SURFACE MODIFICATION OF A BLOOD CONTACTING POLYDIMETHYLSILOXANE MICROFLUIDIC OXYGENATOR

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Abstract

Respiratory distress syndrome (RDS) is an increasingly common complication associated with preterm and term neonates. Current treatment methods use harsh mechanical ventilation to provide sufficient oxygen, often leading to future complications such as bronchopulmonary dysplasia (BPD). Our lab group has conceptualized the 'artificial placenta', a microfluidic lung assist device (LAD) composed of a parallel array of single oxygenator units (SOUs). The LAD is connected to the infant by reopening the umbilical vessels, drawing blood through the pumpless LAD and back to the body, operating within the pressure drop provided by the heart.

The objective of the work reported in this thesis is to modify PDMS surfaces for use in our blood contacting microfluidic LAD. Requirements include: 1) the modification method must be simple and applicable to the complex geometry of the LAD, 2) the surface-modified device must prevent coagulation/thrombosis in blood contact.

In initial work PDMS discs were used to test the effectiveness of the surface-modified PDMS surfaces. PDMS was first modified with polydopamine (PDA) as a 'bioglue' to bond different anti-thrombogenic components to the surface. PDA has been shown to have strong adhesive properties analogous to those of mussels and can be coated on most materials using simple solution coating methods.

To create an anticoagulant surface, a novel covalent antithrombin-heparin (ATH) complex was used. Due to the method of synthesis all of the heparin molecules in ATH have at least one of the specific pentasaccharide sequences required for antithrombin (AT) binding, making it highly effective in the catalysis of thrombin and FXa inhibition by AT. PDMS discs were modified with ATH by incubating in ATH solution. Uptake (0.23 $\mu g/cm^2$) was at the monolayer level. The bound ATH was relatively stable in blood over a 72 h period with 71%

retention. In contrast ATH uptake on bare PDMS (no PDA) was lower (0.17 μg/cm^2) and 76% was removed in 72 h blood contact, suggesting much tighter bonding (presumably covalent) through PDA. The ATH-modified surfaces showed higher adsorption of antithrombin from plasma compared to controls, with ~20 ng/cm^2 of AT adsorbing on the PDMS-PDA-ATH surface compared to ~4 ng/cm^2 for PDMS and PDMS-PDA, suggesting that the heparin component of ATH on the surface retained its anticoagulant activity.

Single oxygenator units (SOU), as the basic components of the LAD device, were modified with ATH using the solution coating method, with PDA as bonding agent, developed for the PDMS discs. ATH uptake, stability and bioactivity were determined as for the discs, but under flow conditions more akin to those expected in clinical use. ATH was taken up in monolayer quantities (0.21 μg/cm^2) and was stable in flowing blood over two days with 76% of the original ATH remaining on the surface. The specific heparin activity of the modified SOUs in terms of AT adsorption from plasma was also similar to that of the PDMS-PDA-ATH discs, with uptake of ~48 ng/cm^2 compared to ~11 ng/cm^2 for PDMS-PDA SOUs. These results demonstrate the efficacy of ATH modification of the basic oxygenator units using PDA as bonding agent.

Modification of PDMS with components of the fibrinolytic system was also investigated with the objective of conferring clot destroying properties on the surface. This approach may be considered as an alternative to clot prevention via ATH modification. It is anticipated that by activating the fibrinolytic pathways on the surface, fibrin clots may be degraded as and when they form. To this end, modification of PDMS discs with tissue plasminogen activator (tPA) was carried out with PDA as bonding agent similar to modification with ATH. tPA was taken up from solution on PDMS-PDA surface in significant quantities (~1.6 μg/cm^2) and was stable in

plasma over 24 h with 70% (~1.1 μg/cm^2) of the original tPA remaining on the surface. Most significantly the tPA modified PDMS was shown to promote clot lysis in a turbidity-based plasma assay system.

It is anticipated that a surface modified with both ATH and tPA would show superior anti-thrombogenic properties by a combination of clot prevention and clot destruction.

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List of Abbreviations

AT	Antithrombin
ATH	Antithrombin-heparin
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
ECMO	Extracoporeal membrane oxygenation
FDP	Fibrin degradation product
Fg	Fibrinogen
GluPlgn	Glutamine-plasminogen
HK	High molecular weight kininogen
HSA	Human serum albumin
LAD	Lung assist device
LWMH	Low molecular weight heparin
LysPlgn	Lysine-plasminogen
MW	Molecular weight
NHS	N-hydroxysuccinimine
NO	Nitric oxide
PBS	Phosphate buffered saline
PDA	Polydopamine
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
Plgn	Plasminogen
Pln	Plasmin
PVDF	Polyvinylidene fluoride
PU	Polyurethane
RDS	Respiratory distress syndrome
SD	Standard Deviation

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SK	Streptokinase
SOU	Single Oxygenator Unit
tPA	Tissue plasminogen activator
UFH	Unfractionated heparin
uPA	Urokinase plasminogen activator
uPAR	Urokinase platelet activator receptor
Vn	Vitronectin

1. Introduction and Objectives

Respiratory distress syndrome (RDS) is a life-threatening disorder affecting newborn infants with underdeveloped lungs that cannot function outside the womb. RDS is among the leading causes of morbidity and mortality in preterm and term infants [1], [2]. Current clinical solutions involve surfactant replacement therapy, assisted ventilation, and extracorporeal membrane oxygenation, although the latter may only be conducted on infants of higher birth weight and is an intrusive process [3]. Therefore, assisted ventilation is a common practice with low birth weight infants. Advancements in the area of ventilation have been made, particularly with high frequency oscillation ventilation and continuous positive airway pressure, although the technology has seemingly plateaued [3]. Furthermore, assisted ventilation used for the treatment of RDS is associated with complications such as the increased risk of lung damage and subsequent bronchopulmonary dysplasia (BPD) [1], [3]–[6]. BPD is common in very low birth weight infants, as one in every three children with birth weight <1000 g who have developed RDS are diagnosed with BPD [5].

Diagnosis of BPD has changed throughout the twentieth and twenty-first centuries as a result of technological advances in mechanical ventilation. Preterm infants are able to survive at lower gestational ages and lower birth weights, facilitating an evolution in the definition of BPD [4,7]. In 1967, a BPD infant was defined as a preterm infant who received more than 150 hours of mechanical ventilation with a high concentration of inspired oxygen [7]. The typical gestational age was between 31-34 weeks. More useful indicators of BPD arose in 1979, with incidence of long-term lung disease suggested by the requirement for oxygen 28 days after birth. By 1988 oxygen use at 36 weeks gestational age was considered indicative of pulmonary disease within the first 2 years of life [7].

The incidence of BPD results in long term morbidity, with children experiencing decreased articulation, language skills, performance IQ, and gross and fine motor skills [8]. Moreover, 15% of preterm infants require at least one rehospitalisation within one year [6]. This equates to US $41 million annually, and the rate of rehospitalisation increases as birth gestational age decreases. As well, the US neonatal ventilator market was $165.1 million in 2012 and is expected to rise to $291.5 million by 2018 [9].

As an approach to the alleviation of infant respiratory distress syndrome, our collaborative research group has conceptualized a lung assist device (LAD) consisting of a microfluidic oxygenator that facilitates gas transfer in flowing blood. The benefits are twofold: oxygen is provided for infants who cannot breathe sufficiently on their own, and the lungs are able to further develop, thereby reducing the risk of rehospitalisation and future complications like BPD. The LAD could be used in conjunction with surfactant replacement therapy or other therapies.

The basic concept, which can be referred to as an “artificial placenta”, is to reopen the umbilical vessels of the infant, allowing blood to pass from the artery through the LAD and back into the body through the vein. Blood passes through the LAD using the natural pressure drop of the heart and no external pump is used. A number of requirements must be met in such a system. First, to avoid overstressing the heart, the volume of blood outside the body cannot exceed 10 mL/kg of body weight (~10%, typically 8-10 mL) [3]. Therefore a microfluidic device, implying a very small holdup volume, is required. The LAD must also be able to sufficiently oxygenate the blood. Polydimethylsiloxane (PDMS) is a material with excellent gas transfer characteristics, and is readily fabricated into complex shapes such as are encountered in microfluidic devices. Accordingly, PDMS was used as the base material for LAD fabrication in this project [10].

Because blood is in direct contact with PDMS in the device, the PDMS surface must be "blood compatible", and in particular blood coagulation and thrombus formation must be avoided. This requirement necessitates modification of the blood contacting PDMS surface.

Blood contact with foreign materials initiates the coagulation cascade through clotting factor activation, and in combination with platelet adhesion and aggregation can lead to thrombus formation [11]. The most common approach to the prevention of coagulation is to attach anticoagulants such as heparin and hirudin to the surface [12]. Heparin molecules that contain a specific pentasaccharide sequence bind to antithrombin (AT), the natural inhibitor of activated clotting factors thrombin (factor IIa) and factor Xa. The AT undergoes a conformational change which allows it to bind and inhibit IIa and Xa very rapidly [13], [14]. The activity of AT (rate of thrombin inhibition) is increased >1000-fold in the presence of heparin [15]. Many methods for surface modification using heparin have been proposed [16-18]. However a serious limitation is that only one third of the heparin in commercially available preparations contains the specific pentasaccharide sequence required for AT binding [19], [20]. To overcome this limitation an antithrombin-heparin (ATH) covalent complex has been developed in which all of the heparin molecules contain at least one pentasaccharide, and it has been shown that, when bound to various surfaces, the ATH complex has greater thrombin inhibiting activity than heparin alone [20]–[22]. ATH has been used in the work reported in this thesis for the modification of PDMS.

Another approach to coagulation-resistant surfaces is through activation of the body's clot lysing or fibrinolytic system. This is the system which acts in hemostasis to break down the hemostatic plug (including clot) when, following vessel repair, it is no longer needed. In fibrinolysis plasminogen activators such as tissue plasminogen activator (tPA) convert the

zymogen plasminogen into its active form, plasmin, which rapidly lyses blood clots by degrading the fibrin mesh [23]–[25]. Modifications of blood contacting surfaces using plasminogen or tPA have the potential to effectively remove any thrombus that forms on the surface.

In previous research a number of methods have been used to modify PDMS surfaces such as hydrosilylation, oxygen plasma treatment, and chemical grafting [26]. Because our LAD is a complex microfluidic device a simple modification method that does not expose the surface to harsh organic solvents is desirable. Polydopamine (PDA) is a recently discovered "bio-glue" with strong adhesive properties analogous to those of mussels. When dopamine in aqueous solution is subjected to alkaline conditions, oxidation occurs and self-assembled layers of PDA form on surfaces, organic or inorganic, in contact with the solution [27]. The PDA has strong adhesive properties that can be exploited for surface functionalization.

The objective of the work reported in this thesis was to modify PDMS surfaces using methods that can be applied to a microfluidic LAD. The specific surface modifications used aimed to reduce thrombogenicity by attachment of ATH, and promote fibrinolysis by attachment of tPA and plasminogen. PDA was used as a 'bioglue' to bond the biomolecules to the PDMS. Surface chemical and physical properties, durability/stability of the modified surfaces in blood, and interactions of the surfaces with blood proteins relevant to the intended functions are reported.

2. Literature Review

2.1 Development of a Microfluidic Oxygenator

A leading cause of neonatal mortality and morbidity is respiratory insufficiency, leading to diagnosis of respiratory distress syndrome (RDS). RDS occurs in infants with insufficient or dysfunctional pulmonary surfactant, resulting in collapsed alveoli and hypoxic conditions [28]. Furthermore, the likelihood of RDS increases in preterm infants with very low birth weight. Between 2010 and 2011, 7.1% of all infants in Canada were born prematurely (<37 weeks gestation) with 6.1% of all infants considered very low birth weight (500-2499 g) [29]. Equivalently in the US in 2014, 1 in 10 infants was born prematurely [30].

Developments in neonatal ventilators and surfactant replacement therapy have increased the survival of low birth weight infants experiencing RDS, but ventilation is often associated with bronchopulmonary dysplasia (BPD) [7,8]. One in three infants in the US diagnosed with RDS develop BPD, resulting in long term morbidity that includes decreased language skills and articulation, performance IQ, and motor skills [8].

Extensive research in mechanical ventilation has aided the development of high frequency oscillation ventilation and continuous positive airway pressure to mitigate the damage caused to the infant, but the technology has seemingly plateaued [3]. Another approach to RDS treatment is extracorporeal membrane oxygenation (ECMO). In this technique blood is pumped through an extracorporeal oxygenator to facilitate gas exchange in the blood. ECMO is often used as a last resort since the procedure is highly invasive and can be performed only on infants weighing >2000 g [3].

In a 2014 review article, Potkay outlines the potential for artificial lungs using micrometer-scale blood channels over traditional ventilation or ECMO for treatment of acute

respiratory distress syndrome [31]. While not all of these devices are intended for neonatal use, similar design and manufacturing techniques are evident. Soft lithography is a commonly used technique to create the microchannel body that is subsequently bound to a gas transfer membrane [32], [33]. Gas transfer occurs primarily across the membrane.

Commercial oxygenators are mainly of the hollow fibre type. However, microfluidic artificial lungs are superior to hollow fibre designs because of the potential for increased oxygen transfer, low blood priming volume, and uniform flow distribution [31]. Flow distribution through hollow fibre oxygenators enhances mixing and consequently gas exchange but is non-uniform and subjects the blood to non-physiological conditions. Although not yet clinically used, the potential for artificial lungs based on microfluidic blood channels is high.

Similar to the artificial lung, the artificial placenta concept is that of a lung assist device (LAD) for blood oxygenation specifically of low birth weight infants. The LAD oxygenates blood taken from and returned to the infant using the umbilical vessels, thus mimicking the oxygenation function of the placenta. Oxygen is transferred from air to the blood across a thin membrane. A number of design constraints must be taken into account. First, access to the umbilical vessels is required [2], [3]. Second, the LAD must be driven by the natural pressure drop of the heart. Third, it must have a low priming volume to minimize extracorporeal blood volume and avoid cardiac high-output failure when priming with saline. Therefore a microfluidic device is appropriate. Although the lungs of the infant are still developing, some lung function is still available and the LAD is not required to be the sole provider of oxygen; the gas exchange properties of the device (membrane material, surface area) can thus be varied depending on the needs of the infant. Finally, the blood contacting surfaces of the LAD must be non-thrombogenic to avoid the necessity for systemic anticoagulation [3].

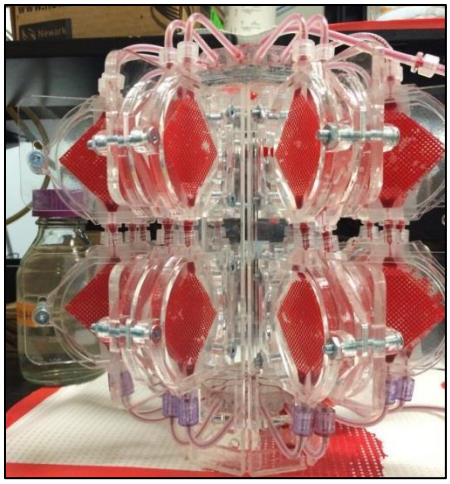

Figure 1 - The Lung Assist Device. An iteration of the LAD, composed of a parallel array of oxygenator units.

The LAD used in the present work was designed as a parallel array of oxygenator "units" (Figure 1) [2]. A single oxygenator unit (SOU) consists of a grid of microfluidic channels formed by pillars "attached" to the unit body (Figure 2). The original design was such that one oxygenator unit could provide oxygen for 0.1 kg of body weight (i.e. a 1 kg infant would require an LAD having 10 oxygenator units) [2]. In subsequent design iterations the oxygenation capacity has been modified, but the basic concept remains.

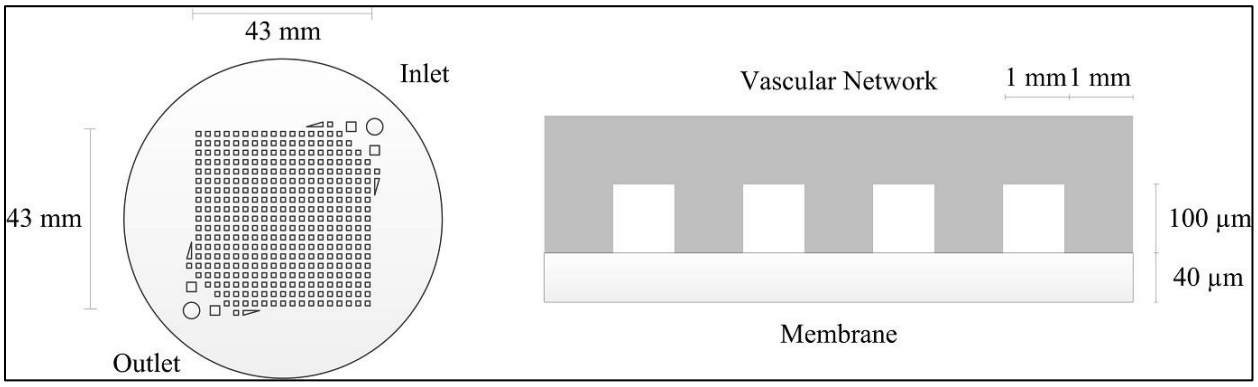

Figure 2 - Schematic of an Oxygenator Unit. The PDMS "vascular network" contains channels formed by pillars. Oxygen plasma treatment is used to facilitate bonding of the membrane to the pillars [34].

Polycarbonate and polydimethysiloxne (PDMS) were investigated as gas transfer membranes [10]. The rates of oxygen and carbon dioxide transfer were highest for porous PDMS membranes. The LADs with this membrane performed better than commercial hollow fiber oxygenators by as much as 3.5-fold; however, porous PDMS was not pursued due to the tendency to form "dead" zones in the oxygenator units with this membrane. Therefore, PDMS, with much higher gas transfer rates compared to PC, was chosen as the membrane for the LAD.

It was desirable to know whether the surfaces of the PDMS oxygenator units could be coated uniformly by contact with aqueous solutions. This question was investigated by measuring human serum albumin (HSA) adsorption [2]. The adsorption of HSA (by physical mechanisms) was shown to be uniform with approximately monolayer coverage.

In previous work surface modification of PDMS with ATH using both physical adsorption and covalent attachment with PEG as a linker/spacer was investigated [35]. ATH attached through physical adsorption could be removed by sodium dodecyl sulfate (SDS), a strong protein eluent. However, covalently attached ATH was much more stable with >80% remaining on the surface after SDS treatment. Nonetheless the particular process used for covalent attachment was complex and required the use of harsh chemical reagents; as such it was considered not suitable for use with the microfluidic device. Therefore a new approach to ATH surface modification was investigated in the work of this thesis.

2.2 Blood Contacting Biomaterials

Blood contacting biomaterials are required for many important applications including intravascular stents, prosthetic heart valves, hemodialysis systems, catheters, and bypass circuits [20]. Biocompatibility is important and is dependent on the desired application and the surrounding environment [11]. In the case of blood contacting devices the material should not

cause blood coagulation, thrombosis, infection or immune response, and should not be degraded by blood contact. Avoidance of coagulation and thrombus formation has so far proved to be the most difficult of these requirements [36]. Biomaterials used for blood contacting applications must be able to interact with blood in such a way that a thrombus does not form, or that the thrombus is rapidly degraded if it should form.

2.2.1 Protein Adsorption

The adsorption of proteins is the first significant event following blood contact with biomaterial surfaces [37], [38]. Protein adsorption is the precursor to subsequent adverse effects that include plasma coagulation, platelet adhesion, platelet activation, immune responses including complement activation, and inflammation. Understanding and thereby being able to control protein adsorption as the initiating event would seem to be of fundamental importance.

2.2.1.1 Surface Modification to Control Protein Adsorption

Over many decades, studies have yielded trends pertaining to the characteristics of blood-material interfaces. Surface interaction with water is of high importance in blood-contacting biomaterials since water is a main component of blood. It is widely believed that hydrophilic surfaces show lower non-specific protein binding than hydrophobic ones. Moreover, adsorption on hydrophobic surfaces is essentially irreversible [11], [39]. Hydrophobic surfaces also cause hemolysis and promote platelet adhesion to a greater extent than hydrophilic surfaces [39].

Surface modification of biomaterials to avoid protein adsorption can be accomplished using a variety of strategies. Modification with polyethylene glycol (PEG) is a common approach. PEG incorporation increases surface hydrophilicity and thus decreases overall protein adsorption and cell adhesion [40]. Many techniques have been used for PEG modification including surface grafting, simple adsorption, and covalent attachment [40]. Important PEG

properties that affect protein resistance are chain length and functional end group [41]. PEG can also be used as a linker-spacer to attach bioactive molecules to the surface. For example, hirudin was immobilized on gold via PEG to create a surface that inhibits the procoagulant enzyme thrombin and is also generally protein resistant [12].

Materials modified with zwitterionic polymers have also been shown to be protein resistant. Zwitterionic-based biomaterials including betaines such as poly(carboxybetaine) or poly(sulphobetaine) are capable of interacting with water through hydrogen bonding and have the ability to immobilize desired biomolecules through functional groups [42]. For example, poly(carboxybetaine) contains carboxylate-anion groups that may readily interact with biomolecular amino groups. Zwitterionic surfaces have shown low non-specific protein adsorption, decreased platelet deposition, and in-vitro corrosion resistance [42], [43]

Another aspect of controlling protein adsorption is promotion of the adsorption of specific proteins thus providing specific bioactivity. Examples include the inhibition of coagulation through modifications using heparin, hirudin (which, respectively adsorb antithrombin an thrombin) and other antithrombotic molecules, or modification with lysine to promote fibrinolysis through adsorption of plasminogen and tissue plasminogen activator [40], [41].

2.2.2 Coagulation

A major requirement for a blood contacting surface is to avoid the activation of coagulation [36], [39]. Prevention of coagulation has proved to be very difficult and has yet to be achieved despite many years of intensive research.

2.2.2.1 Coagulation Cascade

Blood coagulation involves complex sequences of enzyme-substrate reactions involving cascades and feedback loops. Traditional descriptions of coagulation are in terms of the intrinsic and extrinsic pathways, also known as the contact factor and tissue factor pathways, respectively. These initiating pathways eventually converge to a "common" pathway, leading ultimately to fibrin formation. An overview of the coagulation pathways is shown in Figure 3.

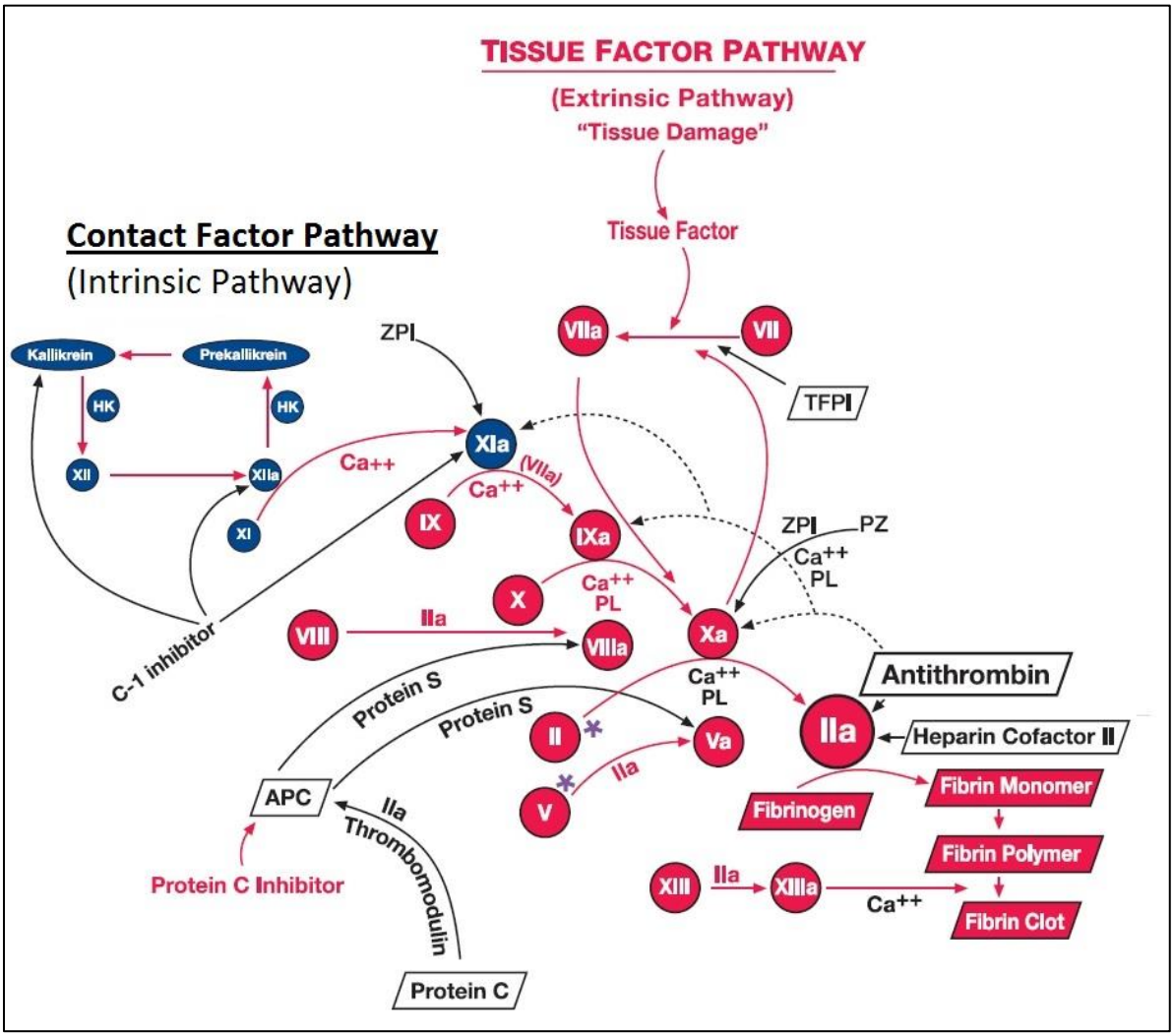

Figure 3 - Overview of the Coagulation Pathways. Adapted from [44].

The intrinsic pathway of coagulation begins with activation of FXII to its enzymatically active form FXIIa [45]. This occurs through a complex set of interactions among FXII, prekallikrein and high molecular weight kininogen (HK) involving reciprocal activation between kallikrein and FXII (Figure 3). FXIIa then converts the zymogen FXI to FXIa, and the cascade continues through FIXa, FXa, and thrombin, to fibrin formation.

The extrinsic pathway is activated upon damage to vascular tissue, e.g. endothelial cells, releasing tissue factor (TF) which activates circulating FVII to FVIIa. The TF-FVIIa complex proteolytically cleaves FIX to FIXa and factor X to FXa. FIXa works in conjunction with non-enzymatic FVIIIa to increase conversion of FX to FXa.

Factor X activation occurs via both pathways. In the resulting common pathway FXa interacts with the non-enzymatic cofactor FVa to form the prothrombinase complex consisting of FXa, FVa, Ca^{2+} and negative phospholipids on cell (e.g. platelet) surfaces. The prothrombinase complex converts the zymogen prothrombin (FII) to thrombin (FIIa) on cells expressing exposed TF [23]. FXa is also capable of dissociating from the prothrombinase complex allowing interaction with cofactors exposed on neighbouring cells.

2.2.2.2 Thrombin

Thrombin (FIIa) is a 35.5 kDa serine protease and is a key player in blood coagulation [46]. The zymogen form prothrombin has a molecular weight of 70 kDa and contains an N-terminal Gla domain, two kringle domains, and an active serine protease domain that includes the C-terminal [46], [47]. Prothrombin undergoes multiple post-translational modifications before conversion to active thrombin [46]. First, removal of a signal peptide from the N-terminal exposes the Gla domain, which is then modified by γ-glutamyl carboxylase. Following modification thrombin is formed from prothrombin by liberation of the active serine protease at

Arg271 from the Gla-domain and the two kringle domains. Thrombin cleaves Arg-Gly bonds in sequences GlyGlyGlyValArg-GlyPro and PhePheSerAlaArg-GlyHis in fibrinogen resulting in the release of fibrinopeptides A and B, respectively [46], [48]. Intermolecular bonds then form between fibrin molecules creating a fibrin mesh.

The ability to regulate thrombin is key to regulating coagulation. Thrombin amplification occurs rapidly and is a result of multiple feedback loops working together to covert prothrombin to thrombin (Figure 4). Thrombin provides positive feedback in the generation of FVIIIa, which in complex with previously formed FIXa converts FX to FXa. Thrombin also provides positive feedback in the conversion of platelet-derived FV to FVa thus increasing the prothrombinase activity of the prothrombinase complex.

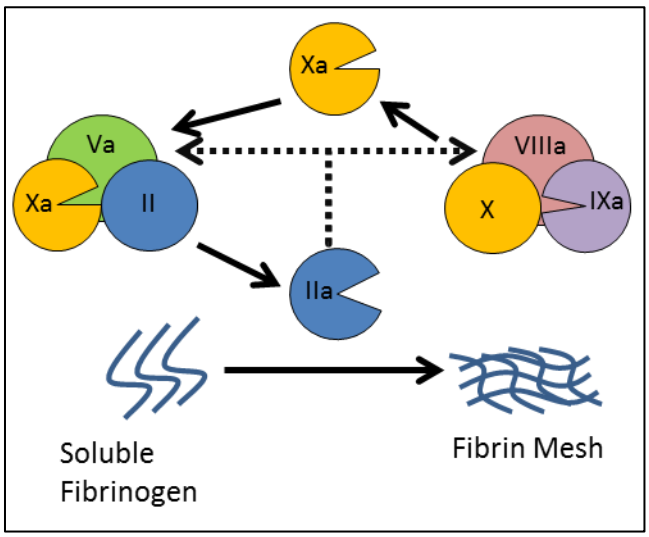

Figure 4 - Thrombin Amplification. Thrombin is involved in feedback loops which rapidly enhance thrombin generation increasing the rate of coagulation. Adapted from [49].

Inhibition of thrombin is the target of several anticoagulant drugs including dabigatran, and indirectly heparin [50], [51]. Other drugs inhibit vitamin K, or inhibit thrombin indirectly by

targeting FXa. Examples are the vitamin K antagonist warfarin and the FXa inhibitors rivaroxaban or edoxaban [52].

2.2.2.3 Fibrinogen

The major role of thrombin in the coagulation cascade is the conversion of soluble fibrinogen to insoluble fibrin. Fibrinogen is a large protein of molecular weight 340 kDa consisting of three pairs of peptide chains, the Aα, Bβ, and γ-chains, held together by disulfide bridges (Figure 5).

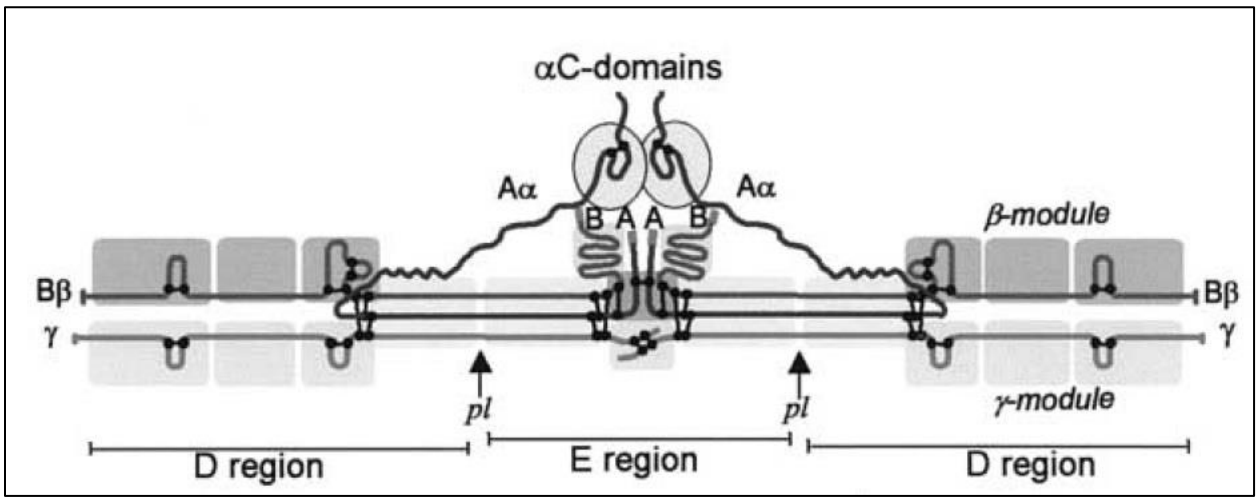

Figure 5 - Structure of Fibrinogen. Fibrinogen molecule showing homologous pairs of chains, Aα, Bβ, and γ. Plasmin cleaves fibrin(ogen) in the D-domain marked pl. Adapted from [53].

The three pairs of chains form a dimer that consists of two identical subunits [23], [53], [54]. Peptides and subunits are connected through disulfide bonds. The structure may be viewed in terms of four main sections: two identical C-terminal D-domains, a central E-domain containing all six N-terminals including fibrinopeptides A and B, and an αC-domain consisting of the Aα C-terminals (Figure 5).

Thrombin cleaves fibrinogen at the N-terminal of the Aα and Bβ chains, resulting in the release of short chain N-terminal fibrinopeptide A (16 amino acid residues) and fibrinopeptide B (14 amino acid residues). Upon release of the fibrinopeptides, fibrinogen is converted to a fibrin monomer. Fibrin monomer molecules then engage intermolecularly in a "knob/hole" interaction, with E domains and D domains of individual fibrin molecules forming a long chain fibrin protofibril [53], [55]. The E and D domain interactions between fibrin molecules are shown in Figure 6, and are driven by self-assembly.

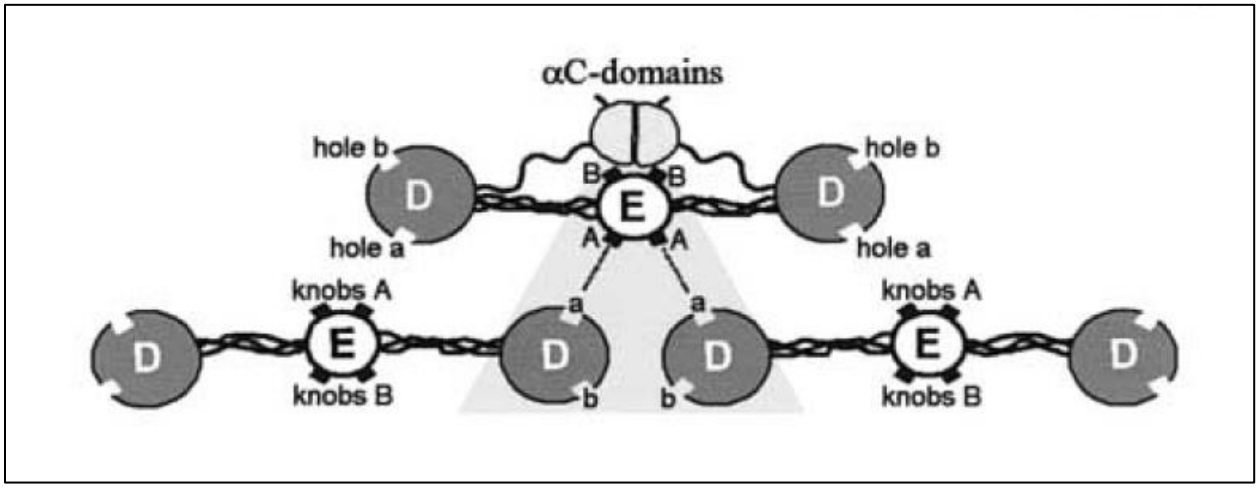

Figure 6 - DD:E knob-hole interaction between fibrin molecules. Upon release of fibrinopeptides A and B, fibrin molecules interact between the D and E domains in a knob/hole interaction to form a fibrin protofibril. The αC-domains in fibrinogen interact intramolecularly, and in fibrin intermolecularly. Adapted from [53].

Fibrin polymer stabilization is completed by crosslinking of the protofibrils and is dependent on activated factor XIII (FXIIIa) for this interaction. Factor XIII is cleaved by thrombin to form FXIIIa, a plasma transglutaminase in which calcium-dependent reactions form isopeptide bonds between specific γ-carbamoyl groups of glutamine residues and ε-amino groups of lysine residues [53]. Between fibrin(ogen) molecules, the C-terminal γ-chain of one molecule interacts with two adjacent γ-chain glutamine residues of another molecule forming a covalent

bond. The same bond can be formed between the C-terminal αC-domains, and between α- and γ-chains. Crosslinking is irreversible due to the covalent nature of the bonds [56].

2.2.2.4 Platelets

Platelets are non-nucleated blood cells that are crucially involved in blood coagulation-thrombosis via the catalysis of several steps in the coagulation cascade and by forming aggregates. Platelets are not activated under normal conditions in the circulation, but upon endothelial damage collagen and von Willebrand Factor in the subendothelium are exposed and promote platelet adhesion and subsequent activation [23]. Thrombin and ADP aid in platelet activation. Activated platelets secrete granules that contain coagulation components fibrinogen and HK, as well as ADP that further propagates platelet activation [57].

It is well established that platelets adhere readily to blood contacting materials. Adhesion occurs through interactions between receptors on the platelet surface and specific amino acid sequences (e.g. RGD) in adsorbed proteins, including fibrinogen, fibronectin, vitronectin, and von Willebrand factor (vWF) [58]. The glycoprotein receptors GPIIb/IIIa and GPIb/IX are of particular importance [59]. When platelets are activated, the GPIIb/IIIa receptor undergoes a conformational change and binds to RGD sequences in adsorbed proteins. To resist platelet adhesion and activation, surfaces that release nitric oxide (NO) have been developed. NO inhibits platelet activation and aggregation and has been shown to reduce thrombotic events on various biomaterials, including polyurethanes (PU) and silicones [40], [60], [61].

2.3 Inhibition of Coagulation

Coagulation is an essential physiologic process allowing damaged blood vessels to heal without loss of blood or reduction in blood flow in the circulation. However, coagulation is also a causative factor in disease states such as pulmonary embolism and deep vein thrombosis, and

of direct relevance to the work reported in this thesis, on the surface of blood contacting materials.

A number of molecules are available as inhibitors of coagulation. Anticoagulants can be administered orally, or injected intravenously or subcutaneously. Oral anticoagulants include the vitamin K antagonist warfarin, as well as non-vitamin K antagonist anticoagulants that directly inhibit thrombin or FXa [52]. Injected anticoagulants include unfractionated heparin (UFH), and low molecular weight heparins (LMWHs) ranging in molecular weight from 1.8-12 kDa [19], [20]. Heparin acts by increasing antithrombin (AT) activity by >1000-fold, and is used extensively in the treatment of cardiovascular disease [15], [20].

2.3.1 Natural Anticoagulants

A number of "natural" anticoagulants are present in the body. Activated protein C in the blood, for example, forms a complex with protein S to cleave non-enzymatic cofactors FVa and FVIIIa, inhibiting tenase activity for which FVIIIa is essential and prothrombinase activity for which FVa is essential [45]. Other natural anticoagulants include tissue factor pathway inhibitor, a Kunitz-type inhibitor, and antithrombin, a serine protease inhibitor. Antithrombin is among the most effective coagulation inhibitors due to its specificity for thrombin, and is discussed further in the following section.

2.3.2 Antithrombin

Antithrombin (AT) is a member of the serpin (serine protease inhibitor) family and acts to inhibit FIXa, FXa, FXIa, FXIIa, and most importantly, thrombin, in an irreversible manner [20], [45].

AT is synthesized in the liver and circulates in plasma at a concentration of approximately 0.15 mg/mL with a half-life of 3 days [62]. The 58 kDa molecule contains 432

amino acids and three disulfide bonds. The structure of AT is similar to that of many other serine proteinase inhibitors, such as α_1-proteinase inhibitor, α_2-antiplasmin, and heparin cofactor II [15]. Several forms of AT exist depending on post-translational glycosylation [20]. AT has four N-glycosylation sites with the amino acid sequence Asn-Xaa-Ser/Thr [63]. Differences in saccharide branching and terminal N-acetylneuraminic acid residue substitution contribute to the variety of AT molecules [20]. Human AT has α- and β- forms, which are equivalent except for a glycosidically linked carbohydrate side chain at Asn135, which is not present in β-AT. Due to the lack of glycan in β-AT, heparin-binding affinity is increased compared to α-AT [13], [20], [63].

AT is among the more effective anticoagulants since it acts directly on thrombin; however, the antithrombin-thrombin reaction is slow. When AT is complexed with heparin, the rate of inhibition of thrombin is increased 1000-fold and of FXa 300-fold [15], [64].

2.3.3 Heparin

Heparin is a straight chain glycosaminoglycan commonly used as a systemic anticoagulant. Also medical devices such as stents, catheters and heart valves modified with heparin have been used clinically [18], [65]. Heparin is composed of repeating dissacharide units of 1-4 linked α-D-glucosamine (predominantly N-sulfated) and uronic acid (α-D-iduronate), as shown in Figure 7 [65]. Commercial unfractionated heparin is derived from natural heparin but has varying chain lengths from 5-30 kDa. The result is a mix of heparin molecules with an average molecular weight of 20 kDa.

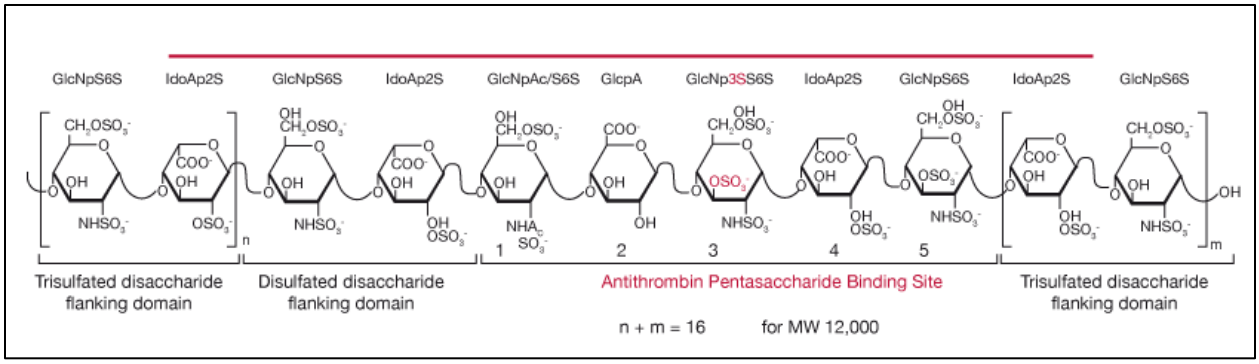

Figure 7 - Structure of Heparin. Heparin is composed of a repeated disaccharide unit. One-third of commercial unfractionated heparin contains at least one pentasaccharide sequence required for antithrombin interaction. From Heparin Science [http://heparinscience.com/structure_heparin.html].

Heparin is an effective anticoagulant due to its ability to bind AT and heparin cofactor II, thereby greatly accelerating the inhibition of various coagulation factors including thrombin [65], [66]. Binding is through a specific pentasaccharide sequence found on approximately one-third of heparin molecules in commercial UFH preparations (Figure 7). For inhibition of FXa, the pentasaccharide sequence alone suffices largely; however, for thrombin inhibition longer heparin chains are required due to the fact that they must bridge across both AT and thrombin in forming the complex (Figure 8) [19], [20].

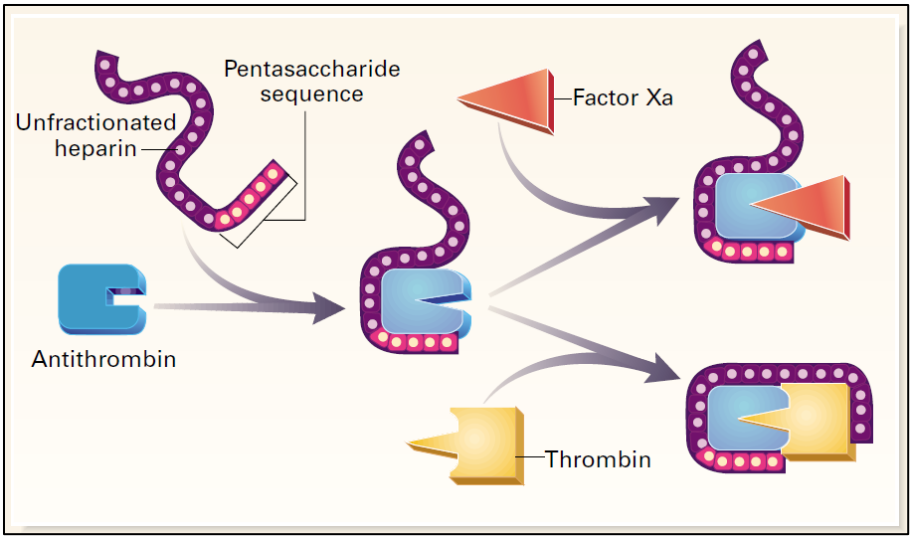

Figure 8 - Heparin Pentasaccharide Sequence. Heparin facilitaties antithrombin interaction using a specific pentasaccharide sequence found on one-third of heparin molecules in unfractionated heparin. Only the pentasaccharide sequence is required for catalysis of FXa inhibition. Catalysis of thrombin inhibition requires the pentasaccharide sequence as well as a longer chain heparin molecule that can bind both antithrombin and thrombin [19].

2.3.3.1 Surface Modification using Heparin

Heparinized surfaces have been shown to reduce thrombogenicity, platelet activation and adhesion, and activation of the complement system [17]. In the earliest heparinized surface going back to the 1960s, the heparin was attached via electrostatic interactions taking advantage of the high negative charge on heparin. A graphite substrate was first coated with positively charged benzalkonium chloride followed by heparin [13], [16]. Heparin bound in this manner was found to be easily lost by ion exchange upon exposure to blood [67].

Many methods of attachment have been devised over the intervening years. Two popular commercial heparinized surfaces are the Duraflo II and the Carmeda® BioActive Surface [68]. The Duraflo II material is based on a heparin–surfactant complex that is soluble in organic solvents. The key attribute of this approach is the simple modification procedure, i.e. coating from solution, enabled by the organic-soluble form of the heparin. The Carmeda® BioActive Surface uses so-called end-point covalent attachment of heparin to create a surface in which the

pentasaccharide sequences are preserved and activity is maximized. In this modification the surface is primed with alternating cationic and anionic polymer layers. The heparin is modified with a reactive aldehyde group that forms a Schiff base by reaction with the amino groups in the priming polymers. The Schiff base is then reduced to give stable covalent bonds to the surface [69], [70].

Many other heparin attachment techniques have been reported, for example through the use of linker/spacer molecules such as PEG or chitosan, or by activating the substrate by treatment in an oxygen plasma [18], [35]. Heparinized surfaces have been used in heart valves, cardiopulmonary bypass circuits, and stents, among other applications [16], [18]. It is generally believed that heparinized surfaces decrease surface thrombogenicity [68], although clinical experience with heparinized systems has been mixed [71].

2.3.3.2 Limitations of Heparin

Although heparin is widely used for systemic anticoagulant therapy, it does have limitations. It is produced in the mucosa of the intestine and lung. Commercial UFH is derived from natural heparin and has a distribution of chain length in the range of 5-30 kDa [20]. Furthermore, only one-third of heparin molecules in UFH have the pentasaccharide sequence required for interaction with AT [72].

UFH has an intravenous half-life in the range of 18-60 min and is removed by interaction with hepatic cells in the liver and by glomerular filtration in the kidneys [20]. Therefore, maintenance of heparin level requires continual infusion [21]. The low and variable half-life of circulating UFH limits its effectiveness and may lead to adverse anticoagulant responses [20], [73]. Furthermore, in a small number of patients heparin use can cause thrombocytopenia and long term use may lead to osteoporosis [21].

The limitations of UFH led to the development of LMWHs, such as nadroparin, enoxaparin, and dalteparin. These are formed by depolymerization of UFH, resulting in LMWHs having higher circulating half-lives and decreased non-specific interactions with plasma proteins [73]. LMWH molecules are eliminated from the body only by excretion through the kidneys, whereas UFH may bind to other proteins, or be otherwise processed by the liver or kidneys. LMWH has a lower associated risk of hemorrhage than UFH; however, inhibition of thrombin can be variable and overdose cannot be fully reversed.

2.3.4 Antithrombin-Heparin Covalent Complex

A novel covalent antithrombin-heparin (ATH) complex with significantly higher anticoagulant activity than UFH has been developed by Berry et al [21]. The half-life of ATH in the circulation is 2.7 days, i.e., substantially higher than that of UFH. The reaction rate of ATH with thrombin is faster than that of UFH and AT separately because the rate-limiting UFH–AT binding step is not required [72]. Also all of the heparin molecules in ATH have at least one pentasaccharide sequence. Studies comparing the activity of ATH to a simple solution of UFH and AT have shown the superior anticoagulant activity of ATH [21], [22], [74]–[76].

The mode of action of ATH is based on two mechanisms: direct inhibition of thrombin by interaction with the AT moiety and indirect, catalytic activity by interaction with the heparin moiety. ATH reacts directly with thrombin in such a way that an intermediate non-covalent complex is formed, which is then transformed into an irreversible covalent complex [22]. Fibrin-bound thrombin is also inhibited by ATH at a much faster rate than by UFH and AT separately [74]. The rate of inhibition of thrombin by ATH was 2-3 fold lower in the presence than in the absence of fibrin, whereas the comparable reduction for a mixed solution of AT and heparin was

57-fold. It was shown that ATH interacts with fibrin such that inhibition of fibrin-bound thrombin goes through a thrombin-ATH complex that remains bound to fibrin.

ATH has the ability to access and inhibit FXa in the prothrombinase complex as demonstrated on artificial phospholipid surfaces, activated platelet surfaces, and activated red blood cell surfaces [77], [78]. Mixed UFH and AT solutions under the same conditions showed substantially slower reactions rates of FXa inhibition, suggesting the covalent linkage in ATH is critical for access and inhibition of surface bound FXa incorporated in the prothrombinase complex [79].

2.3.4.1 Synthesis of Antithrombin-Heparin Complex

The basic 2-stage process for ATH synthesis is shown in Figure 9. In order for the AT-H covalent bond to form, heparin requires a pentasaccharide sequence and a terminal aldose. In the first, slow stage, electrostatic interactions and hydrogen bonding occur between AT allosterically and the heparin pentasaccharide sequence, giving a noncovalent complex with a highly active form of AT. In the second stage the terminal aldose in heparin binds to an amino group in AT forming a Schiff base which is reduced with $NaBH_3CN$ resulting in a covalent complex containing a stable covalent AT-H bond [20].

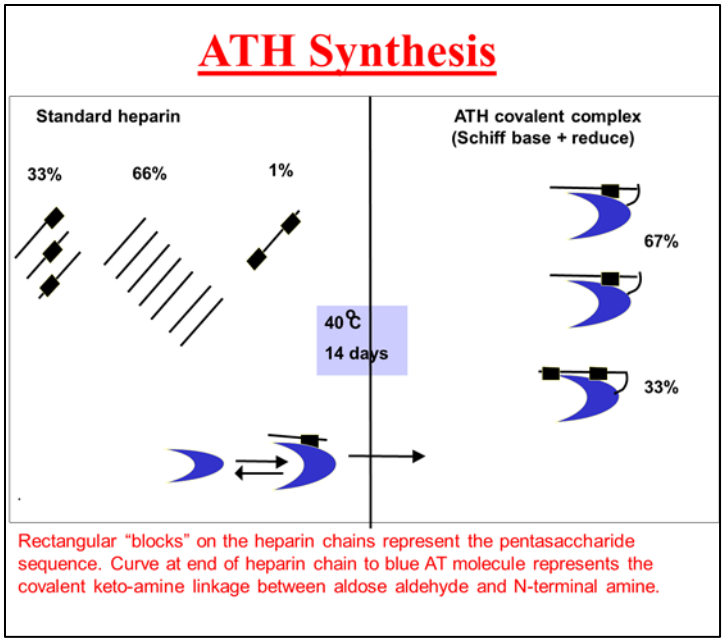

Figure 9 - ATH Synthesis. The two-stage synthesis of ATH occurs with the formation of a non-covalent AT-heparin complex with heparin molecules that contain the required pentasaccharide sequence. A covalent bond then forms between the terminal aldehyde in heparin and an amino group in AT. Courtesy of Leslie Berry.

Only 10% of UFH has a free terminal aldose group [22]. Therefore, a 200 molar excess of UFH relative to AT is used. AT and UFH are heated to 40^{o}C in PBS for 14 days, giving the non-covalent complex with a yield of approximately 40% on the basis of the starting materials. During this time, a Schiff base spontaneously forms between the terminal aldose in heparin and an AT lysyl ε-amino group [22]. Stabilization occurs through Amadori rearrangement if there is a free hydroxyl group on the C_2 of the terminal sugar. Any Schiff base not stabilized after 14 days is reduced by treatment with $NaBH_3CN$ for 5 hours. Purification of ATH is achieved by two-step chromatography using hydrophobic butyl-sepharose chromatrography followed by anion exchange DEAE-sepharose chromatography [72].

2.3.4.2 Surface Modification using Antithrombin-Heparin Complex

Heparin has been used to treat many thromboembolic diseases, for example reducing mortality and morbidity in cases of deep vein thrombosis and pulmonary embolism [21]. However, due to the limitations of heparin limitations such as risk of bleeding and short half-life, new anticoagulants are continually being developed. In particular heparin has proved to be inadequate for the treatment of thrombotic complications in RDS. ATH with a longer half-life and greater anticoagulant activity was seen as a potential solution [72]. In the context of the present work ATH has been investigated for use as a coating agent on various biomaterial surfaces.

Methods for surface modification with ATH have been developed for several substrates including gold, polyurethane (PU), and poly(dimethyl siloxane) (PDMS) [35], [80]–[82]. ATH was covalently immobilized on PU catheters which were evaluated using a chronic rabbit jugular vein model [83], [84]. The ATH coated catheters remained open up to 106 days whereas control and commercial heparin-coated catheters occluded in less than 8 days and 4 days, respectively. ATH catheters also showed decreased cell and protein binding, increased anti-FXa activity, and reduced concentrations of thrombogenic activity markers compared to controls and heparin-coated catheters.

Using gold as a model substrate, ATH was attached directly, or via PEG as a linker to provide resistance to nonspecific protein adsorption [82]. Analogous UFH coated gold surfaces were prepared for comparison. The ATH surfaces bound significantly higher amounts of AT from plasma than the analogous UFH surfaces, despite having lower surface densities of the active molecules. In other work PU was grafted with PEG of varying molecular weight and ATH was attached covalently to the free PEG chain ends [81]. The properties of these surfaces were

found to be optimal using a PEG molecular weight of 600 Da. This surface gave the best balance between resistance to nonspecific protein adsorption and heparin activity. ATH was also attached to PDMS surface with PEG as a linker/spacer [35], and again the modified surfaces showed good protein resistance and anticoagulant activity.

2.4 Fibrinolysis

Fibrinolysis is the physiologic process of clot destruction or lysis. Hemostasis following vascular damage gives rise to a hemostatic plug with fibrin as a major component. As the vessel wall repairs itself the plug is no longer needed and fibrinolysis is activated to lyse the plug and allow completion of vessel wall repair. The major components of fibrinolysis are the zymogen plasminogen, its activated form plasmin, plasminogen activators, and plasmin inhibitors. Ultimately, fibrinolysis results in conversion of insoluble fibrin into soluble fibrin degradation products (FDPs). In the work reported in this thesis attempts were made to harness the fibrinolytic process as a means of alleviating clot formation on PDMS surface.

2.4.1 Plasminogen and Plasmin

Plasmin is the active serine protease responsible for the conversion of fibrin into FDPs. Its circulating precursor is the inactive zymogen plasminogen, a 92 kDa single chain glycoprotein with five homologous kringle domains and an active site [54], [85]. The native form of plasminogen has an N-terminal glutamic acid (Glu-plasminogen); however, the closed conformation of Glu-plasminogen can be converted to a more open conformation through limited plasmic digestion [85]. The newly formed variant has an N-terminal lysine, valine, or methionine and is termed Lys-plasminogen. Plasmin is capable of cleaving its own zymogen Glu-plasminogen to form Lys-plasminogen [54]. Although both Glu- and Lys-plasminogen are substrates for plasminogen activators, Lys-plasminogen in the open conformation is more readily converted to plasmin. The domain structure of plasminogen is shown in Figure 10.

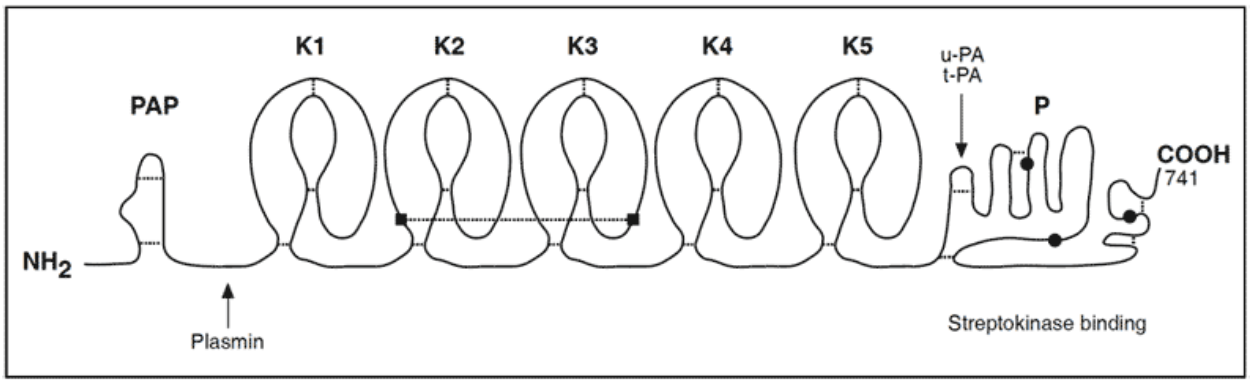

Figure 10 - Domain Structure of Plasminogen. Plasminogen has five kringle domains and an active site. Glu-plasmin(ogen) is converted to Lys-plasmin(ogen) by removal of the preactivation peptide (PAP). Plasminogen activators activate plasminogen to plasmin in the C-terminal region, where amino acid residues His603, Asp646, and Ser741 comprise the active site pocket (P) [http://diapharma.com/plasminogen-plg/].

Plasminogen activators cleave plasminogen at the Arg561-Val562 peptide bond resulting in a two-chain plasmin molecule [85]. The Arg561-Val562 peptide bond is concealed in Glu-plasminogen and exposed in Lys-plasminogen, resulting in slower conversion for Glu-plasminogen [54]. Upon conversion to active plasmin, Glu- and Lys-plasminogen retain their N-terminal in the heavy chain (Glu- and Lys-plasmin, respectively) with the five kringle domains and the C-terminal in the light chain where the active site is located.

Fibrin contains many lysine residues that promote high-affinity binding of plasminogen and tissue plasminogen activator (tPA), bringing the two into close proximity and providing a platform for tPA conversion of plasminogen to plasmin [23], [54]. Plasminogen binds with high-affinity to lysine residues, and in particular to C-terminal lysine residues in which the ε-amino and carboxylic acid groups are free [86]. Cleavage of fibrin by plasmin results in fragments containing C-terminal lysine residues due to preferential cleavage at Lys-Xaa peptide bonds [86]. Initially fibrin contains no C-terminal lysine residues; however, cleavage by plasmin exposes these residues progressively, thereby accelerating fibrinolysis [54], [55], [86].

The two D-domains in fibrinogen contain the C-terminal portions of the Bβ- and γ-chains, as well as a portion of the Aα-chain. The peptides are structured such that the D- and E-domains are connected through a triple helical coiled structure with irregular sections that are readily cleaved by plasmin (Figure 5) [53]. Plasmin cleavage at these sites produces FDPs of various sizes. The smallest FDP consists of two D-domains and one E-domain and is known as a D-dimer [55]. After fibrin degradation, FDPs are removed from the circulation via the liver.

2.4.2 Plasminogen Activators

Three types of plasminogen activators may be distinguished: urokinase plasminogen activator (uPA), streptokinase (SK), and tissue-type plasminogen activator (tPA). Urokinase plasminogen activator is associated with the cell surface receptor urokinase plasminogen activator receptor (uPAR) [54]. uPA has the ability to convert plasminogen to plasmin; however, this occurs typically in the extravascular environment (extracellular matrix). Therefore, uPA activity is commonly associated with extracellular proteolytic activity and the inflammatory response. Interaction with uPAR gives uPA signal-inducing ligand properties, and is mediated by inflammatory tissue necrotic factor found in endothelial cells and keratinocytes [87].

Streptokinase (SK) is a 47 kDa single chain polypeptide isolated from β-hemolytic streptococci and was the original molecule used in thrombolytic therapy [23], [54], [88]. Unlike tPA and uPA, SK activates plasminogen indirectly first by forming a 1:1 stoichiometric complex causing a conformational change in plasminogen, and exposing the active site [23]. The SK-plasmin(ogen) complex remains unaffected by the endogenous inhibitor α_2-antiplasmin [89]. The active site in the modified plasminogen molecule catalyzes the cleavage of the Arg561-Val562 peptide bond forming active plasmin [23]. SK acts on circulating plasminogen and can be a

useful thrombolytic therapeutic agent due to its low cost and increased half-life compared to tPA and uPA [88].

2.4.2.1 Tissue Plasminogen Activator

Tissue plasminogen activator (tPA), which is released from endothelial cells in tissue damage, is the most potent of the three plasminogen activators [24]. The 70 kDa serine protease consists of an active single chain polypeptide that is cleaved by plasmin through hydrolysis of the Arg275-Ile276 peptide bond to form a two-chain molecule [85].

tPA has five domains connected through 17 disulfide bridges [90]. As shown in Figure 11, four domains comprise the heavy chain of tPA: the light chain contains the active site. The finger domain is located in the N-terminal region of tPA (heavy chain); this is linked to an epidermal growth factor-like (EGF) domain, which is linked to two kringle domains. These four domains are connected to the light chain containing the active site. Plasmin and kallikrein cleave the molecule at the junction between the heavy and light chains, converting tPA to a two-chain molecule, both chains of which are proteolytically active.

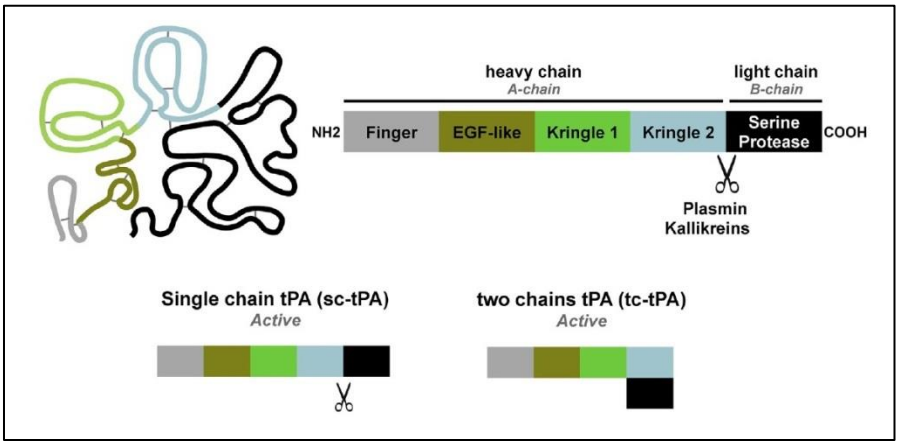

Figure 11 - Structure of tPA. tPA consists of a heavy chain and a light chain. The heavy chain contains an N-terminal finger domain, an EGF-like domain, and two kringle domains. The light chain contains the active site. Cleavage of single chain tPA by plasmin or kallikrein produces a two chain tPA molecule that remains proteolytically active. Adapted from [90]**.**

The N-terminal finger domain is involved in fibrin binding in the formation of the ternary fibrin-plasminogen-tPA complex, and can also interact with some cell membrane receptors [90]. The EGF domain specifically binds to and activates EGF receptors on endothelial cells. Lysine binding sites are contained in the two kringle domains; however, the lysine binding site in kringle 1 is not functional and its role is yet to be elucidated. Kringle 2 has an active lysine binding site with high affinity for fibrin, and is also involved in tPA binding to other cell receptors. Finally, the light chain in tPA contains the active serine protease triad His332, Asp371, and Ser478 [90]–[92].

The proteolytic activity of tPA in the conversion of plasminogen to plasmin is increased up to three orders of magnitude in the presence of fibrin [53]. Therefore, fibrin itself plays a crucial role in the initiation and propagation of fibrinolysis.

2.4.3 Role of Fibrin in Fibrinolysis

Fibrin interaction with plasmin(ogen) and tPA supports initiation and propagation of fibrinolysis by providing a "platform" for the conversion of plasminogen to plasmin via tPA [53]. tPA reactivity with plasminogen increases up to three orders of magnitude in the presence of fibrin. This catalytic effect on plasminogen activation is observed for fibrin but not fibrinogen. Fibrin also helps to protect against plasmin inhibition by α_2-antiplasmin; however, once released from degraded fibrin fragments, plasmin is readily inhibited by α_2-antiplasmin thereby confining fibrinolysis locally to the blood clot.

Fibrin interacts with tPA and plasminogen through binding sites as shown in Figure 12. tPA binding occurs in a lysine-independent manner to the γ-chain of the D-domain. The peptide region γ312-324 interacts most likely with the finger domain of tPA and is initially cryptic in

fibrinogen [53]. Only after fibrinogen cleavage by thrombin does the binding site become exposed.

The peptide sequence Aα148-160 in the D-domain of fibrin interacts with both tPA and plasminogen in a lysine-dependent manner (lys157 being vital to the process) [53]. This region is cryptic in fibrinogen, but after cleavage by thrombin to release fibrinopeptides A and B the lysine binding site is exposed and has equal affinity for tPA and plasminogen. However, due to the much higher plasma concentration of plasminogen compared to tPA, the lysine binding site interacts predominantly with plasminogen.

Another lysine binding site in the αC-domain of the Aα-chain at Aα221-610 also binds both tPA and plasminogen, and is also hidden in fibrinogen but exposed in fibrin. This lysine binding site is thought to be crucial for initiation of fibrinolysis due to the fact that both tPA and plasminogen bind non-competitively to this site, bringing the two into close proximity [53], [54].

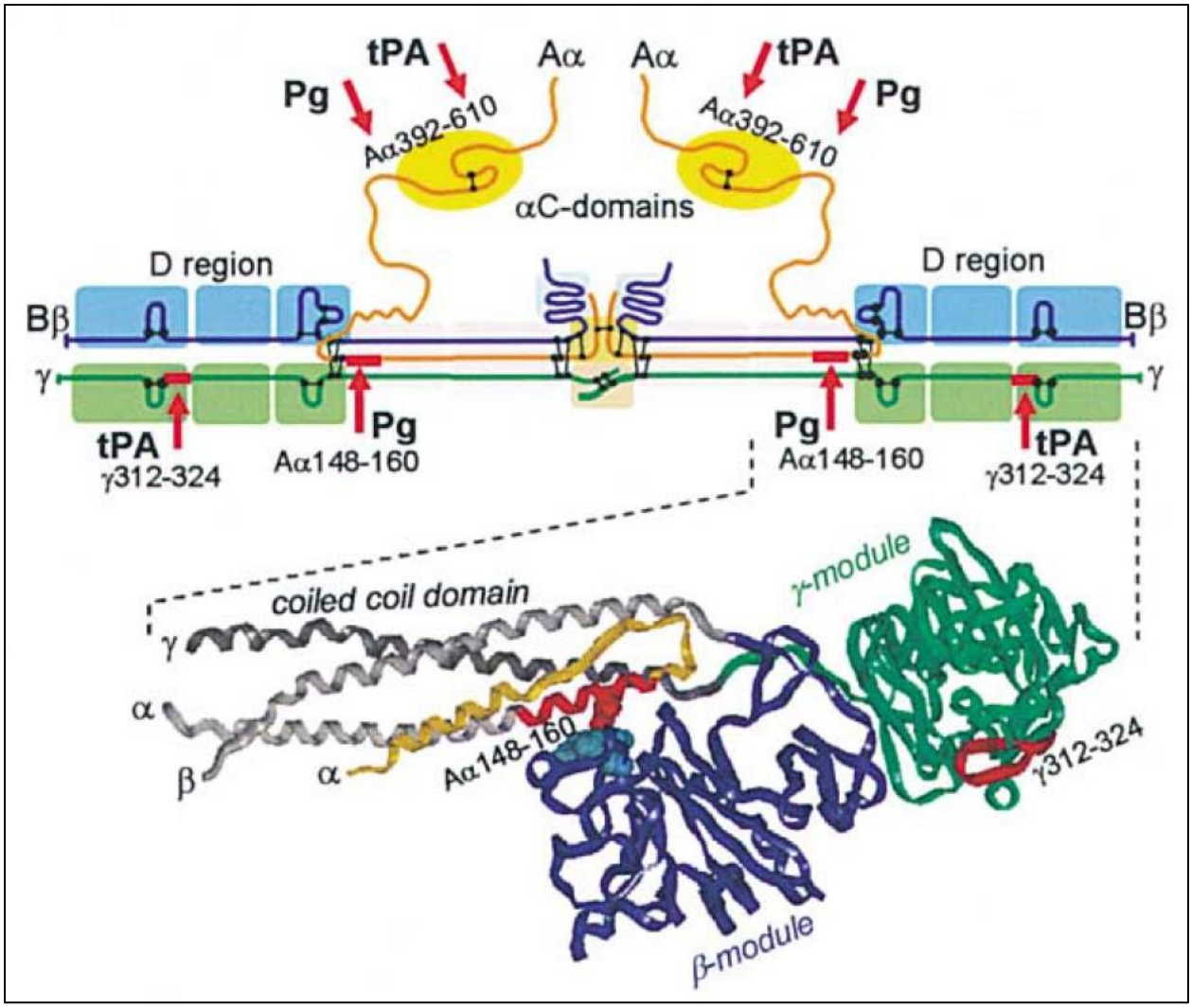

Figure 12 - Plasmin(ogen) and tPA Binding Sites in Fibrin. tPA binds in a lysine-independent manner to the γ-chain. Plasminogen saturates the Aα148-160 lysine binding site. Both tPA and plasminogen bind to Aα392-610 non-competitively, bringing the two into close proximity. Adapted from [53].

2.4.4 Inhibition of Fibrinolysis

Two serpins responsible for inhibition of fibrinolysis are α_2-antiplasmin and plasminogen activator inhibitor-1 (PAI-1). α_2-antiplasmin inhibits plasmin directly in solution at a high rate; however, plasmin bound to fibrin is inhibited much more slowly [54]. PAI-1 acts on tPA and uPA with fast kinetics.

Other fibrinolysis inhibitors include thrombin-activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase B that is slowly activated by thrombin. The cellular receptor thrombomodulin facilitates activation of TAFI by thrombin [85]. Once activated, TAFI removes C-terminal lysine and arginine residues from fibrin, thereby eliminating the ability of plasmin(ogen) and tPA to bind and slowing fibrin degradation.

2.4.5 Surface Modification for Promotion of Fibrinolysis

As discussed above, fibrinolysis *in vivo* is mediated by the binding of plasminogen to C-terminal lysine residues in fibrin. The concept of a fibrinolytic biomaterial based on this mechanism was proposed by Woodhouse and Brash [84]. In that work lysine was attached to polyurethane by reaction with sulfonate groups in the PU and plasminogen adsorption was found to be similar on sulfonated surfaces with and without lysine. Silica glass surfaces containing lysine and sulfonate groups were also developed [93], [94]. Plasminogen adsorption was high for both of these surfaces compared to unmodified silica glass; however, plasminogen interaction on sulfonated silica glass was likely not through the lysine binding site as ε-amino caproic acid did not inhibit plasminogen adsorption [94]. This was not the case for the lysine-on-silica surface, which also showed a preference for plasminogen over fibrinogen. Studies of the ability of plasminogen adsorbed on sulfonated silica glass or lysinized silica glass to lyse fibrin clots showed that clot lysis was greater on the lysinized surface, i.e. when the plasminogen was adsorbed via its lysine binding sites [93].

Further work along these lines used photochemical methods to attach lysine to PU giving surfaces with a much higher density of lysine [95]. It was shown that plasminogen adsorbed preferentially from plasma to these surfaces, and indeed that monolayer quantities of plasminogen were adsorbed. Moreover, the surface-bound plasminogen was readily converted to

active plasmin by tPA [96]. It was also shown that 70% of the surface-bound plasminogen was exchangeable with plasminogen in plasma, indicating that the highly plasminogen-specific lysine surface could maintain its activity on a continuing basis.

Lysine-containing surfaces were also evaluated in a modified Chandler loop experiment using whole human blood [97]. Lysine-modified polyethylene tubing was pre-adsorbed with tPA and then exposed to non-anticoagulated flowing whole blood in the Chandler loop. Clotting and clot lysis were observed visually. On control surfaces (no lysine) it was found that clotting was initiated and continued until the tubing was occluded and blood flow ceased. On the lysine surfaces, fibrin generated initially was lysed within minutes.

More recent research on lysine-modified surfaces used PEG or poly(2-hydroxyethyl methacrylate) (polyHEMA) as linker-spacers and as elements to provide resistance to non-specific protein adsorption [41], [98]–[100]. Compared to controls these surfaces showed decreased fibrinogen adsorption, indicating protein resistance, and increased plasminogen adsorption. However, plasminogen adsorption and clot lysis were somewhat hindered on the PEG-lysine surface [98]. Lysine density was higher and clot lysis was more rapid on polyHEMA-lysine surfaces compared to PEG-lysine surfaces [91].

2.5 Polymers used for Blood Contact

Polymeric materials, either naturally occurring (cellulose, collagen, DNA) or synthetic (polyethylene, silicones, polyurethanes) are widely used for biomedical applications, and in particular for blood contacting applications. Availability, material properties, and ease of fabrication into complex shapes, are among the attractive attributes of synthetic polymers [101].

Polymers are in general composed of repeating monomer units [101]. The monomers undergo either addition (chain reaction), or condensation (stepwise) reactions to create long-

chain polymers. Free radical polymerization of double bond monomers (eg. styrene) is the most common type of chain polymerization [11]. A free radical initiates the reaction by opening the double bond forming a monomeric radical. Propagation then follows forming a long polymer chain with a terminal free radical. Mutual termination of radicals stops the growing chains leaving the polymers as the end product. Condensation reactions occur in a step growth manner where two monomers react to form a covalent bond while releasing water or other small molecule. The reaction is complete upon depletion of the limiting monomer. A wide variety of polymer structures are available, including homopolymers, random copolymers, graft copolymers, and block copolymers. Most polymer molecules are readily formed into bulk solids and can be processed to give virtually any geometric shape.

Polymeric materials can be hydrophobic, i.e. resistant to water contact (non-wetting), or hydrophilic and water wetting. Hydrophobic polymers used as biomaterials include silicones, polyethylene (PE), polypropylene (PP), polytetrafluoroethylene (PTFE), and poly(methylmethacrylate) (PMMA). More hydrophilic polymers include poly(vinylchloride) (PVC) and copoly(lactic-glycolic acid) (PLGA). Fully hydrophilic and water wetting polymers include poly(ethylene glycol) (PEG/PEO) and poly(vinyl pyrrolidone).

2.5.1 Polydimethylsiloxane

Silicone polymers, termed polysiloxanes, are polymers with a backbone of alternating silicon and oxygen atoms [11]. The simplest polysiloxane, polydimethylsiloxane (PDMS), has two methyl groups attached to each silicon atom. The structure of PDMS is shown in Figure 13.

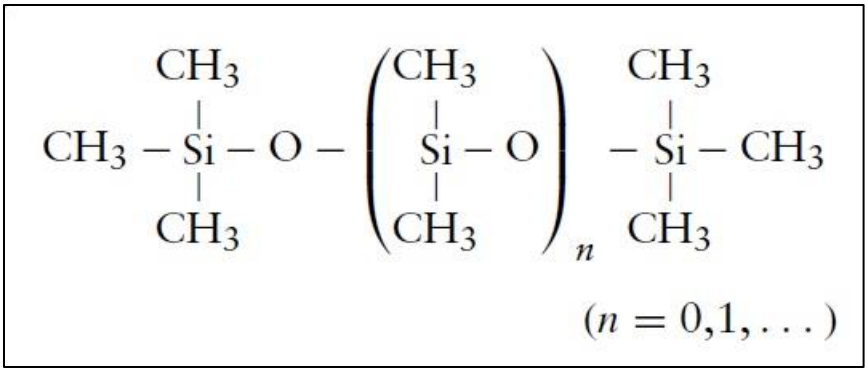

Figure 13 - Structure of PDMS. PDMS consists of a repeating silicone-oxygen backbone with two methyl groups attached to each silicon atom. The chain ends are silicons with three attached methyl groups [11].

PDMS is used in many biomedical applications due to its good biocompatibility properties including non-toxicity and bioinertness. It also has good thermal conductivity and elasticity, is highly permeable to gases such as oxygen, and is relatively simple and inexpensive to produce [11], [32], [33], [102]. PDMS has been used in the construction of blood oxygenators for heart-lung bypass systems, vascular stents, catheters, bioadhesives, tissue reconstruction, and drug delivery [26]. Due to its suitable mechanical and optical properties (transparency down to 280 nm) PDMS is used in contact lenses [103]. Other applications currently being investigated include microfluidic analytic devices and electrophoresis systems [32], [104].

For the LAD discussed in this thesis PDMS was chosen as the base material because of its excellent gas transfer properties, suitable mechanical properties, and ease of fabrication. PDMS is commonly used for the construction of microfluidic devices. With the advent of soft lithography, a master mold can be fabricated and used multiple times to create identical configurations, including microfluidic channels [32]. PDMS microfluidic channels produced by soft lithography can be made with extremely smooth surfaces as determined by the quality of the mold, and with a high degree of accuracy [32], [33].

2.5.2 Surface Modification of Polydimethylsiloxane

Although PDMS is relatively inert in tissue contact generally, it does provoke coagulation in blood contact. To improve blood compatibility the surface must be modified. Modification techniques include physical methods such as gas plasma and laser treatment, and chemical methods such as surface grafting.

Modification with PEG is commonly used due to increase the wettability of PDMS and thus decrease protein adsorption and cell adhesion. PDMS has been modified with PEG in a variety of ways [105], [106]. PEG has also been used as a linker/spacer to immobilize biomolecules on PDMS surface. For example, heparin was immobilized on PDMS via an N-succinimidyl carbonate PEG spacer [107].

Surface modification of PDMS microfluidic devices poses a unique set of difficulties. PDMS is not water wettable; this causes difficulties when using procedures that require treatment with aqueous fluids [32]. Using techniques such as oxygen plasma, chemical vapour deposition, layer-by-layer deposition, hydrosilylation, or bulk modification, microfluidic PDMS devices have been prepared for various applications [32], [104]. For example, bulk modification of PDMS with carbon nanotubes increases thermal and electrical conductivity and mechanical stability as required for sensors, valves, and pumps [104]. For the LAD discussed in this thesis, the surface modification of PDMS should not require harsh solvents that would damage the material. Most importantly, the modified material should be anti-thrombogenic. Two approaches to surface modification of PDMS for anti-thrombogenicity were used in the work reported: attachment of ATH to prevent clot formation and modification with lysine/plasminogen/tPA to lyse clots that may form.

2.6 Polydopamine

In recent years, there has been much interest in polydopamine (PDA) as a "bioglue" for attachment of bioactive molecules to biomaterial surfaces. This application of PDA is bioinspired and is based on the well-known strong adhesion of mussels to many substrates, both organic and inorganic. PDA plays a major role in mussel adhesion [27]. Under alkaline conditions dopamine coated on a surface (eg. by dip coating from solution) self-polymerizes to form a thin layer of PDA, providing a glue for attachment of biomolecules (Figure 14). The exact mechanism of dopamine polymerization is still debated, but is based on the Raper-Mason mechanism of melanin formation [108]–[110]. The self-assembled PDA layer can form on virtually any surface including metal, ceramic and polymer surfaces [27]. Soluble oxygen is required but other oxidants such as Fe^{3+}or Cu^{2+} are also able to facilitate dopamine oxidation [111].

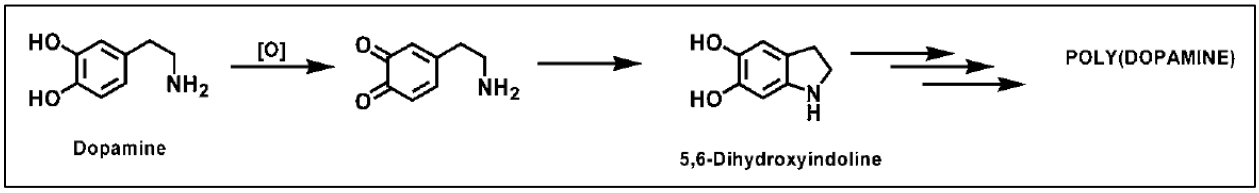

Figure 14 - Dopamine Polymerization. Dopamine polymerizes in aqueous alkaline conditions to form polydopamine. The exact mechanism is still debated. Adapted from [109].

PDA is effective as an adhesive in many applications. The simplicity of the coating method (immersion in dopamine solution) constitutes a significant advantage. Dopamine polymerization does not require harsh solvents or reagents and can be performed at ambient temperature using atmospheric oxygen. PDA coatings are possible on virtually all substrates, organic or inorganic [27], [112]. Furthermore, ad-layer deposition is possible on PDA coated surfaces through Michael addition or Schiff base reactions to further functionalize the coating

[27], [112]. In the work reported in this thesis PDA was used as a linker to attach biomolecules such as ATH, plasminogen, and tPA to the PDMS surfaces of the LAD.

3. Materials and Methods

3.1 Materials

PDMS was prepared using the Sylgard® 184 Silicone Elastomer kit from Dow Corning (Midland, MI). Dopamine hydrochloride was from Sigma-Aldrich (Oakville, ON).

Fibrinogen was from Enzyme Research Laboratories (South Bend, IN). Antithrombin was from Affinity Biologicals Inc. (Ancaster, ON). Enoxaparin (LMW heparin) and tPA were obtained from McMaster University Medical Centre (Hamilton, ON). Glu- and Lys-plasminogen and lysine were from Sigma-Aldrich (Oakville, ON). ATH was obtained from the Thrombosis & Atherosclerosis Research Institute (Hamilton, ON). Bovine serum albumin (BSA) was from Sigma-Aldrich (Oakville, ON).

$Na^{125}I$ was from the McMaster Nuclear Reactor (Hamilton, ON). Iodogen iodination reagent and Slide-A-Lyzer cassettes were from Pierce Biotechnology (Rockford, IL).

Precast polyacrylamide gels, prestained protein standards, Coomassie Blue stain, 4x Laemmle sample buffer, AP conjugate substrate kit, and electrophoresis buffer were from BioRad (Mississauga, ON). iBlot transfer kits were from ThermoFisher Scientific. Antibodies for Western blotting were from Cedarlane Labs (Burlington, ON).

Chromogenic substrates S2251 and S2288 were from Diapharma (West Chester, OH). Substrate CBS 31.39 was from Diagnostica Stago (Asnières sur Seine, France).

Expired fresh frozen human plasma and human erythrocyte concentrate were obtained from Transfusion Medicine at Hamilton Health Sciences and were used according to the policies of Canadian Blood Services.

3.2 Methods

3.2.1 Surface Preparation

3.2.1.1 PDMS Discs

PDMS surfaces were prepared using the Sylgard® 184 Silicone Elastomer kit with a 10:1 ratio of elastomer base to curing agent. The components were poured into a Petri dish to form a thin layer, placed in a vacuum for 30 min to remove air bubbles, then cured for 2 days at room temperature. PDMS approximately 0.5 mm thick was cut into discs using a 6 mm diameter punch.

3.2.1.2 PDMS Oxygenator Units

In brief, single oxygenator units (SOUs) were fabricated by first preparing the SOU body with microfluidic channels using soft lithography. PDMS membranes were prepared by spin coating a thin layer of PDMS onto an aluminum wafer. Elastomer base and curing agent from Slyguard® 184 Elastomer kit were mixed and poured onto the wafer. The membrane was produced by spin coating the wafer at room temperature for 10 s at 500 rpm, followed by 30 s at 4000 rpm to achieve a uniform PDMS coating. The wafer was then placed in the oven at 60^{o}C for 4 h to cure the membrane. The PDMS membrane was then attached to the body following gas plasma treatment of the surfaces to facilitate bonding. Using a high power expanded plasma cleaner and plasma flow gas mixer (Harrick Plasma, Ithaca, NY), the SOU body and membrane surfaces were activated in an oxygen plasma chamber. The components were placed in the plasma chamber and the system evacuated. When the pressure reached 200 mTorr, oxygen was admitted, raising the pressure until a steady value between 500-550 mTorr was reached. The plasma power was then raised to “high” for 2 min. The power was then turned off, the chamber vented, and the surfaces brought into contact. The bonded SOU was kept in an oven at 70^{o}C for 24 h.

3.2.1.3 Polydopamine Modification

PDMS Discs: A solution of dopamine hydrochloride (1 mg/mL) was prepared in phosphate buffered saline (PBS) at pH 8.5. PDMS discs were immersed in 50 mL of dopamine solution in a glass beaker. The beaker was placed on a shaker for 24 h, allowing the dopamine to polymerize and form a PDA layer on the discs. After polymerization, the discs were rinsed three times (5 min each time) in PBS, pH 7.4.

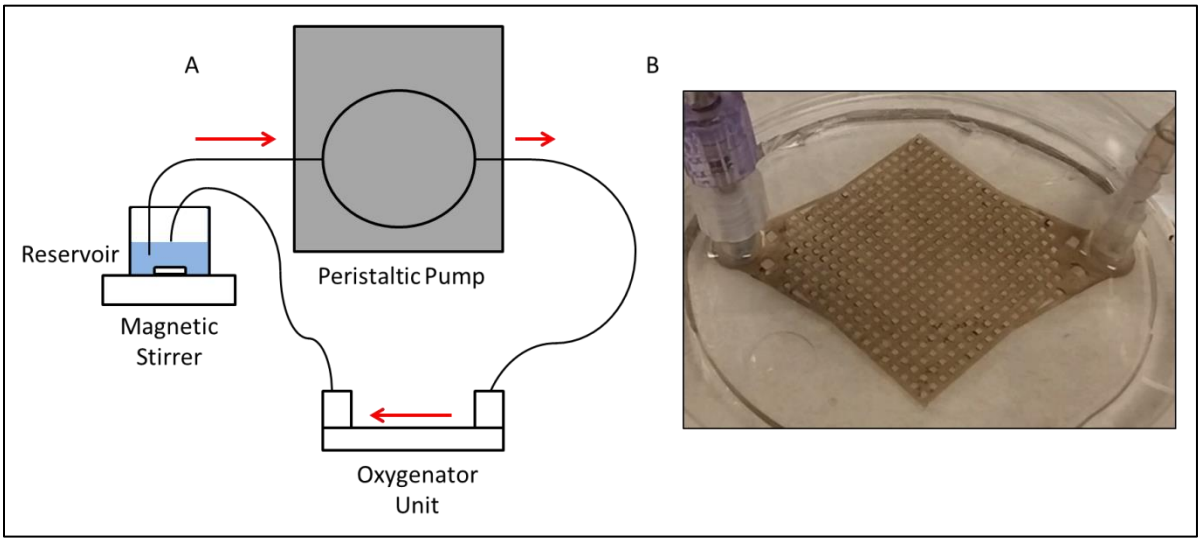

Figure 15 - Continuous loop used for PDA surface modification of oxygenators. A) Dopamine solution was pumped through oxygenator units and back to the reservoir. B) SOU coated with PDA.

PDMS SOUs: PDA modification of SOUs was carried out under flow conditions. 50 mL of a 1 mg/mL dopamine hydrochloride solution in PBS, pH 8.5, was prepared in a 50 mL glass beaker with a magnetic stirrer. Up to four SOUs were connected in series and the solution was circulated through the units using a peristaltic pump at 3 mL/min for 24 h (Figure 15). The solution was returned to the beaker reservoir, creating a closed loop. After PDA polymerization, three 5 min rinse cycles with PBS, pH 7.4, were performed followed by multiple manual rinses using a 3 mL syringe to remove loosely molecules.

3.2.1.4 Modification of Surfaces with Bioactive Components: ATH, Plasminogen, tPA

PDMS Discs: For the 6 mm discs, 200 μL of component solution with concentrations from 0.1-2 mg/mL were prepared in PBS, pH 7.4 and added to the wells of a 96-well plate. Following three 5 min washes in PBS, pH 7.4, the discs were incubated in the solution for times from 3 to 24 h. After the incubation period, three 5 min rinses with PBS were performed.

PDMS SOUs: Modification of oxygenator units with the bioactive species was carried out using the flow system shown in Figure 15, with 1 to 4 oxygenator units in series. Following three 5 min rinses with PBS, pH 7.4, the ATH, plasminogen or tPA solution was circulated through the loop for times from 3 to 24 h as described for the PDA modification (Figure 15). The units were then rinsed three times (5 min each time) by filling with PBS, pH 7.4, and additional multiple times using a 3 mL syringe to ensure complete removal of loosely bound protein.

3.2.2 Protein Radiolabeling

Protein labeling with radioiodine is a common laboratory technique used to monitor protein interactions, location, and characteristics [39]. Radioiodine is attached via reaction with tyrosine residues in the protein. The protein can then be detected and quantified by radioactivity measurements.

For experiments using radiolabeled AT, ATH, tPA, plasminogen, fibrinogen, and BSA, 10% of the protein in the system under study (plasma, solution in buffer) was labeled with ^{125}I while the other 90% of the protein was unlabeled. The radioactive portion acts as a tracer of the whole population. Protein solutions were prepared in PBS, pH 7.4. Surfaces were incubated in the solution for 3 to 24 h followed by three 5 min rinses in PBS, pH 7.4. Radioactivity was determined using a Wizard Automatic Gamma Counter from Perkin Elmer (Waltham, MA).

3.2.2.1 Iodogen Method

ATH, tPA, and plasminogen were labeled with $Na^{125}I$ using the Iodogen method. 1-2 mg of protein and 5 µL of the $Na^{125}I$ were added to an Iodogen vial containing 10 µg iodination reagent and reacted for 15 min. The reaction mixture was then transferred to a Slide-A-Lyzer dialysis cassette. The dialysis buffer was changed three times over a 48 h period to remove unbound isotope.

3.2.2.2 Iodine Monochloride Method

Fibrinogen and BSA were radiolabeled using the iodine monochloride method [113]. 10 mg protein, 22.3 µL ICl reagent, 5 µL $Na^{125}I$, and 240 µL glycine buffer were mixed and allowed to react for 5 min. The reaction mixture was then passed through an AG 1x4 anion exchange column to remove unbound iodide.

3.2.2.3 Free ^{125}Iodide Ion Content of Radiolabled Protein Preparations

In a centrifuge vial, 100 µL of radiolabeled protein solution was added to 900 µL of 1 mg/mL BSA solution (PBS, pH 7.4). A separate set of centrifuge vials were identically prepared and 500 µL of trichloroacetic acid in water (20% w/v) was added, followed by centrifugation for 2 min at 1000 rpm to precipitate the protein. The radioactivity of all vials was measured. Free iodide content was expressed as the supernatant radioactivity divided by the radioactivity of the protein solution. Values less than 5% were observed in all experiments.

3.2.3 Protein Adsorption/Desorption Experiments

Protein adsorption experiments were conducted in buffer (PBS pH 7.4) or human plasma. Radiolabeled protein was added to buffered protein solution or plasma. For experiments using plasma, radiolabeled protein was added to plasma at 10% of the endogenous level. Surfaces (6 mm discs) were incubated in the radiolabeled protein solution or plasma for times from 3 to 24 h, and then rinsed three times with buffer (5 min each time). Radioactivity was determined using

the Wizard Automatic Gamma Counter. Similar experiments were performed with oxygenator units under flow conditions using the setup shown in Figure 15. The uptake of ATH on the discs and oxygenators was measured in a similar manner.

To determine the durability/stability of the ATH and tPA surface modifications in contact with blood and plasma, ATH and tPA were labelled with $Na^{125}I$. Modified disc surfaces were prepared as described above, but using the radiolabeled proteins, and then incubated in human plasma or blood. Samples were taken for evaluation at times from 3 to72 h. They were then rinsed with buffer (3 times, 5 min each time) and radioactivity measured to determine desorption. Similar experiments were performed on oxygenator units modified with ATH using the setup shown in Figure 15.

3.2.4 Water Contact Angles

Water contact angles on PDMS-PDA and PDMS-PDA-ATH surfaces were measured with Milli-Q water using the Drop Shape Analyzer – DSA 100 apparatus from Kruss (Hamburg, Germany).

3.2.5 Western Blots

Western blots were performed on eluates from both 6 mm PDMS discs and oxygenator units after contact with plasma. Six discs of each surface were incubated in 200 µL each of plasma for 3 h in a 96 well plate. They were then rinsed with buffer (3 times, 5 min each time) and incubated in 2% sodium dodecyl sulfate (SDS) solution (250 µL) containing β-mercaptoethanol (SDS: β-mercaptoethanol, 20:3) to elute surface bound proteins. Discs were then removed from the wells and the eluates from each surface collected and pooled.

For oxygenator units 5 mL of plasma was circulated in a closed loop for 3 h at a flowrate of 3mL/min using a peristaltic pump (Figure 15). Only one oxygenator was used in these

experiments. After plasma incubation, oxygenator units were rinsed with buffer (3 times, 5 min each time) followed by multiple manual rinses using a 3 mL syringe. Oxygenator units were then individually incubated for 15 min in 2 mL of 2% SDS:β–mercaptoethanol, 20:3.

Eluates were run on reduced Sodium Dodecyl Sulfate-Polyacrylaminde Gel Electrophoresis (SDS-PAGE) (12% gels) to separate the proteins. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane using the Invitrogen iBlot 7-Minute Blotting System (Israel, WV).

PVDF membranes were incubated with non-fat milk solution for 45 min to block unbound sites. Primary antibodies specific to the protein of interest and secondary antibodies conjugated to alkaline phosphatase were diluted at 1:1000 in TBS. Membranes were incubated in primary antibody for 1 h, rinsed 5 min three times, and incubated in secondary antibody for 1 h. Proteins were visualized by treatment with colour development solution (BioRad, Mississauga, ON).

3.2.6 Plasmin Determination by Chromogenic Substrate S2251 Assay

Chromogenic substrate S2251 is a substrate specific for active plasmin and streptokinase-activated plasminogen. When active plasmin is added to a solution of chromogenic substrate S2251 the substrate is cleaved (Figure 16) and colour is generated. The extent of substrate cleavage, and thus the plasmin concentration, can be determined by measuring the absorbance at 405 nm.

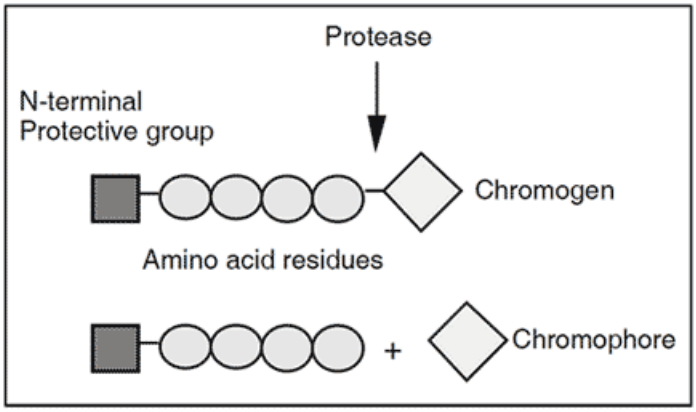

Figure 16 - Chromogenic Substrate Mechanism. Enzymes cleave the chromogenic substrate at a recognizable amino acid sequence. A precipitate is released that changes the reflection of visible light [http://diapharma.com/heparin/].

3.2.6.1 Measurement of Active tPA Activity on tPA-modified Surfaces using Chromogenic Substrate S2251

In 96 well plate wells, 180 µL plasminogen (0.1 mg/mL in PBS, pH7.4) was mixed with 20 µL chromogenic substrate S2251, giving a final substrate concentration of 5 mM. PDMS, PDMS-PDA, and PDMS-PDA-tPA discs were then added to the wells and absorbance at 405 nm was measured every min for 60 min.

3.2.6.2 Active Plasmin Measurement

PDMS, PDMS-PDA, and PDMS-PDA-Plgn (Lys or Glu) discs were evaluated for plasmin activity using chromogenic substrate S2251. Plasminogen-treated discs were rinsed with buffer, 5 min three times, and then incubated in 200 µL tPA solution (0.1 mg/mL in PBS, pH 7.4) for 30 min to activate the surface bound plasminogen to plasmin. The discs were then rinsed with buffer, 5 min three times. In a 96-well plate, 180 µL PBS and 20 µL chromogenic substrate S2251 were added to each well, giving a final substrate concentration of 5 mM. Discs were then added to the wells and absorbance at 405 nm was measured over a 60 min period.

3.2.7 tPA Determination by Chromogenic Substrate S2288 Assay

Chromogenic Substrate S2288 has specificity for active tPA. PDMS, PDMS-PDA, and PDMS-PDA-tPA discs were evaluated for tPA activity using chromogenic substrate S2288. In a 96 well plate, 180 µL of PBS, pH 7.4, and 20 µL of chromogenic substrate S2288 were mixed, giving a final substrate concentration of 5 mM. Discs were then placed in the wells and absorbance at 405 nm was measured every min for 60 min.

3.2.8 ATH Surface Activity Determination by Chromogenic Substrate CBS 31.39 (Anti-FXa Assay)

Chromogenic substrate CBS 31.39 is specific for FXa and was used to determine the activity of surface-bound ATH compared to that of heparin in solution. Experiments were performed in a 96-well plate with absorbance measurements taken at 405nm. PDMS surfaces were modified with either PDA or PDA-ATH. Discs were incubated in 140 µL AT solution (1 mg/mL in PBS, pH 7.4) for 10 min to allow AT-heparin binding. Excess FXa was then added to the wells (3.5 mg/mL, 70µL) and incubated for 5 min. 60 µL of the solution was then transferred to adjacent wells containing 60 µL of 5mM CBS 31.39. Absorbance at 405 nm was measured to determine the quantity of FXa not inhibited by AT. CBS 31.39 assays were performed using a series of heparin solutions of known concentration. A standard curve, A-405 vs heparin concentration, was created from which anti-FXa activity could be expressed in terms of mass of active heparin (ng/cm^2).

3.2.9 Plasma Clotting and Clot Lysis Experiments

Experiments were conducted to measure the effects of the surface modifications on clotting and clot lysis. For these experiments recalcified citrated human plasma was prepared by mixing citrated plasma in a 1:1, v/v ratio with 0.025 M calcium chloride.

3.2.9.1 Plasma Coagulation Time in Oxygenator Units

Citrated plasma (2 mL) and 0.025 M $CaCl_2$ (2 mL) were mixed in a Falcon tube. The recalcified plasma was immediately drawn into a closed flow circuit (Figure 17) consisting of Tygon® PVC tubing, a peristaltic pump, a pressure transducer, and a single oxygenator unit. Approximately 2 mL total volume was used per circuit, and the remaining plasma was used as a control to ensure that coagulation occurred (data not shown).

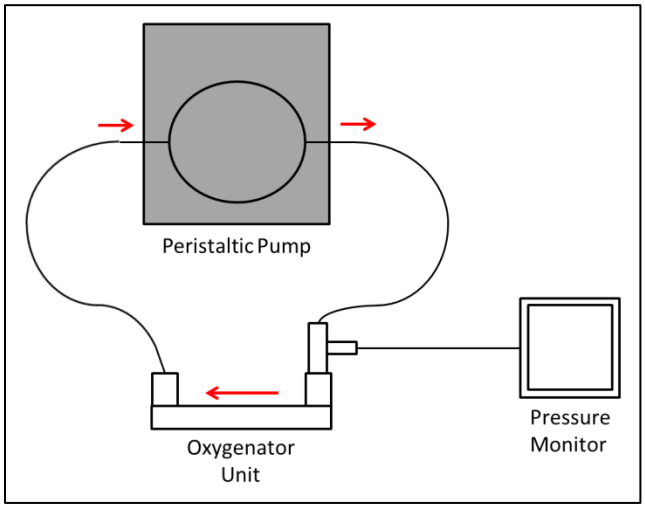

Figure 17 - Closed loop circuit used for plasma coagulation experiments. Recalcified citrated plasma was introduced into the loop. Air bubbles were removed. The time to coagulation, as assessed by pressure change, was measured.

All of the surfaces in the circuit in contact with plasma were modified with the respective surface coating. The pressure was recorded over a 60 min period or until delamination of the membrane occurred in the oxygenator unit.

3.2.9.2 Clot Lysis

Citrated human plasma was used to determine the fibrinolytic activity of the surface modified discs. The method is based on turbidity changes in the plasma due to clot formation (increase in turbidity) and clot lysis (decrease in turbidity) [96].

In a 96 well plate, 100 µL citrated plasma and 100 µL 0.025M $CaCl_2$ were mixed. PDMS, PDMS-PDA, PDMS-PDA-tPA, and PDMS-PDA-(Glu or Lys)-plasminogen discs were then placed in the wells and absorbance at 405 nm was measured every min for 60 min.

3.3 Statistical Analysis

Data were analyzed using Student's t-test, or ANOVA followed by Tukey post-hoc analysis when necessary. Significant differences were defined at $p<0.05$. Data are presented as mean ± standard deviation (SD).

4. Results and Discussion

4.1 ATH-Modified PDMS Discs

4.1.1 ATH Surface Density

PDMS discs were modified with PDA (1 or 2 mg/mL dopamine in PBS, pH 8.5, 24 h) and then incubated in radiolabeled ATH solution for 3 h. Three ATH concentrations: 0.1, 1.0 and 2 mg/mL (PBS buffer pH 7.4) were used. The data are shown in Figure 18.

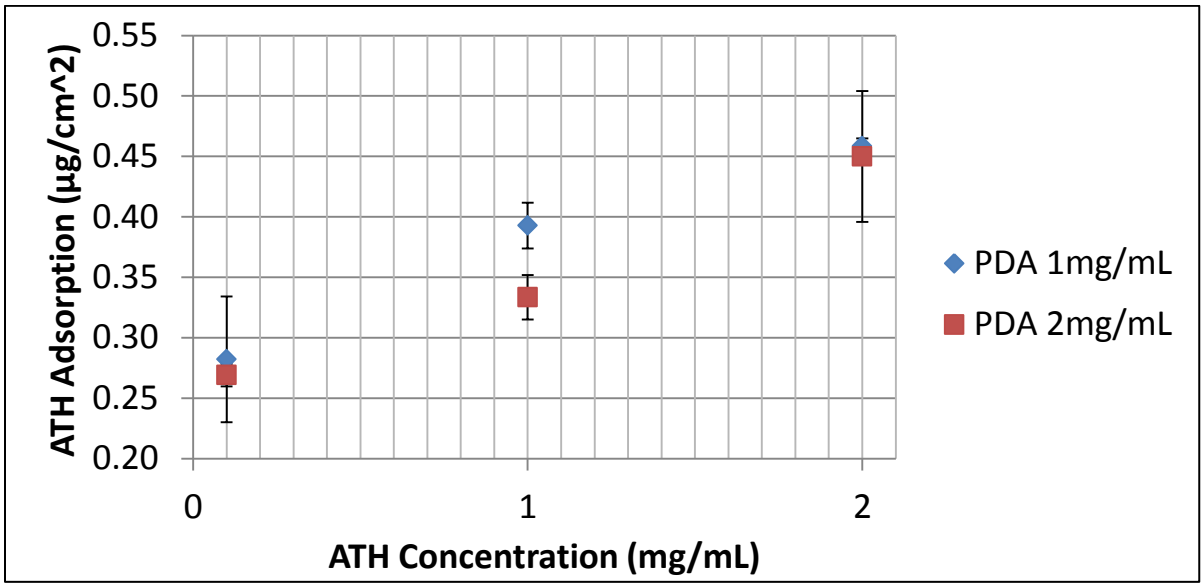

Figure 18 - ATH adsorption to PDMS-PDA surfaces. Data are mean±SD, n=2.

It is seen that ATH adsorption increased with increasing concentration of ATH. By and large the dopamine concentration used in the PDA modification step did not affect ATH uptake, although at 1 mg/mL, uptake was, unexpectedly, greater for the lower dopamine concentration ($p<0.05$). At 1 mg/mL dopamine, the ATH adsorption level increased from 0.27 to 0.45 µg/cm^2, while at 2 mg/mL dopamine, adsorption increased from 0.28-0.46 µg/cm^2 as the ATH concentration increased from 0.1 to 2.0 mg/mL. Typical quantities in protein monolayers are in the range of 0.1-0.5 µg/cm^2, depending on size and orientation [11]. Although the adsorption-concentration curves did not appear to be leveling off at the higher concentration, the values

suggest near monolayer formation across the concentration range 0.1 to 2 mg/mL. From an economical perspective (ATH is expensive) and from previous results showing the efficacy of ATH surfaces modified at 0.1 mg/mL, this concentration was chosen as the "standard" for ATH modification in this work [35], [81].

4.1.2 Water Contact Angles

Water contact angles were measured on modified PDMS discs to determine changes in surface wettability. Many studies have shown unmodified PDMS to be strongly hydrophobic with an average water contact angle greater than 105^{o} [99], [114], [115]. As described, PDMS discs were modified with PDA by incubating in dopamine solution (1 or 2 mg/mL in PBS, pH 8.5) for 3 or 24 h. Discs were modified with ATH by incubating PDA-modified discs in ATH solution (0.1 mg/mL in PBS pH 7.4) for 3 h. Water contact angles were measured for all surfaces. The data are shown in Figure 19.

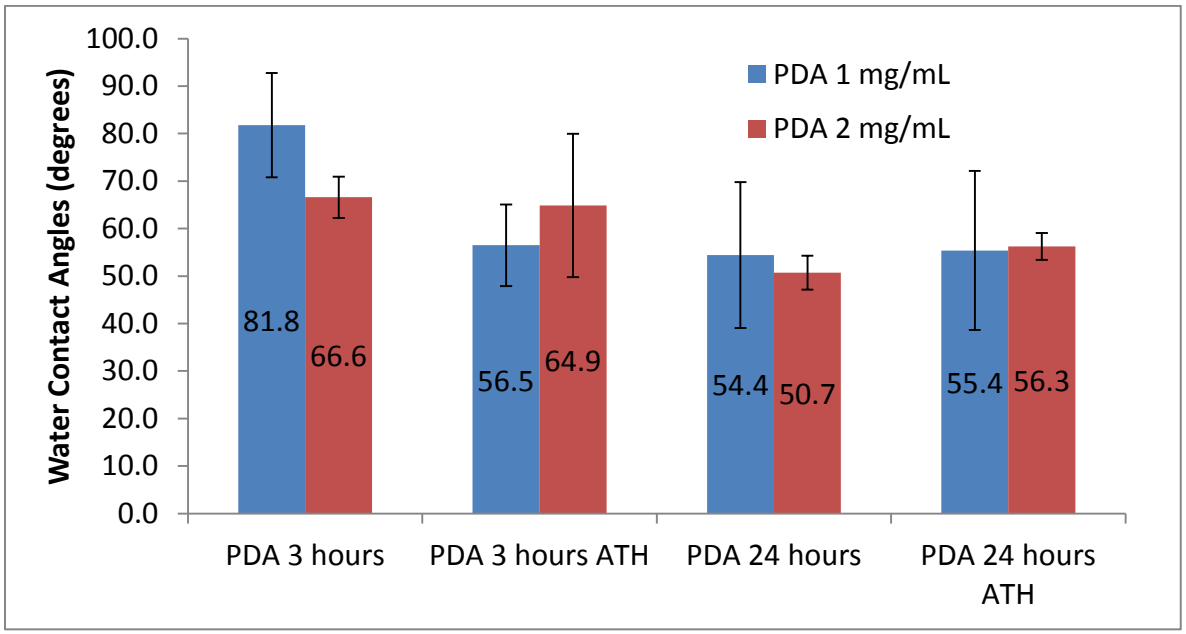

Figure 19 - Water contact angles of PDMS discs modified with PDA or PDA-ATH. Data are mean±SD, n≥4 [34].

The water contact angles on all of the PDMS-PDA, and PDMS-PDA-ATH surfaces were lower than values reported in the literature for unmodified PDMS, indicating a decrease in hydrophobicity following PDA, and PDA-ATH modification [99], [116]. Increasing the dopamine incubation time from 3 to 24 h caused a decrease in water contact angle from 81.8^{o} to 54.4^{o} using 1 mg/mL dopamine ($p<0.05$), and from 66.6^{o} to 50.7^{o} using 2 mg/mL dopamine ($p<0.05$). These data indicate greater PDA surface coverage on PDMS at higher dopamine concentration. The angles on PDA surfaces modified with dopamine at 1 and 2 mg/mL were not significantly different for either 3 h or 24 h incubation ($p>0.05$), suggesting that PDA coverage does not vary under these various conditions and that a concentration of 1 mg/mL provides sufficient surface coverage. However, the surface prepared by incubating in dopamine at 1 mg/mL for 3 h showed a water contact angle of 81.8°, slightly higher than for PDA modified PDMS reported in the literature [114], [115], suggesting incomplete surface coverage under these conditions. Therefore, based on the water contact angles and ATH adsorption data from Figure 18, PDA modification was performed routinely by incubating in 1 mg/mL dopamine for 24 h.

ATH modification of PDA surfaces had little effect on the water contact angles. An exception was the surface prepared by incubation in 1 mg/mL dopamine for 3 h for which the angle decreased from 81.8^{o} to 56.5^{o} upon attachment of ATH ($p<0.05$). Also a slight increase in contact angle from 50.7^{o} to 56.3^{o} was observed for the 2 mg/mL, 24 h PDA surface ($p<0.05$). The contact angles of the ATH surfaces were nonetheless still reduced compared to that of unmodified PDMS. This was also observed for PU surfaces modified with ATH using PEG as a linker [81]. It is possible that on the surfaces where ATH modification did not change the contact angle, ATH coverage may be low. It is noted that the ATH concentration used in preparing these

surfaces was 0.1 mg/mL and as shown in Figure 18 coverage could be increased by using a higher concentration. As will be shown below and as reported by Leung et al, however, ATH bioactivity was achieved on surfaces prepared with 0.1 mg/mL ATH solution [35].

4.1.3 Stability of ATH on Modified Surfaces

An important aspect of the ATH-modified surfaces is the stability of the bioactive component ATH on the surface. ATH-modified surfaces were tested for stability when exposed to human plasma and blood.

4.1.3.1 ATH Stability in Plasma and Resistance to SDS

PDMS discs were modified directly with ATH by incubating in ATH solution (0.1 mg/mL in PBS, pH 7.4, 10% ^{125}I-labeled) for 3 h, or modified first with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h) and then with radiolabeled ATH. Radioactivity of the surfaces were measured at one of three time points: after ATH incubation to determine initial ATH adsorption, after overnight plasma incubation to determine ATH desorption into plasma, and after a subsequent 2 h incubation in 2% SDS to determine stability in contact with a potent surfactant. The data are shown in Figure 20 [35].

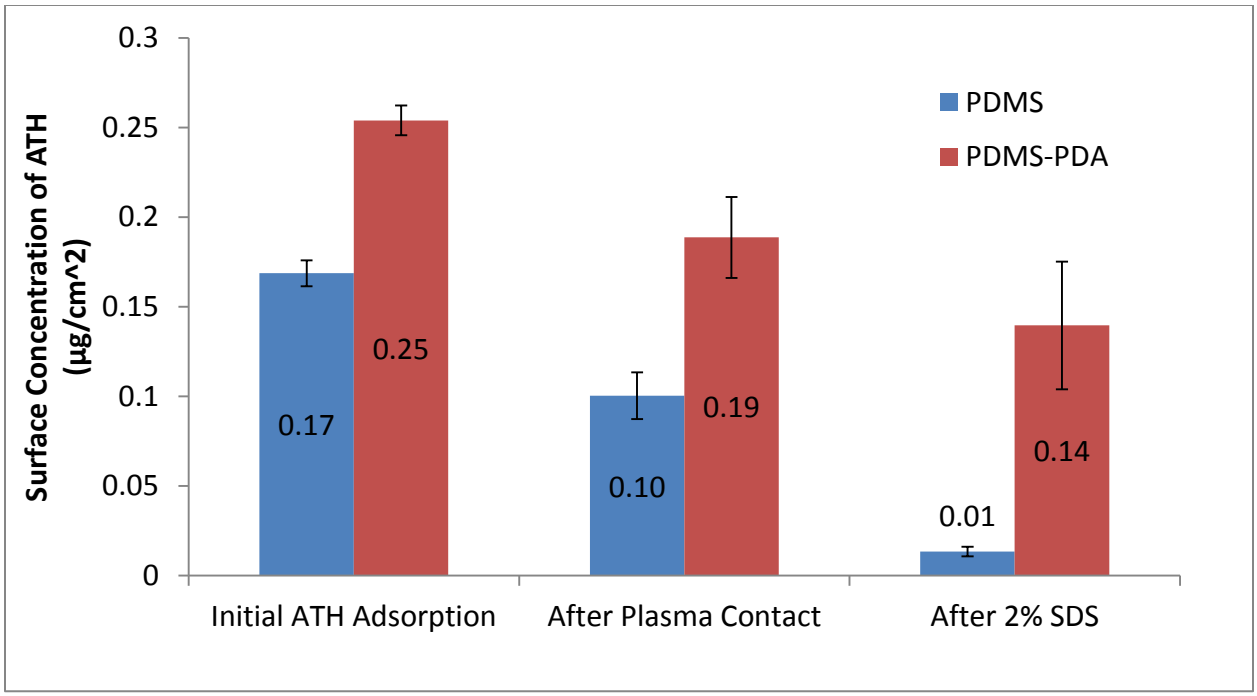

Figure 20 - ATH Stability in Plasma. Data are mean±SD, n=6 per surface [35].

Initial adsorption onto PDMS-PDA surfaces was 0.25 µg/cm^2, similar to the corresponding data in Figure 18. Adsorption was significantly higher on PDMS-PDA than on unmodified PDMS: 0.25 vs 0.17 µg/cm^2 ($p<0.05$). Following plasma exposure, 76% (0.19 µg/cm^2) of the initially adsorbed ATH remained on the PDMS-PDA surface, while only 59% (0.10 µg/cm^2) remained on the PDMS. Subsequent exposure to SDS removed virtually all of the remaining ATH from the unmodified PDMS surface whereas a significant quantity (0.14 µg/cm^2) remained on the PDMS-PDA, suggesting much greater stability for the PDA-modified surface.

In summary, the PDMS surface modified with PDA adsorbed higher levels of ATH than unmodified PDMS, and ATH bound through PDA remained relatively stable, even when exposed to SDS, a very strong detergent. These results show the potential for durability of PDMS-PDA-ATH surface coatings in blood contact.

4.1.3.2 ATH Stability in Blood

PDMS discs were modified directly with ATH (0.1 mg/mL ATH in PBS, pH 7.4, 10% radiolabeled with ^{125}I-ATH, 3 h), or with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h) followed by radiolabeled ATH. After modification, surfaces were incubated in blood for 24, 48, or 72 h, radioactivity measured, and the adsorbed quantity of ATH determined. The data are shown in Figure 21.

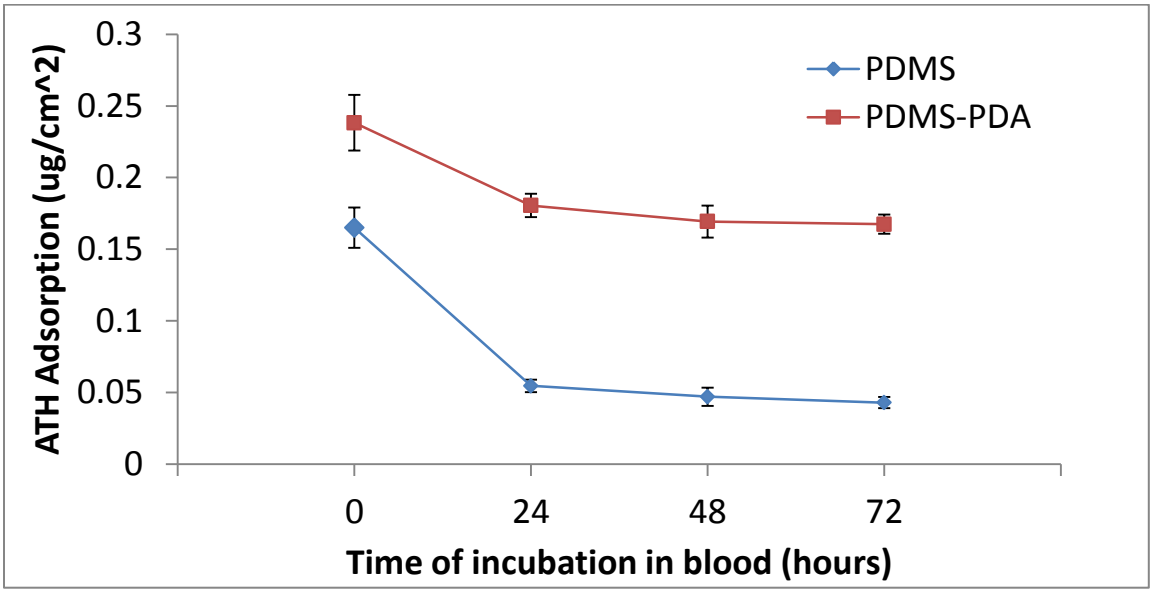

Figure 21 – ATH Stability in Blood. PDMS and PDMS-PDA surfaces were modified with ^{125}I-ATH and incubated in blood for up to 72 h. ATH desorption into the blood was determined. Data are mean±SD, n=6 [35].

ATH adsorption on PDMS-PDA (0.24 μg/cm^2) was significantly higher than on unmodified PDMS (0.17 μg/cm^2) ($p<0.05$). After 24-72 h of blood incubation, 71% of the originally adsorbed ATH remained on PDMS-PDA, whereas only 24-29% remained on the unmodified PDMS. Thus after the initial 24 h when 30% of the ATH was lost, the remaining ATH was largely retained out to 72 h. For the unmodified PDMS initial uptake was lower and retention was less over the same time period, presumably due to the bonding mechanism. ATH

can bind covalently through amine groups on PDA, whereas only physical interaction occurs with PDMS.

4.1.4 Protein Adsorption

4.1.4.1 BSA Adsorption from Buffer to ATH Modified Surface

Albumin is a highly abundant plasma protein in blood and a good "marker" for non-specific protein adsorption [39]. BSA solutions (10% ^{125}I-labeled) were prepared in PBS, pH 7.4 at concentrations of 0.1, 0.2, 0.5 and 1 mg/mL. Unmodified PDMS and PDMS modified with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h) or PDA-ATH (0.1 mg/mL ATH in PBS, pH 7.4, 24 h) were incubated in the BSA solutions for 3 h and protein adsorption was measured. The data are shown in Figure 22.

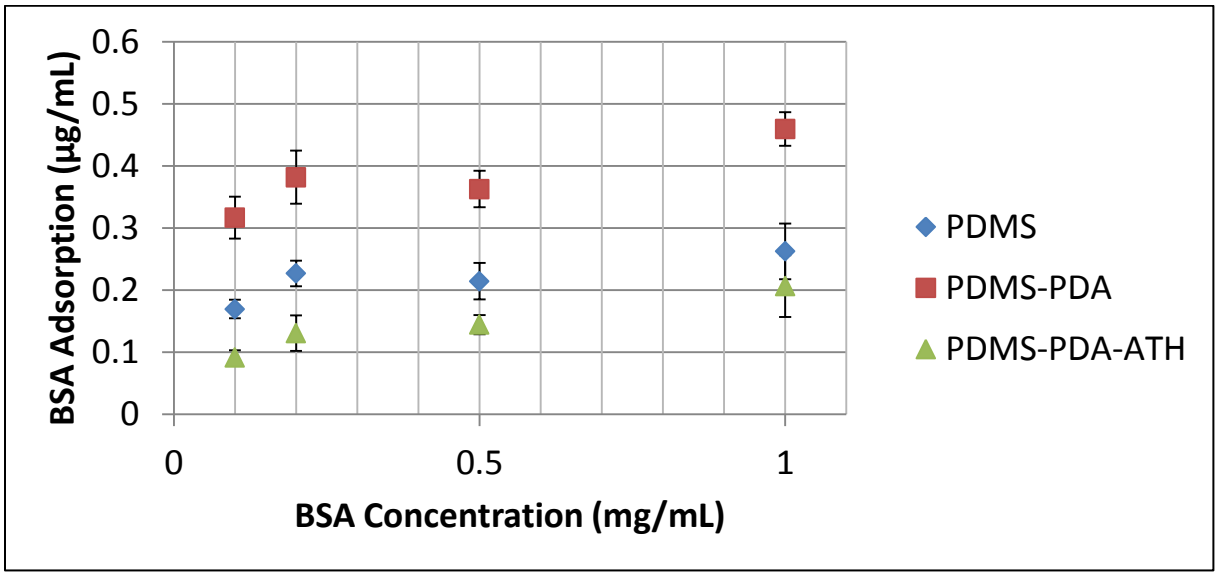

Figure 22 - BSA adsorption from buffer to PDMS and modified PDMS surfaces, adsorption time 3 h. Data are mean±SD, n=6.

BSA adsorption on PDA-modified surfaces ranged from 0.32 to 0.46 µg/cm^2, and was higher than on unmodified PDMS ($p<0.05$), and PDA-ATH-modified PDMS ($p<0.05$). Adsorption on unmodified PDMS was slightly higher than on PDMS-PDA-ATH ($p<0.05$) with

values ranging from 0.17 to 0.26 μg/cm^2 on unmodified PDMS and from 0.09 to 0.21 μg/cm^2 on PDMS-PDA-ATH. However, the difference between these two surfaces at 1 mg/mL was not statistically significant ($p>0.05$). For each surface BSA adsorption increased significantly from one concentration to the next over the studied range from 0.1 to 1 mg/mL ($p<0.05$).

Amino and catechol groups exposed on PDA-modified surfaces have the ability to bind proteins non-specifically, and these surfaces have been shown to have high levels of overall protein adsorption [110]. In agreement with these previous results, the data in Figure 22 show that PDMS-PDA adsorbed significantly more BSA than the other two surfaces. Thus unmodified PDMS and PDMS-PDA-ATH adsorbed up to 0.26 and 0.21 μg/cm^2 BSA, respectively, compared to up to 0.46 μg/cm^2 on PDMS-PDA. Indeed PDMS-PDA adsorbed more at the lowest concentration studied (0.32 μg/cm^2 at 0.1 mg/mL) than did the PDMS and PDMS-PDA-ATH at the highest concentration. Modification of PDA surfaces with ATH caused a decrease in adsorption to a level below that on ummodified PDMS, indicating that ATH modification covered all protein adsorption sites on the PDMS-PDA surface. Of the three surfaces, PDMS-PDA-ATH showed the lowest BSA adsorption, indicating that ATH can inhibit non-specific protein adsorption somewhat when attached to PDMS through PDA with the ability to mask the underlying PDA layer, and thereby potentially improve blood compatibility compared to unmodified PDMS.

4.1.4.2 BSA Adsorption from Buffer to Modified PDA Surfaces "Backfilled" with BSA and LMW Heparin

Using PDA as a bioglue to bond ATH to PDMS was shown to be effective in reducing non-specific BSA adsorption from buffer (Figure 22); however, due to the low concentration of ATH used for modification (Figure 18), there may still be regions of exposed PDA. Treatment of

the ATH-modified surfaces with LMWH or BSA, i.e. "backfilling", was investigated as a possible means of covering exposed PDA and reducing non-specific protein adsorption.

PDMS-PDA surfaces (1 mg/mL dopamine in PBS, pH 8.5, 24 h) and PDMS-PDA-ATH surfaces (0.1 mg/mL ATH in PBS, pH 7.4, 24 h) were prepared. A second, similarly prepared set of PDMS-PDA-ATH surfaces was further modified for 3 h with either enoxaparin (LMWH) (1 mg/mL in PBS, pH 7.4) or BSA (1 mg/mL in PBS, pH 7.4). Surfaces were then exposed to radiolabeled BSA solution (1 mg/mL BSA in PBS, pH 7.4, 10% labeled) for 3 h and adsorption was determined. The data are shown in Figure 23.

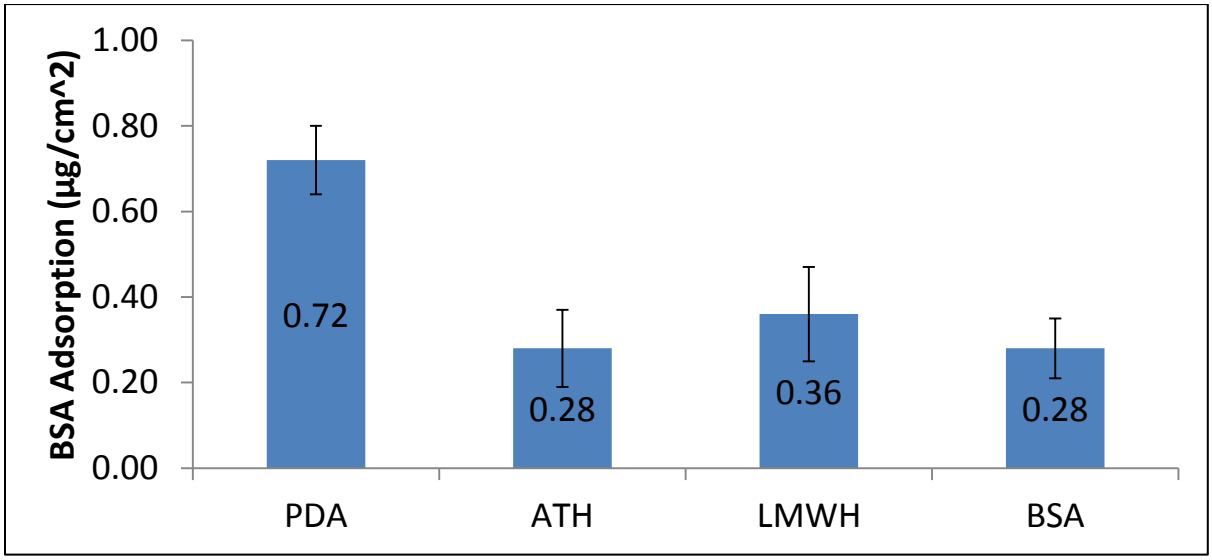

Figure 23 - BSA adsorption to PDMS backfilled surfaces. PDMS-PDA, PDMS-PDA-ATH, and PDMS-PDA-ATH backfilled surfaces were incubated in 1 mg/mL labeled BSA solution for 3 h. Data are mean±SD, n=12.

The absolute levels of BSA adsorption to the PDMS-PDA, and PDMS-PDA-ATH surfaces were slightly higher than those reported in Figure 22, but adsorption on the ATH surface was again much lower than on the PDA surface. BSA adsorption was higher on PDMS-PDA (0.72 µg/cm^2) than on the other three surfaces ($p<0.05$). However adsorption on the backfilled

surfaces was not different than on the PDMS-PDA-ATH. Thus backfilling PDMS-PDA-ATH with LMWH or BSA did not decrease BSA adsorption compared to PDMS-PDA-ATH ($p>0.05$).

4.1.4.3 Fibrinogen Adsorption from Buffer to ATH-Modified Surfaces.

Fibrinogen (Fg) is a common marker for non-specific protein adsorption as well as a key player in coagulation and thrombosis. Fibrinogen adsorption from blood is rapid and is one of the first proteins to adsorb onto biomaterials, being replaced later by molecules of higher affinity such as HK [117]. To investigate nonspecific fibrinogen adsorption, unmodified PDMS, PDMS-PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h), and PDMS-PDA-ATH (0.1 mg/mL ATH in PBS, pH 7.4, 24 h) surfaces were incubated in labeled fibrinogen solution (1 mg/mL in PBS, pH 7.4) for 3 h and adsorption was determined. The data are shown in Figure 24.

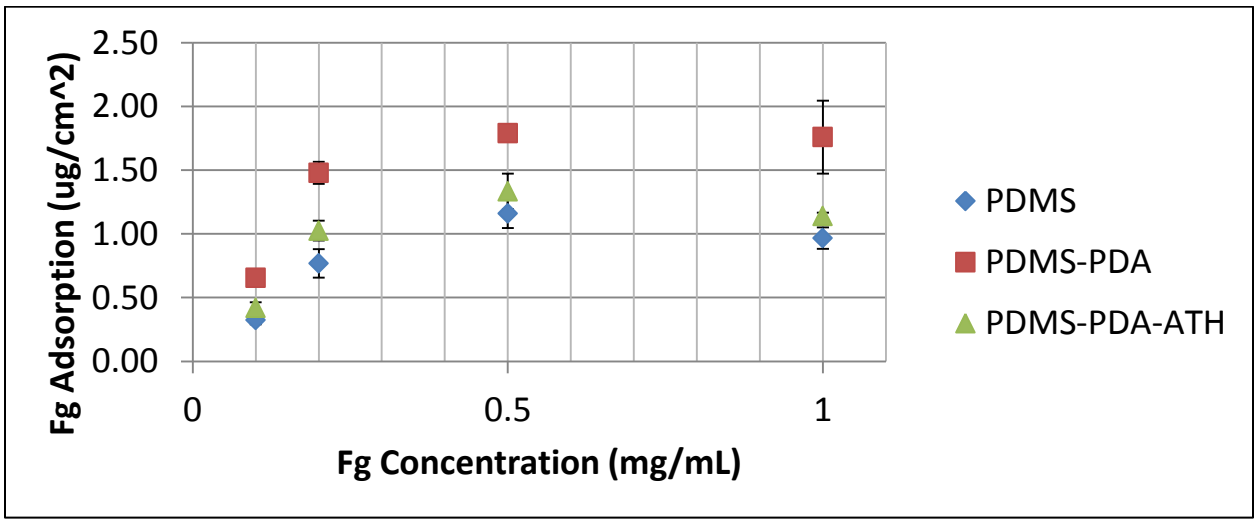

Figure 24 - Fibrinogen adsorption to PDMS, PDMS-PDA, and PDMS-PDA-ATH surfaces from ^{125}I-Fg solutions in buffer (concentration 0.1 to 1 mg/mL, 3 h exposure). Data are mean±SD, n=6.

Fibrinogen adsorption was higher on the PDMS-PDA surface than on the unmodified PDMS and PDMS-PDA-ATH surfaces at all three concentrations studied ($p<0.05$), with values in the range of 0.65 to 1.79 μg/cm^2. The trend is similar to that seen for albumin (Figure 22). No

differences were observed between the unmodified PDMS and PDMS-PDA-ATH surfaces ($p>0.05$), except at 0.2 mg/mL fibrinogen ($p<0.05$). Adsorption ranged from 0.33-1.16 μg/cm^2 on unmodified PDMS and 0.42-1.33μg/cm^2 on PDMS-PDA-ATH. The high fibrinogen adsorption on PDMS-PDA surfaces may contribute to coagulation or cell adhesion if in a plasma or blood multi-protein environment.

4.1.4.4 Fibrinogen Adsorption from Buffer to Modified PDA Surfaces "Backfilled" with BSA and LMW Heparin

Fibrinogen adsorption to modified surfaces backfilled with LMWH and BSA as described in section 4.1.4.2 was measured using radiolabeled fibrinogen. These experiments are analogous to the albumin adsorption experiments on these surfaces reported above (section 4.1.4.2). The backfilled surfaces were exposed to radiolabeled fibrinogen solution (1 mg/mL, PBS, pH 7.4, 10% labeled) for 3 h and adsorption was measured. The data are shown in Figure 25.

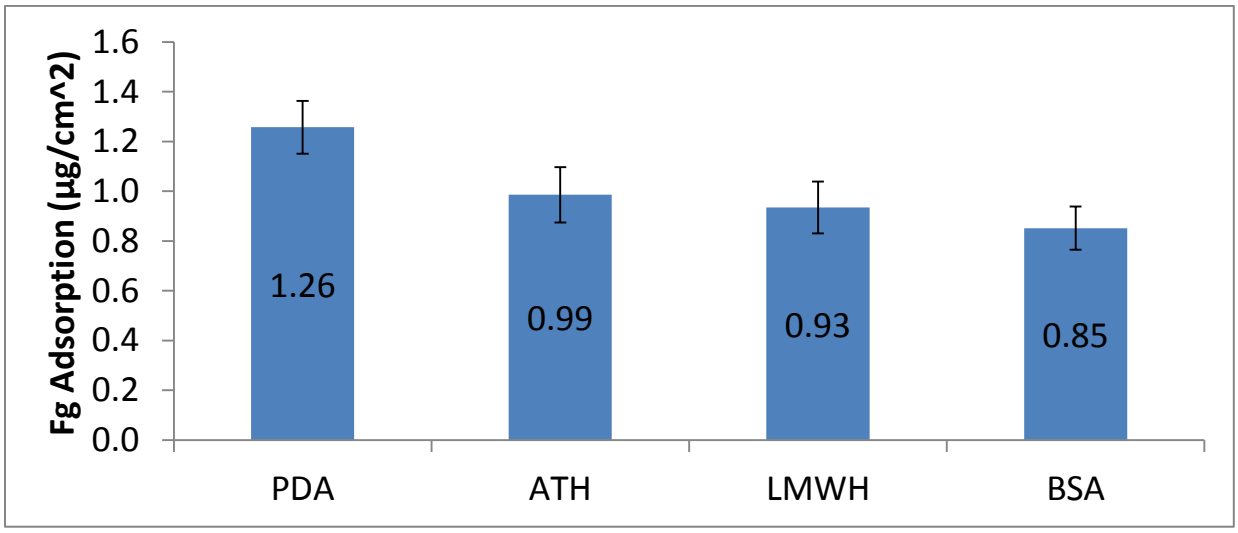

Figure 25 - Fibrinogen adsorption to LMWH- and BSA-backfilled surfaces. Data are mean±SD, n=12 per surface.

Compared to the data in Figure 24, fibrinogen adsorption levels on PDMS-PDA and PDMS-PDA-ATH surfaces were slightly lower; however, adsorption on PDMS-PDA-ATH was again lower than on PDMS-PDA. Adsorption on PDMS-PDA (1.26 μg/cm^2) was greater than on the other three surfaces ($p<0.05$). Adsorption on the backfilled surfaces was similar to that on the precursor PDMS-PDA-ATH surface. However, adsorption on the BSA-backfilled surface (0.88 μg/cm^2) was significantly lower than on the precursor (0.99 μg/cm^2, $p<0.05$). Thus backfilling with LMWH did not improve performance with respect to fibrinogen adsorption, considered as a marker of non-specific adsorption. Only a small decrease in fibrinogen adsorption was observed for the BSA-backfilled surface.

Both BSA and Fg adsorption were similar for PDMS-PDA-ATH surfaces whether backfilled or not. The data in Figure 25 and Figure 23 in combination suggest that backfilling is ineffective in reducing nonspecific adsorption to the PDMS-PDA-ATH surface. This approach was, therefore, not pursued further.

4.1.5 Bioactivity of Surface-bound ATH

4.1.5.1 Antithrombin Adsorption from Plasma

An important aspect of surface-attached ATH is whether it retains its heparin-like anticoagulant activity. To investigate this question for PDA-linked ATH, the adsorption of antithrombin from plasma was studied. It is expected that active heparin will bind and activate antithrombin as a prelude to thrombin binding and inhibition.

Discs of unmodified PDMS, PDMS-PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h), and PDMS-PDA-ATH (0.1 mg/mL ATH in PBS, pH 7.4, 3 h) were incubated in human plasma for 3 h. The plasma contained radio-iodinated antithrombin at 10% of the physiological level (0.15 mg/mL) [62]. AT adsorption was determined from surface radioactivity measurements. The data

are shown in Figure 26 [35].

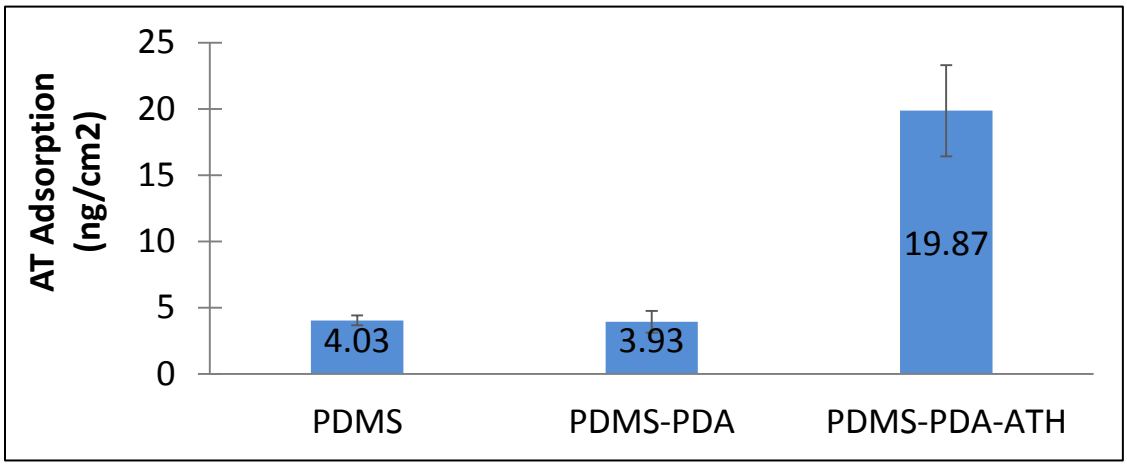

Figure 26 - AT adsorption from plasma to PDMS surfaces. Data are mean±SD, n=6 [35].

It is seen that adsorption on PDMS-PDA-ATH was higher than on unmodified PDMS and PDMS-PDA surfaces, with values of 19.87, 4.03 and 3.93 ng/cm^2, respectively.

When AT is irreversibly bound to thrombin and to a lesser extent FXa, affinity for the heparin moiety decreases and another molecule of AT can compete for heparin binding [20], [75]. Thus, it seems that surface-bound ATH may be oriented in such a way that the heparin moiety facilitates AT-thrombin interactions, releasing the irreversibly bound thrombin-antithrombin complex and allowing the heparin moiety to bind another solution phase AT molecule.

The concentration of AT in plasma is low (0.15 mg/mL) as evidenced by the very low AT binding seen on the PDA modified surface [62]. It is likely that other plasma proteins were adsorbed to the PDA surface due to higher binding affinity and/or higher plasma concentration. The selective adsorption of AT to PDMS-PDA-ATH shows that surface-bound ATH retains its heparin-like bioactivity when linked to PDMS through PDA.

4.1.5.2 Anti-FXa Activity

PDMS surfaces were modified with PDA (1 mg/mL dopamine in PBS, pH 8.5) or with PDA-ATH (0.1 mg/mL ATH in PBS, pH7.4). In multiwell plates, the surfaces were incubated in a solution of AT (0.14 mL, 1 mg/mL, AT in PBS, pH 7.4) for 10 min to allow AT to bind to the heparin moiety of ATH, forming ATH-AT complex on the surface. Excess FXa (70 µL, 3.5 mg/mL, in PBS, pH 7.4) was then added and the solution incubated for 5 min. In the presence of heparin, AT inhibits FXa at a high kinetic rate. A 60 µL portion of the solution was transferred to fresh wells containing 60 µL of chromogenic substrate CBS 31.39, which is active against FXa. Absorbance at 405 nm, representing the amount of residual FXa not inactivated by ATH, was measured. The data, shown in Figure 27, provide an estimate of anti-FXa activity in terms of the mass density of active heparin (ng/cm^2) by comparison to a standard curve (not shown) created using known concentrations of heparin [35].

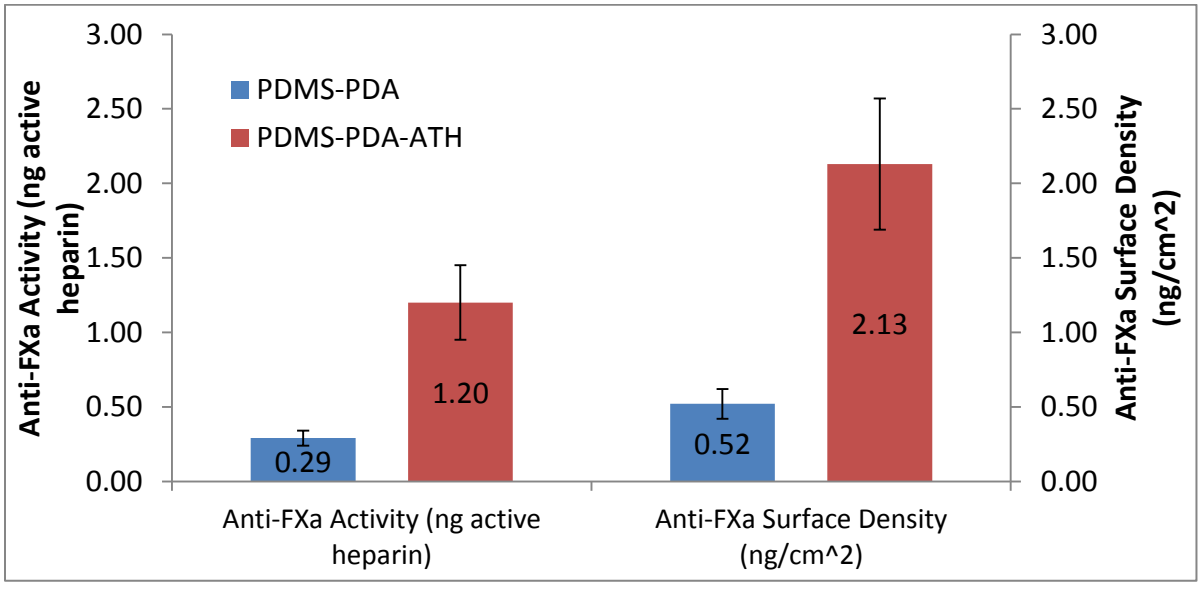

Figure 27 - Anti-FXa activity of PDMS-PDA and PDMS-PDA-ATH surfaces. Surfaces were incubated in AT and then in excess FXa. Residual FXa activity was determined using chromogenic substrate CBS 31.39. Absorbance was measured at 405 nm and compared to that on a standard curve constructed using known concentrations of heparin. Anti-FXa activity is expressed as mass of active heparin. Data are mean±SD, n=8 [35].

The anti-FXa assay is normally used to assess the activity of fluid phase anticoagulants but was modified in this work to measure the activity of surface-bound ATH. Since no anticoagulant species are present on PDMS-PDA, the "apparent" anti-FXa activity on this surface is attributed to non-specific binding of FXa (to exposed PDA) which is then detected in the chromogenic substrate assay [112]. The PDMS-PDA-ATH surface had activity equivalent to 2.13 ng/cm^2 active heparin. This anti-FXa activity is clearly due to the anticoagulant activity of the heparin moiety in ATH since the surface without ATH had only nominal "activity", attributed to non-specific adsorption of FXa.

4.1.6 Western Blots

To investigate the interactions of the surfaces with a broader range of plasma proteins, Western blots were performed on the eluates from surfaces after plasma contact. Antibodies directed against albumin, fibrinogen, AT, and vitronectin were used in these experiments. Five different surfaces were examined: PDMS-PDA, PDMS-PDA-ATH prepared using 0.1 or 1 mg/mL ATH solution (referred to as PDMS-PDA-ATH-0.1 and PDMS-PDA-ATH-1, respectively) and PDMS-PDA-ATH-0.1 backfilled with LMWH or BSA as described above. Six discs of each type were incubated in plasma for 3 h. Surface-bound proteins were then eluted and Western blots performed on the eluates. The blots are shown in Figure 28.

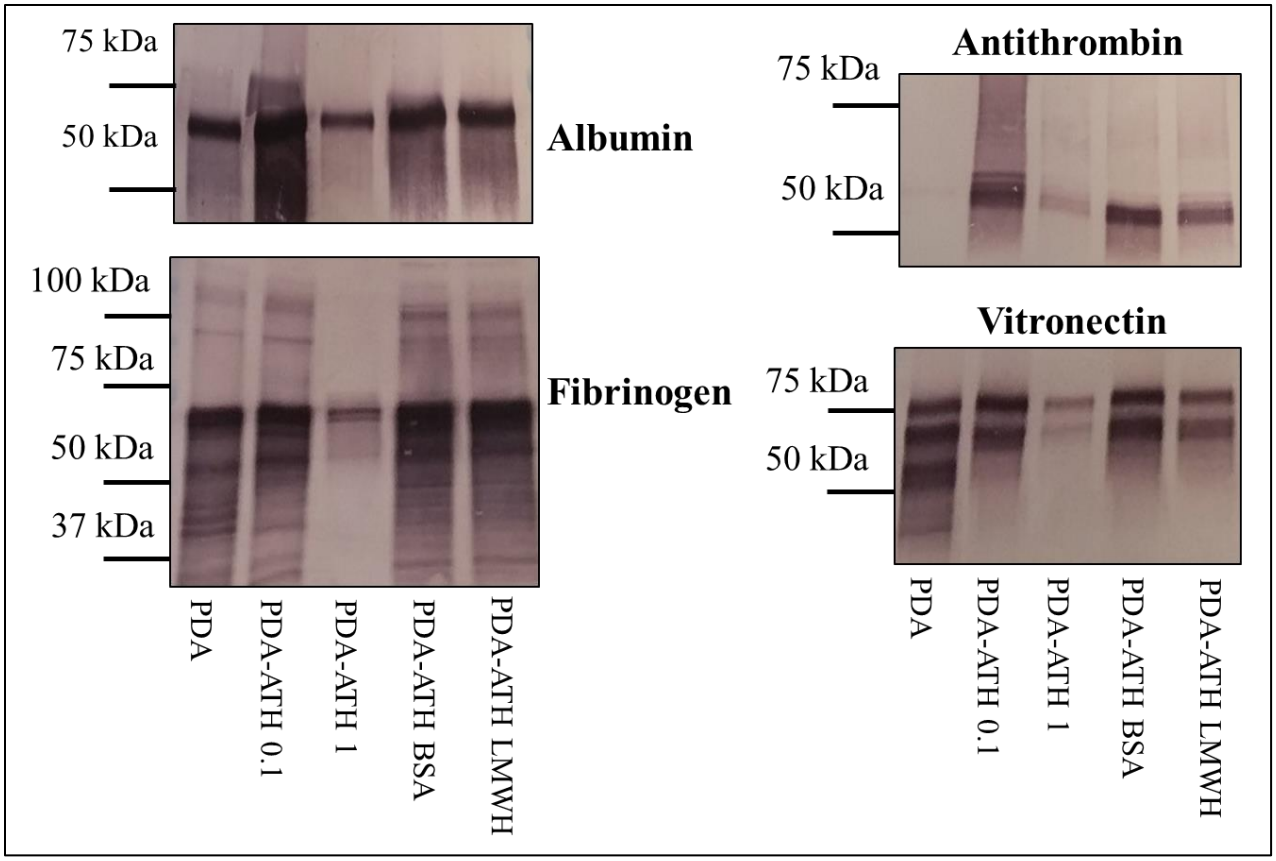

Figure 28 - Western blot analysis of proteins eluted from modified PDMS surfaces following 3 h plasma incubation. Antibodies directed against human albumin, fibrinogen, antithrombin, and vitronectin were used.

Unsurprisingly, albumin, the most abundant of the plasma proteins, was present in all of the eluates, with bands appearing at 66 kDa. Fibrinogen was also seen in all of the eluates. Fibrinogen has a molecular weight of 340 kDa, and is composed of two identical subunits containing Aα, Bβ, and γ chains, which are observed as three lower molecular weight bands at about 66, 56 and 48 kDa in reduced SDS-PAGE. The data in Figure 28 confirm the results from the radiolabeled albumin and fibrinogen experiments shown in Figure 22 and Figure 24, respectively.

Vitronectin is a 75 kDa glycoprotein present in plasma at a concentration of about 300 μg/mL and interacts with a number of proteins and other species in blood including fibrinogen,

kininogens, plasminogen, plasminogen activator inhibitor, and cell surface receptors such as interleukin receptors and urokinase plasminogen activator receptor [118]. It is well known as a protein which, when adsorbed on biomaterial surfaces, facilitates the adhesion of blood cells, particularly platelets. In addition it has been found to be adsorbed on a wide range of surfaces exposed to plasma [119]–[121], and hence was of interest in the present work. Vitronectin appearing as two bands of molecular weight 75 and 65 kDa was clearly present on all five surfaces as shown in Figure 28 [118], [120].

Antithrombin constitutes a relatively small fraction of the total plasma protein complement, with a concentration of 150 μg/mL [62]. As shown in Figure 26, AT adsorbed with high specificity on the ATH-modified surfaces as expected in the presence of active heparin. Bands at 58 kDa in the immunoblots confirmed the adsorption of AT on the ATH surfaces (Figure 28), but no band was seen for the PDMS-PDA precursor surface. PDA-ATH surfaces backfilled with LMWH or BSA showed AT band intensities similar to PDMS-PDA-ATH-0.1. That AT adsorption occurred on all ATH-modified surfaces, but not on the PDA precursor, suggests that AT was adsorbed to the ATH surfaces via the specific heparin-AT binding mechanism.

Blot responses on the PDMS-PDA-ATH-0.1 surface and the surfaces backfilled with LMWH or BSA were not significantly different, in agreement with the results of the radiolabeled BSA and fibrinogen experiments shown in Figure 23 and Figure 25.

The bands for all four proteins on the PDMS-PDA-ATH-1 surface were systematically weaker than on all of the others. It is unclear whether any significance should be attached to these differences. They may be artefactual based on some inadvertent experimental inconsistency.

4.1.7 Summary of ATH-Modified PDMS Discs

The use of PDA as a bioglue has been shown to be feasible as a method to attach ATH to PDMS. ATH uptake on PDMS-PDA surface increased with increasing ATH solution concentration, but heparin-like function was evident on surfaces prepared at ATH concentrations as low as 0.1 mg/mL. Because some exposed PDA, with the potential to adsorb plasma proteins non-specifically, may be present on ATH-modified surfaces, backfilling these surfaces with LMWH or BSA was investigated. However, this approach was ineffective in reducing BSA and fibrinogen adsorption. It was shown that ATH bound to PDMS via PDA retained its heparin-like activity as assessed by antithrombin uptake from plasma. AT uptake was demonstrated using both radiolabeled AT experiments and Western blotting.

4.2 ATH-Modified Oxygenator Units

The data presented above using PDMS discs as substrates shows the efficacy of the PDA bioglue method for modification with ATH. In this section work to modify our microfluidic oxygenator units with ATH and to evaluate their performance in terms of blood interactions is described. Oxygenator units were tested under conditions, including flow conditions, more akin to those that would be encountered in the clinic. ATH uptake on PDMS-PDA modified oxygenators, the stability of the ATH surfaces in blood, and the anticoagulant activity of the ATH surfaces were investigated.

4.2.1 ATH Uptake from Buffer and Stability in Blood

PDMS oxygenator units were modified with PDA by incubation in 1 mg/mL dopamine solution under flow using a peristaltic pump (PBS, pH 8.5, 24 h, 3 mL/min). They were then exposed to radiolabeled ATH solution under flow (0.1 mg/mL, PBS, pH 7.4, 24 h, 3mL/min, 10% ^{125}I-ATH). The units were then evaluated in one of two ways: (1) ATH uptake was measured, or (2) the units were exposed to flowing blood (4 mL/min) for 2 days (blood refreshed

after 24 h) and the remaining quantity of surface-bound ATH was determined. The results are shown in Figure 29.

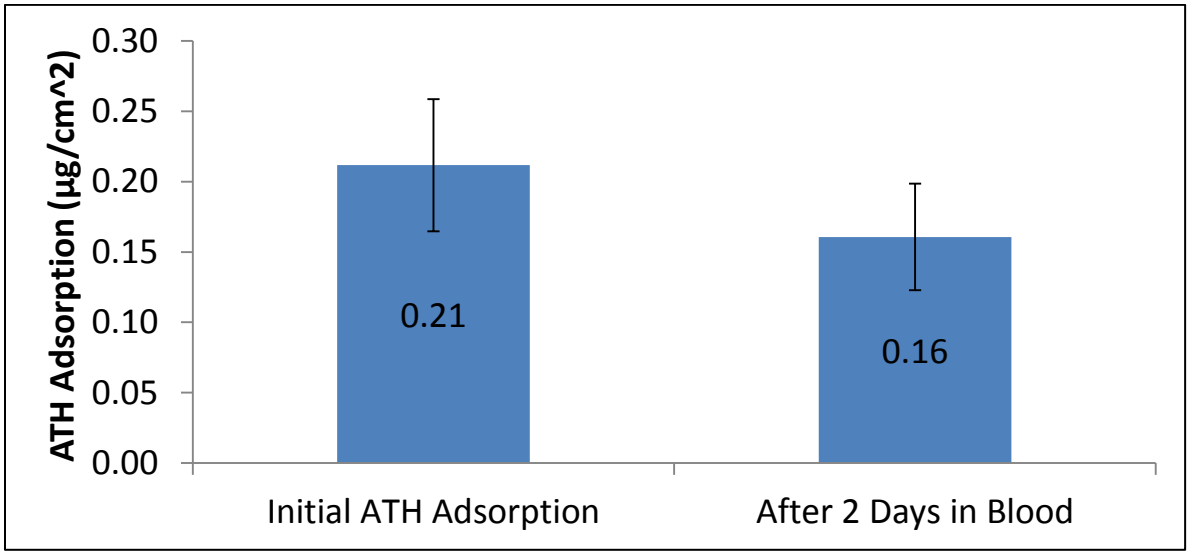

Figure 29 - ATH adsorption to PDA-treated oxygenator units from buffer and stability in blood. Data are mean±SD, n≥7.

Uptake of ATH on the PDMS-PDA oxygenator surfaces was determined to be 0.21 µg/cm^2. This value is comparable to that observed on PDMS discs under static conditions (Figure 18) [35]. After two days of blood circulation through the ATH-modified device the ATH level decreased to 0.16 µg/cm^2, reflecting a statistically significant loss ($p<0.05$). Nonetheless 76% of the initial ATH remained after 2 days of blood contact. These data are in agreement with results from experiments on PDMS discs [35]. Thus ATH appears to remain reasonably stable when subjected to the stress of flowing blood, including protein- and cell-surface interactions, for 2 days, indicating that PDA may be suitable as a bonding agent for ATH on the oxygenator surfaces.

4.2.2 Antithrombin Adsorption from Plasma to ATH-modified Oxygenator Units

To determine if surface bound ATH in the oxygenator units retained its heparin-like bioactivity, AT uptake from plasma was measured. Radiolabeled AT was added to plasma at 10% of normal physiological level. PDMS oxygenator units were modified with PDA (1 mg/mL, 24 h, 3 mL/min), or with PDA followed by ATH (0.1 mg/mL, 24 h, 3 mL/min). The oxygenator units were exposed to flowing (4 mL/min) AT-radiolabeled plasma for 3 h and AT adsorption was determined. The results are shown in Figure 30.

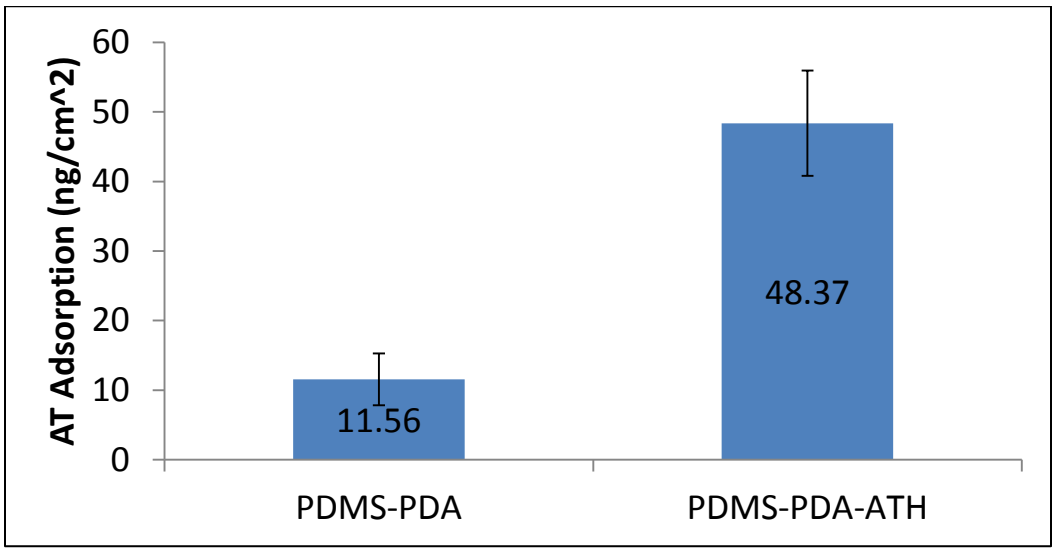

Figure 30 - AT adsorption from plasma to oxygenator units. Oxygenator units modified with PDA, or PDA-ATH were incubated in plasma containing radiolabeled AT for 3 h. Data are mean±SD, n≥4.

The PDMS-PDA-ATH oxygenators bound significantly more AT from plasma than the PDMS-PDA oxygenators by a factor of greater than four with values of 48.37 and 11.56 ng/cm^2, respectively ($p<0.01$). These results are in agreement with the adsorption data for discs shown in Figure 26. AT, as a relatively minor component of plasma, must compete for adsorption sites with the other proteins, many of which are adsorbed to these surfaces as shown by the data from experiments using disc samples (Figure 22, Figure 24, Figure 28). The fact that AT adsorption is

greater on the ATH surface indicates that the interactions are specific, that the heparin component of the ATH is active, and that anticoagulant activity can be expected for this surface.

4.2.3 Western Blots

To investigate the interactions of a variety of proteins under flow conditions in the microfluidic geometry of the oxygenator, Western blots were performed on eluates after plasma contact. Blots were obtained using antibodies against albumin, fibrinogen, and AT. Four surface types were studied: unmodified PDMS, PDMS-PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h, 3 mL/min), PDMS-PDA-ATH-0.1 (0.1 mg/mL ATH in PBS, pH 7.4, 24 h, 3 mL/min), and PDMS-PDA-ATH-1 (1 mg/mL ATH in PBS, pH 7.4, 24 h). Oxygenators (one unit of each surface type) were exposed to flowing plasma (3 mL/min) for 3 h in the closed circuit apparatus shown in Figure 15. Surface-bound proteins were eluted and Western blots performed on the eluates. The results are shown in Figure 31.

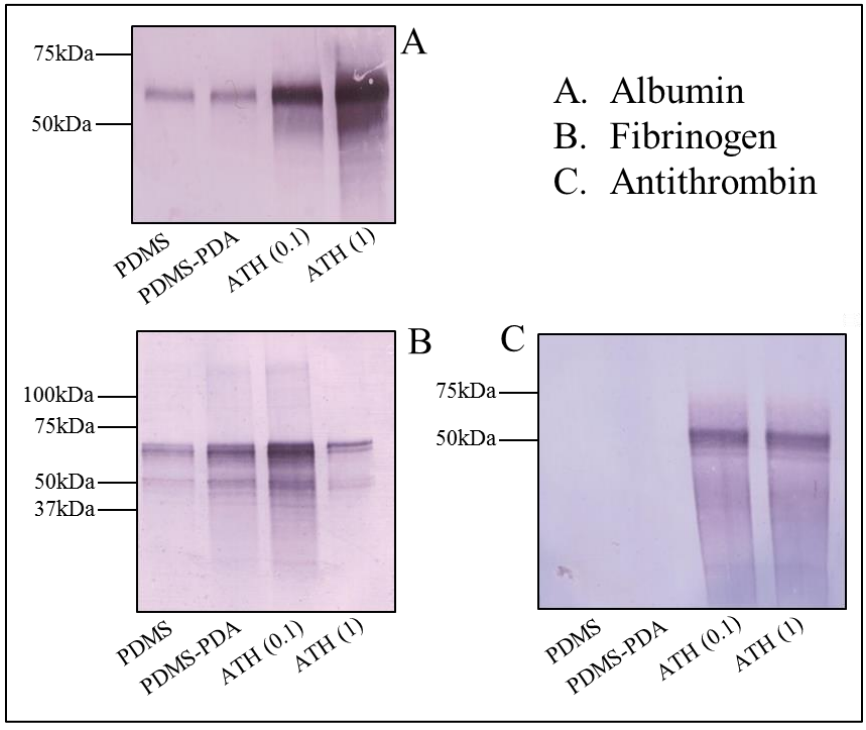

Figure 31 - Western blot analysis of proteins eluted from oxygenator units following plasma contact under flow. ATH modifications were performed using 0.1 or 1mg/mL incubation concentration.

Contrary to the blots from disc samples shown in Figure 28, the albumin blots for PDMS-PDA-ATH-0.1 and PDMS-PDA-ATH-1 oxygenators showed similar band intensities. The albumin band intensities for PDMS and PDMS-PDA oxygenator units were similar to each other but much lower than those for the ATH modified unit. Fibrinogen was present in the eluates from all four oxygenator types, with the highest band intensities for PDMS-PDA and PDMS-PDA-ATH-0.1. The PDMS-PDA-ATH-1 unit showed a relatively weak fibrinogen blot response similar to the disc sample (Figure 28).

AT is an important protein in this context due to its role in inhibition of thrombin via specific interaction with heparin. As seen in Figure 31, AT was not detected in the eluates from the PDMS and PDMS-PDA oxygenators. In contrast, bands at 58 kDa on the PDMS-PDA-ATH-0.1 and PDMS-PDA-ATH-1 blots indicate the specific binding of AT from plasma, and suggest that ATH on these surfaces retains its heparin-like activity.

4.2.4 Coagulation Times of Plasma in Contact with Oxygenator Units

The coagulation times of plasma in contact with the oxygenators was determined as a more direct measure of the anticoagulant effect of surface immobilized ATH. Citrate-anticoagulated plasma was mixed in a 1:1 ratio by volume with 0.025 M calcium chloride to allow clotting to occur [96]. The re-calcified plasma was immediately placed in a closed loop circuit consisting of a single oxygenator unit, a pressure transducer, a peristaltic pump, and connecting tubing (minimized to limit exposure of the plasma to surfaces other than those of the oxygenator). Clot formation could be observed as an abrupt change in pressure in the closed system due to blockage of flow in the microchannels.

Oxygenator units modified with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h, 3 mL/min), and PDA-ATH (0.1 mg/mL ATH in PBS, pH 7.4, 24 h, 3 mL/min) were evaluated. The coagulation times are shown in Figure 32 and Figure 33.

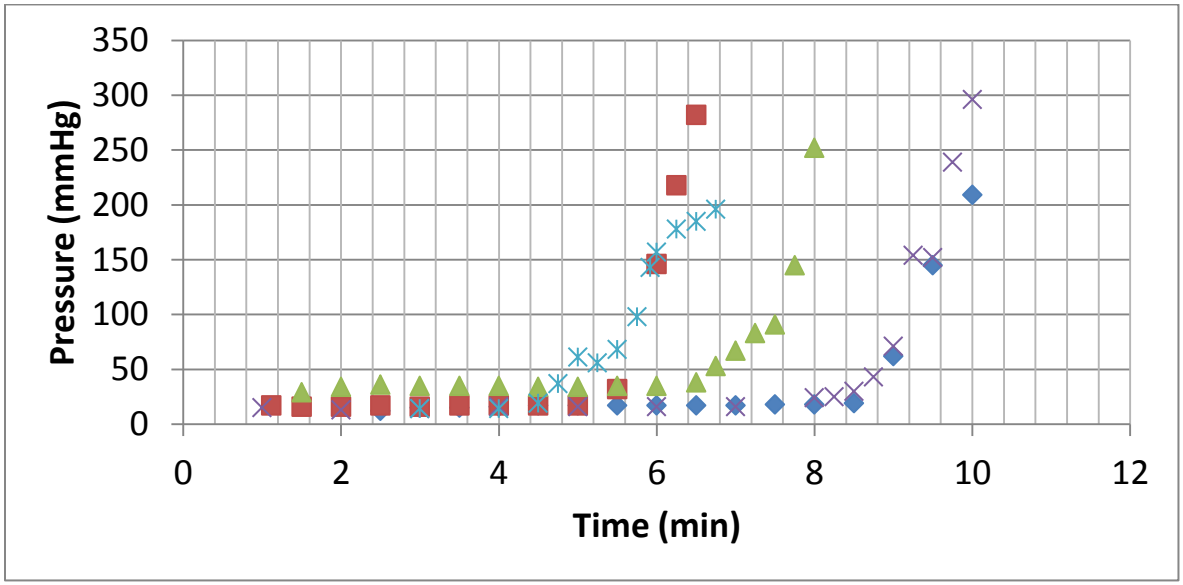

Figure 32 - Clotting times of plasma in contact with PDMS-PDA oxygenator units. Clotting was observed with respect to pressure change in a closed flow loop. Data for 5 independent experiments are shown. Average clotting time = 6.8±2.0 min.

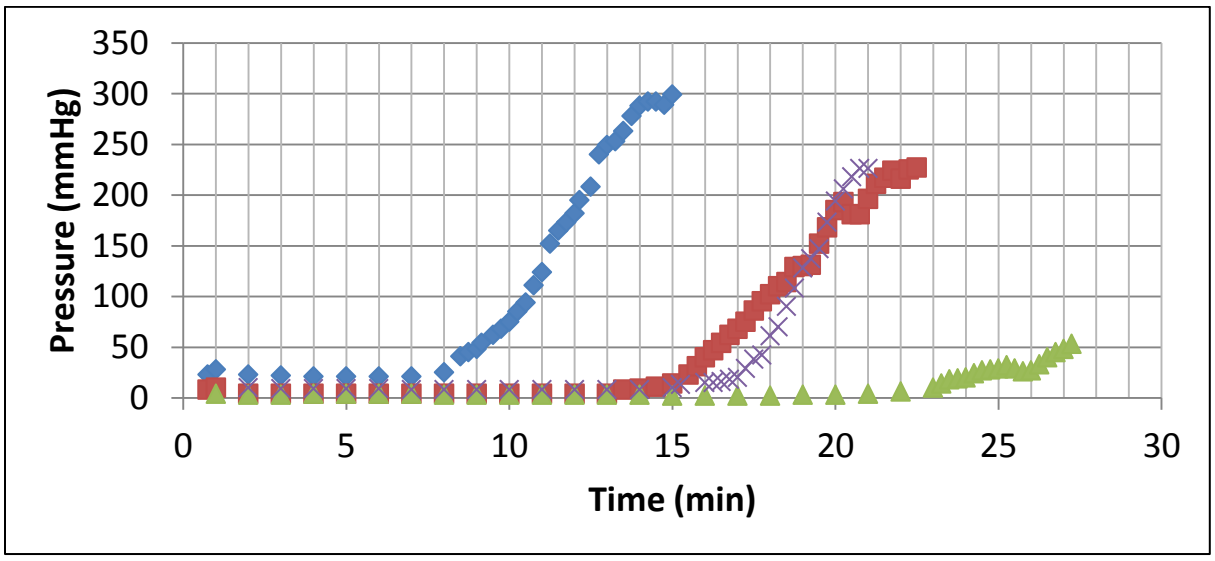

Figure 33 – Clotting times of plasma in contact with PDMS-PDA-ATH oxygenator units. Clotting was observed with respect to pressure change in a closed flow loop. Data for 5 independent experiments are shown. Average clotting time = 15.9±6.2 min.

The experimental design aimed to reduce as many variables as possible by using a closed circuit with all surfaces appropriately modified; however, adding back calcium to citrated plasma will inevitably activate the coagulation cascade. Therefore, the most appropriate measure is the time at which initiation of coagulation occurs, as evidenced by a sharp increase in pressure. For the PDMS-PDA oxygenator units (Figure 32) this time varied over the range of 5 to 9 min, with an average of 6.8 ± 2.0 min. For PDMS-PDA-ATH oxygenator units (Figure 33) the coagulation times were in a wider range from 8 to 23 min, with an average of 15.9 ± 6.2 min. Coagulation was most often observed at the inlet of the oxygenator units as shown in Figure 34, causing an increase in pressure as detected by the pressure transducer.

While the difference in clotting time between the ATH-modified and unmodified oxygenators was not statistically significant ($p>0.05$), clot formation was delayed longer on the ATH-modified device. These findings suggest that the catalytic effect of ATH to inhibit thrombin remains intact when attached to the oxygenator surfaces and evaluated under flow conditions.

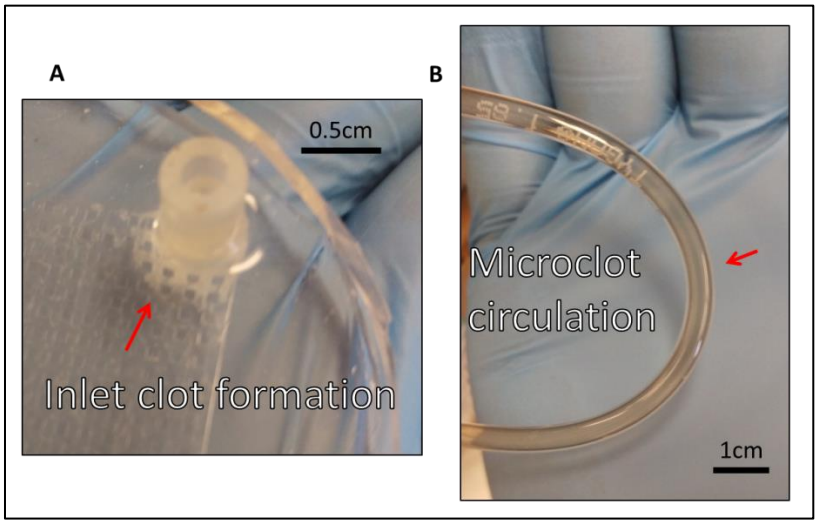

Figure 34 – Clot formation within the closed flow circuit. A) Clotting was often observed at the inlet of oxygenator units. B) Microclots flowing through a segment of connecting tubing.

4.2.5 Summary of ATH-Modified Oxygenator Units

Investigation of PDMS oxygenator units, as opposed to simple PDMS discs, is needed to determine the efficacy of ATH modification in a situation closer to the actual use of the device. Oxygenator units were surface-modified with PDA and ATH and their properties (ATH adsorption, stability, bioactivity) investigated. The protocols were modified to accommodate the complex geometry of the oxygenator units, and were carried out under flow conditions in a closed circuit. The oxygenators behaved similarly to discs of analogous materials prepared and evaluated under static conditions.

ATH bonded to the oxygenators via PDA was retained at the 76% level after 2 days of blood contact under flow. Heparin-like activity as reflected by antithrombin adsorption from plasma was retained by the surface immobilized ATH, and the clotting times of plasma in contact with the ATH modified oxygenators were prolonged compared to those of controls. It is concluded that the simple solution coating methods developed in this work using PDA as a bonding agent are effective for the modification of the oxygenator surfaces.

4.3 Plasminogen and tPA Modified PDMS Discs

The goal of ATH modification is to *prevent* clotting/thrombosis on the inner channels of the microfluidic oxygenator. Another approach to the problem of foreign-surface induced thrombosis is to *destroy* the thrombus as it forms and before it causes harm. The fibrinolytic system in the body is “designed” to do this, or more precisely, to break down hemostatic plugs (equivalent to thrombi) that are formed in the repair of damaged blood vessels (see section 2.4 literature review for details). This approach has been developed in previous work from this lab as discussed in section 2 above, and was explored in the present work for application to the microfluidic oxygenator [96], [122]. In brief, PDMS-PDA discs were modified with plasminogen

or tPA and the fibrinolytic activity of the modified discs was assessed using various *in vitro* assays.

4.3.1 Plasminogen Adsorption from Buffer

The plasma protein plasminogen is a zymogen that is readily converted to its active form plasmin by plasminogen activators. Plasminogen binds to fibrin with very high affinity at C-terminal lysine residues [53]. tPA also binds to fibrin at these residues [53]. Previous research has shown that plasminogen adsorption from plasma is high on polymer surfaces modified with lysine in which the ε-amino and carboxylic acid groups are free as in C-terminal lysine [95]–[97]. In addition the adsorbed plasminogen is readily converted to plasmin on these surfaces.

In the present work, lysine was bound to PDMS discs using PDA as bonding agent. Lys-plasminogen was then attached to the lysine by exposure to plasminogen solution, using radiolabeled plasminogen to allow determination of the surface density. In brief, PDMS discs were modified with PDA by exposure to 1 mg/mL dopamine in PBS, pH 8.5, for 24 h. The PDA discs were incubated in lysine solutions of concentration 0.5, 1 and 2 mg/mL in PBS, pH 7.4, for 24 h. Discs were then incubated in radiolabeled plasminogen solution (0.1 mg/mL in PBS, pH 7.4, 10% labeled) for 3 h and plasminogen adsorption was determined. The data are shown in Figure 35.

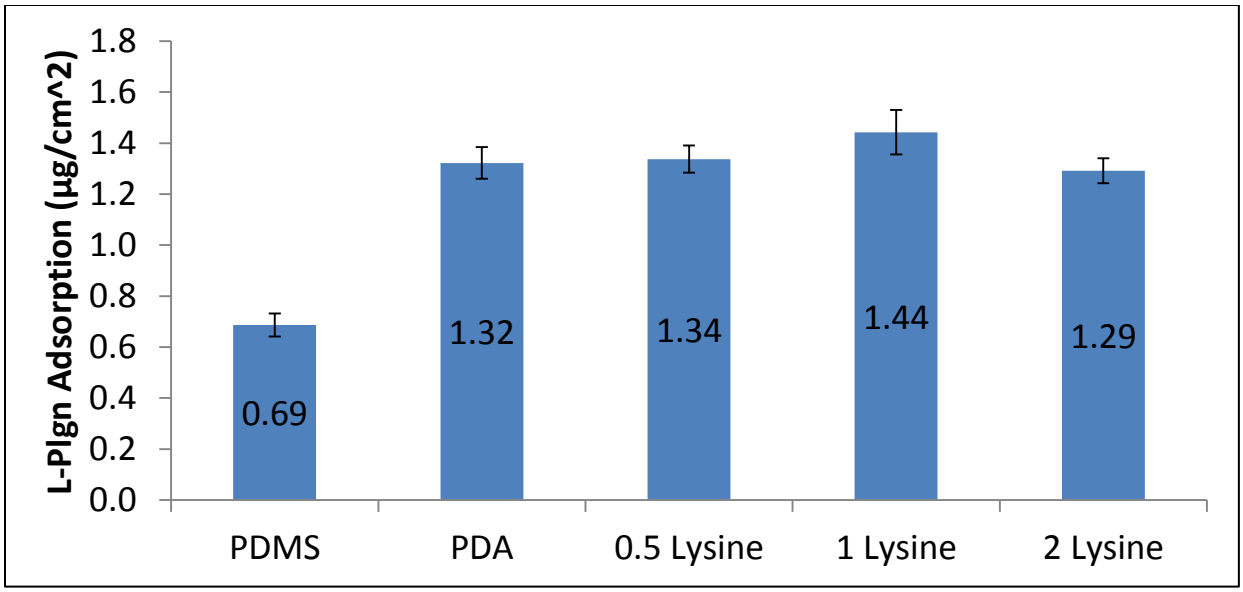

Figure 35 - LysPlgn adsorption to PDMS discs. Unmodified PDMS, PDMS-PDA, and PDA-Lysine surfaces were incubated in ^{125}I-labeled LysPlgn solution. Plasminogen adsorption was determined. Data are mean±SD, n=3.

Unmodified PDMS adsorbed significantly less plasminogen than all other surfaces ($p<0.05$). The quantities adsorbed to PDMS-PDA and the three PDMS-PDA-Lys surfaces were the same within experimental error ($p<0.05$); thus the lysine concentration used in the modification procedure had no effect on plasminogen adsorption. The similar adsorption seen on the PDA and lysine surfaces was unexpected since, as mentioned, lysine is known to be specific for plasminogen binding. Specificity requires, however, that lysine have the ε-amino and carboxylic acid groups free. While it is expected that lysine binds to PDA via its amino groups, it is unknown whether the ε-amino or α-amino groups are involved, and to the extent that ε-amino groups are used in attaching to PDA, specificity for plasminogen will be absent. Also plasminogen bound through ε-lysine changes conformation to an open form more susceptible to tPA activation [53], [86]. The conformation of the plasminogen on the lysine surfaces may thus be such that it is more susceptible to activation by tPA than the plasminogen on the PDMS-PDA

surface. Another unknown is the extent to which plasminogen is bound to the PDMS-PDA-Lys surfaces via exposed PDA, in which case it is not expected to be primed for activation by tPA.

Due to these uncertainties in the condition of plasminogen adsorbed to the lysine surfaces, PDMS-PDA surfaces modified directly with plasminogen (no lysine intermediate) were studied. PDMS discs were modified with PDA (1 mg/mL dopamine, PBS, pH 8.5, 24 h) and then with Glu- or Lys-plasminogen (0.1 mg/mL, PBS, pH 7.4, 24 h). Surfaces were then incubated in tPA solution (0.1 mg/mL, PBS, pH 7.4, 30 min) to activate surface bound plasminogen to plasmin and plasmin activity was determined using chromogenic substrate S2251. The data were scattered and only low levels of activity were observed (data not shown). Variable results were also observed in a clot lysis assay (not shown). Direct attachment of plasminogen was therefore not pursued further. Instead, surfaces modified directly with tPA were investigated.

4.3.2 tPA Uptake from Buffer and Stability in Plasma

A surface modified with tPA in blood contact may be expected to activate plasminogen, convert it to plasmin and lyse fibrin that may be present. Therefore, surfaces modified with tPA through PDA were studied as possible fibrinolytic surfaces.

PDMS discs were modified with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h). The PDA discs were then incubated in radiolabeled tPA solution (0.1 mg/mL in PBS, pH 7.4, 10% labeled) for 24 h. The discs were rinsed, radioactivity was measured, and tPA adsorption determined. Other tPA-adsorbed discs were incubated in plasma for 3, 6, 9, 12 or 24 h. The remaining surface-bound tPA was then determined. The results are show in Figure 36.

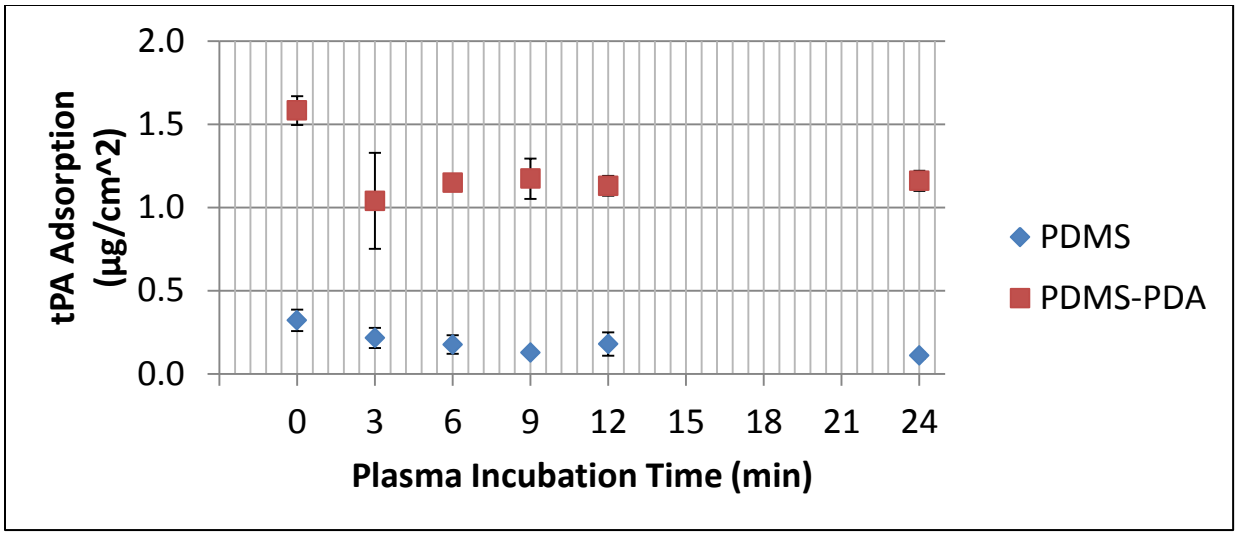

Figure 36 - Unmodified PDMS and PDMS-PDA surfaces were incubated in radiolabeled tPA solution. tPA adsorption was determined. Discs were then exposed to plasma for up to 24 h and residual tPA was determined. Data are mean±SD, n≥3.

tPA adsorption increased from 0.32 µg/cm^2 on unmodified PDMS to 1.58 µg/cm^2 on PDMS-PDA, a nearly 5-fold increase ($p<0.05$). Furthermore the PDMS-PDA-tPA surface experienced only modest tPA desorption over the first 3 h of incubation in plasma. Thereafter, coverage remained stable at around 1.1 µg/cm^2 up to 24 h ($p>0.05$). Some loss of tPA from unmodified PDMS occurred over 24 h incubation but differences from time point to time point were not significant ($p>0.05$).

Clearly the PDMS-PDA surface has greater capacity for tPA binding compared to unmodified PDMS. Moreover, tPA bound to PDMS through PDA was relatively stable in plasma over 24 h. It seems likely that loosely bound tPA was desorbed into the plasma during the initial 3 h of incubation, after which no additional desorption occurred. Indeed around 70% of the originally adsorbed tPA remained bound to the surface after 24 h plasma incubation. The residual level of ~1 µg/cm^2 suggests that a stable monolayer of tPA resistant to plasma was

formed via bonding with PDA. Therefore, using PDA to create a stable fibrinolytic surface on PDMS may be possible. The bound tPA must of course retain its ability to activate plasminogen to plasmin.

4.3.3 Activity of Surface-Immobilized tPA (S2288 and S2251 assays)

Chromogenic substrate S2288 is cleaved by active tPA, and chromogenic substrate S2251 is cleaved by active plasmin. tPA activity on surfaces can be measured directly by incubating in a solution of S2288. tPA can also be measured indirectly using S2251 by incubating the surface in a plasminogen/S2251 solution, and measuring the colour change due to plasmin generation.

4.3.3.1 Chromogenic Substrate S2288

Chromogenic substrate S2288 solution (200 µL, 5 mM in PBS, pH 7.4) was placed in 96-well plate wells. PDMS discs modified with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h) or with PDA, then tPA (0.1 mg/mL tPA in PBS, pH 7.4, 24 h) were placed in the wells and absorbance at 405 nm was measured at 1 min intervals for 60 min. The data are shown in Figure 37.

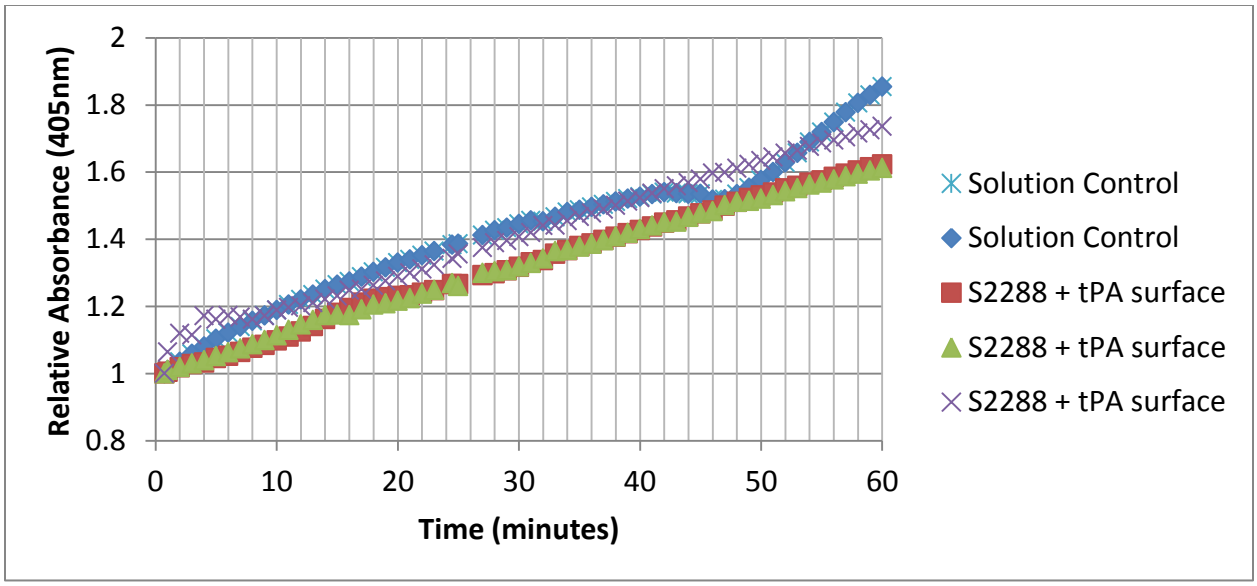

Figure 37 - tPA activity of PDMS-PDA-tPA surface and solutions of tPA containing the same quantity of tPA with respect to chromogenic substrate S2288. The data for all experiments are constrained to an absorbance value of 1.0 at zero time to facilitate comparison.

Unmodified PDMS and PDMS-PDA surfaces showed no activity (data not shown). From the tPA adsorption data in Figure 36 and using a disc area of 0.565 cm^2, the mass of tPA on the PDMS-PDA discs was estimated at 0.9 mg. This mass of tPA was then used to prepare tPA solutions used as controls to compare activity on the surface with that of the same quantity in solution. As seen in Figure 37, tPA bound to PDMS-PDA surfaces was active against chromogenic substrate S2288. Moreover, tPA on the surface showed a level of activity similar to that of the same quantity of tPA in solution, indicating that the process of surface attachment via PDA did not affect the activity of the tPA.

4.3.3.2 Chromogenic Substrate S2251

Chromogenic substrate S2251 is active against plasmin and was used in this work as an indirect measure of tPA activity based on its ability to convert plasminogen to plasmin.

A solution containing chromogenic substrate S2251 and Glu- or Lys-plasminogen (200 µL, 5 mM S2251, 2 µg plasminogen, PBS, pH 7.4) was placed in 96-well plate wells. PDMS discs modified with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h) or with PDA, then tPA (0.1 mg/mL tPA in PBS, pH 7.4, 24 h) were placed in the wells and absorbance at 405 nm was measured at 1-min intervals for 60 min. As for the S2288 experiments, solutions of tPA containing the same quantity of tPA as the surface samples, were used as controls to compare activity on the surface with that in solution. The results are shown in Figure 38.

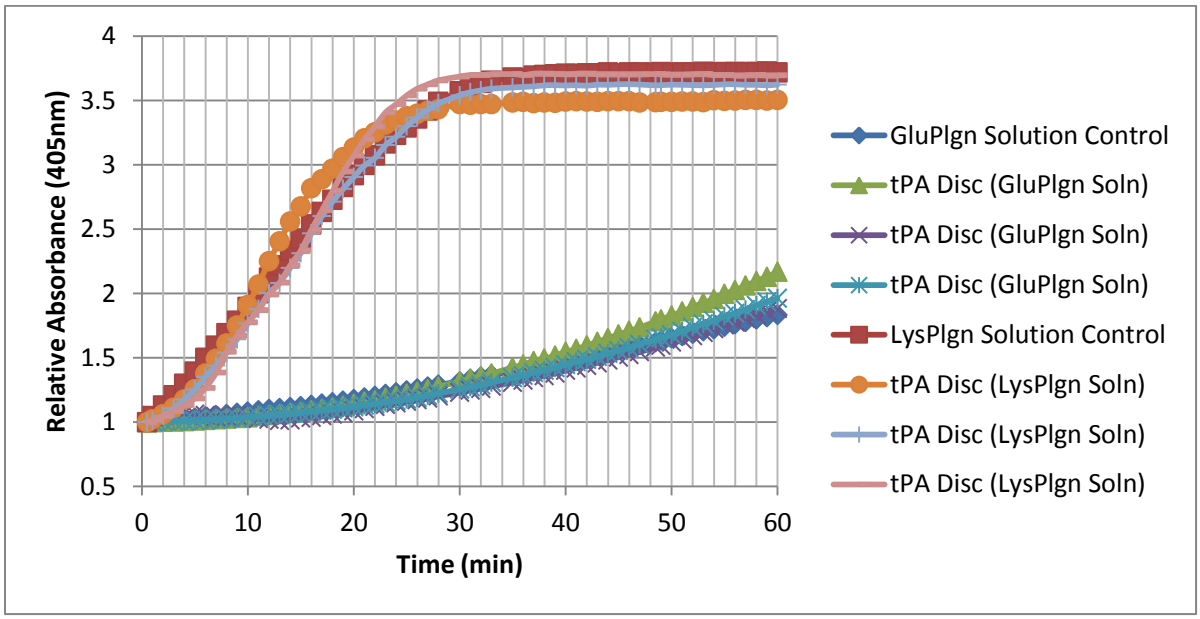

Figure 38 - tPA activity of PDMS-PDA-tPA surface with respect to its ability to convert plasminogen to plasmin. Plasmin activity was measured against chromogenic substrate S2251. The data for all experiments are constrained to an absorbance value of 1.0 at zero time to facilitate comparison.

Neither Glu-plasminogen nor Lys-plasminogen (no tPA) nor tPA alone showed any activity towards chromogenic substrate S2251; also the unmodified PDMS and PDMS-PDA surfaces showed no activity (data not shown). S2251 was, however, cleaved by the PDMS-PDA-tPA surface in the presence of plasminogen, thus showing that the surface-immobilized tPA

retained its ability to convert plasminogen to plasmin. Both Glu-plasminogen and Lys-plasminogen were converted. Predictably the Lys-plasminogen surface was significantly more active than the Glu-plasminogen surface due to the higher activity of Lys-plasmin [85]. In the experiments with Lys-plasminogen, substrate cleavage was "saturated" after 30 min, presumably due to complete cleavage of chromogenic substrate S2251 molecules within the well. As for the S2288 experiments, surface attached tPA showed activity similar to that of tPA in solution for both Glu- and Lys plasminogen. These results, from both the S2288 and S2251 experiments, indicate that tPA attached to PDMS via PDA does not lose activity compared to tPA in solution, suggesting that its conformation is not significantly altered and that its orientation on the surface is favourable for access to the different substrates, S2288 and plasminogen.

4.3.4 Clot Lysis

The experiments described above showed that immobilized tPA retained its bioactivity as assessed relative to the synthetic substrates S2288 and S2251. To investigate the activity of surface attached tPA in an manner more relevant to its fundamental function of fibrinolysis, the ability of the surfaces to lyse clots formed in plasma was evaluated. Citrated plasma in contact with the surfaces was recalcified and clot formation and lysis were monitored by measurement of the plasma turbidity over time.

Citrated plasma in 96-well plate wells was recalcified with 0.025M calcium chloride (1:1 v/v, total volume 200 µL). The plate was shaken for 15 s once per minute for 3 min. PDMS discs modified with PDA (1 mg/mL dopamine in PBS, pH 8.5, 24 h) or with PDA, then tPA (0.1 mg/mL tPA in PBS, pH 7.4, 24 h) were then placed in the wells. Absorbance at 405nm was recorded at 1 min intervals for 60 min. The results are shown in Figure 39. Clot formation is seen as an increase, and clot lysis as a decrease in absorbance.

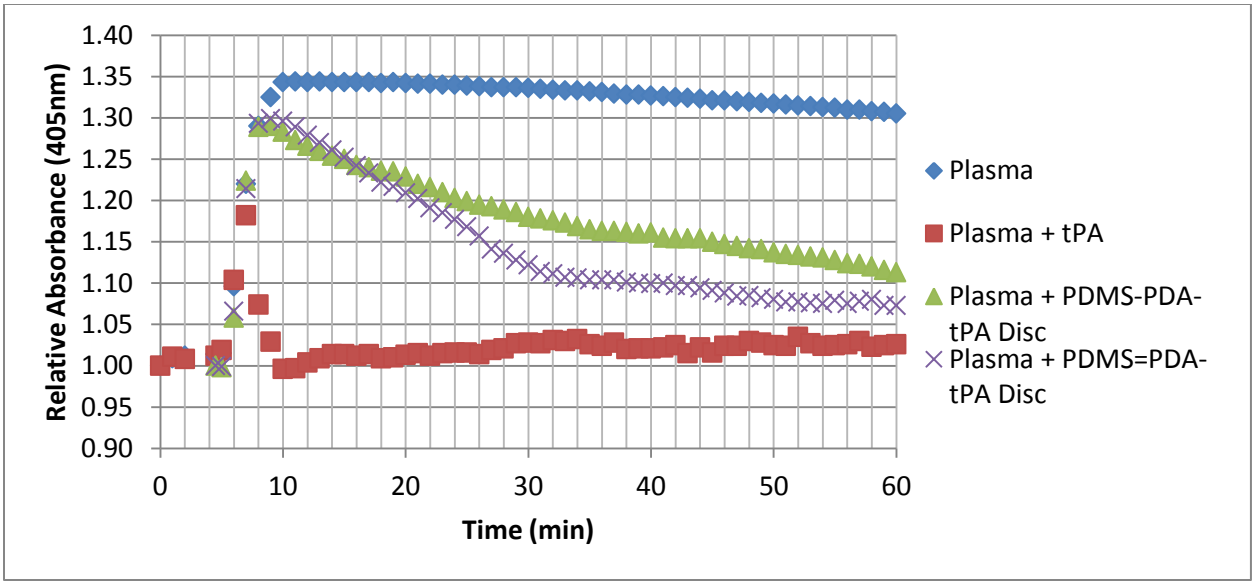

Figure 39 - Clot lysis activity of PDMS-PDA-tPA surfaces. Clot formation is seen as an increase and clot lysis as a decrease in absorbance. The data for all experiments are constrained to an absorbance value of 1.0 at zero time to facilitate comparison.

Unmodified PDMS surfaces and PDMS-PDA surfaces provoked clot formation but not clot lysis (data not shown). For the PDMS-PDA-tPA surfaces two main controls were used, the first being re-calcified citrated plasma. For this control (Figure 39) the absorbance increased sharply after a lag period of ~5 min, and then remained essentially constant over the following 55 min. The second control consisted of recalcified plasma to which tPA was added in a quantity equal to that on the tPA modified surface, estimated as described above for the chromogenic substrate experiments. For this control a very sharp, but small increase in absorbance was observed after ~5 min, with a rapid return to baseline over the following 4 min, indicating minimal clot formation and rapid clot lysis.

For the PDMS discs modified with tPA using PDA as bonding agent, clotting was again initiated after ~5 min and continued until ~8 min, after which the absorbance decreased gradually and significantly over the following 50 min, indicating clot lysis. It is presumed that in these experiments plasminogen in plasma in close proximity to the surface, or physically adsorbed to

the surface was converted to plasmin. These data show that tPA retained its fundamental fibrinolytic activity when immobilized on PDMS-PDA surface.

It is noted that the activity of tPA in the fluid phase was higher than that of surface-attached tPA, as indicated by much more rapid clot lysis in the former case. A number of factors may contribute to the slower clot lysis of surface immobilized tPA. For example, in fibrinolysis *in vivo*, tPA and plasminogen bind to fibrin at C-terminal lysine residues in fibrin to initiate plasminogen conversion to plasmin [53]. This interaction may be slowed or even totally inhibited when the tPA is immobilized. The generation of plasmin would then be due to the conversion of plasminogen molecules not bound to fibrin, and would be greatly slowed given that the fibrin-catalyzes conversion of plasminogen to plasmin occurs at a rate up to three orders of magnitude higher than the uncatalyzed reaction [55].

4.3.5 Summary of Plasminogen and tPA modified Discs

By modifying the surface with components of the fibrinolytic system to produce plasmin, fibrin generated by blood surface interactions may be degraded as it forms. Studies on surfaces modified with plasminogen or tPA using PDA as bonding agent were conducted. Plasminogen-modified surfaces treated with tPA showed variable and very low activity against the plasmin-specific chromogenic substrate S2251, and in a clot lysis assay. tPA adsorbed well to PDMS-PDA surface and was retained at the 73% level after 24 h contact with plasma. Surfaces modified with tPA showed activity against the tPA-specific chromogenic substrate S2288. Also when exposed to plasminogen, these surfaces generated plasmin as assessed by activity against chromogenic substrate S2251. The tPA-modified surfaces also showed fibrinolytic activity in a direct plasma clot lysis assay. These results, although they should be considered preliminary,

show that PDMS may acquire fibrinolytic properties by modification with tPA using PDA as bonding agent.

5. Summary and Conclusions

The work reported in this thesis was concerned with developing a thromboresistant silicone material for a microfluidic blood oxygenator used to treat neonatal infants suffering from respiratory distress syndrome. A two-fold strategy was explored: (1) modification with an antithrombin-heparin (ATH) complex to prevent or minimize clot formation, (2) modification with tissue plasminogen activator (tPA) to promote clot lysis. The modifying molecules were attached to the substrate PDMS via a layer of the bonding agent polydopamine (PDA).

As a preliminary to modification of PDMS microfluidic oxygenators, a first series of experiments was carried out using more geometrically simple PDMS discs. The use of PDA as a bioglue to attach biomolecules to a variety of substrates has been studied extensively over the past decade [27]. A PDA layer is formed on the surface by incubation with dopamine at alkaline pH. In this work, dopamine concentrations of 1 or 2 mg/mL in PBS, pH 8.5, and incubation times of 3 and 24 h were investigated. Water contact angles on the PDA surfaces were as low as 51^{o} compared to $>105^{o}$ on unmodified PDMS. Little effect of the dopamine concentration on contact angle was observed, but the angle did decrease with incubation time from 3 to 24 h. It was concluded that PDA surface coverage was optimum at a dopamine concentration of 1 mg/mL and an incubation time of 24 h. In terms of contact angle, the attachment of ATH (presumed to be through the amino groups of the AT moiety) to the PDMS-PDA surface was found to be unaffected by the dopamine concentration in the range of 1 to 2 mg/mL.

ATH adsorption to PDMS-PDA surfaces increased with increasing ATH concentration and reached 0.46 $\mu g/cm^2$ at 2 mg/mL ATH; however, good bioactivity of the resulting PDMS-PDA-ATH surfaces was observed using the lower concentration of 0.1 mg/mL ATH. Given the limited availability and high cost of ATH, PDMS-PDA-ATH surfaces prepared at the lower

concentration were used in most of the work reported. Modification of PDMS-PDA with ATH did not cause any significant change in water contact angle, presumably reflecting no difference in hydrophilicity between the two surfaces.

Bovine serum albumin (BSA) and fibrinogen adsorption to PDMS-PDA surfaces was significantly higher than to PDMS-PDA-ATH surfaces, indicating that ATH "covered", at least partially, the PDA layer, which is expected to show high nonspecific protein adsorption [109]. However adsorption levels of these "nonspecific" proteins to PDMS-PDA-ATH were still significant. Attempts to suppress this nonspecific adsorption by "backfilling" with BSA or low molecular weight heparin (LMWH) were unsuccessful.

On the other hand, AT adsorption from plasma to PDMS-PDA-ATH was much greater than on PDMS-PDA. PDMS-PDA-ATH adsorbed ~20 ng/cm^2 of AT, PDMS-PDA ~4 ng/cm^2, and unmodified PDMS ~4 ng/cm^2. This 5-fold increase in AT adsorption on the ATH surface compared to the precursor PDA surface suggests that AT adsorption to ATH was biospecific, that the heparin moiety of ATH retained its bioactivity and was appropriately oriented to bind AT. This conclusion was supported by Western blots of eluted surface-bound proteins after plasma incubation. Responses to antithrombin antibody were observed for the eluates from PDMS-PDA-ATH but not for those from PDMS-PDA.

Building on the work using 6 mm PDMS discs, microfluidic PDMS oxygenator units were modified with PDA and ATH. Modifications were done using dopamine solutions and ATH solutions under flow conditions. PDA modification was straightforward and good surface coverage was achieved. PDA-treated oxygenators adsorbed ATH at a level of 0.21 μg/cm^2, similar to that observed on PDMS discs. Moreover, 76% of the bound ATH remained on the

surface after 2 days of contact with flowing blood, indicating good stability of ATH attached via polydopamine. Antithrombin uptake from plasma to the ATH modified oxygenators was at a level of ~48 ng/cm^2 compared to ~12 ng/cm^2 on precursor PDMS-PDA oxygenators. Western blots of eluates from ATH-modifed oxygenators after plasma contact were strongly positive for antithrombin. In contrast, no antithrombin was present in the eluates from precursor PDMS or PDMS-PDA oxygenators. Preferential adsorption of antithrombin from plasma is a strong indication that the heparin moiety of the surface bound ATH retained its specific activity. Clotting times of plasma flowing through the oxygenators in a closed circuit were determined based on the sharp increase in pressure as clot formation occurred. The clotting time was prolonged from an average of 6.8 min for PDMS-PDA oxygenators to 15.9 min for PDMS-PDA-ATH oxygenators. These results for the oxygenators show that simple solution-based modification methods using PDA to bond ATH to the blood contacting surfaces are effective in endowing the devices with heparin-like anticoagulant activity.

As indicated, a second strategy was pursued to alleviate clotting/thrombosis on PDMS surface, namely modification with components of the fibrinolytic system to promote clot lysis. PDMS discs were modified with plasminogen or tPA using PDA as bonding agent.

Plasminogen uptake on PDMS-PDA discs reached a level of ~1.3 μg/cm^2, suggesting monolayer coverage. PDMS-PDA surface modified with lysine (a specific ligand for plasminogen) took up quantities of plasminogen similar to those on PDMS-PDA. It may be that plasminogen bound via lysine is more susceptible to activation by tPA, as in clot lysis *in vivo*, but given the increased complexity in preparing the lysine surface and the apparent lack of improvement compared to the simpler PDMS-PDA, the lysine surface was not pursued. PDMS-PDA-(Glu or Lys)- plasminogen surfaces showed only limited fibrinolytic activity as assessed by

their ability to cleave the plasmin-specific chromogenic substrate S2251 when treated with tPA and by a direct clot lysis assay.

tPA uptake from solution on PDMS-PDA reached a level of ~1.6 μg/cm^2, again indicating complete surface coverage. Approximately 70%, still in the monolayer range, remained after 24 h contact with plasma. The PDMS-PDA-tPA surfaces showed significant activity against the tPA-specific chromogenic substrate S2288, and against chromogenic substrate S2251 after treatment with plasminogen to generate plasmin. This surface was also clearly active in the direct plasma clot lysis assay. These results show that PDMS with tPA attached via PDA has good potential as a fibrinolytic surface.

As a final overall conclusion it appears that a PDMS surface modified with both ATH and tPA as described above, might be thromboresistant via a combination of anticoagulant and fibrinolytic properties. Additional work will be required to verify this conclusion.

6. Recommendations for Future Work.

The surface modification approaches described in this thesis show promise for the development of thromboresistant PDMS. Future work should seek to validate these approaches for application to the design of a blood-compatible PDMS-based microfluidic blood oxygenator and lung assist device (LAD).

- Additional work on disc samples at the *in vitro* level is required to develop a more complete understanding of blood-material interactions, and should include a more comprehensive investigation of the interactions of plasma proteins including coagulation factors such as thrombin and factor X, and proteins of the fibrinolytic pathway. A large gap in knowledge exists currently with respect to platelet interactions, and studies of platelet adhesion, activation and aggregation should be carried out.
- Additional *in vitro* experiments using oxygenator units and complete LADs, consisting of multiple oxygenator units, should be conducted. Further development of the techniques to modify the surfaces of these geometrically complex devices is needed. Modifying an entire LAD will undoubtedly present unanticipated challenges and will most likely require changes to the protocols to achieve a functioning surface. Blood interaction studies as described for disc samples, and including platelet interaction experiments, should also be performed on oxygenators and LADs.
- Following *in vitro* "validation", animal experiments will be required to evaluate the performance of the surface-modified LAD *in vivo*, specifically with respect to thromboresistance. Animal experiments (piglet model) have been performed previously using various LAD designs [2]. In these experiments the animals were heparinized and the focus was on gas transfer rather than coagulation/thrombosis.

- Work to date has been on surfaces modified with either ATH or tPA. In future work surfaces should be modified with both ATH and tPA to incorporate both anticoagulant and fibrinolytic properties, the expectation being that ATH would largely prevent fibrin generation and tPA would lyse any small amounts that might form. Preliminary studies showed that tPA and ATH retained their bioactivity when bound individually to PDMS through PDA. Future experiments should be carried out to determine whether activity is preserved when the molecules are present together on the surface. Studies will also be required to determine the optimum surface composition in terms of the ATH:tPA ratio. I*n vitro* and animal experiments as described for the "individually" modified surfaces and devices should also be performed on the "dually" modified ones.

References

[1] S. Shankaran, A. A. Fanaroff, L. L. Wright, D. K. Stevenson, E. F. Donovan, R. A. Ehrenkranz, J. C. Langer, S. B. Korones, B. J. Stoll, J. E. Tyson, C. R. Bauer, J. A. Lemons, W. Oh, and L. A. Papile, "Risk factors for early death among extremely low-birth-weight infants," *Am. J. Obstet. Gynecol.*, vol. 186, no. 4, pp. 796–802, 2002.

[2] N. Rochow, A. Manan, W. I. Wu, G. Fusch, S. Monkman, J. Leung, E. Chan, D. Nagpal, D. Predescu, J. Brash, P. R. Selvaganapathy, and C. Fusch, "An integrated array of microfluidic oxygenators as a neonatal lung assist device: In vitro characterization and in vivo demonstration," *Artif. Organs*, vol. 38, no. 10, pp. 856–866, 2014.

[3] N. Rochow, E. C. Chan, W. I. Wu, P. R. Selvaganapathy, G. Fusch, L. Berry, J. Brash, A. K. Chan, and C. Fusch, "Artificial placenta - Lung assist devices for term and preterm newborns with respiratory failure," *Int. J. Artif. Organs*, vol. 36, no. 6, pp. 377–391, 2013.

[4] R. S. Amin and M. J. Rutter, "Airway Disease and Management in Bronchopulmonary Dysplasia," *Clin. Perinatol.*, vol. 42, no. 4, pp. 857–870, 2015.

[5] American Lung Association, "Respiratory Distress Syndrome and Bronchopulmonary Dysplasia," pp. 111–116, 2008.

[6] M. a Underwood, B. Danielsen, and W. M. Gilbert, "Cost, causes and rates of rehospitalization of preterm infants.," *J. Perinatol.*, vol. 27, no. 10, pp. 614–619, 2007.

[7] N. L. Maitre, R. a Ballard, J. H. Ellenberg, S. D. Davis, J. M. Greenberg, A. Hamvas, G. S. Pryhuber, and Prematurity and Respiratory Outcomes Program, "Respiratory consequences of prematurity: evolution of a diagnosis and development of a comprehensive approach.," *J. Perinatol.*, vol. 35, no. 5, pp. 313–21, 2015.

[8] B. A. Lewis, L. T. Singer, S. Fulton, A. Salvator, E. J. Short, N. Klein, and J. Baley, "Speech and language outcomes of children with bronchopulmonary dysplasia," *J. Commun. Disord.*, vol. 35, pp. 393–406, 2002.

[9] V. Laxmi, *Anesthesia and Respiratory Devices: Global Markets*, no. April. Wellesley, MA: BCC Research, 2013.

[10] W.-I. Wu, N. Rochow, E. Chan, G. Fusch, A. Manan, D. Nagpal, P. R. Selvaganapathy, and C. Fusch, "Lung assist device: development of microfluidic oxygenators for preterm infants with respiratory failure.," *Lab Chip*, vol. 13, no. 13, pp. 2641–50, 2013.

[11] B. D. Ratner, A. S. Hoffman, F. J. Schoen, and J. Lemons, *Biomaterials Science : A Multidisciplinary Endeavor*. 2004.

[12] S. Alibeik, S. Zhu, and J. L. Brash, "Surface modification with PEG and hirudin for protein resistance and thrombin neutralization in blood contact," *Colloids Surfaces B Biointerfaces*, vol. 81, no. 2, pp. 389–396, 2010.

[13] L. Mourey, J. P. Samama, M. Delarue, J. Choay, J. C. Lormeau, M. Petitou, and D. Moras, "Antithrombin III: structural and functional aspects," *Biochimie*, vol. 72, no. 8, pp. 599–

608, 1990.

[14] R. Skinner, J. P. Abrahams, J. C. Whisstock, a M. Lesk, R. W. Carrell, and M. R. Wardell, "The 2.6 A structure of antithrombin indicates a conformational change at the heparin binding site.," *J. Mol. Biol.*, vol. 266, no. 3, pp. 601–9, 1997.

[15] A. J. Schulze, R. Huber, W. Bode, and R. A. Engh, "Structural aspects of serpin inhibition," *FEBS Lett.*, vol. 344, no. 2–3, pp. 117–124, 1994.

[16] L.-C. Hsu, "Principles of heparin-coating techniques," *Perfusion*, vol. 6, no. 3, pp. 209–219, 1991.

[17] P. Olsson, J. Sanchez, T. E. Mollnes, and J. Riesenfeld, "On the blood compatibility of end-point immobilized heparin," *J. Biomater. Sci. Polym. Ed.*, vol. 11, no. August 2015, pp. 1261–1273, 2000.

[18] S. Murugesan, J. Xie, and R. J. Linhardt, "Immobilization of Heparin: Approaches and Applications," *October*, vol. 8, no. 2, pp. 80–100, 2008.

[19] J. I. Weitz, "Low-Molecular-Weight Heparins," *N. Engl. J. Med.*, vol. 337, no. 10, pp. 688–699, 1997.

[20] L. Berry and A. Chan, "Improving Blood Compatibility," *Biomater. Fabr. Process. Handb.*, pp. 535–73, 2007.

[21] A. K. C. Chan, L. Berry, P. Klement, J. Julian, L. Mitchell, J. Weitz, J. Hirsh, and M. Andrew, "A novel antithrombin-heparin covalent complex: Antithrombotic and bleeding studies in rabbits," *Blood Coagul. Fibrinolysis*, vol. 9, no. 7, pp. 587–595, 1998.

[22] A. Chan, L. Berry, H. O'Brodovich, P. Klement, L. Mitchell, B. Baranowski, P. Monagle, and M. Andrew, "Covalent antithrombin-heparin complexes with high anticoagulant activity: Intravenous, subcutaneous, and intratracheal administration," *J. Biol. Chem.*, vol. 272, no. 35, pp. 22111–22117, 1997.

[23] R. F. . Zwaal and H. C. Hemker, *Blood Coaguation*, 1st Editio. New York: Elsevier Inc, 1986.

[24] D. Collen and H. R. Lijnen, "Tissue-type plasminogen activator: a historical perspective and personal account.," *J. Thromb. Haemost.*, vol. 2, no. 4, pp. 541–546, 2004.

[25] David Morton Waisman, *Plasminogen: Structure, Activation, and Regulation*, 1st Editio. New York: Springer Science, 2003.

[26] F. Abbasi, H. Mirzadeh, and A. A. Katbab, "Modification of polysiloxane polymers for biomedical applications: A review," *Polym. Int.*, vol. 50, no. 12, pp. 1279–1287, 2001.

[27] P. B. Lee, H.; Dellatore, S.M; Miller, W.M; Messersmith, "重点文献Mussel-Ispired Surface Chemistry for Multifunctional coating," *Science*, vol. 318, no. 2007, pp. 426–430, 2007.

[28] Y. Ozsurekci and K. Aykac, "Oxidative Stress Related Diseases in Newborns," *Oxid. Med. Cell. Longev.*, vol. 2016, 2016.

[29] C. I. for H. Information, "Highlights of 2010 – 2011 Selected Indicators Describing the Birthing Process in Canada," *Heal. Indic. Reports*, vol. 2005, no. Figure 2, pp. 1–7, 2011.

[30] Center for Disease Control, "Preterm Birth." [Online]. Available: http://www.cdc.gov/reproductivehealth/maternalinfanthealth/pretermbirth.htm. [Accessed: 12-Jul-2016].

[31] G. Wagner, A. Kaesler, U. Steinseifer, T. Schmitz-Rode, and J. Arens, "Comment on 'The promise of microfluidic artificial lungs' by J. A. Potkay, Lab Chip, 2014, **14** , 4122–4138," *Lab Chip*, vol. 16, no. 40, pp. 1272–1273, 2016.

[32] H. Makamba, J. H. Kim, K. Lim, N. Park, and J. H. Hahn, "Surface modification of poly(dimethylsiloxane) microchannels," *Electrophoresis*, vol. 24, no. 21, pp. 3607–3619, 2003.

[33] J. Kuncová-Kallio and P. J. Kallio, "PDMS and its suitability for analytical microfluidic devices," in *Annual International Conference of the IEEE Engineering in Medicine and Biology - Proceedings*, 2006, pp. 2486–2489.

[34] J. M. Leung, L. R. Berry, H. M. Atkinson, R. M. Cornelius, D. Sandejas, N. Rochow, P. R. Selvaganapathy, C. Fusch, A. K. C. Chan, and J. L. Brash, "Surface modification of poly(dimethylsiloxane) with a covalent antithrombin–heparin complex for the prevention of thrombosis: use of polydopamine as bonding agent," *J. Mater. Chem. B*, vol. 3, no. 29, pp. 6032–6036, 2015.

[35] J. Leung, L. Berry, J. Brash, and A. Chan, "Surface modification of polydimethylsiloxane with a covalent antithrombin-heparin complex to prevent thrombosis," *J. Biomater. Sci. Polym. Ed.*, vol. 25, no. 8, pp. 786–801, 2014.

[36] J. L. Brash, "Exploiting the current paradigm of blood–material interactions for the rational design of blood-compatible materials," *J. Biomater. Sci. Polym. Ed.*, vol. 11, no. 11, pp. 1135–1146, 2000.

[37] J. L. Brash, C. F. Scott, P. ten Hove, P. Wojciechowski, and R. W. Colman, "Mechanism of transient adsorption of fibrinogen from plasma to solid surfaces: role of the contact and fibrinolytic systems.," *Blood*, vol. 71, pp. 932–939, 1988.

[38] C. F. Scott, "Mechanism of the participation of the contact system in the Vroman effect. Review and summary.," *J. Biomater. Sci. Polym. Ed.*, vol. 2, no. 3, pp. 173–181, 1991.

[39] J. L. Brash, "Hydrophobic Polymer Surfaces and Their Interactions With Blood," *Ann. N. Y. Acad. Sci.*, vol. 283, no. 1 The Behavior, pp. 356–371, 1977.

[40] S. Li and J. J. D. Henry, "Nonthrombogenic approaches to cardiovascular bioengineering.," *Annu. Rev. Biomed. Eng.*, vol. 13, pp. 451–75, 2011.

[41] D. Li, H. Chen, W. Glenn McClung, and J. L. Brash, "Lysine-PEG-modified polyurethane

as a fibrinolytic surface: Effect of PEG chain length on protein interactions, platelet interactions and clot lysis," *Acta Biomater.*, vol. 5, no. 6, pp. 1864–1871, 2009.

[42] S. Jiang and Z. Cao, "Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological applications," *Adv. Mater.*, vol. 22, no. 9, pp. 920–932, 2010.

[43] S. H. Ye, Y. S. Jang, Y. H. Yun, V. Shankarraman, J. R. Woolley, Y. Hong, L. J. Gamble, K. Ishihara, and W. R. Wagner, "Surface modification of a biodegradable magnesium alloy with phosphorylcholine (PC) and sulfobetaine (SB) functional macromolecules for reduced thrombogenicity and acute corrosion resistance," *Langmuir*, vol. 29, no. 26, pp. 8320–8327, 2013.

[44] Enzyme Research Laboratories, "The ERL Cascade," *Enzyme Research Laboratories*. [Online]. Available: http://56i7f1v7i3s29dmdj3pej5ng.wpengine.netdna-cdn.com/wp-content/uploads/2013/10/ERL-Cascade.pdf. [Accessed: 15-Dec-2015].

[45] H. H. Versteeg, J. W. M. Heemskerk, M. Levi, and P. H. Reitsma, "New fundamentals in hemostasis.," *Physiol. Rev.*, vol. 93, no. 1, pp. 327–58, 2013.

[46] E. W. Davie and J. D. Kulman, "An overview of the structure and function of thrombin," *Seminars in Thrombosis and Hemostasis*, vol. 32, no. SUPPL. 1. pp. 3–15, 2006.

[47] N. Pozzi, D. Bystranowska, X. Zuo, and E. Di Cera, "Structural architecture of prothrombin in solution revealed by single molecule spectroscopy," *J. Biol. Chem.*, vol. 291, no. 35, pp. 18107–18116, 2016.

[48] H. Nar, "The role of structural information in the discovery of direct thrombin and factor Xa inhibitors," *Trends Pharmacol. Sci.*, vol. 33, no. 5, pp. 279–288, 2012.

[49] P. Liaw and A. Fox-Robichaud, "Blood Vessels & Host Defence Mechanisms," in *MS733 McMaster University Course Notes*, 2015.

[50] A. K. Verma, "Dabigatran etexilate: A new thrombin inhibitor," *Med. J. Aust.*, vol. 192, no. 7, pp. 407–412, 2010.

[51] R. I. Handin, "The History of Antithrombotic Therapy," *Hematol. Oncol. Clin. North Am.*, vol. 30, no. 5, pp. 987–993, 2016.

[52] M. B. Steed and M. T. Swanson, "Warfarin and Newer Agents," *Oral Maxillofac. Surg. Clin. North Am.*, vol. 28, no. 4, pp. 515–521, 2016.

[53] L. Medved and W. Nieuwenhuizen, "Molecular mechanisms of initiation of fibrinolysis by fibrin," *Thromb. Haemost.*, vol. 89, no. 3, pp. 409–419, 2003.

[54] C. Longstaff and K. Kolev, "Basic mechanisms and regulation of fibrinolysis," *J. Thromb. Haemost.*, vol. 13, no. S1, pp. S98–S105, 2015.

[55] M. W. Mosesson, "Fibrinogen and fibrin structure and functions.," *J. Thromb. Haemost.*, vol. 3, no. 8, pp. 1894–1904, 2005.

[56] J. W. Weisel and R. I. Litvinov, "Review Article Mechanisms of fi brin polymerization and clinical implications," *Blood*, vol. 121, no. 10, pp. 1712–1719, 2013.

[57] D. Woulfe, J. Yang, and L. Brass, "ADP and platelets: The end of the beginning," *J. Clin. Invest.*, vol. 107, no. 12, pp. 1503–1505, 2001.

[58] M. B. Gorbet and M. V. Sefton, "Biomaterial-associated thrombosis: Roles of coagulation factors, complement, platelets and leukocytes," *Biomater. Silver Jubil. Compend.*, vol. 25, pp. 219–241, 2006.

[59] J. Schymeinsky, A. Mócsai, and B. Walzog, "Neutrophil activation via beta2 integrins (CD11/CD18): molecular mechanisms and clinical implications.," *Thromb. Haemost.*, vol. 98, no. 2, pp. 262–273, 2007.

[60] D. J. Smith, D. Chakravarthy, S. Pulfer, M. L. Simmons, J. A. Hrabie, M. L. Citro, J. E. Saavedra, K. M. Davies, T. C. Hutsell, D. L. Mooradian, S. R. Hanson, and L. K. Keefer, "Nitric oxide-releasing polymers containing the [N(O)NO]- group," *J. Med. Chem.*, vol. 39, no. 5, pp. 1148–1156, 1996.

[61] H.-W. Jun, L. J. Taite, and J. L. West, "Nitric Oxide-Producing Polyurethanes," *Biomacromolecules*, vol. 6, no. 2, pp. 838–844, 2005.

[62] J. Conard, F. Brosstad, M. Lie Larsen, M. Samama, and U. Abildgaard, "Molar Antithrombin Concentration in Normal Human Plasma," *Pathophysiol. Haemost. Thromb.*, vol. 13, no. 6, pp. 363–368, 1983.

[63] L. J. Berliner, *Thrombin Structure and Function*, vol. 53, no. 9. 1992.

[64] L. Jin, J. P. Abrahams, R. Skinner, M. Petitou, R. N. Pike, and R. W. Carrell, "The anticoagulant activation of antithrombin by heparin.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, no. 26, pp. 14683–8, 1997.

[65] M. C. Z. Meneghetti, A. J. Hughes, T. R. Rudd, H. B. Nader, A. K. Powell, E. a. Yates, and M. a. Lima, "Heparan sulfate and heparin interactions with proteins," *J. R. Soc. Interface*, vol. 12, no. 110, p. 20150589, 2015.

[66] B. Casu, A. Naggi, and G. Torri, "Re-visiting the structure of heparin," *Carbohydr. Res.*, vol. 403, pp. 60–68, 2015.

[67] N. A. Platé and L. I. Valuev, "On the mechanism of enhanced thromboresistance of polymeric materials in the presence of heparin," *Biomaterials*, vol. 4, no. 1, pp. 14–20, 1983.

[68] V. L. Gott and R. L. Daggett, "Serendipity and the development of heparin and carbon surfaces.," *Ann. Thorac. Surg.*, vol. 68, no. 3 Suppl, pp. S19-22, 1999.

[69] Medtronic, "Carmeda BioActive Surface," 2012.

[70] X. Liu, L. Yuan, D. Li, Z. Tang, Y. Wang, G. Chen, H. Chen, and J. L. Brash, "Blood compatible materials: State of the art," *J. Mater. Chem. B*, vol. 2, no. 35, pp. 5718–5738,

2014.

[71] G. M. Palatianos, C. N. Foroulis, M. I. Vassili, G. Astras, K. Triantafillou, E. Papadakis, A. A. Lidoriki, E. Iliopoulou, and E. N. Melissari, "A prospective, double-blind study on the efficacy of the bioline surface-heparinized extracorporeal perfusion circuit," *Ann. Thorac. Surg.*, vol. 76, no. 1, pp. 129–135, 2003.

[72] S. Patel, L. R. Berry, and A. K. C. Chan, "Analysis of inhibition rate enhancement by covalent linkage of antithrombin to heparin as a potential predictor of reaction mechanism," *J. Biochem.*, vol. 141, no. 1, pp. 25–35, 2007.

[73] R. L. Bick, E. P. Frenkel, J. Walenga, J. Fareed, and D. A. Hoppensteadt, "Unfractionated heparin, low molecular weight heparins, and pentasaccharide: Basic mechanism of actions, pharmacology, and clinical use," *Hematol. Oncol. Clin. North Am.*, vol. 19, no. 1, pp. 1–51, 2005.

[74] L. R. Berry, D. L. Becker, and A. K. C. Chan, "Inhibition of Fibrin-Bound Thrombin by a Covalent Antithrombin-Heparin Complex," *J Biochem*, vol. 132, no. 2, pp. 167–176, 2002.

[75] N. Paredes, A. Wang, L. R. Berry, L. J. Smith, A. R. Stafford, J. I. Weitz, and A. K. C. Chan, "Mechanisms responsible for catalysis of the inhibition of factor Xa or thrombin by antithrombin using a covalent antithrombin-heparin complex," *J. Biol. Chem.*, vol. 278, no. 26, pp. 23398–23409, 2003.

[76] S. Patel, L. R. Berry, and A. K. C. Chan, "Covalent antithrombin-heparin complexes," *Thromb. Res.*, vol. 120, no. 2, pp. 151–160, 2007.

[77] I. Stevic, H. H. W. Chan, A. Chander, L. R. Berry, and A. K. C. Chan, "Covalently linking heparin to antithrombin enhances prothrombinase inhibition on activated platelets," *Thromb. Haemost.*, vol. 109, no. 6, pp. 1016–1024, 2013.

[78] I. Stevic, H. H. W. Chan, L. R. Berry, A. Chander, and A. K. C. Chan, "Inhibition of the prothrombinase complex on red blood cells by heparin and covalent antithrombin-heparin complex," *J. Biochem.*, vol. 153, no. 1, pp. 103–110, 2013.

[79] I. Stevic, L. R. Berry, and A. K. C. Chan, "Mechanism of inhibition of the prothrombinase complex by a covalent antithrombin-heparin complex," *J. Biochem.*, vol. 152, no. 2, pp. 139–148, 2012.

[80] K. N. Sask, L. R. Berry, A. K. C. Chan, and J. L. Brash, "Polyurethane modified with an antithrombin-heparin complex via polyethylene oxide linker/spacers: Influence of PEO molecular weight and PEO-ATH bond on catalytic and direct anticoagulant functions," *J. Biomed. Mater. Res. - Part A*, vol. 100 A, no. 10, pp. 2821–2828, 2012.

[81] K. N. Sask, L. R. Berry, A. K. C. Chan, and J. L. Brash, "Modification of polyurethane surface with an antithrombin-heparin complex for blood contact: Influence of molecular weight of polyethylene oxide used as a linker/spacer," *Langmuir*, vol. 28, no. 4, pp. 2099–2106, 2012.

[82] K. N. Sask, I. Zhitomirsky, L. R. Berry, A. K. C. Chan, and J. L. Brash, "Surface modification with an antithrombin-heparin complex for anticoagulation: Studies on a model surface with gold as substrate," *Acta Biomater.*, vol. 6, no. 8, pp. 2911–2919, 2010.

[83] P. Klement, Y. J. Du, L. R. Berry, P. Tressel, and A. K. C. Chan, "Chronic performance of polyurethane catheters covalently coated with ATH complex: A rabbit jugular vein model," *Biomaterials*, vol. 27, no. 29, pp. 5107–5117, 2006.

[84] J. S. Suwandi, R. E. M. Toes, T. Nikolic, and B. O. Roep, "Inducing tissue specific tolerance in autoimmune disease with tolerogenic dendritic cells," *Clin. Exp. Rheumatol.*, vol. 33, pp. 97–103, 2015.

[85] D. C. Rijken and H. R. Lijnen, "New insights into the molecular mechanisms of the fibrinolytic system.," *J. Thromb. Haemost.*, vol. 7, no. 1, pp. 4–13, 2009.

[86] U. Christensen, "C-terminal lysine residues of fibrinogen fragments essential for binding to plasminogen," *FEBS Lett.*, vol. 182, no. 1, pp. 43–46, 1985.

[87] R. L. Medcalf, "Fibrinolysis, inflammation, and regulation of the plasminogen activating system," *J. Thromb. Haemost.*, vol. 5, no. SUPPL. 1, pp. 132–142, 2007.

[88] A. Banerjee, Y. Chisti, and U. C. Banerjee, "Streptokinase - A clinically useful thrombolytic agent," *Biotechnol. Adv.*, vol. 22, no. 4, pp. 287–307, 2004.

[89] A. Kunamneni, T. T. A. Abdelghani, and P. Ellaiah, "Streptokinase - The drug of choice for thrombolytic therapy," *J. Thromb. Thrombolysis*, vol. 23, no. 1, pp. 9–23, 2007.

[90] M. Hébert, F. Lesept, D. Vivien, and R. Macrez, "The story of an exceptional serine protease, tissue-type plasminogen activator (tPA)," *Rev. Neurol. (Paris).*, pp. 1–12, 2015.

[91] Y. S. Rathore, M. Rehan, K. Pandey, and G. Sahni, "First Structural Model of Full-Length Human Tissue-Plasminogen Activator : A SAXS Data-Based Modeling Study," pp. 496–502, 2012.

[92] D. Pennica, W. E. Holmes, W. J. Kohr, R. N. Harkins, G. A. Vehar, C. A. Ward, W. F. Bennett, E. Yelverton, P. H. Seeburg, H. L. Heyneker, and D. V. Goeddel, "Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli.," *Nature*, vol. 301, pp. 214–221, 1983.

[93] K. A. Woodhouse, J. I. Weitz, and J. L. Brash, "Lysis of surface-localized fibrin clots by adsorbed plasminogen in the presence of tissue plasminogen activator.," *Biomaterials*, vol. 17, no. 1, pp. 75–7, 1996.

[94] K. A. Woodhouse, J. I. Weitz, and J. L. Brash, "Interactions of plasminogen and fibrinogen with model silica glass surfaces: adsorption from plasma and enzymatic activity studies.," *J. Biomed. Mater. Res.*, vol. 28, no. 4, pp. 407–15, 1994.

[95] W. G. McClung, D. L. Clapper, S. P. Hu, and J. L. Brash, "Adsorption of plasminogen from human plasma to lysine-containing surfaces," *J. Biomed. Mater. Res.*, vol. 49, no. 3, pp. 409–414, 2000.

[96] W. G. McClung, D. L. Clapper, S. P. Hu, and J. L. Brash, "Lysine-derivatized polyurethane as a clot lysing surface: Conversion of adsorbed plasminogen to plasmin and clot lysis in vitro," *Biomaterials*, vol. 22, no. 13, pp. 1919–1924, 2001.

[97] W. G. McClung, D. E. Babcock, and J. L. Brash, "Fibrinolytic properties of lysine-derivatized polyethylene in contact with flowing whole blood (Chandler Loop model)," *J. Biomed. Mater. Res. - Part A*, vol. 81, no. 3, pp. 644–651, 2007.

[98] H. Chen, Y. Zhang, D. Li, X. Hu, L. Wang, W. G. McClung, and J. L. Brash, "Surfaces having dual fibrinolytic and protein resistant properties by immobilization of lysine on polyurethane through a PEG spacer.," *J. Biomed. Mater. Res. A*, vol. 90, no. 3, pp. 940–946, 2009.

[99] H. Chen, L. Wang, Y. Zhang, D. Li, W. G. McClung, M. A. Brook, H. Sheardown, and J. L. Brash, "Fibrinolytic poly(dimethyl siloxane) surfaces," *Macromol. Biosci.*, vol. 8, no. 9, pp. 863–870, 2008.

[100] D. Li, H. Chen, S. Wang, Z. Wu, and J. L. Brash, "Lysine-poly(2-hydroxyethyl methacrylate) modified polyurethane surface with high lysine density and fibrinolytic activity," *Acta Biomater.*, vol. 7, no. 3, pp. 954–958, 2011.

[101] F. Zia, K. M. Zia, M. Zuber, S. Tabasum, and S. Rehman, "Heparin based polyurethanes: A state-of-the-art review," *Int. J. Biol. Macromol.*, vol. 84, pp. 101–111, 2016.

[102] A. Alrifaiy, O. A. Lindahl, and K. Ramser, "Polymer-based microfluidic devices for pharmacy, biology and tissue engineering," *Polymers (Basel).*, vol. 4, no. 3, pp. 1349–1398, 2012.

[103] H. Chen, M. A. Brook, and H. Sheardown, "Silicone elastomers for reduced protein adsorption," *Biomaterials*, vol. 25, no. 12, pp. 2273–2282, 2004.

[104] J. Zhou, D. A. Khodakov, A. V. Ellis, and N. H. Voelcker, "Surface modification for PDMS-based microfluidic devices," *Electrophoresis*, vol. 33, no. 1, pp. 89–104, 2012.

[105] J. Zhou, H. Yan, K. Ren, W. Dai, and H. Wu, "Convenient method for modifying poly(dimethylsiloxane) with poly(ethylene glycol) in microfluidics," *Anal. Chem.*, vol. 81, no. 16, pp. 6627–6632, 2009.

[106] S. Lee and J. Vörös, "An aqueous-based surface modification of poly(dimethylsiloxane) with poly(ethylene glycol) to prevent biofouling," *Langmuir*, vol. 21, no. 25, pp. 11957–11962, 2005.

[107] H. Chen, Y. Chen, H. Sheardown, and M. A. Brook, "Immobilization of heparin on a silicone surface through a heterobifunctional PEG spacer," *Biomaterials*, vol. 26, no. 35, pp. 7418–7424, 2005.

[108] M. E. Lynge, "Recent developments in poly (dopamine) - based coatings for biomedical applications," *Nanomedicine*, vol. 10, pp. 2725–2742, 2015.

[109] D. R. Dreyer, D. J. Miller, B. D. Freeman, D. R. Paul, and C. W. Bielawski, "Perspectives

on poly(dopamine)," *Chem. Sci.*, vol. 4, no. 10, pp. 3796–3802, 2013.

[110] R. Luo, L. Tang, S. Zhong, Z. Yang, J. Wang, Y. Weng, Q. Tu, C. Jiang, and N. Huang, "In vitro investigation of enhanced hemocompatibility and endothelial cell proliferation associated with quinone-rich polydopamine coating," *ACS Appl. Mater. Interfaces*, vol. 5, no. 5, pp. 1704–1714, 2013.

[111] J. Liebscher, R. Mrówczyński, H. A. Scheidt, C. Filip, N. D. Haìdade, R. Turcu, A. Bende, and S. Beck, "Structure of polydopamine: A never-ending story?," *Langmuir*, vol. 29, no. 33, 2013.

[112] P. Zhou, Y. Deng, B. Lyu, R. Zhang, H. Zhang, H. Ma, Y. Lyu, and S. Wei, "Rapidly-deposited polydopamine coating via high temperature and vigorous stirring: Formation, characterization and biofunctional evaluation," *PLoS One*, vol. 9, no. 11, pp. 0–9, 2014.

[113] L. C. Knight, S. A. Olexa, L. S. Malmud, and A. Z. Budzynski, "Specific uptake of radioiodinated fragment E1 by venous thrombi in pigs," *J. Clin. Invest.*, vol. 72, no. 6, pp. 2007–2013, 1983.

[114] C. M. Liu, R. P. Liang, X. N. Wang, J. W. Wang, and J. D. Qiu, "A versatile polydopamine platform for facile preparation of protein stationary phase for chip-based open tubular capillary electrochromatography enantioseparation," *J. Chromatogr. A*, vol. 1294, pp. 145–151, 2013.

[115] R. P. Liang, X. Y. Meng, C. M. Liu, and J. D. Qiu, "PDMS microchip coated with polydopamine/gold nanoparticles hybrid for efficient electrophoresis separation of amino acids," *Electrophoresis*, vol. 32, no. 23, pp. 3331–3340, 2011.

[116] S. G. Ghoreishi, F. Abbasi, and K. Jalili, "Hydrophilicity improvement of silicone rubber by interpenetrating polymer network formation in the proximal layer of polymer surface," *J. Polym. Res.*, vol. 23, no. 6, p. 115, 2016.

[117] L. Vroman, A. L. Adams, G. C. Fischer, and P. C. Munoz, "Interaction of high molecular weight kininogen, factor XII, and fibrinogen in plasma at interfaces.," *Blood*, vol. 55, no. 1, pp. 156–9, 1980.

[118] D. I. Leavesley, A. S. Kashyap, T. Croll, M. Sivaramakrishnan, A. Shokoohmand, B. G. Hollier, and Z. Upton, "Vitronectin--master controller or micromanager?," *IUBMB Life*, vol. 65, no. 10, pp. 807–18, 2013.

[119] J. M. van Raamsdonk, R. M. Cornelius, J. L. Brash, and P. L. Chang, "Deterioration of polyamino acid-coated alginate microcapsules in vivo.," *J. Biomater. Sci. Polym. Ed.*, vol. 13, no. 8, pp. 863–884, 2002.

[120] A. R. Jahangir, W. G. McClung, R. M. Cornelius, C. B. McCloskey, J. L. Brash, and J. P. Santerre, "Fluorinated surface-modifying macromolecules: Modulating adhesive protein and platelet interactions on a polyether-urethane," *J. Biomed. Mater. Res.*, vol. 60, no. 1, pp. 135–147, 2002.

[121] J. E. Babensee, R. M. Cornelius, J. L. Brash, and M. V. Sefton, "Immunoblot analysis of

proteins associated with HEMA-MMA microcapsules: Human serum proteins in vitro and rat proteins following implantation," *Biomaterials*, vol. 19, no. 7–9, pp. 839–849, 1998.

[122] K. A. Woodhouse and J. L. Brash, "Adsorption of plasminogen from plasma to lysine-derivatized Polyurethane surfaces," *Biomaterials*, vol. 13, no. 15, pp. 1103–1108, 1992.

Appendix A

A.1 Publications

J. M. Leung, L. R. Berry, H. M. Atkinson, R. M. Cornelius, **D. Sandejas**, N. Rochow, P. R. Selvaganapathy, C. Fusch, A. K. C. Chan, and J. L. Brash, *Surface modification of poly(dimethylsiloxane) with a covalent antithrombin–heparin complex for the prevention of thrombosis: use of polydopamine as bonding agent*, J. Mater. Chem. B, vol. 3, no. 29, pp. 6032–6036, 2015.

A.2 Presentations

Biomedical Engineering Annual Symposium, McMaster University, January 7-8, 2016

Presentation of thesis research. Awarded 2nd place presentation.

Surface Modification using Polydopamine to adhere a novel anticoagulant Antithrombin-Heparin Complex to the surface of PDMS Oxygenators used as a Lung Assist Device for Neonates
Darren Sandejas, Jennifer Leung, Rena Cornelius, Niels Rochow, Harpreet Matharoo, Leslie Berry**,** Gerhard Fusch, Helen Atkinson, Ravi Selvanapathy, Christoph Fusch, Anthony Chan, John Brash

Pediatric Academic Societies Meeting, San Diego, CA, April 25-28, 2015

Accepted abstracts. Poster presentation presented by a member of our lab group.

Artificial placenta: Developing a method for polydopamine (PDA) as a bioglue to attach coavalant antitrombin heparin (ATH) complex for hemocompatibility
Jennifer Leung, Leslie Berry, Rena Cornelius, Niels Rochow, **Darren Sandejas**, Gerhard Fusch, Ravi Selvaganapathy, Christoph Fusch, Anthony Chan, John Brash.

Artificial Placenta: Surface Modification of Single Oxygenator Units (SOU) using Polydopamine (PDA) as bioglue for anticoagulant Antithrombin-Heparin (ATH) Complex
Darren Sandejas, Rena Cornelius, Leslie Berry, Niels Rochow, Harpreet Matharoo, Gerhard Fusch, Helen Atkinson, Ravi Selvaganapathy, Anthony Chan, Christoph Fusch, John Brash.

Society for Biomaterals Annual Meeting, Charlotte, NC, April 15-18, 2015

Accepted abstract. Personally presented my work as a poster presentation.

Surface Modification using Polydopamine to adhere a novel anticoagulant Antithrombin-Heparin Complex to the surface of PDMS Oxygenators used as a Lung Assist Device for Neonates
Darren Sandejas, Jennifer Leung, Rena Cornelius, Niels Rochow, Harpreet Matharoo, Leslie Berry, Gerhard Fusch, Helen Atkinson, Ravi Salvanapathy, Christoph Fusch, Anthony Chan, John Brash.

I was selected as a competitor in the annual business competition. Graduate students across North America submitted an abstract to be accepted for entry into the SFB Business Competition. Selected participants created an in-depth business plan to bring

their research to market (technology, intellectual rights, regulatory approach, manufacturing, etc.).

Novel Lung Assist Device for Term and Preterm Neonate
Darren Sandejas, Harpreet Matharoo

Canadian Perinatal Research Meeting, Montebello, Quebec, February 24-27, 2015
Accepted abstracts. Poster presentation presented by a member of our lab group.

Artificial placenta: Developing a method for polydopamine (PDA) as a bioglue to attach coavalant antitrombin heparin (ATH) complex for hemocompatibility
Jennifer Leung, Leslie Berry, Rena Cornelius, Niels Rochow, **Darren Sandejas**, Gerhard Fusch, Ravi Selvaganapathy, Christoph Fusch, Anthony Chan, John Brash.

Artificial Placenta: Surface Modification of Single Oxygenator Units (SOUs) using Polydopamine (PDA) as bioglue for anticoagulant Antithrombin-Heparin (ATH) Complex
Darren Sandejas, Rena Cornelius, Leslie Berry, Niels Rochow, Harpreet Matharoo, Gerhard Fusch, Helen Atkinson, Anthony Chan, Ravi Selvaganapathy, Christoph Fusch, John Brash.

A.3 Scholarships and Awards

The Cecil and Yvette Yip Graduate Bursaries ($2500) Sept. 01, 2014-Aug. 31, 2015

2nd Place Oral Presentation Award
Biomedical Engineering Symposium, McMaster University, Jan. 2015

Biomedical Engineering Travel Award ($500) April 2015

Graduate Management Consulting Association
Winner of the Beginner Case Competition, University of Toronto, Nov. 2014