Materials and Methods

Animal Model

To induce atherosclerosis in mice, 4-6 week old male ApoE-null mice (The Jackson Laboratory, Bar Harbor, ME), were fed a Western diet (TD-88137, Harlan Laboratories, Madison, WI) for 3 months continuously. Following 3 months of Western diet, mice were continued on diet for 1 more month with treatments of either 1 ml/kg saline, control NP, or PPACK-NP three times per week for a total of 12 doses (Fig. 1B). In animals used for plaque permeability measurements, a 1ml/kg dose of crown ether (CE) NP was administered 2 hours prior to sacrifice for single detection by MRI/MRS (see below). Blood was harvested from the left ventricle and serum was submitted to the Washington University Department of Comparative Medicine for cholesterol measurements.

Nanoparticle Formulation

Therapeutic Nanoparticles: PPACK-NP were formulated using previously described emulsification and conjugation techniques.[1] Briefly, NP were first formulated as carboxyl-terminated perfluorocarbon nanoparticles composed of a 20% (vol/vol) perfluorooctylbromide (PFOB) core, 2% (wt/vol) surfactant, 1.7% (wt/vol) glycerin, and water. The surfactant used in this formulation consisted of 99% egg phosphatidylcholine, EPC (Avanti Polar Lipids, Alabaster, AL) and 1 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000], DSPE-PEG2000-COOH (Avanti Polar Lipids, Alabaster, AL). Following emulsification of the mixed surfactant, PFOB, glycerin, and water, the resulting precursor nanoparticle was activated for coupling of PPACK to the surface carboxyl groups with the use of 2 mg/ml 1-ethyl-3-(3-dimethylaminopropyl carbodiimide HCl, EDC (Pierce, Rockford, IL). Amine coupling of PPACK to the nanoparticle was then completed with the addition of 12.5 mg/ml PPACK (American Peptide Company, Sunnyvale, CA) to the activated precursor formulation following overnight mixing. The conjugated nanoparticle formulation was then dialyzed against MilliQ water with a MWCO of 3000-5000 for 4 hours with stirring. Size and zeta potential were measured on a ZetaPlus analyzer (Brookhaven Instruments Corporation, Holtsville, NY) and were determined to be 245.3 ± 3.5 nm and -6.13 ± 0.64 mV, respectively.

Imaging Nanoparticles: NPs were formulated as described above with the following composition: 20% (vol/vol) perfluoro-15-crown-5-ether (CE) core, 2% (wt/vol) surfactant, 1.7% (wt/vol) glycerin, and water. The lipid composition for the imaging nanoparticles consisted of 99% egg phosphatidylcholine, EPC, and 1 mol% 1,2-dipalmitoyl-sn-glycero-3- phosphoethanolamine (Avanti Polar Lipids, Alabaster, AL, USA).

^{19}F Magnetic Resonance Spectroscopy For Quantification of Nanoparticle Deposition in Plaques

Fluorine magnetic resonance spectroscopy (^{19}F-MRS) enables absolute quantification of plaque endothelial barrier disruption (Fig. 1C) by measuring the deposition of fluorine-core nanoparticles into aortic intimal plaques as described in previous work.[2,3] There are several practical advantages of this method over traditional

staining approaches. The semipermeant 250 nm diameter PFC NP circulating in vivo provide a *functional* and selective metric for plaque endothelial barrier damage as they do not penetrate adjacent vascular segments devoid of plaque. Nor do they register the very early stages of endothelial dysfunction (e.g., weakening of tight junctions), but rather correspond to a later stage of endothelial disruption that correlates directly with procoagulant activity.[3] The measurement is fully quantitative based on 19F spectroscopy, and objective because an entire unprocessed aortic segment is utilized without need for subjective selection of regions of interest or interpretation of selected microscopic sections. Finally, the permeation of NP is entirely passive and does not involve cell trafficking after NP uptake as it operates similarly ex vivo in excised and formalin fixed specimens.[2,3]

Measurements were carried out on an 11.7T Varian MR scanner using a custom-built single solenoid RF coil. All measurements were conducted with the following parameters in each fluorine measurement: TR = 2.5s, 1024 signal averages, and a total scan time of ~42 minutes per sample. A reference standard of 0.1% trifluoroacetic acid (TFA) was included in each aorta sample to allow for absolute quantification of the perfluro-crown ether signal from the nanoparticles compared to the known amount of TFA.[4] For each sample, the detected amount of nanoparticles was normalized based on the weight of the aorta.

Photochemical Injury of the Carotid Artery to Assess Vascular Procoagulant Activity

Mice were anesthetized with ketamine (87 mg/kg) and xylazine (37 mg/kg) followed by isolation of the right common carotid artery through a midline cervical incision. A Doppler ultrasound probe (Transonic Systems, Inc., Ithaca, NY) was placed on the carotid artery to monitor blood flow rate for the duration of the experiment. Mice were administered a dose of 50mg/kg Rose Bengal (Sigma-Aldrich, St. Louis, MO) in saline to initiate thrombus growth following illumination of the injury site with a 1.5 mW 540 nm HeNe laser. The injury procedure concluded upon achieving a >85% decrease in measured carotid blood flow rate that was maintained for >5 minutes, indicative of stable occlusion of the carotid artery. Time to carotid occlusion was measured as a metric of coagulability where increased time to occlusion indicated increased potential for coagulation (Fig. 1D).

Cell Culture

Human aortic endothelial cells (HAECs) were obtained from Lifeline Cell Technology (Frederick, MD) and cultured in VascuLife EnGS Endothelial Cell Culture Medium (Lifeline Cell Technology), which was composed of Vasculife Basal Medium supplemented with 0.2% Endothelial Cell Growth Supplement, rhEGF (5 ng/ml), ascorbic acid (50 µg/ml), L-glutamine (10 mM), hydrocortisone hemisuccinate (1 µg/ml), heparin sulfate (0.75 U/ml) and 2% fetal bovine serum (FBS). THP-1 cells were obtained from ATCC and cultured in RPMI-1640 supplemented with L-glutamine (300 µg/ml) and 10% FBS. Cells were used at passages 2-4 for all experiments.

Inflammatory Signaling Events

PAR-1 Assay: A previously described method[5,6] was utilized on HAECs to assay the inhibition of PAR-1 activation by thrombin. HAECs were incubated with 10 nM human thrombin (Haematologic Technologies, Essex Junction, VT) in assay medium (consisting of Vasculife Basal Medium) supplemented with either PBS, 10 nM PPACK,

0.8 nM plain PFOB NPs, or 0.8 nM PPACK NP for 2 hours with N=3 per group. The molar amounts of treatments were determined based on a 1:1 ratio of thrombin to inhibitor. Following incubation of HAECs with thrombin and each respective treatment group, HAECs were washed three times using PBS without Ca^{2+} and Mg^{2+} followed by cell harvesting with Nonenzymatic Cell Dissociation Solution (Sigma-Aldrich, St. Louis, MO). The harvested cells were washed and pelleted in FACS Incubation Buffer (0.5% bovine serum albumin in PBS without Ca^{2+} and Mg^{2+}), followed by staining with a phycoerythin-labeled antibody against residues 35-46 of the PAR-1 receptor, corresponding to the cleavage site of PAR-1 (SPAN12, Beckman-Coulter, Brea, CA) for 30 minutes. Following staining, cells were washed in FACS Incubation Buffer three times each prior to and following fixation with 1% paraformaldehyde. Samples were analyzed on a BD FACScan Analytic Flow Cytometer, with ten thousand events collected per sample. Data was analyzed with FlowJo Collectors Edition.

Surface Tissue Factor Activity Assay: To measure the activity of surface exposed tissue factor on thrombin-activated cells, a functional assay of factor Xa generation was utilized.[7] Cells were plated in 96 well plates at 30,000 cells per well and allowed to adhere overnight. Following overnight acclimation, cells were exposed to thrombin at 1 U/ml or 4 U/ml in serum-free medium containing equimolar amounts of either saline, free PPACK, control NP or PPACK-NP for 6 hours. Following stimulation with thrombin and each respective treatment, cells were rinsed three times with TF Activity Assay Buffer containing 50 mM HEPES, 150 mM NaCl, and 5 mM $CaCl_2$ at pH 7.5. Cells were then exposed to Factor VIIa (4 nM, Haematologic Technologies) and Factor X (300 nM, Haematologic Technologies) in 150 µl of TF Activity Assay Buffer for 30 minutes. Following factor VIIa/X incubation, the reaction conversion was stopped with 5 µl of 100 mM EDTA. Generated Factor Xa was then assayed with the addition of 100 µl of 500 µM Chromogenix S-2222 (Diapharma, West Chester, Ohio), and allowed to incubate for 20 minutes. Cleavage of S-2222 was stopped with 10 µl of 30% acetic acid and the plate was then read at 405 nm on a BioRad Model 550 Microplate Reader.

NF-kB Assay: HAECs and THP-1 cells were seeded on to coverslips at 100,000 cells/coverslip. For THP-1 seeded coverslips, coverslips were treated with 0.01% poly-L-lysine solution for 15 minutes, followed by 2 hours of drying prior to cell seeding. Following overnight incubation to allow for cell attachment, cells were stimulated with either thrombin alone or thrombin treated with PPACK, control NP, or PPACK-NP for 6 hours. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS. Cells were stained for phosphorylated p65 and IkB as markers of NF-kB activation using a phospho-p65 primary antibody (1:200 dilution, ab28856, Abcam, Cambridge, MA) or an IkB primary antibody (1:200 dilution, ab7217, Abcam) and and Dylight 488 secondary antibody (1:500 dilution, ab96899, Abcam). Coverslips were mounted onto slides with DAPI loaded mounting medium to counterstain for nuclei and cells were imaged using fluorescent microscopy.

TAT and sVCAM Assays: For measurements of thrombin-antithrombin (TAT) complexes and soluble VCAM-1 (sVCAM-1), blood was harvested from the left ventricle and serum was stored for ELISA analysis. TAT-complexes were measured on serum[8] using an ELISA kit (ab137994, Abcam) as per the manufacturers instructions. For sVCAM-1 measurements, serum was assayed using an ELISA kit (MVC00, R&D Systems Inc., Minneapolis, MN) as per the manufacturers instructions

Histological Analyses

For evaluation of gross plaque deposition in the aortic arch, aortas were removed a pinned *en face* for staining of plaques with Sudan IV. Tissues were fixed in 10% formalin for 24 hours, then stained with Sudan IV. Quantification of positive fat staining was carried out in the aortic arch for saline treated mice (N=5) and PPACK-NP treated mice (N=7) using ImageJ. Arch segments were selected using the top 1/3 portion of the aorta and uniformity of segments for analysis was verified through ImageJ measurements of total surface area for the region of interest. For histological analysis of plaque characteristics, tissues were stored in in optical cutting medium (OCT) and frozen sectioned at a thickness of 5 μm. Antibodies against Tissue Factor (1:100 dilution, sc30201, Santa Cruz Biotechnology, Dallas, Texas), phospho-p65 (1:200 dilution, ab28856, Abcam), von Willebrand Factor (1:100 dilution, ab11713, Abcam), and macrophages (MOMA-2, 1:100 dilution, ab33451, Abcam) where used to stain tissue sections. Quantification of positive staining for tissue factor, phospho-p65 and macrophages was accomplished using ImageJ with N=3 mice per treatment group. Additionally, secondary confirmation of macrophage staining was conducted with an antibody against the F4/80 antigen (1:100 dilution, ab6640, Abcam) along with isotype control staining (Rat IG2b, 1:100, ab18536, Abcam) to rule out nonspecific binding of the MOMA-2 antibody to mouse aortic tissue.

Activated Partial Thromboplastin Time

For measurement of activated partial thromboplastin time (APTT), blood was drawn from the ventricle at the time of sacrifice into a syringe containing 4% sodium citrate for APTT measurements at a final dilution of 1 part sodium citrate to 9 parts whole blood. Blood samples were centrifuged for 15 minutes at 1000g and the supernatant was saved for APTT measurements. APTT kits (HemosIL, Lexington, MA) were used as per the manufacturers instructions.

Pharmacokinetics of PPACK-NP using in vivo ^{19}F MRI

To elucidate the pharmacokinetics of PPACK-NP, C57/BL6 mice under anthestheia (Ketamine, 54mg/kg/hr and Xylazine (8.2mg/kg/hr)) were administered a 1mL/kg bolus dose of plain PFC-NP, PEGylated PFC-NP or PPACK-NP using a jugular vein catheter and ^{19}F MR signal in the tail was measured on a 4.7T MR imaging system (Varian). The ^{19}F spectra was obtained using a custom-built four-turn solenoid coil through ^{19}F spectroscopy (256 signal averages, TR=1.05s). The decay of in vivo ^{19}F signal intensity detected in the tail was fit to a bi-exponential curve, with the resulting exponents interpreted as half-lives in a two compartment model. For in situ imaging of nanoparticle biodistribution, mice were given a single bolus dose of PFC-NP and sacrificed two hours after administration. Proton (TR=0.5s, TE=0.02s, 256x256 matrix, field of view = 8 cm x 5 cm, 2.2 minute acquisition time) and ^{19}F (TR=0.8s, TE=0.121s, 64 signal averages, 32x32 matrix, field of view = 8 cm x 5 cm, 2 cm image depth, acquisition time = 27.3 minutes) spin echo images were obtained post-mortem on a 11.7T MR imaging system (Varian).

Statistics

All statistical tests were performed on R, version 3.0.1. Student's *t* test was utilized for all data, with $p<0.05$ denoting statistical significance. The Pearson's product-moment correlation test was utilized to determine the relationship between NP accumulation and carotid occlusion time. Error bars represent standard error of the mean.

Study Approval

All animal experimental procedures were performed with approval from the Washington University Animal Studies Committee.

Work Cited

1. Myerson J, He L, Lanza G, Tollefsen D, Wickline S. Thrombin-inhibiting perfluorocarbon nanoparticles provide a novel strategy for the treatment and magnetic resonance imaging of acute thrombosis. *J. Thromb. Haemost.* 2011;9(7):1292–1300. doi:10.1111/j.1538-7836.2011.04339.x.

2. Zhang H, Zhang L, Myerson J, Bibee K, Scott M, Allen J, Sicard G, Lanza G, Wickline S. Quantifying the evolution of vascular barrier disruption in advanced atherosclerosis with semipermeant nanoparticle contrast agents. *PLoS ONE* 2011;6(10):e26385–e26385. doi:10.1371/journal.pone.0026385.

3. Palekar RU, Jallouk AP, Goette MJ, Chen J, Myerson JW, Allen JS, Akk A, Yang L, Tu Y, Miller MJ, Pham CTN, Wickline SA, Pan H. Quantifying progression and regression of thrombotic risk in experimental atherosclerosis. *FASEB J* 2015;29(7):3100–3109. doi:10.1096/fj.14-269084.

4. Morawski AM, Winter PM, Yu X, Fuhrhop RW, Scott MJ, Hockett F, Robertson JD, Gaffney PJ, Lanza GM, Wickline SA. Quantitative “magnetic resonance immunohistochemistry” with ligand-targeted (19)F nanoparticles. *Magn Reson Med* 2004;52(6):1255–1262. doi:10.1002/mrm.20287.

5. Chen J, Vemuri C, Palekar RU, Gaut JP, Goette M, Hu L, Cui G, Zhang H, Wickline SA. Antithrombin nanoparticles improve kidney reperfusion and protect kidney function after ischemia-reperfusion injury. *Am. J. Physiol. Renal Physiol.* 2015;308(7):F765–F773. doi:10.1152/ajprenal.00457.2014.

6. Tull SP, Bevins A, Kuravi SJ, Satchell SC, Al-Ani B, Young SP, Harper L, Williams JM, Rainger GE, Savage COS. PR3 and elastase alter PAR1 signaling and trigger vWF release via a calcium-independent mechanism from glomerular endothelial cells. *PLoS ONE* 2012;7(8):e43916. doi:10.1371/journal.pone.0043916.

7. Takeya H, Gabazza EC, Aoki S, Ueno H, Suzuki K. Synergistic effect of sphingosine 1-phosphate on thrombin-induced tissue factor expression in endothelial cells. *Blood* 2003;102(5):1693–1700. doi:10.1182/blood-2002-11-3607.

8. Hyacinth HI, Adams RJ, Greenberg CS, Voeks JH, Hill A, Hibbert JM, Gee BE. Effect of Chronic Blood Transfusion on Biomarkers of Coagulation Activation and Thrombin Generation in Sickle Cell Patients at Risk for Stroke. *PLoS ONE* 2015;10(8):e0134193. doi:10.1371/journal.pone.0134193.