

**ENGINEERING NOVEL BIOMATERIALS AND
EVALUATING THE USE OF NOVEL THERAPEUTICS IN
CURRENT CLINICAL DEVICES TO MITIGATE MEDICAL
DEVICE-INDUCED THROMBOSIS**

By

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To my grandparents, Evelyn and Gregory. Thank you for the interminable support, love, and most importantly, lots of laughter.

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List of Abbreviations

ACD	Acid citrate dextrose
ACT	Activated clotting time
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP	Activated platelet
aPTT	Activated partial thromboplastin time
ASA	Acetylsalicylic acid (Aspirin)
ATP	Adenosine triphosphate
BMS	Bare metal stent
BSA	Bovine serum albumin
CDI	1-1'-carbonyldiimidazole
cGMP	Cyclic guanosine monophosphate
COX-1	Cyclooxygenase-1
CVD	Cardiovascular disease
DAPT	Dual antiplatelet therapy
DES	Drug-eluting stent
DOACs	Direct oral anticoagulants
ECM	Extracellular matrix
ECMO	Extracorporeal membrane oxygenator
EPC	Endothelial progenitor cell
EPCR	Endothelial cell protein C receptor
ePTFE	Expanded polytetrafluoroethylene
F	(clotting) Factor
FACS	Fluorescence-activated cell sorting
FT	Freeze-thaw
GA	Glutaraldehyde
GP	Glycoprotein
GTP	Guanosine triphosphate
HK	High molecular weight kininogen

ICAM	Intercellular adhesion molecule-1
ID	Inner diameter
IH	Intimal hyperplasia
LDL	Low density lipoprotein
MPO	Myeloperoxidase
NHP	Non-human primate
NO	Nitric oxide
PAD	Peripheral artery disease
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PF4	Platelet factor 4
PK	Prekallikrein
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PT	Prothrombin time
PVA	Poly(vinyl alcohol)
RP	Resting platelet
SD	Standard deviation
SEM	Scanning electron microscope
SMC	Smooth muscle cell
STMP	Sodium trimetaphosphate
SV	Saphenous vein
TAT	Thrombin-antithrombin
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TNFα	Tumor necrosis factor alpha
TXA₂	Thromboxane A2
VCAM	Vascular cell adhesion molecule
VKAs	Vitamin K antagonists
vWF	von Willebrand factor

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Abstract

Cardiovascular diseases (CVD) such as ischemic heart disease and peripheral artery disease, continue to be the leading cause of death worldwide. The primary underlying cause of these diseases is atherosclerosis, the deposition of cholesterol, lipids, and calcium leading to plaque formation within the blood vessel wall, causing thickening and hardening of the tissue and obstruction of blood flow. Implantable medical devices, including vascular grafts and stents, can be used to reintroduce blood flow downstream of the obstructed area. The current gold standard for surgical intervention in CVD is the use of autologous vessels to bypass the obstructed flow. However, patients often lack suitable vessels requiring the use of synthetic graft materials. Current clinically used synthetic materials have shown great success for large-diameter vascular applications, although, they tend to exhibit poor patency rates at small-diameters ($\leq 6\text{mm}$) due to two common failure mechanisms: thrombosis and neointimal hyperplasia. This work uses two approaches to addressing biomaterial-induced thrombosis: one focused on alterations of a vascular graft surface to inhibit material-induced thrombosis and the other focused on examining the efficacy of a novel antithrombotic therapy on a widely used medical device. Work in this dissertation assesses: (1) the properties of hydrogels with a biomimetic surface to encourage *in situ* endothelialization intended for vascular graft applications, (2) *in vitro* hemocompatibility and endothelializability of modified hydrogel biomaterials, (3) *ex vivo* thrombus formation on the modified hydrogels, (4) the *in vitro* role of the contact pathway in nitinol stent thrombosis, and (5) nitinol stents in the presence of blood coagulation contact pathway inhibitors *ex vivo*. This dissertation describes novel biomaterials and therapeutics designed for vascular applications for the advancement of cardiovascular medicine.

Chapter I: Introduction

1.1 Vascular biology

The cardiovascular system is composed of many vessels throughout the body, which serve several critical biological functions, including transport of nutrients and oxygen to tissues, immune regulation, and blood pressure and volume control via the movement of the fluid medium, blood. These vessels are comprised of three distinct layers, an inner endothelialized layer known as the tunica intima, which is in contact with flowing blood, a middle vascular media primarily constituted of smooth muscle cells, collagenous and elastic fibers, which adjust vascular tone, and the adventitia, which makes up the outermost layer of the vessel wall providing protection for the inner layers. There are three main types of blood vessels that are involved in systemic circulation: arteries, veins, and capillaries. Arteries carry blood moving from the heart to the peripheral tissues, while veins host the blood moving from peripheral tissues to the heart. Capillaries reside between arteries and veins and play a role in transporting oxygen, nutrients, and signaling molecules from the arteries to the cell in the surrounding tissues, while supporting diffusion of carbon dioxide, metabolic wastes, and signaling molecules produced by the cells to the veins [1].

1.2 Endothelial cell biology and basement membrane ECM

The tunica intima of the vessel wall is composed of a monolayer of endothelial cells (ECs) which sit on an 80nm thick basal lamina. The apical surface of ECs are

exposed to flowing blood and circulating cells while the basolateral surface is adhered to a three-dimensional extracellular matrix made up of numerous molecules including collagenous bundles and elastic fibers, separating the cells from surrounding tissue. ECs sit along the axis of a blood vessel and have been described to be roughly 10–30µm in width, 30–50µm long, and have a thickness of 0.1–10µm. ECs are anchored to the extracellular matrix (ECM) via focal adhesions consisting of transmembrane integrin adhesion molecules connected to the cytoskeleton of the cell and other ECs via tight and adherens junctions [2]. The main components of the vascular ECM are laminin, elastin, collagen type IV, fibronectin, and proteoglycans. Laminin and collagen type IV play a large role in EC structural support, as these molecules self-assemble into sheet-like networks [3].

Vascular ECs have the unique ability to sense and respond to hemodynamic changes. Under the influence of unidirectional flow, ECs tend to exhibit an elongated morphology, aligning with the direction of blood flow. Under non-uniform or disturbed flow, ECs exhibit a characteristic cobblestone morphology, also seen in static cell culture [4]. In addition to the morphological changes in response to blood flow, ECs also exhibit changes in gene expression and electrochemical activity [5,6]. When ECs are subjected to an increase in unidirectional fluid shear stress, the tangential force acting on a surface, there is an upregulation of the NO, a vasodilator, release from the cell.

1.3 Cardiovascular disease and atherosclerosis

Atherosclerosis is the dominant cause of cardiovascular diseases such as myocardial infarction and stroke. Despite the onset and insurgence of the coronavirus starting in 2019, cardiovascular diseases remain the deadliest worldwide [7,8].

Atherosclerosis is characterized by the presence of fibroinflammatory lipid plaque lesions (**Fig 1.1**), also known as atheromas, on the vessel wall that can protrude into the

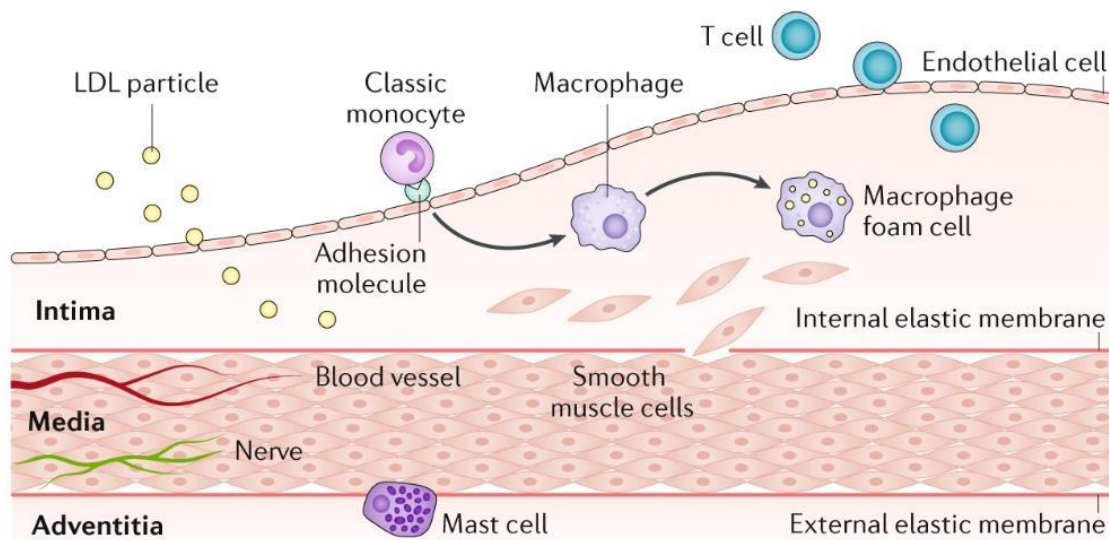


Figure 1.1: Schematic of the development of atherosclerosis.

The progression of an atherosclerotic lesion is shown in a simplified form, developing from a normal blood vessel (far left) to a vessel with an atherosclerotic plaque (right half). LDL-low density lipoprotein. Adapted from Libby, P., Buring, J.E., Badimon, L. *et al.* *Atherosclerosis*. Nat Rev Dis Primers 5, 56 (2019). Reprinted with permission from Springer Nature.

lumen. This process causes stiffening and hardening of the vessel wall. Once the disease has progressed to advanced stages, it often requires clinical intervention to maintain blood flow to distal tissues [9]. The underlying causes of atherosclerosis vary widely, and fatty acid streaking, an early sign of a potential lesion, can begin as early as childhood.

1.3.1 Endothelial cell dysfunction in atherosclerosis

EC activation and subsequent dysfunction is an early event of plaque formation and can be the result of several different conditions including hypertension, hyperlipidemia, and disturbed blood flow at vessel branch points and areas of high curvature. Low shear stress and disturbed flow have been associated with an increase in expression of various endothelial cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin, indicators of vascular inflammation [5]. Furthermore, EC dysfunction plays a role in the progression of atherosclerosis and associated thrombosis by promoting leukocyte adhesion (via cell adhesion molecules), foam cell formation, smooth muscle cell (SMC) infiltration, and platelet activation [10]. The activation of ECs promotes the recruitment of circulating monocytes to the surface of ECs. Cell adhesion molecules mediate monocyte rolling and transmigration into the vessel wall. Monocytes then differentiate into macrophages, which phagocytose an oxidized form of LDL and result in foam cells. Foam cells are a defining characteristic of the development and progression of atherosclerosis [11]. Furthermore, the activated endothelium attracts and activates platelets (discussed in Section 1.5.1 EC regulation in platelet activation and aggregation), leading to an increase in prothrombotic factors such as plasminogen activator inhibitor-1 (PAI-1) and thrombin, causing the plaque to become unstable and potentially leading to rupture [12].

1.4 Hemostasis and Thrombosis

1.4.1 Platelets

Platelets are anuclear blood cells that are produced primarily by megakaryocytes in the bone marrow via a process known as thrombopoiesis. Despite the absence of a nucleus, platelets contain organelles and structures similar to that of a cell. Platelets also contain granules, which hold a variety of biochemical mediators that are released upon platelet activation. These cells are roughly 2-4 μ m in diameter and circulate in the bloodstream in a quiescent (resting) state. Although platelets have a relatively short lifespan of 7-10 days in humans before being cleared by the spleen and liver, they are involved in several diverse biological processes such as tissue repair, inflammation, and host defense [13]. Many studies have shown that platelets exhibit heterogeneity in structure, size, and thus, function, which likely aids in the various roles that these cells can serve [14,15]. However, the main physiologic role of platelets is in hemostasis, a blood loss prevention defense mechanism that occurs when the vascular endothelium has been interrupted. Platelets participate in this process via four distinct steps including adhesion, activation, secretion, and aggregation [16].

Upon injury of the vascular endothelium, the subendothelial matrix is exposed to flowing blood, which leads to recruitment and tethering of platelets to collagen and von Willebrand Factor (vWF), as well as other basement membrane proteins, via different glycoproteins and protein complexes [17]. Platelet adhesion is then followed by activation. Attachment to collagen and vWF is one of the strongest stimulators of platelet activation; however, platelets can be activated by a number of biochemical and physical stimuli [18]. Upon activation, platelets undergo a process called secretion, where

platelets release procoagulant molecules stored in granules such as ADP, ATP, and TXA_2 [19]. Platelet activation also causes activation of the glycoprotein (GP) IIb/IIIa receptor, which plays a large role in platelet aggregation. This receptor binds fibrinogen, which then forms a bridge to neighboring activated platelets, resulting in a hemostatic plug composed of platelet aggregates [20].

1.4.2 Extrinsic pathway of coagulation

Concomitant to the action of platelets upon vessel injury, the extrinsic pathway of blood coagulation (**Fig 1.2**) is initiated on the surface of tissue factor (TF) in the subendothelial matrix residing near the vessel wall injury in a process often referred

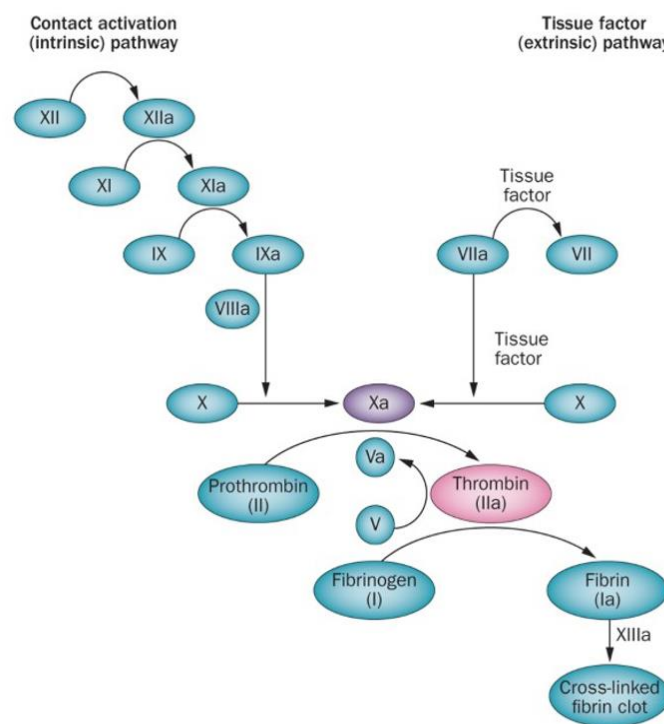


Figure 1.2: The blood coagulation cascade.

Adapted from Sabir, I., Khavandi, K., Brownrigg, J. *et al. Nat Rev Cardiol* 11, 290–303 (2014). Reprinted with permission from Springer Nature.

to as secondary hemostasis. TF binds to and activates coagulation factor (F) VII into FVIIa. These factors form a complex that subsequently catalyzes the activation of FIX and FX. Next, activated FX binds to a cofactor, FVa, forming a complex that catalyzes the conversion of prothrombin (FII) into thrombin (FIIa) [21]. Thrombin serves many roles in the process of blood clotting, including enhancing platelet activation and catalyzing the conversion of fibrinogen into fibrin. Fibrin then undergoes polymerization to form a fibrin mesh, a main structural component of a clot. The process of blood clotting via the extrinsic pathway as a result of a vessel injury is a highly regulated process, and once the vessel injury is resolved, then clots undergo a process called fibrinolysis, where fibrin networks are degraded by plasmin. This allows the clots to persist as long as they are necessary to the vessel injury.

1.4.3 Intrinsic (contact) pathway of coagulation

In addition to the extrinsic pathways of coagulation, the formation of thrombin and ultimately fibrin can also be initiated via the intrinsic, or contact, pathway of coagulation (**Fig 1.2**). Coagulation FXII, the primary initiator of the contact pathway, is an 80kDa zymogen that can be activated into the serine protease FXIIa by binding to a surface via a conformation change in the protein. The FXII zymogen consists of a heavy chain and a light chain connected via a sulfide bond, which remains intact upon activation [22]. FXIIa then activates FXI into FXIa and prekallikrein (PK) into kallikrein. Kallikrein then forms a complex with the protein high molecular weight kininogen (HK) leading to reciprocal activation of FXII [23]. Additionally, FXI can also be activated by thrombin via a positive feedback mechanism, further contributing to thrombin and fibrin generation. Interestingly,

FXII has been shown to play a role in thrombosis (described below) and inflammation; however, FXII has no known role in normal hemostatic clot formation [24].

1.4.4 Dysregulation of hemostasis: Thrombosis

In the sections above, the process of hemostatic clot formation upon disruption of the vascular endothelium under healthy conditions is discussed. While the processes of hemostasis are well-regulated, their dysfunction can lead to pathological aberrant clot formation called thrombosis. Despite having controversial origins, thrombosis has historically been described by Virchow's triad, which lists three factors as the main contributors to the pathology: 1) abnormalities in blood composition, 2) abnormalities in blood flow, and 3) vessel wall injury [25]. Thrombosis can occur in the venous system, the arterial system, or result from the presence of foreign material, such as a bacterial infection, tumor, or medical device.

1.5 The dynamic role of ECs in hemostasis and thrombosis

1.5.1 EC regulation in platelet activation and aggregation

As the primary barrier between tissues and blood flow, the vascular endothelium plays an integral role in regulating hemostasis and blood coagulation. ECs express and release many factors that contribute to and inhibit platelet activation (**Fig 1.3**). One of the major contributors to regulating platelet adhesion to ECs is nitric oxide (NO). When the endothelium is healthy and intact, endothelial isoform of NO synthase generates NO, a cell membrane permeable gas, which is continuously synthesized and released. This

gas can diffuse into platelets and activate the enzyme guanylate cyclase, which catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The increase in cGMP decreases intracellular calcium, GPIIb/IIIa expression, and P-selectin expression [26], regulating platelet function and limiting activation and adhesion.

Another important factor in regulating platelet activity on the endothelium is vWF, a multimeric adhesion glycoprotein. vWF is synthesized within the endoplasmic reticulum of ECs and processed by the Golgi complex. This secreted molecule is essential for platelet adhesion to both the surface of ECs and collagen at sites of vascular injury. Upon disruption of the endothelial surface, vWF forms long strings (up to several millimeters in length) on the surface of endothelial cells resulting in ultra-large vWF multimers that readily form high-strength bonds with platelets via the platelet receptor glycoprotein Ib-V-IX. There are other mechanisms and factors involved in EC regulation of platelet activity including prostacyclin, which inhibits calcium mobilization and granule release from platelets, and ectonucleotidase CD39/NTPDase1, which hydrolyzes ATP and ADP, preventing platelet aggregation [27].

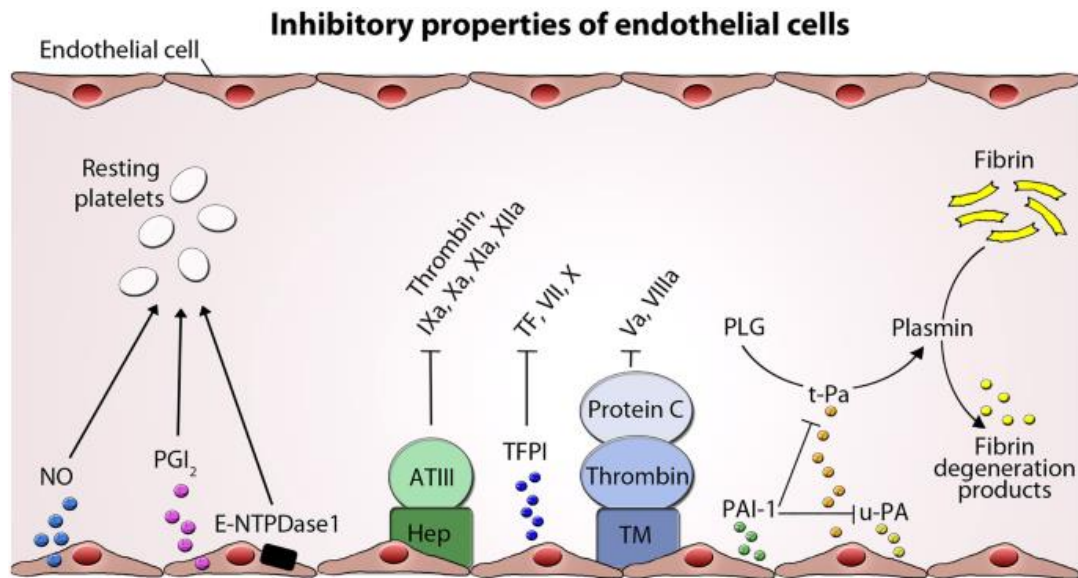


Figure 1.3: Inhibitory properties of endothelial cells.

Inhibition of platelet function and coagulation by several endothelial molecules and factors and the targeted coagulation factors are shown. ECs express NO and PGI₂, and E-NTPDase1, which inhibit platelet adhesion and aggregation. Heparin-like molecules (Hep) expressed on the endothelial surface serve as a cofactor for antithrombin inactivating several coagulation factors. ECs express TFPI, which limits the action of TF. Thrombomodulin (TM) binds thrombin activating protein C and degrades factor Va and VIIIa. Adapted from Neubauer, K., Zieger, B. Endothelial cells and coagulation. *Cell Tissue Res* (2021). No special permissions required for reprint.

1.5.2 EC regulation in blood coagulation

In addition to modulating platelet activity in blood clotting, ECs also participate in the regulation of activation of various components of the blood coagulation cascade (**Fig. 1.3**). Under normal physiologic conditions, the healthy intact endothelium inhibits coagulation through the expression of natural anticoagulants including TFPI, EPCR, thrombomodulin, and heparin-like proteoglycans. TFPI is a tissue factor pathway

inhibitor, which inhibits TF-initiated thrombin and fibrin production. EPCR is an EC surface receptor that acts in conjunction with thrombomodulin to activate protein C. Activated protein C inhibits the action of FVa and FVIIIa, downregulating further thrombin production. Heparin-like proteoglycans serve as a platform for antithrombin III binding, which inactivates thrombin activity. These mechanisms are all in play under healthy conditions; however, when the endothelium becomes activated, ECs can also exhibit procoagulant activity.

It has been shown that cultured ECs can express TF in the presence of pro-inflammatory molecules, such as lipopolysaccharide, tumor necrosis factor- α (TNF- α), and thrombin [28–30]. These studies show how activated ECs can upregulate thrombin production via the extrinsic pathway [31].

1.6 Cardiovascular devices to treat CVD

1.6.1 Stents

For advanced stages of atherosclerotic diseases, such as ischemic heart disease and peripheral artery disease, vascular interventions and operations become vital and often involve the use of autologous vessels or synthetic medical devices. Stents are small mesh tubes made of metal alloys or polymers, which are inserted into a diseased vessel using a catheter. These devices are often used for revascularization in vessels that are less than 70% occluded [32]. Despite many improvements in stent technology, this intervention has not been shown to reduce the risk of death or heart attack without additional side effects such as acute coronary syndrome for cardiac applications [33]. Stents, specifically bare metal stents (BMS), tend to result in a high occurrence of

restenosis, negating the initial treatment and often requiring additional medical care for the patient. Restenosis is a primary cause of stent failure and occurs as a result of a vascular remodeling and healing process known as neointimal hyperplasia [34]. Neointimal hyperplasia is characterized by the proliferation and migration of vascular SMCs into the tunica intima layer, where the device resides. This leads to vascular wall thickening and the gradual loss of luminal patency (opening), restricting blood flow [35]. In more recent years, drug-eluting stents (DES) have been presented as a treatment for CVD that reduces the incidence of hyperplasia. These stents consist of a polymer coating loaded with anti-proliferative agents, such as sirolimus and paclitaxel, which are released over time [36]. DES address the SMC proliferation associated with hyperplasia. However, the anti-proliferative agents also inhibit EC migration onto the device, which is ultimately required for biological integration. DES have been found to increase the expression of TF, leading to high incidence of thrombosis, another primary cause of device failure. Furthermore, the anti-proliferative drugs associated with DES have been shown to lead to downstream EC dysfunction and hypersensitivity reactions to the polymer material [37,38].

1.6.2 Vascular grafts

An alternative treatment for ischemic heart disease and peripheral artery disease caused by atherosclerosis is bypass grafting, where an autologous vessel or synthetic graft material is surgically implanted to bypass the atherosclerotic occlusion. This invasive surgical option has been thought to be the optimal intervention for patients requiring long-term revascularization [39]. For small diameter applications such as coronary bypass, the use of autologous veins or arteries continue to be the gold

standard for this procedure (**Fig 1.4**) [40]. The internal thoracic artery and radial artery have been shown to have superior patency rates compared synthetic graft materials and to the saphenous vein, which continues to be the most commonly used vessel for bypass grafting [39]. Failure rates for the use of a saphenous vein autograft, particularly in coronary artery bypass grafting, has been shown to be roughly 50% after 10 years [41]. Despite the preferred use of autologous vessels, these graft sources can be limited due to lack of suitable vessels within patients due to vascular disease or donor site morbidity [42].

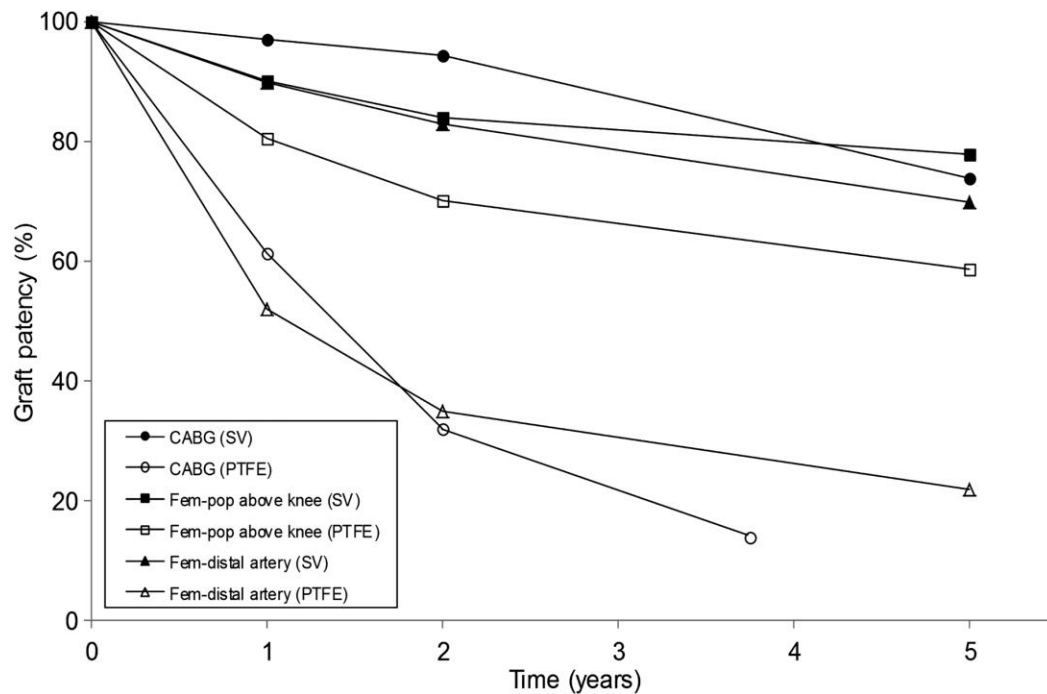


Figure 1.4: Patency rates for small-diameter vascular bypass procedures using the saphenous vein (SV) and polytetrafluoroethylene (PTFE) conduits.

Adapted from Pashneh-Tala, Samand *et al.* *Tissue engineering. Part B, Reviews* vol. 22,1 (2016): 68-100. No special permission required for reprint.

When autologous vessels are unavailable, synthetic vascular grafts made from non-biodegradable polymers, such as expanded polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (Dacron), or biologically-derived materials may be used as an alternative. Synthetic grafts have been successful long-term when used for large-diameter applications (>6mm). However, synthetic grafts fail at small diameters due to thrombosis, mismatched mechanical properties, aneurysmal failure, calcification, chronic inflammatory response, and neointimal hyperplasia [43].

1.6.2.1 Thrombosis and intimal hyperplasia are the main contributors to graft failure

Thrombosis has been shown to occur at any point along the lumen of the graft but is most likely to occur at anastomoses, the surgical site at which the synthetic graft is attached to the native vasculature [44]. The anastomoses is an area that is subjected to a prothrombotic combination of suturing, endothelial injury (causing high expression of TF), and disturbed blood flow [45]. Thrombosis along the luminal surface of the graft is the result of many mechanisms, including platelet attachment and activation, activation of the contact pathway of coagulation, and TF-initiated aberrant clotting. In addition to thrombosis, neointimal hyperplasia is also most prominent at the graft anastomoses, with a key mechanical factor being compliance mismatch. Compliance of a graft refers to the circumferential elasticity of the material when pressure is applied, and mismatch refers to the compliance of the synthetic conduit being different from the native vasculature [46]. Current engineering strategies are focused on mitigating the effects of these failure mechanisms.

1.7 Antithrombotic strategies

1.7.1 Mechanisms of medical device-induced thrombosis

The general biology and causes of thrombosis as well as the role pathology plays in the context of medical devices was briefly discussed in Sections 1.4 and 1.6.2. This section will discuss the mechanisms of thrombosis in the presence of a synthetic vascular device. Upon implantation or placement of a blood-contacting vascular device, the surface is rapidly coated in proteins via adsorption from the blood. The process of protein adsorption is highly dynamic and can be altered by the chemical and physical properties of the material surface and the proteins present. Amongst these proteins are fibrinogen, vWF, fibronectin, FXII, HK, and PK. Thrombus formation on the surface of a device is the result of mechanisms similar to those described in Section 1.4. Fibrinogen is one of the first plasma proteins to adhere to a device surface, allowing for mediation of platelet adhesion. Adhered platelets then become activated and release granules containing prothrombotic factors such as ADP, ATP, and TXA₂, activating surrounding platelets, ultimately resulting in aggregation. In tandem, adsorbed FXII undergoes autoactivation and reciprocal activation by HK and PK, leading to increased thrombin generation and fibrin production. Thrombin locally upregulates the activation of platelets and FXI, further promoting clot formation on the surface of the device. Platelet aggregates on the surface are stabilized by fibrin strands, forming a thrombus [47].

1.7.2 Endothelialization of synthetic grafts

Most mechanisms of graft failure have been attributed to the absence of a functional endothelium, which naturally regulates pathways that lead to graft failure, on the synthetic graft surface. With roughly one-fifth of patients whom require revascularization lacking suitable autologous vessels, the development and improvement of synthetic conduits continues to be an intense area of research [42,48].

Some researchers have taken the approach of conventional tissue engineering of vascular grafts, where ECs are first cultured *in vitro* then seeded onto scaffolds prior to implantation. This method is known as *in vitro* endothelialization of a synthetic graft and was thought to be advantageous, as a patient's own autologous cells could be harvested and applied to the conduit, thus limiting chronic inflammatory response [49]. Despite the promise and personalization of this method, it comes with many drawbacks including the high cost and time it takes to harvest and culture a patient's cells and the decrease in cell viability after implantation [50].

These disadvantages have led many researchers to take the approach of *in situ* endothelialization, which is achieved by modifying the luminal surface of synthetic grafts to improve biological integration and combat device failure. The most widely used approach has been to chemically or physically modify the surface of the lumen of synthetic scaffolds to encourage and direct endothelialization [51]. Cellularization of the synthetic graft surface can occur via migration of ECs from surrounding native vasculature or by the adhesion and proliferation of circulating ECs, often referred to as endothelial progenitor cells (EPCs). Chemical modifications to encourage EC migration and EPC attachment include the immobilization or release of molecules targeted at EC integrins, including ECM or ECM-mimicking molecules, as these reside in the native

basolateral environment of ECs (discussed further in Chapter 3 of this dissertation) [52]. Additionally, alterations in the topography of the synthetic graft surface can increase the surface area of the material, and, thus, increase the number of bioactive molecules that can be immobilized or adsorbed. Furthermore, anisotropic topographic patterning of the graft surface can influence ECs into an elongated shape in the absence of flow, leading to a beneficial atheroprotective phenotype, as well as direct EC migration and enhance the strength and adhesion of EC barrier junctions [53,54].

1.7.3 Currently available therapeutics to combat vascular device-induced thrombosis

Vascular device-related thrombosis is associated with significant morbidity and mortality and increased healthcare costs. Several strategies have been employed to mitigate this aberrant clotting including alterations of the biomaterial surface described in Section 1.6. Another approach to minimizing thrombosis is systemic administration of antiplatelet therapies and/or anticoagulants. However, because these pharmaceuticals target the same molecules required for hemostatic clot formation, a major risk and side effect of this treatment is bleeding [55].

Antiplatelet agents, such as acetylsalicylic acid (aspirin, ASA) and clopidogrel, are commonly used for the prevention of stent thrombosis [56]. ASA blocks the production of the platelet agonist TXA₂ by irreversibly inhibiting cyclooxygenase 1 (COX-1) via acetylation of serine-530 [57]. Clopidogrel inhibits platelet activation by selectively binding to P2Y₁₂, an ADP receptor on the platelet surface, thereby inhibiting the action of ADP, which would otherwise activate adjacent platelets and promote aggregation [58].

TXA₂ and ADP are critical components in hemostatic clot formation; thus, their inhibition can lead to detrimental bleeding upon any slight disruption of the vascular endothelium.

Pharmacological inhibition of the intrinsic and extrinsic coagulation pathways is also implemented to minimize device-related thrombosis (summarized in Fig 1.5). These anticoagulants can inhibit coagulation enzymes in either a direct or indirect manner [47]. Heparin has been a long time standard antithrombotic therapy and is used for the inhibition of thrombin and FXa via the activity of antithrombin. Heparin has not only been used for systemically administered anticoagulation but has also been immobilized on vascular device surfaces to combat thrombosis [59]. This drug can be used alone, however, is often administered in conjunction with other antithrombotics, particularly,

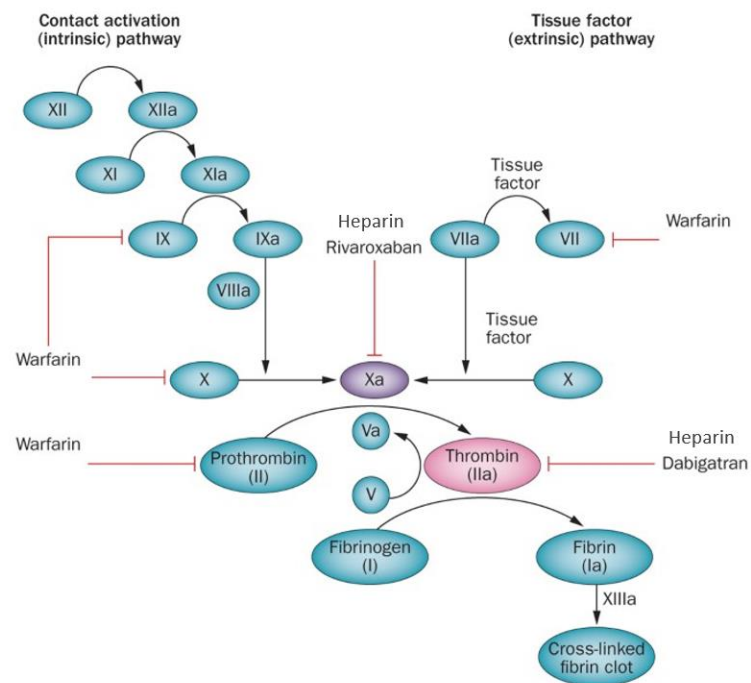


Figure 1.5: Coagulation cascade targets of select anticoagulants.

Adapted and modified from Sabir, I., Khavandi, K., Brownrigg, J. *et al. Nat Rev Cardiol* 11, 290–303 (2014). Reprinted with permission from Springer Nature.

antiplatelet agents [60]. Another class of anticoagulants are vitamin K antagonists (VKAs) such as warfarin, which inhibit the synthesis of the vitamin K-dependent clotting factors, FII, FVII, FIX, and FX. Aside from the major bleeding side effects, patients receiving VKAs long-term are sensitive to systemic fluctuations in dietary vitamin K, which can alter the response of the drugs [61]. Furthermore, drugs such as heparin and warfarin require close monitoring and frequent adjustment of dosage to maintain a therapeutic range. Direct oral anticoagulants (DOACs) such as rivaroxaban, which target FXa and thrombin, were developed to address some of the challenges observed with VKAs [62]. These drugs can be administered in fixed doses, their action is not altered by dietary vitamin K intake, and routine drug monitoring is not required [63].

Despite the historical use and improvement of clinically used anticoagulants, dangerous bleeding side effects persist. As a result, novel safe anticoagulants are emerging and targeting contact pathway proteins such as FXI and FXII, has been a promising approach due to the limited role these factors play in hemostatic clot formation. It has also been reported that patients with congenital FXII deficiency do not have abnormal bleeding, as patients with other clotting factor deficiencies, which often result in hemophilias [22,24,64]. Research supporting the use of FXI and FXII inhibitors for device-related thrombosis includes the use of monoclonal antibodies directed against FXIIa to prevent clotting in rabbit [65] and non-human primate extracorporeal membrane oxygenator (ECMO) models [66] were found to be as effective as heparin. The knockdown of FXI or FXII has shown therapeutic benefit in different thrombotic pathologies [67,68]; however, more work must be done to determine if targeting contact pathway activation would be an effective strategy for mitigating thrombosis associated with various vascular devices.

1.8 Dissertation aims

This dissertation aims to better understand the effects of altering the material properties of hydrogels on thrombosis and understanding how contact pathway-targeted therapies can alter nitinol stent-induced thrombosis.

Aim 1: Characterize the effect of various crosslinkers on PVA hydrogel thrombogenicity *in vitro*. Aim 1 addresses the hypothesis that the type and process of hydrogel crosslinking can change how the material responds to different components of the blood. This is the first published support that toxic chemical crosslinkers can modify FXII activation and platelet attachment to PVA hydrogels. Work pertaining to Aim 1 is presented in Chapter II, and has been published in *Cardiovascular Engineering and Technology* [69].

Aim 2: Engineer a biomimetic hydrogel and characterize the material properties, ability to support endothelial cell attachment, and quantify thrombogenicity *in vitro* and in an *ex vivo* non-human primate model. This aim seeks to exploit biology for novel biomaterial design and tests how immobilization of a collagen-mimicking peptide on the surface impacts material-induced thrombosis. This aim uses contact angle and x-ray spectroscopy to characterize the hydrogel surface properties and then evaluates thrombogenicity *in vitro* by measuring fibrin formation, FXII activation, and static platelet adhesion. Endothelial cell attachment in static culture is also assessed. Additionally, tubular modified hydrogels are evaluated under physiologic whole blood flow in a non-human primate model. Work pertaining to Aim 2 is presented in Chapter III and has been published in *Frontiers: Bioengineering and Biotechnology* [70].

Aim 3: Investigate the role of FXII in nitinol stent-induced thrombosis. This aim addresses the hypothesis that inhibition of the contact pathway is an effective approach to treating vascular device-related thrombosis. The roles of FXII and FXI are examined *in vitro* and under physiologic whole blood flow in an *ex vivo* model. This aim addresses coagulation, platelet activation, and several aspects of inflammation in stent thrombosis. Work pertaining to Aim 3 is presented in Chapter IV.

CHAPTER II: Evaluation of the effect of crosslinking method of poly(vinyl alcohol) hydrogels on thrombogenicity¹

2.1 Abstract

Crosslinked poly(vinyl alcohol) (PVA) is a biomaterial that can be used for multiple cardiovascular applications. The success of implanted biomaterials is contingent on the properties of the material. A crucial consideration for blood-contacting devices is their potential to incite thrombus formation, which is dependent on the material surface properties. The goal of this study was to quantify the effect of different crosslinking methods of PVA hydrogels on *in vitro* thrombogenicity. PVA was manufactured using three different crosslinking methods: 30% sodium trimetaphosphate (STMP), three 24hr freeze-thaw cycles (FT), and 2% glutaraldehyde-crosslinked (GA) to produce STMP-PVA, FT-PVA and GA-PVA, respectively. Expanded polytetrafluoroethylene (ePTFE) was used as a clinical control. As markers of thrombus formation, the degree of coagulation factor (F) XII activation, fibrin formation, and platelet adhesion were measured. The GA-PVA material increased FXII activation in the presence of cofactors compared to vehicle and increase platelet adhesion compared to other PVA surfaces. The STMP-PVA and FT-PVA materials had equivalent degrees of FXII activation, fibrin formation and platelet adhesion. This work supports crosslinker dependent

¹ This work was originally published in *CVET*. Bates NM, Puy C, Journey PL, McCarty OJT, Hinds MT. Evaluation of the Effect of Crosslinking Method of Poly(Vinyl Alcohol) Hydrogels on Thrombogenicity. *Cardiovasc Eng Technol*. 2020;11(4):448-455. doi:10.1007/s13239-020-00474-y © 2020 The authors.

thrombogenicity of PVA hydrogels and advances our understanding of how the manufacturing of a PVA hydrogel affects its hemocompatibility.

2.2 Introduction

Synthetic vascular grafts are required to replace damaged or occluded vessels when autologous veins are unavailable [71]. Despite this beneficial treatment, small diameter vascular grafts have a tendency to fail due to thrombosis. The development of hemocompatible cardiovascular biomaterials remains an unmet clinical need. There has been an increasing interest in the use of hydrogels, including poly(vinyl alcohol) (PVA), for cardiovascular biomaterial purposes [72–75]. PVA is a water-soluble polymer that can be physically or chemically crosslinked into a hydrogel. This material has been used widely for biomedical applications due to its desirable properties including tunable mechanical properties, amenability to surface modifications and compliance [72–74].

The hemocompatibility of PVA has been widely deliberated [55,72,73,75–81]. However, there are stark differences in manufacturing of PVA hydrogels for different biomedical applications. The size, shape, and density of the materials all influence the interactions with the components of blood. The crosslinking method of hydrogels can potentially change the bulk and surface properties of the material [72], including thrombogenicity. Thrombogenicity is particularly important when developing blood-contacting biomaterials, such as vascular grafts.

There are multiple methods of crosslinking hydrogels, including physical and chemical processes. The common chemical crosslinking agents for PVA are aldehydes, such as glutaraldehyde (GA) [82]. The aldehyde functional group reacts with the hydroxyl group on PVA forming an ester bond. Another chemical crosslinking method was designed by Chaouat et al., who were the first to engineer a PVA-based vascular graft using sodium trimetaphosphate (STMP), a food grade crosslinker [75]. In this process, the hydroxyl groups on PVA undergo phosphoesterification by STMP, which

leads to the availability of phosphate groups in PVA to react with other hydroxyl groups to form a crosslinked network. Additionally, PVA can be physically crosslinked through a series of freeze-thaw (FT) cycles, which is a process that relies on the hydrogen bonding and the crystallinity of the polymer [83,84].

The introduction of different chemical groups onto PVA can affect how blood components interact with the hydrogel, an important concern for blood-contacting devices. Thus, we hypothesized that the method of crosslinking PVA would alter the biomaterial thrombogenicity. It is widely accepted that the contact pathway of blood coagulation is activated by artificial surfaces. Blood coagulation factor (F) XII can bind to artificial surfaces, inducing a conformational change of FXII which leads to activation of the zymogen into its protease form, FXIIa [23,85]. FXIIa then activates prekallikrein (PK) into kallikrein, which along with high molecular weight kininogen (HK), amplifies FXIIa production [86]. FXIIa then activates FXI into FXIa, which ultimately leads to fibrin formation, one of the main structural components of a thrombus. In addition to fibrin, platelets are a major component of a thrombus. Platelets can bind to adsorbed fibrinogen and become activated, promoting thrombosis. This work compares the effects of the crosslinking method on the *in vitro* hemocompatibility, including the effect on the contact pathway, fibrin formation, and platelet attachment.

2.3 Materials and Methods

2.3.1 Hydrogel manufacturing

A final concentration of 10% (w/v) aqueous PVA (Sigma) was used for all hydrogels. The STMP-PVA was manufactured as previously described [75], 15% (w/v)

sodium trimetaphosphate (STMP, Sigma) was added to aqueous PVA followed by 30% (w/v) sodium hydroxide and cured as films. The FT-PVA was manufactured by pouring a 10% aqueous PVA solution as a film, which was then frozen at -20°C for 18hr and thawed at RT for 6hr three times. The FT-PVA samples were then set at RT for 14h before placing in oven and increasing temperature by 10°C every 2hr until 80°C was reached. The FT-PVA samples remained at 80°C for 20hr before use. The glutaraldehyde crosslinked PVA (GA- PVA) was manufactured using a solution with a final concentration of 2% glutaraldehyde (Sigma) and 10% aqueous PVA. Ethanol (95%) treated expanded polytetrafluoroethylene (ePTFE, Gore) was dipped in the 2% glutaraldehyde crosslinking PVA solution three times with 30min of drying in between dips. The GA-PVA samples were dried overnight before additional drying in oven at 70°C for 2hr. After preparation all materials were punched with a 5mm biopsy punch. Control materials of glass (positive control for FXII activation) and unmodified ePTFE (clinical standard control) were used.

2.3.2 Factor XII Activation

The amount of factor XII (FXII) activation was quantified using a method modified from Puy et al [87]. A 200µL solution of FXII (200nM, Haematologic Technologies, Inc., Essex Junction, VT, USA) was incubated at 37°C alone in 25mM HEPES, pH 7.4, 150mM NaCl, and 0.1% BSA vehicle buffer or with the cofactors PK (50nM, Enzyme Research Laboratories, Inc., South, IN, USA) and HK (50nM, Enzyme Research Laboratories, Inc., South, IN, USA) in the vehicle buffer in the absence or presence of PVA biomaterials or glass as a control in a 96-well plate. Samples were removed and quenched with 50µg/ml soybean trypsin inhibitor (Sigma-Aldrich, St Louis, MO, USA) to

limit further FXIIa production, followed by quantification of FXIIa generated by measuring the rates of Spectrozyme-FXIIa (American Diagnostica, Inc., Stamford, CT, USA) hydrolysis at 405nm absorbance using an Infinite M200 spectrophotometer (Tecan, Männedorf, Switzerland). A standard curve of FXIIa was used to convert the absorbance to FXIIa concentration. Six individual experiments were performed for this study.

2.3.3 Fibrin Generation and Fibrinolysis

Each PVA biomaterial, ePTFE as a clinical control, and a blank well were incubated in 50µl of pooled platelet-poor plasma (PPP, International Society of Thrombosis and Haemostasis Scientific and Standardization Committee Lot 4) with 50µl HEPES buffered saline (HBS, 25mM HEPES and 150mM NaCl, pH 7.4) and 3nM tissue plasminogen activator (tPA), followed by the addition of 50µl CaCl₂ (8mM). Changes in turbidity of the solution were measured as absorbance at 405nm and documented over time at 37°C. Fibrin generation initiation times were defined by a 5% increase over the baseline absorbance. The 5% increase was chosen as a standard, comparable time point because it was a consistent point above the noise floor of the citrated plasma. The rates of fibrin generation were defined as the maximum slopes and the rates of fibrinolysis were characterized as the minimum slopes. Six individual experiments were performed for this study.

2.3.4 Washed platelet and platelet-rich plasma preparation

The work herein required human blood to isolate plasma and platelets, which was done in accordance with Oregon Health & Science University Institutional Review

Board (IRB) approval. The subjects gave informed written consent to their participation in these studies and the work adheres to the Declaration of Helsinki.

Human venous whole blood was drawn from healthy donors into sodium citrate, as previously described [88]. In brief, the whole blood was centrifuged at 200g for 20min to obtain platelet-rich plasma (PRP). In select studies, purified platelets were isolated from PRP by further centrifugation at 1000g for 10min in the presence of 0.1 μ g/ml prostacyclin. The pellet was resuspended in HEPES/Tyrode buffer (129mM NaCl, 0.34mM Na₂HPO₄, 2.9mM KCl, 12mM NaHCO₃, 20mM HEPES, 5mM glucose, 1mM MgCl₂; pH 7.3) containing 0.1 μ g/ml prostacyclin. The platelets were washed once via centrifugation (1000g for 10min) and resuspended in HEPES-Tyrode buffer.

2.3.5 Platelet adhesion quantification

The static platelet adhesion was quantified using a method modified from Vaníčková et al [89]. A 200 μ L solution of PRP or purified washed platelets suspensions (5.0×10^8 platelets/mL) were incubated on each material in a microtiter plate for 1hr at room temperature. The samples were then rinsed three times with phosphate buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA) to remove all non-adherent platelets. The amount of platelets attached to the surface of each material was measured using the platelet acid phosphatase activity with a calibration curve using platelet solutions ($0-5.0 \times 10^8$ platelets/mL). Three individual experiments were performed using isolated purified platelets and six individual experiments were performed using platelet rich plasma.

2.3.6 Scanning Electron Microscopy

Samples were prepped identically to those in the purified platelet adhesion studies but were examined using scanning electron microscopy (SEM). After incubation with platelets, the samples were rinsed three times with PBS before fixing for 30min at room temperature using Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde and 0.1M sodium phosphate buffer, pH 7.4 containing 0.1M sucrose), after which they were rinsed three times with PBS. The samples were then dehydrated using ethanol:water solutions (35, 50, 70, 80, 90, 95, 100% ethanol) for 10min each. The samples were further dehydrated with a treatment of hexamethyldisilazane (Sigma) solutions (50 and 100%) for 10min each, before drying overnight. The samples were mounted onto studs then sputter coated with gold palladium alloy at 48mA for 30sec in 50mTorr argon (Desk II, Denton Vacuum). The conformation of platelets adhered to each samples was observed using FEI ESEM Quanta 200 scanning electron microscope and was imaged at 10keV.

2.3.7 Atomic Force Microscopy

Atomic Force Microscopy (AFM) scans were performed on dry STMP-PVA samples using Bruker Fastscan-C silicon nitride cantilevers with silicon probe tips (5nm tip radius, 0.8N/m force constant) and a Dimension Fastscan Bio Icon System (Bruker Nano Surfaces, Santa Barbara, CA). Scans were acquired over a $5\mu\text{m} \times 5\mu\text{m}$ area in Peakforce Tapping Mode. The samples were prepared using a 6mm biopsy punch and secured to a glass slide using double-sided tape before scanning.

2.3.8 Statistics

The data are shown as means $\pm$ SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism v 8.2.1 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Probability values of $p < 0.05$ were considered to be statistically significant.

2.4 Results

2.4.1 Effects of PVA biomaterials on factor XII activation

In purified systems, we first studied the ability of PVA biomaterials to both autoactivate FXII alone and amplify FXII activation in the presence of cofactors. FXII autoactivation was not detected on any of the crosslinked PVA hydrogels as compared to glass surfaces. In the presence of the cofactors PK and HK, an increase in the degree of FXII activation was observed on GA-PVA as compared to vehicle. A similar increase was observed for the clinical and positive controls, ePTFE and glass, respectively (**Fig 2.1**).

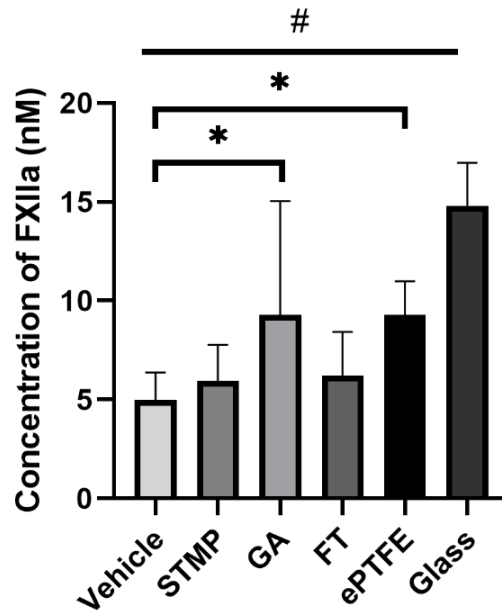


Figure 2.1: Time dependent FXII activation in the presence of PK and HK on crosslinked biomaterials after 60min.

Data are shown as means $\pm$ SD. Statistical analyses were performed using a one-way ANOVA with Tukey's post hoc testing (n=6). * indicate significant increase from vehicle (p<0.05). # indicates an increase from all other sample means (p<0.01)

2.4.2 Effects of PVA biomaterials on fibrin generation and fibrinolysis

We next studied the effects of the PVA biomaterials on the formation and degradation of fibrin in platelet-poor plasma. The initiation time of fibrin formation remained roughly 1000sec for all three PVA biomaterials and plasma alone. An average initiation time of fibrin formation of 797 ± 33 sec was recorded on ePTFE. There were no statistical significant differences in the rate of fibrin generation and fibrinolysis between ePTFE, the three crosslinked PVA biomaterials and the plasma control (**Fig 2.2**).

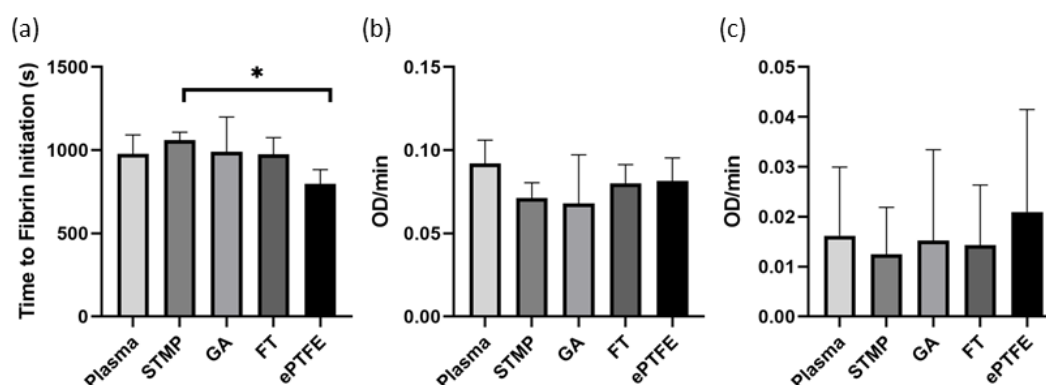


Figure 2.2: Fibrin formation dynamics in the presence of biomaterials.

(a) Fibrin initiation time on crosslinked PVA hydrogels. (b) Rate of fibrin generation on biomaterials shown as optical density over time (OD/min). (c) tPA induced fibrinolysis in the presence of biomaterials shown as optical density over time (OD/min). The absolute value was taken to make decreasing rates positive for the fibrinolysis data. Data are shown as means $\pm$ SD. Statistical analyses were performed using a one-way ANOVA (n=6).

2.4.3 Effects of PVA biomaterials on platelet adhesion

We then studied the extent to which PVA biomaterials supported platelet adhesion. Purified platelets or platelet-rich plasma was incubated on crosslinked biomaterials or ePTFE and the degree of adhesion quantified using an acid phosphatase assay. There was significantly higher platelet attachment to GA-PVA as compared to STMP-PVA in both the purified platelet system (**Fig 2.3a**) and in the platelet-rich plasma (**Fig 2.3b**). A similar degree of platelet adhesion was recorded on STMP-PVA and FT-PVA materials and ePTFE in both the purified platelet and platelet-rich plasma systems. These findings were further corroborated by visual inspection of the biomaterial surface by scanning electron microscopy. A robust degree of individual platelets and platelet aggregates were observed on GA-PVA and individual platelets on ePTFE (**Fig 2.4**). The

analysis of the AFM scan (**Fig 2.5**) indicated the surface roughness average (R_a) was 4.60nm for STMP-PVA.

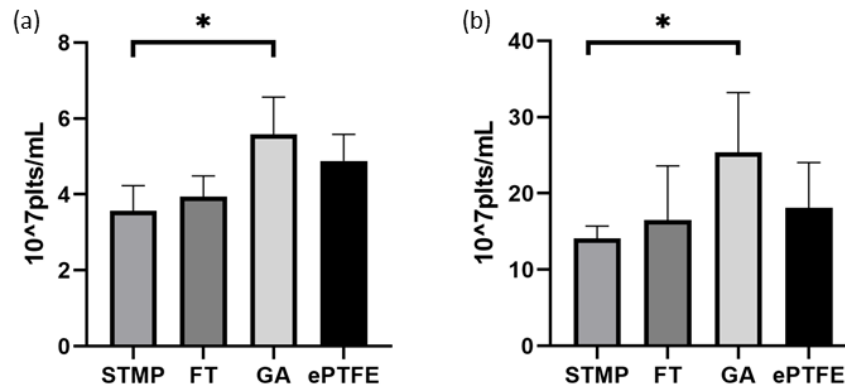


Figure 2.3: Static platelet adhesion presented as the number of platelets present per mL of lysis buffer.

(a) Isolated platelet adhesion onto crosslinked PVA biomaterials (n=3). (b) Static platelet adhesion onto crosslinked biomaterials in platelet-rich plasma (n=6). Data are shown as means $\pm$ SD. Statistical analyses were performed using a one-way ANOVA with Tukey's post hoc testing. * indicate significant difference ($p < 0.05$)

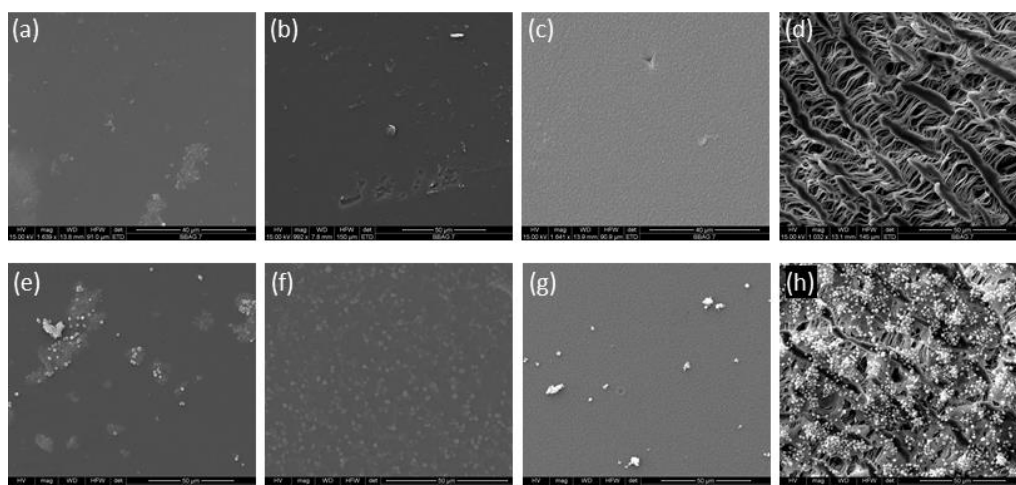


Figure 2.4: Representative SEM micrographs of each biomaterial before (top row) and after (bottom row) isolated platelet incubation.

(a, e) STMP-PVA; (b, f) GA-PVA; (c, g) FT-PVA; and (d, h) ePTFE-PVA. All scale bars = 50nm.

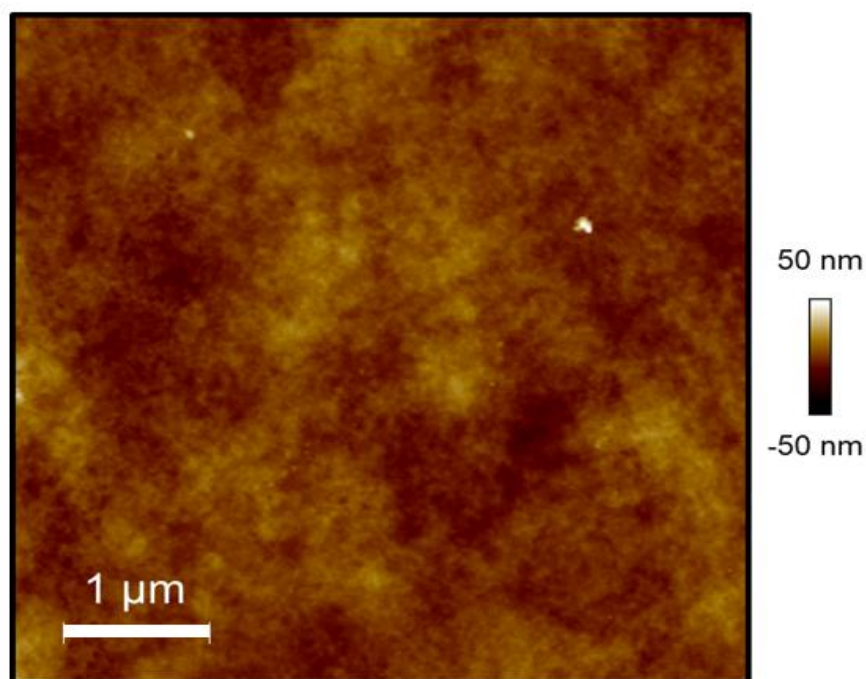


Figure 2.5: Topographic characterization of the surface of STMP-PVA using AFM.

The dimensions of the scan are $5\mu\text{m} \times 5\mu\text{m}$. The surface had roughness average (R_a) is 4.6nm and the total surface area of the sample is $25.248\mu\text{m}^2$.

2.5 Discussion

PVA hydrogels have many desirable properties for cardiovascular applications including modifiable surfaces and tunable mechanical properties. However, the extent to which a change in the bulk properties of PVA hydrogels alters thrombogenicity remains poorly understood. This work investigated how FXII activation, fibrin generation, and platelet adhesion are affected *in vitro* due to physically and chemically crosslinked PVA hydrogels.

The crosslinking method of PVA can change the surface properties of the biomaterial, thus altering thrombogenicity. Here we studied the effects of chemically crosslinking the PVA with STMP and GA, as well as a physically crosslinking PVA with consecutive FT cycles, on hemocompatibility. The use of STMP as a crosslinking agent leads to the formation of durable PVA scaffolds with mechanical properties similar to native human arteries that are non-toxic to cells [79,90,91]. STMP crosslinked PVA biomaterials have demonstrated low platelet adhesion and long thrombin generation times [75]. The low platelet adhesion and low fibrin formation on the surface of STMP crosslinked PVA have also been shown in an *ex vivo* arteriovenous shunt model [72,80,81,92]. *In vivo* studies using STMP crosslinked PVA vascular prosthesis showed 100% patency after 7 days of implantation in a rat abdominal aorta model [93]. Glutaraldehyde crosslinked PVA hydrogels have insufficient mechanical strength to act as a material alone and therefore have been studied as a coating [94]. These glutaraldehyde crosslinked PVA hydrogels, were reactive to platelets in a canine arteriovenous (AV) shunt model even with the addition of heparin to the material surface [94]. FT crosslinked PVA hydrogels are highly elastic, porous and spongy when compared to other crosslinking methods [83,84,95]. The preparation of PVA biomaterials via this cryotropic treatment has been a preferred method due to the absence of potentially toxic chemical crosslinking agents. Yet the effect of FT crosslinked PVA on thrombus formation was not known. There are advantages of chemically crosslinking PVA, including the prevention of hydrogel dissolution and controllable mechanical properties. However, the leaching of some chemical agents, such as glutaraldehyde and formaldehyde, can lead to toxic microenvironments and decreased hemocompatibility.

We studied activation of the contact pathway by the three different crosslinked PVA hydrogels. FXII autoactivation was not detected on any of the crosslinked PVA surfaces. Plasma proteins are adsorbed onto surfaces within seconds of encountering blood. Amongst these proteins is FXII, which is activated into FXIIa upon binding to a surface [86]. The PVA hydrogels are highly hydrophilic, which has been shown to minimize protein adsorption [96]. The surface of glass is negatively charged, which is known to activate FXII at a high rate [23]. The surface chemistry of ePTFE consists of a fluorinated carbon-hydrogen backbone making the material hydrophobic. Hydrophobic surfaces encourage protein adsorption. FXII is also activated on surfaces in the presence of kallikrein (K) and high molecular weight kininogen (HK). In these cofactor mediated FXII activation studies, GA-PVA and ePTFE significantly increased the amount of FXIIa after 1hr (**Fig 2.1**). The increased amount of FXIIa is likely due to the hydrophobicity of the ePTFE surfaces. The addition of glutaraldehyde into PVA polymer networks introduces small hydrophobic regions into the hydrogel, while the STMP crosslinker and physical crosslinking of the PVA maintain their hydrophilicity. Despite these findings, it must be noted that continuous activation of FXII by biomaterial surfaces does not always lead to continuous downstream activation of the coagulation system [86,87]. Thus, FXII activation alone in purified systems on biomaterial surfaces is not a complete predictor of biomaterial thrombogenicity. For a complete understanding of biomaterial thrombogenicity, the coagulation system must be studied in conjunction with platelet activation.

In the contact pathway, FXIIa subsequently activates downstream coagulation factors, ultimately leading to fibrin production. This work showed ePTFE reduced fibrin generation initiation time compared to STMP-PVA in human PPP, indicating that the

ePTFE more rapidly initiated fibrin formation (**Fig 2a**). All of the crosslinked PVA biomaterials had initiation times and rates of fibrin generation equivalent to the vehicle. Thus, FXIIa activity did not correlate to rate of fibrin generation on PVA crosslinked materials supporting previous work that FXII activation alone does not equate to clot formation. However, the shorter initiation time of fibrin generation for ePTFE did correlate with increased FXII activation in the presence of PK and HK. As expected, once fibrin formation is initiated, the fibrin clot grew at the same rate and had the same rate of fibrinolysis for all of the materials. Thus, in these static conditions, the density of the fibrin clot was likely unchanged by the underlying material.

When materials contact blood, the materials are immediately coated with blood proteins, particularly fibrinogen. Platelets can then bind to adsorbed fibrinogen via the glycoprotein IIb/IIIa receptor (GPIIb/IIIa), which leads to platelet activation. Activated platelets, along with fibrin, are the main structural components of a thrombus. In this work, we used a static acid phosphatase detection assay to quantify the amount of platelets attached to each material. There was increased platelet adhesion to GA-PVA compared to STMP-PVA (**Fig 2.3**), in both isolated platelet and platelet rich plasma assays, which supports the platelet reactivity reported in the literature [82,97]. In previous studies glutaraldehyde crosslinked PVA-coated polyethylene (PE) was shown to have higher platelet reactivity and attachment, when compared to the shunt tubing alone in a canine arteriovenous shunt model [94]. Whereas, STMP crosslinked PVA was shown to have reduced platelet attachment when compared to clinical and biological positive controls in a non-human primate arteriovenous shunt model [72,80]. The difference in the animal models of these previous studies is noteworthy. Canine platelets are known to be more reactive to procoagulants than human platelets, which could

account for these differences. Additionally, the differences in platelet adhesion to the crosslinked PVA materials in these studies may be due to how platelet reactivity was measured. In the canine arteriovenous shunt model, platelets were indirectly measured by platelet regeneration as defined by cyclooxygenase activity after a single dose of aspirin. In the baboon model, platelets were radiolabeled, and real-time accumulation was measured using a nuclear imaging camera. However, this current work shows that the PVA crosslinker also has an effect on platelet adhesion.

The work presented here has shown that changes in the bulk PVA hydrogel network can lead to changes in platelet attachment to the surface, specifically the GA-PVA had increased platelet attachment; however, the absence of surface characterization of the crosslinked materials is a limitation of this study. The surface of each material was qualitatively examined using SEM. There were no visible differences in the STMP and GA crosslinked PVA surfaces. The FT-PVA samples did appear to have a more crystalline surface than the STMP-PVA and GA-PVA hydrogel surfaces. The surface morphology of FT-PVA surfaces has been quantified previously using AFM [98]. In these previous studies, hydrogels prepared from 10% PVA, which were subjected to 15 FT cycles, were found to have a roughness average of approximately 80nm and a significant decrease in roughness was observed as the number of FT cycles increased. If data obtained by Pramanick *et al.* were extrapolated to 3 FT cycles, as was examined in the present work, the roughness of the PVA hydrogel surface would exceed a roughness average of 80nm. We quantified the surface roughness of STMP-PVA using AFM and have found that the surface has a roughness average approximately 4.6nm (**Fig 2.5**). The surface of ePTFE has been reported to have a roughness average of approximately 150nm [99]. While these data cannot be statistically compared, taken

together, they suggest that the increase in platelet adhesion to GA-PVA is unlikely due to an increase in surface area due to surface roughness.

2.6 Conclusion

This work supports the hypothesis that the crosslinker in PVA hydrogel networks affects individual components of coagulation *in vitro*. A limitation of this study is that the quantities of FXIIa in solution compared to protein adsorbed onto the material surfaces were not measured directly. Additionally, these studies were all performed *in vitro* in the absence of flow. Future studies should incorporate the crosslinked PVA materials into flow systems to study hemocompatibility. Furthermore, differentially crosslinked PVA materials can be investigated in shunt models or whole blood systems, which are more physiologically representative to predict *in vivo* performance. This work is an important step toward understanding how the design of cardiovascular biomaterials can impact thrombogenicity.

Chapter III: Bioconjugation of a collagen-mimicking peptide onto poly(vinyl alcohol) encourages endothelialization while minimizing thrombosis²

3.1 Abstract

Poly(vinyl alcohol) hydrogel, PVA, is a suitable material for small-diameter vascular grafting. However, the bioinert properties of the material do not allow for in situ endothelialization, which is needed to combat common graft failure mechanisms, such as intimal hyperplasia and thrombosis. In this work, the surface of planar and tubular PVA was covalently modified with a collagen-mimicking peptide, GFPGER. The surface of modified PVA was characterized by measuring contact angle and x-ray photoelectron spectroscopy. Endothelial cell attachment to GFPGER-modified PVA was quantified and qualitatively examined using immunohistochemical staining. Then, *in vitro* hemocompatibility testing was performed by quantifying platelet attachment, coagulation factor XII activation, and initiation of fibrin formation. Finally, an established *ex vivo*, non-human primate model was employed to examine platelet attachment and fibrin formation under non-anticoagulated, whole blood flow conditions. GFPGER-modified PVA supported increased EC attachment. *In vitro* initiation of fibrin formation on the modified

² This work was originally published in *Frontiers: Bioengineering and Biotechnology*. Bates NM, Heidenreich HE, Fallon ME, et al. Bioconjugation of a Collagen-Mimicking Peptide Onto Poly(vinyl alcohol) Encourages Endothelialization While Minimizing Thrombosis. *Front Bioeng Biotechnol.* 2020;8:621768. Published 2020 Dec 18. doi:10.3389/fbioe.2020.621768 © The authors.

material was significantly delayed. *Ex vivo* thrombosis assessment showed a reduction in platelet attachment and fibrin formation on GFPGER-modified PVA. Overall, GFPGER-modified PVA encouraged cell attachment while maintaining the material's hemocompatibility. This work is a significant step toward the development and characterization of a modified-hydrogel surface to improve endothelialization while reducing platelet attachment.

3.2 Introduction

Development of novel, synthetic, small-diameter vascular grafts for treatment of life-threatening cardiovascular diseases has been limited by the challenge of mitigating common failure mechanisms, such as hyperplasia and thrombosis. Currently available and widely used synthetic materials include Dacron® and expanded polytetrafluoroethylene (ePTFE), which are primarily used in bypass surgeries for large diameter (>6mm) vessels [100–102]. Current synthetic materials fail at small diameters, likely due to a mechanical mismatch with native tissues and a lack of an endothelium to promote a healthy, blood-contacting surface. Alternatively, autologous tissues can be used for small-diameter prostheses; however, patients in need of treatment are often critically ill and may not have suitable vessels for this application. Thus, there remains an unmet clinical need for synthetic biomaterials which are hemocompatible and capable of supporting an endothelium.

One synthetic cardiovascular biomaterial under development for vascular tissue engineering applications is poly(vinyl alcohol), PVA, a hydrogel that is amenable to surface modification and has tunable mechanical properties. Previous work thoroughly characterized the mechanical properties of the PVA grafts and found that PVA hydrogels

have an elastic modulus ranging from 250 to 500kPa [73,103], which is similar to the rabbit femoral artery that has a modulus of 230kPa [93]. These hydrogels have a modifiable circumferential compliance, which can mimic native vasculature. PVA compliance ranged between 3–7% [73,103], which is comparable to the rabbit femoral artery of 6% [93]. Despite PVA having suitable mechanical properties for cardiovascular applications, the bioinert surface, critical for preventing thrombosis, also prevents the material from being endothelialized in situ after implantation [79]. The native vascular endothelium regulates thrombosis and prevents intimal hyperplasia by secreting inhibitory factors, such as nitric oxide and prostacyclin [54]. Therefore, a widely accepted approach for attaining long term success of vascular grafts is to improve hemocompatibility of synthetic graft surfaces by encouraging in situ endothelialization. Chemical modification of bioinert devices like PVA hydrogels, can encourage endothelialization.

To generate a biomimetic tissue engineered surface, the addition of extracellular matrix-derived peptides has been shown to increase endothelial cell (EC) attachment. However, a major drawback is that many of these peptides also support unwanted platelet attachment. For decades, work in this area focused on immobilizing peptides based on the Arg–Gly–Asp (RGD) motif in fibronectin; however, there have been mixed reports on platelet adhesion to these sequences [72,80,104–107]. Similarly, the hexapeptide, Gly-Phe-Pro-Gly-Glu-Arg (GFPGER), based on the GFOGER motif within collagen, where the O represents hydroxyproline [108,109], has been studied for its ability to support EC attachment. Both of these sequences specifically recognize $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, which are known to bind ECs and both bind and upregulate platelet activation. The incorporation of GFPGER into materials has been shown to promote cell

growth and, while in solution, the GFPGER sequence bound platelets but did not cause activation of the platelets [110]. The effect on platelet attachment and aggregation due to a covalently linked GFPGER surface is unknown.

Previous work from our group examined the biomimetic effects of mixing extracellular matrix (ECM) proteins and peptides, including GFPGER, into a hydrogel network to enhance EC attachment [80]. We found that the GFPGER peptide minimized platelet attachment *in vitro* when compared to PVA-collagen hydrogels. However, antiplatelet monotherapy was required to minimize platelet adhesion under flow in our established *ex vivo*, non-human primate, whole blood, shunt model. This was likely due to the random presentation of amino acids on the hydrogel surface. The possibility of the binding domain being buried within the hydrogel network also suggested the need for a more specific and well-controlled surface binding approach. We hypothesized that covalent attachment of GFPGER to the surface of PVA hydrogels would encourage endothelialization of the material, while mitigating thrombotic responses. The current work presents a systematic examination of this biomimetic tissue-engineered surface for cell attachment and thrombotic potential of PVA hydrogels with GFPGER at various concentrations conjugated to the surface using a 1,1'-carbonyldiimidazole (CDI) linker. This work is a significant step toward the development of hemocompatible off-the-shelf devices for the treatment of cardiovascular disease.

3.3 Materials and Methods

3.3.1 PVA film and tube manufacturing

Planar PVA films and tubes with an inner diameter (ID) of 4mm were manufactured, as previously described [73,111]. In brief, PVA films were manufactured by adding 15% sodium trimetaphosphate (STMP, Sigma-Aldrich, St. Louis, MO) to aqueous 10% PVA (Sigma-Aldrich) followed by 30% sodium hydroxide. The solution was cast into tissue culture-treated well plates at a 1.5:1 volume to surface area ratio ($\mu\text{L}/\text{mm}^2$). Films were left to crosslink in a sterile incubator at room temperature with 95% humidity followed by drying in a biosafety cabinet. Films were rehydrated under sterile conditions in 10X phosphate buffered saline (PBS, Fisher Scientific, Waltham, MA) followed by 1X PBS and subsequent deionized water. Prior to modification, samples were dried in an oven overnight. PVA films were then covalently modified with GFPGER (Sigma-Aldrich) peptides. The surface of the PVA films was activated using 100mg/mL CDI (Sigma-Aldrich) in dimethyl sulfoxide (DMSO, Sigma-Aldrich) for 1hr then briefly rinsed three times with 1X PBS before adding GFPGER (0, 15, 30, 60, and 120 $\mu\text{g}/\text{mL}$) in PBS solutions at 37°C overnight (GFPGER₀, GFPGER₃₀, GFPGER₆₀, and GFPGER₁₂₀, respectively). Modified films were rinsed with 1X PBS three times before use.

For the fabrication of tubular PVA samples, a cylindrical mold with an outer diameter of 3.75mm was coated with a thin PDMS film (Dow Corning, Midland, MI). The mold was treated with air plasma and then was immediately immersed in a solution of crosslinking PVA. Dip casting was performed repeatedly for 12 dips, with a drying duration of 15–30min between each dip. The tubes were then cured at 18–20°C for 3 days before rehydration in PBS and DI water, as described above, then thoroughly

rinsed in DI water overnight. For GFPGER modification, rehydrated and dried PVA tubes were immersed in a 100mg/mL CDI solution followed by 0, 15, or 120 μ g/mL GFPGER solution, as described for the films. Samples were briefly rinsed three times with PBS in between immersions. PVA films were used for surface characterization, cell attachment, and *in vitro* platelet attachment and fibrin formation assays. Tubular samples were used for *ex vivo* thrombosis testing in whole blood.

3.3.2 Surface characterization

3.3.2.1 Quantification of conjugated GFPGER peptide on the surface of PVA

PVA films in a 96-well plate were modified with GFPGER as described above (GFPGER₀, GFPGER₃₀, GFPGER₆₀, and GFPGER₁₂₀) with the inclusion of a five percent supplementation of each peptide concentration with fluorescent labeled GFPGER (GFPGER{Lys(5-FAM)}, GenScript USA, Piscataway, NJ). The modified films were rinsed with 1X PBS three times before being placed in an Infinite M200 spectrophotometer (Tecan, Männedorf, Switzerland). Fluorescent peptides conjugated to the surface of the PVA films were excited at 488nm and emission was read at 525nm. A standard curve of 5% fluorescent-labeled GFPGER at a range of peptide concentrations was used to quantify the peptide on the surface.

3.3.2.2 Captive bubble contact angle

Static contact angle was measured by using a captive bubble method with an optical contact angle system (OCA20, Future Digital Scientific Corp). GFPGER-modified samples were immersed in DI water, and an air bubble (4 μ L) was injected into the water

with a syringe. The bubble was allowed to attach to the surface of the samples and imaged by a camera. The contact angle on each surface was then calculated using SCA20 software (DataPhysics Instruments USA Corp., Charlotte, NC).

3.3.2.3 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) was used to obtain the elemental composition on the surfaces of the PVA samples. XPS survey spectra were measured using a VG ESCALab 250 with a monochromatic Al K-alpha X-ray source (1486.6eV), and high resolution spectra of C1s were obtained using a standard magnesium X-ray source (1253.6eV) at Waterloo Advanced Technology Laboratory (University of Waterloo, Waterloo, ON).

3.3.3 Endothelialization of surfaces

3.3.3.1 Cell isolation

Carotid arteries were harvested from baboons, and primary endothelial cells (ECs) were isolated, as previously described [112]. Artery lumens were filled and incubated for 5min with 600U/mL collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) then massaged and flushed into collagen type I (Corning Inc., Corning, NY)-coated tissue culture treated well plates containing Vasculife basal medium (Lifeline Cell Technology, Frederick, MD) supplemented with 18% fetal bovine serum (FBS, Hyclone, Logan, UT). Cell cultures were grown to confluence before sorting with Dynabeads (Invitrogen, Carlsbad, CA) for CD31+ cells per the manufacturer's protocol. Sorted cells were expanded then stored in freezing media (50% Vasculife basal medium,

40% FBS, and 10% DMSO). Cells thawed for experimentation were maintained in 10% FBS Vasculife basal medium and used at the fourth or fifth passage.

3.3.3.2 Cell seeding and quantification

Rehydrated peptide-modified PVA films were incubated in FBS for 1hr at 37°C before seeding ECs at a density of 0.2M cells/mL in 10% FBS Vasculife basal medium. For cell quantification, cells were cultured for 48hrs, rinsed, and frozen dry at -20°C before further lysing with 0.02% sodium dodecyl sulfate in sodium citrate buffer. DNA in the lysate was quantified using a PicoGreen assay per the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were similarly seeded onto tissue culture treated plastic wells to serve as a positive control to the PVA films. Treatment group cell quantities were calculated as a percent confluence from this control.

3.3.3.3 Immunostaining

Cells were incubated on PVA samples for 48hrs then stained, as previously described [81]. In brief, cells adhered to samples were fixed using 3.7% paraformaldehyde then stained for VE-cadherin (primary antibody, Santa Cruz Biotechnology, mouse IgG1 monoclonal, 1:100, 1hr and IgG1 Alexa-488 as a secondary (Invitrogen, goat polyclonal, 1:500, 30min)), and DAPI (Invitrogen, 1:10000, 5min) as a nuclear stain. Samples were imaged with a Zeiss LSM 880 inverted confocal microscope system. Low-magnification immunofluorescent (IF) images were collected using a 10X PlanApo objective NA = 0.45. Z-stacks are presented in figures as maximum intensity Z-projections and post-processing of all images was performed using FIJI (SciJava) [113,114].

3.3.4 Thrombogenicity of surfaces

3.3.4.1 *Washed platelet and platelet-rich plasma preparation*

Human venous whole blood was drawn from healthy donors into sodium citrate, as previously described [88]. In brief, whole blood was centrifuged at 200g for 20min to obtain platelet-rich plasma (PRP). In select studies, purified platelets were isolated from PRP by further centrifugation at 1000g for 10min in the presence of 0.1 μ g/mL prostacyclin. The pellet was resuspended in HEPES/Tyrode's buffer (129mM NaCl, 0.34mM Na₂HPO₄, 2.9mM KCl, 12mM NaHCO₃, 20mM HEPES, 5mM glucose, 1mM MgCl₂; pH 7.3) containing 0.1 μ g/mL prostacyclin. The platelets were washed once via centrifugation (1000g for 10min) and resuspended in HEPES-Tyrode's buffer.

3.3.4.2 *Static platelet adhesion quantification*

Static platelet adhesion was quantified using a method modified from Vaníčková et al. to incorporate PVA films [89]. Purified washed platelets suspensions (5.0×10^8 platelets/mL) were incubated on peptide-modified PVA films, in the absence of ECs, in a microtiter plate for 1hr at room temperature. Samples were then rinsed three times with PBS to remove all non-adherent platelets. The amount of platelets attached to the surface of each material was quantified by measuring platelet acid phosphatase activity using a calibration curve of platelet solutions ($0-5.0 \times 10^8$ platelets/mL).

3.3.4.3 *Coagulation quantification*

Pooled platelet-poor plasma (PPP, ISTH SSC Lot 4) was incubated with peptide-modified PVA, in the absence of ECs, films followed by the addition of CaCl₂ (8mM). Absorbance at 405nm was documented over time. Fibrin generation lag times were

defined by a 5% increase over baseline absorbance. Rates of fibrin generation were defined by maximum slopes.

Activation of coagulation factor XII (FXII, 200nM, Haematologic Technologies, Inc., Essex Junction, VT, USA) in the presence of coagulation enzymes generated on GFPGER-modified samples, in the absence of ECs, was measured in a purified system using a chromogenic substrate, Spectrozyme-FXIIa (American Diagnostica, Inc., Stamford, CT, USA), as previously described [69].

3.3.4.4 Whole blood platelet and fibrin adhesion under flow quantification

Rehydrated GFPGER-modified tubes, in the absence of ECs, (prepared from 15 or 120µg/mL GFPGER solutions) were incorporated into a chronic, femoral arteriovenous shunt via connective silicone tubing in an established non-anticoagulated, non-human primate model [72,80]. Juvenile male baboons (9-12kg) underwent a minor surgical procedure, cannulation of the superficial femoral artery and vein, for the placement of a chronic arteriovenous (AV) shunt. The AV shunts were composed of implanted sterilized silicone rubber tubing (3.0mm ID, SIL-TEC, Technical Products, Inc, Lawrenceville, GA). Single devices were connected to the implant with Silastic tubing (4mm ID, Dow Chemical, Midland, MI) (**Supplemental Fig 3.1**). Upstream of the device was a flow probe (Transonic, Ithaca, NY) to measure blood velocity, and downstream of the device was a tubing clamp to control the flow at a steady rate. Autologous platelets and homologous fibrin were labelled with indium-111 (¹¹¹In) and iodine-125 (¹²⁵I), respectively. Samples were subjected to whole blood flow at 100mL/min and platelet accumulation onto the samples was quantified in real-time by measurement of ¹¹¹In radiation using a Brivo NM615 nuclear imaging camera (General Electric, Boston, MA).

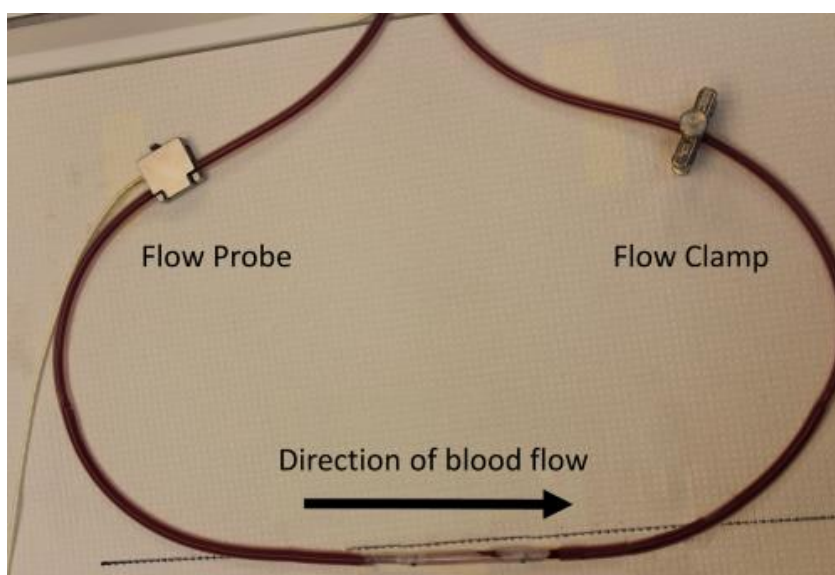


Figure 3.1: Shunt study set up

A shunt was placed between the femoral artery and the femoral vein of a non-human primate (not pictured) using Silastic tubing. The sample to be tested was placed between the tubing using connectors, such that blood flow was continuous through the *ex vivo* shunt loop. Whole blood was used in this study without the presence of anticoagulant or antiplatelet therapies. Flow rate was controlled at 100mL/min using the downstream flow clamp and measured using the upstream flow probe. This figure has been modified to remove all informal labeling using Photoshop.

The amount of fibrin was quantified as an end-point measurement of ^{125}I radiation approximately 30 days post experiment, once the ^{111}In decayed, using a 1480 Wizard gamma counter (PerkinElmer, Waltham, MA). CDI-active PVA without peptide (GFPGER₀) and plain PVA were used as negative controls, collagen-coated expanded polytetrafluoroethylene (Collagen, 1mg/mL equine collagen type I, Chrono-log Corp.) was used as a positive control and clinical grade ePTFE (IMPRA®, Bard Peripheral Vascular, Inc., Tempe, AZ) with an ID of 4mm was used as a clinical control.

After whole blood testing, the tubes were rinsed with PBS and fixed with 3.7% paraformaldehyde (PFA) for 48-72hrs before additional rinsing and storage. Some of the samples used in the shunt studies were examined with micro-computed tomography (MicroCT). The samples were prepared as described previously [115]. Briefly, PVA samples were filled with Microfil® polymer to render the lumens radiopaque. Since the Microfil® polymer is incompatible with ePTFE, ePTFE samples were soaked in Lugol's (Sigma-Aldrich) solution to render the thrombus radiopaque.

Shunt studies were performed in a male Papio anubus baboon (10.5kg), which was under the care of the Oregon National Primate Research Center (ONPRC) staff. All studies abided by guidelines provided by the National Research Council and the Committee on Care & Use of Laboratory Animals of the Institute of Laboratory Animal Resources. All studies were approved by the ONPRC Institutional Animal Care and Use Committee.

3.3.4.5 Micro-computed tomography analysis

MicroCT (Inveon, Siemens) imaging was used to analyze thrombus area over the length of the samples (volume) in the small-diameter vascular grafts formed under flow by whole blood in *ex vivo* shunts, as described previously [115]. Imaging was performed with 2x2 binning, 220 projections, at an exposure of 660 ms/projection. Thrombus physical characteristics were determined with the Amira® software package (FEI, version 5.2.2) by a trained operator blinded to specific sample treatments. The software also generated 3-dimensional (3D) volume renderings of either the thrombus for ePTFE grafts or the lumen for collagen-coated and PVA grafts. The software-calculated volumes and lengths of each sample was used for subsequent analysis. Amira® was utilized, as previously described [115], to segment specific regions of interest (ROIs) in

each cross-sectional image. For PVA samples, the ROI was the luminal area of the graft. ePTFE samples were segmented for the thrombi as the ROI, and collagen-coated ePTFE samples contained ROIs of both the luminal and thrombus areas. 3D representations of either the lumen (PVA and collagen-coated ePTFE) or thrombus (ePTFE) of the grafts were generated from ROI summations, and the surface areas of each cross-sectional slice were subsequently measured. These cross-sectional data were then utilized in analyzing average thrombus generation over the entire graft surface. The cross-sectional areas obtained from each slice were used to generate topography maps of the thrombus surface for each individual sample. This allows for characterization of the thrombus surface and quantification of the intrasample variability of the thrombus over the length of a single sample.

3.3.5 Statistical Methods

Data are presented as mean $\pm$ standard deviation (SD) for all studies. Probability values of $p < 0.05$ were considered to be statistically significant. To determine statistical significance of captive bubble contact angle ($n=3$), XPS ($n=3$), peptide quantification ($n=5$), lag time and rate of fibrin generation ($n=3$), static *in vitro* platelet adhesion ($n=3$), and *ex vivo* end-point fibrin accumulation data ($n=6$), one-way analysis of variance (ANOVA) tests were performed. To determine statistical significance for *ex vivo* platelet data, a repeated measures ANOVA was performed on surface modified PVA samples ($n=6$). Platelet reactivity of collagen-ePTFE, ePTFE, and unmodified PVA controls samples ($n=2$) were confirmed and compared to historic data, but these controls were not included in the statistical analysis. All studies were tested for ANOVA assumptions using Levene's and qqplots and, when necessary, data were natural log transformed. A

subset of the *ex vivo* samples were tested with microCT (n=4 for GFPGER₀, GFPGER₁₅, and GFPGER₁₂₀) using a one-way ANOVA between the average luminal cross-sectional area (volume/length) for each test group. SDs of the volume data generated over the entire graft length for each single sample (generated from per slice area data) were also compared with a one-way ANOVA to determine if intrasample variability was significantly different between test groups.

3.4 Results

3.4.1 Surface characterization

3.4.1.1 *Peptide quantification*

GFPGER peptides were supplemented with 5% fluorescent-labeled peptide to quantify the amount of GFPGER immobilized onto the surface of PVA at each concentration. A significant increase in peptide quantity was found on GFPGER₃₀, GFPGER₆₀, and GFPGER₁₂₀ when compared to unmodified samples (**Fig 3.2**). Additionally, GFPGER₆₀ and GFPGER₁₂₀ were significantly increased from GFPGER₁₅, and GFPGER₁₂₀ was significantly increased from GFPGER₃₀.

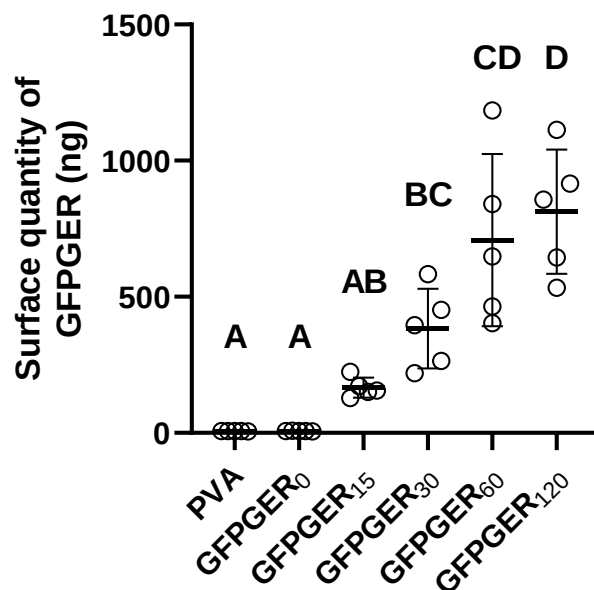


Figure 3.2: Quantification of GFPGER peptide on the surface of modified PVA samples.

The quantity of immobilized GFPGER peptide on the surface of each PVA sample was determined by fluorescent peptide supplementation and is shown as mean $\pm$ SD (n=5). Statistical analysis was performed using a one-way ANOVA test with Tukey's HSD post hoc testing. Groups which do not share a letter indicate statistical significance ($p < 0.05$).

3.4.1.2 Contact angle

Contact angle did not exhibit a significant difference among all test groups (**Fig 3.3**). The surfaces of PVA and GFPGER modified PVA samples all remained hydrophilic, with contact angles ranging from 44.8° to 51.7°.

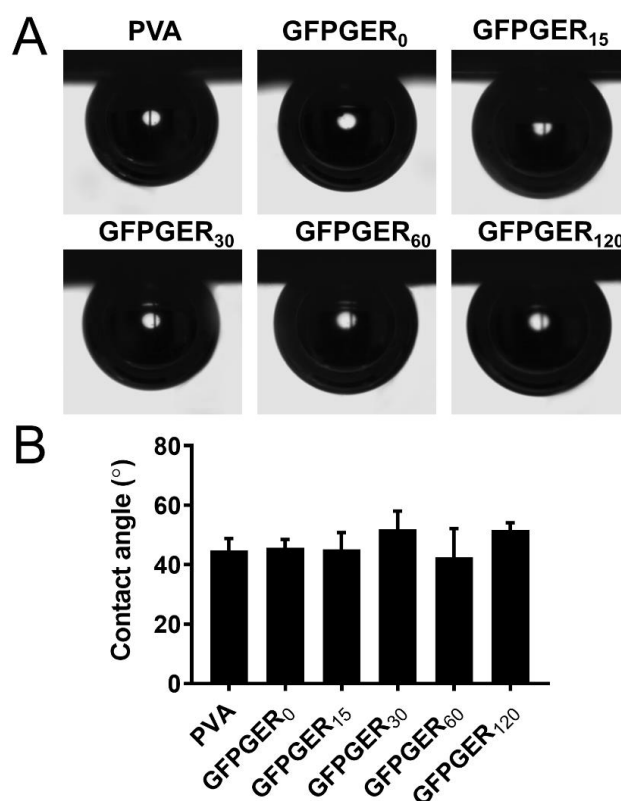


Figure 3.3: Contact angle of PVA films modified with different concentrations of GFPGER.

The contact angle was measured using a captive bubble method. Representative images (A) and contact angle data (n=6) are shown as mean $\pm$ SD (B). Statistical analysis was performed using a one-way ANOVA test. No significant difference was observed among all groups.

3.4.1.3 XPS

XPS survey spectra of PVA and GFPGER modified PVA are shown in **Fig 3.4A**. GFPGER and CDI contain nitrogen and, thus, all modified PVA groups showed significant N1s peaks. **Table 3.1** and **Fig 3.5** showed the atomic percentage of C, O, and N on different samples. GFPGER₁₂₀ had significantly higher C% than GFPGER₃₀ and GFPGER₀, and significantly lower O% than all the other groups. GFPGER modified PVA samples had significantly higher N% than GFPGER₀.

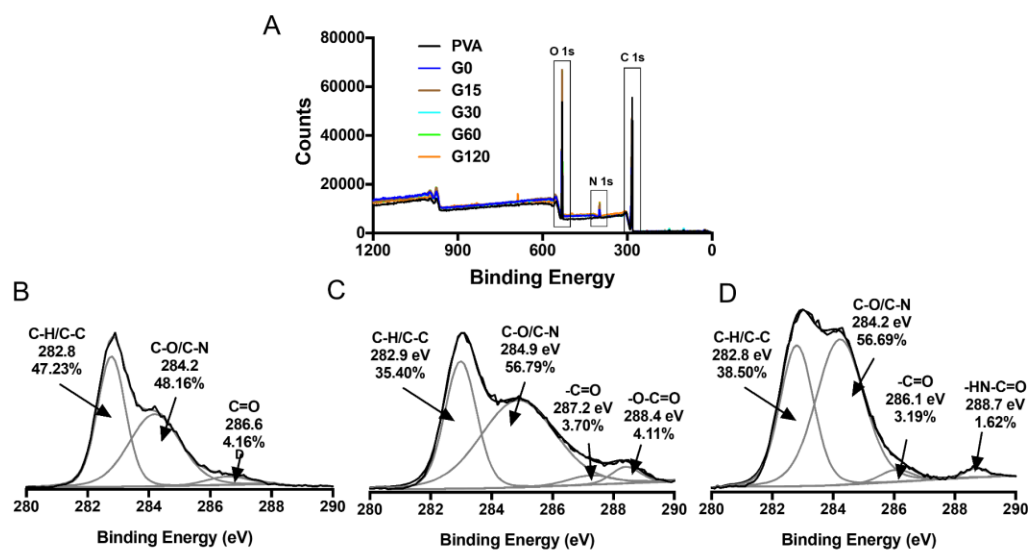


Figure 3.4: (A) Survey XPS spectra of PVA and all GFPGER-modified PVA samples.

High resolution of carbon on plain PVA (B), GFPGER₀ (C), and GFPGER₁₂₀ (D) samples.

Fig 3.4(B-D) shows the high-resolution spectra of carbon on PVA, GFPGER₀, and GFPGER₁₂₀ samples. High-resolution spectrum of carbon on PVA showed characteristic peaks of the PVA structure at 282eV, 284eV, and 286eV from

Table 3.1: XPS results of PVA films modified with different concentration of GFPGER.

Atomic percentage of carbon, nitrogen, and oxygen. Data (n=3) are shown as mean $\pm$ SD (N.D.= not detected).

Atomic %	C	O	N
PVA	76.8 $\pm$ 2.0	23.1 $\pm$ 2.0	N.D.
GFPGER ₀	73.9 $\pm$ 1.9	22.7 $\pm$ 2.1	3.3 $\pm$ 0.3
GFPGER ₁₅	71.4 $\pm$ 1.0	23.9 $\pm$ 0.8	4.6 $\pm$ 0.2
GFPGER ₃₀	74.0 $\pm$ 1.6	21.8 $\pm$ 1.6	4.11 $\pm$ 0.1
GFPGER ₆₀	72.2 $\pm$ 1.7	23.2 $\pm$ 1.9	4.6 $\pm$ 0.3
GFPGER ₁₂₀	78.6 $\pm$ 2.2	17.3 $\pm$ 1.9	4.17 $\pm$ 0.3

C-C/C-H, C-O, and C=O, respectively. In addition to the characteristic PVA peaks, GFPGER₀ also showed a peak at 288eV from the -O-C=O bond in the imidazole carbamate intermediate groups. GFPGER contains NH-C(=O) groups, and the peak at 288.7eV observed from GFPGER₁₂₀ samples further confirmed the presence of GFPGER on the samples.

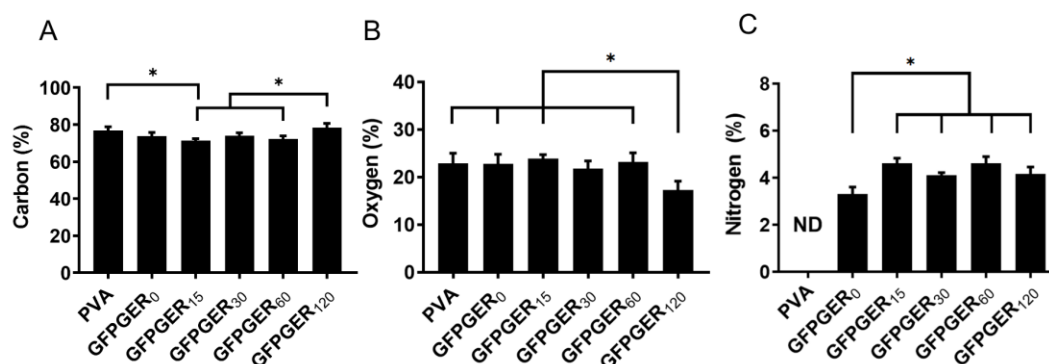


Figure 3.5: XPS measured atomic percentages of PVA films modified with different concentrations of GFPGER.

XPS was used to measure the percentage of carbon, oxygen, and nitrogen on GFPGER-modified surfaces. Atomic percentages (n=3) are shown as mean $\pm$ SD for all samples for carbon (A), oxygen (B), and nitrogen (C). Statistical analysis was performed using a one-way ANOVA with Tukey's HSD post hoc testing. * indicates statistical significance ($p < 0.05$). ND = not detected.

3.4.2 Endothelialization of Surfaces

We examined the ability of PVA to attach ECs after the hydrogel was modified with GFPGER peptide. ECs that adhered to samples were quantified using PicoGreen® as a percentage of cells adhered to tissue culture plastic and observed qualitatively using fluorescent staining. Only the GFPGER-modified samples (15, 60, and 120 $\mu\text{g/mL}$) showed a significant increase in EC attachment when compared to GFPGER₀ (**Fig 3.6A**). These results were also reflected qualitatively in the obtained stained images (**Fig**

3.6B). GFPGER₁₂₀ showed particularly robust VE-cadherin staining suggestive of strong cell-cell interactions. Adherent ECs were not detected nor observed on plain PVA samples, similar to results from our work [80,116].

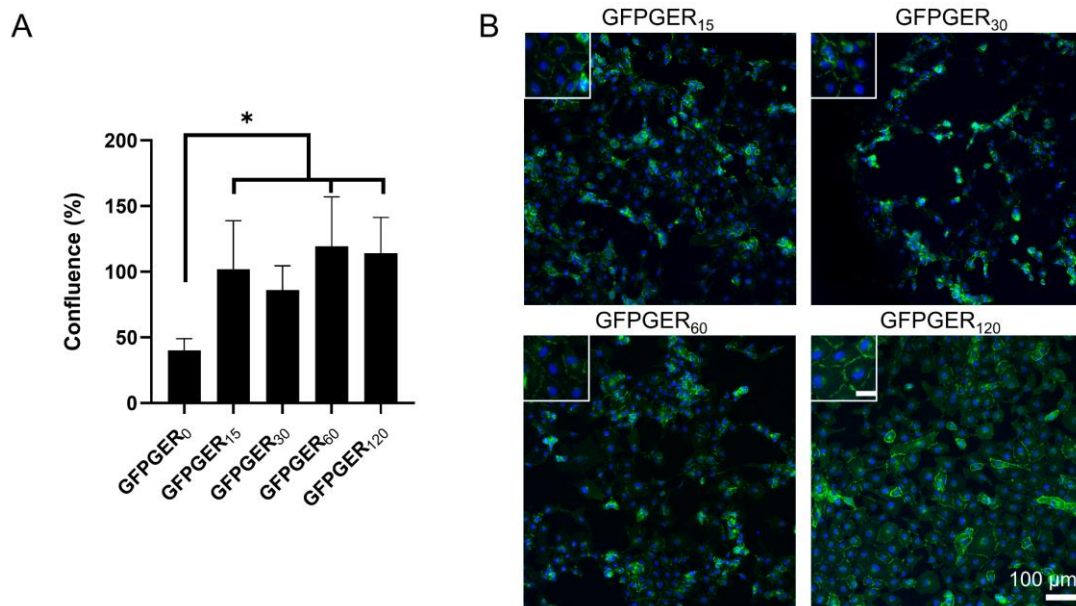


Figure 3.6: EC quantification and visualization after 48hr on GFPGER-conjugated PVA.

Cell attachment increased on modified PVA compared to unmodified PVA (A). Data (n=4-6) are presented as a percent increase from ECs adherent on a tissue culture plastic (TCP) control, where ECs were 100% confluent. Statistical analysis was performed using a one-way ANOVA with Tukey's HSD *post hoc* testing. * indicates statistical significance (p<0.05). Representative staining of baboon carotid ECs on GFPGER-conjugated PVA films after 48hr showed adherent ECs on all modified PVA samples, while no cells were observed on unmodified PVA (B). Green=VE-cadherin (primary antibody and IgG1 Alexa-488 as a secondary) and blue=DAPI as a nuclear stain. Inset scale bar=30μm.

3.4.3 Thrombogenicity of Surfaces

3.4.3.1 Static *in vitro* platelet adhesion quantification

We studied the extent to which peptide-modified PVA samples supported static platelet adhesion using a solution of purified human platelets *in vitro*. There were no statistical differences found (Fig 3.7).

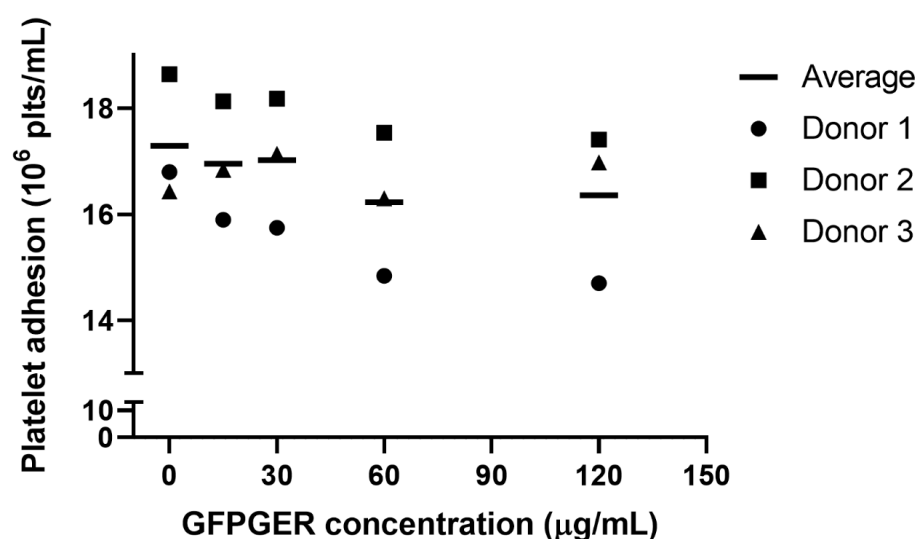


Figure 3.7: Quantification of static platelet adhesion of purified platelets onto GFPGER-conjugated PVA samples.

Platelet adhesion after 1hr, under static, purified conditions, was measured on the surface of GFPGER-modified PVA samples. Data (n=3) are shown as individual biological replicates with means overlaid. Statistical analysis was performed using a one-way ANOVA test. No significant difference was observed among all groups.

3.4.3.2 Fibrin clotting time, rate of fibrin generation in vitro, and FXII activation

We quantified the time to fibrin clot formation in PPP and the rate of fibrin formation on peptide-modified PVA samples. GFPGER-modified samples all had prolonged times to initiation of fibrin generation when compared to CDI only-modified samples (**Fig 3.8A**). There were no significant differences observed in the rate of fibrin generation in the presence of GFPGER-modified PVA films when compared to the baseline (**Fig 3.8B**).

To determine if the contact pathway was playing a role in the difference of time to initiation of fibrin formation, activation of FXII was measured (**Fig 3.8C**). There were no significant differences observed between unmodified and GFPGER-modified PVA samples.

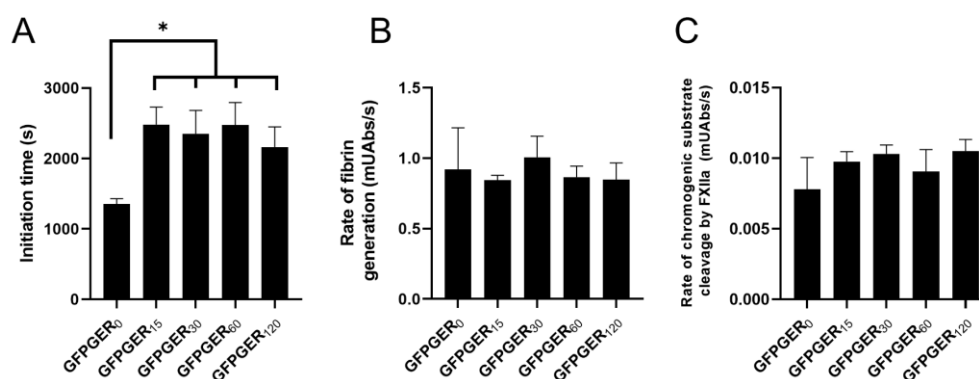


Figure 3.8: Quantification of *in vitro* coagulation on GFPGER-conjugated PVA samples.

Coagulation potential of GFPGER-modified samples was determined by measuring fibrin initiation time, rate of fibrin generation, and activity of coagulation factor XII from static coagulation assays. Fibrin initiation lag times in platelet-poor-plasma in the presence of GFPGER-conjugated PVA samples showed prolonged initiation times on GFPGER-modified samples (A). No difference in rate of fibrin generation on GFPGER-conjugated PVA samples was measured (B). There was no difference in FXIIa produced by GFPGER-conjugated PVA samples, shown as rate of cleavage of a chromogenic substrate, when compared to unmodified (C). Data (n=3-6) are shown as mean $\pm$ SD. Statistical analysis was performed using a one-way ANOVA with Tukey's HSD post hoc testing. * indicates statistical significance (p<0.01).

3.4.3.3 Whole blood platelet and fibrin adhesion under flow quantification

We then examined platelet and fibrin deposition onto GFPGER-modified tubes from whole, flowing blood with *ex vivo* thrombosis testing. We observed a significant decrease in platelet attachment onto GFPGER-modified samples when compared to the CDI only-modified samples for both concentrations of tested conjugated peptide, low (15 μ g/mL) and high (120 μ g/mL) (**Fig 3.9A**). Unmodified PVA samples were also quite low compared to the positive and clinical controls and consistent with both

concentrations of GFPGER modifications. We observed a similar significant decrease in fibrin formation on GFPGER₁₅ and GFPGER₁₂₀ when compared to the CDI-only samples (Fig 3.9B).

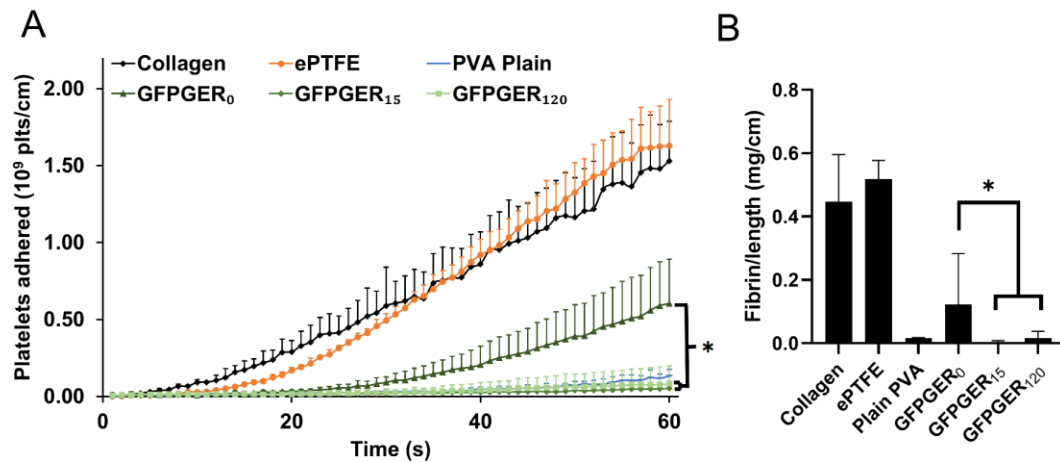


Figure 3.9 Whole blood, ex vivo shunt testing.

Tubular GFPGER-modified PVA (prepared from 15 or 120 $\mu\text{g/mL}$ GFPGER solutions, $n=6$) and unmodified but CDI-active PVA ($n=6$) were tested in an ex vivo shunt model. Collagen-coated ePTFE ($n=2$), ePTFE ($n=2$), and plain PVA ($n=2$) were run to compare to historical control data. (A) Platelet accumulation was quantified over 60min exposure to whole blood. Data are shown as mean $\pm$ range or SD. Statistical analysis was performed only on the modified PVA samples using a repeated measures ANOVA with Tukey's HSD *post hoc* testing. GFPGER₀ was found to be significant from GFPGER₁₅ ($p<0.001$) and from GFPGER₁₂₀ ($p=0.001$), while GFPGER₁₅ was not significant from GFPGER₁₂₀. (B) Fibrin data were quantified and normalized per centimeter of axial length for all grafts at 60min. Data ($n=2-6$) are shown as mean $\pm$ range/SD. Statistical analysis was performed using a one-way ANOVA with Tukey's HSD *post hoc* testing, * $p<0.001$.

3.4.3.4 MicroCT

Three-dimensional renderings of either the lumen (PVA and collagen-coated ePTFE) or thrombus (ePTFE) area along the length of the samples (volume) were generated based on cross-sectional ROI segmentation of each graft type (**Fig 3.10**). The measurement of thrombus area along the length of each individual graft as a percentage of the maximal luminal area was evaluated and representative samples are shown in **Fig 3.11**. This representative GFPGER₀ sample (**Fig 3.10A**) had an average thrombus area percentage of $12.10 \pm 3.99\%$ of the maximal luminal area, the representative GFPGER₁₅ sample (**Fig 3.11B**) had an overall thrombus area percentage of $3.66 \pm 1.11\%$, and the representative GFPGER₁₂₀ sample (**Fig 3.11C**) had an overall thrombus area of $2.68 \pm 1.41\%$. By capturing the area per sample slice, this method of analysis allows for the study of variability of an individual thrombus. The intrasample variability was quantified as the standard deviation of the individual thrombus area as a percent of the maximal luminal area of each sample type. These standard deviations were averaged and compared between all the PVA samples (n=4) resulting in variabilities of GFPGER₀ ($4.17 \pm 0.87\%$), GFPGER₁₅ ($3.10 \pm 2.84\%$), and GFPGER₁₂₀ ($4.26 \pm 2.39\%$). These variability data were not statistically significant. The average Amira® luminal area for each type of PVA modifications (n=4) were also compared and showed no statistical significance between the three GFPGER-modified groups (**Fig 3.12**).

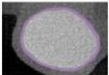
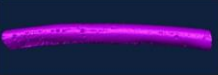
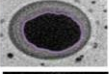
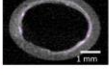
Proximal	MicroCT Cross-Section	Side View of Reconstructed Image	Type	Treatment	Selection
			PVA	Plain	Lumen
			PVA	GFPGER ₀	Lumen
			PVA	GFPGER ₁₅	Lumen
			PVA	GFPGER ₁₂₀	Lumen
			ePTFE	Collagen	Lumen
			ePTFE	-	Thrombus

Figure 3.10: Three-dimensional surface volume renderings of microCT cross-sectional ROI selections using Amira® showing thrombi in the lumen of tested samples.

All grafts from *ex vivo* whole blood testing were 4-5mm ID. PVA and ePTFE samples were 4cm long and collagen-coated ePTFE samples were 2cm long. ROI selection from a blinded, trained user is shown in purple, designating the user-selected masking range. Scale bar for microCT cross-sectional images and volume renderings are 1mm and 1cm, respectively.

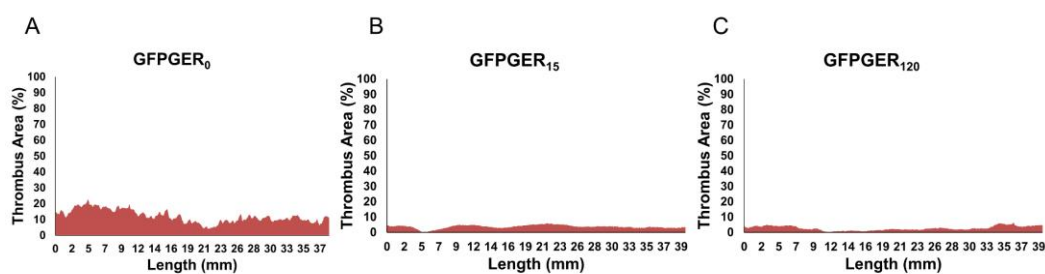


Figure 3.11: Quantification of the cross-sectional thrombus area as a percentage of the maximal luminal area per image slice over the entire graft length for a representative sample of GFPGER₀ (A), GFPGER₁₅ (B), and GFPGER₁₂₀ (C).

Samples illustrate the variability seen in thrombus over the length of a single graft after 1hr of whole, flowing blood. Samples conjugated with GFPGER (B and C) had less thrombus and less observed variability than the control sample with only a vehicle control (A).

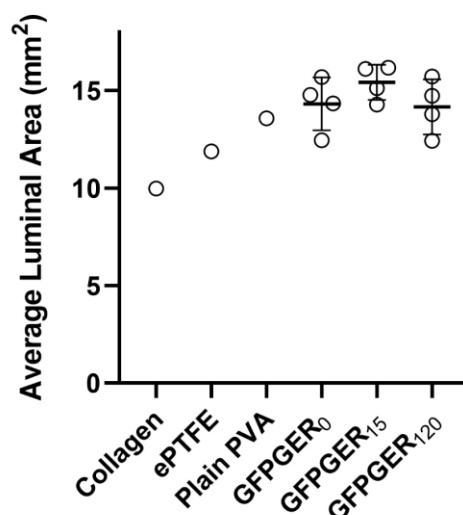


Figure 3.12: Average luminal area of grafts after exposure to whole flowing blood in the *ex vivo* shunt.

Collagen (n=1), ePTFE (n=1), plain PVA (n=1), GFPGER₀, GFPGER₁₅, and GFPGER₁₂₀ (all covalently-modified GFPGER PVA samples were n=4) grafts were analyzed for the average cross-sectional area per slice of the lumen. Data are presented as the mean $\pm$ SD of the average luminal area. Statistical analysis was performed only on experimental (GFPGER) samples using a one-way ANOVA. No statistical differences were observed.

3.5 Discussion

Successful integration of synthetic small-diameter vascular grafts *in vivo* is impeded by a lack of in situ endothelialization and material-vessel compliance mismatch leading to neointimal hyperplasia and thrombosis and potential loss of patency. Studies of compliant PVA biomaterials suggest that the low thrombogenicity of the material makes it a promising vascular graft material [72,75,80,92,117] However, in the absence

of surface modifications, PVA is unable to support EC attachment and growth. We hypothesized that the covalent attachment of a collagen-mimicking peptide to PVA would enhance EC adhesion, without compromising hemocompatibility of the material. Thus, we modified PVA hydrogel surfaces using CDI chemistry with the collagen-mimetic peptide, GFPGER, a low-cost, commercially available peptide, which specifically recognizes $\alpha1\beta1$ and $\alpha2\beta1$ integrins. We performed characterization of this modified surface as well as thorough *in vitro* and *ex vivo* hemocompatibility testing to assess the thrombotic potential of the material.

Using CDI as a crosslinker enabled a concentration dependent increase in GFPGER covalently bound to the PVA surface. Upon examination of the surface chemistries present on the modified hydrogels, we found an increase in C% and N%, which indicated the introduction of amine groups onto the hydrogel, while the contact angle remained unchanged when compared to unmodified PVA. CDI has been used as a chemical crosslinker to modify the surface of polymers and hydrogels, including PVA, with full length proteins [92,117,118]. CDI reacts with hydroxyl groups to form highly reactive, anchoring carbamate groups, which are then available to react with proteins or peptides for immobilization. Nuttelman et al. have previously used CDI to covalently attach fibronectin, an ECM protein found to play a role in cell adhesion and proliferation, to PVA [118]. In their work, an 11-carbon spacer, bromoundecanoic acid, was added to the PVA hydrogel in an intermediate step before CDI activation, followed by the addition of fibronectin. Using this chemical scheme, they found that fibroblast cell attachment, proliferation, and migration were all significantly increased on fibronectin-conjugated PVA when compared to the unmodified material. However, the additional acid wash step is cumbersome and unnecessary, as shown by Rizwan *et al.*, in which CDI was used to

covalently attach gelatin, another cell-adhesive protein, directly to PVA [117]. In this prior study, CDI alone was used to aminate the surface of PVA before protein modification and the presence of gelatin on the PVA surface after the covalent modification was confirmed using Fourier transform infrared spectroscopy and XPS. Their surface characterization results showed a significant increase in nitrogen on the surface, which, like the results in the current study, indicate the presence of amine groups on the material surface. While some peptides, such as REDV [119], require spacers in order to retain their bioactivity, we successfully grafted GFPGER to PVA using CDI and the peptide remained active. Additionally, it is unlikely that using CDI chemistry to immobilize peptides to the surface of PVA would alter the mechanical properties of the material as was shown by previous work from our group [117], whereas there is more potential for physical alteration of the material when mixing peptides into the hydrogel network. To our knowledge, the work presented here is the first time that CDI has been used to covalently attach an ECM-mimicking peptide to the surface of PVA.

In our cell studies, we found that GFPGER-conjugated PVA supported the formation of an endothelial layer. This result is similar to cell attachment results obtained in our previous GFPGER-mixed PVA studies, where we found greater cell adhesion to GFPGER-mixed PVA samples when compared to PVA modified with collagen and other proteins and peptides [80]. In the current study, EC adhesion to most concentrations of GFPGER-modified PVA was significantly increased when compared to the CDI-only PVA baseline (GFPGER₀), and a confluent layer of ECs was seen at the lowest and highest concentrations of GFPGER-conjugated samples. Despite the surface characterization which suggested that the GFPGER had not saturated the surface at the lower concentrations, it was interesting to see that all the examined concentrations had

substantially increased cell adhesion that was not significantly different from other GFPGER concentrations. Plain PVA is biologically inert and does not support cell attachment alone [111,117]. The EC attachment attained is likely through the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, which are well known to interact with GFPGER [108]. This cell attachment is in agreement with the work of several groups, including ours [80,110,117]. GFPGER has been shown to support cell attachment and spreading on several surfaces, including a collagen-mimetic protein with GFPGER incorporated, Scl2-GFPGER, coated onto tissue culture plastic [108] and a Scl2-GFPGER incorporated PEG hydrogel [120]. However, we were able to successfully conjugate GFPGER alone directly to a hydrogel surface via a simple chemical scheme, eliminating the need for site-directed mutagenesis or in-house protein synthesis.

ECs have been shown to have a distinct cobblestone appearance when cultured *in vitro* [121]. Despite being cultured under identical conditions, the morphology of ECs on the different densities of GFPGER-conjugated PVA varied. ECs on GFPGER₁₅ were less spread and dominated by their nucleus whereas cells on GFPGER₁₂₀ appear to have spread more with a larger cytoplasm and the characteristic VE-cadherin expression of *in vitro* cultured confluent ECs. The difference in peptide density was also demonstrated by the distinct cell wall borders observed around cells on hydrogels with the highest concentration of GFPGER. The increase in confluency and altered morphology on GFPGER₁₂₀ indicates that higher peptide concentrations may lead to phenotypic alteration of ECs. Hydrogels with a higher density of peptide have more binding sites for EC integrins, which encourages EC spreading to a greater degree than lower densities of EC adhesive peptide. However, this phenomenon was not observed in GFPGER-mixed PVA studies [80]; thus, more studies would need to be performed to

understand the relationship between conjugated-peptide density and EC morphology and phenotype.

Importantly, the CDI-GFPGER modification of PVA did not increase platelet attachment and aggregation nor did the modification initiate clotting via the coagulation cascade. In our evaluation of GFPGER-conjugated PVA hydrogels, we found that all GFPGER-conjugated samples delayed the initiation time of fibrin clotting of PPP and decreased the fibrin formation *ex vivo* when compared to the baseline material (GFPGER₀). The lack of a significance difference found in the analysis of FXII activation on PVA samples suggested that contact pathway was not a potential coagulation initiation mechanism. However, more studies will have to be done to determine the mechanism of fibrin formation inhibition on GFPGER-modified samples. *In vitro* and *ex vivo* assessment of platelet attachment onto GFPGER-conjugated samples indicated no alteration of the PVA hemocompatibility. MicroCT analysis of thrombi formed on PVA samples after *ex vivo* testing showed minimal thrombus formation. These results contrast what is known about collagen-platelet interactions [122–125] and our previous GFPGER-mixed PVA studies [80], where we found significantly higher platelet attachment to GFPGER-mixed samples when compared to unmodified PVA. The GFPGER binding site to $\alpha 2\beta 1$ integrin on platelets is known to contribute to platelet activation and aggregation [108,126]. The divalent cation in the $\alpha 2$ I-domain of the integrin coordinates with the glutamate in collagen or collagen-mimicking peptides, leading to a conformational change in the receptor followed by activation. Rich et al. predicted that the ligand-binding site in the $\alpha 1$ subunit, which is found on ECs, is longer and more flexible than the $\alpha 2$ subunit [109,127], which is present on the platelet surface. Previous studies found that GFPGER has slightly less affinity for the $\alpha 2$ subunit, when

compared to GFOGER, the native integrin binding sequence in collagen [126]. This decreased affinity may be enough to minimally bind platelets and not activate key pathways leading to aggregation. Additionally, when GFPGER is directly bonded to the surface of PVA, as with the CDI linker, the glutamate that participates in integrin coordination is always available, leading to a controlled interaction between the material and biological components. Mixing the peptide into the PVA, as was done previously [80], buries some of the key groups needed for integrin recognition, which likely lead to non-specific binding of both cells and platelets [80]. Thus, CDI-mediated binding of GFPGER is a promising surface modification of blood contacting materials, which does not activate platelets.

Ultimately, the work presented herein supported our hypothesis that covalent attachment of GFPGER to the surface of PVA hydrogels would encourage endothelialization of the material, while attenuating thrombosis. We presented a systemic characterization and evaluation of GFPGER-conjugated PVA hydrogels using *in vitro* and *ex vivo* experimentation. We have shown that covalently modifying PVA with a low cost, widely available commercial peptide, GFPGER, for use as a synthetic vascular graft material may prevent thrombosis, while promoting *in situ* endothelialization of the material. A limitation of this work is the short time duration in which thrombogenesis is studied. Future work will include longer shunt study times and implantation studies using established rabbit (small animal) carotid bypass grafting [73] and non-human primate (large animal) aortoiliac bypass grafting [128] implant models both with an end-to-side methodology to increase pre-clinical understanding of material integration. These models provide the best approach for recapitulating clinical flow dynamics, which is frequently neglected in other vascular graft animal models [129].

Additionally, further *in vitro* work will be done to investigate specific EC phenotype and SMC proliferation on GFPGER-conjugated PVA. The promising results reported here with our novel synthetic biomaterial support the potential of this covalently linked surface modification either to coat off-the-shelf cardiovascular materials or for tissue-engineered constructs, supporting endothelialization without aggregating platelets.

Chapter IV: Evaluation of the role of FXII in a nitinol stent non-human primate model³

4.1 Abstract

Nitinol stents are a mainstay for treatment of cardiovascular diseases, which persist as the leading cause of death worldwide with high rates of morbidity and mortality. Despite advances in interventional cardiology and prevention of restenosis, detrimental clinical consequences of the administration of systemic antithrombotics, which are required during stent placement, remain. Because current clinically used antithrombotics target factors that are essential for normal hemostasis, abnormal bleeding continues to be a side effect of these treatments. It has been thought that targeting the contact pathway of coagulation might be an ideal alternative to common anticoagulants. In this work, we tested the efficacy of a humanized blocking anti-factor XII antibody, h5C12, on stent thrombosis. First, we characterized the h5C12 using aPTT, PT, and ACT coagulation assays and examined platelet accumulation and fibrin formation on collagen and TF surfaces under physiological flowing whole blood. We then measured the activity of FXIIa in the presence of stents and stents pre-seeded with platelets in vitro. We examined the effect of different contact pathway inhibitors on fibrin clotting time in the presence of stents and stents pre-seeded with platelets in vitro. Lastly, we used an established non-human primate model of acute thrombogenesis with stents deployed at two diameters, 4mm and 8mm, to examine the impact of h5C12

³ This work is in preparation for a simultaneous submission to *Science Translational Medicine* as a primary article by Bates, NM, Wallisch, M, Jordan, KR, Johnson, J, Puy, C, McCarty, OJT, Nguyen, KP, Aslan, JE, Gruber, A, Hinds, MT, Anderson, DEJ.

under different shear rates. Additionally, we measured platelet reactivity using fluorescence activated cell sorting, platelet factor 4, thrombin-antithrombin levels, and myeloperoxidase activity in samples collected from the animal studies. Results showed high efficacy of h5C12 in functional characterization tests. In vitro investigation into stent thrombosis showed that FXII is activated in high quantities in the presence of stents and h5C12 abrogates this activity. Our ex vivo results showed a slight decrease in platelet attachment and fibrin formation on stents in h5C12-treated animals. Overall, h5C12 had a minimal impact on platelet-driven thrombosis mechanisms but did show effectiveness in coagulation pathway driven thrombosis. This work is an important step forward in developing novel antithrombotics targeting the contact pathway of coagulation.

4.2 Introduction

Stent placement is a common clinical intervention with broad indications including peripheral arterial disease, acute limb ischemia, carotid disease, coronary disease, aneurysm, and neurovascular disease. Despite advances in interventional cardiology [130–133] and antithrombotic therapies [134,135], these widely used vascular stents induce significant thrombus formation with severe clinical consequences [136–138]. Stent thrombosis contributes to up to 20% of all myocardial infarctions post vascular intervention and has an associated risk of death four times greater than that of ST segment elevation myocardial infarction [139].

In addition to subacute thrombotic events, long-term interactions between the hemostatic system and device surfaces also contribute to device failure. The mechanisms of stent thrombosis are multifactorial, with platelet attachment and activation, initiation of coagulation pathways, activation of immune responses, and fibrin deposition all playing a role in the condition [140]. Early and late thrombotic events may be related to blood stasis, stent underexpansion, incomplete healing of surrounding tissue, and inadequate neointimal coverage of the device [56,139–141]. Frequently, stent-induced thrombosis requires reinterventions to maintain patency, resulting in additional procedures and additional risk to the patients [142,143].

Pharmacologic agents aimed at preventing thrombus formation on stents are foundational therapies for the prevention of stent failure and progressive vascular disease. Efforts to address stent failure have focused on generating non-fouling surfaces of vascular devices or implementing antithrombotic strategies; however, current clinical biomaterials remain ineffective and current therapeutics come with a high risk of bleeding [144–147]. Drugs targeting blood coagulation proteins and specific platelet

receptors increase device complications and the risk of dangerous bleeding side effects due to inhibition of factors required for the formation of hemostatic clots.

Targeting the intrinsic blood coagulation pathway has been thought to be an ideal approach for the development of novel antithrombotic therapeutics without the bleeding risk associated with dual antiplatelet and current direct oral anticoagulant therapies [148]. Coagulation factor (F)XII of the intrinsic pathway plays no known role in hemostatic clot formation but has been implicated in vascular device-related thrombus formation [136,149,150]. FXII is a zymogen that can be activated (FXIIa) upon contact with a foreign surface, such as a medical device, or by forming a complex with prekallikrein and a cofactor, high molecular weight kininogen. FXIIa subsequently activates FXI into FXIa, and this activation continues to further downstream coagulation factors, ultimately leading to fibrin production and thrombus formation on the device surface.

Herein, we examine the role of FXII in stent-induced thrombosis and platelet function by using a novel, humanized anti-FXII monoclonal antibody. We use an established and versatile non-human primate (NHP) *ex vivo* arteriovenous shunt model to examine the efficacy of a novel blocking anti-FXII antibody and study acute thrombogenesis in the setting of stent thrombosis. We characterize the humanized FXII antibody *in vitro* and examine its function in coagulation initiation and propagation on collagen-coated and tissue factor-coated grafts in flowing whole blood *ex vivo*. We then use the non-human primate model of experimental acute thrombogenesis on nitinol stents to examine the role of contact pathway inhibition. To address the potential role of shear stress in stent thrombosis, we use two stent diameters (4mm and 8mm) to generate different blood shear rates. We hypothesize that inhibition of FXII will limit

platelet and fibrin quantities on the stent surface. The results of this work will help understand how stent failure initiates and progresses to inform treatment of clinical stenting for vascular diseases.

4.3 Materials and Methods

4.3.1 Manufacturing of monoclonal antibodies

The anti-FXII antibody, h5C12, was produced by complementarity determining region grafting from the murine precursor antibody 5C12 [66] and stably expressed in a manufacturing Chinese hamster ovarian (CHO) cell line. Expression of the antibody in a 50-L single-use bioreactor was followed by purification of the antibody by Protein A chromatography, followed by both cation and anion exchange chromatography.

4.3.2 Functional characterization of humanized 5C12 monoclonal antibody and evaluation of antibody reactivity in NHP

Whole blood and coagulation studies were performed as saline controls or with h5C12 dosing at 9mg/kg given 30min prior to the first study of each week. Activated partial thromboplastin time (aPTT) of h5C12-inhibited platelet-poor plasma was measured using a SynthASil (Instrumentation Laboratory, Bedford, MA, USA) and a KC4 Analyzer (TCoag, Ltd, Wicklow, Ireland) in duplicate, as previously described [66]. Activated clotting time (ACT) of whole blood inhibited with h5C12 was measured in duplicate using LupoTek KCT (r2-Diagnostics, South Bend, IN, USA). Prothrombin time (PT) assay was conducted with Dade® Innovin® (Siemens Healthcare Diagnostics,

Flanders, NJ, USA), according to the manufacturer's protocol, and the PT of h5C12 inhibited plasma samples was quantified using a KC4 Analyzer. To characterize h5C12 under physiologic conditions, 2.0cm long collagen- or tissue factor (TF)-coated expanded polytetrafluoroethylene (ePTFE, 4.0mm I.D.; WL Gore & Associates, Flagstaff, AZ) vascular graft segments were interposed in series between segments of silicone rubber tubing extending a surgically-placed chronic arteriovenous (AV) shunt of the baboon (detailed below), as previously described [151]. Whole blood studies were performed in a single animal with saline controls or with h5C12 dosing at 9mg/kg given 30min prior to the first study of each week. Experiments were performed on three consecutive days, at one week, and at two weeks, in the same animal post dosing. In these experiment, collagen- and TF-coated grafts were exposed to flowing h5C12-inhibited whole blood continuously for 60min. Platelet accumulation and fibrin deposition onto the collagen and TF grafts were quantified (quantification methods further detailed below).

4.3.3 *In vitro* coagulation studies

4.3.3.1 *Washed platelet and PPP preparation*

Washed platelets and pooled platelet-poor (PPP) plasma were obtained by human venous whole blood drawn from healthy donors into sodium citrate, as previously described [88]. In brief, whole blood was centrifuged once at 200g for 20min before further centrifugation at 1000g for 10min in the presence of 0.1µg/mL prostacyclin to obtain PPP and a pellet of platelets. The pellet was resuspended in HEPES/Tyrodé's buffer (129mM NaCl, 0.34mM Na₂HPO₄, 2.9mM KCl, 12mM NaHCO₃, 20mM HEPES, 5mM glucose, 1mM MgCl₂; pH 7.3) containing 0.1µg/mL prostacyclin. The platelets were

washed once more via centrifugation (1000g for 10min) and resuspended in HEPES-Tyrode's buffer before use.

4.3.3.2 Real-time FXIIa generation in a purified system and in pooled human plasma

Activation of coagulation factor XII (FXII, 200nM, Haematologic Technologies, Inc., Essex Junction, VT, USA) on the surface of stents alone, stents seeded with washed platelets (resting platelets, RP), and stents seeded with activated platelets (AP) was measured in real time in a purified system (FXII, prekallikrein (PK), and high molecular weight kininogen (HK) only) and in human plasma using a chromogenic substrate, Spectrozyme-FXIIa (American Diagnostica, Inc., Stamford, CT, USA). Washed platelets were stimulated with 30 μ M of thrombin receptor activator peptide 6 (TRAP6, Tocris Bristol, UK,) to generate AP. A HEPES-buffered solution of FXII, PK, and HK or pooled PPP was added to stents, with or without platelets, followed by the addition of Spectrozyme-FXIIa. Cleavage of the chromogenic substrate was measured over one hour at 405nm absorbance using an Infinite M200 spectrophotometer (Tecan, Männedorf, Switzerland).

4.3.3.3 Fibrin clotting time in pooled human plasma

Anti-FXI neutralizing antibodies m1A6 and m14E11 were generated as previously described [87,152,153]. Before the start of the experiments, PPP was treated with FXI or FXII antibodies, m1A6 (40 μ g/mL), m14E11 (40 μ g/mL), or h5C12 (40 μ g/mL) for 10min. PPP, m1A6-treated PPP, m14E11-treated PPP, h5C12-treated PPP, FXI-deficient PPP, or FXII-deficient PPP (<1% FXI, <1% FXII, Haematologic Technologies, Inc., Essex Junction, VT, USA) was incubated with stents or stents seeded with AP

followed by the addition of CaCl_2 (8mM). Fibrin clotting was observed by a change in turbidity (absorbance at 405nm) documented over one hour. Fibrin clotting times were defined by the time at which there was a 10% increase over baseline absorbance.

4.3.4 *Ex vivo* whole blood shunt study

4.3.4.1 *Anticoagulation for shunt studies*

Whole blood studies (described below) and coagulation studies were performed as controls or with h5C12 dosing at 9mg/kg given 30 min prior to the first study of each week. To confirm reactivity of the drug for stent studies, blood samples were collected into 3.2% citrate at a one to ten ratio daily prior to whole blood shunt studies. aPTT, PT, and ACT were performed using the same method as in Section 4.3.2. aPTT was expected and confirmed to be at least twice the baseline average.

4.3.4.2 *Real-time platelet and end-point fibrin quantification*

Nitinol stents (Admedes, Livermore, CA) were deployed into fluorinated ethylene propylene (FEP, Zeus Inc., Orangeburg, SC) heat shrink tubing and connected to Silastic tubing (4mm ID, Dow Chemical, Midland, MI) with silicone adhesive (Factor II, Inc., Lakeside, AZ). The final diameter of the deployed stents was 4.2mm or 7.9mm, referred to as 4mm and 8mm, respectively (**Fig 4.1B**). The Silastic™ tubing was connected to an exteriorized, arteriovenous shunt placed in a chronic implant in a juvenile, male *Papio anubus* baboon (13kg). Animal work followed guidelines from the National Research Council and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources. Animals were under the full care and supervision of the staff at the Oregon National Primate Research Center (ONPRC), and

studies were approved by the ONPRC Institutional Animal Care and Use Committee. In the current studies, stents were placed centrally in the silicone tubing and exposed to whole flowing blood, controlled at 100mL/min using a clamp distal to the device (**Fig 4.1A**) and measured with an upstream flow probe (Transonic, Ithaca, NY). This resulted in a mean wall shear rate of 265s^{-1} in the 4mm stents and 33s^{-1} in the 8mm stents. This model has been well established in our lab to dynamically measure both platelet attachment over time and endpoint fibrin accumulation [66,70,71,154,155]. Briefly, autologous platelets are labelled with In-111 and quantified every minute for 60min with a Brivo NM615 (General Electric, Boston, MA). Homologous fibrin was labeled with I-125 and included in the flow loop. After the In-111 decayed, the I-125 was quantified with a 1480 Wizard gamma counter (PerkinElmer, Waltham, MA).

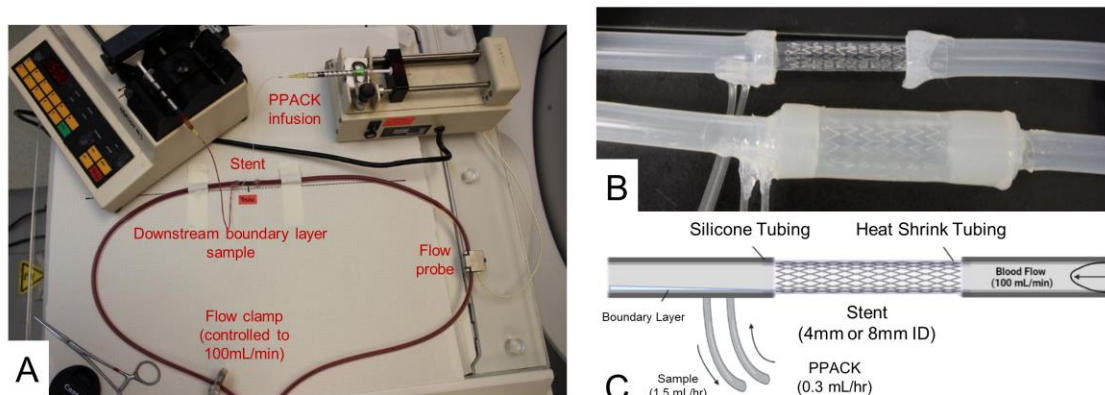


Figure 4.1: Stent deployment and blood collection device.

Nitinol stents were deployed at final diameters of 4mm and 8mm into heat shrink tubing and connected to Silastic™ tubing with silicone adhesive. Whole blood was allowed to flow through the device for 60min at a fixed rate of 100 mL/min for the entirety of each study by distal clamping. Blood samples were drawn from the concentration boundary layer by a syringe pump 1cm downstream from the acutely developing thrombi. PPACK was infused 3mm proximal to the sample port to prevent the sample port from occluding during the study.

4.3.5 Downstream sampling

Throughout the 60min study, whole blood samples were taken for thrombosis characterization after interaction with the stent surface. This procedure was modified from previous work with our shunt model [154]. A bulk blood sample (hereafter referred to as “0min”) was drawn into 10% (v/v) citrate and 9% (m/v) PPACK. Samples from 0-30min (referred to as “30min”) and 30-60 (referred to as “60min”) were dynamically sampled immediately downstream from the stent with a steady pull at 1.5mL/hr into 10% by volume citrate to provide a concentrated boundary layer sample (**Fig 4.1C**). PPACK (Sigma, St. Louis, MO) was pumped immediately upstream of the sample at 0.3mL/hr to prevent clotting in the sampling tubing. These samples were used for fluorescence-activated cell sorting (FACS) analysis and assays for PF4 (R&D systems, Minneapolis, MN), thrombin-antithrombin complexes (TAT) (Siemens Healthcare Diagnostics, Los Angeles, CA), and myeloperoxidase (MPO) (Cell Technology, Hayward CA).

4.3.6 FACS

Whole blood for FACS testing was diluted 1:4 with HEPES/Tyrodes (HT) buffer and stimulated for 20min with collagen-related peptide (CRP, 0, 0.1, and 1 μ g/ml) at 37°C in the presence of CD62-PE (BD Pharminogen,) or lactadherin-FITC (Haematologic Technologies, Catalog # BLAC-FITC). 1 μ g/mL CRP stimulation was used as a positive control for platelet reactivity to stimuli. For analysis of platelet-leukocyte aggregates, diluted whole blood was incubated with CD45-APC (BD Bioscience, Catalog # 561290) and CD41-FITC (Biolegend Catalog, # 303704) for 20min at 37°C. Samples were fixed and stored in 2% PFA at 4°C until analysis with a BD FACS Canto II Flow Cytometer.

4.3.7 ELISAs

Blood samples from the concentration boundary layer were centrifuged to obtain PPP and subsequently frozen at -80°C. Plasma platelet factor 4 (PF4) and thrombin-antithrombin complexes (TAT) were measured using ELISA kits from R&D Systems (Minneapolis, MN, USA) and Siemens (Flanders, NJ, USA), respectively. Plasma myeloperoxidase (MPO) was measured using a fluorogenic assay kit from Cell Technology (Davis, CA, USA). All experiments were performed according to the manufacturers' instructions.

4.3.8 Statistics

Data are presented as mean $\pm$ standard deviation (SD) for all studies. Probability values of $p < 0.05$ were considered to be statistically significant. Blood coagulation testing (aPTT, PT, ACT) was compared with a one-way ANOVA to investigate the effects of h5C12 ($n=6$). Platelet accumulation onto collagen-initiated or TF-initiated surfaces analysis, formation, FACS analysis, PF4 ELISA, TAT ELISA, and MPO activity were analyzed using a two-way ANOVA with factors of treatment and time ($n=3-6$). Analysis of fibrin formation on collagen-initiated or TF-initiated surfaces was performed using a Kruskal-Wallis one-way ANOVA. *Ex vivo* platelet quantification analysis was performed as a repeated measures two-way ANOVA with drug and time as factors for each stent size ($n=6$). *Ex vivo* fibrin quantification analysis was performed using a one-way ANOVA ($n=6$). *In vitro* FXIIa generation was tested as a one-way ANOVA with Tukey's *post hoc* testing ($n=3-5$). Fibrin clotting time in PPP to investigate the effects of drug in the presence of stents and platelets on stents was tested using a two-way ANOVA with drug and condition (plasma, stent, and stent with platelets) as factors and Tukey's *post hoc*

testing (n=3-6). All studies were tested for ANOVA assumptions using Levene's and quantile-quantile plots and, when necessary, data were natural log transformed.

4.4 Results

4.4.1 Characterization of humanized anti-FXII antibody in an *ex vivo* non-human primate model

Because collagen and TF in the subendothelial matrix are exposed upon vascular injury, to confirm reactivity of the drug for shunt studies, we tested the efficacy of h5C12 in reducing thrombus formation on collagen-coated and TF-coated grafts in an otherwise anticoagulated, flowing whole blood system. We first measured aPTT, PT, and ACT of baboon blood samples treated with h5C12. The h5C12 treatment caused significant increases in aPTT ($F=34.141$, $p<0.001$), PT ($F=12.926$, $p=0.005$), and ACT ($F=245.595$, $p<0.001$) (**Fig 4.2**). ACT and aPTT both had a greater than 2-fold difference during daily evaluation. aPTT went from $31\pm0.67s$ to $77\pm19s$ with h5C12 treatment. ACT increased from $116\pm5.0s$ to $299\pm28s$. The increase in PT, while statistically significant, would not be considered clinically increased from a baseline of $8.6\pm0.19s$ to $9.0\pm0.24s$.

Perfusion of h5C12-treated baboon blood over collagen-coated grafts showed significant, but modest, decreases in platelet deposition up to 384hrs post h5C12 administration ($F=46.72$, $p<0.001$) (**Fig 4.3A**) and significant decreases in fibrin formation in experiments performed up to 48hrs post h5C12 administration ($p<0.01$) (**Fig 4.3B**). The drug had no effect on platelet deposition (**Fig 4.3C**) or fibrin formation (**Fig 4.3D**) initiated by the TF coating.

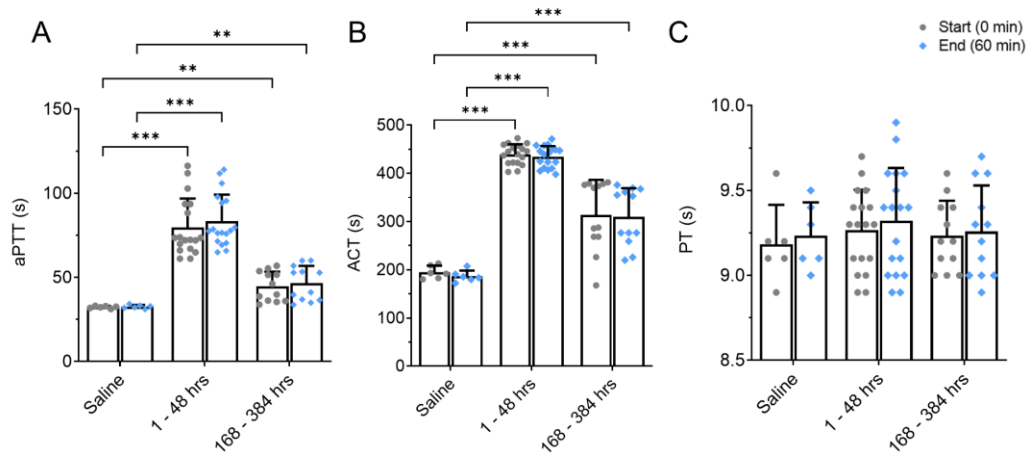


Figure 4.2: Coagulation measurements during functional characterization of h5C12 in thrombosis experiments.

Blood samples were taken at the start (0min, grey dots) and end (60min, blue diamonds) of the graft experiment and aPTT (A), ACT (B), and PT (C). Saline was the control treatment (n=6 for each graft), 1 – 48hrs refers to experiments performed 1 to 48 hours post h5C12 administration (n=18), 168-384hrs refers to experiments performed 1–2 weeks post-administration (n=12). Administration of h5C12 significantly increases both aPTT and ACT for up to 384hr, while it does not affect PT. Statistical analyses were performed as a two-way ANOVA with factors of treatment and time, **p<0.01, ***p<0.001.

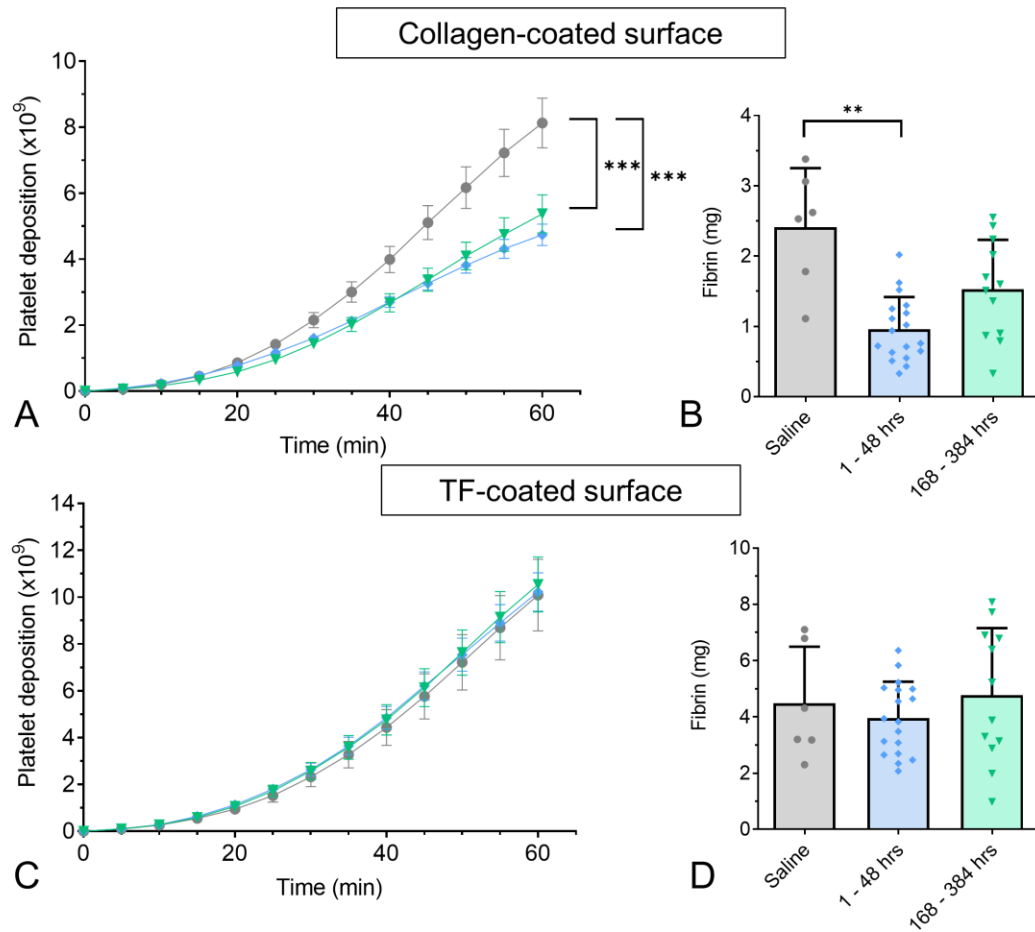


Figure 4.3: Effect of FXII inhibition by h5C12 on platelet deposition and fibrin formation during thrombus formation on collagen- (A&B) or tissue factor-coated (C&D) graft devices.

Coated ePTFE grafts (n=6) were inserted into the external loop of an AV shunt and platelet deposition within the total thrombus was recorded within the graft itself (head thrombus) and the adjacent downstream tail thrombus. For control experiments (n=6) 0.5ml/kg saline was given 1hr before the start of the experiments. Administration of 9mg/kg h5C12 was given I.V. and experiments were performed 1-384hrs post-administration (n=18 for 1-48hrs, n=12 for 16-384hrs). FXII inhibition by h5C12 significantly reduces thrombi growth and size on collagen-coated surfaces but has no effect on thrombi initiated by tissue factor coating (***p<0.001, two-way ANOVA with factors of treatment and time, GraphPad Prism 9). Fibrin content (mg) in grafts coated with either collagen (B) or tissue factor (D). Terminal fibrin content was quantified after 60min of thrombus growth in the coated grafts. Fibrin content is reduced in collagen-coated grafts following h5C12, but not in tissue factor-coated grafts (**p<0.01, one-way ANOVA with Kruskal-Wallis test).

4.4.2 Effect of stents in real time FXIIa generation in a purified system and in pooled human plasma *in vitro*

To evaluate the role of FXII activation in stent thrombosis, the generation of FXIIa in the presence of stents, stents and resting platelets, and stents and activated platelets was measured in a purified system containing only FXII, PK, and HK, and in pooled human plasma. The presence of the stent alone significantly increased the generation of FXIIa, as shown by higher FXIIa activity in cleaving a chromogenic substrate, in the purified system (Fig 4.4A, $p=0.0125$) and in PPP (Fig 4.4B, $p=0.0007$). In the purified system, the presence of activated platelets on stents significantly reduced the activity of FXIIa when compared to the stent alone (Fig 4.4A, $p=0.0038$), which was not observed in PPP. In PPP, the presence of RP (Fig 4.4B), but not AP, had significantly higher FXIIa activity on stents, trending toward a reduction in FXIIa activity on stents seeded with AP compared to stents alone.

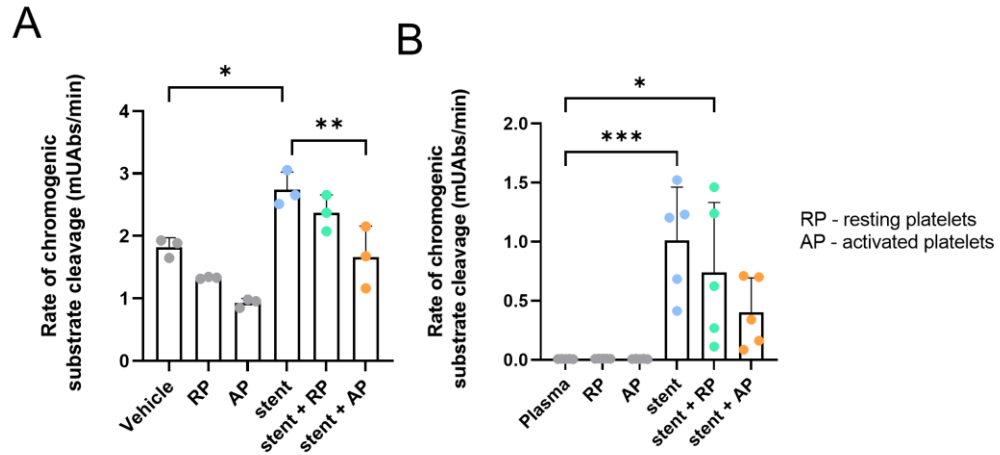


Figure 4.4: Time dependent FXII activation and activity in a purified system of only PK, HK, and FXII (A) and in pooled human platelet-poor plasma (B) in the presence of stents and stents pre-seeded with platelets after 60min.

FXIIa activity in a purified system was increased in the presence of stents compared to all control conditions (vehicle, RP, and AP). The presence of platelets reduced FXIIa activity compared to the stent alone; this reduction was trending downward for stents with RP and showed a statistically significant decrease in stents with AP. Similarly in plasma, FXIIa activity was significantly higher in the presence of stents compared to all control conditions. However, the presence of platelets did not alter this response. Data are shown as mean $\pm$ SD. Statistical analyses were performed using a one-way ANOVA with Tukey's *post hoc* testing (n=3-5), *p<0.05, **p<0.01, ***p<0.001.

4.4.3 Effect of stents in fibrin clotting times *in vitro*

We next evaluated the role of FXII activation in stent thrombosis by measuring fibrin generation in the presence or absence of stent surface, with and without platelets. Using a two-way ANOVA, with factors of surface and inhibitor, without the interaction term (examination of main effects only), stents (p<0.0001) and stents with platelets

($p < 0.0001$) showed a significant reduction in fibrin clotting time (**Fig 4.5**) compared to plasma alone, suggesting an increase in thrombogenicity in the presence of a stent and stent with activated platelets. The blocking anti-FXII antibody, h5C12, and the blocking anti-FXI antibodies, m14E11 and m1A6, treatment of plasma all significantly ($p = 0.003$, $p = 0.007$, and $p < 0.0001$, respectively) prolonged fibrin clotting time compared to vehicle. However, drug-treated plasmas were not as effective in prolonging clotting time as FXII- and FXI-depleted plasmas ($p < 0.0001$ and $p < 0.0001$).

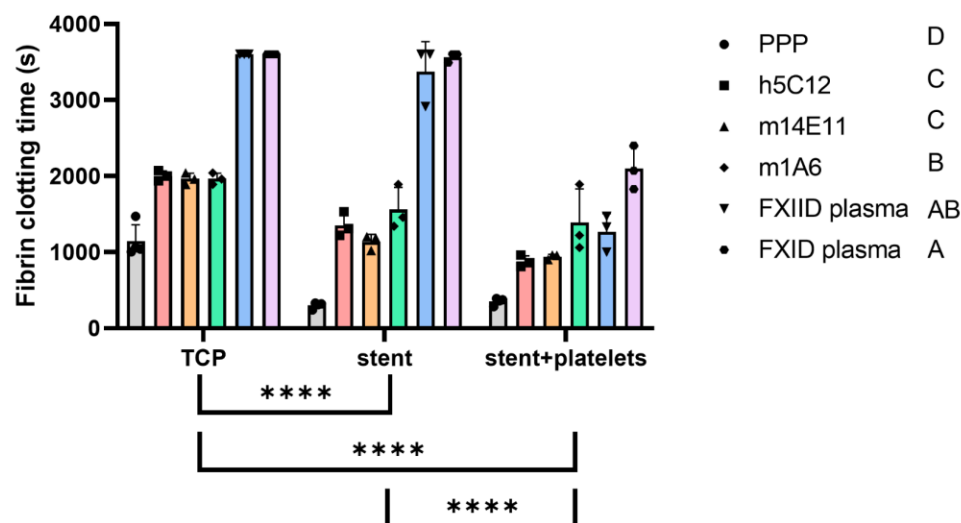


Figure 4.5: *In vitro* fibrin clotting times in the presence of stents and stents pre-seeded with platelets in drug-treated or contact pathway-depleted plasma.

Overall, stents decreased fibrin clotting time compared to the tissue culture plastic (TCP) controls, with the exception of stents in FXI- and FXII-depleted plasma. The clotting time in the presence of platelets on the stent surface was significantly decreased compared to the vehicle controls and stents alone, indicating a prothrombotic profile. Both drug-treated plasmas and FXI- and FXII-depleted plasmas significantly delayed fibrin clotting in the presence of stents alone compared to no drug (PPP). Statistical analysis was performed as a two-way ANOVA (without interaction) with drug or depletion and surface as factors. For surfaces, **** $p < 0.0001$, and for drug-treated or depleted plasma, groups which do not share a letter indicate statistical significance ($p < 0.05$).

4.4.4 Whole blood shunt study

Blood treated with h5C12 prior to the shunt studies showed a significant delay in contact pathway-driven clotting (**Fig 4.6**). Whole blood testing showed a significant decrease in platelet attachment with a one-way repeated measures ANOVA for 8mm stents with h5C12 (**Fig 4.7D**) ($F=8.807$, $p=0.014$), but a non-significant decrease for 4mm stents by the h5C12 treatment (**Fig 4.7A**) ($F=4.598$, $p=0.058$). Fibrin formation (**Fig 4.7 B&E**) was not significantly different for any of the groups but showed a decrease trend with h5C12 treatment in the 4mm stents. Visual inspection of the thrombus on the stent (**Fig 4.7 C&F**) did suggest a reduction in thrombosis on the h5C12 stents with no downstream tail forming on any of the treated samples.

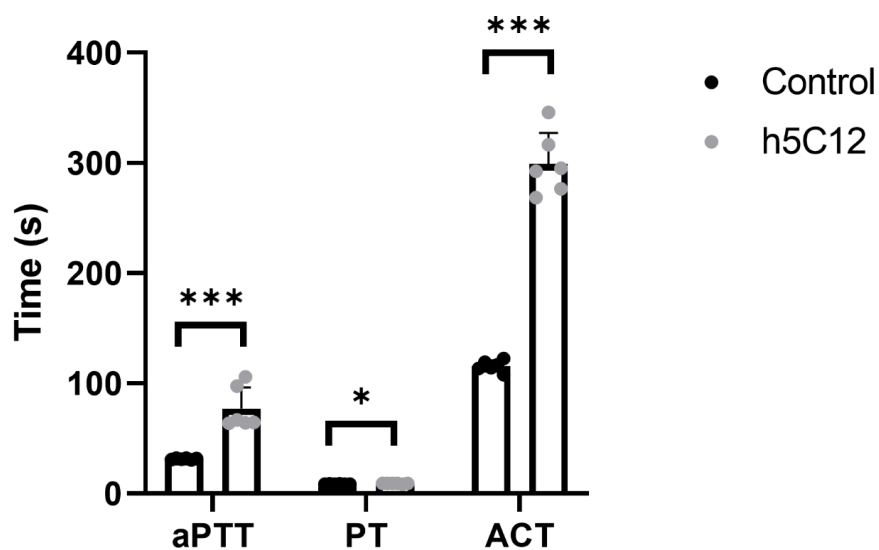


Figure 4.6: Coagulation measurements prior to stent thrombogenesis experiments.

Blood samples were collected and tested just prior to stent shunt studies.

Administration of h5C12 significantly increases both aPTT and ACT. A slight increase in PT was observed in h5C12-treated plasma, however, the clotting time remained low. Statistical analyses were performed as a one-way ANOVA (n=6), *p<0.05 and *** p<0.001.

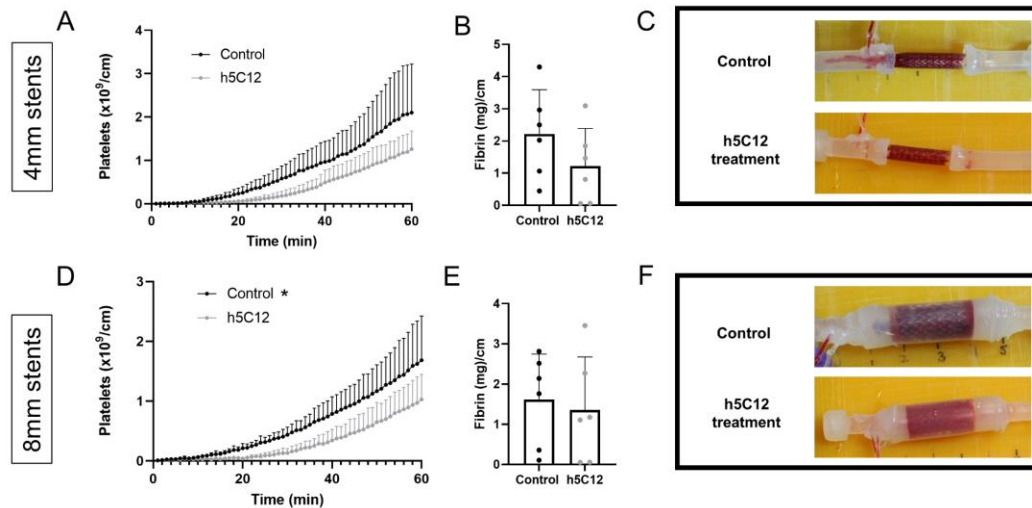


Figure 4.7: Whole blood *ex vivo* shunt testing of stent-induced thrombogenesis.

4mm and 8mm stents were tested in an NHP *ex vivo* shunt model with the stent alone being the control and the h5C12-treated blood being the test group (n=6). Platelet accumulation was quantified on (A) 4mm stents and (D) 8mm stents over 60min exposure to whole blood with and without h5C12 treatment. Data are shown as mean $\pm$ SD. Statistical analysis was performed using a repeated measures two-way ANOVA with Tukey's *post hoc* testing, *p<0.05. Fibrin data were quantified and normalized per centimeter of the axial length for (B) 4mm stents and (E) 8mm stents at 60min. Data are shown as mean $\pm$ SD. Statistical analysis was performed using a one-way ANOVA. No significant differences were observed in the fibrin samples at 4mm or 8mm. Representative images of thrombi formed on (C) 4mm and (F) 8mm stent surfaces were recorded by photograph. There appears to be a reduction in overall thrombus formation in h5C12-treated samples.

4.4.4.1 Effect of stent-initiated thrombus development in thrombin generation, local platelet activation, and inflammation and leukocyte activation

Thrombin generation was measured by TAT complexes in plasma collected downstream from 4mm and 8mm stents. TAT concentration was significantly increased over time in the presence of the 4mm (**Fig 4.8A**, 0min compared to 60min, $p=0.0002$ and 30min compared to 60min, $p=0.0004$) and 8mm (**Fig 4.8B**, 0min compared to 60min, $p<0.0001$, and 30min compared to 60min, $p<0.0001$) nitinol stents. The h5C12 treatment was effective in abrogating the generation of TAT in 4mm ($p=0.0044$) and 8mm ($p<0.0001$) stent samples, however, only at the 60min time point.

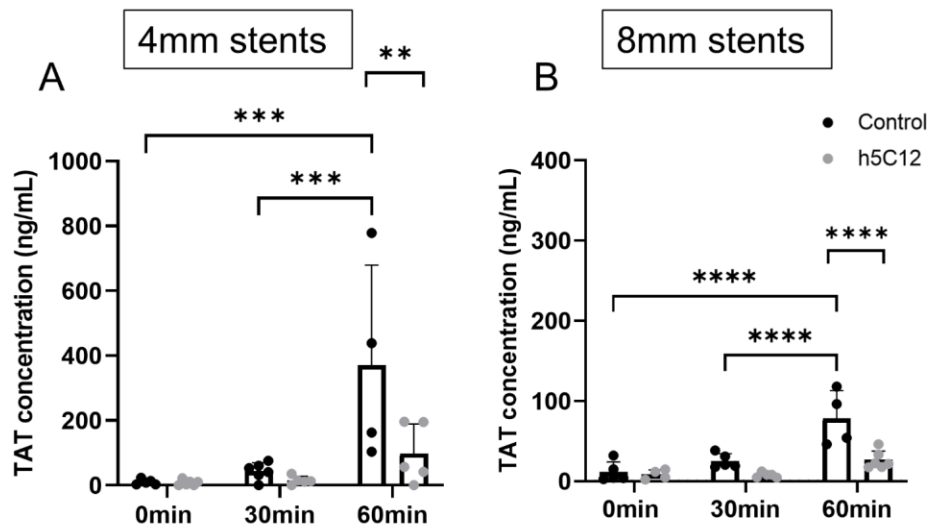


Figure 4.8: TAT quantification in plasma collected downstream of 4mm and 8mm stents in the NHP *ex vivo* shunt model.

The concentration of TAT increased over time in control samples. The h5C12 treatment was able to abrogate this response at the 60min time point and TAT in h5C12-treated samples remained low over the 60min study. Data are shown as mean $\pm$ SD. Statistical analysis was performed as a two-way ANOVA with time and treatment as factors and Tukey's *post hoc* testing, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Local platelet activation was measured by PF4 concentration and P-selectin exposure in samples collected downstream of 4mm and 8mm stents. The presence of a nitinol stent significantly increased the quantity of PF4 over time in 4mm (**Fig 4.9A**, 0min compared to 60min, $p = 0.0003$ and 30min compared to 60min, $p = 0.0279$) and 8mm stents (**Fig 4.9B**, 0min compared to 60min, $p = 0.0301$) but was unaffected by the h5C12 treatment. To test platelet reactivity and sensitivity to a low dose stimulus in the presence of stents, we measured P-selectin, with and without $0.01\mu\text{g/mL}$ CRP stimulation, by FACS. Without CRP stimulation, the presence of 4mm (**Fig 4.9C**) and 8mm (**Fig 4.9D**) stents significantly increased the amount of P-selectin over time (4mm:

$p=0.0007$ for 60min compared to 0min and $p=0.0052$ for 30min compared to 60min, 8mm: $p=0.0223$ for 60min compared to 0min and $p=0.0333$ for 30min compared to 60min) and this was unaffected by the h5C12 treatment. No difference in P-selectin expression over time was observed in the CRP stimulated samples at 4mm or 8mm. The positive control samples (1 μ g/mL CRP) showed that platelets were highly reactivity to CRP stimulation and this did not change in samples treated with h5C12 (data not shown).

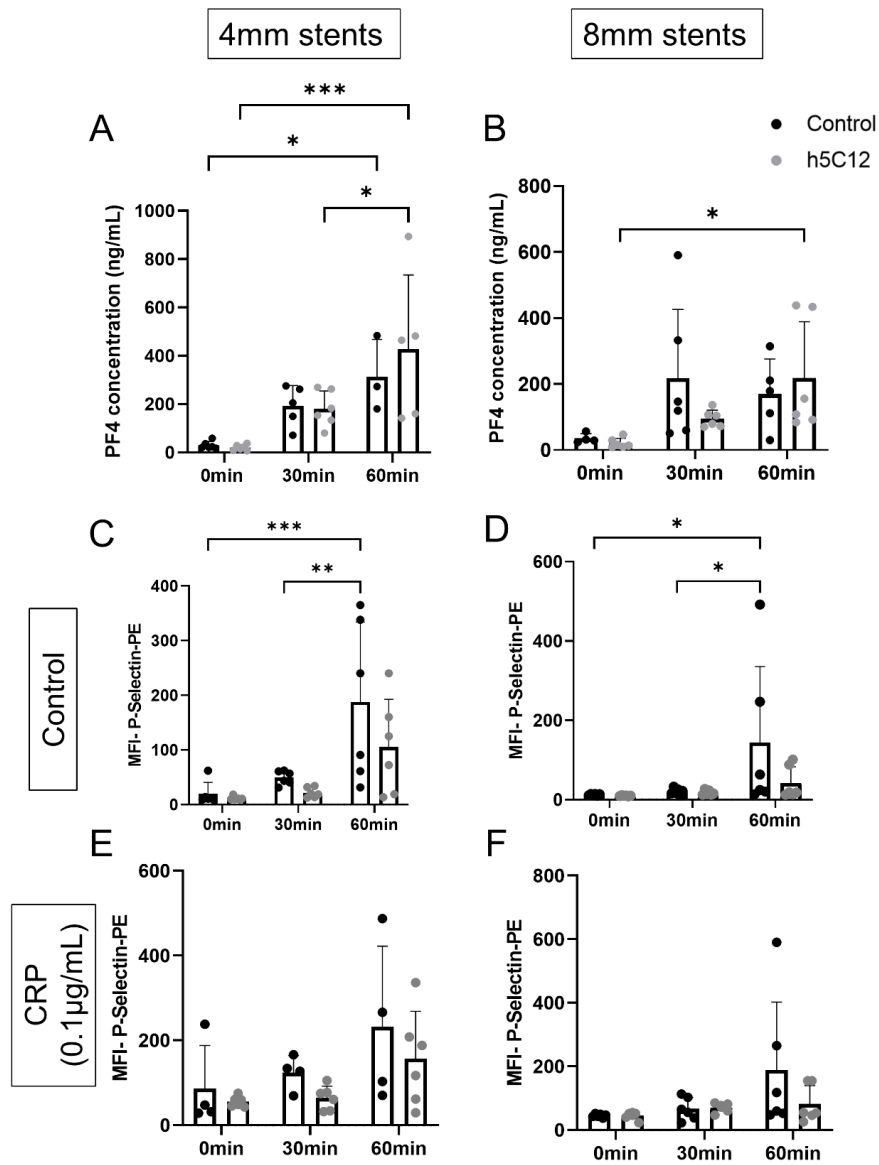


Figure 4.9: Quantification of the effect of h5C12 on platelet activation as measured by platelet factor 4 (PF4) and P-selectin expression, with and without CRP (0.1µg/mL) stimulation, on platelets exposed to 4mm and 8mm stents using fluorescence-activated cell sorting (n=4-6).

Data are shown as mean $\pm$ SD. Statistical analysis was performed as a two-way ANOVA with Tukey's *post hoc* testing. (A&B) PF4 increased over time in the presence of 4mm and 8mm stents. (C&D) P-selectin was significantly increased in control samples exposed to 4mm and 8mm stents, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. CRP stimulation at 0.1µg/mL had no effect on P-selectin release.

To assess inflammation and leukocyte activation, we measured platelet-leukocyte aggregates (PLAs) (**Fig 4.10A**) and MPO activity in plasma collected downstream of 4mm and 8mm stents. The cell population of CD41+/CD45+ (PLAs) was significantly increased in the 4mm control condition (**Fig 4.10B**) over time ($p=0.0263$ for 30min compared to 0min and $p=0.0044$ for 60min compared to 0min). The 8mm stent results showed no effect of time or drug (**Fig 4.10D**). MPO activity significantly increased over time in the presence of 4mm and 8mm stents with the treatment of h5C12 (**Fig 4.10C&E**, 4mm: 0min compared to 30min, $p<0.0001$, 0min compared to 60min, $p=0.0003$, 8mm: 0min compared to 60min, $p<0.0001$, 0min compared to 60min, $p<0.0001$) and without (4mm: 0min compared to 30min, $p<0.0001$, 0min compared to 60min, $p<0.0001$, 30min compared to 60min, $p=0.0096$, 8mm: 0min compared to 30min, $p=0.0007$, 0min compared to 60min, $p=0.0423$). Mitigation of MPO activity in h5C12-treated sampled was observed in 4mm ($p=0.0271$) and 8mm ($p<0.0001$) stent samples at the 30min time point.

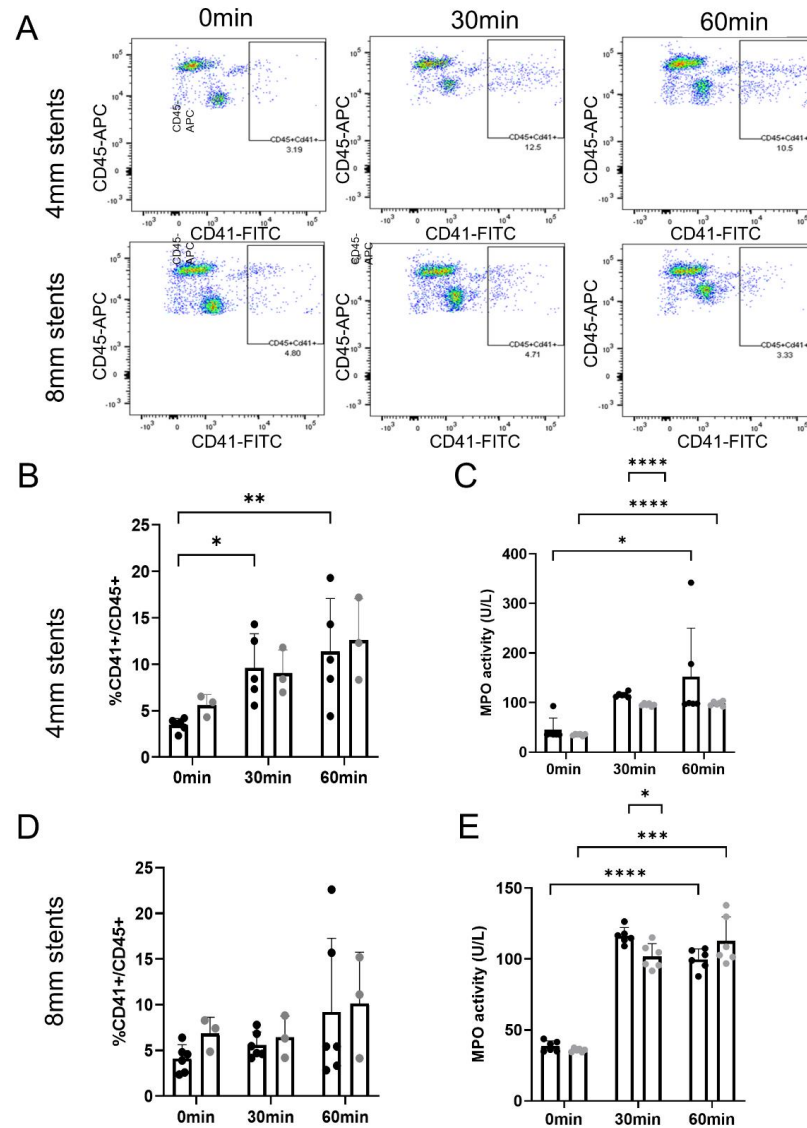


Figure 4.10: Effects of h5C12 treatment on platelet-leukocyte interactions in the presence of 4mm and 8mm stents as shown by fluorescence-activated cell sorting.

(A) Representative images of the gating strategy used to identify the cell population that was positive for platelets (CD41+) and leukocytes (CD45+) in samples at 0min, 30min, and 60min. (B&D) Quantification of platelet-leukocyte aggregates, shown as %CD41+/CD45+, at 0min, 30min, and 60min in 4mm and 8mm stents (n=3-6). Data are shown as mean $\pm$ SD. Statistical analysis was performed using a two-way ANOVA, with factors of time and drug, and Tukey's *post hoc* testing. Control 4mm samples increased significantly with time (*p<0.05 and **p<0.01) and h5C12 was unable to abrogate this response. There were no differences seen in 8mm samples. (C&E) MPO activity increased over time in 4mm and 8mm samples and h5C12 was effective at decreasing this response but only at the 30min time point.

4.5 Discussion

We hypothesized that inhibited FXII in the presence of stents would reduce the main structural components of a thrombus, platelets and fibrin, on the surface of the device. In this study, we show that treatment with a FXII inhibitor, h5C12, modestly reduced platelet accumulation onto the surface of nitinol stents deployed at 4mm and 8mm in a non-human primate model of acute thrombogenesis. This inhibitor acts by binding directly to FXII and FXIIa, thus, inhibiting the proteolytic activity of the enzymatic form (FXIIa). There has been interest in targeting the contact pathway for antithrombotic therapies due to FXII being the primary initiator of the pathway resulting in thrombin and fibrin formation. FXII is known to autoactivate on foreign surfaces, including medical devices, implicating the protein in pathological thrombus formation. Additionally, FXIIa has no known role in the normal hemostatic clotting process, as it has been observed that FXII-deficient patients have no bleeding abnormalities; thus, inhibition of the enzyme is unlikely to cause bleeding diathesis [156–158].

Herein, we showed that our newly characterized humanized form of an anti-FXII antibody, h5C12, was highly effective in prolonging the clotting time of whole blood and plasma using aPTT and ACT assays. The h5C12 treatment did not alter clotting time of the PT assay, as this test is associated with TF-initiated clotting, whereas aPTT and ACT are associated with contact pathway-initiated clotting. This is further supported by our results showing h5C12 decreases platelet deposition up to 384hrs after treatment and fibrin formation up to 48hrs after treatment on a collagen-coated surface but having no effect on a TF-coated surface. The potency of h5C12 in prolonging clotting was similar to the antithrombotic activity of FXII inhibitors generated by other groups [65,67,159].

Despite extensive research on contact pathway inhibition and device-induced thrombosis, to our knowledge, this study is the first to examine the role of FXII in stent-induced thrombosis by treating with a neutralizing antibody. Previously, selective depletion of contact pathway factors XII and XI with antisense oligonucleotides (ASO) was used to assess catheter-associated thrombosis in rabbits [67]. ASO were used to reduce the production of contact pathway proteins in the liver. The ASO treatment successfully prolonged the time to occlusion by thrombi forming within the catheter, which was positioned in the jugular vein of the rabbits. These data provide support for a role of FXII, and other contact pathway proteins, in device-related thrombosis.

Larsson *et al.*, showed similar results using an FXIIa neutralizing antibody, 3F7. This antibody was tested in an experimental thrombosis model, induced by injection of a 10% iron chloride solution into the carotid artery, in mice and rabbits [65]. They found that these animals were protected from thrombosis when treated with 3F7 and did not exhibit any aberrant bleeding profiles when compared to saline and FXII-deficient animal controls. This same antibody was examined in an extracorporeal membrane oxygenation (ECMO) cardiopulmonary bypass system in rabbits. The ECMO system has high blood to surface contact and many components that can lead to platelet and contact pathway activation, contributing to greater incidence of clotting compared to other medical devices [160,161]. Larsson *et al.* found that their antibody significantly reduced platelet attachment and fibrin formation within the extracorporeal circuit, and animals were protected from bleeding when compared to heparin [65]. This study provides important evidence of the efficacy of contact pathway inhibition in medical device-induced clotting; however, it is difficult to predict performance of the same drug in humans using smaller animals. More recent work by Wallisch *et al.* extended the Larsson *et al.* study by

examining the effect of a neutralizing anti-FXII antibody, m5C12, in an ECMO model in baboons [66]. This work also showed a significant reduction in platelet attachment and fibrin formation within the ECMO system when compared to no treatment and to heparin treatment, without increased bleeding.

A role for FXII in stent-induced thrombosis is supported by our *in vitro* data showing that FXIIa activity is increased in the presence of a stent, in a purified system and in human plasma, when compared to the vehicle control. We also tested the effect of platelets on stents in FXIIa activity. There was a significant reduction in FXIIa activity in the presence of activated platelets and a downward trend in the presence of resting platelets. This is likely due to the presence of FXIIa inhibitors on the platelet surface. One known FXIIa inhibitor is histidine-rich glycoprotein (HRG), which circulates in plasma and resides in the α -granules of platelets [162]. Upon platelet activation, HRG is released and can remain associated with the platelet membrane, where it can bind to and inhibit different coagulation factors, including FXIIa. We saw a significant increase in FXIIa activity in the presence of stents compared to a plasma control; however, the variability observed in these studies suggests modulation of FXIIa by factors in plasma and from platelets.

To investigate the role of the contact pathway and potential mechanisms in stent thrombosis, we examined the effects of various contact pathway inhibitors (h5C12, m14E11, and m1A6) on fibrin clotting time *in vitro* in the presence of stents and stents seeded with platelets. We observed roughly a doubling of clotting time in control samples when plasma was inhibited with any contact pathway drug, which was similar to the results we saw in aPTT and ACT studies when treated with h5C12. In FXII- and FXI-depleted plasmas, fibrin clotting was completely prevented in control samples over the

hour-long study. These depleted plasmas were just as effective in prolonging clotting in the presence of a stent, indicating a strong role for the contact pathway in stent expedited fibrin clotting *in vitro*. However, when platelets are seeded onto the stent surface, fibrin clotting time was decreased in both inhibitor-treated plasmas and contact pathway factor depleted plasma, compared to the stent alone. These results indicate that the platelet surface is altering fibrin formation independent from the coagulation cascade. It has been recognized that the platelet surface can regulate thrombin production and activity via platelet membrane bound factors such as FVIIa and phosphatidylserine [163,164]. Therefore, it is likely that these factors on the platelet surface are upregulating thrombin production, thus, increasing the time to fibrin clotting.

Historically, the composition and structure of thrombi formed in the arterial and venous systems have been considered to be distinct [165]. Arterial thrombi form under high shear rates ($300\text{--}800\text{ s}^{-1}$ for large arteries) and have been described as “white” clots, with reference to a composition primarily of platelets and fibrin. Whereas, venous thrombi form under lower shear rates ($20\text{--}200\text{ s}^{-1}$) and have been described as “red” clots, with reference to a composition primarily of fibrin and red blood cells. This theory has been widely debated, and clinical data often contradicts the idea of distinct arterial and venous clot compositions [166–172]. Additionally, shear rate is known to play a role in platelet activation and aggregation, with high shear rates increasing these processes, and there is support that the efficacy of some antithrombotics, such as aspirin, is shear dependent [173]. Due to the impact of different shear rates on thrombus formation, we measured platelet accumulation and fibrin deposition onto nitinol stents at two different shear rates, 265 s^{-1} and 33 s^{-1} . Despite h5C12 having similar effects on aPTT and ACT assays to previous results [66], in current our work, we only saw a modest decrease in

platelet accumulation onto the surface of 4mm and 8mm stents in baboons with only the 8mm devices showing a significant statistical change. Fibrin deposition on stent surfaces varied widely in both the control devices and the h5C12-treatment group; however, there is a greater downward trend with h5C12 treatment in 8mm stents. These nuanced changes in the two major structural components of clots may be due to the limited range of shear rates explored. Since large arteries have a shear rate of roughly 550s^{-1} , we may need to investigate higher shear rates to see differences in platelet attachment, fibrin formation, and efficacy of h5C12.

We were interested in the effect of h5C12 treatment on platelet function after being exposed to 4mm and 8mm stents under whole blood flow. To measure PLAs, P-selectin expression, and lactadherin in whole blood, samples were collected from a sampling output just downstream of the stent deployed in the shunt. This previously described downstream sampling (DSS) [154,174] allows for collection of blood from a concentration boundary layer, where systemic factors circulating in blood are more concentrated than blood collected from a bulk draw or venipuncture draw. In all conditions, h5C12 was unable to abrogate the increase in PLAs and P-selectin expression. Therefore, it is unlikely that FXII is playing a role in platelet-leukocyte aggregation or in platelet P-selectin expression in the setting of stent thrombosis. We also measured PF4 in plasma spun down from whole blood collected from the DSS set-up and found that h5C12 treated blood was not different from vehicle control samples. This indicates that h5C12 treatment is unlikely to affect platelet activation leading to the release of PF4 in the environment of stent thrombosis. To examine the overall generation of thrombin, TAT complexes were measured in plasma from the DSS set up. There was a significant decrease in the quantity of TAT at the 60min time point, where

TAT concentration is the greatest in the control condition, in the presence of both 4mm stents and the 8mm stents. These data support that the h5C12 treatment is negatively affecting global thrombin production, which could account for the decrease in platelet attachment and propagation leading to thrombus tail formation from the stent surface.

Overall, the results of this study support an unassertive role for FXII in stent-induced thrombosis under physiological whole blood flow. We presented a functional characterization of a humanized anti-FXII neutralizing antibody, h5C12, and a systematic evaluation of the efficacy of the antibody in the context of stent thrombosis *in vitro* and under flow *ex vivo*. A limitation of these work is the lack of inclusion of a clinical pharmacological control such as heparin. Future work will include the examination of current clinical standards for preventing thrombosis during stent placement alongside with h5C12. Additionally, we will study the effects of inhibition of other factors of the contact pathway, namely FXI. Epidemiological data show that FXI deficiency in humans is thromboprotective and patients deficient in FXI exhibit mild bleeding abnormalities, typically in the context of trauma or major surgery [175,176]. Moreover, our *in vitro* fibrin clotting data suggest a greater role for FXI in stent-related clotting. Despite the humble effect h5C12 had on stent thrombosis, this work is a significant contribution to our knowledge of the role of the contact pathway in medical device-induced thrombosis.

Chapter V: Future directions

The studies conducted in this dissertation have examined the *in vitro* hemocompatibility of PVA hydrogels with different crosslinkers (Chapter II), characterized the surface properties and measured the *in vitro* and *ex vivo* hemocompatibility of a biomimetic PVA hydrogel (Chapter III), and tested the utility of a novel anticoagulation therapy targeting the contact pathway of coagulation in the setting of nitinol stent-induced thrombosis (Chapter IV). This work provides a foundation for future studies in hydrogel-based biomaterial and contact pathway-targeted pharmacological engineering. Future directions for this research would include 1) an *in vivo* assessment of GFPGER-modified PVA hydrogels and 2) investigation of inhibition of coagulation FXI in the setting of nitinol stent-induced thrombosis.

5.1 *In vivo* assessment of GFPGER-modified PVA

Chapter III of this dissertation shows the results of exposing PVA hydrogels modified with a collagen-like peptide, GFPGER, via immobilization to the hydrogel surface, to whole blood flowing at a physiologically relevant rate (100mL/min). Many studies have shown that PVA hydrogels alone do not accumulate a significantly high quantity of platelets, nor fibrin, after one hour of exposure to flowing whole blood *ex vivo* [72,73,80,116,117]. This was supported by the results seen in Chapter III, and the presence of the GFPGER peptide did not alter this hemocompatibility. However, our *ex vivo* assessment of the biomaterial is a simplified model compared to an *in vivo* environment. Additionally, the performance of the material is only tested in the setting of acute thrombosis within one hour. When a vascular graft device is implanted *in vivo*, the

material is not only exposed to whole blood but is also exposed to surrounding native tissues. It is important to investigate GFPGER-modified PVA graft performance *in vivo* to properly assess biological integration and neointimal hyperplasia formation.

Previous work by Yao *et al.* used an end-to-side implantation technique to insert unmodified PVA grafts into rabbits at the right carotid artery. They found that after one month of implantation, the PVA graft was only 40% patent, and ultrasound detection showed minimal to no blood flow through the grafts. Additionally, no cells were observed on the luminal surface of unmodified PVA grafts as shown by hematoxylin and eosin staining [92]. These data indicate a prothrombotic profile and poor biological integration of unmodified PVA after one month of implantation in rabbits. This work showed an improvement in PVA graft performance *in vivo* when the hydrogel was modified with a sulfated polysaccharide, fucoidan, that exhibited antithrombotic properties [92]. Due to the promising results of the GFPGER-PVA grafts we observed in our *ex vivo* shunt model, it is likely that this modification will support biointegration via the migration and adhesion of ECs onto the material. In future studies, GFPGER-PVA should be implanted into non-human primates using an end-to-side surgical technique for one month. Patency of the graft during implantation should be monitored using ultrasound. Upon explantation of the graft, H&E staining should be performed to assess biointegration and endothelialization of the lumen. Furthermore, H&E staining should additionally be used to assess the resolution of the foreign body response.

5.2 Inhibition of FXI in nitinol stent thrombosis

Chapter IV describes work that addresses the unmet clinic need of finding safer antithrombotic therapies that minimize unwanted bleeding in the setting of stent

thrombosis. The blood coagulation contact pathway is initiated by the activation of FXII into FXIIa, which subsequently activates FXI into FXIa. FXIa then goes on to activate factors in the common pathway, leading to thrombin and fibrin generation. Research has been focused on targeting the activity of these proteases, as they have no known role in normal hemostasis, meaning they are not required to form a blood clot upon vascular injury [177–180]. The roles of these factors have been extensively studied and have been thought to act as secondary promoters of blood coagulation. Similar to FXII-deficient patients, FXI-deficient patients have mild bleeding abnormalities and this typically occurs upon major trauma or surgery [181–183]. There has also been support that FXI and FXII play a large role in thrombosis [184]. In the *ex vivo* work performed in Chapter IV, we showed that inhibiting FXII and FXIIa had a modest effect on stent-associated thrombogenesis as measured by real-time platelet accumulation and endpoint fibrin deposition onto stents. To investigate the mechanism of clotting on stents, *in vitro* experiments were performed to measure the impact of inhibiting FXI and FXII on fibrin clotting in the presence of stents and stents pre-seeded with platelets. Our *in vitro* data showed that inhibition of FXI was slightly more effective at preventing fibrin clotting in the presence of stents and stents pre-seeded with platelets (**Fig 4.5**). When stents were placed in plasma that was completely depleted of FXI and FXII, fibrin clotting was delayed to a greater extent than the factor-inhibited plasma. FXI-depleted plasma had the longest delay in fibrin clotting time in the presence of stents and platelets, suggesting a greater role for FXI in clotting in the presence of platelets. These data support the use of an anti-FXI antibody in the setting of stent thrombosis.

5.2.1 Preliminary study shows h1A6 treatment decreases platelet attachment and fibrin formation on 4mm and 8mm stents

To test the hypothesis, inhibition of FXI will reduce platelet attachment and fibrin formation in acute stent thrombogenesis, we performed a single *ex vivo* study similar to that in Chapter IV. For this work, a humanized anti-FXII antibody, h1A6, was given to non-human primates at a dose of 0.5mg/kg 30min prior to stent thrombogenesis studies. Blood was drawn just prior to the study and coagulation tests were performed. aPTT and ACT clotting times were doubled in the h1A6-treated sample while PT was unchanged (n=5 for controls and n=1 h1A6, **Fig 5.1**).

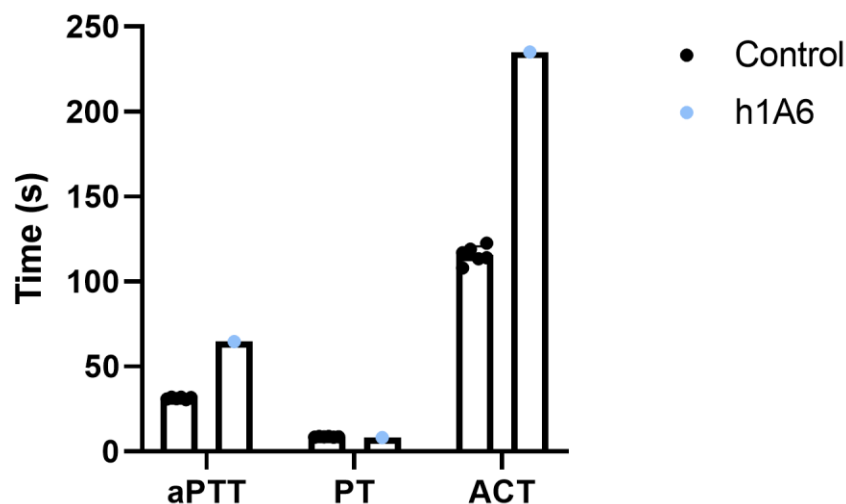


Figure 5.1: Coagulation measurements prior to stent thrombogenesis experiments.

Blood samples were collected and tested just prior to stent shunt studies. Administration of h1A6 increased both aPTT and ACT, with no change in PT observed (n=5 for controls, n=1 for h1A6).

We then tested 4mm and 8mm stents in our *ex vivo* model of acute thrombogenesis, with the same methods as in *Section 4.3.4.2*. We found that h1A6-treatment reduced platelet accumulation on the surface of 4mm and 8mm stents and that fibrin formation was also reduced on these surfaces compared to the control samples (**Fig 5.2**).

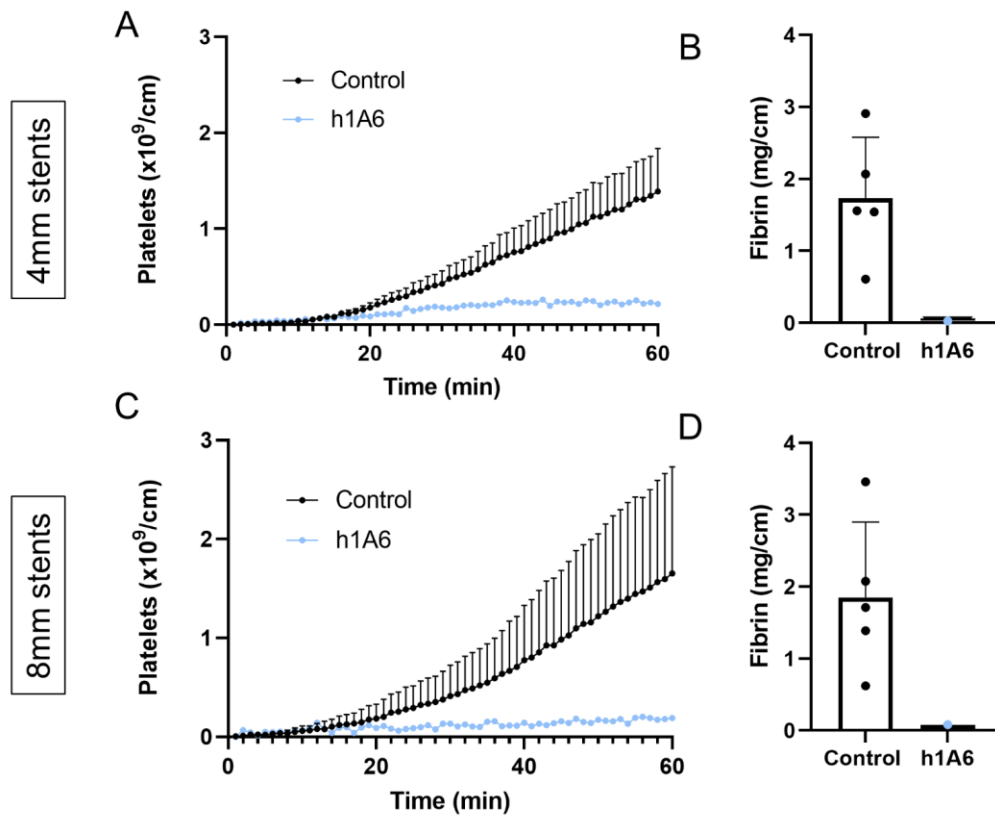


Figure 5.2: Whole blood *ex vivo* shunt testing of stent-induced thrombogenesis.

4mm and 8mm stents were tested in an NHP *ex vivo* shunt model with the stent alone being the control (n=5) and the h1A6-treated blood being test group (n=1). Platelet accumulation was quantified on (A) 4mm stents and (C) 8mm stents over 60min exposure to whole blood with and without h1A6 treatment. Fibrin data were quantified and normalized per centimeter of the axial length for (B) 4mm stents and (D) 8mm stents at 60min.

Visual examination (**Fig 5.3**) of stent samples post *ex vivo* testing confirmed the quantitative platelet and fibrin deposition results, as a decrease in thrombus on both stent samples was observed.

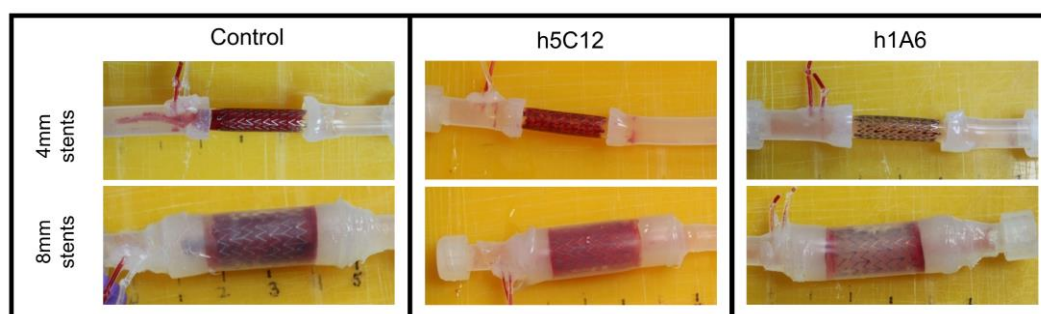


Figure 5.3: Modified from Fig 4.7C&F above. Representative images of thrombi formed on 4mm and 8mm stent surfaces were recorded by photograph.

There appears to be a reduction in overall thrombus formation in h1A6-treated samples.

FXI has the unique ability to be activated into FXIa by thrombin, independent of FXIIa activation of FXI, further driving the production of thrombin [185,186]. This activity contributes to local platelet activation by thrombin. Thus, FXII inhibition alone may not be effective in platelet-driven thrombosis processes. Polyphosphates, which are released by platelets, can enhance thrombin-mediated FXI activation [187]. Additionally, FXI activation has been shown to be enhanced in the presence of nucleic acids [188]. Given that FXI has been shown to contribute to additional mechanisms of promoting thrombosis, it is important to consider this coagulation factor as a therapeutic target. The preliminary results shown in this chapter, combined with those from Chapter IV, are promising and show a potential strong role for the contact pathway in acute stent-induced thrombosis.

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