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Supplementary Information for

Targeted polyelectrolyte complex micelles treat vascular complications *in vivo*.

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Supplementary Materials and Methods

All studies were conducted in accordance with protocols approved by the Institutional Biosafety Committee (IBC) or Institutional Animal Care and Use Committee (IACUC) of The University of Chicago. All experimental animals were assigned unique identifiers to blind experimenter to genotypes and treatment. A block randomization method was used to assign experimental animals to groups on a rolling basis to achieve adequate sample numbers for each experimental condition.

Generation of *miR-92a^{TG}*, *miR-92a$^{EC\text{-}TG}$* and *miR-92a$^{EC\text{-}TG}$/Apoe$^{-/-}$* mouse lines

miR-92a^{TG} (Inducible Rosa-CAG-miR-92a transgenic) mice, in which miR-92a expression can be induced by the *Cre-loxP* system, were generated by inserting the mmu-miR-92a precursor sequence after a PGK-neo-4xPA stop cassette in the ROSA26 locus in the C57BL/6 mice. The PGK-neo-4xPA stop cassette contains a *LoxP*-flanked selection and a transcriptional stop cassette, enabling transcriptional silencing of a downstream mmu-miR-92a precursor. This embryonic stem (ES) cell-based approach to generate transgenic mouse line using the PGK-neo-4xPA stop cassette has been described in detail by Mao et al. (1). Briefly, we engineered the ES cells with the PGK-neo-4xPA-mmu-miR-92a cassette insertion in the ROSA26 locus. PCR- and Southern blot-verified ES cells were used for microinjection into the blastocyst embryos to generate chimeric mice which were then bred with wild-type C57BL/6 mice to determine the germ-line transmission. PCR primers were designed to identify founders harboring the transgene in the ROSA26 locus. This founder was crossed to wild-type C57BL/6J mice to obtain *miR-92a^{TG}* +/- offspring; the F1 *miR-92a^{TG}* +/- progeny was then crossed to generate homozygous *miR-92a^{TG}* mice. F2 *miR-92a^{TG}* were born at the expected Mendelian frequency and, at baseline, showed no detectable developmental defects or structural anomalies. The preparation of mouse zygotes, microinjection, blastocysts transfer, and initial breeding of the *miR-92a^{TG}* animals were performed by the Transgenic Mouse Core Laboratory in National Taiwan University.

To generate an inducible, endothelial-specific miR-92a transgenic mouse line, *miR-92a^{TG}* mice were bred with *Cdh5(PAC)-CreERT2* (kindly provided by Prof. Ralf H. Adams) to generate *miR-92a$^{EC\text{-}TG}$* transgenic mice. *miR-92a$^{EC\text{-}TG}$* on an *Apoe$^{-/-}$* background mice (*miR-92a$^{EC\text{-}TG}$/Apoe$^{-/-}$*) were generated by crossing *miR-92a$^{EC\text{-}TG}$* mice with *Apoe$^{-/-}$* mice. For activation of the Cre-ERT system, tamoxifen (2 mg/day, dissolved in corn oil) was injected intraperitoneally for consecutive 7 days at the age of 7 weeks. The induction of Cre recombinase for efficient miR-92a overexpression in adult vascular endothelium was described in results section.

Tamoxifen-injected male *miR-92a$^{EC\text{-}TG}$/Apoe$^{-/-}$* and control (*Cdh5(PAC)-CreERT2/Apoe$^{-/-}$)* mice were fed a high-fat diet (Harlan Teklad TD88137) for 10 weeks. Then the animals were sacrificed, blood collected, and heart harvested after pressure perfusion with PBS via left ventricle after severing inferior vena cava. Hearts were fixed in 4% paraformaldehyde for 48 hours and embedded in OCT after being immersed in OCT:sucrose =1:1 mixed solution for 24 hours. Frozen embedded samples were sectioned in 8 μm thickness and stained with an Oil Red O Staining Kit (Sigma-Aldrich, MO, USA and ScienCell, CA, USA) according to the manufacturer's instructions. The plaque areas were calculated using National Institutes of Health ImageJ software. Lesions of the aortic root were quantified as the absolute lesion area.

Material synthesis and purification

VCAM1-targeting peptides (VHPKQHRGC) and polylysine (CK30) were purchased from GenScript (NJ, USA). N_3-PEG-MAL (Molecular weight/Mw=2000) were

obtained from JenKem Technology (Beijing, China). DBCO-PEG12-MAL and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich (St. Louis, USA). The miR-92a inhibitors (miRIDIAN microRNA hsa-miR-92a-3p hairpin inhibitor) and miRNA inhibitor controls (microRNA Hairpin Inhibitor Negative Control #1) (miRIDIAN microRNA Hairpin Inhibitors, Dharmacon, USA) are single-stranded, chemically enhanced oligonucleotides purchased from Dharmacon, Inc. (CO, USA) and Horizon Discovery Biosciences Ltd (Cambridge, UK).

Synthesis of the VHPKQHR-PEG-polylysine polymer

DBCO-PEG12-MAL (0.2 mL, 63.0 mg/mL, DMSO) was added in 2 mL CK30 (25.0 mg/mL, DI) containing TCEP (3.0 mg/mL, DI) and EDTA•2Na (3 mM, DI). The reaction was stirred at room temperature for 24 hrs. Then, 0.5 mL N_3-PEG-MAL (50.0 mg/mL, DMF) was added in the above solution and the reaction continued for another 4 hrs. After that, 0.655 mL solution containing VCAM1-tagerting peptide VHPKQHRGC (20 mg/mL, DI), TCEP (3.0 mg/mL, DI) and EDTA•2Na (3 mM, DI) was subsequently added into the reaction and stirred for another 24 hrs. The products were intensively dialyzed (MWCO=3500) with DI water and lyophilized to obtain the VHPKQHR-PEG-polylysine polymer. The non-targeted polylysine-PEG polymers were obtained without adding the VHPKQHRGC peptides into the solutions. The reactions were conducted under N_2 atmosphere.

Formation of the polyelectrolyte complex micelles

The VHPKQHR-PEG-polylysine polymer (1.0 μL, 1 mg/mL, DI) was first diluted into 50 μL solution and then added into 50 μL aqueous solution containing 0.5 μg miR-92a inhibitor or miRNA inhibitor control; the solution was then mixed gently and incubated for 30 min at room temperature to form the polyelectrolyte complex micelles. The non-targeting micelles were prepared in an identical procedure except that the VHPKQHR-PEG-polylysine polymer was replaced with the PEG-polylysine polymer.

Micelle characterization

Transmission electron microscope (TEM) images were obtained from FEI Spirit LaB6 Routine Electron Microscope with a voltage of 120 kV located in the Advanced Electron Microscopy Facility at The University of Chicago. NMR spectrum was recorded by a 400 MHz NMR instrument (Bruker, Germany). The hydrodynamic size and zeta potential were measured by Zetasizer Nano ZS90 (Malvern, UK).

Gel retardation assays

The polyelectrolyte complex micelles were prepared at different mass ratio of 0.25, 0.5, 1, 2, 3 (VHPKQHR-PEG-polylysine: miRNA inhibitor). 10 μL of the complexes were loaded on 1 % agarose gel containing 10 μg/mL ethidium bromide (EthBr) and electrophoretically separated for 15 min at 100 V. The gel was visualized with a UV illuminator (Bio-Rad).

Ethidium bromide (EthBr) competitive binding assay

miR-92a inhibitors (1.0 μL, 1 mg/mL, DI) was first diluted into 150 μL aqueous solution and then incubated with 150 μL EthBr (0.04 μg/mL, DI). 5.0 μL of VHPKQHR-PEG-polylysine polymer (1 mg/mL, DI) was then added into the above solution, followed by 20 min incubation at room temperature. The fluorescence of the solution was measured with an excitation at 470 nm and emission at 500-700 nm by a Tecan microplate reader (Thermo Fisher). The EthBr aqueous solution and EthBr-stained naked miR-92a inhibitor were used as controls.

Isothermal titration calorimetry (ITC) assays

The ITC experiments were conducted using a MicroCal iTC200 isothermal titration calorimeter (GE Healthcare) under a constant 25.17 ºC. The solution was first degassed at room temperature for 30 min prior to experiments. The cell and syringe were pre-rinsed with DI water. Solution of the VHPKQHR-PEG-polylysine polymer (107.0 μM, DI) was injected into the solution containing miR-92a inhibitors (13.3 μM, DI) in 19 potions at 4 min intervals (2 μL in each injection) to allow equilibration of the system. The heat signals were recorded per potions accordingly. As a reference, VHPKQHR-PEG-polylysine polymer solution (107.0 μM, DI) was injected into DI water and recorded the heat. The titration heat to form the polyelectrolyte complex micelles was calculated by subtracting the reference.

Cell culture

Human aortic endothelial cells (HAECs) were obtained from Lonza (NJ, USA) and cultured in EGM2 containing 2 % FBS at 37 ºC under 5 % CO_2. The cells at passage 5-8 were used for the experiments.

Quantitative real-time PCR (qPCR)

Total RNA was extracted by a Direct-zol RNA MiniPrep isolation kit (Zymo, Research). 0.2 μg mRNA was reverse-transcribed into cDNA through a high capacity reverse-transcription kit (Life Technologies). 0.1 μg miRNA were reverse-transcribed into cDNA through Taqman MicroRNA reverse transcription kit (Life Technologies). The cDNA was amplified by LightCycler 480 II (Roche) system using a SYBR Green as probe. Absolute quantification of the gene of interest was normalized to the geometric mean of β-actin, GAPDH and Ubiquitin. For miR-92a quantification, it was normalized to snoU6 RNA.

Delivery of miR-92a inhibitors to inflamed endothelial cells *in vitro*

HAECs were seeded in a 24-well plate overnight to reach 80-100 % confluence before the experiments. Endothelial inflammation was induced by LPS treatment (200 ng/mL) for 3 hrs at 37 ºC, verified by markedly increased E-selectin, CCL2, IL6, and VCAM-1 analyzed by qPCR. PBS- or LPS-treated HAECs were incubated with 2 μg miR-92a inhibitor or inhibitor control in the naked form or complexed by 4 μg VHPKQHR-PEG-polylysine polymers. After 30 min incubation of the miRNA inhibitor, the culture media were removed and 500 μL fresh culture medium was added into cells. The cells were then harvested 24 hrs later. miR-92a levels were detected by qPCR.

The cellular uptake of Dye 547-labelled miRNA inhibitor control (Dharmacon), delivered in the naked form or in complex with the micelles, by HAECs was further determined by confocal laser scanning microscopy (CLSM, Leica SP5 2-Photon, Germany) and flow cytometry (BD LSRFortessa). For CLSM imaging, HAECs were seeded in a μ-slide 8 well (Ibidi, 80827) with glass bottom dishes for 24 hours and then were treated with 0.5 μg Dye 547-labelled miRNA inhibitor Ctrl in the naked form or encapsulated in the micelles. After 30 min incubation, the culture medium was removed, and the cells were washed with PBS. Then 200 μL of paraformaldehyde (4 % in PBS) were added in each well to fix the cells for 15 min and then the cells were incubated with Hoechst 33342 (2 μg/mL in PBS) for 10 min to stain the nucleus. The fluorescence images were taken by 40x oil immersion objective using CLSM. For flow cytometry analysis, the HAECs were seeded in 12-well plate for 24 hours and then were treated with 1.5 μg Dye 547-labelled miRNA inhibitor Ctrl in the naked form or in encapsulation by the micelles. After 30 min incubation, the culture medium was removed, and the cells

were washed with PBS and suspended in PBS for flow cytometry analysis. For the VCAM-1 blocking assay, the HAECs were pre-treated with excess free VCAM1-targeting peptides (3 mg, 2.83 μmol) before the treatment of micelles.

Delivery of miR-92a inhibitors to inflamed endothelial cells *in vivo*

All mouse procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Chicago. Partial carotid artery ligation was surgically preformed to introduce acute disturbed flow activating endothelial cells in the left carotid artery, as described in previous studies (2). Briefly, male 7-9 weeks old *Apoe*$^{-/-}$ C57BL/6 mice (The Jackson laboratory) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) mixed solution. A ventral midline incision (~ 5 mm) in the neck was opened by a tinny scissor (F.S.T 14090-11). The left carotid artery (LCA) was exposed by blunt dissection under microscopy. Then, the left external carotid, internal carotid, and occipital artery were ligated with 6-0 silk suture while the superior thyroid artery remains intact. After that, the incision was closed with Nylon monofilament suture (Air-Tite products) and the cut was smeared with Betadine to prevent infection. After the surgery, the mice were fed high-fat diet.

The *in vivo* biodistribution of miRNA inhibitor, delivered in the naked form or by the polyelectrolyte complex micelles, was determined in *Apoe*$^{-/-}$ mice subjected to partial carotid artery ligations (PCAL). 24 hrs after the PCAL, *Apoe*$^{-/-}$ mice were intravenously injected with Dye 547-labeled miRNA inhibitor Ctrl (4 mg/kg bogy weight) in the naked form or encapsulated in the non-targeting or VCAM1-tageting micelles. 24 hrs after the injection, the mice were sacrificed and extensively perfused with PBS. Ligated left carotid arteries and non-ligated right carotid arteries were fixed by 4 % paraformaldehyde for 15 min and collected for *en face* imaging to detect the distribution of Dye 547-labelled miRNA inhibitor Ctrl in the vasculature. The fixed carotid artery was stained with Alexa fluor-488 labelled CD31 antibody (R&D, FAB6874G) and mounted on glass slides using fluorescence mounting medium containing DAPI (Abcam) to counterstain nucleus. The fluorescence images were taken by confocal laser scanning microscopy (CLSM, Leica SP5 2-Photon, Germany).

Pharmacokinetics of miRNA inhibitor *in vivo*

Adult *Apoe*$^{-/-}$ mice were intravenously injected with Dye 547-labeled miRNA inhibitor Ctrl (2 mg/kg bogy weight) in the naked form or encapsulated in the non-targeting or VCAM1-tageting micelles. At various predetermined time intervals (0, 30, 60, 120 and 240 min) after the injection, ~ 50-100 μL blood was drawn by submandibular bleeding. The plasma was obtained by centrifuging the blood at 5000 rpm for 10 min. Fluorescence intensity of Dye 547-miRNA inhibitor Ctrl in plasma was recorded at emission and excitation wavelengths of 540 nm and 580 nm after diluting 15 μL supernatant with 45 μL PBS. Pharmacokinetics was calculated by normalizing the fluorescence intensity of each time point with the initial (0 min) time point.

Biodistribution of miRNA inhibitor *in vivo*

Adult *Apoe*$^{-/-}$ mice were intravenously injected with Dye 547-labeled miRNA inhibitor Ctrl (2 mg/kg bogy weight) in the naked form or encapsulated in the non-targeting or VCAM1-tageting micelles. 24 hrs after the injection, mice were euthanized and the heart, lung, liver, spleen and kidney were harvested for the miRNA biodistribution analyses. Fluorescence imaging of organs was conducted using an in vivo imaging system (IVIS 200, PerkinElmer).

Treatments of aortic root atherosclerosis in *Apoe*$^{-/-}$ mice by miR-92a inhibitor delivered by VCAM1-targeting polyelectrolyte complex micelles

Male *Apoe*$^{-/-}$ mice were fed a high-fat diet (Harlan Teklad TD88137) starting at week 16 for 4 weeks. Two weeks after the start of high-fat diet (week 18), mice were subjected to one tail vein injection of the following agents: 1) PBS, 2) naked miR-92a inhibitors, 3) VCAM1-targeting micelles encapsulating miRNA inhibitor controls, and 4) VCAM1-targeting micelles encapsulating miR-92a inhibitors. Mice were intravenously administered with miRNA inhibitor at 8 mg/kg body weight or 4 mg/kg body weight and sacrificed at week 20 for lesion analyses. The animals were sacrificed, blood collected, and heart harvested after pressure perfusion with PBS via left ventricle after severing inferior vena cava. Hearts were fixed in 4% paraformaldehyde for 48 hours and embedded in OCT after being immersed in OCT:sucrose =1:1 mixed solution for 24 hours. Frozen embedded samples were sectioned in 8 μm thickness and stained with an Oil Red O Staining Kit (Sigma-Aldrich, MO, USA and ScienCell, CA, USA) according to manufacturers' instructions. The plaque areas were calculated using National Institutes of Health ImageJ software.

Treatments of carotid atherosclerosis induced by acute disturbed flow in *Apoe*$^{-/-}$ mice by miR-92a inhibitor delivered by VCAM1-targeting polyelectrolyte complex micelles

PCAL as described above and in previous studies, were conducted in male *Apoe*$^{-/-}$ mice (2). Mice were fed with high-fat diet (Harlan Teklad TD88137) after PCAL. Mice were subjected to three tail vein injections of the following agents: 1) PBS, 2) naked miR-92a inhibitors, 3) VCAM1-targeting micelles encapsulating miRNA inhibitor controls, and 4) VCAM1-targeting micelles encapsulating miR-92a inhibitors. Intravenous injections were conducted at day 5, day 8, and day 11 after the PCAL surgery. Each injection contained PBS or miR-92a inhibitor at 2 mg/kg body weight, delivered by the naked form or by VCAM1-targeting polyelectrolyte complex micelles. Injections of miRNA inhibitor control encapsulated in the VCAM1-targeting micelles served as an additional control. 14 days after the PCAL surgery, the mice were anesthetized to collect the blood and perfused with PBS. The LCA and contralateral carotid artery were excised and fixed by 4 % paraformaldehyde for 15 min, following by the treatment of 30 % sucrose to dehydrate overnight. The tissues were then embedded with optimal cutting temperature (OCT, Tissue Tek) and sectioned into slices with a thickness of 8 μm. To visualize the atherosclerosis plaque in LCA and the contralateral carotid artery, the sections were stained with Oil red O according to the protocol previously reported (2).

References for Supplementary Materials and Methods

1. J. Mao, J. Barrow, J. McMahon, J. Vaughan, A. P. McMahon, An ES cell system for rapid, spatial and temporal analysis of gene function in vitro and in vivo. *Nucleic Acids Res* **33**, e155 (2005).
2. D. Nam *et al.*, Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. *Am J Physiol Heart Circ Physiol* **297**, H1535-1543 (2009).

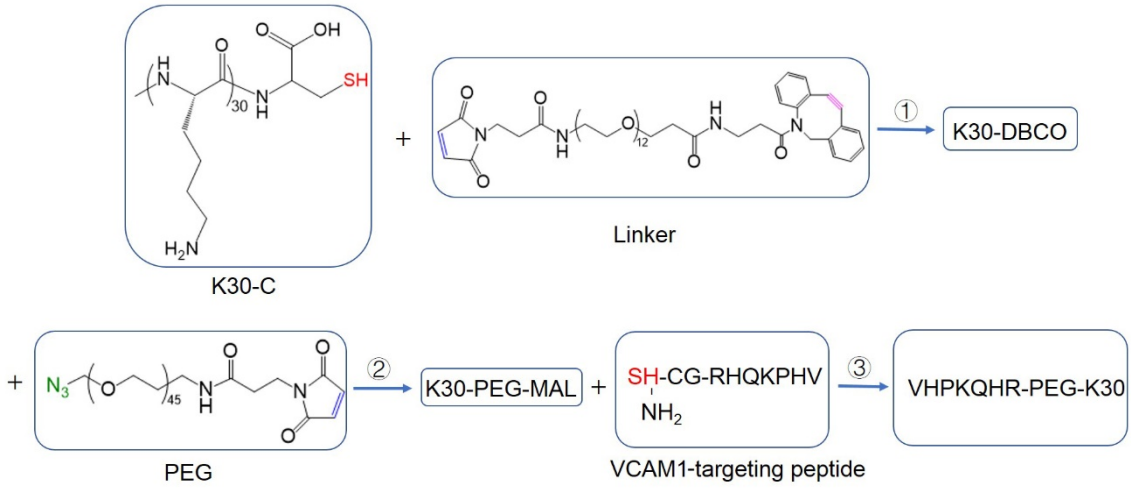

Figure S1. The chemical synthetic route of the VHPKQHR-PEG-K30 polymers. First, Sulfhydryl K30 (K30-C) conjugates to the maleimide group at the linker which has a terminal DBCO functional group, forming K30-DBCO. Second, K30-DBCO conjugates to PEG via DBCO-N_3 click chemistry to form K30-PEG-MAL. Third, VHPKQHR conjugates to the K30-PEG-MAL via the thiol-maleimide reaction to form the VHPKQHR-PEG-K30 polymers.

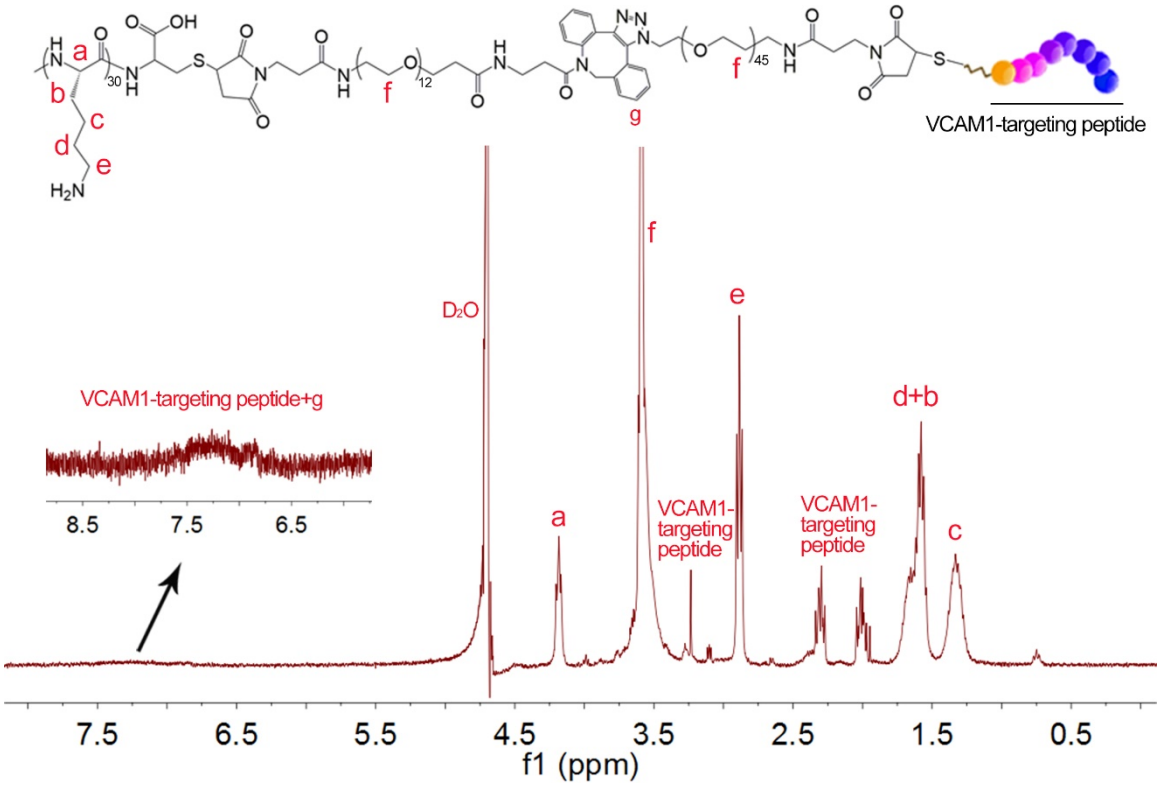

Figure S2. Nuclear magnetic resonance (NMR) spectroscopy of the VHPKQHR-PEG-K30 polymers.

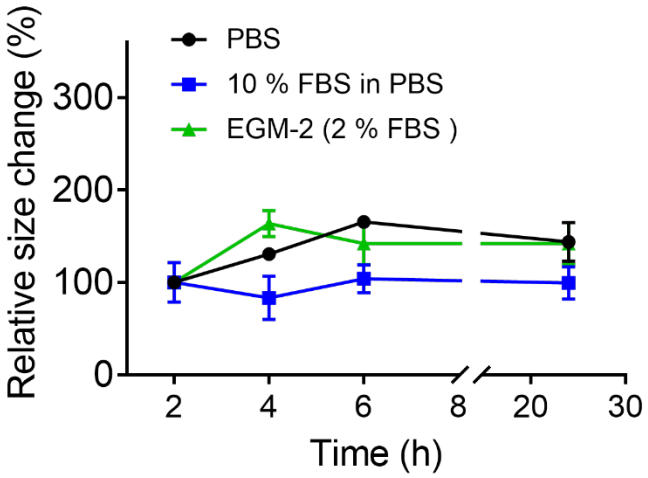

Figure S3. The relative size of the VCAM1-targeting, miRNA inhibitor-encapsulated polyelectrolyte complex micelles in PBS, PBS containing 10 % FBS, and EGM-2 medium containing 2 % FBS for 24 hrs. All error bars are means ± standard deviation.

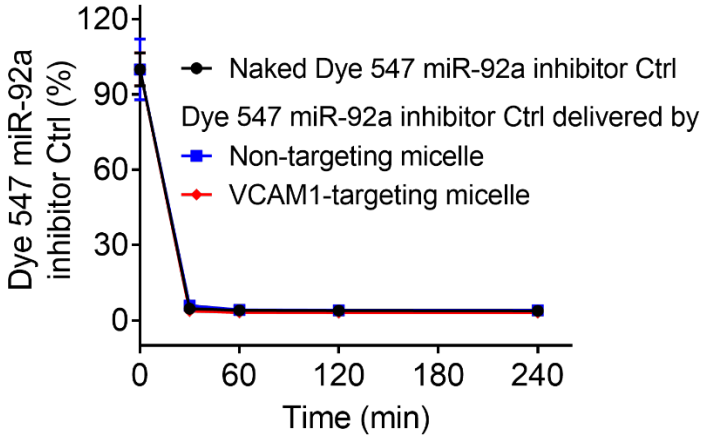

Figure S4. The pharmacokinetic profile of Dye 547-labeled miRNA inhibitor Ctrl in the circulation. The plasma terminal half-life of Dye 547-labeled miRNA inhibitor Ctrl was not significantly altered by the micelles. The majority of the miRNA inhibitor, whether in the naked form or in complex with the micelles, was cleared in the plasma in an hour (n= 3 biological samples). All error bars are means ± standard deviation.

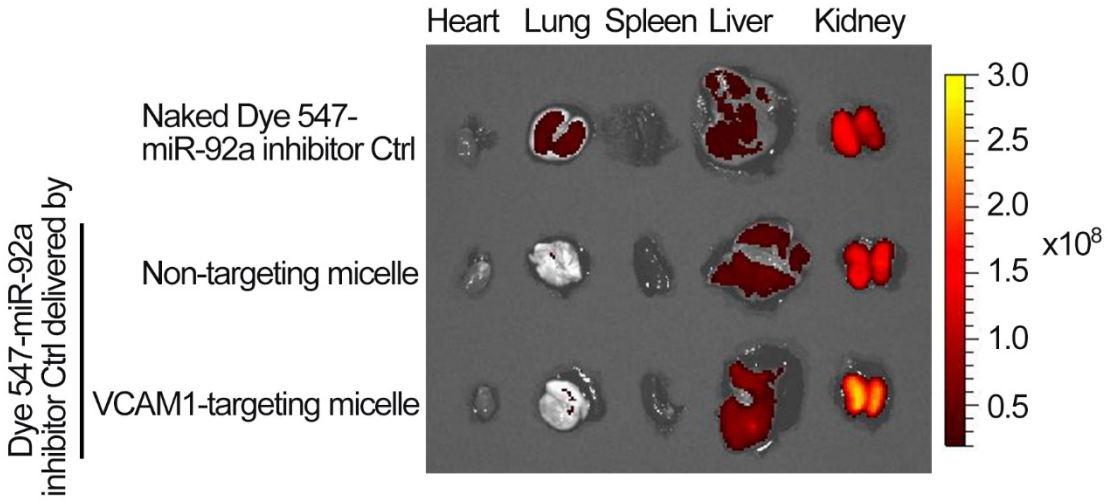

Figure S5. Representative biodistribution images of Dye 547-labeled miRNA inhibitor Ctrl delivered in the naked form or in complex with the polyelectrolyte complex micelles in mice 24 hours after a single intravenous dose (2 mg/kg body weight). Major organs were excised and collected, and fluorescence imaging of organs was conducted using an IVIS 200 imaging system.

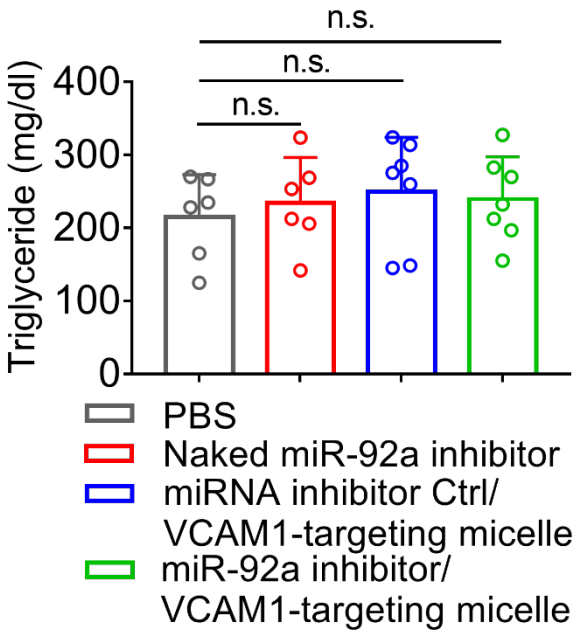

Figure S6. Plasma triglyceride levels in *Apoe*$^{-/-}$ mice subjected to PBS or miRNA inhibitors (8 mg/kg body weight) delivered in the naked form or by VCAM1-targeting polyelectrolyte complex micelles. Plasma triglycerides were not affected by injections of the therapeutic agents (n= 6-7 biological samples). Statistical significance determined by multiple unpaired one-tailed t-test. All error bars are means ± standard deviation. n.s., not significant.

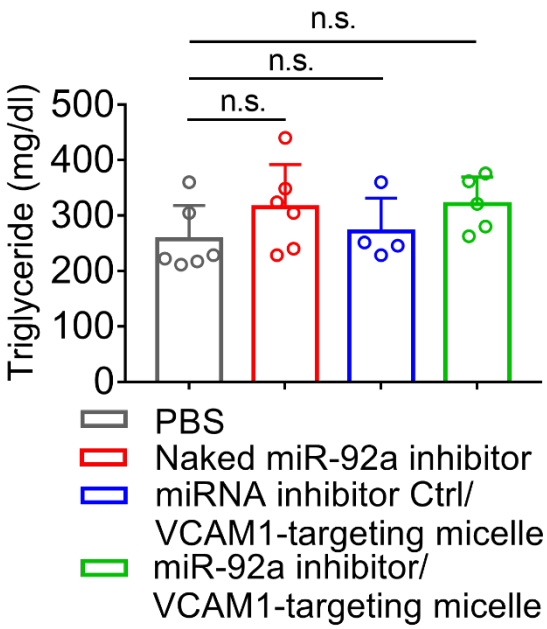

Figure S7. Plasma triglyceride levels in *Apoe*$^{-/-}$ mice subjected to PBS or miRNA inhibitors (4 mg/kg body weight) delivered in the naked form or by VCAM1-targeting polyelectrolyte complex micelles. Plasma triglycerides were not affected by injections of the therapeutic agents. (n= 4-6 biological samples). Statistical significance determined by multiple unpaired one-tailed t-test. All error bars are means ± standard deviation. n.s., not significant.

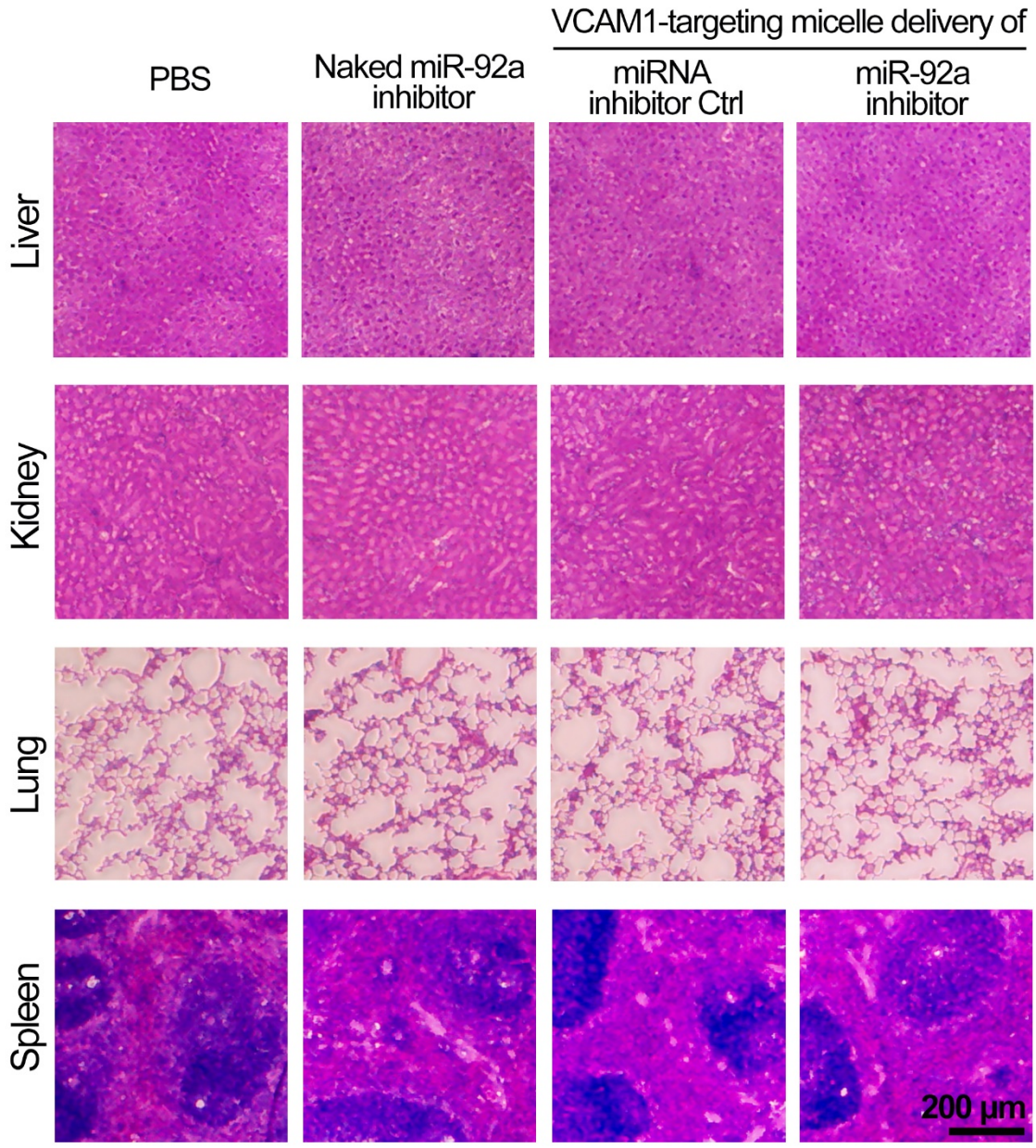

Figure S8. Histological sections of liver, kidney, lung, and spleen in *Apoe*$^{-/-}$ mice subjected to three doses of PBS or miRNA inhibitors (2 mg/kg body weight) delivered in the naked form or by VCAM1-targeting polyelectrolyte complex micelles. *Apoe*$^{-/-}$ mice were subjected to PCAL first and the therapeutic agents were administered, in the naked form or in complex with VCAM1-targeting micelles, via the tail vein on day 5, 8, and 11 after the surgery. Mice were sacrificed on day 14.