Supporting Information (SI)

Multi-Functional Nanoparticles Facilitate Molecular Targeting and miRNA Delivery to Inhibit Atherosclerosis in ApoE$^{-/-}$ Mice

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SUMMARY: Here, we demonstrate novel lipid nanoparticles carrying microRNA inhibitors (anti-miR) that are targeted specifically to inflamed endothelium in pro-atherogenic regions in mice, thereby preventing atherosclerosis without causing off-tissue target effects, a major hurdle in using microRNA modifiers to treat diseases.

Contents

Supporting Information (SI)

1. Study design and materials. The main goal of this study was to develop and utilize lipid nanoparticles that 1) are coated with cationic lipids (CCLs) to enhance the nucleotide-based anti-miR transfection to endothelial cells *in vitro* and *in vivo*, 2) carry the VHPK peptide post-inserted after the CCL preparation to ensure proper placement of the peptide on the CCL surface, and 3) encapsulate anti-miR-712 within CCL for its efficient escape from the lysosomal compartment in the cell. All *in vitro* studies were carried out using at least three biologically independent replicates while *in vivo* therapeutic and targeting studies were carried out with n=5 mice based on our previous experience for this type of studies. For atherosclerosis studies using the partial ligation model, n=7 was chosen based on a Power Calculation study. Lastly, for the determination of the systemic toxicity of the particles in wild type mice, n=20 was divided among five groups (CCL-anti-miR-712, VHPK-CCL-anti-miR-712, and VHPK-CCL-mismatched, VHPK-vacant liposomes and saline) (n=4 per group). For all mouse studies, only male mice were used for reproducibility within a gender.

Materials. Hydrogenated soy phosphatidylcholine (HSPC), cholesterol (chol), 1,2-distearoylglycero-3 phosphatidylethanolamine-*N*-polyethylene glycol-2000 (DSPE-PEG2k), and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Sepharose CL-4B and Boric acid were purchased from Sigma (St. Louis, MO, USA). Sephadex G-25 and G-75 were from GE Healthcare (Uppsala, Sweden). Triethylammonium acetate (TEAA) solution was supplied by Calbiochem (La Jolla, CA, USA). Fmoc-amino acids and peptide coupling reagents were purchased from NovaBiochem, with the exception of Fmoc-NH-(PEG)27-COOH, which was purchased from EMD Chemicals (Darmstadt, Germany). 5'-NH_2-modified anti-miRNA composed of locked nucleic acid (LNA) and phosphorothioate backbone was purchased from Exiqon North America (Woburn, MA). A series of Alexa dye-NHS ester were purchased from Life Technologies (Grand Island, NY). All other chemicals of analytical grade quality were purchased from Sigma-Aldrich (Milwaukee, WI) and VWR (Brisbane, CA).

2. Labeling of anti-miRNA with Alexa555- and FAM-NHS ester. 5'-C6-NH_2-modified anti-miRNA (1 mg) was dissolved in 0.1 M sodium borate buffer (pH 8.5, 500 µL) and added to either Alexa555- or FAM-NHS ester in anhydrous DMSO (50~80 µL). The mixture was allowed to stir at room temperature overnight and then was quenched by 0.1 M TEAA solution (pH 7.5). The fluorescently-labeled anti-miRNA was purified by high performance liquid chromatography (HPLC; Column: Phenomenex, Clarity 5u Oligo-RP, 250 x 10 mm) with 5% of solvent B gradually increased to 60% over 30 min at 60°C (solvent A: 95% of 0.1 M TEAA, solvent B: methanol). All fractions containing the fluorescently-labeled anti-miR-712 were combined, evaporated, and lyophilized for subsequent desalting by Sephadex G-25.

3. Preparation of VHPK-lipo-PEG-peptide. Peptide VHPK (Boc-VHPKQHRGGSK(ivDde)GC) was prepared using standard Fmoc mediated solid-phase peptide synthesis on Rink amide MBHA resin (500 mg) using HCTU (3 eq.) and DIPEA (6 eq.) at room temperature for 30 – 60 min. Standard Fmoc-amino acids were used for the syntheses with the exception of Fmoc-Lys(ivDde)-OH and Boc-Val-OH for the VHPK peptide. ivDde was removed using 10% hydrazine in DMF (3 x 10 min). Once the identity of the linear peptide was confirmed by MALDI (VHPK calculated: 1388.7208 $gmol^{-1}$, observed: 1389.8727 $gmol^{-1}$), VHPK-lipo-PEG-peptide (VHPK-LPP) was prepared by solid phase conjugation of Fmoc-NH-$(PEG)_{27}$-COOH (x3) followed by Fmoc-Lys(Fmoc)-OH and two stearic acids, following our method described in [1].

Crude LPP (~11 mg/run) was purified using semi-preparative high performance liquid chromatography (HPLC) using a gradient starting at 50:50 (water : acetonitrile, 0.05% TFA) and ending at 10:90 after 30 minutes. Column temperature was maintained at 60 °C during HPLC purification of the LPP. The purified LPP was confirmed by MALDI: VHPK-LPP calculated MW: 5964.7829 $gmol^{-1}$, observed MW: 5966.7144 $gmol^{-1}$ (SI Figure S2A).

4. Post-insertion of VHPK lipo-PEG-peptide into CCL-anti-miR-712. Lipo-PEG-peptide (LPP) containing VHPK (VHPK-LPP) was dissolved in an appropriate amount of chloroform and the chloroform was removed under a stream of nitrogen to form a film of dried peptide. The remaining trace amount of chloroform was removed under vacuum at 37-40ºC for 2 hr. CCL-anti-miR-712-FAM was then added to the dried LPP. The mixture was incubated for 1 hr at 37-40ºC with continuous slow stirring. The resulting VHPK-CCL-anti-miR-712-FAM and nontargeted CCL-anti-miR-712-FAM were purified using Sepharose CL-4B columns and separated from nonencapsulated anti-miR-712-FAM and free VHPK-LPP. The amount of LPP incorporated into the CCLs was measured by HPLC. The HPLC column used for the analysis was Phenomenex Jupiter C4, 5 µm, 250 x 4.6 mm). A standard curve was obtained from a known concentration of LPP solution by area integration from HPLC chromatogram. Prepared targeted-CCLs (20 µL) were injected to HPLC running under a gradient from 50% acetonitrile (0.05 % trifluoroacetic acid) to 90% acetonitrile (0.05% trifluoroacetic acid) for 30 minutes. The LPP peak area was integrated from the chromatogram and the amount of LPP was calculated from the standard curve.

The incubation time required to achieve the maximum insertion of VHPK-LPP into CCL-anti-miR-712 was studied by monitoring the particle surface charge and measuring the binding efficiency of the purified VHPK-CCL-anti-miR-712-FAM to HUVECs *via* flow cytometry (SI Figure S4). Incubation for 0.5 hr was found to be sufficient for insertion of VHPK-LPP and HUVEC binding was not affected by further incubation to 24 hr. Taken together, 1 hr incubation was considered to be optimal for VHPK-LPP post-insertion.

***5. In vitro* miRNA knockdown and targeted gene effects of CCLs.** Immortalized mouse aortic endothelial cells (iMAECs) were used for this study. For imaging, $2.5x10^5$ cells were seeded onto gelatin coated coverslips in a 6-well plate. Cells were pretreated with 3 ng/mL of mouse TNFα to induce expression of surface VCAM1. To induce the expression of miR-712, cells were transfected with 20 nM pre-miR-712 or control pre-miR as described in methods below. To study the effect of CCLs on miR-712 and its target genes, cells were incubated with either Alexa555-labeled-anti-miR-712 encapsulated in VHPK-CCLs or in non-targeting CCLs for various time points.

***6. In vitro* imaging of cell-CCL interactions.** For imaging IMAECs, cells were washed with ice cold DPBS including 0.9 mM calcium and 0.5 mM magnesium (DPBS+/+) supplemented with 1% w/v bovine serum albumin (BSA) X3 and images were acquired using an inverted fluorescence microscope. Post imaging, the regular culture media was replaced and cells were kept in a CO_2 incubator for 24 hr and total RNA was extracted to study expression of miR-712 and its target gene TIMP3 and RECK by qPCR. All *in vitro* experiments included non-CCL exposed controls as well as a naked Alexa555 labeled-anti-miR-712 control.

For imaging interactions with human umbilical vein endothelial cells (HUVECs), cells were obtained from Lonza (Portsmouth, NH, USA) and cultured in endothelial growth medium (EGM, Lonza) supplemented with 0.1% (v/v) human endothelial growth factor (hEGF), 0.1% (v/v) hydrocortisone, 0.1% (v/v) GA-1000, 0.4% (v/v) bovine brain extract (BBE) and 10% (v/v) fetal

bovine serum (FBS), all also from Lonza, inside a 95% air/5% CO_2 atmosphere incubator maintained at 37°C. HUVECs from passages 5 to 7 were used for these experiments. For Nikon fluorescence microscopy, HUVECs were seeded at 2×10^5 cells/dish in collagen-coated 35 mm tissue culture dishes 24 hr before experiments. Liposomes or CCLs were added to each 4°C pre-chilled cell dish at a final concentration of 100 µg lipid/mL in cold Dulbecco's phosphate buffered saline with 0.9 mM calcium and 0.5 mM magnesium (DPBS+/+) supplemented with 1% w/v bovine serum albumin (BSA). After a 15 min incubation on ice, cells were washed twice with cold DPBS+/+ and incubated at 37°C for 2 hr in 0.2 % w/v BSA in DPBS+/+. After 2 h, the cell incubation buffer was changed to complete medium for another 22 hr and cell plates were imaged after 24 hr.

7. Mouse partial carotid ligation surgery. All animal procedures carried out for this study were approved by the Animal Care and Use Committees at Emory University and University of California at Davis. Male C57BL/6 and *ApoE*$^{-/-}$ mice (Jackson Laboratory, Bar Harbor) were fed *ad libitum* with standard chow diet until surgery at 8 to 9 weeks of age. Mice were anesthetized with 3.5% isoflurane initially and then 1.5 to 2% during the entire procedure and underwent partial ligation. The LCA bifurcation was exposed by blunt dissection and three of four caudal LCA branches (left external carotid, internal carotid, and occipital arteries) were ligated with 6-0 silk sutures, leaving the superior thyroid artery intact. The contralateral RCA was left intact as an internal control. Following surgery, analgesic buprenorphine (0.1 mg/kg) was administrated and the animals were fed a Paigen's high-fat diet (HFD; Science Diets) containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid[2] for 2 weeks.

8. Evaluation of systemic toxicity of CCL-antimiR-712 and VHPK-CCL-antimiR-712.

***In vitro* complement activation:** CCL and VHPK-CCL encapsulating anti-miR-712 prepared in PBS were mixed with complement-preserved human serum at 2 mg/mL final lipid concentration and incubated at 37ºC for 30 min. Following termination of reaction by EDTA, C3a-desArg production in serum was quantified *via* ELISA (BD Biosciences OptEIA C3a ELISA kit, #550499).

Single dose toxicity by CBC: Three types of CCLs: CCL-anti-miR-712, VHPK-CCL-anti-miR-712 and VHPK-CCL-mismatched, and VHPK-vacant liposomes (not containing anti-miR-712) were prepared and administrated intravenously into C57BL/6 mice at 1 mg anti-miR-712/kg-body weight (n=5 per group). For VHPK-vacant liposomes, the lipid concentration was matched with VHPK-CCLs. Control animals received an equivalent volume of saline. Animals were weighed and euthanized after 24 hr. Blood was collected and major organs such as heart, liver, and spleen were dissected and weighed. Blood samples were analyzed for complete blood count (CBC) and blood chemistry as a measure of liver/kidney function.

Multi-dose toxicity: ApoE-/- C57BL/6 mice underwent partial carotid ligation and were fed a high fat diet from the day following the surgery. VHPK-CCL-anti-miR-712 or control saline was administrated *via* tail vein injection at 1 mg of anti-miR-712 per kg animal body weight on day 0, 3, 7, 10, 14 starting 2 days after surgery. Weight was obtained at 0, 3, 7, 10, 14 days (n=8, Figure S10), and major organs were weighed (n=8, Figure S10) and blood and tissue harvested after euthanasia on day 18. The major organs were processed for hematoxylin and eosin (n=8,

Figure S10) and blood processed for blood chemistry and complete blood cell count (n=8, Figure S10).

Cytokine levels: Following treatment termination on day 18, plasma was isolated from blood and processed for cytokine detection. Anti-inflammatory and pro-inflammatory cytokines such as IL-1B, IL-10, IL-6, IL-12, GM-CSF, IL-5, IFN-γ, TNF-alpha, IL-2, IL-4 were measured using the Cytokine Mouse 10-Plex Panel for Luminex® Platform (Invitrogen, ELISA kit, #LMC0001).

9. Radiolabeling, biodistribution and pharmacokinetics of VHPK-CCL-anti-miR-712

^{64}Cu radiolabeling of liposomes: The preparation of 6-BAT (^{64}Cu chelator) incorporated into VHPK-liposomes followed a previously reported method replacing DSPE-PEG2k-OMe with 5 mol% VHPK-lipopeptide.[3] Liposomes prepared in 0.1 M ammonium citrate buffer (pH5.5) were added to $^{64}CuCl_2$ in 0.1 M ammonium citrate (100 µL). Reaction mixtures were incubated for 40 min at 30 °C for 45 min until radioTLC showed a completion of reaction. EDTA (0.1 M, 20 µL) was added and kept at room temperature for additional 10 min. ^{64}Cu-labeled liposomes were then isolated through a size exclusion column (Sephadex-G75, superfine) preincubated in DPBS (1x). Isolated liposomes were collected and kept at room temperature until the dose was injected to animal on the same day of operation. For ^{64}Cu labeling of CCLs, 1 mol% 6-BAT lipid and VHPK-lipopeptide were simultaneously post-inserted into CCLs. The resulting VHPK-CCLs incorporating 6-BAT lipid were added to $^{64}CuCl_2$ in 0.1 M ammonium citrate buffer (pH 5.5). The rest of procedure followed the labeling of VHPK-liposomes as described above.

Biodistribution of VHPK-CCL and VHPK-liposomes: VHPK-CCL (321 ± 11.1 µCi, n=4) and VHPK-liposomes (309 ± 3.66 µCi, n=4) were intravenously administered through the tail vein of the FVB mouse. At 4 hours after injection, mice were euthanized and perfused with saline. Interesting organs, such as the blood, urine, heart, lungs, liver, spleen, kidneys, bone, muscle, cecum, small intestine, and stomach were harvested and radioactivity in those organs was measured in a gamma counter.

Half-life of CCLs and liposomes in blood: To obtain the half-life ($t_{1/2}$) of VHPK-CCL and VHPK-liposomes, images acquired for 30 minutes were reconstructed with a specified temporal resolution (15 sec x 8 frames (2 min), 60 sec x 8 frames (8 min), 180 sec x 5 frames (15 min), 300 sec x 1 frame (5 min)). ROIs were drawn in the hot spot on heart chamber with 4~6 pixel size. The half-life ($t_{1/2}$) was calculated by one phase decay curve with Prism software (the maximum mean value shown at 30 sec after injection was used as first curve fit value).

10. Isolation of endothelial-enriched RNA. Following partial carotid ligation in C57BL/6 mice, animals were sacrificed by CO_2 inhalation and extensively pressure-perfused for 10 min with saline containing heparin using a needle inserted into the heart. After perfusion, carotids were dissected out and carefully cleaned of periadventitial fat. Then the aortic lumen was quickly flushed (takes less than 2-3 seconds) with 150 µL of QIAzol lysis reagent (QIAGEN) using a 29-gauge syringe (Kendal) into a microfuge tube as we previously described.[4, 5] The eluate was then used for intimal RNA isolation using miRNeasy Mini kit (QIAGEN) according to the manufacturer's instruction. Following the intimal RNA flushing, the remaining abdominal aorta was used to prepare RNA from media and adventitia. The remaining tissue was snap-frozen in liquid nitrogen, pulverized by mortar and pestle, lysed in QIAzol lysis reagent, and total

RNA was isolated using the miRNeasy Mini kit. For each experiment, the LC and GC regions from two to four mice were pooled. A panel of markers genes for endothelium (PECAM1), smooth muscle (αSMA) and immune cells (CD45) was used to determine the enrichment of endothelial RNA from the preparation, and confirmed that the endothelial-enriched fraction did not contain a discernible contamination of RNAs from smooth muscle cells and immune cells (SI Figure S12).

11. Plaque lesion analysis. Aorta and carotid arteries were isolated *en bloc* as described above from ligated ApoE$^{-/-}$ mice fed a high-fat diet for 2 weeks. RCA and LCA were photographed using a CCD camera (Moticam 2500, Motic) attached to a dissection microscope (EMZ-8TRD, MEIJI Techno) at 3x magnification and the opaque area covered by plaque and total artery area of LCA were quantified using NIH ImageJ software.

12. Quantitative real-time PCR (qPCR). Intimal RNAs from three LCAs or RCAs were pooled to obtain ~30 ng total RNA [5]. Total RNA was polyadenylated and reverse transcribed for use in a two-step qRT-PCR using the NCode miRNA First-Strand cDNA Synthesis and qRT-PCR kits (Invitrogen). The resulting cDNA was subjected to qPCR using the NCode universal reverse primer in conjunction with a sequence-specific forward primer for selected miRNAs. RNU6B were used as the internal control using a ABI StepOne Plus qPCR machine. The PCR conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 4 s and 60 °C for 30 s. For measurement of mRNA targets, qPCR was performed on selected genes using Brilliant II SYBR Green QPCR Master Mix (Stratagene) with custom designed primers using 18S as house-keeping control. Fold changes between LCA and RCA were determined for all targets using the $\Delta\Delta$Ct method. Sequences for primers used for miRNA expression and mRNA expression studies were previously published by us.[6, 7]

Mature and precursor miRNA assays were performed using MicroRNA quantification was performed by SYBR green qPCR assay using miScript reverse transcription kit (Qiagen) according to the manufacturer's instructions. qPCR was performed using miScript SYBR Green PCR kit (Qiagen) with miScript universal primer (U6B) and the miRNA-specific forward primers and relative fold change was calculated. The specific mature and pre-miR primers were purchased from Qiagen. Each reaction was performed in duplicate with a final volume of 20 µL containing 2 µL of the cDNA, 10 µL of 2× SYBR Green PCR Master mix, of 2 µL and 10x miScript universal primer and 10x miScript primer assay. The amplification profile was denaturation at 95 °C, 15 min, followed by 40 cycles of 94 °C, 15 s; 55 °C, 30 s, and 70 °C, 30 s.

13. *en face* staining. C57BL/6 mice were sacrificed by CO_2 inhalation and perfused first with saline containing heparin and then with formalin. Aortas were carefully excised, dissected free of surrounding fat tissue, fixed in 4% paraformaldehyde, and tissue samples from LCA and RCA as well as the greater and lesser curvature of the aortic arch taken. Samples were counterstained using DAPI (Sigma) and mounted on glass slides using fluorescence mounting medium (Dako). Samples were imaged using a Zeiss LSM 510 META confocal microscope (Carl Zeiss).

14. Immunohistochemistry. Following treatment, ApoE$^{-/-}$ mice were euthanized and perfused with saline containing heparin as described above. LCA and RCA were collected *en bloc* along with the heart, aortic arch, trachea, esophagus, and surrounding fat tissue. Tissue was embedded in optimal cutting temperature (OCT) compound (Tissue-Tek), frozen on liquid nitrogen and stored at –80°C until used. To visualize atherosclerosis development, Oil red O

staining was carried out using frozen sections using preheated Oil Red O stain at 60°C for 6 minutes. The slides were differentiated in 85% propylene glycol for 1 in and rinsed with 2 changes of distilled water and counterstaining was done using modified Mayer's Hematoxylin for 1 min. Sections were fixed in a 1:1 mixture of methanol/acetone for 10 min at RT and then blocked (1 hr, at RT) using 10% (v/v) donkey serum in PBS. Immunohistochemical staining was carried out using following antibodies: TIMP3 (Abcam) (1:100) and RECK (cell signaling) (1:100) overnight at 4°C. Secondary staining for TIMP3 and RECK was performed using DyLight 549-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch) (1:250). Samples were imaged using a Zeiss LSM 510 META confocal microscope (Carl Zeiss).

15. *in situ* zymography. Gelatinolytic activity was analyzed in unfixed cryostat sections (8 µm thick) using DQ-gelatin as a substrate (Molecular Probes) as we recently described. DQ-gelatin was dissolved in a concentration of 1 mg/ml in water and then 1:10 diluted in reaction buffer according to the manufacturer's protocol. The mixture (50 µL) was put on top of the sections and incubated for 6 hr at room temperature. Proteolytic activity was detected as green fluorescence (530 nm) by Zeiss fluorescence microscopy.

16. Transient transfections of naked anti-miRs. Immortalized mouse aortic endothelial cells were transiently transfected with naked anti-miR-712 (400nM; Exiqon), mismatched anti-miR control (400 nM; Exiqon), pre-miR-712 (20 nM; Ambion), or control-pre-miR (20 nM; Ambion) using Oligofectamine (Invitrogen), following manufacturer's protocol as described previously.[7, 8]

17. Statistical analyses. Statistical analyses were carried out with Graph-Pad Prism (GraphPad Software). All error bars reported are SEM unless otherwise indicated. Pairwise comparisons were performed using one-way Student *t*-tests. Multiple comparisons of means were performed using 1-way ANOVA followed by Tukey's multiple comparison tests. Differences between groups were considered significant at p values below 0.05. All miRNA array data were analyzed with GenomeStudio software (Illumina).

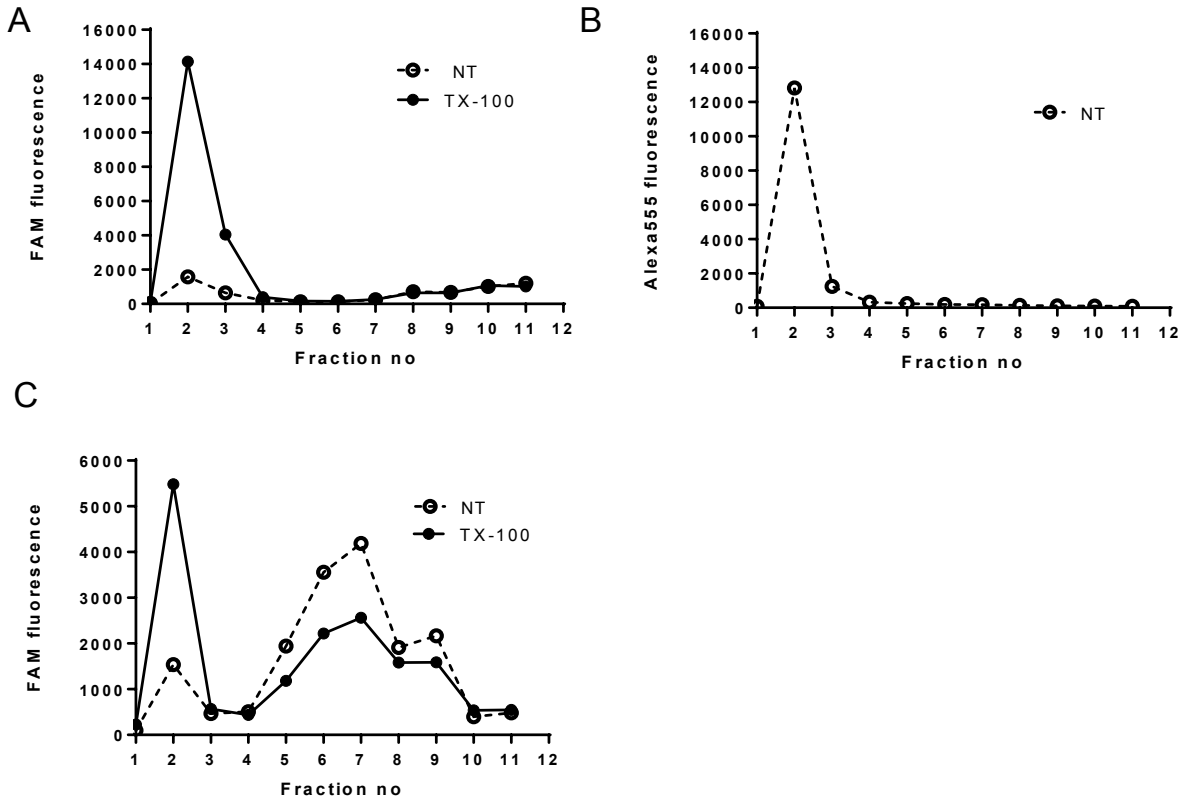


Figure S1. Elution profile of anti-miR-712-fluorophore encapsulated within non-targeted CCLs containing FAM- or Alexa 555- conjugated anti-miR-712. A) Separation CL-4B column profile of CCL-anti-miR-712-FAM and B) CCL-anti-miR-712-Alexa555. C) Incorporation of 5 mol% DSPE-PEG5000-Maleimide into lipid coating of CCLs to prepare Mal-CCL-anti-miR-712-CCL. In A and C, samples from the collected fractions were treated with 0.25%Triton X-100 (TX-100) for 20 min at 50°C or kept at room temperature for 20 min (NT). Anti-miR-712 within CCLs was eluted at void volume on a Sepharose CL-4B column in fractions 2 and 3, whereas non-encapsulated anti-miR-712 was eluted in fractions 5-9. FAM-conjugated anti-miR-712 was quenched when loaded within the CCLs and used to test *in vitro* stability. Alexa555-conjugated anti-miR-712 is not quenched and was used in subsequent *in vivo* and *ex vivo* studies of encapsulated anti-miR-712.

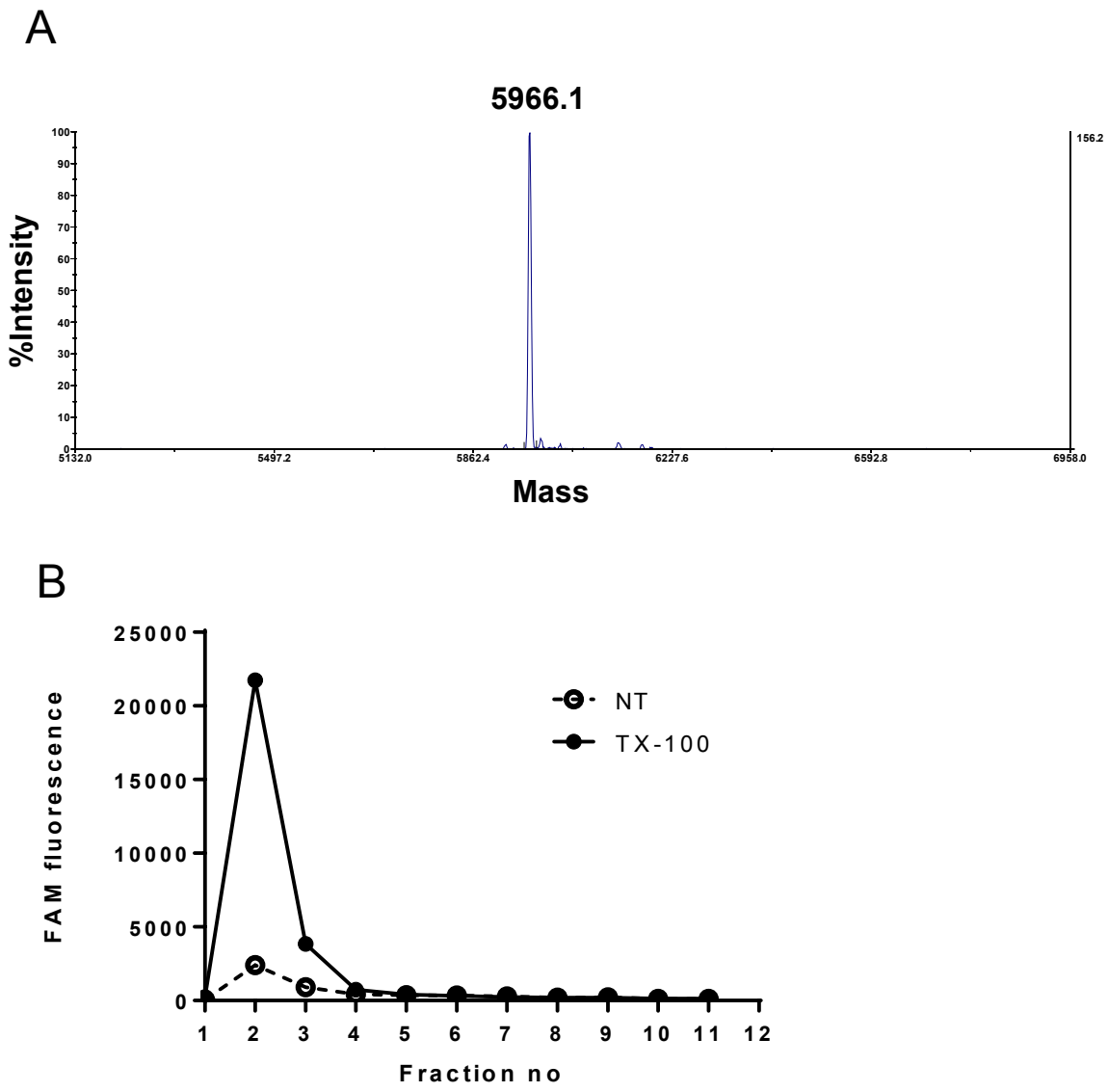


Figure S2. Characterization of the VHPK-lipopeptide (VHPK-LPP) and VHPK-CCLs. A) Matrix-assisted laser desorption/ionization spectrums (MALDI) of VHPK-LPP. MALDI spectrums were obtained under linear mode with sinapinic acid as a matrix. (Calculated MW: 5964.7829 gmol^{-1}, observed MW: 5966.7144 gmol^{-1}) B) Elution profile of anti-miR-712-FAM encapsulated within VHPK-CCL (after post-insertion of VHPK-LPP into CCL-anti-miR-712-FAM). Samples from the collected fractions were treated with 0.25%Triton X-100 (TX-100) for 20 min at 50°C. Anti-miR-712 within CCLs was eluted at void volume on a Sepharose CL-4B column in fractions 2 and 3, whereas non-encapsulated anti-miR-712 was eluted in fractions 5-9.

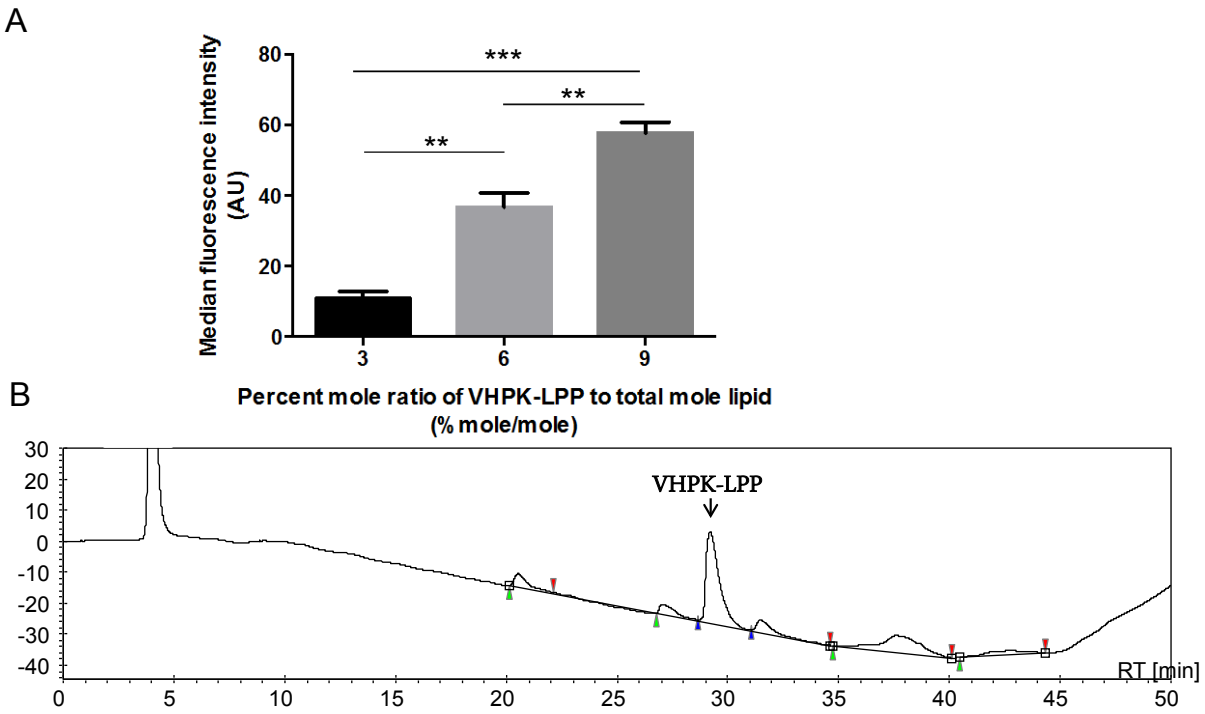


Figure S3. Post-insertion of VHPK-LPP into CCLs and quantification of VHPK incorporated on the surface of CCLs. A) Effect of increased mole ratio of VHPK-LPP post-inserted into CCL-anti-miR-712-FAM on binding to HUVECs. CCL-anti-miR-712-FAM were incubated with VHPK-LPP at 3, 6, and 9 mol% to total lipid, purified using Sepharose CL-4B column, and incubated with HUVEC cells as described in the Methods. The fluorescence intensity of FAM associated with HUVEC cells was quantified with flow cytometry. ** $p<0.01$ and *** $p<0.001$ as determined by Student's t-test. B) HPLC chromatogram of VHPK-CCLs. VHPK-CCL-anti-miR-712 (20 μL) were injected to HPLC and eluted using a 30-min linear gradient from 50% acetonitrile (0.05 % trifluoroacetic acid) to 90% acetonitrile (0.05% trifluoroacetic acid). VHPK-LPP peak area was integrated from the chromatogram and the amount of VHPK-LPP was calculated. The mole percent of VHPK was calculated 4.9 mol% from a standard curve obtained with VHPK-LPP.

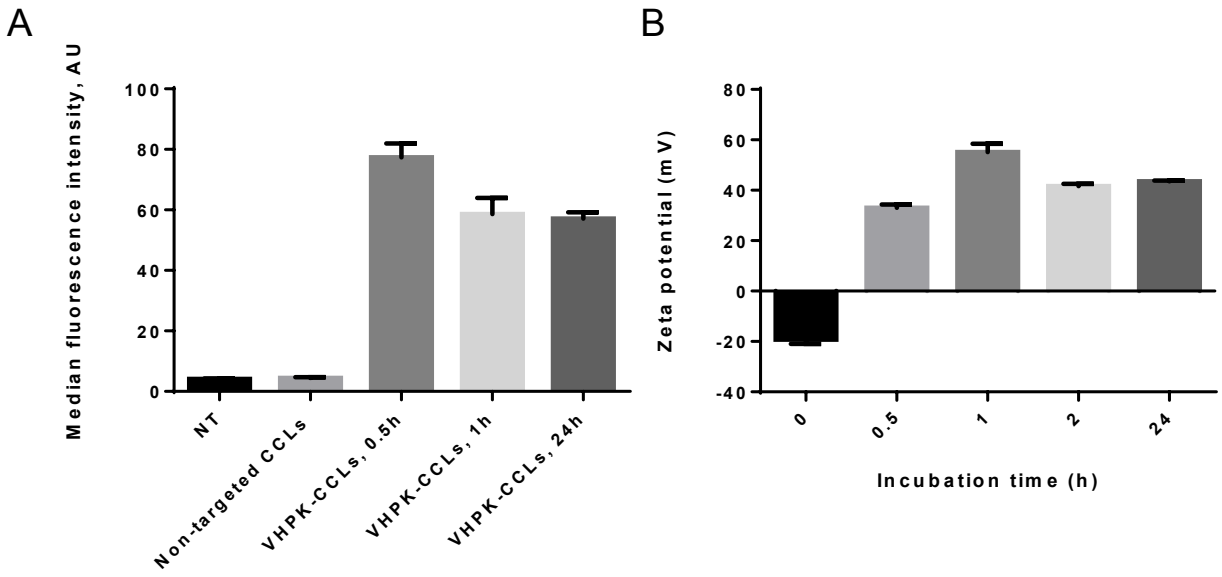
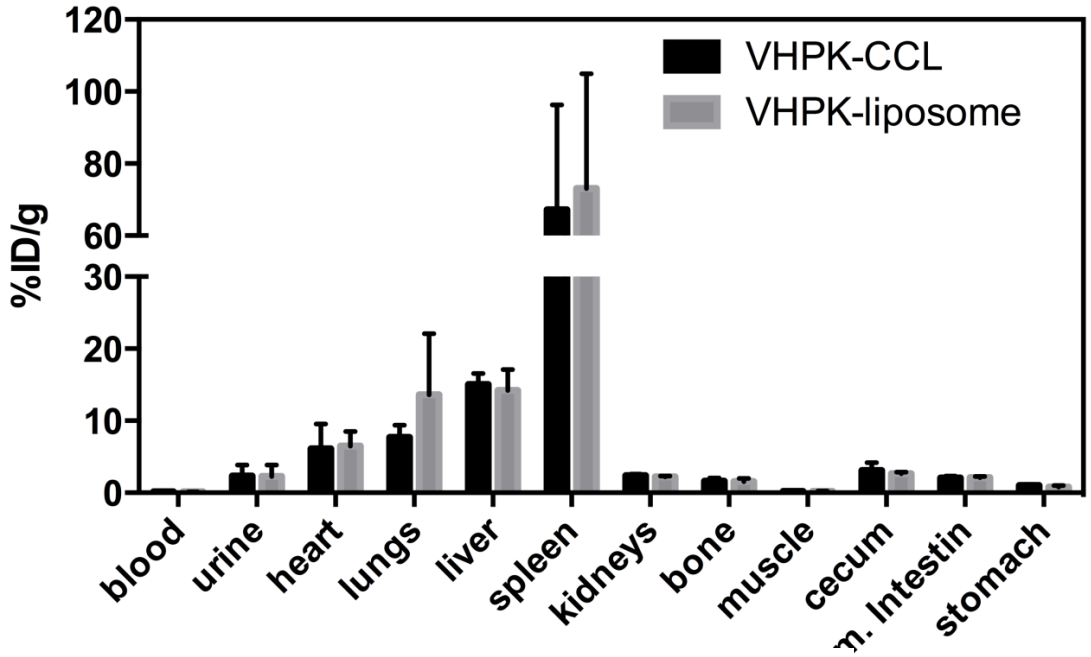


Figure S4. Effect of incubation time of VHPK-LPP with CCL-anti-miR-712-FAM (to form VHPK-CCL-anti-miR-712-FAM). A) FAM fluorescence intensity of VHPK-CCL-anti-miR-712-FAM bound to HUVECs was quantified by flow cytometry as a function of the incubation period of VHPK-LPP and CCL-anti-miR-712-FAM. At each time point, 100 µL of the mixture was applied to a Sepharose CL-4B column and the fluorescence of purified VHPK-CCL-anti-miR-712-FAM was matched. HUVECs were incubated with approximately 1.5 nmol of anti-miR-712 in 1 mL of DPBS +/+ supplemented with 1% w/v BSA for 15 min on ice. Cells were washed and FAM fluorescence associated with HUVECs was measured by flow cytometry. B) As a function of the incubation period of VHPK-LPP and CCL-anti-miR-712-FAM, the zeta potential of the purified VHPK-CCL-anti-miR-712-FAM was analyzed by the Malvern Zeta-Sizer.

A



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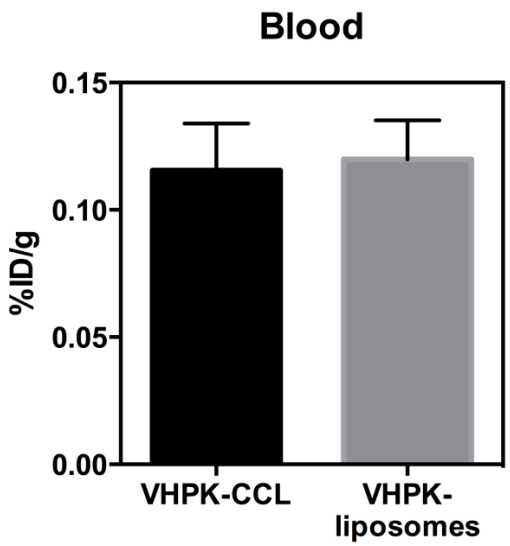

Figure S5. Biodistribution of VHPK-CCL and VHPK-liposomes in FVB mice (n=4 per each group). A) Biodistribution. B) Blood circulation. Liposomes were intravenously administrated through tail vein. At 4 hours after injection, mice were euthanized and perfused with saline before organ/tissue dissection.

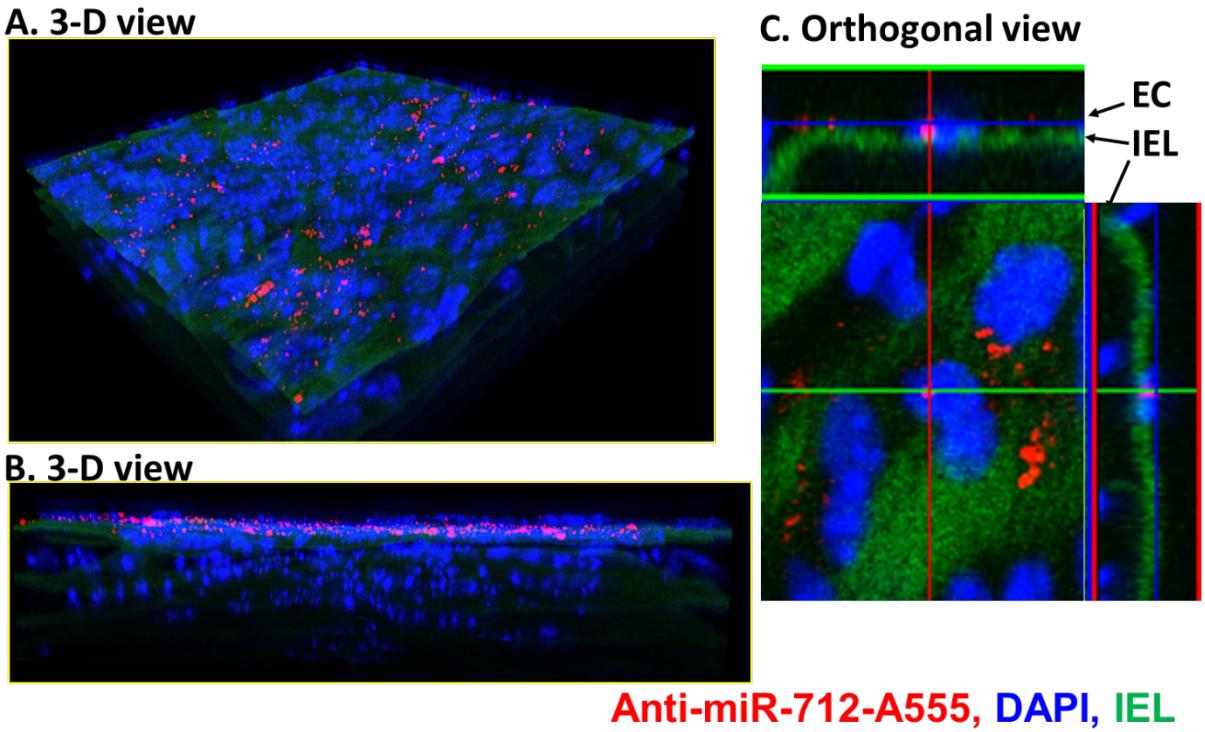

Figure S6. ***Ex vivo*** **confocal assessment of intracellular targeted delivery of VHPK-CCL-Anti-miR-712-Alexa555 to endothelial cells after tail vein injection in mice with partial ligation of LCA. At 4 days post-partial ligation surgery, C57BL/6 mice were injected with VHPK-CCL-anti-miR-712-labeled with Alexa555** ***via*** **the tail vein. After 1 hr, mice were sacrificed, carotid arteries dissected out and analyzed by** ***en face*** **confocal imaging. Images are shown either in two different angles of 3-D views (A, B) or orthogonally (C). Representative images (n=3) are shown. IEL: internal elastic lamina. EC: endothelial cells.**

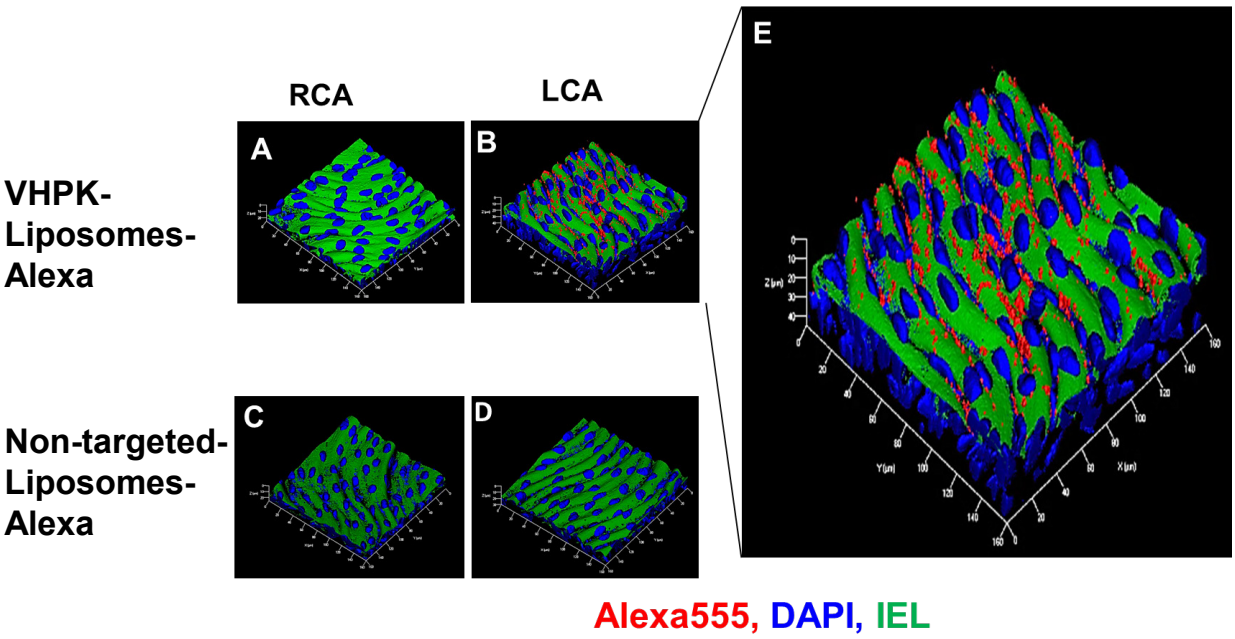

Figure S7. ***Ex vivo*** **confocal assessment of systemic targeted delivery of A-B) VHPK-liposomes encapsulating Alexa555 and C-D) non-targeted liposomes encapsulating Alexa555 in mice using the partially ligated LCA model. At 4 days post-partial ligation surgery, C57BL/6 mice were injected** ***via*** **the tail vein with each liposomal formulation. After 1 hr, mice were sacrificed, carotid arteries dissected out and analyzed by** ***en face*** **confocal imaging. Images are shown 3-D views and E is a magnified view of B.**

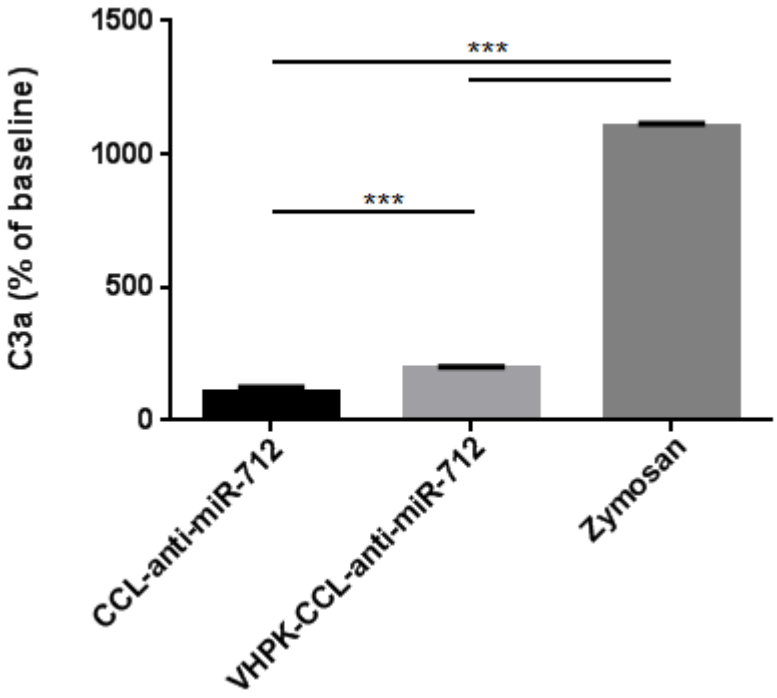

Figure S8. *In vitro* complement activation of CCL and VHPK-CCL. One-way ANOVA with Tukey's multiple comparisons test was performed. * ($p<0.001$). VHPK-CCL activated more serum C3a than CCL, however, the levels were 5.7- and 10-fold significantly lower than the level of activation with the positive zymosan control ($p<0.001$), respectively.**

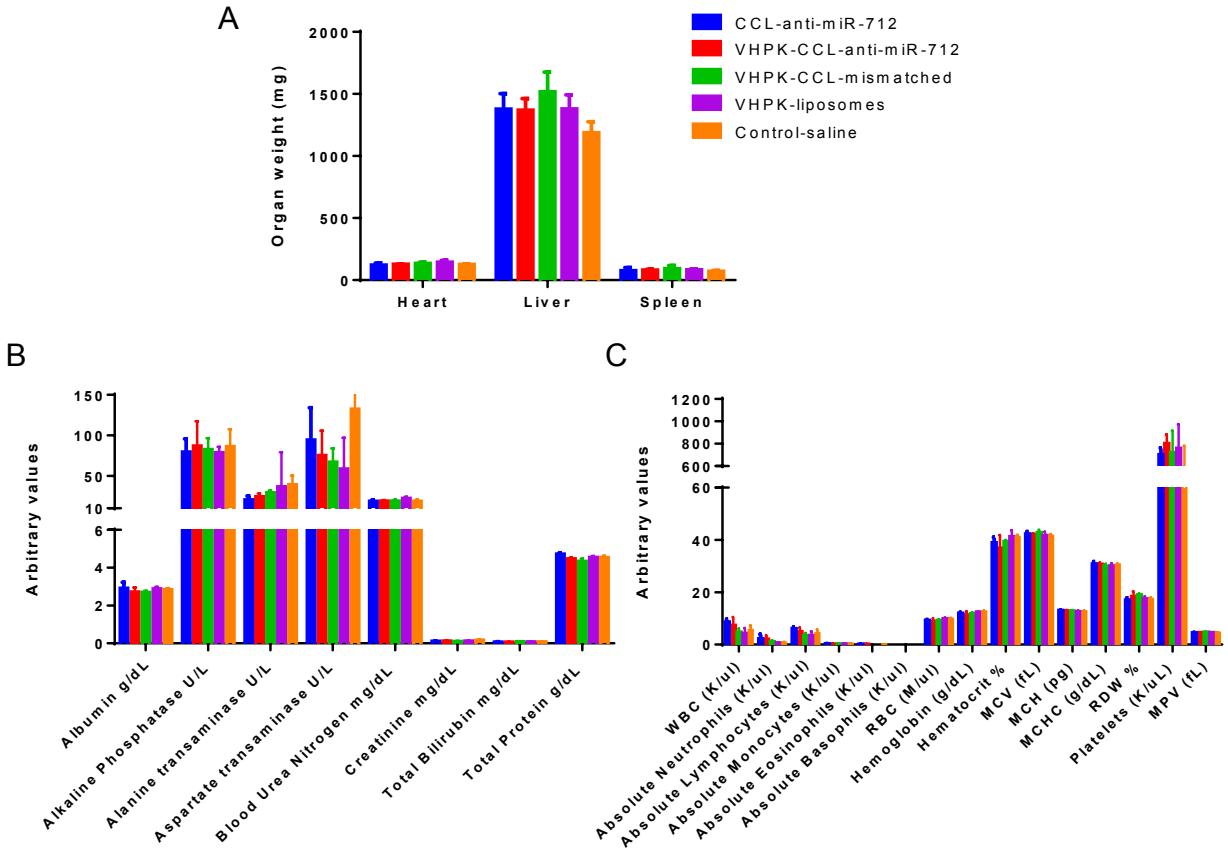

Figure S9. Evaluation of acute toxicity 24 hours following a single injection of either CCLs, VHPK-CCLs encapsulating anti-miR-712 or the scrambled ("mismatched") oligonucleotide, VHPK-vacant liposomes or saline. C57BL/6 mice were intravenously injected with saline or the nanoparticles. After 24 hr, mice were euthanized, organs were dissected and weighed (A), and blood was drawn and analyzed for (B) blood chemistry and (C) a complete blood cell count.

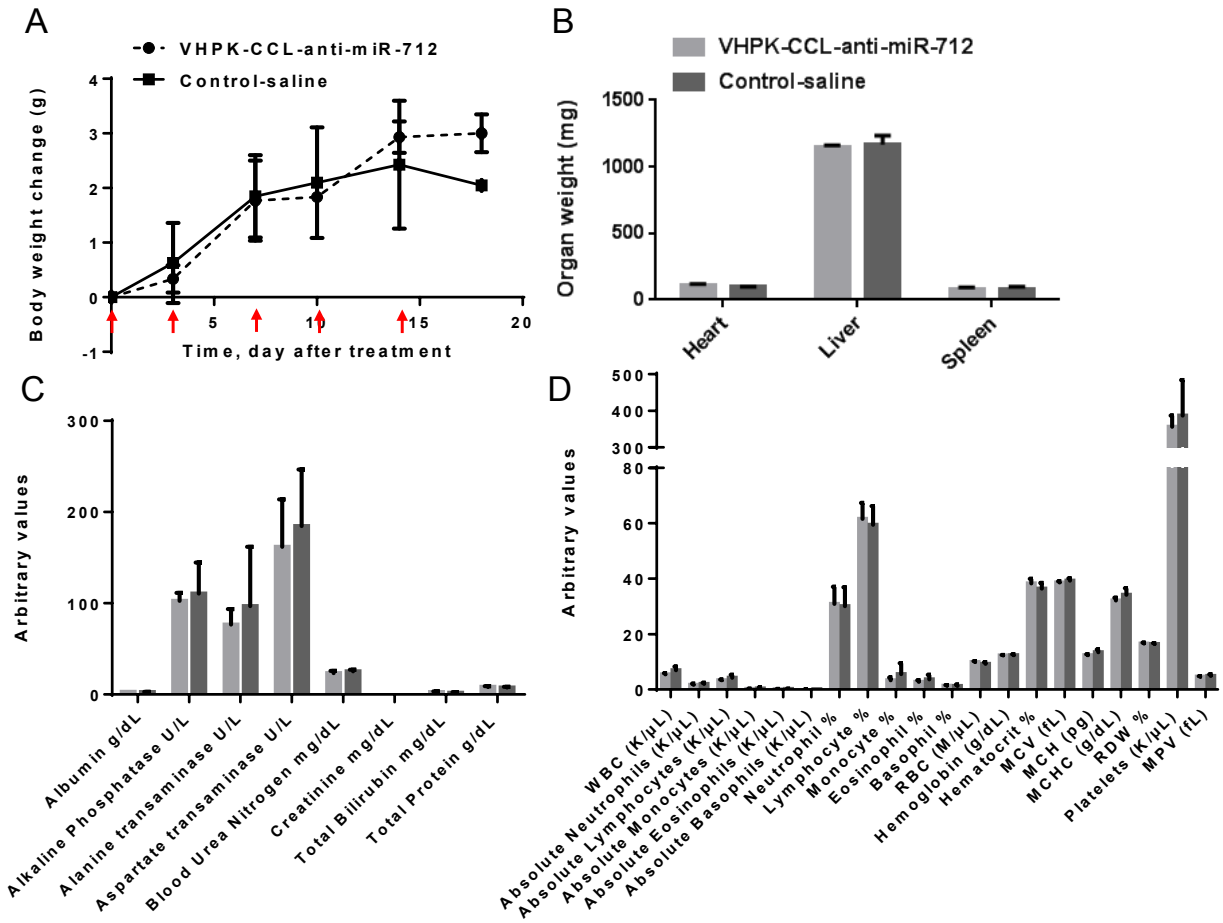

Figure S10. Evaluation of toxicity associated with multiple administrations of VHPK-CCL-anti-miR-712 or control (saline) in the ApoE -/- C57BL/6 mouse (n=8). A) Body weight. The red arrows indicate days on which treatment was administered. No change in body weight was detected during the 18-day treatment as compared to saline-treated control animals. B) Following euthanization on day 18, major organs such as heart, spleen, and liver had similar weights compared to control animals. C-D) Blood chemistry and complete blood count revealed no difference between VHPK-CCL-treated and saline-treated animals.

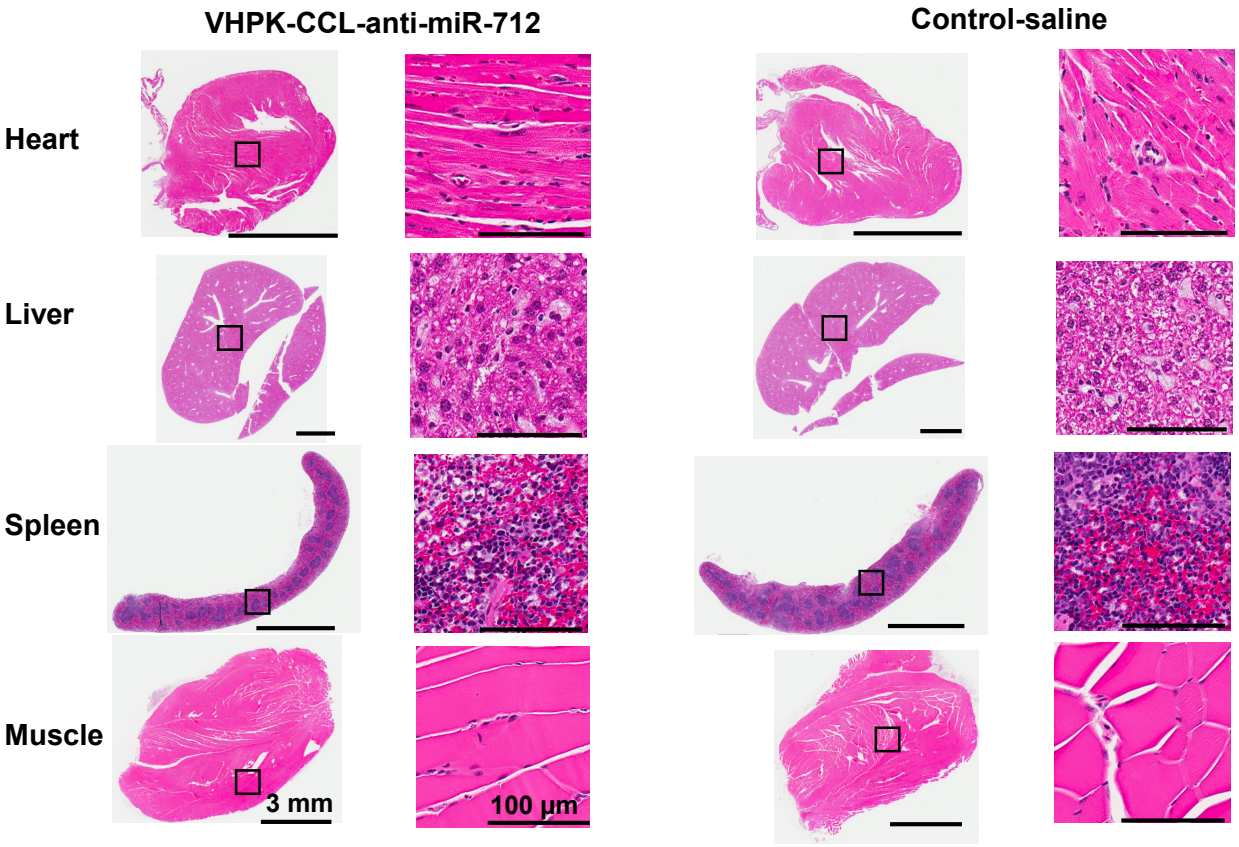

Figure S11. Histological sections of heart, liver, spleen and skeletal muscle treated with 4 doses of VHPK-CCL-anti-miR-712 compared to control animals, which received saline. Scale bars represent 3 mm and 100 µm in whole organ/tissue and magnified images, respectively.

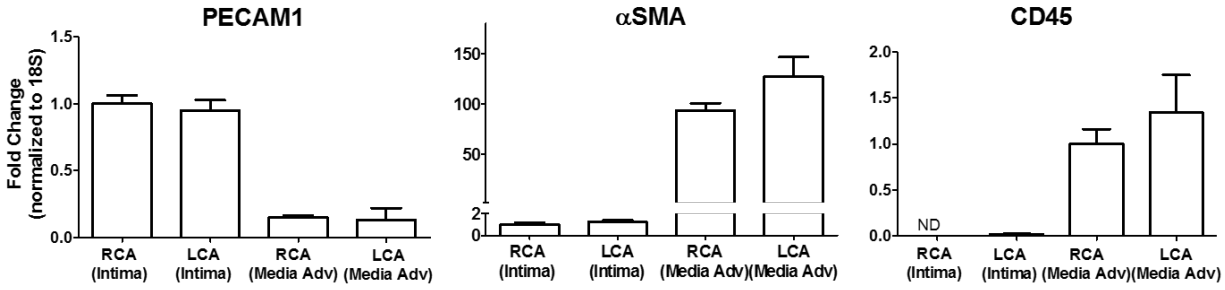

Figure S12. Endothelial-enriched total RNAs were obtained by flushing the LCA and RCA at 2 days post-partial carotid ligation. Following endothelial-enriched RNA preparation, the left-over samples representing media + adventitial regions were also prepared. To determine the purity of endothelial RNAs, qPCR was carried out using PECAM1, αSMA and CD45, marker genes for endothelial, smooth muscle, and immune cells, respectively, and fold-change was determined by normalizing against 18S as an internal control. ND: not detected.

Table S1. Biodistribution of VHPK-CCL and VHPK-liposomes in FVB mice (n=4 per each group). ^{64}Cu-VHPK-CCL and VHPK-liposomes were intravenously administrated through the tail vein. At 4 hours after injection, mice were euthanized and perfused with saline before organ/tissue dissection.

	VHPK-CCL			VHPK-liposomes		
Organs	**Mean**	**Stdev**	**N**	**Mean**	**Stdev**	**N**
blood	**0.12**	**0.04**	**4**	**0.12**	**0.03**	**4**
urine	**2.32**	**1.52**	**4**	**2.27**	**1.61**	**4**
heart	**6.13**	**3.41**	**4**	**6.49**	**2.01**	**4**
lungs	**7.70**	**1.68**	**4**	**13.6**	**8.50**	**4**
liver	**15.05**	**1.51**	**4**	**14.22**	**2.86**	**4**
spleen	**67.12**	**29.20**	**4**	**73.03**	**31.90**	**4**
kidneys	**2.36**	**0.23**	**4**	**2.22**	**0.08**	**4**
bone	**1.61**	**0.45**	**4**	**1.55**	**0.47**	**4**
muscle	**0.19**	**0.01**	**4**	**0.19**	**0.03**	**4**
cecum	**3.09**	**1.09**	**4**	**2.68**	**0.22**	**4**
sm. intestine	**2.06**	**0.27**	**4**	**2.09**	**0.19**	**4**
stomach	**1.01**	**0.14**	**4**	**0.78**	**0.25**	**4**

Table S2. Serum lipid profile

Following partial carotid ligation, ApoE$^{-/-}$ mice were fed a high-fat diet for 2 weeks. Mice were then treated with either VHPK-CCL-anti-miR-mismatched, VHPK-CCL-anti-miR-712, or CCL-anti-miR-712 (twice a week *via* tail-vein injection) for 2 weeks. Blood collected immediately following sacrifice was stored frozen until assayed to measure total cholesterol, light-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides at Emory Cardiovascular Specialty Laboratories.

Group (n)	Total Cholesterol (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	Triglycerides (mg/dL)
VHPK-CCL-anti-miR-MMC (5)	457.2±117.4	60.2±23.1	24.12±3.4	259.6±36.6
VHPK-CCL-anti-miR-712 (5)	403.4±180.6	25.4±5.9	18.0±6.6	189.0±34.1
CCL-anti-miR-712 (5)	403.8±99.5	26.4±5.8	19.5±7.4	125.6±37.1

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