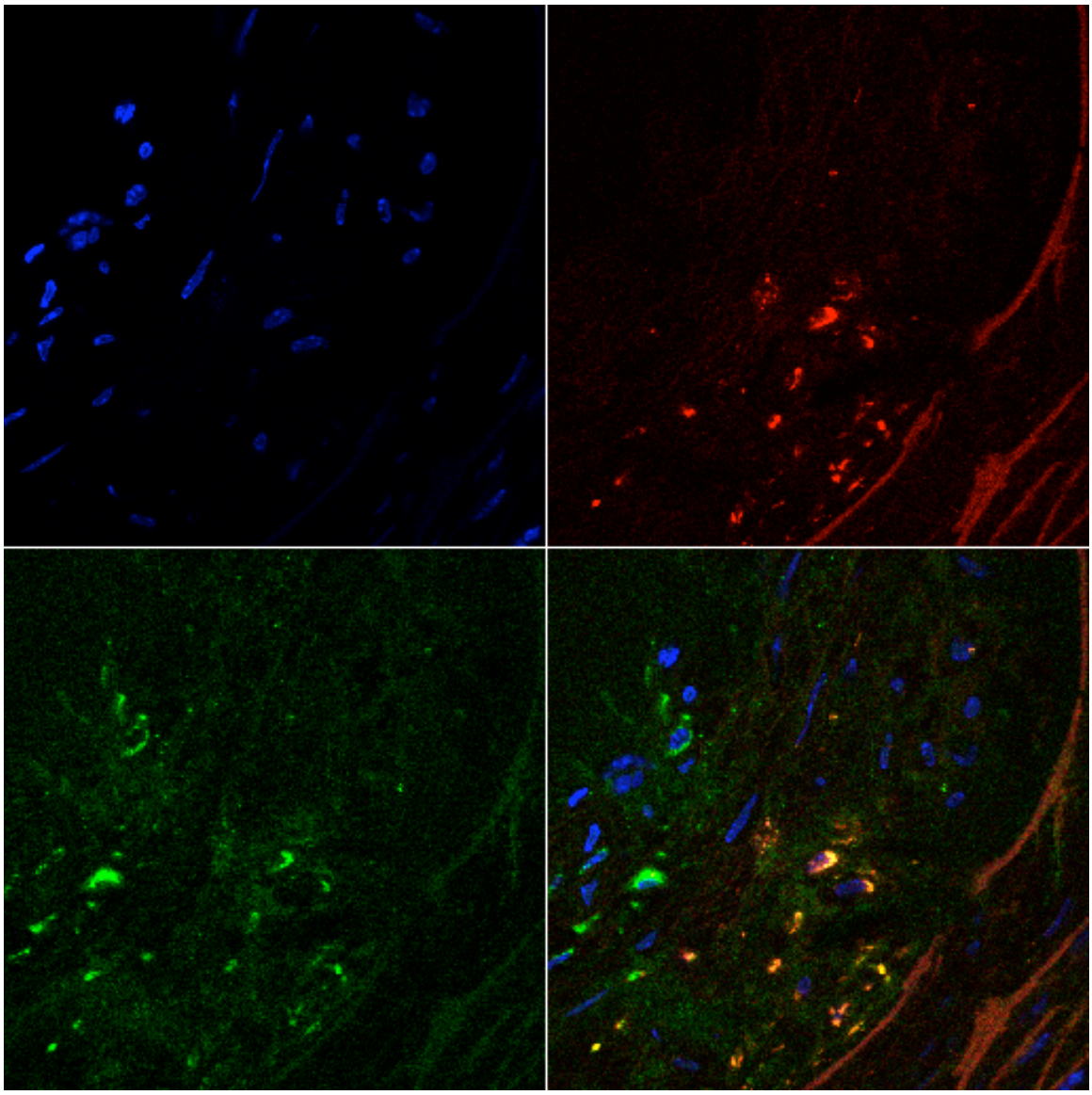

Figure S6 Confocal microscopy image of and aortic section taken from a mouse injected with Au-HDL. Clockwise from bottom left: CD68 macrophage staining, DAPI staining for nuclei, rhodamine from FeO-HDL and a merged image.

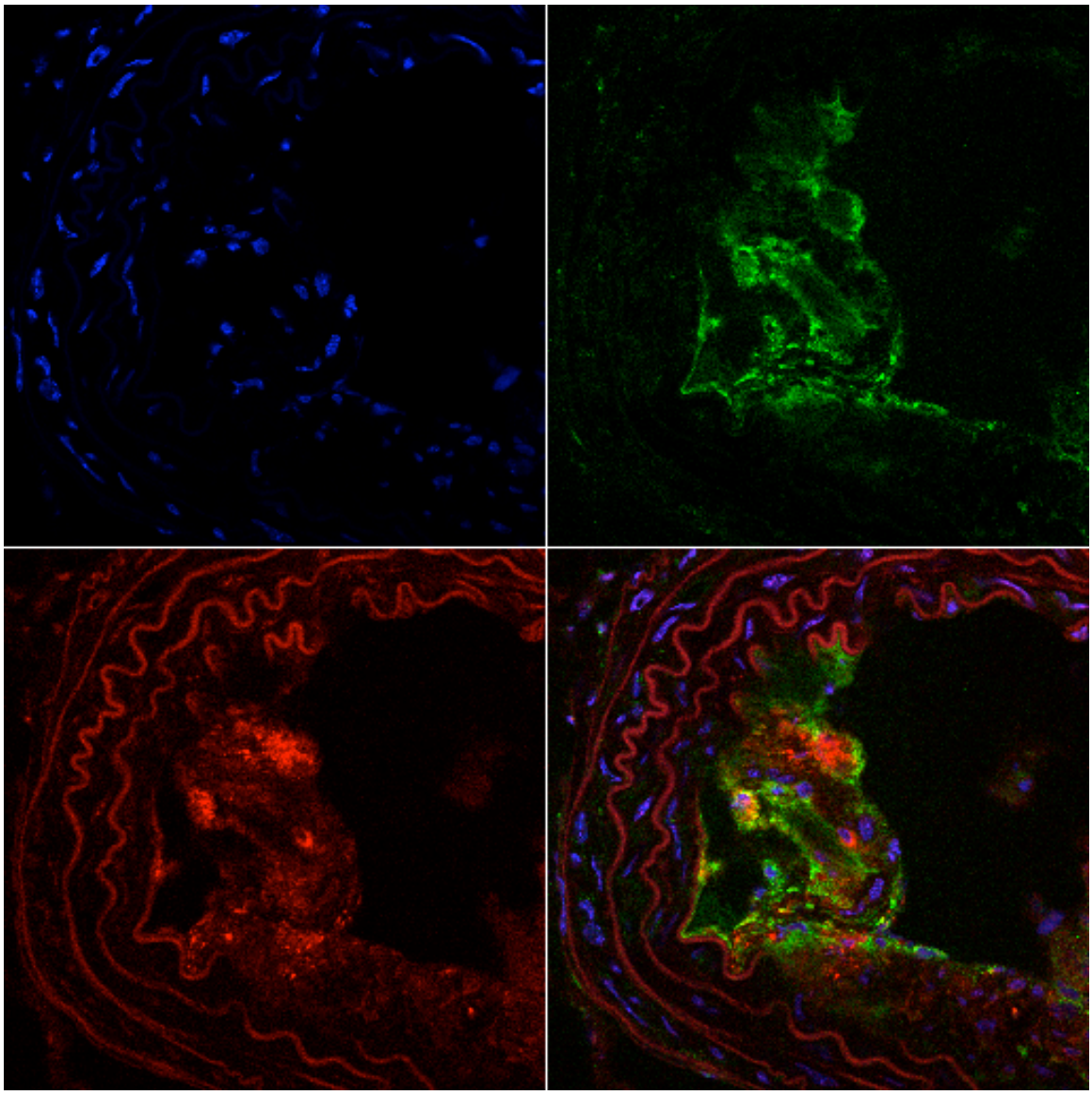

Figure S7 Confocal microscopy image of and aortic section taken from a mouse injected with Au-HDL. Clockwise from bottom left: CD68 macrophage staining, DAPI staining for nuclei, signal from QD-HDL and a merged image.

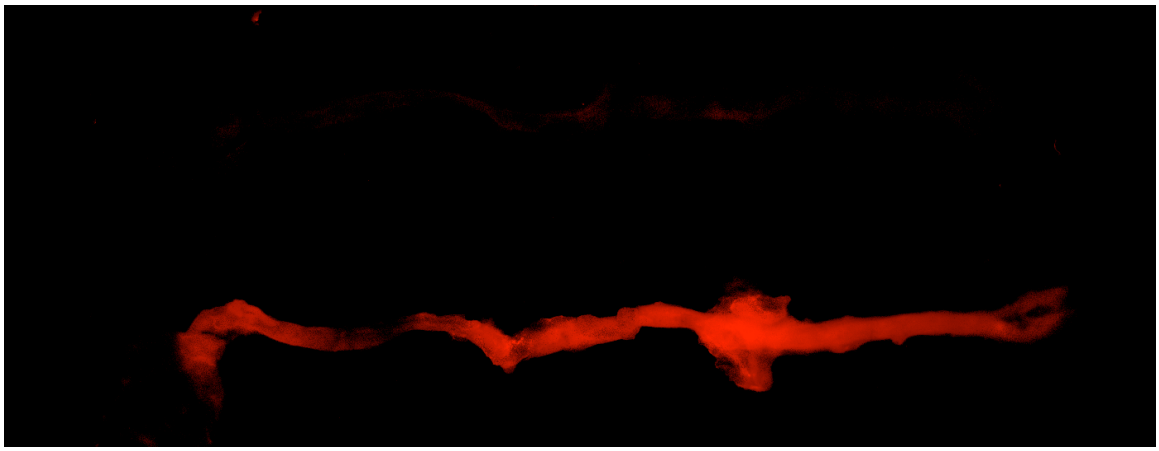

Figure S8 Fluorescence image of the excised aorta of apoE-KO mice injected 24 hours previously with saline (top) and FeO-HDL (bottom).

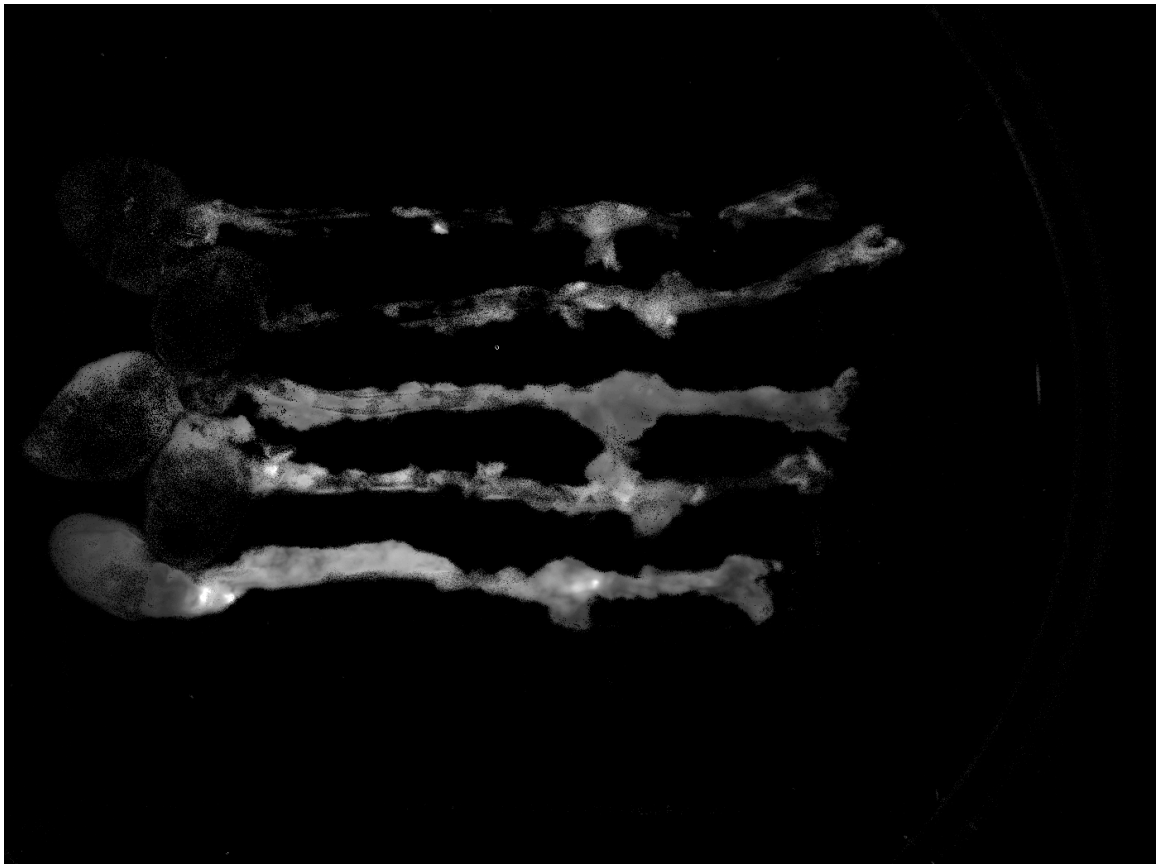

Figure S9 Fluorescence image of aortas of apoE-KO mice injected 24 hours previously with (from top) saline, Au-PEG, Au-HDL, Au-HDL and Au-HDL.

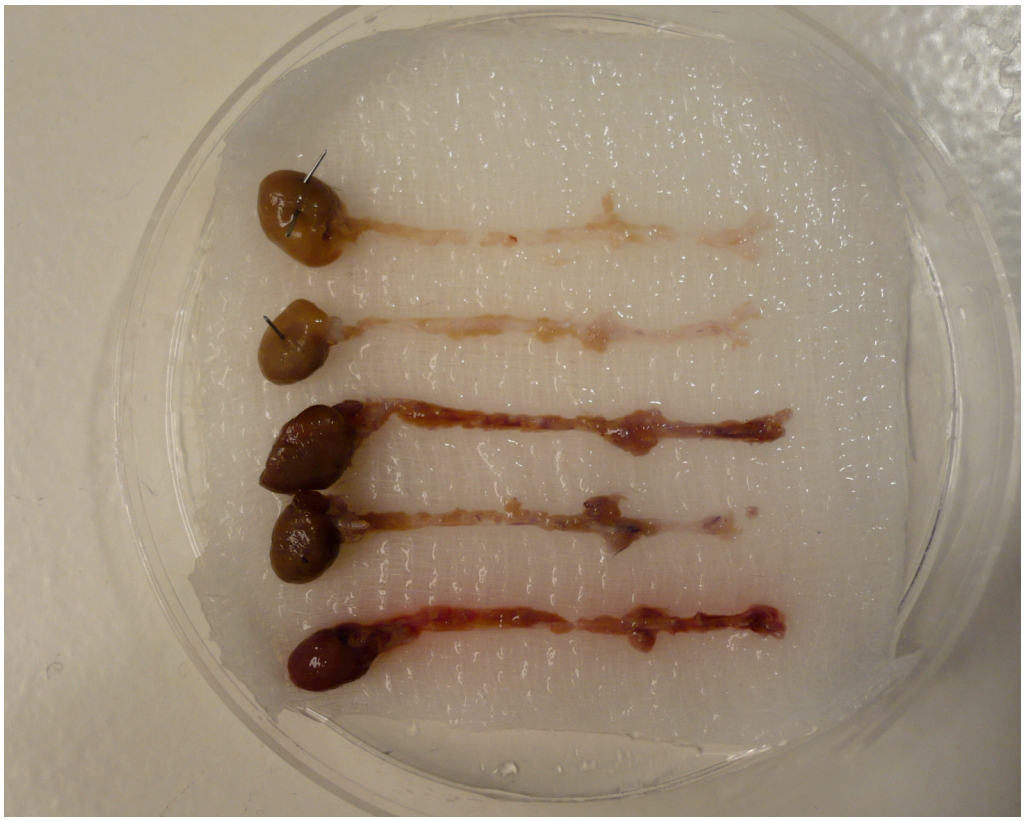

Figure S10 Photograph of the excised heart and aorta of apoE-KO mice injected 24 hours previously with (from top) saline, Au-PEG, Au-HDL, Au-HDL and Au-HDL. The bottom three aortas are red in color due to the uptake of Au-HDL (gold nanoparticles are red).

References

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