Molecularly Targeted Nanoparticles for Modulation of Inflammatory Mediators in Atherosclerosis

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Molecularly Targeted Nanoparticles for Modulation of Inflammatory Mediators in Atherosclerosis

by

Rohun Palekar

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF THE DISSERTATION

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By

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Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2015

Professor Samuel A. Wickline, Chair

The enzyme thrombin has been demonstrated through experimental and clinical studies to play a crucial role in mediating both inflammation and thrombosis in atherosclerosis. The cellular effects of thrombin in promoting atherosclerosis involve the activation of signaling pathways that result in the secretion of a host of various chemokines, cytokines, cell adhesion molecules, etc. that promote vascular inflammation. Due in part to thrombin and other pro-atherogenic molecules, this prolonged inflammatory state in atherosclerosis results in the deterioration of the endothelium, increasing the risk of focal thrombosis. Current treatment strategies to address the role of thrombin in atherosclerosis, despite efficacy of anticoagulant activity, suffer from significant bleeding side effects. Thus, the goal of this dissertation was to address the central role that thrombin plays in atherosclerosis and thrombosis through the application of perfluorocarbon nanoparticles carrying the direct thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone, to impart focal anti-inflammatory and
anticoagulant effects at sites of high thrombin activity. In this work, we demonstrate the ability of thrombin-inhibiting nanoparticles and liposomes to form localized "anticlotting" surfaces that inhibit the growth of clots both in vivo and on ex vivo applications on bare metal vascular stents. We also investigate the use of quantitative fluorine magnetic resonance spectroscopy ($^{19}$F-MRS) to demonstrate an inverse relationship between endothelial barrier integrity and propensity for thrombosis. We show that increased exposure of an atherosclerotic animal to an inflammatory stimulus (e.g. cholesterol) over time decreases endothelial barrier integrity and increases hypercoagulability, however resolution of these symptoms after removal of the inflammatory stimulus can be tracked using $^{19}$F-MRS. Furthermore, we demonstrate through proof-of-concept imaging studies, the possibility for non-invasive imaging of endothelial barrier disrupted atherosclerotic plaques. These concepts in mind, our final objective was to utilize the ability to deliver localized thrombin inhibition with thrombin-inhibiting nanoparticles and demonstrate their ability to limit the effect of vascular inflammation on loss of endothelial barrier integrity, as measured by $^{19}$F-MRS. Our results indicate that within one month of treatment with thrombin-inhibiting nanoparticles, ApoE-null atherosclerotic mice presented with diminished endothelial barrier loss, reduced hypercoagulability, and an overall 22.5% decrease in aortic arch plaque deposition.

Future work on this platform would involve the improvement of dosing regimens that may address earlier time points of atherosclerosis development that may have a greater effect on induction of vascular inflammation. Nevertheless, these results demonstrate for the first time, the utility of focal thrombin inhibition as a means for a limiting vascular inflammation and hypercoagulability in atherosclerosis.
Chapter 1: Introduction
1.1 Atherosclerosis, Inflammation and Coagulation

Atherosclerosis and associated acute vascular syndromes represent the underlying cause of strokes and heart attacks, which cause the greatest patient mortality in the Western world.\textsuperscript{1} Atherosclerosis is a highly inflammatory disease characterized by the development of fatty plaques in the intimal layer of arteries. Early stages of atherosclerosis are initiated by the accumulation of oxidized low-density lipoprotein (oxLDL) in the vessel wall, resulting in endothelial damage that promotes the generation of thrombin as a wound healing response which in turn, promotes the expression of cytokines and chemokines that promote the recruitment of inflammatory cell types, e.g. monocytes and T lymphocytes. This prolonged inflammatory state induces further thrombin generation as a response to local inflammation, establishing a feedback loop of hypercoagulability and inflammation with thrombin playing a central role.\textsuperscript{2}

1.1.1 Thrombin and Protease-Activated Receptors

The enzyme thrombin plays a central role coagulation, inflammation and atherosclerosis and vascular pathophysiology through several pleiotropic functions. Within the coagulation cascade, thrombin functions in converting fibrinogen into fibrin, along with various feedback mechanisms within the coagulation cascade that amplify clot formation and thrombin generation including activation of Factor V, Factor VIII, Factor XI and Factor XIII.

Along with its role in the coagulation cascade, thrombin has been implicated as a powerful signaling effector through its actions on a family of G-protein coupled receptors (GPCRs) known as protease-activated receptors (PARs), namely PAR-1. The PAR-1 receptor is expressed on the surface of a wide range of cell types, including endothelial cells, vascular smooth muscle cells, macrophages, and platelets among others. Thrombin exerts its effect on
PAR-1 (EC$_{50}$ ~ 50 pM) through binding to the N-terminal extracellular domain of the receptor, cleaving after the Arg41 residue to expose the N-terminal tethered ligand sequence SFLLRN that subsequently activates the PAR-1 receptor and downstream signaling pathways. The effects of PAR-1 activation on cells by thrombin has been demonstrated to be a powerful effector of vascular physiology.

Important molecular signaling events induced by thrombin and PAR-1 activation depend in part on the NF-$\kappa$B pathway. Activation of NF-$\kappa$B has previously been shown to be a powerful contributor to the progression of atherosclerotic disease, mediating the expression of various cytokines and chemokines implicated in atherosclerosis, including but not limited to, vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), monocyte chemoattractant protein 1 (MCP-1), platelet derived growth factor (PDGF) and tissue factor (TF). Activation of NF-$\kappa$B occurs upon cleavage of PAR-1 by thrombin (Fig. 1-1) followed by signaling through G-proteins ($G_{\alpha \text{q}}$ and $G_{\beta \gamma}$) to stimulate IKK, which in turn results in the phosphorylation and degradation of I$\kappa$B, thus releasing the p65 homodimer that can then translocate to the nucleus and facilitate the transcription of inflammatory genes.

1.1.2 The Role of the Endothelium in Homeostasis

The inflammatory state of the vasculature initiated by accumulation of oxLDL and propagated by inflammatory mediators such as thrombin has quantifiable detrimental effects on the endothelium. This inflamed environment promotes the formation of a dysfunctional endothelium that is characterized by weakened or broken tight junctions that allow increased permeability to macromolecules (such as secreted cytokines and chemokines), with further
breakdown of the endothelial barrier through loss of endothelial cells due to apoptosis\textsuperscript{3} or senescence\textsuperscript{4}, thus predisposing patients to focal atherothrombotic events.\textsuperscript{5}

Maintenance of the endothelial barrier is a normal part of homeostasis. The endothelium serves as a natural anticoagulant barrier in its healthy state, but becomes procoagulant in the event of endothelial dysfunction and loss of endothelial cells. In fact, endothelial cell loss as evaluated through measurements of endothelial cells circulating in the blood has been correlated with an increased incidence of cardiovascular disease and thrombosis. Clinical studies on patients with coronary artery disease\textsuperscript{6}, acute myocardial infarction\textsuperscript{7}, and peripheral arterial disease\textsuperscript{8} all demonstrate increased circulating endothelial cell counts associated with increased severity of disease.

While circulating endothelial cells as a potential biomarker for endothelial damage and atherosclerotic severity remain important avenues of research, the specificity of such measurements is limited. An approach that might be useful to facilitate medical management of vascular diseases would be to enable more precise identification of plaques that are at risk of acute thrombosis, or "hypercoagulable plaques". Existing methods such as intravascular ultrasound (IVUS), optical coherence tomography (OCT) and near-infrared spectroscopy have all been previously used clinically to identify "at risk" plaques, however these methodologies are largely unable to detect the >1/3 of lesions that occlude as a result of plaque erosions\textsuperscript{5} rather than rupture of thin-capped fibrous atheromas (TCFAs).\textsuperscript{9} These erosions are largely undetectable prior to histopathological analysis of patient samples and remain an important target for the development of non-invasive diagnostic techniques.
1.1.3 Existing Strategies for Atherothrombosis Therapy

Existing antithrombotic treatments such as heparin have been effective in limiting the activity of thrombin, as measured primarily through its inhibitory effect on coagulation. However, the use of heparin has several limitations. First, as with many existing antithrombotic treatments, systemically delivered heparin results in significant side effects with respect to risk of hemorrhage and in some specific cases, development of a condition known as heparin-induced thrombocytopenia that is characterized by the generation of antibodies against heparin-platelet-factor-4 complexes, which in turn activate platelets and induce thrombosis.\textsuperscript{10} Second, the antithrombotic actions of heparin are largely based on its interactions with cofactors for effective anticoagulant effect. Inhibition of thrombin (and Factors Xa, XII, XI, IX and the TF-VIIa complex) by heparin occurs through interaction of thrombin with a complex consisting of heparin and antithrombin III.\textsuperscript{11} One interesting drawback of heparin in its interaction with thrombin is the ability of heparin to serve as a bridge between exosite 2 on thrombin (i.e., the heparin binding domain) and fibrin. In this "fibrin-bound" state, the enzymatic activity of thrombin is protected to some extent, and thus the fibrin-bound thrombin serves a potent source of enzymatic activity that is largely not inhibited by heparin-antithrombin III complexes.\textsuperscript{12}

A new class of anticoagulants known as "direct thrombin inhibitors" or DTIs has recently gained favorability due to their improved efficacy and safety profiles. As the name implies, this class of inhibitors aims to address the crucial role of thrombin in mediating coagulation by directly binding to the active site, or exosites on the thrombin enzyme to inhibit its activity. These DTIs improve upon prior anticoagulant treatments, especially regarding specificity, because they function as inhibitors of thrombin that bind directly to the active site without the need for a cofactor and exert inhibitory effects on both free and fibrin-bound thrombin.
Several direct thrombin inhibitors have been approved by the FDA for inhibiting thrombosis in selected indications, and more recently some have been evaluated for inhibiting the inflammatory signaling activity of thrombin in atherosclerosis. For instance, inhibitors such as bivalirudin (Angiomax, The Medicines Company) have been approved and successfully used for treatment of unstable angina.\(^{13}\) Other inhibitors such as orally administered dabigatran (another active site inhibitor) have been approved for the prevention of stroke in patients with atrial fibrillation along with pulmonary embolism (PE) and venous thromboembolism (VTE).\(^{14}\) Recent works have also explored the utility of orally administered dabigatran\(^ {15-17}\) as a treatment for atherosclerosis in mice, which have demonstrated diminished plaque deposition and vascular inflammation with continuous administration over the course of high cholesterol feeding of ApoE knockout mice.

A notable drawback in these uses of DTIs is that the systemic administration of these drugs results in significant bleeding tendencies. Accordingly, in the present work we propose that the use of nanoparticles as a method of drug delivery for \textit{focal suppression} of thrombin activity would occur by concentrating the anticoagulant moieties to hypercoagulable plaque sites and concomitantly by rapid diminution of systemic anticoagulant effects by clearance of residual circulating nanoparticles that would improve the safety profile.

\textbf{1.2 Nanotechnology for Drug Delivery and Imaging}

One significant advantage of nanoparticles is their ability to serve as targeted agents for imparting imaging contrast or drug delivery through passive targeting as a result of the enhanced permeability and retention effect (EPR)\(^ {18}\), or through surface modifications where nanoparticles can be functionalized with targeting moieties (i.e. antibodies, peptides) to accomplish ligand-
directed targeting.\textsuperscript{19,20} Additionally, surface modifications can include attachment of drugs, imaging agents or polyethylene glycol (PEG) chains of various lengths to impart favorable tissue half-lives. Through site-specific targeting and modulation of pharmacokinetic (PK) parameters, highly concentrated doses of drugs and imaging agents can be delivered to a disease site with diminished systemic side effects, offering the opportunity to accomplish increased efficacy of imaging and therapy, as exemplified by nanoparticulate formulations of doxorubicin that have already achieved FDA-approval for clinical use (i.e. Doxil/Caelyx).\textsuperscript{21}

For effective clinical diagnostic utility, nanoparticles must be designed to achieve high stability, selective binding, low toxicity, and favorable contrast-to-noise tissue enhancement. The design of nanoparticles must be well controlled to allow for reproducible scale-up of synthesis that would preserve the purity of the formulation, while maintaining uniform potency. Certain trade-offs exist in the design of nanoparticles for effective drug delivery and imaging, where surface functionalization to promote favorable biodistribution and PK parameters may interfere with ligand-targeting strategies. Furthermore, sizing considerations are pertinent where deeper penetration into affected tissues may be advantageous.\textsuperscript{22} Despite these considerations, the use of nanoparticles as diagnostic contrast agents presents a unique opportunity to facilitate non-invasive molecular imaging of biological processes such as inflammation and thrombosis by targeting of important pathogenic molecules.

1.2.1 Magnetic Resonance Imaging and Spectroscopy

Magnetic resonance imaging is based on the principle of the nuclear magnetic resonance (NMR) phenomenon. When placed in a magnetic field ($B_0$), the spins of electrons and protons in a sample will orient themselves either parallel or antiparallel to $B_0$, corresponding to lower or
higher energy states, respectively. The distribution of parallel and antiparallel spins is generally equally distributed outside of a magnetic field, resulting in a net magnetization vector of zero, but upon encountering the $B_0$ magnetic field, this distribution is altered, resulting in a net non-zero magnetization vector that precesses at some frequency (i.e., the Larmor frequency) depending on the field strength of the magnet and the physical properties (i.e., the gyromagnetic ratio) of the particular nucleus being studied. Upon external radiofrequency excitation, these spins can absorb energy and be “tilted away” from the direction of the $B_0$ field. After ceasing the radiofrequency pulses, the spins then return to their original state in a process referred to as relaxation. Using a detection coil that is tuned specifically to the Larmor frequency of the nuclei of interest, it is possible to register the radiofrequency energy released upon return of these spins back to equilibrium as this relaxation generates an oscillating current in the detection coil that can then be processed and displayed as a Free Induction Decay (FID). A signature spectrum for a compound of interest can then be generated through Fourier transform of the FID. This generated spectrum is quantitative in that the integral of each generated peak is proportional to the number of nuclei in the sample, and serves as the basis for the quantitative magnetic resonance spectroscopy used in this dissertation.

By the external application of magnetic field gradients to the aligned spin system, it is possible to encode spatial localization by altering the frequency and phase of the nuclei contained in a sample of interest. The generation of an image is based on signal intensities in each given voxel that in turn is affected by numerous parameters including the type of pulse sequence/imaging parameters (repetition time, TR; echo time, TE; flip angle, etc.), that affect the intrinsic relaxation rate of a particular sample. Signal contrast in MRI, often defined by “T1” (spin-spin) or “T2” (spin-lattice) relaxation times, depends on the nature of the pulse sequence
used to excite and then measure the relaxing spins. Exogenous contrast agents may be employed to alter local T1 or T2 relaxation times to produce highly enhanced tissue contrast as compared to the expected background T1 or T2 signals.\textsuperscript{23}

1.2.2 Fluorine Imaging and Spectroscopy with Perfluorocarbon Nanoemulsions

Fluorine magnetic resonance imaging (\textsuperscript{19}F MRI) and spectroscopy (\textsuperscript{19}F MRS) utilize unique properties of fluorinated compounds that are advantageous for NMR studies over other non-proton (\textsuperscript{1}H) molecules.\textsuperscript{24} First, \textsuperscript{19}F NMR exhibits a large chemical shift range of \textasciitilde 300 ppm that allows multiple fluorinated compounds to be detected simultaneously without risk of signal overlap. The gyromagnetic ratio of fluorine nuclei is relatively close to that of \textsuperscript{1}H (42.57 MHz/T vs. 40.07 MHz/T respectively). This 6\% difference along with a relative sensitivity of 0.83 is favorable, as the MRI signal for fluorine would be the most sensitive of all non-proton molecules used for MRI.\textsuperscript{25} Furthermore, the use of fluorinated compounds for MRI/MRS as exogenous contrast agents in biologic tissue offers the considerable advantage of virtually zero background signal thereby markedly enhancing conspicuity of the contrast agent whose signature is unique as compared with that of water protons. Thus, these properties make fluorine an attractive candidate for quantitative molecular imaging.

Our lab has pioneered the development of fluorinated nanoparticles as ultrasound\textsuperscript{26,27} and MRI contrast agents.\textsuperscript{28-30} These nanoparticles consist of a perfluorocarbon (PFC) core surrounded by a stabilizing lipid-surfactant monolayer, resulting in nanoparticles ranging in diameter from 200-250 nm (Fig. 1-2A). The outer lipid shell can be functionalized with drugs, targeting, or imaging agents through covalent conjugation\textsuperscript{31,32} or insertion of compounds into the membrane.\textsuperscript{33-37}
In addition to varying the type of targeting agents, it is possible to substitute a variety of perfluorocarbon cores, with differing spectral signatures. Perfluorocarbon molecules consist primarily of hydrocarbons that can be fluorinated by complete substitution of hydrogen atoms with fluorine atoms. Numerous such fluorocarbons exist, and two are routinely used in our laboratory for diagnostic imaging and spectroscopy, namely perfluoro-15-crown-5 ether (PFCE, $\text{C}_{10}\text{F}_{20}\text{O}_5$) and perfluoroctylbromide (PFOB, $\text{CF}_3-(\text{CF}_2)_6-\text{CF}_2\text{Br}$). The characteristic spectrum for PFOB consists of 8 distinct peaks, which contrasts with the single peak spectrum for PFCE. The presence of a single peak for PFCE makes it an ideal candidate for spectroscopy and imaging as signal to noise ratio is greatly improved over that of PFOB due to the total measured energy being confined to a single peak rather than being split into multiple peaks that are more challenging to register simultaneously. As previously discussed, the large chemical shift range of fluorine allows for the detection of multiple fluorinated compounds without signal overlap and as such, detection of PFCE nanoparticles and PFOB nanoparticles is possible in the same sample (Fig. 1-2B), as demonstrated by Morawski et al.\textsuperscript{38} Furthermore, because the relative $^{19}\text{F}$ spectroscopic signal is proportional to the amount of perfluorocarbon emulsion in a sample (Fig. 1-2C), concentration maps of targeted perfluorocarbon nanoparticles can be generated, allowing for quantitative monitoring of molecular processes based on estimation of the number of binding sites occupied by the nanoparticles. (Fig. 1-2D-F).

1.2.3 Advantages and Disadvantages of Perfluorocarbon Nanoparticles

PFC nanoparticles exhibit minimal toxicity and favorable biocompatibility. One early use of perfluorocarbons in clinical medicine was as an artificial blood replacement, which recognized their ability to dissolve and carry oxygen, with some formulations having reached FDA approval in the past.\textsuperscript{39} Adverse effects of perfluorocarbons is very low, due in part to the
extremely high in vivo tolerance of administrated PFCs, with an LD50 ranging from 30-41g PFC/kg body weight. Symptoms of toxicity are relatively mild as well, characterized by “flu-like” symptoms, with resolution within 12 hours. In the case of circulating PFC nanoparticles for molecular imaging or therapy, those not bound to a specific target rapidly accumulate in the liver and spleen, with detection possible after several minutes post-injection and up to 24 hours. Subsequently, the lipid components are recycled by various plasma carriers and the perfluorocarbons are exhaled through the lungs and cleared via the reticuloendothelial system. It is important to note the role of species differences in the secretion and clearance of large nanoparticles especially as they relate to rodent PK models, as these differences can affect interpretation of clearance data. Furthermore, the tissue half-lives of perfluorocarbons in vivo must be considered and potentially corrected for in instances of serial imaging where long tissue residence times (i.e. PFOB) could complicate imaging studies.

1.3 Applications of Nanotechnology for Cardiovascular Imaging and Drug Delivery

Nanoparticle applications for cardiovascular imaging and drug delivery are varied based on specific disease targets. Here we will review selected major targets of nanoparticles as they relate to atherosclerosis, with a focus on perfluorocarbon nanoparticle-based treatments.

1.3.1 Thrombosis

Acute onset of occlusive thrombosis is the proximate cause of mortality in cases of heart attack and stroke. Accordingly, numerous studies have examined the deployment of nanoparticle-based imaging contrast agents for detection of thrombi. Destabilization of plaques due to rupture or erosion can result in disruption of the normal hemostatic barrier of the endothelium, resulting in the initiation of the coagulation cascade, which in turn generates
thrombin and other coagulation factors, facilitating fibrin deposition and platelet activation. The ability to image thrombi noninvasively offers important clinical diagnostic information and bears implications for therapeutic management of acute ischemic syndromes. Although methods exist to characterize certain aspects of thrombi (e.g. thrombus age, methemoglobin content, among others), the potential to image specific molecular epitopes associated with thrombi could allow for improved localization of emerging thrombi and subsequent responses to therapies designed to inhibit thrombotic activity.

In 1997, Lanza et al. reported fibrin as a target for nanoparticle-based molecular imaging of clots with the use of MRI, and described a novel paramagnetic, nanoparticle based contrast agent targeting fibrin for T1-weighted imaging of clots. The nanoparticle used in this study was a perfluorocarbon nanoparticle laced with various concentrations of Gd-DTPA, and conjugated to a fibrin specific antibody NIB 1H10 that was highly specific for fibrin. Initial work demonstrated the utility of these Gd-loaded PFC nanoparticles for T1-weighted imaging of plasma clots in vitro at 4.7T. Later work confirmed direct binding of nanoparticles to fibrin with scanning electron microscopy (Fig. 1-3A-B), and demonstrated the ability to image fibrin bound nanoparticles both in ex vivo human endarterectomy samples at 4.7T (Fig. 1-3C), and in vivo using a clinical 1.5T MRI scanner (Fig. 1-3D-E). Additionally, Morawski et al. quantified thrombin binding with the use of F MRI in human carotid endarterectomy specimens that employed fibrin-targeted PFC nanoparticles.

Recent work from Myerson et al. demonstrated the potential of the perfluorocarbon nanoparticle platform for imaging and treating developing thrombi. In this work, perfluorocarbon nanoparticles were conjugated to a direct thrombin inhibitor, D-phenylalanyl-L-prolyl-L-arginyl-chloromethylketone (PPACK) and delivered in vivo in a mouse model of acute thrombosis in the
carotid artery. Ex vivo $^{19}$F MRI at 11.7T illustrated the ability of these PPACK nanoparticles to selectively bind to the surface of thrombin-bearing clots, allowing for imaging of areas of high thrombin activity (Fig. 1-3F-H). This anti-thrombin nanoparticle platform has since been modified to include FDA-approved bivalirudin as the thrombin-binding epitope, which increases the specificity for thrombin over and above that of PPACK.

A perfluorocarbon nanoparticle system utilizing direct thrombin inhibitors (PPACK and Bivalirudin) not only enables imaging of active thrombi as previously discussed, but simultaneously effects a potent localized antithrombotic property at sites of acute and active clotting with minimal systemic side effects. The same mechanism of thrombin binding that allows for successful imaging of clots with fluorine MRI establishes an dense “anticlotting surface” where single nanoparticles presenting thousands of inhibitor molecules lay down in a monolayer over the active thrombus and further inhibit the activity of clot-bound thrombin. This effect has been observed for both PPACK and bivalirudin perfluorocarbon nanoparticles; and extension of the system to liposomal analogues of this system explored in Chapter 2 of this thesis. This propensity of rapid targeting of acute thrombi also suggests the potential for thrombolytic therapy with nanoparticles. For example, Marsh et al. employed fibrin targeting of streptokinase-loaded perfluorocarbon nanoparticles to induce rapid clot dissolution within 60 minutes of treatment, as tracked by ultrasound imaging.

Other relevant applications of perfluorocarbon nanoparticles broadly related to thrombosis pertain to acute ischemic kidney injury in which compromised microvascular blood flow leads to death of proximal tubular cells and renal failure. For example, Hu et al. demonstrated the utility of perfluorocarbon nanoparticles in assessing the extent of perfusion in the renal microvasculature following acute kidney injury. Furthermore, recent work by Chen &
Vemuri, et al. demonstrated the ability of the aforementioned thrombin-inhibiting nanoparticles to limit kidney damage following ischemia/reperfusion injury by preventing clotting and maintaining renal blood flow. Perfluorocarbon nanoparticles may be a particularly useful tool for imaging applications in the kidney as contrasted with conventional gadolinium-based agents in the evaluation of renal compromised patients, because perfluorocarbon nanoparticles are not cleared through the kidney, but rather through the liver and spleen. Additionally, perfluorocarbon nanoparticles do not suffer the complications from nephrogenic systemic fibrosis that restrict the use of gadolinium agents in situations of existing renal dysfunction.

1.3.2 Endothelial Permeability and Neovasculature

Zhang et al. reported the ability of non-targeted perfluorocarbon nanoparticles to depict endothelial damage caused by a high cholesterol diet in a fat-fed rabbit model of atherosclerosis. The passive diffusion of these nanoparticles through disrupted endothelial barriers in vivo was quantified with $^{19}$F MRS, where the concentration of detected nanoparticles in aortic plaques was increased in rabbits fed a high-cholesterol diet for 7-14 months compared to minimal deposition in plaques of rabbits fed cholesterol for only 3 months. In this study, imaging of nanoparticles retained in endothelium-disrupted intimal plaques was demonstrated with ex vivo $^{19}$F MRI at 11.7T (Fig. 1-4A-B).

Perfluorocarbon nanoparticles have also been used to track endothelial progenitor cells as demonstrated by Partlow et al. In this work, perfluorocarbon nanoparticle-labeled stem/progenitor cells acquired from cord blood samples after fetal delivery can be tracked to tumor neovasculature and quantified in vivo with $^{19}$F MRI/MRS. Other groups have demonstrated the utility of perfluorocarbon emulsions for the tracking stem cells in vivo further
demonstrating the potential of fluorine imaging for non-invasive evaluation of tissue regenerative strategies.\textsuperscript{56,57}

Other studies have focused on depiction of neovasculature in atherosclerotic plaques. Taking advantage of the “leaky” nature of developing vasculature, several albumin-binding paramagnetic agents have been utilized for imaging of permeable neovasculature. Cornily et al. evaluated the use of gadocoletic acid trisodium salt, or “B-22956/1” which binds with high affinity to albumin, and evaluated its use in atherosclerotic rabbits. They demonstrated the ability of their albumin-binding agent to produce increased enhancement of plaques over and above enhancement produced by Gd-DTPA alone, using T1-weighted MR sequences.\textsuperscript{58} More recent work by Phinikaridou et al. involved the use of gadofosveset, a clinically approved albumin-binding T1 MRI agent for the evaluation of permeable endothelium in plaques\textsuperscript{59} and the tracking of interventional therapies (minocycline, ebselen) in reducing plaque burden.\textsuperscript{60}

Winter et al. first demonstrated the use of a targeted T1 agent using Gd-loaded perfluorocarbon nanoparticles targeted to the $\alpha_v\beta_3$ integrin that is associated with plaque neovasculature. This study showed the potential for in vivo assessment of angiogenesis and neovasculature development using a 1.5T clinical MR scanner (Fig. 1-4C).\textsuperscript{61} Targeting of the $\alpha_v\beta_3$ integrin also enables therapeutic applications of nanoparticles such as the focal inhibition of angiogenesis that is critical for plaque growth\textsuperscript{62}, by targeting the $\alpha_v\beta_3$ integrin that is highly expressed in neovasculature. In this study, $\alpha_v\beta_3$-targeted perfluorocarbon nanoparticles were loaded with an anti-angiogenic agent (fumagillin) and tested for efficacy in atherosclerotic rabbits. Administration of fumagillin-loaded $\alpha_v\beta_3$-targeted perfluorocarbon nanoparticles resulted in a reduction of plaque neovasculature by 50-75% following a single pulsed dose, with a sustained effect for up to 3 weeks following initial therapy.\textsuperscript{63} Additionally, molecular targeting
of nanoparticles to $\alpha_\beta_3$ integrins has allowed for the tracking of anti-angiogenic therapy in the treatment of peripheral vascular disease using MRI as a readout.\textsuperscript{64}

Targeting of the $\alpha_\beta_3$ integrin also has proven therapeutic utility in animal models of restenosis as shown by Cyrus et al. with local catheter delivery of rapamycin-loaded $\alpha_\beta_3$-targeted nanoparticles that inhibited restenosis following balloon overstretch injury in rabbits without the need for stenting.\textsuperscript{35} In this study, the inhibition of restenosis was observed only for integrin-targeted delivery of the drug-loaded nanoparticle, but statistically insignificant for the non-targeted version of the drug loaded nanoparticle. The targeting and retention of nanoparticles allowed for site specific delivery of rapamycin through “contact-facilitated drug delivery”\textsuperscript{65}, while the addition of gadolinium on the nanoparticle surface allowed for effective T1-weighted imaging of drug delivery to the treatment area.

1.3.3 Inflammatory Mediators

The role of inflammation in atherosclerosis has been recognized with markers of inflammation being investigated as potential targets for therapeutic intervention.\textsuperscript{66} The early phases of atherosclerosis involve the attachment and recruitment of inflammatory immune effector cells (e.g., monocytes), which contribute to plaque development. The mechanism of inflammatory cell recruitment involves the expression of cellular adhesion molecules on the inflamed endothelium, such as vascular cell adhesion molecule (VCAM-1), intracellular adhesion molecule (ICAM-1) or P-selectin, among others.\textsuperscript{2}

Fluorine imaging also has been utilized to image VCAM-1 upregulation in atherosclerosis, with the use of PFC nanoparticles as carriers for VCAM-1 targeting peptides previously developed by Kelly et al.\textsuperscript{67} Initial work in this area was accomplished by Southworth
and Kaneda, et al. using this anti-VCAM-1 nanoparticle for the imaging of renal inflammation in hyperlipidemic mice, demonstrating significant amounts of VCAM-1 targeted nanoparticles in inflamed kidneys from fat-fed ApoE null mice as compared to minimal deposition in kidneys of control wild-type mice (Fig. 1-5A-C). Pan et al. extended this functionality by designing a novel linker system for rapidly post-formulation functionalization of lipidic (and PFC) nanoparticles. In this work, VCAM-1 targeting peptides were linked to a membrane inserting peptide based on the amphipathic cationic peptide melittin, that enabled rapid insertion of a VCAM targeting peptide into the perfluorocarbon nanoparticle surfactant membrane in a simple mixing procedure. These VCAM-1 targeted nanoparticles were utilized in the ApoE null model of atherosclerosis, where VCAM-1 targeted particles were preferentially detected in the aortas of ApoE null mice as compared to minimal signal from aortas from wild-type mice (Fig. 1-5D).

1.4 Conclusions

The therapeutic and diagnostic techniques used to identify and treat atherosclerosis should address not just the late clinical presentations of atherosclerosis (plaque rupture, erosions, thrombosis), but also should be poised to identify early indicators such as vessel hypercoagulability and inflammation, with a goal to apply and direct therapies to protect vascular endothelial function. How focal inhibition of thrombin might alter the course of disease progression in atherosclerotic subjects remains unknown. For our purposes, we ask specifically can focal thrombin inhibition limit the detrimental effects of a diet-induced inflammatory state on endothelial health and plaque procoagulant activity, while limiting systemic side effects that are characteristic of conventional anticoagulants? Accordingly, the goal of this dissertation is to examine the utility of nanoparticle-based approaches for quantifying endothelial barrier integrity and plaque thrombotic risk, and evaluate the use of nanoparticles for focal administration of
thrombin inhibitors to limit the procoagulant and proinflammatory effects of thrombin in atherosclerotic mice. We address these objectives in the following chapters:

• CHAPTER 2: Characterize the ability of thrombin-inhibiting liposomes to provide local anti-clotting activity through the generation of clot-inhibiting surfaces on damaged and thrombosed arteries.

• CHAPTER 3: Demonstrate the utility of thrombin-inhibiting perfluorocarbon nanoparticles in forming anti-clotting surfaces on bare metal stents in preventing thrombosis.

• CHAPTER 4: Evaluate changes in endothelial barrier integrity in response to proatherogenic diet and elucidate the relationship of endothelial barrier integrity to hypercoagulability in atherosclerotic mice with the use of perfluorocarbon nanoparticles.

• CHAPTER 5: Demonstrate the effect of thrombin-inhibiting perfluorocarbon nanoparticles on preserving endothelial barrier integrity and limiting vessel procoagulant and proinflammatory activity in atherosclerotic mice.
1.5 Copyright Acknowledgement

Portions of this chapter were adapted verbatim with permission from a review article published in the journal *Nanomedicine* titled "Molecular Imaging of Atherosclerosis with Nanoparticle-based Fluorinated MRI Contrast Agents". The full citation is listed below:

Figure 1-1: Activation of the NF-kB pathway by thrombin. Thrombin cleavage of PAR-1 stimulates IKK, followed by dissociation and degradation of IkB from the IkB-p65 complex. The p65-homodimer is then phosphorylated and facilitates transcription of inflammatory genes that promote atherosclerosis.
Figure 1-2: (A) Schematic of perfluorocarbon nanoparticles. (B) Sample fluorine \(^{19}F\) spectra demonstrating unique signatures of two types of perfluorocarbon nanoparticles—perfluoro-15-crown-5 ether (PFCE) and perfluorooctylbromide (PFOB). (C) Fluorine signal measured using \(^{19}F\) magnetic resonance spectroscopy (\(^{19}F\)-MRS) is proportional to the volume of perfluorocarbon in a sample. Using this observation, it is possible to take samples such as a (D) human endarterectomy specimens and (E) image deposited nanoparticles with \(^{19}F\)-MRI to generate a (F) concentration map to locate differential deposition of nanoparticles within a sample. (Reprinted with permissions from \(^{38}\))
Figure 1-3: Imaging with thrombus-specific nanoparticles. Scanning electron micrographs depict fibrin clot (A) without nanoparticle treatment, with arrows depicting fibrin fibrils and (B) with addition of fibrin-targeted perfluorocarbon nanoparticles, with arrows depicting fibrin-bound nanoparticles. (C) T1-weighted gradient echo images of human carotid endarterectomy specimens treated with fibrin-targeted nanoparticles (left) demonstrating contrast enhancement with nanoparticle treatment compared to control (right). (D) In vivo demonstration of T1-weighted contrast enhancement at thrombus site with fibrin-targeted nanoparticles compared with (E) control thrombus in the contralateral external canine jugular vein (Reprinted with
permission from 31) (F-H) Ex vivo $^{19}$F imaging at 11.7T of thrombin-targeted PFC nanoparticles bound to mouse thrombi. (F) Proton scan of excised mouse carotid artery with occlusive thrombus, with (G) overlay of $^{19}$F MR image collected in (H). (Reprinted with permission from 32)
Figure 1-4

Figure 1-4: Imaging of endothelial permeability and neovasculation. (A) Sagittal 3D rendering of nanoparticle deposition measured at 11.7T in a rabbit aorta excised following 12 months of cholesterol feeding and 12 hours of nanoparticle circulation in vivo. (B) Cross sectional $^{19}$F image of nanoparticle deposition in atherosclerotic plaque. Concentration mapping illustrates depth of penetration and local concentration in the rabbit atherosclerotic plaque following 9 months of cholesterol feeding and 2 hours of in vivo nanoparticle circulation. (Reprinted with permission from 54) (C) Localization of $\alpha_v\beta_3$-targeted nanoparticles to areas of plaque angiogenesis in atherosclerotic rabbits. Top panel shows region of interest, with cross sectional images of aorta prior to administration of nanoparticles (Pre), following nanoparticle administration (Post), and the application of a segmented aortic wall mask (Segmented) used to quantify T1-weighted contrast enhancement (Enhancement). (Reprinted with permission from 61)
Figure 1-5: Imaging of Inflammatory Mediators. Ex vivo $^{19}$F MRI of atherosclerotic mouse kidneys (top row) or wild-type mouse kidneys (bottom row) with (A) proton scan for anatomical detail, (B) $^{19}$F image, and (C) overlay of $^{19}$F image on proton image demonstrating increased accumulation of VCAM-1 targeted PFC-nanoparticles in atherosclerotic kidneys over wild-type kidneys, corresponding to differences in VCAM-1 expression in atherosclerotic vs. wild-type subjects. (Reprinted with permission from 68) (D) Targeting of PFC-nanoparticles using VCAM-1 targeting peptides increases binding of nanoparticles in atherosclerotic mouse aortas, as
measured with $^{19}$F magnetic resonance spectroscopy at 11.7T. (Reprinted with permission from ""
1.6 References


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in early-stage atherosclerosis with alpha(v)beta3-integrin-targeted nanoparticles. 


Chapter 2: Generation of Sustained Anticlotting Barriers with Thrombin-Targeted Liposomes
Chapter Overview

The goal of the present work was to design and test an acute-use nanoparticle-based antithrombotic agent that exhibits sustained local inhibition of thrombin without requiring a systemic anticoagulant effect to function against acute arterial thrombosis. To demonstrate proof of concept, we functionalized the surface of liposomes with multiple copies of the direct thrombin inhibitor, D-phenylalanyl-L-prolyl-L-arginy1-chloromethyl ketone (PPACK), which exhibits high affinity for thrombin as a free agent, but manifests too rapid clearance in vivo to be effective alone. The PPACK-Liposomes were formulated as single unilamellar vesicles, with a diameter of 170.78 ± 10.59 nm and a near neutral charge. In vitro models confirmed the inhibitory activity of PPACK-Liposomes, demonstrating a $K_I$ of 172.6 nM. In experimental clots in vitro, treatment of formed clots completely abrogated any further clotting upon exposure to human plasma. The liposomes were evaluated in vivo in a model of photochemical-induced carotid artery injury, resulting in significantly prolonged arterial occlusion time over that of controls (69.06 ± 5.65 min for saline treatment, N=6, 71.33 ± 9.46 min for free PPACK treated; N=4, 85.75 ± 18.24 min for precursor liposomes; N=4, 139.75 ± 20.46 min for PPACK-Liposomes; \( P = 0.0049, \) N=6). Systemic anticoagulant profiles revealed a rapid return to control levels within 50 minutes, while still maintaining antithrombin activity at the injury site. The establishment of a potent and long-acting anticoagulant surface over a newly forming clot with the use of thrombin targeted nanoparticles that do not require systemic anticoagulation to be effective offers an alternative site-targeted approach to the management of acute thrombosis.
2.1 Introduction

The development of new pharmaceutical agents to treat acute arterial thrombosis remains a subject of active investigation to meet the continuing medical need for safe and controllable but powerful anticoagulation.\(^1\)-\(^3\) The deployment of direct thrombin inhibitors such as dabigatran recognizes the central role that thrombin plays in clot formation by proteolytic cleavage of fibrinogen into fibrin, but also its role in activation of platelets and Factor XIIIa, among others.\(^4\),\(^5\) Many of these treatment options display small therapeutic windows, with concerns for deleterious side effects such as bleeding that hamper their clinical adoption.\(^4\)-\(^7\)

Previous studies have suggested the potential of nano-scaled drug delivery systems as antithrombotic agents.\(^8\)-\(^11\) Such treatments would allow for effective, highly localized antithrombotic activity with potentially fewer risks for systemic side effects. Of these candidate nanoscale constructs, liposomes offer many advantages, including their biocompatibility and biodegradability with a history of suitability for regulatory approval.\(^12\)-\(^14\) Indeed, liposomes already have achieved clinical translation through the recent labeling of a number of FDA-approved formulations (e.g., Doxil, DaunoXome, DepotDur, Ambisome). Moreover, the potential for encapsulation of drugs within liposomes together with surface conjugation of anticoagulant agents offers a design format that could enable synergistic combination therapy.\(^15\)

Herein, we describe an antithrombotic agent formed through the conjugation of direct thrombin inhibitors to the surface of unilamellar liposomes. The use of a direct thrombin inhibitor conjugated to the surface of the liposome would allow for high affinity and avidity binding and targeting to thrombin at the site of acute thrombosis. Furthermore, we postulated that the construct would remain active at the site of acute thrombosis for hours by forming an
anticlotting surface after binding, while manifesting only a very transient systemic anticoagulant effect, which should mitigate the potential for hemorrhage without the need for an antidote. For proof of concept, the inhibitor used in this study is a short chain peptide, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (PPACK), which inhibits thrombin through alkylation of a histidine residue at the active site.\textsuperscript{16}
2.2 Materials and Methods

2.2.1 Liposome Preparation

Unilamellar liposomes were created using standard protocols as previously described. A lipid mixture containing 94 mol% egg phosphotidylcholine, EPC (Lipoid, Newark, NJ), 4 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, DPPE (Avanti Polar Lipids, Alabaster, AL), and 2 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000], DSPE-PEG-COOH (Avanti Polar Lipids, Alabaster, AL) was dissolved in chloroform. For fluorescent liposomes, the lipid mixture contained 93.9 mol% EPC, 4 mol% DPPE, 2 mol% DSPE-PEG-COOH, and 0.1 mol% 1,1’-Dioctadecyl-3,3,3’,3’-Tetramethylindocarbocyanine Perchlorate, DiI (Invitrogen, Carlsbad, CA). The lipid mixture at 10 mg/ml was placed in a heated water bath and dried into a film using rotary evaporation. The resulting lipid film was further dried in a vacuum oven at 40°C overnight to ensure complete evaporation of any residual organic solvent.

After drying, the lipid film was hydrated in phosphate buffered saline (WashU Tissue Culture Lab, St. Louis, MO) and sonicated at level two on a Branson probe sonicator for 2 minutes to resuspend the lipids and generate multilamellar vesicles (MLVs). The MLVs were subjected to extrusion through stacked polycarbonate/polyethylene filters (Whatman Inc, Piscataway, NJ) with a pore size of ~200 nm to generate a suspension of PEGylated, carboxy-terminated unilamellar liposomes.

2.2.2 PPACK-Liposome Formulation

PPACK conjugation was accomplished using amine-carboxyl coupling. Activation of carboxyl groups was accomplished using EDCI, 1-ethyl-3-(3-dimethylaminopropyl)
carbodiimide HCl (Pierce, Rockford, IL), added in excess at 2 mg/ml to the suspension of carboxy-terminated liposomes and mixed for 15 minutes. PPACK (American Peptide Company, Sunnyvale, CA) was added to the activated precursor liposomes at 12.5 mg/ml and allowed to mix overnight. Excess EDCI and uncoupled PPACK was removed from the suspension using dialysis tubing of MWCO 3000-5000 (Spectrum Labs, Rancho Dominguez, CA) and allowed to filter for four hours.

2.2.3 Liposome Characterization

The sizes of the liposomes with or without PPACK incorporated were analyzed by dynamic light scattering (Brookhaven Instruments Corp., Holtsville, NY). Zeta potential values were determined with a PALS Zeta Potential Analyzer (Brookhaven Instruments Corp., Holtsville, NY). Liposomes were diluted in MilliQ Water for zeta potential measurements. Data was acquired in the phase-analysis light scattering mode following solution equilibration at 25°C. For transmission electron microscopy evaluation of liposomes, samples of precursor and PPACK-liposomes were deposited onto formvar-coated Cu grids, followed by application of the vanadium-based NanoVan negative stain (Nanoprobes, Yaphank, NY). The liposomes were visualized using an FEI Spirit TEM at 120 kV.

Conjugation of PPACK to precursor liposomes was confirmed through RP-HPLC quantification of uncoupled PPACK recovered from the supernatant after centrifugation of predialysis PPACK-Liposomes mixed with Cleanascite lipid adsorption reagent (Biotech Support Group, Monmouth, NJ). PPACK elution was accomplished using a C18 reverse phase column (Grace, Deerfield, IL) on a Waters HPLC system. Eluent A was 0.1% Trifluoroacetate (TFA) in water, Eluent B was 0.1% TFA in acetonitrile, and a linear gradient (10-30% B) was established.
over 20 minutes. PPACK elution was measured at a wavelength of 215 nm. Determination of the liposomal PPACK concentration was accomplished through mass balance of uncoupled PPACK subtracted from total PPACK added during the conjugation step.

The Stewart assay\(^{19}\) was employed to determine liposome concentration through the measurement of total phospholipid content of the liposome sample. For the phospholipid content measurements, 100 µl of liposomes was diluted up to 2 ml with chloroform, followed by addition of 2 ml 0.1 M ammonium ferrothiocyanate. The mixture was vortexed for 1 min, and the organic phase was removed and measured at 488 nm using a Shimadzu UV-1601 UV-Vis spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The results were plotted against a standard curve of known lipid concentrations to determine liposomal phospholipid concentration. From this data, given the size of the liposome, liposome concentration was calculated accordingly.

2.2.4 In Vitro Inhibition of Thrombin Activity

A Chromozym TH, Tosyl-Gly-Pro-Arg-4-nitranilide acetate (Roche Applied Science, Indianapolis, IN) assay was utilized to quantify PPACK-mediated inhibition of thrombin in accordance with existing methods.\(^{20}\) Varying amounts of free PPACK or PPACK-Liposomes were incubated for 1 min at room temperature with 100 µl of 12 nM human \(\alpha\)-thrombin (Haematologic Technologies, Essex Junction, VT). The thrombin/liposome mixture was then added to 500 µl of 100 µM Chromozym TH in Tris buffer. Thrombin activity against the Chromozym TH substrate was assessed by measuring optical absorbance at 405 nm, indicative of liberation of the 4-nitranaline group of Chromozym TH. To quantify the activity of uninhibited thrombin, the change in absorbance per minute was recorded. The kinetics of PPACK-thrombin
inhibition was fit to a model of thrombin inhibition by hirudin to estimate an apparent inhibition constant $K_I^{21}$. Assuming binding to the enzyme causes a significant decrease in inhibitor concentration, the variation of the steady-state velocity can be described in terms of $K_I$: 

$$v_s = \frac{v_0}{2E_t} \left[ \left( \frac{(K_I + I_tE_t)^2}{4K_I^2 + 4K_IE_tE_t} \right)^{\frac{1}{2}} \cdot (K_I + I_tE_t) \right]$$

where the steady state velocity, $v_s$, is shown as a function of $v_0$, the velocity in the absence of an inhibitor; $I_t$, the total inhibitor concentration; $E_t$, the total enzyme concentration; and $K_I$, the rate constant where the substrate and the inhibitor compete for binding to the active site, as is the case with PPACK inhibition of thrombin.

To test PPACK-Liposome activity against clot-bound thrombin, clots were made by combining 2.25 µl of 1U/µl bovine thrombin (Sigma-Aldrich, St. Louis, MO), 16.5 µl of 500 mM CaCl$_2$ and 281.5 µl of human plasma, using the cap of a 1.5 ml eppendorf tube as a template. The clots were then transferred to strips of nitrocellulose and aged in saline at 37°C for 2 hours on an orbital shaker. After incubation, 50 µl of either saline, 12.5 mg/ml free PPACK, precursor liposomes or PPACK-Liposomes were added and allowed to incubate with the clots for 1 hour. After liposome incubation, the clots were rinsed with saline and weighed. The clots were then submerged in a mixture of 3.77 ml of human plasma and 220 µl of 500 mM CaCl$_2$ and allowed to incubate for intervals of 5, 15, 30, 60 and 180 minutes. At the conclusion of each time point, the clots were rinsed in saline and weighed.

For the visual determination of PPACK-liposome binding to clot-bound thrombin, the previously described protocol was slightly altered. Clots were made as previously described, combining thrombin, CaCl$_2$ and human plasma in a template. The clots were aged in saline for 2
hours at 37°C on a shaker, followed by the addition of either non-targeted or PPACK-functionalized fluorescent liposomes. The fluorescent liposomes were allowed to incubate for 1 hour at 37°C. After incubation, the clots were rinsed in saline and transferred to 6-well plates with fresh saline for imaging. Competition experiments were accomplished with the addition of 50 µl of 12.5 mg/ml PPACK followed by 2 hours of incubation and a subsequent saline rinse prior to the addition of fluorescent PPACK-Liposomes. Clots were imaged using a Xenogen in vivo imaging system (IVIS) Spectrum Workstation (Caliper Life Sciences, Hopkinton, MA) at excitation and emission wavelengths of 535 and 580 nm, respectively. Radiant efficiency was also measured for the imaged clots to gather a quantitative assessment of PPACK-Liposome binding. Additionally, untreated clots were fixed and embedded in paraffin for histological analysis of fibrin content.

Platelet aggregometry was carried out to assess the inhibition of thrombin-mediated platelet activation by PPACK-Liposomes. Blood collection protocols were approved by the Washington University Institutional Review Board. Human donor whole blood was collected via venipuncture into a syringe containing 3.2% sodium citrate for anticoagulation (1:10 citrate to blood ratio). Platelet-rich plasma (PRP) obtained by centrifuging the anticoagulated whole blood at 180g for 10 minutes at room temperature. The supernatant was carefully collected and the remaining material was further centrifuged at 1200g for 20 minutes at room temperature to obtain platelet-poor plasma (PPP).²²,²³ PPP was used as the standard for 100% light transmission. Platelet aggregometry was conducted on a Model 300 Payton Aggregation System (Payton Scientific, Inc., Buffalo, NY) at 37°C. Briefly, 500 µl of PRP was mixed with 5 µl of either water, 150 µM PPACK, precursor liposomes, or PPACK-Liposomes and stirred at 1000 RPM in the aggregometer. The mixture was allowed to sit to ensure a stable baseline reading and upon
stabilization, 5 µl of 500 µM thrombin (5 µM final concentration) was added to the PRP mixture and allowed to aggregate fully.

2.2.5 In vivo Inhibition of Thrombin Activity

The antithrombotic efficacy of PPACK-Liposome was defined according to their ability to delay the onset of thrombosis in C57BL/6 mice that were subjected to photochemical injury of the carotid artery. The mice were sedated with ketamine (87mg/kg)/xylazine(13mg/kg), followed by isolation of the carotid artery through a midline cervical incision. To follow the kinetics of occlusion after injury initiation, an ultrasonic Doppler probe (Transonic Systems Inc, Ithaca, NY) was placed on the carotid artery to measure the blood flow rate. Saline, free PPACK at 12.5 mg/ml, precursor liposomes (without surface modifications) or PPACK-Liposomes were delivered via tail vein injection as a 1ml/kg bolus 10 minutes before the injection of photosensitive Rose Bengal dye in phosphate-buffered saline to induce arterial injury upon exposure to laser light. A 1.5 mW 540 HeNe laser was focused on the isolated carotid artery. The experiment was terminated upon observation of the flow rate decreasing to < 15% of the original flow and maintaining that level for >5 minutes. The injured carotids were removed and preserved in paraffin for histological analysis of clot composition using the Carstairs stain for fibrin and platelets.24

For observation of liposome localization to the injury site, fluorescent PPACK-Liposomes were administered and the right carotid artery was exposed to photochemical injury for 30 minutes. The mice were euthanized and the injured carotid arteries were excised and embedded in Optimal Cutting Temperature (OCT) medium and sectioned for fluorescence microscopy.
2.2.6 Activated Partial Thromboplastin Time

To measure the effects of PPACK-Liposome treatment on activated partial thromboplastin time (APTT), a series of C57BL/6 mice were injected with a bolus of PPACK Liposomes with terminal left ventricle blood draws at 10, 20, 50, and 100 minutes post-injection. The mice were sedated with ketamine (87mg/kg)/xylazine(13mg/kg) and blood was drawn from the left ventricle into a syringe with 4% sodium citrate for anticoagulation (1:10 citrate to blood ratio). Blood samples were subsequently spun in a microcentrifuge for 15 minutes at 1000g. After centrifugation, the plasma was aspirated and frozen for future use. APTT kits (HemosIL, Lexington, MA) were used to obtain data for each time point. 100 µl of APTT reagent (colloidal silica with a mixture of synthetic phospholipids, buffer and preservatives) was added to 100 µl of thawed plasma and allowed to incubate for 3 minutes. 100 µl of 25 mM calcium chloride was then added to the reagent-plasma mixture and APTT was determined through mechanical agitation of the mixture.

2.2.7 Biodistribution

DiI-labeled PPACK-Liposomes were utilized to assess biodistribution and avenues of liposome clearance after 2 hours of circulation. C57BL/6 mice were given either no injection to serve as a control, or DiI-PPACK-Liposomes (1ml/kg) via tail-vein injection. Mice were euthanized 2 hours post-injection and perfused with saline. The liver, spleen, and kidneys were removed and imaged in a Xenogen IVIS Imaging Workstation as previously described for imaging DiI-labeled liposomes. Total radiant efficiency was measured to give a quantitative measurement of liposome accumulation in the organs.
2.2.8 Statistics

In vivo thrombin inhibition and in vitro clot growth data was analyzed using the Kruskal-Wallis ranked sum test, where $P < 0.05$ demonstrates statistical significance. For all other data, a two-sided Wilcoxon ranked sum test was used, where $P < 0.05$ demonstrates statistical significance. Error bars represent standard error of the mean.
2.3 Results

2.3.1 PPACK-Liposome Synthesis and Characterization

After sonication-extrusion treatment of lipid films, liposomes were synthesized with exposed terminal carboxy-PEG ends by incorporation of DSPE-PEG-COOH. The carboxy-terminated ends were activated with EDCI to form amide bonds with the N-terminus of PPACK. (Fig. 2-1A) The resulting PPACK-Liposome complex, along with the bare liposomes were analyzed with dynamic light scattering to determine the particle size of the resulting formulations. The bare precursor carboxy-terminated liposomes exhibited a mean hydrodynamic diameter of 163.2 ± 3.89 nm, while the PPACK-liposomes exhibited a mean hydrodynamic diameter of 170.78 ± 10.589 nm. (Fig. 2-1B) TEM images were also obtained to observe liposome morphology. (Fig. 2-2) Zeta potential was used to further characterize the stability of the liposome formulations. The precursor liposomes exhibited a zeta potential of -31.92 ± 11.43 mV, while the PPACK-terminated liposomes exhibited a zeta potential of -13.45 ± 2.00 mV. (Fig. 2-1B) Because of the positively charged amino acid in the PPACK peptide, this difference in zeta potential confirms successful conjugation of PPACK to the surface of the liposome. In addition, RP-HPLC quantification (Fig. 2-3A) of liposomal PPACK concentration, combined with Stewart assay quantification (Fig. 2-3B) of liposome concentration indicated the coupling of 6254.24 ± 1245.14 PPACK peptides per liposome.

2.3.2 In Vitro Inhibition of Thrombin

The chromogenic substrate Chromozym TH was utilized to measure thrombin activity in vitro. Varying amounts of free PPACK or PPACK-Liposomes were added to a solution of thrombin in order to demonstrate a dose-dependent inhibition of thrombin. After a one-minute
incubation time, the thrombin/inhibitor mixtures were added to a solution of Chromozym TH in Tris buffer and the cleavage of Chromozym TH was allowed to occur. The results of the Chromozym TH assay were further used to estimate the apparent $K_i$ for PPACK inhibition of thrombin. The Chromozym TH data was fit to a standard model of tight-binding inhibition of thrombin by hirudin, as previously described. The results showed a $K_i$ of liposome-conjugated PPACK was 172.6 nM, and that of free PPACK was 12.07 nM. (Fig. 2-4)

Next, an in vitro assay was developed to study the ability of PPACK-Liposomes to inhibit clot-bound thrombin and prevent further growth of clots after plasma incubation. (Fig 2-5A) Standardized clots were produced in vitro by incubating thrombin, plasma and CaCl$_2$ together in a template for one minute. These clots were then removed from the template and affixed to nitrocellulose paper, followed by exposure to buffer for 2 hours (Fig. 2-5B. Then, clots (3-6 clots per group) were exposed to either 5 ml of buffer or 12.5 mg/ml free PPACK, Precursor Liposomes, or PPACK-Liposomes in buffer (50 µl in 5 ml of PBS) for 60 minutes, washed three times, and then incubated in plasma for selected intervals to observe clot growth according to clot weight (Fig. 2-5A, C-F). After 15 minutes of incubation in plasma, the weight of the saline control (Fig. 2-5C) and precursor liposomes (Fig. 2-5E) clots grew to greater than 500% of the initial weight of the pre-incubation clot, totally filling the well of a 6-well plate with new clot. A similar, but more gradual increase in clot size was observed for clots treated with free PPACK, finally reaching a size approximately 800% of the initial clot weight at the end of the 180-minute plasma exposure time (Fig. 2-5D). This significant increase was observed for all subsequent time points and was potentially limited by the restrictions of the size of the well in the plate. Clots treated with PPACK-Liposomes did not grow significantly over 180 minutes (Fig. 2-5F).
Accordingly, PPACK-Liposomes completely prevented new clot growth, or “regrowth,” as compared to the control treatments.

To confirm the localization of the PPACK-Liposomes to the clots, liposomes were synthesized with the incorporation of the carbocyanine dye, DiI. Clots (N=6 per group) were grown and incubated with non-targeted liposomes, PPACK-functionalized liposomes, or no treatment. In addition, one group of clots was treated with 12.5 mg/ml free PPACK to block thrombin in the clot, followed by treatment with PPACK-Liposomes to observe any nonspecific interactions of PPACK-Liposomes with the clots. The clots were then imaged with an IVIS Spectrum Workstation after washing away excess, non-bound particles. PPACK-Liposomes exhibited significantly higher amounts of bound particles compared to the control liposomes and the competition group (Fig. 2-6A), as confirmed by a quantitative measurement of total radiant efficiency. (Fig. 2-6B)

PPACK-Liposomes also displayed the ability to effectively inhibit thrombin-mediated activation of platelets through platelet aggregometry measurements. In the presence of water or precursor liposomes, where PPACK is completely absent, platelets rapidly aggregated upon addition of thrombin at a concentration of 5 µM in the cuvette. In the control group where free PPACK is present in the PRP, platelets exhibited no aggregation throughout the duration of the experiment. PPACK-Liposomes demonstrated a similar ability to ablate platelet aggregation. (Fig. 2-7)

2.3.3 Inhibition of Thrombus Formation in vivo

To delineate the efficacy of the PPACK-Liposomes in vivo, injury of the carotid artery of C57Bl/6 mice was induced by the traditional photochemical/laser method in mice each treated
with either saline (N=6), free PPACK (N=4), Precursor Liposomes (N=4) or therapeutic PPACK-liposomes (N=6). The efficacy of liposomes for inhibition of thrombosis was measured by denoted time required to reach complete occlusion. Precursor liposomes with no PPACK functionality served as the liposome control. When injected 10 minutes prior to induction of arterial trauma, the control liposomes yielded an occlusion time of 85.75 ± 18.24 min. This exhibited no significant increase over the saline-treated group of 69.06 ± 5.65 min or the free PPACK group of 71.33 ± 9.46 min. In addition, the free PPACK group showed no significant increase over the saline treated group. In the PPACK-Liposome treated mice, occlusion time was prolonged to 139.75 ± 20.46 min and was significantly increased compared to all other groups. (Fig. 2-8A) Additionally, the activity of PPACK-Liposomes appeared to exhibit a continuous effect at the injury site as determined by flow rate monitoring throughout the course of the injury. (Fig. 2-8B) Site-specificity and fluorescent PPACK-Liposome accumulation in the clot was observed through fluorescence microscopy evaluation (Fig. 2-8C) of partially formed clots (Fig. 2-8D) in excised carotid arteries. The results of this study indicate that the addition of PPACK to the surface of the liposomes provides the needed functionality to serve as a thrombin inhibitor in vivo.

Activated partial thromboplastin time (APTT) testing was carried out in mice treated with PPACK-Liposomes. (Fig. 2-9) Liposome treatment resulted in an initial prolongation of APTT up to 65.98 ± 19.59 seconds at 10 minutes after the administration of PPACK-Liposomes. Prolonged APTT was observed 20 minutes after treatment, but followed by a return to the control range within 50 minutes after treatment. The normalization of systemic APTT values did not affect the prolonged therapeutic action at the site of injury since in vivo inhibition of thrombus formation was observed beyond 2 hours in the carotid artery.
2.4 Discussion

The tripeptide agent PPACK is recognized as a potent candidate for antithrombotic applications due to its high affinity for thrombin and low systemic toxicity with an LD$_{50}$ of $>$50 mg/kg. However, its fast clearance rate ($T_{1/2} \sim 3$ minutes) in vivo precludes clinical application.$^{16}$ To overcome the rapid systemic clearance, we conjugated PPACK to the surface of a liposome, which yields an agent that manifests prolonged anti-thrombin activity at the site of an acute clotting event, yet its systemic anticoagulant activity rapidly wanes after injection. The anticoagulant nanostructure design has the advantage of exposing multiple anticoagulant sites on the surface of the liposome to achieve exceptional thrombin avidity and markedly enhanced local anticoagulation efficacy. Moreover, the aqueous compartment of the liposome offers the potential of encapsulating drugs for possible localized combination anticoagulation or thrombolytic therapy.

PPACK-Liposomes are capable of binding to and inhibiting clot-bound thrombin as shown by the in vitro clot growth assays and fluorescent imaging (Fig. 2-6). Remarkably, PPACK-Liposomes completely prevented further clot growth in this system, which offers a distinct advantage over heparin that is unable to associate with clot-bound thrombin due to masking of the heparin-binding site on fibrin-bound thrombin.$^{25,26}$ The establishment of a highly focal, long-lasting multivalent anticlotting surface atop a newly forming clot appears to sequester procoagulant materials from serum clotting factors that otherwise might continue to promote coagulation through activation of Factor VIII or other factors.$^{27}$

In vivo experiments of carotid occlusion time post-injury confirmed the efficacy of the PPACK-Liposomes, demonstrating an ability to prolong the formation rate of occlusive arterial
thrombi after the administration of one pre-injury bolus treatment. The use of multiple dosing regimens and treatment of pre-formed thrombi in vivo will be the subject of future investigation to advance towards clinical translation and establish a maximum effective dose.

The prolonged occlusion times in the Rose Bengal mouse model are a consequence not only of the inhibition of thrombin at the site of injury, but also reduced platelet activation and aggregation that depends on thrombin interacting with various platelet receptors, including GPIbα, PAR-1 in humans, and PAR-4 in the mouse. This activation of platelets by thrombin is completely inhibited in the presence of PPACK-Liposomes as confirmed by platelet aggregometry. (Fig. 2-7) This dual functionality likely amplifies the potency of the agent at the site of acute clotting without disrupting alternative mechanisms of platelet activation that would be useful to maintain hemostasis elsewhere. Moreover, despite the prolonged activity of PPACK-Liposomes at the site of injury, the systemic action of PPACK-Liposomes rapidly declines between 20-50 minutes after injection according to activated partial thromboplastin times. This rapid diminution of systemic anticoagulant effect is due to the removal of a sufficient amount of circulating thrombin-targeted liposomes by the liver and spleen (Fig. 2-10) to drop blood pool levels below that required to inhibit further clotting while still providing an enormous focal pool of PPACK at the site of thrombosis to immediately inactivate any subsequently released thrombin.

The limitations of PPACK as an antithrombotic agent also comprise its broader specificity as a protease inhibitor beyond thrombin, although the rapid clearance of the unbound liposomes mitigates this concern. This point might also apply equally to the concern of the irreversible nature of the interaction with the active site on the thrombin molecule. Nevertheless, it is probable that alternative anticoagulant molecules such as bivalirudin could serve a similar
purpose when conjugated to a liposome to enhance specificity in a reversible interaction if required. A pluripotent design with multiple specific factor inhibitors could promote synergism for the formed anticlotting surface without having to completely attenuate any one factor alone.

The use of a liposomal structure may occlude the interaction of some of the PPACK due to potential steric and mobility effects of liposome-bound PPACK, limiting the freedom of PPACK to invade the active site of thrombin. This possibility may explain the increased $K_e$ of the PPACK conjugated liposome versus free PPACK noted in the model-based analyses of affinity, as not all of the included PPACK would be active against thrombin as long as the liposome remained intact. However, due to the high avidity achieved by including copies of PPACK, high local activity should be maintained as shown by the in vivo clot inhibiting experiments.

Previous reports have suggested the potential of nanometer-sized particles to act as effective anticoagulants in vivo, in some cases involving surface modifications to promote anticoagulant activity. However, the use of liposomes as anticoagulant structures has the advantage that they are established as a vehicle for biomedical applications with an abundance of FDA-approved liposomal formulations. Furthermore, the opportunity to encapsulate drugs or imaging agents ultimately could facilitate detection and localized treatment of acute thrombi in emergency situations. This benefit is vital—especially when triage to medical versus surgical therapy might necessitate ensuring normalized systemic clotting parameters at the time of decision, which is possible with this agent without the need for an “antidote”.
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Figure 2-1: (A) Schematic of EDCI-mediated PPACK conjugation to carboxyl-terminated liposomes. (B) Size and zeta potential of carboxyl-terminated liposomes and PPACK-Liposomes. Results indicate a hydrodynamic diameter of 163.2 ± 3.89 nm and zeta potential of -31.92 ± 11.43 mV for the carboxyl-terminated liposomes. PPACK liposomes exhibit a diameter of 170.78 ± 10.59 nm and zeta potential of -13.45 ± 2 mV. The rise in zeta potential is indicative of the increased positive surface charge on the liposomes after PPACK conjugation.
Figure 2-2: (A) TEM Image of precursor carboxyl-terminated liposomes. Scale bar denotes 100 nm. (B) TEM Image of PPACK-Liposomes. Scale bar denotes 100 nm. Both samples were negatively stained with the vanadium-based stain NanoVan.
Figure 2-3: (A) Standard curve of PPACK quantified by RP-HPLC. The standard curve was used to determine the amount of uncoupled PPACK left over after the conjugation step. (B) Stewart Assay standard curve to quantify the amount of phospholipids in the liposome sample. The Stewart Assay quantification was then used to determine the liposome concentration given the overall size of the liposome measured by dynamic light scattering. Both assays were then used in tandem to determine the number of PPACK molecules per liposome (6254.24 ± 1245.14 PPACK peptides per liposome).
Figure 2-4: Demonstration of inhibition of thrombin activity against the chromogenic substrate Chromozym TH. PPACK-Liposomes exhibited an inhibition constant of 172.6 nM compared to an inhibition constant of 12.077 nM for free PPACK.
Figure 2-5. In vitro clot assay demonstrating the inhibition of clot-bound thrombin by PPACK-Liposomes. (A) Measurement of clot growth after incubating saline, free PPACK, Precursor liposomes or PPACK-Liposome-treated clots in plasma for selected time points. Clots treated with saline, free PPACK and Precursor Liposomes all exhibited significant growth after 3 hours of plasma incubation compared to PPACK-Liposome treated clots. (*P < 0.05) (B-F) Representative images of clots before (B) and after (C-F) plasma incubation, comparing clots from all treatment groups after 3 hours of plasma incubation.
Figure 2-6: (A) Fluorescent images of clots treated with either saline, DiI-labeled carboxyl-terminated liposomes (Non-Targeted) or DiI-labeled PPACK-Liposomes (Targeted). Competition experiments refer to prior inhibition of clot-bound thrombin with free PPACK followed by treatment with DiI-labeled PPACK-Liposomes. (B) Measurement of Total Radiant Efficiency demonstrating that liposome binding to clot was significantly increased with PPACK functionalization. (**P<0.005, N = 6 for non-targeted, PPACK liposomes, and competition groups) Additionally, competition experiments demonstrated specificity through low association of PPACK-Liposomes with clots previously treated with free PPACK.
Figure 2-7

Figure 2-7: The effect of PPACK-Liposomes on thrombin-mediated aggregation of human platelets was evaluated using platelet aggregometry, where 100% light transmittance represents 100% aggregation. Platelet-rich plasma (PRP) containing either (A) water, (B) Precursor Liposomes, (C) free PPA, (D) PPACK-Liposomes was exposed to thrombin and allowed to aggregate. The red arrow indicates the addition of thrombin to the PRP. The presence of PPACK or PPACK-Liposomes in the PRP abolishes thrombin-mediated activation of platelets.
Figure 2-8: (A) Time to occlusion of carotid artery with the Rose Bengal dye injury model in mice treated with Saline, free PPACK, Precursor Liposomes or PPACK-liposomes. Occlusion time was significantly increased for the PPACK-Liposome group over all other groups (**P<0.005) demonstrating potent antithrombotic activity in vivo. (B) Representative measurements of flow rate through the injured carotid artery throughout the duration of the in vivo photochemically-induced thrombus formation model. The formation of the thrombus continues over the entire course of the experiment—a process that is gradually delayed with PPACK-Liposome treatment. The time course for thrombus formation was largely unchanged for saline, Free PPACK and Precursor Liposomes. (C) Localization of PPACK-Liposomes (white arrows) at site of initial clot formation. (D) H&E stain of neighboring section showing initial formation of clot and endothelial damage. Scale bars represent 100 µm.
Figure 2-9: Activated partial thromboplastin time for PPACK-Liposome treated mice demonstrating a significant increase in APTT (*P<0.05) compared to the normal range 10 and 20 minutes after PPACK-Liposome injection, followed by a return to the control range within 50 minutes after injection.
Figure 2-10: Biodistribution of PPACK-Liposomes after 2 hours of circulation time. The light grey bars represent baseline organ fluorescence from mice that received no injections. The clearance of PPACK-Liposomes from the circulation appears to be mediated by the liver and spleen.
2.6 References


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Chapter 3: Inhibition of Stent Thrombosis using Thrombin-Inhibiting Perfluorocarbon Nanoparticles
Chapter Overview

Despite significant advances in intravascular stent technology, safe prevention of stent thrombosis over prolonged periods after initial deployment persists as a medical need to reduce device failure. The objective of this project was to assess the potential of perfluorocarbon nanoparticles conjugated with the direct thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone (PPACK-NP) to inhibit stent thrombosis.

In a static model of stent thrombosis, 3 mm x 3 mm pieces of stainless steel coronary stents were cut and adsorbed with thrombin to create a procoagulant surface that would facilitate thrombus development. Following treatment with PPACK-NP or control NP, stents were exposed to platelet poor plasma (PPP) or platelet rich plasma (PRP) for set time points up to 60 minutes. Measurements of final clot weight in grams were utilized for assessing the effect of nanoparticle treatment on limiting thrombosis. Additionally, groups of stents were exposed to flowing plasma containing various treatments (saline, free PPACK, control NP and PPACK-NP) and generated thrombi were stained and imaged to investigate the treatment effects of PPACK-NP under flow conditions.

The static model of stent thrombosis utilized in this study indicated a significant reduction in thrombus deposition with PPACK-NP treatment (0.00067 ± 0.00026g, N=3) compared to control NP (0.0098 ± 0.0015g, N=3, p = 0.026) in PPP. Exposure to PRP demonstrated similar effects with PPACK-NP treatment (0.00033 ± 0.00012g, N=3) versus control NP treatment (0.0045 ± 0.00012g, N=3, p = 0.000017). In additional studies, stents were exposed to both platelet rich plasma pretreated with vorapaxar and PPACK-NP, which illustrated adjunctive benefit to oral platelet inhibitors for prevention of stent thrombosis. Additionally, an
in vitro model of stent thrombosis under flow conditions established that PPACK-NP treatment significantly inhibited thrombus deposition on stents.
3.1 Introduction

Stroke, myocardial infarction, and limb loss from peripheral vascular disease are significant causes of cardiovascular morbidity and mortality in the U.S. that often are treated with interventions requiring one or more stents. Over the past few decades, significant technological advances have been made in these interventional treatments, yet all such therapies continue to fail a significant portion of patients due to thrombosis as the final common endpoint. In fact, recent reports from the Dual Antiplatelet Therapy (DAPT) study have demonstrated that the current standard of dual antiplatelet therapy (P2Y₁₂-receptor inhibitor plus aspirin) for prevention of thrombotic complications following placement of drug-eluting stents may require extended use beyond 1 year following stenting to prevent thrombosis, instead of the commonly prescribed 6-12 months.

Despite this demonstration of a reduction of risk of stent thrombosis and major adverse cardiovascular and cerebrovascular events with therapy for >1 year, the prolonged treatment regimen harbors an increased risk of bleeding, suggesting a clear medical need for new antithrombotic and antiplatelet agents that provide increased efficacy in preventing thrombotic complications, while minimizing bleeding risk. These studies also highlight the clinically significant rate of stent thrombosis for intravascular stents in the carotid, coronary and peripheral circulation. Intravascular stents continue to experience thrombosis at rates in coronary circulation of 0.9% at 30 days post-implantation with second-generation drug eluting stents, but highly variable rates in the peripheral circulation with failure rates up to 25%. Significantly, the immediate incidence of death or myocardial infarction is 64.4% for coronary stent thrombosis and in the peripheral circulation stent thrombosis can result in limb loss, stroke, renal failure or bowel ischemia. Aggressive and prolonged pharmacological therapy with platelet inhibitors is
required to mitigate the prothrombotic tendency of stents until they become endothelialized and more resistant to thrombosis, but at the cost of significant and sustained bleeding risk. Indeed the published data indicate a severe bleeding risk of 1.7% and moderate bleeding risk of 2.1% over 28 months with the highest risk during the first year and significant additional mortality with moderate bleeding events.\(^6\) We propose an alternative solution to maintaining stent patency while minimizing bleeding risk by exerting a \textit{localized} anticlotting effect devoid of any sustained \textit{systemic} anticoagulant effect. To address this clinical need, this study assessed the ability of anti-thrombin PFC NPs to prevent thrombosis on thrombin treated stents in both static and flow in-vitro models.

The agents utilized in this study consist of perfluorocarbon nanoparticles (PFC-NP) with a diameter of 160.5 ± 2.6 nm that were conjugated to the direct thrombin inhibitor PPACK (D-phenylalanyl-L-prolyl-L-arginy1 chloromethylketone) to form antithrombotic nanoparticles, PPACK-NP (Fig. 3-1A).\(^7\) These nanoparticles were formulated to carry \textasciitilde{}13650 PPACK moieties per particle, and already have demonstrated favorable safety profiles in vivo with respect to bleeding risk, with APTT and mouse tail vein bleeding times normalized within 60 minutes following intravenous administration. Furthermore, PPACK-NP are effective antithrombotic agents in mouse models of arterial thrombosis, where prior work has demonstrated that PPACK-NP treatment more than doubles the time to total thrombotic occlusion of the carotid artery after dye-laser injury by directly inhibiting thrombin activity at the site of injury. Additionally, as thrombin accumulated, PPACK-NP continually bound and inactivated all exposed thrombin molecules ultimately forming an "anti-clotting" surface (Fig. 3-1B).\(^8\) The anti-thrombin activity of the nanoparticle PPACK system in vivo is greatly improved over that of free PPACK due to an increased circulating half-life (t\(_{1/2}\) \textasciitilde{} 3 min for free inhibitor\(^9\)
vs. $t_{1/2} \sim 105$ min for PPACK-NP as a direct consequence of conjugation of the active PPACK moiety to the stable nanoparticle.\textsuperscript{10} Even though PPACK is a potent direct and irreversible inhibitor of thrombin by covalently coupling to the active site of the protease, its lack of acceptable pharmacokinetics render it essentially useless as a clinical anticoagulant unless coupled to a nanosystem to extend its clearance time.

Herein, we sought to define the utility of PPACK-NP for inhibiting stent thrombosis in both static and dynamic models of thrombosis in vitro. PPACK-NP were assayed under conditions of stent exposure to platelet poor (PPP) and platelet rich plasma (PRP), and also tested in conjunction with Vorapaxar treatment to delineate an additive antithrombotic effect of PPACK-NP, which we hypothesize will occur through decreased thrombin mediated protease activated receptor-1 (PAR-1) platelet activation with PPACK-NP and through Voraxapar as it directly blocks PAR-1 along with diminished activity of thrombin in cleaving fibrinogen as a consequence of PPACK-NP therapy, in the prevention of stent thrombosis. Dynamic models of stent thrombosis were utilized to generate thrombi on stents under flow conditions, and PPACK-NP were tested for their ability to prevent the formation of an occlusive thrombus following infusion of thrombin within a flow loop. The goal was to elucidate the beneficial effects of PPACK-NP in the prevention of stent thrombosis that might support further development of both novel nanoparticle coatings for stents and subsequent intravenous treatment regimens to prevent or ameliorate thrombosis of stents or other intravascular prosthetics.
3.2 Materials and Methods

3.2.1 Nanoparticle Formulation

Nanoparticles were synthesized according to previously established emulsification techniques. Briefly, the precursor PFC-NP were composed of a 20% (vol/vol) perfluoro-15-crown-5-ether (CE) core, 2% (wt/vol) surfactant, 1.7% (wt/vol) glycerin, and water. The surfactant used in the 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 surfactant, CE, glycerin and water, the resulting precursor PFC NP was activated for coupling to the free carboxyl groups using 2 mg/mL 1-ethyl-3-(3-dimethylaminopropyl carbodiimide HCl, EDC (Pierce, Rockford, IL). Amine coupling of PPACK to the nanoparticle surface was accomplished with the subsequent addition of 12.5 mg/ml PPACK (American Peptide Company, Sunnyvale, CA) to the activated precursor nanoparticle and the conjugation reaction was allowed to proceed overnight. Following conjugation, the excess EDC and uncoupled PPACK was removed from the nanoparticle suspension using dialysis tubing of MWCO 3000-5000 (Spectrum Laboratories, Rancho Dominguez, CA) and allowed to filter for 4 hours.

3.2.2 Static Model of Stent Thrombosis

For the static in vitro model of stent thrombosis, expired Guidant® bare metal stents constructed of 316L stainless steel, were acquired from the Washington University in Saint Louis’ Department of Medicine. Stents were expanded and cut into 3 x 3 mm sections and placed into wells of a 12-well plate. To model potential biofouling and accumulation of prothrombotic
material on stents following deployment, the stent sections were incubated with 10 µl of 1 U/µl bovine thrombin (Sigma-Aldrich, St. Louis, MO) for 1 hour. Following incubation, the stents were rinsed with saline to remove any unadsorbed thrombin. To confirm the presence of thrombin on the stent surface, the activity of adsorbed thrombin on the stent surface was tested against a chromogenic substrate for thrombin, S-2238 (Diapharma, West Chester, OH). Stents with or without adsorbed thrombin were incubated with 100 µl of 333 µM S-2238 for 30 minutes, after which the reacted S-2238 solution was transferred to wells of a 96-well plate and absorbance was measured at 405 nm.

To test the efficacy of PPACK-NP treatment in this static model, thrombin-adsorbed stent sections were treated with 10 µl of either saline, free PPACK (12.5 mg/ml), control NP, or PPACK-NP and allowed to incubate for 1 hour. In this and subsequent experiments, the control NP used were the precursor PFC NP prior to amine coupling of PPACK. Following treatment, the stents were then washed with saline to remove any non-stent associated nanoparticles. The treated stents were then exposed to, platelet poor plasma (PPP), generated through the addition of 500 mM CaCl$_2$ to aliquots of expired human plasma. In the investigation of the effect of dual treatment with platelet inhibitors, platelet rich plasma (PRP) was made through the dilution of expired human platelet concentrate (ZenBio, Research Triangle Park, NC) in PPP, resulting in a total platelet concentration of 300,000 platelets/ml. For test groups including platelet inhibitors, PRP was pretreated with Vorapaxar (Axon Medchem LLC, Reston, VA) at a final concentration of 50 nM. The treated stents were incubated with 10 µl of PPP, PRP or PRP/Vorapaxar for designated time points of 5, 15, 30, and 60 minutes at 37°C, $N=3$ for each group at each time point. At each of these time points, stents were weighed to determine clot weight following plasma incubation.
3.2.3 Ultrasound Imaging of Thrombosed Stent Sections

Samples were placed in room temperature saline at the focal zone of a high-frequency ultrasound scanner (Vevo 660 with RMV-704 40-MHz probe, VisualSonics, Toronto, ON, Canada). The probe was mounted to a computer-controlled gantry to permit scanning in multiple image planes, where each plane corresponded spatially to a cross-sectional view that was 8.0 mm wide and 1.9 mm deep. Raw radio-frequency (RF) data were acquired and stored as the probe was scanned over the sample surface. The backscattered energy at each point in the digitized waveforms making up an image frame was computed from the log of the sum of the squared RF amplitudes in a center-weighted moving window. These values were mapped to grayscale and scaled to the appropriate physical dimensions to form cross-sectional images of the sample, with acoustically 'bright' areas represented in white and non-echogenic regions in black.

3.2.4 Scanning Electron Microscopy

Stent sections harboring thrombi were prepared for scanning electron microscopy to visualize the presence of deposited fibrin and platelets on the stent surface. Following clot growth, stent sections were each placed into wells of a 12-well plate containing 1 ml of freshly prepared 2% gluteraldehyde (Sigma-Aldrich, St. Louis, MO) overnight at 4°C for fixation. After fixation, the stent sections were serially dehydrated in 1-hour incubations with 10%, 30%, 50%, 70%, 90% and 100% (3x) ethanol. Following dehydration, the stent sections were placed in a vacuum dessicator overnight to remove any residual moisture. The stent sections were then sputter coated with gold for 90 seconds followed by imaging with a Hitachi S-2600H and FEI Nova Nano 2300. ImageJ v1.47 was utilized to determine the diameter of nanoparticles visualized on stents.
3.2.5 In Vitro Flow Model of Stent Thrombosis

To test the ability of PPACK-NP to prevent stent thrombosis under flow conditions, a flow loop was utilized to simulate thrombus formation under flow conditions. The stent was deployed in Tygon tubing and 15 ml of PRP (150,000 platelets/ml) containing 50 µl of either saline, free PPACK (12.5 mg/ml), control NP, or PPACK-NP was allowed to flow in a loop using a peristaltic pump at 50 ml/min. After continuous perfusion of the flow loop with plasma, 10 µl of 1 U/µl bovine thrombin was infused into the flow loop and the plasma was allowed to flow for 10 mins. After 10 minutes, stents were removed from the flow loop, fixed overnight in 10% formalin and stained with picrosirius red for 30 minutes to obtain images of thrombus deposition on the stent.

3.2.6 Effect of PPACK-NP on aortic endothelial cell proliferation

Human aortic endothelial cells (HAECs) were acquired from Lifeline Cell Technology (Frederick MD). HAECs were cultured in VascuLife EnGS Complete Medium (Lifeline Cell Technology) prior to detachment with 0.05% trypsin-EDTA and plating in a 96 well plate at 10,000 cells/well. Following overnight cell attachment, the growth medium was replaced with EnGS complete medium with 1% penicillin-streptomycin-amphotericin (PSA, LS-1085, Lifeline Cell Technology) containing 1 U/ml human thrombin (Haematologic Technologies, Essex Junction, VT) plus equimolar amounts of free PPACK, precursor NP and PPACK-NP. The cells were allowed to incubate for 4 days with gentle shaking to prevent precipitation of nanoparticles. Following 4 days of incubation, proliferation of HAECs was determined using an XTT assay (Biotium Inc., Hayward, CA) as per the manufacturers instructions.
3.2.7 Statistics

All statistical tests were performed on R, version 3.0.1. For the static model of stent thrombosis, the Student’s t-test was utilized, where $p < 0.05$ denotes statistical significance. All error bars are depicted as standard error of the mean.
3.3 Results

3.3.1 Evaluation of PPACK-NP in a static model of stent thrombosis

We initially seeded sections of stents with thrombin to facilitate the further accumulation of procoagulant material on the stent surface. Thrombin adsorption to the stent surface was quantified through incubation of thrombin treated stents with S-2238, a chromogenic substrate that liberates a p-nitroanilide group following specific cleavage by thrombin, thus producing an absorbance peak at 405 nm. Thrombin-treated stents significantly increased the amount of S-2238 cleavage when compared to saline controls (p = 0.00000052, n = 3 per group), confirming the presence of thrombin on stent surfaces (Fig. 3-2A).

The efficacy of PPACK-NP was assayed in comparison to saline, free PPACK and plain NP controls. Initial work was performed with platelet poor plasma (PPP) to isolate the effect of PPACK-NP for inhibiting the formation of primarily fibrin clots (Fig. 3-2B). In the absence of thrombin inhibiting compounds (PPACK or PPACK-NP), we observed that treatment with saline and control NP resulted in a gradual time-dependent increase in clot weight. Treatment of thrombin-adsorbed stents with PPACK or PPACK-NP results in minimal fibrin clot formation, which was indicative of passivation of the procoagulant stent surface.

As thrombin exerts pleiotropic thrombotic and signaling effects, we aimed to explore the role of PPACK-NP for inhibiting platelet activation on stents. Platelet-rich plasma (PRP) was generated through supplementation of platelet concentrate into samples of PPP, with a final concentration of 300,000 platelets/ml used in each experiment. Additionally, separate groups of stents were exposed to Vorapaxar treated plasma to delineate the benefit of PPACK-NP in conjunction with an established platelet inhibitor. PRP exposure to stents (Fig. 3-2C) resulted in
rapid clot formation within 5 minutes of exposure and plateaued for the duration of the experiment in saline and control NP treated groups. PPACK and PPACK-NP treatments resulted in little to no clot deposition on stents following 60 minutes of PRP exposure. This lack of clot deposition on stents was also observed in stents treated with PPACK and PPACK-NP after exposure to platelet-inhibitor treated plasma. In the case of Vorapaxar-treated plasma, PPACK and PPACK-NP inhibited stent thrombosis with greater efficacy than did Vorapaxar alone (Fig. 3-2D).

Ultrasound imaging of clots revealed a dense clot deposited on the surface of stents treated with saline, compared to imperceptible clot deposition in stents treated with PPACK-NP (Fig. 3-2E). The morphology of platelet-rich clots was observed by scanning electron microscopy of stents following 60 minutes of PRP exposure for each stent treatment group. SEM evaluation revealed a dense fibrin network formed on stent surfaces treated with saline (Fig. 3-3A) and control nanoparticles (Fig. 3-3B), with platelets embedded within fibrin clots in the saline treated stents. PPACK treated stents (Fig. 3-3C) demonstrated only a very thin layer of fibrous material deposited on the stent surface, consistent with clot weight measurements confirming very little accumulation of clot on stent surfaces in the static model of stent thrombosis. Interestingly, in PPACK-NP treated stents (Fig. 3-3D), the surface of the treated stents showed the deposition of circular nanostructures and a lack of fibrin or platelets. We hypothesized that the circular particles on the surface of the stent were particles that had adhered to the stent surface, with their flat appearance on SEM due to the vacuum drying step in SEM preparation that results in evaporation of the perfluorocarbon core and subsequent collapse of the particle, leaving a flattened deposit of the collapsed PFC-NP lipid monolayer. The diameter of these deposited particles was observed to be $185.46 \pm 2.92 \text{ nm}$, as measured by ImageJ.
3.3.2 Evaluation of PPACK-NP in an in vitro flow model

Following the evaluation of PPACK-NP in the static stent thrombosis model, we investigated the ability of PPACK-NP to prevent clot formation in flowing, platelet rich plasma (Fig. 3-4). In this model, a flow loop circuit was perfused with platelet-rich plasma at 150,000 platelets/ml together with selected nanoparticle or control solutions. Following complete perfusion of the flow loop, 10 units of thrombin was infused and allowed to flow for 10 minutes, after which the stents were removed and stained with picrosirius red to observe clot deposition. The results of this experiment demonstrate the ability of PPACK-NP to prevent clot deposition on prothrombotic materials such as a bare metal stent.

3.3.3 Effect of PPACK-NP on Endothelial Cell Viability and Proliferation

Because reendothelialization of denuded vascular regions and stent surfaces is crucial to the wound healing response following stent implantation, we sought to define the effect of PPACK-NP on endothelial proliferation. Proliferation was measured using an XTT assay (Fig. 3-5) that revealed a modest but significant decrease in proliferation after 4 days of continuous exposure to thrombin (12.45% decrease, p = 0.011) or thrombin and control NP (11.24%, p = 0.049). PPACK and PPACK-NP treatment restored baseline endothelial proliferation levels under these conditions suggesting an additional benefit of anti-thrombin nanoparticle in accelerating local endothelial proliferation.
3.4 Discussion

Previous experiments have attributed a potential mechanistic explanation for the efficacy of antithrombin nanoparticles, where the presentation of multiple copies of thrombin inhibiting peptides allows for the binding and retention of nanoparticles to clot-bound thrombin. This retention of particles allows for the generation of an “anti-clotting” surface on top of a fresh thrombus that delays or prevents future clot growth. This phenomenon represents an additional benefit of antithrombin nanoparticle treatment, as treatment with free inhibitor only allows for 1:1 binding and would not establish such an antithrombotic surface. Furthermore, as each particle can inactivate thousands of thrombin molecules, the continued surveillance and inactivation of local thrombin results in a sustained thrombin “sponge” effect.

In static conditions, PPACK-NP treated stents exhibited markedly reduced clot burdens than did saline or control nanoparticle both in platelet rich and platelet poor plasma. Ultrasound imaging of stents along with scanning electron microscopy clearly revealed that PPACK and PPACK-NP treated stents presented with essentially no clot deposition as compared to the dense fibrin thrombi formed in control nanoparticle and saline control groups (Fig. 3-2B&C). Furthermore, as antiplatelet therapy is a commonly prescribed as an adjunct to increase stent patency, we investigated the effects of PPACK-NP on treated stents following exposure to platelet inhibitor-treated PRP. As thrombin served as the agonist in these experiments, we tested Vorapaxar (Zontivity, Merck), a recently FDA-approved direct protease-activated receptor-1 antagonist to block the activation of platelets by thrombin. Only a minor effect of Vorapaxar was observed in preventing thrombus formation as compared to thrombus inhibition with PPACK or PPACK-NP (Fig. 3-2D). This superiority of PPACK-NP treated stents over and above Vorapaxar alone suggests that even in the event of diminished platelet activation due to
vorapaxar treatment, the uninhibited effects of thrombin on fibrinogen cleavage still prevails in formation of thrombi at injury sites. These results illustrate a strong potential adjunctive benefit for the use of PPACK-NP either alone or in conjunction with other standard therapies.

We further explored this activity in an in vitro flow loop model to simulate a clinically relevant scenario. Picrosirus red staining of stents following exposure to circulating PRP demonstrated total occlusion of stents after saline and plain NP treatment, as compared to no thrombosis in PPACK or PPACK-NP treated stents (Fig. 3-4). The present results suggest a potential clinical utility for PPACK-NP as either stent coatings or through periodic intravenous administration that would allow for effective antithrombotic activity with minimal bleeding side effects, as previously demonstrated.7

Endothelialization of stents following implantation is a crucial step in healing of injury sites14, and as such, we investigated the effect of PPACK-NP on endothelial proliferation (Fig. 3-5). Thrombin has previously been shown to promote wound healing through generation of VEGF, and with inhibition of thrombin being potentially detrimental to endothelialization. However, several groups have shown a concentration dependent and cell-type dependent effects of thrombin on endothelial cell proliferation, where high concentrations of thrombin (>1U/ml) have a detrimental effect on proliferation of aortic endothelial cells, as opposed to umbilical vein endothelial cells.15-17 Accordingly, we utilized human aortic endothelial cells for measurements of proliferation in response to thrombin stimulation at 1U/ml for 4 days. A modest thrombin-dependent decrease in proliferation was observed for the control group, but basal cell proliferation/viability was restored in the PPACK and PPACK-NP treated groups. These results indicate that thrombin inhibition with PPACK-NP in this in vitro experiment preserved endothelial proliferative potential on the face of high local concentrations of thrombin such as
those found in atherosclerotic plaques and vessel segments undergoing stenting. Additionally, it is possible that treatment with PPACK-NP may have other related effects on the endothelium, namely effects on cell migration and survival. Thrombin's role in vascular pathophysiology, for example, includes the upregulation of NADPH oxidase secretion by vascular smooth muscles cells that may contribute to reactive oxygen species (ROS)-mediated endothelial damage and inhibition of endothelial cell migration. Thus along with the present data, and the effects of thrombin on endothelial proliferation, migration and survival published in the literature, there may be a benefit to thrombin inhibition with PPACK-NP, however this effect remains to be delineated and is the subject of future studies with this therapeutic.

Current recommended systemic strategies for post-stent therapy include anticoagulation and antiplatelet therapy. There has been considerable interest in modification of the implant itself with strategies including coatings, surface modifications, and techniques to more rapidly endothelialize the surface. Specific anti-thrombotic surface modifications with the addition of heparin and bivalirudin have shown promise in prior studies. Of note, Zilver PTX was recently approved and is the first drug-eluting stent approved for treatment of peripheral arterial disease. Unfortunately, drug-eluting devices are plagued by early thrombosis and require prolonged dual antiplatelet therapy with its attendant bleeding risks and costs. Our approach offers a flexible method to employ a potent biocompatible nanoparticle that is functionalized with > 10,000 copies of a relevant anticlotting agent per particle, which could be PPACK in this case, or any other moiety as we have shown already for bivalirudin.

The study has some limitations that merit discussion. As this is an in-vitro experiment with controlled variables, these results cannot be immediately extrapolated to similar efficacy in in-vivo models or in patients, where flow conditions may differ. Additionally, in the static
experiment the clots were followed out to 60 minutes, but to more accurately depict clinical situations more prolonged observations would be needed. Furthermore, bare metal stents are less commonly employed clinically and the effect of these NPs on drug eluting stents will require examination. Finally, persistence of the agent at the site of stenting or vessel injury will need to be assessed.

Overall, this study has shown that anti-thrombin perfluorocarbon nanoparticles can inhibit stent thrombosis in static and flow loop in-vitro models. Future work will explore efficacy in vivo using rabbit models of thrombosis following vascular device implantation to validate the promise of a potentially safer adjunctive solution to the clinical problem of intravascular stent thrombosis.
3.5 Acknowledgements and Copyright

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Figure 3-1: (A) Schematic of perfluorocarbon nanoparticle with surface conjugated PPACK (PPACK-NP). (B) Following implantation, activated thrombin adsorbs to vascular stents forming a pro-thrombotic coating. PPACK-NP binds to and inactivates this adsorbed thrombin transforming the stent surface with a functionally anti-thrombotic coating.
Figure 3-2: (A) S-2238 incubation confirms thrombin adsorption to stent surfaces. (B) Incubation of treated stents with platelet poor plasma results in significant clot deposition following 60 minutes of exposure with non-inhibitor treated stents compared to lack of clot growth on PPACK-NP treated stents. (C) Rapid thrombus deposition following 5 minutes of platelet-rich plasma exposure, compared to no growth on PPACK-NP treated stents for the duration of the experiment. (D) Pretreatment with vorapaxar demonstrates little benefit in preventing stent thrombosis alone, with significantly less clot deposition when used in combination with PPACK-NP. (E) Ultrasound imaging of platelet poor plasma exposed stents confirms lack of clot deposition on PPACK-NP treated stents compared to saline controls. In all panels, *p<0.05, **p<0.005, ***p<0.0005.
Figure 3-3: Scanning electron microscopy of platelet rich plasma exposed stents treated with (A) Saline, (B) Control NP, (C) free PPACK, and (D) PPACK-NP. Stents treated with saline or control NP show deposition of a dense fibrin mesh compared to PPACK or PPACK-NP treated stents. SEM confirms the deposition of PPACK-NP on the stent surface with a measured diameter of $185.46 \pm 2.92$ nm.
Figure 3-4: Picrosirus red staining of thrombosed stents following 10 minutes of stent exposure to flowing plasma containing each treatment group and 10 units of thrombin. In saline and control NP treated plasma, occlusive thrombi were deposited on stents with minimal deposition on stents in PPACK and PPACK-NP treated plasma.
Figure 3-5: Thrombin inhibition allows for maintenance of normal cell viability and proliferation in human aortic endothelial cells compared to non-inhibitor controls. In saline and control NP treated groups, proliferation of endothelial cells was significantly decreased compared to baseline levels of proliferation following 4 days of thrombin exposure.
3.6 Literature Cited


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Chapter 4: Quantifying Progression and Regression of Thrombotic Risk in Experimental Atherosclerosis with Perfluorocarbon Nanoparticles
Chapter Overview

Currently, there are no generally applicable noninvasive methods for defining the relationship between atherosclerotic vascular damage and risk of focal thrombosis. Herein, we demonstrate methods to delineate the progression and regression of vascular damage in response to an atherogenic diet by quantifying the in vivo accumulation of semipermeable 200-300 nm perfluorocarbon-core nanoparticles (PFC-NP) in ApoE null mouse plaques with $^{19}$F magnetic resonance spectroscopy ($^{19}$F MRS). Permeability to PFC-NP remained minimal until 12 weeks on diet, then increased rapidly following 12 weeks, but regressed to baseline within 8 weeks after diet normalization. Markedly accelerated clotting (53.3% decrease in clotting time) was observed in carotid artery preparations of fat fed mice subjected to photochemical injury as defined by the time to flow cessation. For all mice on and off diet, an inverse linear relationship was observed between the permeability to PFC-NP and accelerated thrombosis ($p = 0.02$). Translational feasibility for quantifying plaque permeability and vascular damage in vivo was demonstrated with clinical 3T magnetic resonance imaging of PFC-NP accumulating in plaques of atherosclerotic rabbits. These observations suggest that excessive permeability to PFC-NP may indicate prothrombotic risk in damaged atherosclerotic vasculature, which resolves within weeks after dietary therapy.
4.1 Introduction

Atherosclerosis is the leading cause of death in the developed world, manifesting high morbidity and mortality as a consequence of recurrent acute vascular events that are essentially unpredictable in individuals and frequent despite maximal medical therapy.\textsuperscript{1,2} Recent focus on the pathophysiology of atherosclerosis has shifted to the panoply of inflammatory cell types and necrotic debris that engage a host of prothrombotic signaling events resulting in acute focal clotting and vascular obstruction, unstable angina, and infarction.\textsuperscript{3,4} In territories prone to plaque development, early lesion formation initiates with the development of a proinflammatory endothelium, characterized by weakened tight junctions (<20 nm) that permit the passage of small molecule dyes (e.g. Evans Blue)\textsuperscript{5,6} and albumin\textsuperscript{7-9}. Noninvasive delineation of these very early pathophysiological features that emerge well before clinical events arise has been available for years with application of numerous imaging techniques that appear to presage an increased incidence of events, at least in study populations that harbor traditional coronary risk factors.\textsuperscript{10,11}

We recently reported a magnetic resonance imaging (MRI) and spectroscopic (MRS) approach for delineating the severity of vascular endothelial damage in atherosclerotic vessels by measuring the passive permeation of perfluorocarbon nanoparticles (PFC-NP) into plaques of fat-fed rabbits after only 60-120 minutes of circulation \textit{in vivo}.\textsuperscript{12} Endothelial apoptosis, erosions, and fibrin deposition were observed in these plaques after 6 months on a high cholesterol diet. \textit{Ex vivo} fluorine ($^{19}$F) MRI and MRS depicted the intimal localization and quantity of PFC-NP in rabbit atherosclerotic lesions that appeared after 6 months on diet, and also in diseased human carotid endarterectomy samples that were incubated \textit{ex vivo} with the PFC-NP. However, the related clinically relevant question as to whether increased vascular permeability to PFC-NP in
damaged vessels is correlated with focal prothrombotic risk in genetically-prone models of vascular disease has not been examined.

Accordingly, we sought to answer the following questions: 1) whether prolonged feeding of Western Diet induces a state of increased vascular permeability in ApoE null mice that can be detected and quantified with the use of magnetic resonance spectroscopic and imaging methods; 2) whether the permeability of the endothelium to PFC-NP resolves following cessation of a Western Diet and if such phenomena can be tracked and quantified with PFC-NP imaging methods, and 3) whether vessel thrombotic risk is related directly to diet-induced vascular permeability and how quickly accelerated thrombosis might resolve following Western diet cessation.
4.2 Materials and Methods

4.2.1 Nanoparticle Formulation

Fluorescent perfluorocarbon nanoparticles were formulated according to previously established emulsification techniques. Briefly, the PFC-NP consisted of a 20% (vol/vol) perfluoro-15-crown-5-ether (CE) core, 2% (wt/vol) surfactant, 1.7% (wt/vol) glycerin, and water. The surfactant consisted of 98.8 mol% egg phosphatidylcholine, EPC (Lipoid, Newark, NJ), 1 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, DPPE (Avanti Polar Lipids, Alabaster, AL) and 0.2 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), Rhodamine-PE (Avanti Polar Lipids, Alabaster, AL). The emulsion was modified to replace the crown ether core with perfluorooctylbromide (PFOB) for use as a $^{19}$F MRS reference standard for quantification of nanoparticle concentration in plaques. For in vivo $^{19}$F MRI studies in rabbits, the emulsion was modified to increase the perfluorocarbon and surfactant content to include 4% (wt/vol) surfactant and 40% (vol/vol) CE.

4.2.2 Animal Experimental Model

Mouse feeding regimen: To produce aortic plaques, groups of male 4 to 6-week-old ApoE-null mice were fed either normal chow or Western diet (TD-88137, Harlan Laboratories, Madison, WI) for 2, 3, 4, 5, and 6 months. Two other sets of mice were fed with Western diet for 4 months and then switched to normal chow for either 1 or 2 months, thus serving as experimental “off-diet” time points age-matched with mice continued on Western diet for 5 or 6 months. At each selected time point, a 1 ml/kg bolus dose of nanoparticles was administered via tail vein injection and allowed to circulate for 2 hours to allow for saturation of nanoparticle accumulation in plaques.
Carotid artery thrombosis procedure: Following 2 hours of *in vivo* nanoparticle circulation, mice were anesthetized with a cocktail of ketamine and xylazine (87 mg/kg and 37 mg/kg, respectively) and subjected to photochemical injury of the carotid artery\textsuperscript{13-15} to determine time to vessel occlusion. The right common carotid artery was exposed through a midline cervical incision and blood flow rate was monitored using an ultrasonic Doppler probe (Transonic Systems Inc., Ithaca, NY). The intended injury site was illuminated with a 1.5 mW 540 nm HeNe laser, followed by injection of 50 mg/kg Rose Bengal (Sigma-Aldrich, St. Louis, MO) in saline to initiation of thrombus growth. The experiment was concluded upon achieving a >85% decrease in carotid blood flow rate maintained for >5 min. Time to occlusion was recorded and used to evaluate coagulability. Mice were then sacrificed and the aortas were cleaned and removed for $^{19}$F spectroscopic evaluation of nanoparticle accumulation.

Rabbit feeding regimen: Male New Zealand White rabbits were maintained on an 0.25% cholesterol feed (Cat. 9433, TestDiet, St. Louis, MO) for 9 months. For control images without plaque development, young rabbits were fed normal chow. Both control and atherosclerotic rabbits were anesthetized and given a 1ml/kg intravenous bolus of nanoparticles 2-3 hours prior to $^{19}$F MRI.

4.2.3 $^{19}$F Magnetic Resonance Spectroscopy

Fluorine magnetic resonance spectroscopy (MRS) was utilized to quantify nanoparticle accumulation in aortic plaques *ex vivo*. $^{19}$F MRS was performed on an 11.7T Varian MR scanner with a custom-built single-turn solenoid RF coil, with the following parameters: TR = 2.5 s, 1024 signal averages, with a scan time of ~42 minutes. A fluorine reference standard of 0.1% PFOB emulsion was included with each aorta sample. To quantify plaque accumulation of crown
ether nanoparticles, crown ether signal was compared to the distinct signal of the known PFOB standard. The amount of crown ether nanoparticles was normalized to the weight of the aorta sample.

4.2.4 Aortic Perfusion of PFC-NP and $^{19}$F Magnetic Resonance Imaging Ex Vivo

Aortas from rabbits fed a cholesterol diet for 8 months were excised and cannulated with stub adapters for attachment to a custom-built perfusion system. The perfusion system consisted of a variable flow rate perfusion pump (Cole Parmer, Barrington, IL) with Masterflex tubing connected to stub adapters at either end of the vessel. A 50 ml conical tube served as a reservoir for the perfusate, containing a 1:20 dilution of PFC-NP in saline. The diluted PFC-NP mixture was perfused through the arteries at a flow rate of 30 mL/min for 4 hours. Following perfusion, the isolated aortas were rinsed and fixed in 10% Formalin for 24-48 hours.

Ex vivo $^{19}$F spectroscopy of isolated aorta was performed on an 11.7T Varian scanner (Varian Inc, USA). Prior to ex vivo $^{19}$F imaging, the vessel lumen was filled with 2% agarose gel and stored in saline. Upon MRI, aortas were placed in a 1-cm tube filled with saline. A capillary tube containing diluted (1:200) CE NPs were attached to the tube wall to serve as the external signal standard. All images were acquired using a solenoid radio-frequency coil and a standard fast-spin echo sequence. The coil was first tuned to 1H frequency to acquire a set of multi-slice 1H images. Imaging parameters were: field of view, 2 x 2 cm$^2$; image matrix, 128 x 128; slice thickness, 2 mm; number of slices, 11; TR, 2s; TE, 20ms, etl, 8; number of averages,4; imaging time, 2 min. The coil was then tuned for 19F MRI. A set of 19F images was acquired at the same location as 1H images. Imaging parameters were: field of view, 2 x 2 cm$^2$; image matrix, 32 x 32; slice thickness, 2 mm; TR, 1.2s; TE, 20ms, etl, 8; number of averages, 4096; imaging
time, 5 hour 28 min. The acquired 19F images (matrix size = 32 x 32) were interpolated to 128x128 and overlaid on corresponding 1H images to localize 19F signal of PFC NPs.

4.2.5 In vivo 19F Magnetic Resonance Imaging

To image the aortic wall for detection of nanoparticles permeating into the plaque intima, we employed a 3T clinical whole-body scanner (Achieva, Philips Healthcare, The Netherlands), outfitted with a dual 19F/1H spectrometer system.16 A dual-resonant 19F/1H surface RF coil was used (15×15 cm), which can either transmit or receive at both resonance frequencies simultaneously.17 Imaging was performed ~2h post-injection of 1.0 ml/kg PFC-NP with perfluoro-15-crown-5-ether (PFCE; C_{10}F_{20}O_{5}) core as previously described. To avoid signal contamination from inhaled fluorinated anesthesia, a xylazine (10mg/kg) / ketamine (85 mg/kg) i.m. injection was used for anesthesia induction, which was maintained with a ketamine i.v. infusion (18 mg/kg/hr). A 2D simultaneous 19F/1H gradient echo (FFE) sequence was used with the following parameters: FOV = 128×128 mm, matrix = 96×96, slice thickness = 20 mm, voxel size = 1.33×1.33×20 mm, α = 25°, exBW = 5 kHz centered on single PFCE peak, pBW = 500 Hz, TR/TE = 14/1.72 ms, 1000 NSA, and a scanning time of 33 minutes. Saturation bands proximal and distal to the imaging slice were applied to eliminate 19F signal from the blood pool. The imaging slice was centered on the abdominal aorta, located 2-3 cm distal to the renal artery via an angiogram consisting of a multi-2D time-of-flight gradient echo sequence with the following parameters: FOV = 100×78 mm, matrix = 112×112, slice thickness = 2 mm, α = 60°, TR/TE = 13.54/4.06 ms, 4 NSA, and a scanning time of 3 minutes. After anatomical colocalization of 19F signal was confirmed with the simultaneously acquired 1H image, a high-resolution gradient echo 1H image was used to display the overlaid 19F signal with the following
parameters: FOV = 128×128 mm, matrix = 256×256, slice thickness = 4 mm, voxel size = 0.5×0.5×4 mm, α = 35°, TR/TE = 25.16/7.02 ms, 23 NSA, and a scanning time of 2.5 minutes.

4.2.6 Scanning Electron Microscopy

Freshly harvested aortas from ApoE-/- mice fed a high fat diet for 6 months were speed vacuum dried overnight to allow visualization of superficial cholesterol crystals. Dried tissues were then mounted for imaging. Scanning electron microscope images were acquired with Hitachi S-2600H and FEI Nova Nano 2300.

4.2.7 Histology

For immunofluorescent evaluation of CD31 and thrombin deposition, mouse aortas were harvested and embedded in optical cutting temperature (OCT) medium. Aortas were sectioned and stained with either an anti-CD31 antibody (ab28364, Abcam Inc., Cambridge, MA) or an anti-thrombin antibody (ab92621, Abcam Inc., Cambridge, MA). For Oil Red O staining of rabbit aortas, tissue was embedded in OCT medium and sectioned. Slides were fixed in formalin, and then stained in freshly prepared Oil Red O solution followed by rinsing with 60% isopropanol. Sections were stained with hematoxylin, rinsed, then mounted for microscopy.

4.2.8 Two-Photon Imaging

Aortas were removed from both the normal chow and high cholesterol fed rabbits and incubated en face for 15 minutes with 500 kD FITC-dextran to allow for ex vivo penetration of the FITC-dextran into plaques. The imaging of the aorta samples was performed on a custom-built video rate two-photon microscope equipped with a Chameleon Vision II Ti:Sapphire laser (Coherent, Santa Clara, CA) in the Washington University in St. Louis School of Medicine In
Vivo Imaging core. Fluorescence emission was passed through 480 nm and 560 nm dichroic mirrors placed in series and detected as red (>560 nm), green (480 to 560 nm), and blue (<480 nm) channels by three head-on multi-alkali photomultiplier tubes.

4.2.9 FACS analysis of PFC-NP Uptake by Peripheral Blood Leukocytes and Splenocytes

Mice were injected i.v. with 200 µl of nanoparticles and sacrificed 30 min later. Leukocytes were isolated from mouse spleens and peripheral blood with Histopaque-1119 according to manufacturer's protocol (Sigma, St. Louis, MO). Antibodies against the following molecules coupled to the indicated fluorochromes were used from BD Pharmingen (San Jose, CA), eBioscience (San Diego, CA) or BioLegend (San Diego, CA): FITC anti-Ly-6C (HK1.4; BioLegend), PerCP anti-Ly6G (1A8; BD Pharmingen), APC anti-F4/80 (BM8; eBioscience), APC anti-TCRβ (H57-597; eBioscience), FITC anti-CD19 (1D3; BD Pharmingen). In general, 10^6 cells were blocked with the anti-FcR mAb 2.4G2, stained with the indicated Abs for 20 min at 4°C and then washed and resuspended for FACS analysis. Flow cytometry was performed on the BD FACSCalibur™. Data analysis was performed using BD CellQuest™ Pro software.

4.2.10 Western Blot Analysis of Complement Activation

Western blot analysis of complement activation was performed as described previously. C57BL/6 mice were injected intravenously with 10 µl/g of PBS, plain PFC-NP, and two different species of gadolinium (Gd) loaded PFC (Gd-DTPA and Gd-DOTAP). Gd-DOTAP PFC-NP were utilized as a positive control for complement activation. Thirty minutes following administration of test doses, the mice were sacrificed and blood was collected via the inferior vena cava in EDTA-loaded tubes. Blood samples were centrifuged for 5 min at 4°C to obtain plasma samples. For each sample, 15 µl of plasma was diluted 1:100 in SDS running buffer and fractionated by
SDS-PAGE under reducing conditions to observe complement activation in plasma. For probing of complement activation on the nanoparticle surface, blood samples were centrifuged at 960g for 15 min, and the resulting pellet was rinsed four times in EDTA buffer, resuspended in SDS running buffer and fractionated under reducing conditions with SDS-PAGE. In both plasma and NP-surface analysis, complement activation was probed using anti-C3 (1:10,000 dilution, Valeant Pharmaceuticals International, Aliso Viejo, CA) and anti-Factor H (1:1000 dilution, CompTech, Tyler, TX) primary antibodies. The secondary antibody used was a horseradish peroxidase-conjugated donkey anti-goat IgG (1:10,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA). The resulting bands were visualized with a SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

4.2.11 Statistics

All statistical tests were performed on R, version 3.0.1. Carotid artery occlusion time data was analyzed using Mann-Whitney-Wilcoxon rank sum test. Nanoparticle accumulation trendline analysis was accomplished using a Student’s t test. The Pearson’s product-moment correlation test was utilized to determine the relationship between nanoparticle accumulation and carotid occlusion time. An ANCOVA was performed for analysis of cholesterol, nanoparticle accumulation and carotid occlusion time data. For all statistical tests, p<0.05 denotes statistical significance. Error bars denote standard error of the mean.

4.2.12 Study Approval

All procedures were performed with approval from the Washington University Animal Studies Committee.
4.3 Results

4.3.1 Functional evaluation of vascular permeability after prolonged Western Diet

To elucidate vascular permeability *in vivo* in ApoE null mice subjected to selected durations on a Western diet, PFC-NP were injected i.v. and allowed to circulate for 2 hours prior to excising whole aortas for $^{19}$F-MRS quantification of PFC-NP. A progressive monthly increase in PFC-NP accumulation was observed in the atherosclerotic aortas of mice on Western diet over and above that of mice on normal chow diets, the severity of which reflected the duration of Western diet feeding (Fig. 4-1A). Prior to 3 months of feeding, little accumulation of PFC-NP was observed in either Western diet fed or normal chow groups. However, beyond 3 months, increased accumulation of PFC-NP was noted in aortic plaques of fat fed mice as compared with the normal chow group, with plaque concentrations of PFC-NP reaching $0.201 \pm 0.043 \mu l/g$ aorta (N=3) in mice fed a Western diet for 6 months compared to the age-matched group of ApoE null mice fed normal chow ($0.081 \pm 0.011 \mu l/g$ aorta, N=3).

4.3.2 Tracking regression of vascular permeability to PFC-NP

To delineate the resolution of endothelial permeability after dietary cholesterol reduction, two additional groups of ApoE null mice were fed a Western diet for 4 months and then switched to normal chow for either 1 or 2 months before injecting PFC-NP for spectroscopic evaluation of vascular permeability. Serum cholesterol levels confirmed the effect of dietary replacement, as cholesterol levels rose to $965.45 \pm 201.05$ mg/dl by 5 months in mice on Western diet compared to $403 \pm 39.69$ mg/dl and $461.77 \pm 45.15$ mg/dl for mice on Western Diet for 4 months and then off the Western diet for 1 month or 2 months, respectively (Fig. 4-1B). During this period, no perceptible change in gross aortic plaque coverage was observed between experimental “off-
diet” groups and age-matched Western diet groups (data not shown). Regression of vascular permeability was confirmed by a dramatic decrease in nanoparticle accumulation in mouse aortas by two months after dietary cholesterol lowering (0.065 ± 0.019 µl/g aorta, N=4, p = 0.0009 vs age-matched Western diet group), returning to age-matched ApoE null normal chow fed control levels (p = NS). (Fig. 4-1A). Trend assessment from 4-6 months was performed by regression analysis and demonstrated a significant difference in the slope of the linear fit between the continued progression of vascular permeability in the Western diet fed mice versus the resolution of vascular permeability in those mice removed from the diet (slope: 0.033 ± 0.019 vs -0.026 ± 0.009 for the Western diet group and the mice removed from Western diet, respectively; p = 0.015).

4.3.3 Relationship between vascular permeability and vessel thrombotic potential

As increased endothelial permeability and loss of endothelial cells has been linked to future thrombotic events, we anticipated that potential differences in vessel thrombotic potential might be related to the severity of permeability. Our hypothesis was further supported by the presence of large deposits of intraplaque thrombin (Fig. 4-2A) that may contribute to the generation of local inflammatory and procoagulant functions. Therefore, a standard model of photochemical injury to the carotid artery was used to quantify the propensity to focal thrombosis for the different diet schedules according to the time required to attain complete vessel occlusion, which is inversely related to local procoagulant activity. Vessel injury in ApoE null mice on normal chow for 5 months resulted in an occlusion time of 72.14 ± 5.02 min (N=10), which was equivalent to that previously observed for wild-type C57BL/6\(^\text{14}\); 69.06 ± 5.66 min, N=6; p=NS (Fig. 4-2B, red dashed line). ApoE null mice fed with Western diet for 5 months exhibited dramatically shortened occlusion times of 33.62 ± 3.04 min (N=6) as compared
with the mice on normal chow (N=10) for 5 months (p = 0.004). In mice fed a Western diet for 4 months followed by normal chow for either 1 or 2 subsequent months, occlusion times progressively lengthened back to control levels: 45.33 ± 5.63 min, N=7 (p = 0.005 vs. control) and 68.6 ± 10.79 min (p=NS vs. control), respectively. (Fig. 4-2B) By 2 months after cessation of Western diet, the occlusion time (68.6 ± 10.79 min, N=5) was not significantly different as compared with wild-type C57BL/6 mice, indicating reversible diet-dependent vessel procoagulant activity. Furthermore, occlusion time measurements on ApoE-/- mice fed a Western Diet for 1 month (65.73 ± 23.65, N=3) were not significantly different compared to wild-type C57BL/6 or normal chow ApoE controls. Importantly, an inverse correlation (Fig. 4-3) was observed between nanoparticle accumulation and occlusion time (p = 0.02), indicating that thrombotic potential tracks with increased vascular permeability in this model. Additionally, ANCOVA analysis demonstrated no significant effect of serum cholesterol levels on carotid occlusion time (p = 0.3849) and no significant interaction effect between serum cholesterol levels and plaque nanoparticle accumulation (p = 0.4223), ruling out an independent effect of blood cholesterol itself on occlusion times.

4.3.4 Mechanisms of intimal permeation of nanoparticles

To investigate potential mechanisms of nanoparticle penetration and retention, mouse aortic tissue samples were imaged with scanning electron microscopy to examine the morphology of intimal plaques. In accord with prior reports in rabbit\textsuperscript{12} and human\textsuperscript{18} atherosclerosis specimens that manifest superficial deposits of protruding cholesterol crystals when properly prepared for SEM, we also observed analogous intimal and superficial cholesterol crystals on samples of mouse aortas presenting with plaques (Fig. 4-4A-C). Immunofluorescent staining of CD31 (Fig. 4-4D-F) revealed barely detectable intra-plaque signal, consistent with
prior reports of limited angiogenesis at these earlier time points in atherosclerotic mice, suggesting that passive diffusion of PFC-NP into the intima did not occur through neovascular elements.\textsuperscript{21}

To better define the potential route of intimal penetration of these PFC-NP into plaques, we resorted to the previously validated rabbit model\textsuperscript{12} for two-photon assessment of permeability. Rhodamine-labeled PFC-NP were administered to rabbits fed either normal or high cholesterol chow. Aortic segments were removed from the rabbits following 2 hours of in vivo nanoparticle circulation and incubated with FITC-dextran \textit{ex vivo}, demonstrating prominent accumulation of both FITC-dextran and PFC-NP beyond the lumenal surface of the plaque (Fig. 4-5), which is consistent with prior conclusions that active transport and blood flow are not required to achieve intimal nanoparticle penetration. Furthermore, excised atherosclerotic rabbit aortas were perfused \textit{ex vivo} with PFC-NP on a custom built perfusion system for 4 hours to elucidate the contribution of passive lumenal permeation of NP without the need for active cellular transport or trafficking through vasa vasorum. Fluorine imaging of nanoparticle perfused aortas (Fig. 4-6) demonstrates ample plaque-associated nanoparticles due to passive permeation. To further rule out alternative cellular transport mechanisms, FACS analysis of isolated peripheral blood leukocytes (PBL) (Fig. 4-7A) and splenocytes (Fig. 4-7B) collected 30 minutes after i.v. injection of PFC-NP demonstrated minimal active uptake of the circulating PFC-NP by cell types that might traffic to plaques: 0.26% of PBL and 0.93% of splenocytes. Western blot analysis revealed no complement activation in response to the administration of nanoparticles, militating against immune cell trafficking of complement activated PFC-NP (Fig. 4-8).
4.3.5 Imaging nanoparticle plaque permeation in vivo

In a pilot study, $^{19}$F MRI was performed on rabbits with or without diet-induced atherosclerotic plaques (Fig. 4-9). Figures 5A and D are proton images of abdominal cross-sections showing the position of the aorta in rabbits fed normal chow and Western diet, respectively; 5B and E are the fluorine-only signatures after PFC-NP circulation in vivo for 180 minutes. Note the excellent suppression of lumenal blood $^{19}$F signal in the normal chow rabbits due to saturation band placement (Fig. 4-10) indicating that no signal arises from the circulating PFC-NP. Figures 4-9C and 4-9F are $^{19}$F/$^1$H overlays showing the aortic and adjacent vena cava wall $^{19}$F signals (green) emanating from PFC-NP permeating into arterial plaques, and interestingly into adjacent inflamed venous structures that can accumulate PFC-NP when subjected to the same hyperlipidemic drive. $^{22,23}$ Figure 4-9C demonstrates that in a healthy rabbit, no measurable nanoparticle retention occurs in the aorta or vena cava. Corresponding Oil Red O staining of the imaged area (Fig. 4-9G-H) confirmed the presence of aortic plaques, or lack thereof.
4.4. Discussion

Herein we describe for the first time a quantitative *in vitro* and *in vivo* MRI/MRS approach for delineating the progression and/or regression of atherosclerotic vascular damage that bears a direct relationship to focal thrombotic risk. The use of $^{19}$F MRS/MRI enables quantification of plaque permeability as a consequence of vascular damage induced by Western diet according to measured plaque PFC-NP content (Fig. 4-1A). It is notable here that we employed the *entire aorta* to ensure an objective definition of vascular permeability that avoided any potentially subjective bias of segmental selection. We observed a marked acceleration of endothelial permeability after 12 weeks on a Western diet in ApoE null mice, greatly exceeding that of mice maintained on normal chow. Rapid diminution of permeability to PFC-NP to baseline levels was achieved after only 2 months of dietary management, where ANCOVA analysis demonstrated no significant interaction effect ($p = 0.4223$) of serum cholesterol levels on endothelial permeability, suggesting that some feature of, or response to the dietary regimen other than just serum cholesterol level may have contributed to the observed vascular damage.

Furthermore, we observed a concomitant rapid resolution of the vascular prothrombotic state in ApoE null mice after only 2 months of dietary management. A standard experimental method was used to quantify vessel thrombotic potential by inducing occlusive thrombosis in the carotid artery with Rose Bengal dye and laser injury that operates by generating caustic superoxide anions, which are known to contribute to the pathogenesis and progression of atherosclerosis. We observed that the functional MRI/MRS readouts for vascular permeability were correlated with the severity of prothrombotic risk (Fig. 4-3) as measured by time to total occlusion of the carotid artery after photochemical injury. Furthermore, we found that these correlated measures of vascular permeability and accelerated thrombosis resolve concomitantly
and rapidly to baseline values after dietary normalization. One potential caveat to note here is that the occlusion times were measured in the carotid artery territory, whereas the permeability metrics were acquired from the entire unselected aorta because experimentally it was not possible to acquire both data sets from the same vascular region in a given animal. However, because the disease process is clearly diffuse and progressive, even though some local differences in plaque severity may pertain, the correlative assessment should be informative as to the overall state of the disease process, especially in light of the rapid resolution of both metrics after cessation of the Western diet.

The use of ApoE null mice for the quantification of vascular barrier damage with PFC-NP was advantageous because immunofluorescent staining of aortic plaques for CD31 in our mouse model revealed little intra-plaque neovasculature up to 6 months of continuous Western Diet (Fig 4-4D-F). Prior reports of angiogenesis in mouse plaques as measured by CD31 staining indicate the presence of neovasculature only after 9 months of cholesterol feeding\(^\text{21}\). Lack of plaque neovasculature at our measured time points, in concert with evidence of cholesterol crystals perforating the intima of diseased ApoE null mouse aortas (Fig 4-4A-C), suggests that lumenal entry of PFC-NP through highly permeable endothelial barriers may be the prevailing route of nanoparticle penetration and retention, rather than through vasa vasorum or neoangiogenic routes. However, it is important to note that vasa vasorum/neoangiogenic delivery of nanoparticles may be possible in larger subjects such as rabbits and humans. Our current data utilizing ex vivo nanoparticle perfusion of atherosclerotic rabbit aortas (Fig. 4-6) coupled with prior data utilizing human endarterectomy specimens\(^\text{12}\), suggests that lumenal entry of nanoparticles remains a significant contributor to nanoparticle signal. Furthermore, the likelihood of predominantly lumenal entry and retention of PFC-NP in plaques accords with our prior work
in atherosclerotic rabbits that showed that PFC-NP do not gain access to the intima of atherosclerotic vessels through neoangiogenic routes in early atherosclerosis, but may enter through eroded endothelium in later stages of plaque development. Additionally, investigations of alternative routes of plaque accumulation of PFC-NP through assays for complement activation (Fig. 4-8) or through FACS analysis of nanoparticle uptake in circulating cells (Fig. 4-7) suggests that active trafficking of peripheral blood leukocytes and/or splenocytes harboring PFC-NP is unlikely in this short experimental time window (<2 hours). Thus, the detected $^{19}$F signal is more probably a consequence of passive accumulation of PFC-NP entering through a highly permeable or disrupted endothelial barrier rather than influx of PFC-NP bearing cells.

With respect to potential interpretations of these data in the light of classically described endothelial dysfunction, we note that implications for endothelial dysfunction in the ApoE null model are not especially well defined in a temporal and quantitative sense. Endothelial dysfunction typically is depicted in terms of decreased endothelium-dependent vasodilation. The time course of the development of endothelial dysfunction in ApoE null mice is somewhat debatable, with various studies demonstrating differing responses to acetylcholine (ACh)-induced vasodilation at selected time points after induction of hypercholesterolemia. Moreover, some studies have even reported normal responses of the aortic endothelium at 20-30 weeks of cholesterol feeding, as opposed to other studies demonstrating significant impairment at 14-15 weeks of cholesterol feeding. Thus, speculation as to whether enhanced PFC-NP permeation represents classical endothelial dysfunction as contrasted with more severe endothelial damage or death (e.g., erosions) cannot be supported by the present data set.

Nevertheless, it is clear that these data indicate the possibility of delineating thrombotic risk by quantification of endothelial permeability to PFC-NP that are directly associated with
structural damage to the endothelial lining of vessels where plaques have formed. Other groups have previously explored noninvasive assessments of endothelial permeability in atherosclerotic ApoE null mice at very early time points after fat feeding. For example, recent work by Phinikaridou et al utilized the clinically approved albumin-binding contrast agent gadofosveset (<6 nm diameter) for measurements of endothelial permeability as a consequence of early endothelial dysfunction with MRI. In this work, MRI of mice on a high-fat diet demonstrated accumulation of gadofosveset as early as 4 weeks after inception of a high-fat diet, which correlated well with Evans Blue staining. These results are consistent with prior descriptions of endothelial permeability of Evans Blue and albumin through weakened and/or broken tight junctions. However, in our study at these earlier stages of atherosclerotic disease up to 12 weeks of cholesterol feeding, the endothelium remains impermeable to the PFC-NP and there is no significant increase in vessel hypercoagulability as measured by carotid occlusion times. Together these observations suggest the appearance of more substantial structural changes in the endothelial barrier of these large vessels, when junction widths increase to ~2-3 μm or greater as a consequence of pathological features such as cholesterol crystal perforation (Fig. 4-4A-C) and superficial plaque erosions that accord with markedly enhanced permeability over and above that which might be observed with mild cell junction widening that is associated with classical early measures of endothelial dysfunction. In support of this contention, it has been noted that as atherosclerotic disease progresses, mechanical (i.e. cholesterol crystals) and biological stressors contribute to endothelial apoptosis and subsequent sloughing of endothelial cells, leaving a disrupted or denuded endothelial barrier exposing large multi-micron diameter perforations in the intimal lining, and unfettered access of circulating blood elements to a reservoir of inflammatory cell types, lipids, cytokines, and coagulation factors that enhance prothrombotic
tendencies.\textsuperscript{31} These and prior data suggest that enhanced endothelial permeability to small molecules within the first 3 months of high-fat diet feeding primarily reflects nanoscopic expansion of cell-cell junctions in intact viable, but dysfunctional endothelium, whereas beyond 3 months of high-fat feeding, the endothelial permeability to PFC-NP might reflect more severe vascular damage that would be more quantitatively depictive of a focal hypercoagulable state than would endothelial dysfunction alone.

As prior pathological studies on victims of acute coronary syndromes have established the clinical relevance of eroded or denuded plaques\textsuperscript{32,33}, the ability to non-invasively image endothelial permeability could have clinical relevance. In view of recent clinical reports by Damani et al. of the association of plaque endothelial sloughing and acute vascular syndromes in atherosclerotic patients\textsuperscript{10} and Makin et al, demonstrating increased CECs can be indicative of endothelial damage in patients with severe atherosclerosis\textsuperscript{34}, non-invasive evaluation of endothelial barrier disruption that corresponds directly with local thrombosis potential could be useful for detecting frank vascular damage and for depicting beneficial responses to cholesterol lowering at the vascular tissue level.\textsuperscript{35} In light of these clinical reports, we investigated a potential translatable methodology for \textit{in vivo} delineation of endothelial permeability through imaging of rabbits with or without atherosclerosis in a clinical 3T MRI scanner (Fig. 4-9) outfitted for \textsuperscript{19}F detection. Our results suggest the ability to depict permeable plaques \textit{in vivo} after only \textasciitilde2-3 hours of PFC-NP circulation (Fig. 4-9C). We note here that the voxel size required for recording a sufficient \textsuperscript{19}F signal to enable fluorine MRI necessarily yields a comparatively low resolution fluorine image in the rabbit (see Fig. 4-9B and 4-9E), as contrasted with the higher resolution \textsuperscript{1}H image. Nevertheless, the ability to image voxels containing PFC-
NP at 3T on a multispectral clinical scanner in rabbits suggests that this approach could be implemented on clinical scanners.

Although endothelial dysfunction as currently measured is associated with risk of eventual clinical events in large populations of selected patients\textsuperscript{36,37}, it is not used routinely as a harbinger of thrombotic potential in individual patients. Whether the present noninvasive methods for quantification of endothelial damage and accelerated thrombosis have any value for detecting plaque rupture or vulnerable plaque, at least as these entities are now defined, remains conjectural because their evaluation in animal models is problematic.
4.5 Acknowledgements and Copywrights

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Figure 4-2: (A) $^{19}$F MRS demonstrating time dependence of nanoparticle accumulation in ApoE null mouse aortas with or without a Western diet. Continuous Western diet feeding results in a significant increase in nanoparticle accumulation over age-matched normal chow controls († denotes $p = 0.003$) Return to a normal chow diet restores control levels of nanoparticle accumulation (‡ denotes $p = 0.0009$ vs age-matched Western diet group). "Off Diet" group at 6 months was not significantly different from age-matched normal chow controls. Trend analysis on Western Diet and “Off Diet” groups following 4 months on diet demonstrates significant difference in progression of nanoparticle accumulation ($p=0.015$). (B) Serum cholesterol values for mice on normal chow for 5 months, on Western diet for 5 months, on Western diet for 4 months followed by 1 month or 2 months switched back to normal chow.
Figure 4-2: (A) Immunofluorescent staining confirms abundant intra-plaque thrombin (green) in mouse aortic plaques. Scale bar denotes 100 µm. (B) Return to normal chow progressively increases carotid occlusion times to control values for both normal chow ApoE null mice (leftmost bar) and wild-type mice (dashed line: based on prior published data\textsuperscript{14} (*p=0.0056, **p=0.004, vs. control).
Figure 4-3: Correlation plot of aortic nanoparticle accumulation and carotid occlusion time ($R = -0.64$, $p = 0.02$) demonstrating relationship between vascular permeability and thrombotic potential.
Figure 4-4: (A) SEM of cholesterol crystals densely deposited on surface of aortic plaque. Scale bar: 100 µm (B) SEM of cholesterol crystals on denuded plaque (P) but not on adjacent regular endothelium (E). Scale bar: 40 µm. (C) Higher magnification SEM depicts morphology of cholesterol crystals. Scale bar: 20 µm. (D-F) Immunofluorescent staining for CD31 (green) demonstrates little to no intraplaque angiogenesis in ApoE null fed a Western Diet for (D) 4 months, (E) 5 months, and (F) 6 months. Scale bars denote 50 µm.
Figure 4-5: (A) Penetration of FITC-dextran (green) and PFC-NP (red) into a plaque is revealed with 3-D two-photon microscopy imaging of en face atherosclerotic rabbit aortic tissue. (B) Two-photon microscopy of en face normal rabbit aortic tissue. Scale bar: 50 µm. Tissue autofluorescence is shown in blue. (C) Sideview two-photon microscopy image of atherosclerotic rabbit tissue demonstrating penetration of FITC-dextran (green) and PFC-NP (red) into plaques. (D) Sideview two-photon microscopy image of normal rabbit tissue demonstrating lack of FITC-dextran (green) and PFC-NP (red) penetration through intact tissue lumenal barriers. Scale bar: 50 µm.
Figure 4-6: Fluorine magnetic resonance imaging of excised rabbit aorta at 11.7T following 4 hours of PFC-NP perfusion. (A) Proton image demonstrating intimal plaque deposition. (B) Fluorine image confirms the presence of PFC-NP in the aorta sample. (C) Fluorine image overlaid on the proton image demonstrates localization of PFC-NP in plaques. An external standard (bottom left corner of all three images) allows for quantification of nanoparticle concentration.
Figure 4-7: (A) FACS analysis reveals minimal cellular active uptake of rhodamine-labeled PFC-NP by 0.26% of circulating peripheral blood leukocytes. The RhodPE+ population comprises Ly6C+Ly6G+ cells (myeloid cells) and a small number of CD19+ cells (B cells). (B) FACS analysis reveals minimal active uptake of PFC-NP by 0.93% of splenocytes. The RhodPE+ population comprises F40/80+ and Ly6C+F4/80+ cells (monocytes/macrophages) and a smaller percentage of CD19+ cells (B cells). T cells (TCRβ+) do not take up PFC-NP.
Figure 4-8: Assays for nanoparticle-dependent complement activation following 30 minutes of nanoparticle circulation in mice (A) Western blot analysis of complement component 3 (C3) cleavage in the plasma and on the nanoparticle surface. Administration of plain PFC-NP does not initiate the generation of the C3α chain cleavage product, α2, on both the nanoparticle surface or in the plasma. (B) Plasma and nanoparticle surface-bound Factor H was assayed, indicated no recruitment of Factor H to the nanoparticle surface following plain PFC-NP administration.
Figure 4-9: Cross-sectional $^1$H images at 3T of (A) normal chow rabbit and (D) cholesterol fed rabbit showing location of abdominal aorta (red box). $^{19}$F gradient echo images of perfluoro-15-crown-5-ether nanoparticle $^{19}$F signal in the region of interest for (B) normal chow rabbit and (E) cholesterol fed rabbit. Saturation bands proximal and distal to imaging slice eliminate $^{19}$F signal from blood (See Figure S5). $^{19}$F signal (green) overlaid on $^1$H image showing $^{19}$F signal colocalization for the region of interest in a (C) normal chow rabbit and (F) cholesterol fed rabbit, demonstrating deposition of PFC-NP only in inflamed abdominal aorta (AA) and vena cava (VC). Representative Oil Red O stains of the imaged area showing plaque elements in the (G) normal chow rabbit aorta and the (H) cholesterol-fed rabbit aorta. Scale bars denote 500 μm.
Figure 4-10: Locations of saturation bands used to null fluorine signal from the blood pool. The aorta, shown in red, was imaged with a time-of-flight angiogram.
4.6 Work Cited


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Chapter 5: Anti-Inflammatory and Endothelial Barrier

Protective Effects of PPACK-NP in Atherosclerotic Mice
Chapter Overview

A role for thrombin in the pathogenesis of atherosclerosis has been suggested through clinical and experimental studies revealing a critical link between the coagulation system and inflammation. Although approved drugs for inhibition of thrombin and thrombin-related signaling have demonstrated efficacy, their clinical application to this end may be limited due to significant potential for bleeding side effects. Thus, we sought to implement a plaque-localizing nanoparticle-based approach to interdict thrombin-induced inflammation and hypercoagulability in atherosclerosis.

We deployed a novel magnetic resonance spectroscopic method to quantify the severity of endothelial damage for correlation with traditional metrics of vessel procoagulant activity after dye-laser injury in fat-fed ApoE-null mice. We demonstrate that a one-month course of treatment with anti-thrombin nanoparticles carrying the direct thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone (PPACK-NP): 1) reduces the expression and secretion of proinflammatory and procoagulant molecules, 2) diminishes plaque procoagulant activity without the need for systemic anticoagulation, 3) rapidly restores disrupted vascular endothelial barriers, and 4) retards plaque progression in lesion prone areas.

These observations illustrate the role of thrombin as a pleiotropic atherogenic molecule under conditions of hypercholesterolemia, and suggest the utility of its inhibition with locally acting anti-thrombin nanoparticle therapeutics as a rapid-acting anti-inflammatory strategy in atherosclerosis to reduce thrombotic risk.
5.1 Introduction

Atherosclerosis, the leading cause of death in the Western world, is essentially a disease of inflammation from its inception through the evolution of vulnerable atheromas that eventually break down to induce focally occlusive thrombosis with consequential tissue death in subtended vascular beds. A direct link exists between inflammation and coagulation in atherosclerosis by way of the serine protease thrombin that plays a central role in both clot formation and inflammatory molecular signaling events that may instigate and potentiate plaque development. Thrombin's vascular activity is mediated primarily by a family of G-protein coupled receptors known as protease-activated receptors (PARs). Activation of PAR-1 by thrombin initiates a signaling cascade that promotes proinflammatory, vasomotor, and cellular proliferative effects in various cell types, including endothelial cells, smooth muscle cells, and macrophages, among others. Thrombin signaling promotes the synthesis and release of procoagulant factors such as tissue factor, which establishes the conditions for repeated cycles of endothelial disruption, coagulation, inflammation, and plaque expansion.

Given the abundance of thrombin in atherosclerotic plaques and its recognized contribution to plaque inflammation and hypercoagulability, we sought to investigate the hypothesis that focal inhibition of plaque thrombin to abrogate its inflammatory signaling actions would both attenuate plaque procoagulant activity and facilitate restoration of naturally anticoagulant endothelial vascular barriers. Furthermore, we sought to elucidate direct relationships between thrombin inhibition, regulation of inflammatory signaling, recovery of endothelial barriers, and reduction in thrombotic risk with the use of clinically translatable functional methods for quantifying vascular barrier integrity. Although clinically approved anti-thrombotic pharmaceuticals have been evaluated in experimental primary and clinical secondary
prevention trials\textsuperscript{9}, no information exists as to their ability to directly attenuate focal plaque thrombotic propensity or to improve vascular barrier integrity, which could serve to deter acute vascular syndromes.

To those ends, we have reported recently that atherosclerotic endothelial damage can be quantified nondestructively with the in vivo use of semipermeant perfluorocarbon-core nanoparticles (PFC-NP) that passively diffuse beyond disrupted endothelial barriers in plaques, allowing both fluorine magnetic resonance imaging and quantification of PFC-NP deposition with fluorine magnetic resonance spectroscopy (\textsuperscript{19}F-MRI and \textsuperscript{19}F-MRS, respectively).\textsuperscript{10} Using this method, we have delineated the temporal progression of endothelial barrier disruption in ApoE-null mice as a consequence of a prolonged high fat diet and demonstrated that barrier damage was related directly to the propensity for thrombotic occlusion in the dye-laser vessel injury model. Moreover, dietary management by restoration of a normal chow diet simultaneously recovered vascular barrier integrity and rapidly reduced plaque hypercoagulability within 1-2 months.\textsuperscript{11} Although these nanoparticles previously have been conjugated to selected antithrombin agents to serve as potent and safe antagonists of thrombosis in acute clotting events\textsuperscript{12,13}, their therapeutic potential for chronic control of inflammatory signaling through thrombin inhibition is untested in atherosclerosis.

Accordingly, we hypothesize that the reported capability of PFC-NP to localize to atherosclerotic plaques manifesting disrupted barriers and be retained for prolonged periods\textsuperscript{10,11} would set the stage for the formulation and focal deposition of a reservoir of anti-thrombin nanoparticles in plaques (Fig. 5-1A) to exert prolonged surveillance against and rapid inactivation of any locally generated thrombin. The present work was designed to examine the efficacy and safety of nanoparticle-based strategies for focal inhibition of thrombin (Fig. 5-1A)
in atherosclerosis and to define related inflammatory signaling events in vitro and in vivo that might play a mechanistic role in accelerating vascular endothelial barrier damage and thrombotic risk. The observed efficacy of the approach indicates a significant direct contribution of thrombin signaling to the evolution of atherosclerosis and the emergence of thrombotic risk, and implicates it as a key contributor to endothelial damage in this model.
5.2 Materials and Methods

5.2.1 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. 5-1B). 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.

5.2.2 Nanoparticle Formulation

PPACK-NP were formulated using previously described emulsification and conjugation techniques.12 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.

5.2.3 \(^{19}\)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. 5-1C) by measuring the deposition of fluorine-core nanoparticles into aortic intimal plaques as described in previous work.\(^{10,11}\) 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.\(^{11}\) The measurement is fully quantitative based on \(^{19}\)F 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.\(^{10,11}\)
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 perfluoro-crown ether signal from the nanoparticles compared to the known amount of TFA. For each sample, the detected amount of nanoparticles was normalized based on the weight of the aorta.

5.2.4 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. 5-1D).

5.2.5 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.

5.2.6 Inflammatory Signaling Events

**PAR-1 Assay:** A previously described method\textsuperscript{15,16} 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\textsuperscript{2+} and Mg\textsuperscript{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\textsuperscript{2+} and Mg\textsuperscript{2+}), followed by staining with a phycoerythrin-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. 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 CaCl₂ 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-κB 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-κB 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, ab14877, Abcam).
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 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.

5.2.7 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 (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.
5.2.8 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.

5.2.9 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.

5.2.10 Study Approval

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

5.3.1 Anti-thrombin Nanoparticles Reduce Vascular Procoagulant Activity in vivo

To characterize the effects of PPACK-NP on reducing procoagulant activity, groups of ApoE-null mice were fed a Western diet for 3 months followed by continuation of the diet with treatment (saline, control NP, and PPACK-NP) for 1 additional month. At the conclusion of the treatment/feeding period, mice were subjected to a previously validated\textsuperscript{11,18,19} model for measuring thrombotic risk using photochemical injury of the carotid artery that yields a quantitative metric of coagulant activity (vessel occlusion time) with good dynamic range and monotonic responsiveness to therapeutic agents that affect clotting. Prior to performing the vessel injury, mice were maintained on their diets for 2-3 additional days without further treatment to allow washout of any residual anti-thrombin nanoparticles. After 1 month of PPACK-NP treatment, carotid occlusion times increased significantly over control groups (Fig. 5-2A), with occlusion times reaching 48.71 ± 6.7 min (N=7) compared to saline treatment (26.11 ± 4.63 min, N=9, p=0.005) and control NP treatment (25.55 ± 4.17 min, N=9, p=0.004). We note that the occlusion times in the 1-month PPACK-NP treated group approximated those of previously reported fat-fed ApoE null mice after 2 months off diet, indicating that the PPACK NP therapy may more rapidly attenuate vessel procoagulant activity than does dietary management in this model, despite maintenance of the high fat diet in the PPACK NP treated group.\textsuperscript{11}

5.3.2 Anti-thrombin Nanoparticles Restore Vascular Barrier Integrity in vivo

We tested the ability of PPACK-NP treatment to restore functional endothelial barriers, which concomitantly might be expected to reduce plaque procoagulant activity as previously
reported. Mice were injected with a dose of CE-NP for MR spectroscopy that were allowed to circulate for 2 hours prior to sacrifice, at which time plaque saturation occurs. Following this circulation time, the entire length of the aorta (from the aortic root to the bifurcation) was removed for ex vivo $^{19}$F-MRS measurements at 11.7T. Figure 5-2B illustrates the beneficial effects of PPACK-NP treatment for 1 month on plaque endothelial permeability according to the decreased deposition of CE-NP (0.084 ± 0.009 µl/g aorta, N=7) compared to saline (0.122 ± 0.011 µl/g aorta, N=8, p=0.023) and control NP (0.132 ± 0.013 µl/g aorta, N=10, p=0.014). Using paired samples, we observed an inverse correlation (R = -0.56, Fig. 5-2C) between plaque permeability and vessel procoagulant activity (p=0.004), consistent with our previous observation that thrombotic risk tracks with plaque permeability to PFC-NP.

A subset of mice was allocated for measurements of plaque extent in the aortic arch by conventional Sudan IV staining and computer assisted planimetry. Overall, PPACK-NP treatment resulted in a 22.5% decrease in aortic arch plaque extent (Fig. 5-2D): 40.24 ± 3.21% plaque area for saline-treated mice (N=5) and 31.91 ± 1.69% plaque area for PPACK-NP treated mice (N=7, p=0.03).

5.3.3 Anti-thrombin Nanoparticles Attenuate Inflammatory Signaling Molecules

**PAR-1 responses:** To delineate molecular signaling events responsible for the beneficial effects of PPACK-NP, cell culture studies were used to quantify the responses of activated endothelial and monocytic cell lines to thrombin inhibition. First, as thrombin's effect on cell types is mediated primarily by cleavage of the PAR-1 receptor on cell surfaces, flow cytometry was used to determine the percentage of intact PAR-1 receptors that was left after treatment with thrombin in the various treatment groups. PPACK-NP treatment completely prevented thrombin
cleavage of PAR-1 receptors on human aortic endothelial cells (HAECs), compared to thrombin or thrombin/control NP groups, which manifested significantly decreased PAR-1 expression (N=3 for both groups, p=0.019 and p=0.000005, respectively) as compared to baseline (Fig. 5-3A).

**Tissue Factor responses:** Because PPACK-NPs successfully prevented PAR-1 activation, we tested the downstream signaling effects of PAR-1 activation related to inflammation and coagulation. Expression of tissue factor on the surface of HAECs and THP-1 monocytes in response to thrombin stimulation was assayed using a functional assay of measuring FXa generation as a result of the presence of TF/FVIIa complexes. PPACK-NP prevented thrombin-induced TF expression on the surface of both HAECs (Fig. 5-3B) and THP-1 monocytes (Fig. 5-3C), with no significant increase over baseline TF levels at both concentrations of thrombin utilized (1 U/ml and 4 U/ml). Whole excised aortic arch segments exhibited a marked reduction in TF-positive plaque area after PPACK-NP treatment: 46.75 ± 5.74% (N=3) in PPACK treated mice (Fig. 3D), versus 72.69 ± 5.06% (N=3, p=0.027) for saline treatment and 72.52 ± 4.34% (N=3, p=0.023) for control NP treatment as quantified in immunofluorescent staining using ImageJ (Fig. 5-3E-G).

**NFkB responses:** Because thrombin is known to stimulate NFkB transcriptional regulation of a panoply of inflammatory genes through PAR-1 signaling, we delineated the effect of PPACK-NP on the inhibition of NF-kB activation in HAEC (Fig. 5-4A-E) and THP-1 cells (Fig. 5-4F-J). Thrombin cleavage of PAR-1 results in the activation of Gαq and dissociation of the Gβγ complex, which subsequently results in the parallel activation of PKCσ and PI3-kinase/Akt pathways. These parallel pathways then converge to stimulate IKK, which results in the binding of the p65 homodimer to IκB and subsequent phosphorylation and degradation of
IkB. Activation of the PKCσ pathway results in activation of p38 which in turn, phosphorylates p65 to induce the nuclear translocation and transcriptional activity of the p65.21

Cell cultures were stained for phosphorylated p65 following six hours of thrombin stimulation and treatment. Treatment with PPACK-NP resulted in little to no observable positive staining for intracellular phospho-p65 and preservation of IkB protein (Fig. 5-5) compared to thrombin and thrombin/control NP treatment groups. The preservation of IkB indicates that this cytoplasmic regulatory component of p65/p50 retains control of the preexisting cytoplasmic stores of p65 thereby preventing subsequent p65 phosphorylation and translocation to the nucleus.

Next we quantified pp65 in the endothelium (Fig. 5-6A) and intraplaque regions (Fig. 5-6B), by staining sections of the excised aortic arch for phosphorylated NF-kB p65 (pp65, Fig. 5-6C-E), where increased phosphorylation of p65 indicates increased NF-kB activity. After PPACK-NP treatment, aortic plaques exhibited significantly decreased endothelial pp65 (11.49 ± 3.66%, N=3) compared to saline (33.11 ± 4.05%, N=3, p=0.017) and control NP treatments (33.25 ± 4.33%, N=3, p=0.019). Decreased macrophage pp65 also was observed in plaque regions after PPACK-NP treatment (21.78 ± 3.15%, N=3) compared to saline (43.31 ± 6.55%, N=3) and control NP treatments (47.51 ± 4.59%, N=3). To rule out loss of endothelial pp65 staining due to missing endothelium, selected neighboring slide sections where pp65 was noted to be reduced were stained for vWF, indicating that endothelium was present, thus confirming the specificity for NFkB downregulation by PPACK-NP in endothelium in vivo (Fig. 5-6F-H).

Systemic coagulation and inflammation markers: Thrombin-antithrombin (TAT) complexes are correlative systemic harbingers of procoagulant activity.22 PPACK-NP treatment
significantly reduced serum thrombin-antithrombin (TAT) complexes (5.43 ± 0.64 ng/ml, N=5; p=0.0001 vs. saline, p=0.032 vs control NP) (Fig. 5-7A) versus saline (13.32 ± 1.01 ng/ml, N=6) and control NP (9.96 ± 1.64 ng/ml, N=5, p=NS vs. saline).

Because NF-kB is a known driver of endothelial adhesion molecules, we measured soluble VCAM-1 (sVCAM-1) levels as biomarkers of activated endothelium in atherosclerosis. ELISA analysis of sVCAM-1 (Fig. 5-7B) revealed a modest, but significant decrease in sVCAM-1 with PPACK-NP treatment (1504.88 ± 65.25 ng/ml, N=4) compared to saline treatment (1666.37 ± 12.78 ng/ml, N=5, p=0.029)

Macrophage responses: Excised aortic arch sections were stained for macrophages (Fig. 5-8A-C) and plaque macrophage content was quantified using ImageJ. We observed no significant difference between treatment groups in terms of overall plaque macrophage content (Fig. 5-8D).

Systemic responses to PPACK-NP: APTT measurements conducted on serum collected at the time of sacrifice indicated no persistent non-specific effects of PPACK-NP after the terminal treatment dose 2-3 days prior to sacrifice (Fig. 5-8E). Furthermore, no significant difference was observed in serum cholesterol following the PPACK-NP treatment regimen (Fig. 5-8F).

5.4 Discussion

The principal new observation in this work is that focal inhibition of plaque thrombin in fat-fed ApoE-null mice results in rapid recovery of damaged endothelial barriers and attenuated vascular procoagulant activity in spite of a continued Western diet. These beneficial outcomes were achieved with the use of anti-thrombin nanoparticles that passively permeated plaque
intimal regions after i.v. injection and were focally retained to exert sustained pleiotropic anti-
inflammatory effects. Additionally, the progression of atherosclerotic plaque in lesion-prone
areas of the ascending aorta was forestalled during the 1 month treatment period. Potential
mechanisms for promoting quiescence in activated endothelium related to downregulation of
inflammatory NFkB signaling activity through inhibition of the thrombin-PAR1 signaling are
illustrated in Fig. 5-9.

The direct relationship between thrombotic risk and endothelial barrier disruption (Fig. 5-
2C and Fig. 4-3 in Chapter 4) according to the metrics employed in these models confirms a
primary role for intact endothelium in maintaining vascular homeostasis in atherosclerosis.
Recent reports of the relationship between endothelial damage/sloughing and acute coronary
syndromes in patients\(^24\) recalls original descriptions of hypercoagulable vascular erosions by the
Virmani group\(^25\) and focuses attention on ways to measure and preserve endothelial integrity as a
strategic path to the detection and reduction of thrombotic risk. Prior work in our lab indicates
that the effects of cholesterol feeding elicit both procoagulant effects and barrier disruption only
after some time on a sustained high fat diet: >3 months in ApoE-null mice\(^11\) and >6 months in
NZW rabbits\(^10\). In ApoE-null mice, barrier disruption worsens progressively over time on a
high fat diet, but can resolve rapidly within 2 months after switching to normal chow.\(^11\) Although
leaky vasculature and reduced vasodilatory capacity associated with endothelial dysfunction may
occur within weeks of inception of an atherogenic diet in the ApoE-null model\(^26\), the barrier
disruption and procoagulant activity identified by our nanoparticle permeability metrics emerge
later and may serve as more direct harbingers of thrombotic risk.

With respect to the underlying mechanisms responsible for endothelial damage,
inflammatory signaling and immunomodulatory events orchestrated by various plaque cell types
interacting with activated endothelium have been described in detail. Here we have focused on thrombin as a key instigator of plaque growth and instability contributing to endothelial activation, vessel inflammation, and hypercoagulability, as summarized in Figure 5-9. Surprisingly, after an aggressive one-month treatment period with PPACK-NP following 3 months of initial cholesterol feeding, marked benefits were observed even in the face of persistently elevated serum cholesterol. The role of thrombin not only as a principal prothrombotic agent, but also as an atherogenic molecule is not unexpected given that it drives many of the inflammatory molecules that participate in plaque growth such as NFkB, NADPH oxidase, VCAM-1, PDGF, among many others. Thrombin's role as a proinflammatory molecule through the activation of the NF-kB pathway results in numerous downstream effects that accelerate plaque development, cell infiltration, expression of inflammatory molecules, and promotion of hypercoagulability through stimulation and secretion of procoagulant enzymes.

Of particular interest is the role of endothelial-specific NFkB activation in atherogenesis as demonstrated by Gareus et al. with the use of genetically engineered conditional knockouts of endothelial NFkB that markedly suppressed plaque formation in fat-fed ApoE-/- mice. The seminal observations of very early upregulation of NFkB in lesion prone aortic arch regions by Cybulsky’s group raises the interesting speculation of a role for thrombin even at these incipient time points, particularly in the context of previously documented clusters of intense, albeit small, clusters of endothelial apoptosis and replication in these aortic arch regions even in normal subjects. The ability to achieve focal suppression of NFkB with nanoparticle delivery systems that might abrogate endothelial PAR-1 activation could help to maintain a more quiescent endothelial phenotype (e.g., reduced sVCAM: Fig 5-7B), preserve barrier integrity, and simultaneously reduce paracrine crosstalk with other inflammatory plaque components.
A pleiotropic response to the suppression of thrombin signaling in diverse cell types that participate in atherogenesis is evidenced by modulation of NFkB in THP-1 as well as HAEC cells (Fig. 5-4A-J, and Fig. 5-5), and our previous reports of reduced platelet content in clots that are produced by vessel injury.\textsuperscript{12,13} Regarding platelet activation, synergistic benefits also could accrue by local inhibition of thrombin-PAR-1 signaling through the NFkB axis.\textsuperscript{35} However, as a potential caveat, it is interesting to note that selective inhibition of NFkB in macrophage populations has been associated with \textit{increased} atherosclerosis as contrasted with more specific inhibition of endothelial NFkB.\textsuperscript{36} Although we show that NFkB may be downregulated in representative human monocyte cell lines in vitro by interrupting thrombin/PAR-1 activation (Fig. 5-3A) and in vivo through quantification of pp65 staining in PPACK-NP treated mice, the exact relationships between macrophages and endothelial signaling and responses to this intervention remain to be defined.

Recent experimental reports have explored the role of thrombin and related coagulation enzymes in promoting atherosclerosis with the use of orally administered antithrombotic agents or genetically modified mice for primary prevention of atherosclerosis.\textsuperscript{37-39} All such studies report significant decreases in overall plaque extent and reduced expression of inflammatory mediators. Secondary prevention clinical trials in patients with acute coronary syndromes using oral anti-thrombotic agents have shown very modest effects on subsequent clinical events related to atherosclerosis progression, but at the risk of significantly increased bleeding.\textsuperscript{9} Interestingly, nanoparticle-based thrombin inhibition exhibits similar therapeutic effects to that of the experimental studies mentioned above, but with significantly fewer treatments (12 doses over 4 weeks), no requirement for cholesterol reduction (Fig. 5-8F), and a more promising safety profile.
as coagulation parameters and bleeding times have been shown to normalize within 30-60 minutes after i.v. injection.\textsuperscript{12}

We observed no significant change in plaque macrophage content between treatment groups (Fig. 5-8A-D) in contrast to the 50% decrease in plaque macrophages reported by Hara et al. after 5 months of Xa inhibition.\textsuperscript{40} However, despite our shorter 1-month time window of therapeutic intervention that may not have allowed for reduced plaque macrophage content, our observations of rapid downregulation of NF-kB and downstream inflammatory markers (TF, TAT complexes, sVCAM) (Fig. 5-6C-E), is consistent with the similar observations of Kadoglou et al in dabigatran-treated ApoE-null mice.\textsuperscript{38} These results also accord with previously published data\textsuperscript{41} demonstrating the effect of thrombin and PPACK-thrombin in modulating the expression of TF in human saphenous vein endothelial cells, which is thought to be due to activation of NF-kB.

The optimal dosing interval for this therapy and the duration of the local effect on barrier integrity and procoagulant activity remain to be defined. PPACK-NP exhibit a mean clearance half-life of $105.87 \pm 23.38$ min, compared to clearance half-lives for plain PFC-NP ($181.3 \pm 40.7$ min) and PEGylated PFC-NP ($240.16 \pm 23.42$ min), indicative of only modest effects on pharmacokinetics with selected particle surface modifications. The anticipated clearance mechanism for PFC-NP was the reticuloendothelial system as demonstrated by $^{19}$F-MR imaging of mice post-mortem, which depicted accumulation of nanoparticles in the liver and spleen following 2 hours of nanoparticle circulation prior to sacrifice.\textsuperscript{42} This minor difference in PK may allow for convenient swapping of alternative anticoagulants such as bivalirudin as we have shown previously.\textsuperscript{13} These PK parameters are advantageous for potential clinical applications, as we have previously shown in mice that nanoparticle clearance through the RES results in
reduction of residual circulating (i.e., nontrapped) bioactive conjugated PPACK or bivalirudin moieties within 30-60 minutes to a level below that required to alter systemic clotting and bleeding parameters.\textsuperscript{12,13} The potential disadvantage of intravenous nanotherapy also is notable, but there are a number of clinical scenarios that might benefit from early and aggressive treatment for a period of time before effective cholesterol control could be established. It is also important to note that the particular thrombin inhibitor PPACK may not be entirely specific to thrombin as a serine protease inhibitor, and that local inhibition of other coagulation proteases (e.g. Xa) may in fact exert a synergistic but still locally constrained effect in preventing the activation of thrombin.
5.5 Acknowledgements

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Portions of this chapter were adapted verbatim from an article that is currently under review for publication at the time of submission of this dissertation. The current title and author list are listed below:

Palekar, RU, Jallouk AP, Myerson JW, Pan H, Wickline SA. "Thrombin-targeted nanoparticles restore disrupted endothelial barriers and attenuate thrombotic risk in experimental atherosclerosis".
Figure 5-1: (A) Schematic of PPACK-NP (center panel). Nanoparticles consist of a perfluorocarbon core surrounded by a stabilizing phospholipid monolayer to which PPACK is covalently conjugated. In this work, PPACK-NP act on both the exterior and interior regions of plaques in inhibiting the inflammatory effects of thrombin. Fluorescent microscopy image (right panel) of mouse atherosclerotic plaque at 3 months of cholesterol feeding demonstrates intraplaque accumulation of PPACK-NP (red). Sudan IV staining of the aortic arch of cholesterol fed ApoE mice demonstrates ample plaque deposition serving as a target for PPACK-NP therapy (left panel) (B) Feeding and dosing schedule for ApoE-null mice. During the 4-week treatment period, mice received i.v. treatments three times per week. (C) Schematic representation of $^{19}$F-MRS-based detection of endothelial barrier disruption. Non-targeted CE-NP are administered and circulated for 2 hours. Aortas are removed and $^{19}$F signal is measured. High $^{19}$F signal corresponds to increased endothelial barrier disruption and increased nanoparticle accumulation, whereas low $^{19}$F signal corresponds to decreased endothelial barrier disruption and diminished nanoparticle accumulation. (D) Representative kinetics of photochemically-induced thrombus formation in ApoE-null mice treated with either saline, control NP or PPACK-NP.
Figure 5-2: (A) PPACK-NP treatment (rightmost bar) significantly increases time to occlusion of the carotid artery by 46% over saline (p=0.005) and control NP (p=0.004) treatments. (B) Plaque permeability is reduced with PPACK-NP treatment by 33% compared to saline (p=0.023) and control NP treatments (p=0.014). (C) Paired samples of aortic nanoparticle accumulation measurements and carotid occlusion times demonstrates a significant inverse correlation between the two metrics, confirming prior work indicating a relationship between increased endothelial permeability and increased vessel hypercoagulability. (D) Sudan IV staining of the aortic arch of saline and PPACK-NP treated mice demonstrates a 22.5% decrease in gross plaque deposition with PPACK-NP treatment vs. saline treatment (p=0.03) as quantified with ImageJ.
Figure 5-3: (A) Flow cytometry for intact PAR-1 receptors following thrombin stimulation in the presence of each treatment group demonstrates inhibition of thrombin-mediated cleavage of PAR-1 with PPACK-NP treatment. (B) PPACK-NP inhibit expression of surface tissue factor on HAECs and (C) THP-1 cells in response to stimulation with different concentrations of thrombin (light gray bars, 1U/ml; dark gray bars, 4U/ml). **p<0.005, ***p<0.0005 (D) diminished tissue factor expression in PPACK-NP treated-mice (p=0.027 and 0.023 vs saline and control NP, respectively) as quantified by ImageJ. (E-G) Immunofluorescent staining of tissue factor in ApoE-null mice treated with (E) saline, (F) control NP, and (G) PPACK-NP.
Figure 5-4: Immunocytochemistry for phosphorylated p65 demonstrates inhibition of NF-κB activation in (A-E) HAECs and (F-J) THP-1 cells with PPACK-NP treatment.
Figure 5-5: Immunocytochemistry for IkB on HAECs demonstrates diminished degradation of IkB in response to thrombin stimulation with PPACK-NP treatment, consistent with inhibited activation of NF-kB.
Figure 5-6: ImageJ quantification of (A) endothelial and (B) intraplaque phospho-p65 staining on (C-E) ApoE-null mouse plaques demonstrate significantly less endothelial and macrophage phospho-p65. (F-H) Von Willebrand factor staining was conducted on neighboring sections to rule out loss of endothelial phospho-p65 staining due to missing endothelium in this case.
Figure 5-7: (A) ELISA evaluation of thrombin-antithrombin complexes reveals a significant decrease in detected TAT-complexes following one month of PPACK-NP treatment. (B) ELISA analysis for detection of serum soluble VCAM-1 (sVCAM-1) demonstrated a decrease in detectable sVCAM-1 following one month of PPACK-NP treatment.
Figure 5-8: Immunofluorescent staining for macrophages in ApoE-null mouse plaques treated with (A) saline, (B) control NP, and (C) PPACK-NP revealed no significant difference in (D) detected plaque macrophages as quantified with ImageJ. (E) No significant difference in activated partial thromboplastin time (APTT) between treatment groups indicating no persisting systemic effect of PPACK-NP on coagulation 2-3 days after the penultimate treatment dose. (F) No significant difference was observed on serum cholesterol between treatment groups, indicating that therapeutic effects observed occurred without cholesterol lowering as a consequence of nanoparticle treatment.
Figure 5-9: Schematic depiction of the effect of PPACK-NP on reducing the inflammatory effects of thrombin. Thrombin promotes the release of inflammatory molecules (cell adhesion molecules) and procoagulant molecules (TAT complexes, tissue factor) as demonstrated in this work, through the activation of the NF-κB pathway as measured through IκB degradation and phosphorylation of p65.
5.6 Works Cited


42. Myerson JW. Thrombin-Inhibiting Perfluorocarbon Nanoparticles: A New Class of Therapeutic for Acute Thrombosis Treatment and Diagnosis. 2014.
Chapter 6: Conclusions
The work described in this dissertation confirms the role of thrombin as a potent mediator of inflammation that drives the progression of atherosclerosis as a key factor in the disruption of endothelial barrier integrity. Several groups have noted the importance of thrombin and the role it plays in early plaque development either through genetic knockout studies of thrombomodulin or through various studies involving the use of orally administered anticoagulants during the course of atherosclerotic development in ApoE-/- mice.

In Chapter 2, we investigated the utility of nanoparticles (specifically liposomes) as thrombin inhibitors through the surface conjugation of the direct thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone (PPACK) as a means to delay clot formation without exerting significant systemic anticoagulant effects, which has been described as a "holy grail" for antithrombotic drug development. We demonstrated in this work that these nanoparticulate inhibitors of thrombin manifest high-localized antithrombotic activity at the site of injury, with rapid normalization of systemic anticoagulation parameters (APTT, tail vein bleeding time) after an initial bolus. A key observation in this work is the propensity of nanoparticle-based thrombin inhibitors to bind to areas of active thrombosis to form an "anti-clotting surface" that prevents or delays the formation of additional clot. We observed that this interaction was due in part to interactions of PPACK on the surface of the liposome with thrombin bound to the surface of clots, which is a population of active thrombin that heparin is largely unable to inhibit. Due to the presence of multiple copies of PPACK (6254.24 ± 1245.14 copies per liposome, ~13650 PPACK per perfluorocarbon nanoparticle) on the nanoparticle/liposome surface, a particle that binds to the surface of the clot exposes PPACK to the periphery of the clot, thus preventing the actions of free thrombin at the site of injury.

In Chapter 3, we expanded on the concept of generation anticlotting surfaces with
antithrombin nanoparticles by applying nanoparticles to the surface of thrombin-coated stents to prevent clot deposition upon exposure to plasma. The results of this work demonstrate a novel role for thrombin-inhibiting nanoparticles as inhibitors of thrombosis on vascular devices. Thrombosis is a significant factor in the failure of vascular devices and implants, requiring that patients be maintained on continuous anticoagulation for some time after implantation, leaving them at risk for hemorrhage. Indeed, recent clinical studies have recommended increasing time on dual antiplatelet therapy, underlining the importance of developing alternative methods for preventing thrombosis of vascular implants. The results of our work demonstrate that stent thrombosis might be prevented with the use of thrombin-inhibiting nanoparticles. However it is important to note several limitations of this study. All experiments were carried out using in vitro models, where the in vivo effects of PPACK-NP on stent thrombosis are not yet known. Future work aims to address the in vivo effect of PPACK-NP, however we hypothesize that PPACK-NP treatment could be efficacious based on prior work done on acute models of thrombosis demonstrated in Chapter 2 and in prior work in our lab by Myerson et al.\textsuperscript{6,7}

In Chapter 4, we elucidated the relationship between thrombosis/hypercoagulability as it relates to the integrity of the endothelium. Fluorine magnetic resonance spectroscopy (\textsuperscript{19}F-MRS), was used to detect progressive changes in the permeability of ApoE-/- mouse vascular barriers to 200 nm diameter perfluorocarbon nanoparticles, where increasing passive permeation and retention of nanoparticles was observed as a function of time on a high cholesterol diet. Interestingly, we were able to detect the ability of plaques to "heal" upon removal of an inflammatory stimulus (in this case, dietary cholesterol), according to the observed reduction of nanoparticle deposition in plaques following dietary management. Furthermore, we observed that increased nanoparticle retention in the aorta due to endothelial barrier disruption was correlated
inversely with vessel procoagulant activity, or the time to thrombotic occlusion of the carotid artery after dye-laser injury. This relationship suggests a direct, quantitative and causal link between endothelial damage and propensity for thrombosis. These results are translationally applicable to a current unmet need for novel techniques that can accurately classify plaques at risk of thrombosis, as current noninvasive diagnostic imaging methods are unable to detect the presence of plaque erosions\textsuperscript{8}, which represent over 1/3 of all acutely thrombosed lesions\textsuperscript{9}, and can only be diagnosed post-mortem.

Finally, in Chapter 5, we demonstrated the therapeutic utility of \textit{focal} thrombin inhibition with anti-thrombin nanoparticles for preserving endothelial barrier integrity as a consequence of modulating hypercoagulability and vascular inflammation. Furthermore, we were able to assess the extent of endothelial barrier disruption as measured non-invasively with fluorine (\textsuperscript{19}F) magnetic resonance spectroscopy as described in Chapter 4. This work demonstrates a significant role for nanoparticle-based treatments in forestalling the progression of atherosclerosis simply by inhibiting a fundamental inciting molecule, thrombin. This intervention results in a significant downstream decrease in NF-kB activity in plaques, that is associated with decreased hypercoagulability and restoration of vascular barrier integrity. Interestingly, the content of plaque macrophages remained unchanged irrespective of treatment, at least over this short interval, indicating that the early effects of thrombin inhibition operate by suppressing the activity of macrophages rather than by depleting their numbers to elicit the beneficial effects on plaque growth. Moreover, the results are obtained in the face of continued high blood levels of cholesterol, indicative of the critical role of thrombin in driving inflammation and plaque growth. Future work might focus on longer term intervention to examine the response of macrophage populations with respect to mechanistic efferocytosis, cellular egress, and actual plaque burden.
reduction. Taken together, these results clearly establish a prominent role for thrombin in the very early inflammatory events that promote plaque growth and not just in the later emergence of plaque instability and thrombosis that is the current dogma.
6.1 Work Cited


