INVESTIGATION OF THROMBOSIS AT THE BIOMATERIAL SURFACE
IN LEFT VENTRICULAR ASSIST SYSTEMS

A Thesis in
Bioengineering

by
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ABSTRACT

Cardiovascular disease is the leading cause of death in the United States. With heart transplants in short supply, the use of ventricular assist devices (VADs) is up to almost 20% in patients awaiting heart transplants. Although the use of VADs has increased, thrombosis remains a major problem, occurring in up to 35% of VAD patients. A thrombus may obstruct blood flow, alter fluid dynamics causing damage to blood components, or wash off the surface as emboli and occlude vessels.

Thrombosis at the biomaterial surface in a left ventricular assist system (LVAS) and the effects of implantation time, anticoagulation, fluid mechanics, and biomaterial chemistry were investigated. LVAS were implanted in calves for 3 and 30 days with and without anticoagulation. Surface thrombosis was assessed on the polyurethane blood sacs of LVAS by macroscale examination and microscopy techniques. Platelet adhesion to polymers of different chemistry and activation of bulk platelet suspension were assessed in vitro by immunofluorescence techniques over a range of low shear stresses relevant to pediatric pumps.

Macroscopic thrombosis was lower at 30 days than at 3 days. Anticoagulation had no effect on coverage by macroscopic thrombi: 0.42% and 0.44% for 3-day study, and 0.23% and 0.16% for 30-day study, anticoagulated and nonanticoagulated groups, respectively. Differences in topography of blood sacs with implantation time were observed by scanning electron microscopy and confocal microscopy. Although anticoagulation reduced adhesion of platelet-like structures in the 30-day study, it was ineffective in reducing biologic deposition in the 3-day study. In both studies topography
and platelet and fibrin adhesion varied with sac region, which can be related to shear stress. For quantitative analysis of platelet adhesion on confocal images, fluorescence intensity threshold and particle size filtering criteria were established.

*In vitro* platelet studies were performed on candidate polymers for a pediatric VAD, Biospan® MS/0.4 and Biospan-P®, for shear stresses 0 – 10 dynes/cm². Minimal platelet adhesion was observed on both polymers at all shear stresses, except in regions with possible disturbed flow from the experimental setup. No significant difference in platelet adhesion between polymers was observed except at 5.5 and 8.9 dynes/cm².
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Chapter 1

Introduction

1.1 Biomaterials and Biocompatibility

Implantable medical devices are an important part of modern medicine, with devices available to replace or assist numerous organs throughout the body. The materials used for such implants, the biomaterials, are equally significant. A biomaterial is defined as a nonviable material used in a medical device, intended to interact with biological systems. Selection of a biomaterial for a specific medical application involves understanding the purpose of the biomaterial to determine the desired material properties and biological response to the biomaterial. The required material properties such as mechanical strength, and the desired biological response, such as cell adhesion, will be different depending on the biomedical application of the biomaterial. Biocompatibility, “the ability of a material to perform with an appropriate host response in a specific application”, is a critical property of a biomaterial. In blood-contacting applications, biocompatibility is generally referred to as the hemocompatibility of a biomaterial.

1.2 Hemocompatibility and Thrombosis

Blood contact with a biomaterial involves several host responses: hemostasis and thrombosis, inflammatory, foreign body, wound healing, and immune. Each of these
responses involves a complex series of events. The focus here is hemocompatibility in terms of thrombosis.

Thrombogenicity of a biomaterial can be defined as its propensity to form a thrombus. Thrombosis, the formation of thrombi, can lead to problems with vessel occlusion, turbulent flows (caused by a thrombus attached to a vessel wall altering the normal flow patterns in the vessel), and embolism that can lead to cell damage, stroke and tissue necrosis. A thrombus is an aggregate of blood factors, primarily platelets and fibrin, with entrapment of blood cells. The roles of coagulation factors and platelets are briefly discussed.4

Protein adsorption is well accepted as one of the initial events to occur upon blood contact with a biomaterial. Blood contains hundreds of proteins, several involved in thrombosis. Fibrinogen is the third most abundant protein in blood, present at a concentration between 2 – 4.5 mg/ml. It plays many key roles in thrombosis, mediating platelet adhesion to the biomaterial surface, aggregation of platelets, as well as polymerizing to form fibrin.5 The adsorption and desorption,6,7 conformation,8 molecular activity,9 and surface density of proteins such as fibrinogen that adsorb to the biomaterial surface all influence the events to follow leading to formation of a thrombus. Extensive studies on the interactions of various proteins with different biomaterials have been performed and are discussed in some detail later with regards to polyurethanes (PUs).

Platelets are disc-shaped anuclear bodies approximately 1.5 – 4 μm in diameter and present at a concentration of 150,000 – 350,000 platelets per μl in blood.3 Platelet adhesion to a biomaterial surface, mediated primarily by fibrinogen and Von Willebrand factor, is an important step in thrombosis. Adhered platelets as well as platelets in
suspension can become activated releasing numerous cofactors of the coagulation cascade, including Ca\(^{2+}\) and fibrinogen, amplifying thrombosis.\(^{10}\) Activated platelets also release adenosine diphosphate (ADP), which induces platelet aggregation. Platelet-platelet binding or aggregation is mediated by fibrinogen, again showing the importance of fibrinogen in thrombosis.

Fibrin is a major component of thrombosis and is responsible for entrapping cells in thrombi. Fibrinogen is again a key player, the precursor of fibrin. Fibrinopeptides A and B are cleaved from fibrinogen by the binding of thrombin to fibrinogen, forming fibrin monomers. The polymerization sites on fibrinogen are exposed and lead to formation of fibrin.\(^{11}\)

The responses of proteins and platelets to contact with biomaterials are the focus of many studies\(^{12-15}\) in the development of biomaterials and evaluation of hemocompatibility. In fact, bulk and surface modifications of PUs have been motivated by goals to elicit specific responses from proteins and platelets upon biomaterial contact.

### 1.3 Polyurethanes for Biomedical Applications

PUs are an important class of biomaterials used extensively in blood-contacting medical devices. Biomer\(^{\text{™}}\), a segmented poly(ether urethane urea) (SPEUU), was introduced as a biomaterial in the 1960s. Over the years PU has been proven superior over other biomedical polymers in tensile strength, durability, compliance, lubricity, abrasion resistance, and ease of handling.\(^{3}\) The hemocompatibility of PUs has been equal to or better than existing biomedical polymers. PUs have been shown to adsorb less
protein such as fibrinogen, and exhibit lower platelet activation than polyvinylchloride, polytetrafluoroethylene, and silicone rubber. Attempts to further improve the hemocompatibility of PUs have been made by bulk modification, modifying surface properties such as chemistry and energetics, and grafting molecules such as anticoagulants and nitric oxide releasing agents.

1.3.1 Basic Polyurethane Chemistry

PUs can be divided into several classes based on their chemical composition. PUs are thermoplastic elastomers made up of blocks of hard and soft segments with chain extenders. They contain urethane groups that link the dominant functional groups. The hard segment consists of a diisocyanate linked by a low molecular weight diol or diamine chain extender. Some common isocyanates used in PU synthesis are cyclohexyl diisocyanate (CHDI), 2, 4 toluene diisocyanate (TDI), and methylene bis(p-phenyl isocyanate (MDI). Chain extenders for PUs include ethylene diamine, ethylene glycol, and hexanediol. The soft segment consists of a polyol. Polyols used for the soft segment include polyethylene oxide (PEO), poly(tetramethylene)oxide (PTMO), and polydimethylsiloxane (PDMS). The many different isocyanates, polyols, chain extenders, their combinations, and the ratios of hard to soft segments, provide a wide range of PUs with diverse physical properties.

An example of a commercial PU for biomedical applications, currently used in cardiac assist devices, is Biospan® MS/0.4. This polymer is a SPEUU and consists of methylene bis(p-phenyl isocyanate) (MDI), approximately 2000 molecular weight
poly(tetramethylene oxide) (PTMO) and mixed diamine chain extenders, ethylene diamine (ED) and 1,3-cyclohexanediadmine (1,3-CHD). The chain ends are capped with approximately 2000 molecular weight poly(dimethylsiloxane) (PDMS) lending to 0.4 wt% of the copolymer. Studies have shown this polymer to be chemically stable.

1.3.2 Hemocompatibility of PUs

The diversity of PUs has allowed various bulk and surface modifications to improve their hemocompatibility. Beginning with the use of Biomer as a biomedical PU, additional polyether urethane ureas, polycarbonate urethanes, polyester urethanes, and silicone-PUs have been studied and used for biomedical applications.

Commercial PUs have become widely available for blood-contacting medical devices due to their relatively good hemocompatibility. Vialon™, a proprietary PEUU, was shown to dramatically reduce thrombophlebitis by 46% compared to Teflon for peripheral intravenous infusion cannulae in the late 1980s. Vialon is currently used in systems such as the BD Intima™ IV catheter system by Becton Dickinson. In 1970, Avcothane-51™, a silicone-PU was introduced by Avco Everett Research Laboratory (Everett, MA), and has since been renamed as Cardiothane-51. This polymer was used in the Arrow intraortic balloon pump. Medical grade polyester and polyether urethanes are available by Dow Chemical’s series of Pellethane™ products. The family of polycarbonate urethanes Chronoflex™ by CardioTech International is used for vascular grafts and pump diaphragms. The medical grade PEUU, Biomer, has been replaced by Biospan marketed by The Polymer Technology Group. The Polymer Technology Group
also has polycarbonate urethanes and ureas, polyether urethanes and ureas, and silicone-PUs. Following the development of such an extensive selection of PUs came various surface modifications.

### 1.3.3 Surface Properties and Hemocompatibility of PUs

Surface properties thought to affect hemocompatibility of PUs include chemistry, energetics, charge, hydrophobicity, topography, ratio of hard to soft segments, phase separation, and biological activity. Various techniques have also been developed for surface modification of biomaterials. Surface properties such as chemistry, charge, and hydrophobicity can be modified using self-assembled monolayers (SAMs) to form model systems for evaluation of hemocompatibility.

Various surface chemistries including PEO, styrene, fluorine, and sulfone have been shown to improve hemocompatibility of PUs. Surface chemistry modification of PUs by gas plasma showed a reduction in contact activation of the coagulation cascade for NH$_3$- and N$_2$-treated surfaces but no effect for the O$_2$- and Ar-treated surfaces. PEO resistance to protein adsorption is commonly believed to be due to steric hindrance caused by the strong interaction between PEO and water. In addition to the effects of polymer graft density, chain length, and molar mass, Curie et al. discuss the significance of rearrangement of polymers in adsorption of proteins specifically to PEO. In another study by Nojiri et al. PEO-grafted SPU and 2-hydroxyethyl methacrylate (HEMA)/styrene copolymer were compared to SPU vascular grafts. Three weeks to one month after implantation of the PEO-grafted SPU and control SPU grafts, the adsorbed
protein layers were 1000 to 2000 Å. On the other hand, even after three months, the protein layer adsorbed on the HEMA/styrene graft was less than 200 Å thick. Scanning electron microscope imaging of the graft surfaces also showed greater platelet adhesion and fibrin formation on the PEO-grafted PU and control PU. Hemocompatibility of fluorinated surface-modifying macromolecules was evaluated by Jahangir et al. by measuring adsorption of adhesive proteins including fibrinogen, fibronectin, and vitronectin, and platelet adhesion and activation on PEU. Fibrinogen adsorption was found to be similar for the control PEU and the fluorinated surfaces. Platelet adhesion, however, was significantly reduced on all fluorinated surfaces in comparison to the control PEU.

Hydrophobicity and surface energy of a surface are thought to affect the interaction of water with the biomaterial surface influencing accessibility of the biomaterial surface to proteins. Vogler et al. reported increased coagulation activation with increased water wettability (surface energy). Prime and Whitesides evaluated the adsorption of five proteins, including fibrinogen, to different SAMs including a hydrophobic methyl-terminated SAM and hydrophilic hydroxyl-, maltose-, and hexa(ethylene glycol)-terminated alkanethiols. They found increased protein adsorption with increasing hydrophobicity for all five proteins. For the same hydrophilicity, measured in terms of contact angle, hexa(ethylene glycol) was found most effective at resisting protein adsorption. In a comparison of ten PUs with different surface chemistry and hydrophilicity, including Biospan, two fluorinated PUs, and PEO-containing PUs, Wu et al. reported lowest fibrinogen adsorption and platelet adhesion on the three hydrophilic PEG-PUs. Poussard et al. investigated a new ionic PU and found
that the insertion mode, incorporation of the carboxylic group in the soft or hard segment, affected platelet adhesion. Incorporation of the group in the soft segment increased hydrophilicity and resulted in decreased fibrinogen adsorption and platelet adhesion \(^{22}\).

Textured surfaces can be used to promote one of two responses from blood: 1) minimal thrombosis – protein adsorption and platelet adhesion and 2) formation of pseudo-neointimal layer. Sub-micron sized pillars on PU surfaces have been used to reduce PU surface area for platelet contact, thus reducing platelet adhesion \(^{44}\). On the other hand, surfaces have been textured to encourage formation of stable thrombi to create a passive neointimal cellular lining. PU textured with fibers showed greater cellular deposition compared to the nontextured surfaces after 1 week in ovine arteries \(^{45}\). By 3 weeks post-implantation however, no histological difference was observed between the surfaces. Textured surfaces such as this have been used in cardiac assist pumps \(^{46,47}\).

The distinct properties of hard and soft segments of PU make the ratio of the segments and the degree of phase separation at the surface, properties greatly important to the nonthrombogenicity of PUs. Groth et al. investigated the relationship of hard to soft segment ratio with protein adsorption, lymphocyte adhesion, and platelet adhesion and activation \(^{48}\). They examined PUs synthesized with 4,4′-diphenylmethane diisocyanate (MDI) and polytetramethylene glycols (PTMG) with different hard segment content and composition. Three sets of four PUs with different hard segment content were synthesized: one set in which the ratio of urethane to urea was kept constant, a second set with constant urethane content, and a third with constant urea content. Human serum albumin and fibrinogen adsorption increased with increasing hard segment content and albumin adsorption was dependent on chemical composition. Platelet activation also
increased with increase in hard segment content with a constant urethane to urea ratio and with constant urethane content. However, constant urea content resulted in constant platelet activation, which was lower than for other PUs. PUs with constant urea content also resulted in decreased recognition by a monoclonal antibody for fibrinogen with increased hard segment content, suggesting conformational change in fibrinogen for this set of PUs. Nanophase separation has also been shown to play a role at the surface. Less platelet activation was observed with greater nanophase separation.49

Biologically active50 or passivating surfaces with albumin, anticoagulants and nitric oxide-releasing agents are also thought to improve hemocompatibility. Albumin-coating has been used to passivate surfaces and studies show decreased protein adsorption and thrombogenicity.51 Nitric oxide is present in blood and is well known to inhibit platelet adhesion. Several types of nitric oxide-releasing polymers have been developed.52 Covalent attachment of anticoagulants such as Persantin has been shown to successfully reduce platelet adhesion to PUs.25,53 However, the duration of the anticoagulant effect of such surfaces has been questionable.

1.4 Surface Thrombosis in Ventricular Assist Devices

1.4.1 Cardiovascular Disease and Ventricular Assist Devices

With approximately 4.9 million Americans suffering from congestive heart failure and 13 million with coronary heart disease, cardiovascular disease (CVD) is the leading cause of death in the United States.54 Almost 2,600 people die of CVD each day and the
number of deaths due to CVD alone is about the same as those caused by the next five leading causes of death combined. Over 150,000 of the deaths caused by CVD are of people under the age of 65. In children under the age of 15, CVD is the second leading cause of death. As of February 1, 2006, 3,003 people are on the waiting list for a heart transplant. The supply of heart donors has not been able to meet the demand for heart transplants. VADs provide support for patients unable to receive heart transplants.

VADs supported almost 20% of heart transplant patients in 2003. VADs have been used for short-term support, bridge to transplantation, and destination therapy. They are also proving beneficial in myocardial recovery. The benefits of VADs were proven in a multicenter study, Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH). A 48% decrease in mortality was observed by use of VADs compared to optimal medical management involving pharmacologic treatment. Along with continued improvements in adult VADs, efforts to develop pediatric circulatory support systems are now well underway. VADs are finding use in a larger population and wider range of cardiac failure patients and it is therefore crucial for VADs to perform without failure.

Left ventricular assist systems (LVAS) are designed to assist a failing left ventricle. The demand on the left ventricle that supplies blood to the systemic circulation is much greater than that of the right ventricle, which delivers to the pulmonary circulation. Therefore, a failing left ventricle is more common than a failing right ventricle. Among LVAS is a variety of systems that can be divided into two types: pulsatile flow and continuous flow. As suggested by the names, the two types differ by
the type of blood flow delivery. Pulsatile flow VADs mimic the pumping of a natural heart. On the other hand, continuous flow VADs provide a continuous stream of blood by means of an axial or centrifugal pump. A fully implantable pulsatile electronic LVAS was developed originally at Penn State and became the LionHeart™ LVAS (Arrow International Corporation).\textsuperscript{61} Figure 1.1 shows the components of a pulsatile LVAS similar to the LionHeart LVAS. Many of the blood-contacting components, including the blood sac found in the blood pump assembly, are made of polymeric biomaterials such as PU.

Figure 1.1. Components of fully implantable, pulsatile LVAS. The system consists of inlet (A) and outlet (B) cannulae, blood pump assembly (C), compliance chamber (D), access port (E), motor controller (F), internal (G) and external (H) coils, and external battery pack (I). (After Weiss et al.)\textsuperscript{62}

Improvement of LVAS is possible with consideration of various factors involved in device design and operation. This includes biomaterials, fluid mechanics,
anticoagulation, and the thrombotic response to these factors with time. Surface thrombosis in relation to these factors is the focus of the work presented.

1.4.2 Progression of Thrombosis with Time

Thrombosis is a complex dynamic event involving various components of blood over an extended period. In medical devices such as LVAS, which are intended for short term as well as permanent use, acute and chronic blood responses to biomaterials are important to consider. As protein adsorption is immediate with biomaterial contact, and coagulation and platelet responses rapidly follow, the acute response of blood to biomaterials is critical. However, the chronic responses that occur as the interface evolves are also of great significance in that LVAS are intended for long-term use, from months to years.

1.4.3 Anticoagulants for Thrombosis Prevention

Efforts to reduce occurrence of thrombosis commonly involve pharmacological therapy including anticoagulation. Common anticoagulants, particularly for use with cardiac assist devices, include heparin and warfarin sodium (Coumadin). Anticoagulation therapy must be applied and monitored carefully considering the methods of action and side effects of each anticoagulant.

Heparin is an anticoagulant that takes effect almost immediately and is therefore commonly used perioperatively and immediately after operations. Heparin binds to
antithrombin III and this complex inactivates thrombin by binding to it. Since heparin acts by directly by binding to antithrombin III and inactivating the potent coagulation activator, thrombin, heparin is immediately effective.\textsuperscript{65} Heparin also binds to thrombin, factor Xa, IXa, and XIa, inactivating these coagulation factors as well. However, complications with thrombocytopenia, low platelet count, have been associated with heparin,\textsuperscript{66} and is therefore only used for short-term anticoagulation.

Long-term anticoagulation is maintained by anticoagulants such as warfarin sodium. Warfarin sodium, also known as Coumadin\textsuperscript{®}, interferes with recycling of vitamin K in the liver thereby inhibiting biological activity of vitamin K-dependent coagulation factors.\textsuperscript{65} Specifically, warfarin sodium directly inhibits the interconversion of vitamin K and vitamin K epoxide, inhibiting posttranslational carboxylation of glutamate on coagulation factors. This inhibits calcium-dependent complexing of proteins with cofactors. Several coagulation factors: prothrombin, VII, IX, and X, are dependent on vitamin K for synthesis and are therefore affected by warfarin sodium (Figure 1.2). The indirect pathway by which warfarin sodium acts to inhibit coagulation causes a delay of days in seeing the anticoagulation effects of warfarin sodium.
Although such anticoagulants have been around for decades and have been used successfully, problems with bleeding are common. Even with anticoagulation, patients implanted with medical devices still have complications of thrombosis. New anticoagulants, such as Plavix and Persantin, are continuously being introduced. Therefore, although protocols for administration and monitoring of anticoagulation with heparin and warfarin sodium have been established, the thrombotic responses to these anticoagulants still need assessment.
1.4.4 Role of Fluid Mechanics in Thrombosis

Fluid mechanics has been shown to significantly affect protein adsorption and platelet adhesion to biomaterials. In an *in vivo* study of protein adsorption on PU blood sacs of LVADs, lower protein adsorption including fibrinogen was measured in regions of higher shear stresses.\(^72\) Platelet adhesion to biomaterial surfaces was shown to be shear-dependent, but in distinct manners depending on the mechanism of adhesion, by fibrinogen or von Willebrand factor (vWF).\(^73\) Platelet binding to fibrinogen by the \(\alpha_{\text{IIb}}\beta_3\) integrin on the platelet membrane decreases with increasing shear rate. Savage et al. reported efficient platelet binding to fibrinogen at shear rates of up to 600 to 900 s\(^{-1}\) and minimal adhesion at 1500 s\(^{-1}\).\(^73\) vWF, in contrast, binds platelets via the glycoprotein Ib\(_\alpha\) at shear rates as high as 6000 s\(^{-1}\). Platelet adhesion to vWF immobilized on glass was minimal at 50 s\(^{-1}\) and maximal at 1500 s\(^{-1}\). The ability of the platelet glycoprotein Ib\(_\alpha\) to bind to vWF at higher shear rates appears to be due to fast association and dissociation rates, and high resistance to tensile stress. Although platelet binding to vWF was lower at 50 s\(^{-1}\) than at 1500 s\(^{-1}\) platelet binding to vWF was still comparable to platelet binding to fibrinogen at 50 s\(^{-1}\). Thus, platelet adhesion to the glass surface occurred via both immobilized fibrinogen and vWF at 50 s\(^{-1}\). At 1500 s\(^{-1}\) platelet binding to fibrinogen was minimal and binding to vWF was maximal.

Various studies have reported a range of shear rates critical to platelet adhesion and spreading (Table 1-1). Similar to decrease in platelet adhesion, fibrin adhesion decreases with increased shear stress.\(^73-75\) Shear stresses can be estimated from the reported shear rates as the product of shear rate and viscosity, assuming a viscosity
equivalent to that of blood (0.035 dyn·s/cm²). Platelet adhesion is reported to be minimal beyond critical shear rates varying from 1,000 to 2,000 s⁻¹, corresponding to shear stresses from 35 to 70 dynes/cm² in blood.⁷⁴,⁷⁵ Maximum platelet surface coverage has been reported at shear rates between 50 to 500 s⁻¹, corresponding to shear stresses from 2 to 18 dynes/cm² in blood.⁷³,⁷⁴ Above 50 dynes/cm², platelet aggregation due to shear stress has been reported.⁵ Although the generally accepted relationships between fluid mechanics and thrombotic responses were just described, conflicting results and a varying relationships between platelets and shear stress have been reported. Furukawa et al. reported levels of platelet adhesion at low and high shear stresses (0.1 and 5 dynes/cm²) to be reversed for expanded polytetrafluoroethylene (ePTFE) and silicone.⁷⁶ Platelet adhesion increased with shear stress on silicone but decreased with shear stress on ePTFE. The thrombotic responses to shear stress continue to be investigated as studies clearly show their significance in hemocompatibility.

**Table 1-1. Literature reports a variety of critical values of shear rates for platelet and fibrin responses.**

<table>
<thead>
<tr>
<th>Shear Rate (s⁻¹)</th>
<th>Biological Response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 2000</td>
<td>Minimal platelet adhesion</td>
<td>Balasubramanian and Slack 2002 ⁷⁵</td>
</tr>
<tr>
<td>50 -500</td>
<td>Maximum surface coverage by a single platelet</td>
<td>Savage et al. 1996 ⁷³</td>
</tr>
<tr>
<td>500</td>
<td>Maximum shear rate above which thrombosis is reduced</td>
<td>Hubbell and McIntire 1986 ⁷⁷,⁷⁸</td>
</tr>
<tr>
<td>Increased</td>
<td>Decreased fibrin deposition</td>
<td>Colman 2001 ⁷⁹</td>
</tr>
</tbody>
</table>
The complex fluid flow patterns in medical devices such as LVAS are important to consider in designing such devices. Analysis of fluid mechanics using computational fluid dynamics is common in assessment of blood pump designs. The shear stresses in the adult 70-cc blood sac of the LionHeart LVAS studied here are believed to be high enough to provide effective washing of the surface thereby limiting thrombus deposition. Yet thrombosis continues to be one of the main complications in LVAS such as the LionHeart. Baldwin et al. reported shear stresses in the inlet and outlet regions by the valves in the regurgitant jets as high as 3600 dynes/cm² and 5500 dynes/cm², respectively. All other regions within the blood sac had shear stresses below 500 dynes/cm². Pediatric VADs with lower flows compared to adult VADs create greater risk for thrombosis in pediatric VADs. The majority of Reynold’s shear stresses in pediatric and adult LVAS of similar designs have been reported as < 100 dynes/cm² and < 250 dynes/cm², respectively. In the same study by Bachmann et al. the majority of wall shear stresses were reported as 21 dynes/cm² and 35 dynes/cm² for the pediatric and adult LVAS, respectively. These lower shear stresses in pediatric VADs, resulting in insufficient washing, suggest thrombosis to be more prevalent in pediatric VADs than in adult VADs. Therefore, it is particularly important to consider the thrombotic response in pediatric VADs and to evaluate the fluid mechanics in such devices.

The shear stresses reported in the adult and pediatric blood sacs, when converted to shear rates (using the viscosity and density values provided) can be compared to reported shear rates at which platelet adhesion occurs (Table 1-1). The LVAS shear rates are well above the range of shear rates at which platelet adhesion has been reported to occur. In the adult blood sac, the shear stresses reported are equivalent.
to maximum shear rates of $82,707\ \text{s}^{-1}$, $54,135\ \text{s}^{-1}$, and $7,519\ \text{s}^{-1}$ in the outlet, inlet, and center regions, respectively. The majority of shear rates for the pediatric and adult LVAS calculated from the Reynolds shear stresses reported are $<1,515\ \text{s}^{-1}$ and $<3,759\ \text{s}^{-1}$, respectively. Majority of wall shear rates equivalent to the reported shear stresses are $318\ \text{s}^{-1}$ and $526\ \text{s}^{-1}$ for the pediatric and adult LVAS, respectively. The maximum shear rates and the majority of shear rates in the adult LVAS are below the shear rate of 2000, above which platelet adhesion was reported to be minimal (Table 1-1). The shear rates in the pediatric LVAS, however, may still be low enough for platelet adhesion. Although there are various studies reporting minimal platelet adhesion above specific shear rates, Savage et al. report platelet binding to vWF immobilized on glass at shear rates as high as $6000\ \text{s}^{-1}$, as discussed previously.
Chapter 2
Multiscale Analysis of Chronic Surface Thrombosis \textit{In Vivo} in a Left Ventricular Assist System

2.1 Abstract

Thrombosis limits the success of ventricular assist devices as the demand for alternatives to heart transplants increases. This study mapped the occurrence of thrombosis in a LVAS to better understand the biologic response to these devices. Nine calves divided into two groups were implanted with LVAS for 28 to 30 days. One group was anticoagulated, whereas the second group received no long-term anticoagulation. The blood-contacting poly(urethane urea) (PUU) surfaces of blood sacs in the LVAS were examined for macroscopic thrombi upon retrieval. The sac was partitioned into eight sections and imaged for thrombi by scanning electron microscopy. No difference in thrombosis was observed macroscopically between the groups. Anticoagulation appeared to result in reduction of platelet-like structures, but the presence of fibrin-like structures remained similar between groups. Regional differences correlating with high and low shear stress regions were observed. At the macroscale, fewer thrombi were recorded in the high shear stress ports. At the microscale, features resembling fibrin were observed primarily in the ports and platelet-like features were common in lower shear stress regions. These variations in thrombosis with anticoagulation and location are likely due to varied fluid mechanics within the LVAS blood sac.
2.2 Introduction

Thromboembolism is one of the major complications in VADs, occurring in 3% to 35% of bridge-to-transplant patients, and resulting in strokes in 16% of destination therapy VAD patients of the REMATCH study. A thrombus may obstruct blood flow, alter fluid dynamics causing damage to blood components, or wash off the surface as emboli and occlude vessels. The introduction of foreign materials into contact with blood, changes to fluid flow dynamics caused by the pump, and pharmacologic therapy are all insults to the biologic system involved with use of VADs that may potentially lead to thrombosis. A better understanding of the effects of such changes on thrombogenesis can provide criteria for the improvement of VADs.

Fluid mechanics has been closely associated with thrombosis in VADs and is an important design criterion for reducing thrombogenicity. The relationship between fluid mechanics and thrombosis in VADs is complex, with various blood components each being affected by flow differently. In animal studies comparing adult and pediatric pulsatile LVAS, adult LVAS were found to be thrombus-free while numerous thrombi formed on pediatric pumps. Fluid flow studies of the devices revealed significantly different flow dynamics between the two blood pumps of different sizes but similar geometry. Other studies have also used fluid mechanics analysis techniques to optimize pump design to reduce thrombosis. Such studies confirm the relationship between fluid flow dynamics and thrombosis in blood-contacting devices. This relationship is an important element for consideration in ongoing efforts to develop reduced-size pumps for children and smaller adult patients.
Efforts to reduce occurrences of thrombosis commonly involve pharmacologic therapy such as anticoagulation. This therapy, however, introduces the additional complication of bleeding, with the incidence of hemorrhage having been reported to range from 31% to 60%. Bleeding due to anticoagulation was reported as the cause of death in up to 11% of patients in studies of three device designs. Despite anticoagulation of many VAD patients, thrombosis is still a problem and new anticoagulants are constantly being developed. A closer examination of the thrombotic blood response to anticoagulation in LVAS could mitigate requirements for anticoagulation therapy.

Detection of thrombus formation on biomaterial surfaces focuses on measurement of the primary components of thrombi. Protein adsorption is widely accepted to be the initial response to blood contact with biomaterials. This is followed by platelet adhesion, activation, and aggregation, and fibrin formation culminating in formation of a thrombus. Thus, protein adsorption, platelet response, and fibrin formation on biomaterials are considered important measures of thrombogenicity of biomaterials.

In this study, we investigated in vivo thrombus formation on the blood-contacting surface of PUU blood sacs in LVAS implanted in calves for 28 to 30 days. In vivo studies allow investigation of the blood response under the complex environment found in the LVAS. Macroscopic and microscopic thrombus formation was examined throughout the various regions of the blood sacs. To the best of our knowledge, such a thorough investigation, including mapping regional differences in thrombosis in LVAS pumps, has not yet been reported. Numerous studies have investigated in vitro blood
responses to flow and biomaterials, and in vivo studies discuss occurrences of thrombosis both on macroscopic and microscopic scales. However, these studies do not perform close examination of the location of thrombus formation within the pump. The significance of this comprehensive analysis of thrombosis throughout the blood sacs and its correlation with fluid flow dynamics in the highly complex in vivo environment of the LVAS is discussed.

2.3 Materials and Methods

2.3.1 In Vivo Studies with Bovine Models

In vivo studies of thrombosis in a 70-cc-static stroke volume LVAS with PUU, Biospan MS/0.4 (The Polymer Technology Group, Inc., Berkley, CA) blood sacs fabricated using techniques developed by The Pennsylvania State University were performed in two groups. A total of nine 70-cc LVAS were implanted in Holstein calves for 28 to 30 days. The blood flow rate was controlled to try to maintain flow rates from 5 to 6 l/min. Group 1, consisting of four calves, received postoperative anticoagulation with heparin and warfarin sodium the first 4 postoperative days and just warfarin sodium thereafter. The dosage was adjusted to maintain the prothrombin time (PT), a measure of clotting, at 1.5 to 2 times the baseline PT before surgery. Similar PT monitoring is performed for anticoagulation management in VAD patients. Group 2 consisted of five calves that received no postoperative anticoagulation. These calves were referred to as nonanticoagulated calves. All animals were housed and studies performed in
AAALAC-accredited animal facilities. Veterinary care was administered in accordance with guidelines set forth in The Guide for Care and Use of Laboratory Animals.\textsuperscript{105}

Implants were retrieved in accordance with the guidelines of the 2001 American Veterinary Medical Association Panel on Euthanasia. To avoid stagnant blood, the sacs were flushed with saline followed by 1\% paraformaldehyde (PFA) fixative. This was accomplished by connecting a catheter with tubing to a 1-l bag of saline and two 1-l bags of 1\% PFA. With the device still pumping, the inlet cannula was cut and the catheter connected to the bags of solution was inserted into the inlet cannula. Saline was released into the pump through the inlet cannula, clamping off the inflowing blood simultaneously. The outflow cannula was cut and the distal end of the outflow cannula was clamped off. Saline from the outflow cannula was allowed to drain. The blood sac was flushed with approximately 950 ml saline. One liter of 1\% PFA was then introduced into the pump. As the first 1-l bag of 1\% PFA was emptied, the pump stop command was given, and the second bag of 1\% PFA was allowed to flow until the pump completely stopped. The calf was euthanized and the blood pump was retrieved. The blood sac, filled with 1\% PFA, was fixed for 1 hour, replaced with saline, and stored at 4\textdegree C.
2.3.2 Evaluation of PUU Blood Sacs for Thrombosis

2.3.2.1 Macroscale Evaluation

Macroscale surface evaluation was performed by mapping macroscopic thrombi on the sacs from both in vivo study groups. The sac was examined for thrombi and the size, location, and colors of each were recorded. The color of the thrombus is indicative of the presence or absence of red blood cells entrapped in a fibrin mesh. Drawings of six views of the 70-cc LVAS blood sac were created in Solid Works software (Concord, MA). Color, size, and location of thrombi were sketched onto the Solid Works sac drawings using Adobe Photoshop (San Jose, CA). Thrombus images from each implant were overlaid to identify regional patterns of thrombosis for each study group. The percentage of the sac surface covered by macroscopic thrombi was also calculated for each region.

2.3.2.2 Microscale Evaluation

Microscale surface evaluation of thrombosis was performed using scanning electron microscopy (SEM) to examine surface topography of the explanted blood sac samples. Biologic deposition of protein, platelets, and fibrin was investigated. Negative and positive in vitro controls were also prepared to help identify topographic features observed on the blood sacs by SEM. Immunofluorescent labeling and confocal microscopy were performed to validate the identity of structures observed by SEM.
2.3.2.2.1 *In Vitro* SEM Controls

Seventeen negative control samples of PUU blood sacs that were not exposed to blood were prepared for microscopic surface evaluation by SEM. The samples were cut into approximately 1-cm² pieces from the various regions of the blood sac. Three positive controls of fibrin clots on sac PUU were also prepared. Bovine blood drawn into citrate phosphate dextrose adenine was centrifuged for 20 minutes at 600g at room temperature to isolate the supernatant platelet-rich plasma (PRP). After transferring a portion of the PRP to a tube, the remaining blood was centrifuged at 1,500g for 20 minutes to obtain platelet poor plasma (PPP). Total volumes of 2.5, 5.0, and 6.5 μl of 1 M calcium chloride (CaCl₂) were each added to 0.5 ml PPP covering a 1-cm² sample of blood sac PUU. Samples were incubated for 2 hours at 37°C, rinsed three times with phosphate-buffered saline (PBS), fixed with 1% PFA for 1 hour at 4°C, and rinsed three times with PBS. A positive control of bovine platelets was also prepared by plating PRP diluted with PPP to approximately physiologic concentration on a glass coverslip. The platelets were fixed with 1% PFA for 1 hour at 4°C. All controls were dried with an ethanol series as described in the following section for SEM.

2.3.2.2 LVAS Blood Sacs

The blood sac analysis was divided into eight regions as illustrated in Figure 2.1. Two square samples approximately 0.7 cm by 0.7 cm were cut from each region. The samples were placed in the wells of 24-well polystyrene tissue culture plates (VWR, Bridgeport, NJ) and covered with PBS (pH 7.4, 10mM) until prepared for SEM.
Both \textit{in vitro} control and retrieved blood sac samples from \textit{in vivo} studies were prepared for SEM by drying with a 50\%, 60\%, 70\%, 80\%, 90\%, and 100\% ethanol dehydration series. Each sample was soaked in 2 ml of each ethanol concentration for 10 minutes. The 100\% ethanol was aspirated and samples were left to air dry. Samples were mounted on SEM stubs using carbon tape and coated with a 10-nm-thick gold layer or with an equivalent gold-palladium coating. Imaging was performed on a high voltage Philips XL-20 SEM (Eindhoven, Netherlands) and an AmRay 3200 EcoSEM (Bedford, MA). To obtain images representative of the sac sample’s surface, a minimum of six randomly selected areas at 500x and one 100x image were taken. Additional images at higher magnifications were taken as needed to examine specific features.

\textbf{Figure 2.1.} The 70-cc blood sac. The sac was divided into eight regions: inlet, outlet, inlet side, outlet side, center front, center back, top, and bottom.
2.3.2.2.4 Immunofluorescence Assay

SEM analysis performed is unable to distinguish polymeric from biologic features and cannot distinguish between different biologic components. Therefore, as an additional experimental control, indirect immunofluorescent labeling of platelets and fibrin was performed to confirm the identity of structures observed by SEM. Indirect immunofluorescent labeling involves first binding a primary antibody to the antigen of interest. A secondary polyclonal antibody tagged with fluorescent label is used to bind to the primary antibody, thus indirectly fluorescently tagging the antigen of interest. A blocking agent is incubated with the primary and secondary antibody solutions to minimize nonspecific adsorption of antibodies to the samples. Platelets can be identified by labeling for the αIIb chain on the αIIbβ3 integrin present on the platelet membrane. Fibrin can be labeled with a monoclonal antibody solution that recognizes bovine fibrinogen. The anti-bovine fibrinogen antibody recognizes both fibrinogen and fibrin on the samples. Further detail on indirect immunofluorescent labeling assays is given in Chapter 4 where extensive evaluation of the blood sacs by immunofluorescent labeling is discussed.

A sample and its negative control of a bovine PRP clot were prepared. PRP clots were prepared following a protocol similar to that described previously for PPP clots. In a 24-well tissue culture polystyrene plate (VWR), 1 ml of diluted PRP was added to each of two wells. To each well, a total volume of 15 μl of 1 M CaCl2 was added. Samples were incubated for 2 hours at 37°C, rinsed three times with PBS, fixed with 1% PFA for 1 hour at 4°C, and rinsed three times with PBS. Retrieved sac samples were also
prepared. Two samples (approximately 0.7 cm by 0.7 cm) were cut from each of three of the nonanticoagulated sacs retrieved from calves. The samples were covered with PBS in 24-well tissue culture polystyrene plates (VWR).

Samples of the PRP clots and retrieved sacs were immunofluorescently labeled for platelets and fibrinogen. Antibody solution volumes and incubation times for PRP clots differed slightly from the following given for the sac samples. PBS was replaced with 500 \( \mu l \) 1\% rabbit serum albumin (Sigma, Milwaukee, WI) as a blocking agent. The primary antibodies, 0.5 \( \mu l \) goat anti-bovine fibrinogen (American Diagnostica, Stamford, CT) and 0.75 \( \mu l \) mouse anti-bovine \( \alpha_{IIb}\beta_3 \) (Veterinary Medical Research and Development, Pullman, WA), were added to one sample from each sac. The other sample from each sac was prepared as a negative control by excluding the primary antibody to measure nonspecific labeling of the sample. The samples were incubated overnight at 4°C. The antibody solution was aspirated and samples were rinsed three times with approximately 2 ml of PBS. Samples were covered with 500 \( \mu l \) 1\% rabbit serum albumin. Secondary antibodies were added to each well in the following amounts: 0.5 \( \mu l \) donkey anti-goat IgG-fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories, West Grove, PA) and 5 \( \mu l \) donkey anti-mouse IgG-phycoerythrin (PE) (Jackson ImmunoResearch Laboratories). Samples were incubated for 1 hour at 37°C while protected from light. The antibody solution was aspirated and samples were rinsed with approximately 2 ml distilled water three times.

Each sample was placed on a glass microscope slide in the middle of four small pieces of PUU glued to the slide with cyanoacrylate. A drop of Gel/Mount antifade
mounting medium (Biømeda, Foster City, CA) was placed on the sample. Cyanoacrylate was applied onto the small pieces of PUU and a coverslip was placed over the calf sac sample, and held in place at all four corners with the cyanoacrylate on the PUU pieces. The small PUU pieces were used to hold the coverslip in place over the thick PUU calf sac sample that otherwise would not be held by the mounting medium alone. For the bovine PRP clots, each clot was mounted on top of a piece of PUU cut from a blood sac and no PUU pieces were used to mount the coverslip.

2.3.2.2.5 Confocal Microscopy

Samples were imaged using the 488-nm and 543-nm lasers on a TCS SP2 AOBS confocal microscope (Leica, Deerfield, IL) with a 63x oil immersion objective. Negative controls were imaged for nonspecific and background fluorescence, followed by imaging of the samples labeled with primary and secondary antibodies.

2.4 Results

2.4.1 Clinical Observations

The device for each implant operated normally. The average flow rates for the nine calves were maintained between 5.13 and 6.46 l/min, with standard deviations ranging from 0.74 to 1.56 l/min. The flow rates, however, covered a wider range with values as low as 0.50 l/min and as high as 8.80 l/min (Table 2-1). Many of the minimum values recorded occurred as the pump was turned on the first day of implantation. Few
measurements above 8.0 l/min were recorded. The average PT times of the group 1 anticoagulated calves were successfully maintained between 1.5 to 2 times the baseline values, although occasional deviations from the desired range were seen (Table 2-2). The implant duration was 30 days for eight of the nine implants. One implant was removed at 28 days at which time the calf was in good health and the device was operating without complications.

Table 2-1. Average blood flow rates, standard deviations, and minimum and maximum values recorded for anticoagulated (n = 4) and nonanticoagulated calves (n = 5)

<table>
<thead>
<tr>
<th>Calf</th>
<th>Average Flow Rate (l/min)</th>
<th>Standard Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
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<td>0.50</td>
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<tr>
<td>2</td>
<td>5.13</td>
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<td>0.90</td>
<td>8.80</td>
</tr>
<tr>
<td>3</td>
<td>6.46</td>
<td>0.93</td>
<td>2.30</td>
<td>8.40</td>
</tr>
<tr>
<td>4</td>
<td>6.30</td>
<td>0.97</td>
<td>1.10</td>
<td>8.30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calf</th>
<th>Average Flow Rate (l/min)</th>
<th>Standard Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.40</td>
<td>7.90</td>
</tr>
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<td>2</td>
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<td>0.74</td>
<td>2.23</td>
<td>7.03</td>
</tr>
<tr>
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<td>5.67</td>
<td>0.93</td>
<td>1.20</td>
<td>8.20</td>
</tr>
<tr>
<td>4</td>
<td>6.15</td>
<td>1.10</td>
<td>1.70</td>
<td>8.40</td>
</tr>
<tr>
<td>5</td>
<td>5.82</td>
<td>1.25</td>
<td>1.10</td>
<td>8.40</td>
</tr>
</tbody>
</table>
2.4.2 Macroscopic Thrombosis Observations

Overlay images of macroscopic thrombosis mappings for each group of anticoagulated and nonanticoagulated calf blood sacs are shown in Figure 2.2. Little macroscopic thrombus formation was seen in the sacs with the exception of one sac from each group. Thrombosis in the inlet and outlet ports was minimal, occurring in only two of the nine blood sacs. In the anticoagulated blood sacs, one sac had a relatively large number of thrombi and the remaining three sacs had few or no thrombi (Figure 2.2A). Similar results were obtained for the five nonanticoagulated calf blood sacs of group 2 (Figure 2.2B). One sac had a relatively large number of thrombi and the remaining sacs

<table>
<thead>
<tr>
<th>Calf</th>
<th>Average Ratio of PT/Base PT</th>
<th>Standard Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.60</td>
<td>0.41</td>
<td>1.02</td>
<td>2.51</td>
</tr>
<tr>
<td>2</td>
<td>2.03</td>
<td>0.60</td>
<td>1.15</td>
<td>3.14</td>
</tr>
<tr>
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<td>0.53</td>
<td>1.18</td>
<td>2.98</td>
</tr>
<tr>
<td>4</td>
<td>1.91</td>
<td>0.63</td>
<td>1.25</td>
<td>3.10</td>
</tr>
</tbody>
</table>

**Table 2-2.** Average prothrombin times (PTs), standard deviations, and minimum and maximum values recorded for anticoagulated (n = 4) and nonanticoagulated calves (n = 5)
had few or no thrombi. Detailed observations for each sac are listed in Table 2-3. No major difference in macroscopic thrombus formation was observed between blood sacs from groups 1 and 2. In all blood sacs, no red thrombus was observed. The total sac surface coverage by macroscopic thrombi was 0.23% and 0.16% for anticoagulated and nonanticoagulated sacs, respectively (Table 2-4).
Figure 2.2. Macroscopic mapping of thrombi. (A) Overlay of the four anticoagulated blood sacs. Thrombi are numbered with the sac numbers. Thrombi on sac 4 with the most thrombi are unlabeled to provide greater visibility of thrombus distribution. (B) Overlay of four nonanticoagulated blood sacs (mapping of thrombi for first sac not included, see text). The unlabeled thrombi were observed on sac 3.
Table 2-3. Observations of macroscopic thrombosis on blood sacs from groups 1 and 2

<table>
<thead>
<tr>
<th>Sac #</th>
<th>Macroscopic Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Group 1: Anticoagulated Calf Blood Sacs</strong></td>
</tr>
<tr>
<td>1</td>
<td>No macroscopic thrombus</td>
</tr>
<tr>
<td>2</td>
<td>Cluster of 0.1 cm diameter white thrombi on the center back below the inlet</td>
</tr>
<tr>
<td>3</td>
<td>Single white thrombus 0.8 cm long by 0.2 cm wide towards the bottom of the inlet side</td>
</tr>
<tr>
<td>4</td>
<td>Numerous small, white thrombi mainly at the bottom, center front, and center back. Three to four macroscopic thrombi on each of the sides below the inlet and outlet. No thrombus on the top region.</td>
</tr>
<tr>
<td></td>
<td><strong>Group 2: Nonanticoagulated Calf Blood Sacs</strong></td>
</tr>
<tr>
<td>1</td>
<td>Several 0.1 to 0.3 cm diameter white thrombi</td>
</tr>
<tr>
<td>2</td>
<td>No macroscopic thrombus</td>
</tr>
<tr>
<td>3</td>
<td>Numerous small, white thrombi on the top, center front, and center back. A few white thrombi on the inlet and outlet sides. No thrombus on the bottom.</td>
</tr>
<tr>
<td>4</td>
<td>Four white thrombi on the inlet side</td>
</tr>
<tr>
<td>5</td>
<td>One 0.2 cm by 0.1 cm white thrombus on the back</td>
</tr>
</tbody>
</table>
2.4.3 Microscopic Thrombosis Observations

2.4.3.1 Confocal Microscopy

To demonstrate the presence of biologic structures on the retrieved sacs, immunofluorescent labeling and confocal microscopy were used. Images showing bovine fibrin and platelets are shown in Figure 2.3. All negative controls showed minimal nonspecific labeling. Figures 2.3A and 2.3B show the structures of bovine fibrin and platelets, respectively, in PRP clots on PUU, and confirm successful labeling of the structures. Similarly, fibrin and platelet structures were observed on the

<table>
<thead>
<tr>
<th>Sac #</th>
<th>Inlet</th>
<th>Outlet</th>
<th>Front Center</th>
<th>Back Center</th>
<th>Bottom</th>
<th>Top</th>
<th>Inlet Side</th>
<th>Outlet Side</th>
<th>Entire Sac</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
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<td>0.00</td>
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<td>0.00</td>
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<td>0.79</td>
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<td>0.53</td>
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<td>0.00</td>
<td>0.70</td>
<td>0.31</td>
<td>0.79</td>
</tr>
<tr>
<td>Average</td>
<td>0.07</td>
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<td>0.13</td>
<td>0.59</td>
<td>0.14</td>
<td>0.00</td>
<td>0.52</td>
<td>0.08</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Table 2-4. Percentage of sac surface coverage by macroscopic thrombi

**Group 1: Anticoagulated Sac...**

**Group 2: Nonanticoagulated Sac...**

**Sac #**

<table>
<thead>
<tr>
<th>Sac #</th>
<th>Inlet</th>
<th>Outlet</th>
<th>Front Center</th>
<th>Back Center</th>
<th>Bottom</th>
<th>Top</th>
<th>Inlet Side</th>
<th>Outlet Side</th>
<th>Entire Sac</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>3</td>
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<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>Average</td>
<td>0.17</td>
<td>0.15</td>
<td>0.05</td>
<td>0.39</td>
<td>0.00</td>
<td>0.08</td>
<td>0.29</td>
<td>0.02</td>
<td>0.16</td>
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</tbody>
</table>
nonanticoagulated calf sac samples (Figures 2.3C and 2.3D).

![Figure 2.3](image)

**Figure 2.3.** Confocal microscopy images of fibrin and platelets. (A) Fibrin in a bovine PRP clot on PUU. (B) Bovine platelets in a PRP clot on PUU. (C) Fibrin on the surface of a blood sac retrieved from a nonanticoagulated calf. (D) A layer of fibrin and a platelet covered with fibrinogen on the surface of a blood sac retrieved from a nonanticoagulated calf. Solid arrows: fibrin. Dotted arrow lines: platelets.

### 2.4.3.2 *In Vitro* SEM Controls

The negative control PUU material without biologic contact was relatively smooth with few microscopic features visible by SEM (Figure 2.4). On one sample, however, in an area approximately 240 μm by 72 μm, a single group of branching strand-like features was observed. This feature was considered an anomaly observed on a single image of 102 images taken at 500x on 17 negative control samples. Positive controls of
PPP clots on PUU consisted of well-formed fibrin meshes with ridges, folds, and branches (Figure 2.4 B). The positive control of PRP plated on glass showed platelets adhered and spreading (Figure 2.4 C). Similar images from the controls were used to identify structures observed on the blood sacs retrieved from calves.

![Figure 2.4. SEM control images.](image)

**Figure 2.4.** SEM control images. (A) Representative negative control of PUU with no biologic contact consists of a primarily smooth surface (imaged at 500X). (B) Positive control of a PPP clot with fibrin mesh illustrates the topography of fibrin observed in several of the blood sacs retrieved from calves (500X). (C) Positive control of PRP plated on glass. Structures resembling the bovine platelets shown here were observed on most blood sacs (5,000X). Solid arrows: fibrin. Dotted arrow lines: platelets.

### 2.4.3.3 LVAS Blood Sacs

Microscopic evaluation by SEM provided information on the topography of the different blood sac regions after 30 days implantation in calves. An array of structures such as circular masses from 1 μm up to 10 μm in diameter and branching fibrils was observed on the surfaces of the anticoagulated and nonanticoagulated blood sacs (Figure 2.5). Rough topography as shown in Figure 2.5A was frequently observed throughout the sacs. The small size of these structures and their uniformity across the surface suggested such topography resulted from the formation of a protein layer. Other
features resembled unspread and fully spread platelets (Figures 2.5B and 2.5C) and fibrin (Figure 2.5D) seen in the positive controls. These structures also closely resemble the platelets and fibrin imaged by confocal microscopy, and will be referred to as platelets and fibrin from hereon. The dark circles in Figure 2.5C were observed frequently and are believed to be pits in the PUU possibly due to polymer composition and fabrication process, although degradation is a possibility as suggested in studies investigating the stability of similar polymers. Features resembling a protein layer and unspread platelets were most abundant and observed in several of the anticoagulated and nonanticoagulated sacs.

In sacs 1, 2, and 4 of the anticoagulated group, rough proteinaceous topography similar to Figure 2.5A was observed in most regions. In sac 1, a low to medium density of platelets as shown in Figure 2.5B was found in all regions except the outlet. The inlets and outlets were densely covered with the fibrillar features shown in Figure 2.5D. In sac 2, a few platelets were present at the top and center front. Similar to sac 1, the inlets and outlets were densely covered with fibrin. On sac 3, few features were observed. Samples from the outlet and center front had low densities of proteinaceous topography. No other relevant structures were observed. Sac 4 had a low density of small proteinaceous structures seen in all regions except the center front. The inlet side had a sparse distribution of features resembling spread platelets. No fibrillar structures were observed.
All five nonanticoagulated group 2 sacs had low to high densities of rough topography, suggesting formation of protein layers in all or most regions. Sac 1 had features resembling unspread platelets in all regions except the inlet. The ports were covered with structures resembling fibrin. In sac 2, the majority of such proteinaceous topography was observed in the inlet, outlet, center back, and top. Platelets about 5 μm in diameter were observed in the centers, sides, and bottom of the sac in medium to high densities. Fibrillar structures were observed mainly in the inlet and to a lesser extent in the outlet side, top, and bottom. In sac 3, the surface appeared to be coated with a protein layer throughout the sac. Unspread platelets were observed at a low density in the inlet and outlet sides. However, structures resembling fully spread platelets were observed in all regions except the inlet and outlet. No fibrin was observed. In sac 4, the highest density of proteinaceous topography was observed in the inlet and outlet. Unspread platelets were observed on some samples from the ports, center front, outlet side, and top. No spread platelets were observed. Fibrillar structures were observed on one sample from the top of the sac. Sac 5 had low to high densities of proteinaceous topography throughout the sac. Platelets were observed on one sample from each of the inlet, center

Figure 2.5. Array of topography observed on surfaces of explanted PUU blood sacs. Topography resembling proteinaceous layers (A), unspread platelets (B), spread platelets (C), and fibrin (D), as seen in positive controls, were observed in the explanted blood sacs. Dotted arrow lines show platelets.
back, and inlet side, and on both samples from the top and bottom regions at low
densities. Fibrillar structures were observed in all samples from the inlet and outlet but
not in any other region.

Anticoagulation of calves from group 1 resulted in differences in microscopic
observations between groups 1 and 2. Figure 2.6 compares representative SEM images
showing the surfaces from different regions of anticoagulated and nonanticoagulated
blood sacs. The inlets and outlets of both groups had fibrin deposition (Figures 2.6A-D).
Fibrin was observed in one or both ports of three of four anticoagulated and three of five
nonanticoagulated blood sacs. In all other regions of the sac, this structure resembling
fibrin was rarely seen. Only one of the nine sacs had fibrin in regions other than the
ports. Deposition of unspread and spread platelets differed between the two groups. The
platelets were observed more frequently in group 2 nonanticoagulated blood sacs
compared to group 1 anticoagulated sacs (Figures 2.6E-P). Only one out of four
anticoagulated sacs showed platelets in most regions, whereas all five nonanticoagulated
sacs had at least moderate coverage by either spread or unspread platelets.
Anticoagulation given to group 1 calves appeared to hinder platelet adhesion but not
fibrin formation, whereas in group 2 nonanticoagulated calves, platelet adhesion was
abundant.
Figure 2.6. Effects of anticoagulation on surface topography of blood sacs. Surface topographies of the eight different regions examined are shown. Surface topography of the anticoagulated sacs (images on left) differed from that of the nonanticoagulated sacs (images on right). Platelet-like features were scarce in all regions of the anticoagulated blood sacs. In contrast, in nonanticoagulated sacs such platelet-like structures were present in abundance in all regions except for the inlets and outlets. However, in both groups of sacs the primary structures observed in the inlets and outlets were branching features similar in structure to fibrin clots. Solid arrows: fibrin. Dotted arrow lines: unspread and spread platelets. (Scale bar = 50 μm)

<table>
<thead>
<tr>
<th>Sac Region</th>
<th>Group 1: Anticoagulated</th>
<th>Group 2: Nonanticoagulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet</td>
<td>![Image A]</td>
<td>![Image B]</td>
</tr>
<tr>
<td>Outlet</td>
<td>![Image C]</td>
<td>![Image D]</td>
</tr>
<tr>
<td>Center Front</td>
<td>![Image E]</td>
<td>![Image F]</td>
</tr>
<tr>
<td>Center Back</td>
<td>![Image G]</td>
<td>![Image H]</td>
</tr>
<tr>
<td>Top</td>
<td>![Image I]</td>
<td>![Image J]</td>
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<tr>
<td>Bottom</td>
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</tr>
<tr>
<td>Outlet Side</td>
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</tbody>
</table>
The location within the blood sac also appeared to affect the observed surface topography. Figure 2.7 shows representative SEM images from the eight sampled regions of the anticoagulated calf blood sac 2, demonstrating the location-dependent surface topography. Fibrin was observed primarily on the inlets and outlets of the blood sacs, as seen in Figures 2.7A and 2.7C. Such structures were absent in all other regions (Figures 2.7B and 2.7D-H). Similar location-dependent topography was observed in the nonanticoagulated calf sacs as shown in Figure 2.8 for sac 5. Fibrin was the main feature that appeared to be location-dependent in all sacs, with these structures being observed almost exclusively in the inlets and outlets of four of the nine sacs. Unspread and spread platelets were least abundant in the inlets and outlets. The differences observed in surface topography with sac location may be a result of differing fluid dynamics between regions.
Figure 2.7. Location-dependent surface topography in blood sac 2 after 30 days in calves with anticoagulation. The main difference was observed in the inlet and outlet ports of several blood sacs. In these regions, fibrin-like structures were seen most abundantly and few platelet-like structures were present.
2.5 Discussion

Precise control of the flow rates in the pumps was difficult because of the presence of a healthy native left ventricle of the calf as well as changes in flow through the pump with movement of the animal from standing to lying down. The effect that the low and high transient blood flow rates may have on thrombogenesis \textit{in vivo} is unclear. It has not been established whether the transient high flow rates are enough to affect thrombosis at the surface. It is unknown if the higher flows can wash biologic deposition
that may occur during periods of lower flow rates. Similarly, the effect of variation in
prothrombin times of the anticoagulated calves on thrombosis is unclear. Although the
average prothrombin times were maintained well above the baseline times, it is unknown
if the few abnormal prothrombin times negate the effectiveness of the anticoagulation
therapy. The consequences of low transient flow rates and prothrombin times on chronic
thrombosis are topics for further investigation.

Evaluation of the anticoagulated and nonanticoagulated sacs showed variations in
thrombus formation with both anticoagulation and location within the sac. At the
macroscopic level, no difference in the number and size of macroscopic thrombi was
observed between anticoagulated and nonanticoagulated blood sacs. However, at the
microscopic level, features resembling platelets were observed less in anticoagulated
sacs. In the high shear stress ports, observations were similar for both anticoagulated and
nonanticoagulated groups at both macroscale and microscale. Anticoagulation
administered in these studies appeared to hinder platelet adhesion but did not reduce the
formation of the fibrillar structures. The anticoagulation of group 2 calves with heparin
and warfarin sodium should have reduced fibrin formation and perhaps indirectly,
reduced platelet adhesion. As described in Section 1.4.3, heparin and warfarin sodium
both hinder the coagulation cascade and inhibit fibrin formation by affecting coagulation
factors involved in the coagulation cascade. Neither anticoagulant acts directly on
platelets. Heparin binds to antithrombin III, which then binds to thrombin, and
inactivates thrombin, a key factor in the common pathway of the coagulation cascade.
Warfarin sodium inhibits full biologic function of the vitamin K-dependent coagulation
factors VII, IX, X, and prothrombin, which taken together are involved in all three
pathways of the coagulation cascade. Warfarin sodium interferes with recycling of vitamin K cycle, in turn inhibiting posttranslational carboxylation of glutamate on coagulation factors, inhibiting calcium-dependent complexing of proteins with cofactors. Heparin and warfarin sodium appeared to have no effect on formation of fibrillar structures in the high shear stress ports but did reduce platelet adhesion in the sacs. Regional differences were observed at both the macroscopic and microscopic scales, with the high shear stress inlet and outlet ports differing from all other regions. At the macroscopic level, fewer thrombi were recorded in the ports. At the microscopic level, features resembling fibrin were observed almost exclusively in the ports and platelet-like features were common in the lower shear stress regions. Such differences in observations between study groups and with sac location may be related to the fluid dynamics in the sacs.

The microscopic observations by SEM indicating region-dependent topography can be compared to region-dependent shear stress measurements reported for the adult 70-cc sac and a 50-cc blood sac. The shear stress measurements in the 70-cc blood sac published by Baldwin et al. under similar operating conditions to our in vivo studies concluded there was a difference in Reynolds shear stress magnitudes in the inlet and outlet compared to the rest of the sac. They showed that the shear stresses in the inlet and outlet regions by the valves in the regurgitant jets were as high as 3600 dynes/cm² and 5500 dynes/cm², respectively. All other regions within the blood sac had shear stresses below 500 dynes/cm². Our study showed regional differences in macroscopic thrombi and microscopic surface topography corresponding with differences in flow found by Baldwin and colleagues, with fibrin observed primarily in regions of shear stresses as
high as 5,500 dynes/cm² in the outlet and 3600 dynes/cm² in the inlet, and platelets adhered to surfaces with shear stresses below 500 dynes/cm². As mentioned previously, adhesion of platelets to the surface was reduced by anticoagulation in the lower shear stress regions of < 500 dynes/cm². However, formation of fibrin in the higher stress regions of the inlets and outlets remained unaffected by anticoagulation. These results suggest that the differences in platelet and fibrin adhesion may be caused by varied fluid dynamics in the blood sacs.

The shear stress reported by Baldwin and colleagues to occur in the adult LVAS are considerably above the values at which thrombus deposition normally occurs. A range of critical shear rates from 1,000 to 2,000 s⁻¹, corresponding to shear stresses from 35 to 70 dynes/cm² in blood, has been reported above which minimal platelet adhesion occurs. Maximum platelet surface coverage has been reported to occur at shear rates between 50 to 500 s⁻¹, corresponding to shear stresses from 2 to 18 dynes/cm² in blood, and similarly fibrin deposition is thought to decrease with increased shear rate. The shear stresses in the adult 70-cc blood sac are believed to be high enough to provide effective washing of the surface thereby limiting thrombus deposition. However, the in vivo results presented here suggest differently, with thrombus formation at the surface occurring even at shear stresses at least six times greater than the shear rate literature reports it being inhibited.

Microscale evaluation in this work consisted of SEM imaging, which provides topographic information. It does not conclusively identify platelets and fibrin and, therefore, should be supplemented with techniques such as immunolabeling to confirm the identity of thrombus components. However, the use of extensive controls both
positive and negative, preliminary confocal images, two in vivo groups with several animals each, and intense surface sampling, should provide ample information for identification of the structures observed by SEM.

2.6 Conclusion

This study illustrates the need for thorough in vivo evaluation of thrombosis in LVAS beyond the macroscopic into the microscale for comparison of regions with varied flow dynamics. Differences between animals that received anticoagulation and those that did not were seen primarily in increased numbers of platelets in the nonanticoagulated group. Results showed strong location dependence within the blood sac at both macroscale and microscale. There appear to be correlations between fluid dynamics and macroscopic/microscopic thrombi within the sac even at these higher-than-physiologic shear stresses. Further investigation of the relationship between thrombosis and levels of shear stress may allow us to establish new parameters for reduction of thrombosis in LVAS.
Chapter 3

Short-term *In Vivo* Study of Surface Thrombosis in a Left Ventricular Assist System

3.1 Abstract

Thrombosis continues to be a major adverse and at times fatal event in patients with left ventricular assist systems (LVAS). To assess acute thrombosis in LVAS, multiscale analysis of surface thrombosis was performed on LVAS blood sacs retrieved following implantation in seven calves for 3 days. Two study groups were evaluated: one group was given heparin and warfarin sodium throughout the study while the second received no postoperative anticoagulation. Upon explantation, the blood sacs were examined for macroscopic thrombi, while microscale thrombosis was assessed using scanning electron microscopy (SEM). Macroscopic thrombi about 1 mm in diameter were seen in all sacs from both groups. Although macroscopic thrombi occurred in all sac regions, SEM revealed differences in microscale topography between the port regions and the other sac regions. The primary structure was spherical particles approximately 400 nm in diameter, found to occur at a lower density in the ports. In contrast, the highest densities of proteinaceous rough topography and fibrillar structures were seen in the port regions. The density distribution of these structures was different in the eight sac regions and anticoagulation therapy appeared to have no effect on surface thrombosis in these short-term LVAS implants.
3.2 Introduction

Current knowledge of thrombosis at biomaterial surfaces includes an understanding that protein adsorption, platelet adhesion, and fibrin formation are affected each in their own ways by factors such as fluid mechanics and biomaterial properties. The biologic series of events that occurs upon blood contact with biomaterials is well documented and has led to anti-platelet and anticoagulation therapy commonly used today. Although many advances have been made, VAD patients are still encountering adverse events of thrombosis, which may lead to death.

A better grasp of the blood response to VADs during the first few days following implantation may be critical to preventing thrombosis and improving success. Blood contact with a biomaterial surface induces protein adsorption to the surface. Protein adsorption is followed by platelet adhesion, activation, and aggregation, and fibrin formation, depending on the proteins that adsorb and their biological activity. The thrombotic response of blood may vary over time as the biological activity and biomaterial surfaces undergo changes. Snyder et al. showed increase in platelet microaggregates after implantation of VADs but values returned to preoperative values within one week postoperation. Platelet activation increased in both sham surgical procedures and VAD implantations. However, in the surgical sham procedures, the surgical effects on platelet activation were seen during the first 17 days postoperatively, after which values returned to preoperative values. On the other hand, in calves implanted with VADs platelet activation remained elevated for the implant durations of almost four weeks. Such temporal changes in the thrombotic response to the surgical
procedure and the VAD can also be measured at the biomaterial surface. In a previous study, we examined long-term surface thrombosis in a left ventricular assist system (LVAS) after implanting the devices in calves for 30 days. To investigate the earlier response of blood in terms of surface thrombosis in LVAS, we now examine the thrombotic response of the body at 3 days to the presence of LVAS.

In this study, we focus on surface thrombosis in the LVAS PUU blood sacs in vivo after 3 days implantation in a bovine model. For thorough evaluation of surface thrombosis, multiscale analysis of the blood sacs was performed. Multiscale analysis involved recording of all macroscopic thrombi and the use of SEM for evaluation of surface topography and adhesion of platelets and fibrin. SEM samples were prepared from the various regions of the blood sac. The relationship between surface thrombosis and anticoagulation (a variable in this study), fluid dynamics (reported to vary throughout the blood sac), and implantation time (by comparison to our previous report for 30-day implants) were examined.

3.3 Materials and Methods

3.3.1 In Vivo Study with LVAS in Calves

Surface thrombosis was evaluated on Biospan MS/0.4 (The Polymer Technology Group, Inc., Berkley, CA) blood sacs that were part of a completely implanted rollerscrew adult LVAS and were fabricated using techniques developed by The Pennsylvania State University. Acute in vivo studies of surface thrombosis in the
LVAS were performed in the bovine model. In total, seven LVAS were implanted, each for 3 days in Holstein calves. The study was performed in two groups. Group 1 was anticoagulated postoperatively. This study group, consisting of three calves, was administered two anticoagulants: heparin and warfarin sodium. Warfarin sodium interferes with the vitamin K cycle, inhibiting posttranslational carboxylation of glutamate on the coagulation factors, and thus inhibiting calcium-dependent complexing of the vitamin K-dependent coagulation factors, prothrombin, VII, IX, and X, with their cofactors. Therefore, proteins must be synthesized and turned over for the anticoagulant to be effective and therefore it is generally not effective for early time periods. However, we administered warfarin sodium in order to best compare this 3-day study to a previous 30-day study in which warfarin sodium was used as the primary anticoagulant. Because it operates by a direct inhibition of thrombin, heparin is a rapidly effective anticoagulation therapy and was also used in both studies. The effect of heparin on anticoagulation was monitored by measuring the activated clotting time (ACT) of the calves’ blood throughout the study. Heparin dose was adjusted to maintain ACTs between 225 and 300 seconds. To monitor effectiveness of anticoagulation by warfarin sodium, prothrombin time (PT), another measure of clotting, was monitored. PT is commonly used clinically to monitor the efficacy of anticoagulation by warfarin sodium. Anticoagulation by warfarin sodium dose was considered effective if PTs reached between 1.5 to 2 times baseline preoperative values. PT values were used to calculate the international normalized ratios (INRs). As previously mentioned, it is doubtful that warfarin sodium therapy will have a measurable effect on clotting in this short time period.
After implantation, ACTs were checked every 2 hrs for the first 6 hrs. When chest tube drainage was more than 50 cc/hr for two consecutive hours or chest tube drainage hematocrit was less than 6% and chest tube drainage was less than 100 cc/hr, heparin was started at 10,000 units/hr. Heparin drip was adjusted to maintain ACT between 225-300 secs. ACTs were taken every 3 hrs thereon. When chest tube drainage reached less than 100cc/8 hrs, warfarin sodium was started at 5 mg/day. The second study group consisted of four calves that were not given any postoperative anticoagulation. Although all animals received heparin intraoperatively, the anticoagulant effect of heparin lasts just a few hours. For all implants, a flow rate of 5 to 6 L/min was targeted, although these devices are intended to supply varying flow rates and absolute control is difficult. All animals were housed and studies performed in AAALAC-accredited animal facilities. Veterinary care was administered in accordance with guidelines in The Guide for Care and Use of Laboratory Animals.

The devices were explanted in accordance with the guidelines of the 2001 American Veterinary Medical Association Panel on Euthanasia. Stagnant blood during implant retrieval was deemed undesirable for thrombosis analysis in the LVAS blood sac and was avoided as described previously. Briefly, prior to stopping the blood pump approximately 1 L of normal saline was flushed through the LVAS blood sac and was immediately exchanged for a minimum of 1 L of 1% paraformaldehyde (PFA) fixative. With the blood sac filled with PFA the LVAS pump was stopped and the animal was euthanized. The LVAS was retrieved and the blood sac was fixed in the 1% PFA for 1 hour from when the pump was stopped. The LVAS pump case was disassembled to
retrieve the blood sac. The PFA in the sac was replaced with saline and the sac was stored at 4°C.

3.3.2 Surface Evaluation of Blood Sacs for Thrombosis

3.3.2.1 Macroscale Surface Thrombosis Evaluation

Upon retrieval of the blood sac, macroscale thrombi adhered to the blood sac were recorded. Thrombi were located by visual observation, and the dimensions and location of each thrombus within the blood sac were measured. The color of each thrombus was also noted. This information was used to map all thrombi on each sac on drawings of six different views of the blood sac. The distribution of thrombi throughout the sac was examined by overlaying thrombus maps of sacs from each study group in order to study spatial patterns and identify regions that are prone to thrombus formation.

The percentage of the sac surface covered by macroscopic thrombi was also calculated. To investigate variation in surface thrombosis throughout the blood sac, the sac was divided into 8 different regions as illustrated in Figure 3.1. The data describing macroscopic thrombi and the surface area of each sac region in Figure 3.1 were used to calculate the percent surface coverage by these macroscopic thrombi in each of the eight regions of the sac. These values were calculated for each sac and the averages were compared for the two study groups.
3.3.2.2 Microscale Surface Evaluation

The blood sac surfaces were evaluated on the microscale by SEM, focusing on biological deposition of proteins, platelets and fibrin clot. Negative and positive in vitro controls were prepared previously\textsuperscript{111} and used to help identify structures observed on the SEM images of the blood sac surface. The retrieved blood sacs were classified into the eight regions discussed previously and shown in Figure 3.1. From each sac, two samples
approximately 0.5 cm² were cut from each of the eight regions of the blood sac identified in Figure 3.1. These samples were kept hydrated in phosphate-buffered saline (PBS, pH 7.4, 10mM) in 24-well polystyrene tissue culture plates (VWR, Bridgeport, NJ).

SEM blood sac samples were dehydrated with a 50, 60, 70, 80, 90, and 100% series of ethanol. Samples were incubated in 2 ml of each ethanol concentration for 10 minutes each at room temperature. After the last 10-minute incubation in 100% ethanol, the solution was aspirated and the samples were allowed to air dry. Carbon tape was used to mount each sample to a SEM stub. Samples were sputter-coated with a 10 nm-thick gold layer or with an equivalent gold-palladium coating. Blood sac surfaces were imaged on either a high voltage Philips XL-20 SEM (Eindhoven, Netherlands) or an AmRay 3200 EcoSEM (Bedford, MA). On each sample, six areas were randomly selected for imaging at 500x to ensure that the surface topography and features representative of the sac sample’s surface were recorded. One lower magnification 100x image was also taken to examine a larger surface area and confirm that the areas imaged at higher magnification were representative. Higher magnification images were taken to examine various structures of interest as necessary.

Analysis of the surface density of the main structures observed on the blood sac surfaces was performed for each region of the blood sac. Due to the small size and irregularity of shape of some structures, and limitations of contrast in SEM images, analysis of thrombosis on the surfaces was limited to visually scoring surface density of structures into one of four categories: 0—None, 1—Low density, 2—Medium density, and 3—High density. Each of the six images taken at 500x magnification for each sample was scored accordingly for each structure.
3.3.2.3 Statistical Analysis

Although the data are scores from 0 to 3, the means were used instead of the medians. The median for scores between 0 and 3 would not be sensitive enough to show differences between sets of data. The large repeated measures design provides ample data for statistical analysis using means. Low sample size in each group is an inherent limitation of large animal in vivo studies. For each sac region, the mean surface density score was calculated using 12 images, 6 images from each of two samples. Statistical analysis of data on surface density of structures on SEM images of the blood sacs was performed with two-way repeated measures analysis of variance (ANOVA). A value of p < 0.05 was considered indicative of statistical significance. All tests were performed using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego, CA).

3.4 Results

3.4.1 Clinical Observations

All devices operated normally and no exceptional circumstances were observed. Average values of flow rates, ACTs, PTs, and INRs for each calf are listed in Table 3-1. The mean flow rates for the seven calves were maintained between 4.71 and 6.85 L/min with standard deviations from 0.95 to 1.69 L/min. The mean (± standard deviation) postoperative ACTs were 247 secs ± 47 secs and 188 secs ± 32 secs for the anticoagulated and nonanticoagulated groups, respectively. The average PTs were
similar for anticoagulated and nonanticoagulated groups at 1.2 ± 0.2 and 1.1 ± 0.2
normalized to the baseline PTs. Similarly, there was no difference in INRs between
anticoagulated and nonanticoagulated groups, with averages of 1.4 ± 0.4 and 1.2 ± 0.4,
respectively. These results are expected given that the mechanism of action of warfarin
sodium is by hepatic inhibition of the interconversion of vitamin K and vitamin K
epoxide, so there is expected to be a lag time between initiation of warfarin sodium
anticoagulation therapy and changes in clotting, as compared to heparin. Heparin is
effective immediately\(^6\) because it binds to antithrombin, and together they inhibit the
action of thrombin, a potent activator of coagulation. Warfarin sodium was clearly
ineffective in this 3-day study as shown by the lack of difference in the PTs and INRs for
the two study groups. However, the calves were successfully anticoagulated by heparin,
as reflected in the difference in the ACTs between the study groups.

Clinical results including flow rates, anticoagulation, kidney infarcts, and platelet
and fibrinogen concentrations for this 3-day study are compared to the 30-day study in
Appendix A.
3.4.2 Macroscale Surface Thrombosis

Macroscopic thrombi were observed on all seven retrieved blood sacs. The majority of thrombi were white, but some red thrombi, suggesting the presence of red blood cells entrapped in fibrin, were located along the inlet and outlet sides and edges of the center of the sacs. Several small thrombi, many approximately 1 mm in diameter, were observed on each sac. Due to the small size of the thrombi, the total surface area covered by macroscopic thrombi in each sac was low, 0.42% and 0.44%, for the anticoagulated and nonanticoagulated groups, respectively (Figure 3.2). The large standard deviations for some measurements are often the result of one sac having one large thrombus or numerous small thrombi. This was particularly notable in the case in
the inlet region of the anticoagulated group, where two sacs had no thrombi in the inlet but one sac had ten thrombi 1 mm in diameter and one very large thrombus with a diameter of 7 mm. Although such differences in macroscopic thrombosis between sacs were observed in both study groups, thrombosis was not consistently higher in particular sacs or specific regions. Percent surface coverage of the sacs by macroscopic thrombi did not differ between the anticoagulated and nonanticoagulated groups. Analysis of the seven blood sacs for macroscopic thrombi showed that no specific region appeared to be more likely to have surface thrombi than any other region of the blood sac.

Figure 3.2. Macroscopic thrombi surface coverage of the blood sacs for the 3 anticoagulated and 4 nonanticoagulated blood sacs of groups 1 and 2. No difference was observed in the average macroscopic thrombus surface coverage of the anticoagulated sacs compared to nonanticoagulated sacs. No region of the blood sac appeared to be more thrombogenic than other regions at the macroscale. Data are presented as mean value ± standard deviation.
3.4.3 Microscale Surface Thrombosis

Various structures, both biologic and polymeric, were observed on the retrieved implants as shown in Figure 3.3. The primary structure observed on the retrieved blood sacs was a spherical particle with a diameter of approximately 400 nm (Figure 3.3A). The structures observed on the retrieved blood sacs were not observed on negative control samples of sacs that had never been in contact with blood. The identity of these particles is not certain, although the structure is consistent with platelet-derived microparticles. Preliminary functional studies using confocal microscopy show the presence of small particles (< 1 μm diameter) on the surfaces that show specificity for the platelet integrin αIIbβ3 (data not shown). Furthermore, a drop in platelet count was observed in all calves and is consistent with platelet consumption and formation of microparticles. However, platelet-derived microparticles can also originate from platelets adherent to the biomaterial surface and so it is not necessary that platelet microparticles be circulating in order to be the identity of the particles observed on the sac surfaces. For the purpose of this study, the particles will simply be referred to as 400 nm particles. Very few structures immediately identifiable as platelets were observed. Although most regions were covered with the 400 nm particles, other regions of the blood sac appeared to be coated with a rough layer of biologic material, most likely protein (Figure 3.3B) and consistent with our previous studies. Fibrillar structures consistent with fibrin were observed in the inlets and outlets but rarely in other regions of the sacs (Figure 3.3C). This localization of these structures primarily to the port regions is also consistent with our previous studies at 30 days. Comparison of this 3-day study
to our previous 30-day study is further elaborated in the Discussion. Dark circles shown in Figure 3.3D were found often and believed to be pits in the polymer, most likely caused by the polymer composition and fabrication process, although biologically-induced degradation\textsuperscript{115} is a possibility. However, we have observed such pitting in the blood sacs immediately after fabrication and therefore we do not believe it to arise from biological contact (data not shown). Larger thrombi, up to tens of μms in diameter, were also observed (Figure 3.3E). Cell-like structures were also seen but were rare.

![Figure 3.3](image)

**Figure 3.3.** Surface topography of the explanted blood sacs and structures observed. Structures including sub-platelet sized particles approximately 400 nm in diameter (A), rough proteinaceous layer (B), fibrillar structures (C), pits in the polymer (D), and thrombi 10-50 μm in diameter (E), were observed. Note differences in scale bars shown for each image: 2 μm (A), 50 μm (B and C), and 10 μm (D and E).

The three dominant structures found in the microscale images of the retrieved blood sacs were: A) particles with approximate diameter of 400 nm, B) proteinaceous topography, and C) fibrillar structures. The presence of these structures was carefully examined for each sac and scored qualitatively by surface densities of 0 (negligible), 1 (low), 2 (medium), and 3 (high), as demonstrated in Figure 3.4 for the 400 nm particles. The average surface density of the dominant structures in each of the eight regions was calculated for each group of sacs and results were compared for the two groups,
anticoagulated and nonanticoagulated (Table 3-2). The larger thrombi were minimal in both groups in all regions and therefore surface density of these larger thrombi was not scored.

Figure 3.4. Examples of scoring surface density of structures on SEM images. The 400 nm diameter particles adhered to the blood sac surface of anticoagulated sac 2 were scored: 0 for negligible surface density (A), 1 for low (B), 2 for medium (C), and 3 for high surface density (D). Other structures observed on the surfaces were scored similarly.

Table 3-2. Density of primary features on blood sac surfaces in anticoagulated and nonanticoagulated calves (Average value ± standard deviation).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Group</th>
<th>Blood Sac Region Mean Surface Density Score (0-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inlet</td>
</tr>
<tr>
<td>400 nm Diameter Particles</td>
<td>Anticoagulated</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>Nonanticoagulated</td>
<td>0.44</td>
</tr>
<tr>
<td>Proteinaceous Topography</td>
<td>Anticoagulated</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>Nonanticoagulated</td>
<td>0.48</td>
</tr>
<tr>
<td>Fibrillar Structure</td>
<td>Anticoagulated</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Nonanticoagulated</td>
<td>0.40</td>
</tr>
</tbody>
</table>
In the anticoagulated group 1, the main structure observed on the sac surfaces was the 400 nm diameter particles. Several samples had high densities of this structure, an example is shown in Figure 3.5. On average, the region with the lowest surface density of these particles was found in the outlet of the anticoagulated blood sacs (average surface density score of 0.03). Low surface density (average density score below 1.00) was also recorded in the center front and back and on the bottom. All other regions had average surface density scores above 1.00 for the three anticoagulated sacs. The proteinaceous structure (Figure 3.3B) was rarely observed on the retrieved blood sac surfaces. However, the highest density of this structure was observed in the outlet, where the 400 nm particles were seen least. The value for the average surface density of this rough topography observed was 0.97 for the anticoagulated sacs in the outlets. This topography was also seen in the inlets of all three group 1 sacs but was rarely seen in the other regions and then mostly at low surface density. The fibrillar structure resembling fibrin (Figure 3.3C) was also concentrated to the inlets and outlets, similar to the proteinaceous topography. Even among the inlet and outlet samples, the fibrillar structure was only observed on one and three samples, respectively, from the six samples imaged from each of the port regions for the anticoagulated sacs. Large thrombi reaching to as wide as 50 μm were observed throughout the anticoagulated sacs but generally they were only observed in small numbers in just a few images.
The four nonanticoagulated sacs of group 2 followed a very similar trend to that of the anticoagulated sacs. Figure 3.6 illustrates this pattern showing representative surface densities of the three most common structures, comparing the outlet port region to other regions of the blood sac. Figure 3.7 summarizes this data as the average surface densities for each of the three dominant structures for the eight sac regions. The particles with diameter of approximately 400 nm were present at the highest density and frequency for the 64 sac samples imaged for the nonanticoagulated group. The inlet and outlet had the lowest surface densities of these particles (average surface density scores of 0.44 and 0.25, respectively). This dominant feature was present on all samples from all other regions in all four nonanticoagulated blood sacs, at higher surface densities than in the inlets and outlets (Figure 3.7A). The proteinaceous topography and fibrillar structures were observed primarily in the inlet and outlet ports. This can be seen in Figures 3.7B

Figure 3.5. The particles shown adhered to the blood sac surface at a high density (a value of 3 on the scale of 0-3) as seen in the inlet side region of explanted anticoagulated blood sac 2 (A). Such high surface density was frequently observed in most regions of the anticoagulated blood sacs. A much lower density of the particles was observed in the outlet and center back of the anticoagulated sacs. Higher magnification image showing the size of the particles is approximately 400 nm (bar = 2 μm) (B).
and 3.7C. Proteinaceous rough surfaces were not seen in any images captured from the center back, inlet side, or outlet side of any of the four blood sacs of group 2. Fibrin-like structures were seen in only one other region besides the ports, on the top region of the sac, and at a low surface density. Larger microscale thrombi were minimal.

Figure 3.6. Similar trends in the surface density of structures on the different regions of the blood sacs were observed for anticoagulated and nonanticoagulated groups. Lower density of particles was observed in the inlet and outlet ports compared to the other regions of the blood sac (A, B), whereas the opposite was true for proteinaceous topography (C, D) and fibrillar structures (E, F). The top three images were from the outlet of the nonanticoagulated sac 3 and the bottom images were from the inlet side, center back, and top regions, respectively, of the same blood sac.
Figure 3.7. Surface density distribution of the main structures observed in the eight regions of the blood sacs retrieved from anticoagulated and nonanticoagulated calves. The density distribution of the three main structures observed in the eight sac regions differed. Adhesion of the 400 nm diameter particles was seen at high density most frequently, but tended to be lower in the inlet and outlet (A). On the other hand, proteinaceous topography (B) and fibrillar structures (C) were seen at a higher density in the inlet and outlet ports. Error bars = standard errors ■ Anticoagulated Average □ Nonanticoagulated Average
Similarity in the trends of surface density distribution of the various structures along the blood sacs was observed between the anticoagulated and nonanticoagulated groups. Overall, the surface topography of the blood sacs recorded by SEM in the inlet and outlet ports appeared to differ from that of all other regions of the blood sac. The ports had higher densities of proteinaceous and fibrillar structures, whereas all other regions were highly populated by the surface particles. Two-way repeated measures ANOVA showed that anticoagulation had no significant effect on surface density of all three structures with p values greater than 0.05 (p = 0.97, 0.89, and 0.96 for 400 nm particles, proteinaceous topography, and fibrillar structure, respectively). However, the surface density of all three structures did differ significantly with blood sac region with p < 0.001 (p = 0.0005, 0.0001, and 0.0023 for 400 nm particles, proteinaceous topography, and fibrillar structure, respectively).

3.5 Discussion

Surface thrombosis was seen at the macroscale regardless of whether or not the calves received anticoagulation. No differences in percent surface coverage of the sacs by thrombi were observed in any individual region of the sac as well as in the entire sac. However, in comparison to our previous study with 30-day implants, short-term macroscopic surface thrombosis in the LVAS was observed in a higher fraction of sacs as well as with higher frequency (larger number of macroscopic thrombi in each sac).111

Microscale surface thrombosis observed in this study was found to be dependent on the location within the blood sac. Anticoagulation was found to have no significant
effect on the surface density of the structures. However, surface density of all three structures was found to be significantly different with blood sac region. The primary structure observed, 400 nm diameter spherical particles, structurally resembles platelet microparticles. The particles, approximately 400 nm in diameter as seen by SEM, appeared to adhere at a lower density in the inlet and outlet ports. On the other hand, the inlet and outlet ports had the highest density of proteinaceous topography and fibrillar structures, similar to our 30-day in vivo experiments. The main location-dependent difference in microscale surface thrombosis was observed between the ports and the rest of the sac.

Differences in surface thrombosis between the ports and the rest of the sac may be related to the variations in shear stresses in these regions. Previous work by Baldwin et al. has shown the shear stresses to be significantly different in the inlet and outlet ports, compared to other regions of the blood sac. Reynolds shear stresses measured in the inlet and outlet ports by the valves were reported to be 5,500 dynes/cm² or less, whereas shear stresses were below 500 dynes/cm² for all other regions of the blood sac. Such shear stresses are thought to be sufficiently high enough to provide effective washing of the surface to prevent and/or wash away any thrombus formation, with minimal platelet adhesion and surface coverage. The inlet and outlet shear stresses appeared to be high enough to minimize adhesion of the 400 nm diameter particles. These particles appeared to adhere at a lower density in the higher shear stress inlet and outlet ports. On the other hand, the inlet and outlet ports had the highest density of proteinaceous topography and fibrillar structures. In the other regions of the sac where higher adhesion of the 400 nm particles but lower adhesion of other structures were observed, shear
stresses are lower than 500 dynes/cm\(^2\). Although there appears to be a correlation between surface thrombosis and fluid dynamics, our results suggest that the dynamic environment of the LVAS is much more complex and requires greater attention to resolve the issue of thrombosis.

Surface topography in the blood sacs differed with implantation time in the comparison of this 3-day study to our previous 30-day study. The 3-day sacs from the two study groups, anticoagulated and nonanticoagulated, had higher blood sac surface area covered by macroscopic thrombi, 0.42% and 0.44%, compared to the 30-day sacs, which were reported as having 0.23% and 0.16% total sac surface coverage by thrombi. On the microscale, the location-dependent occurrence of proteinaceous topography and fibrillar structure was the same for both 3-day and 30-day studies. The localization of the fibrillar structures primarily to the port regions observed in this 3-day study was also observed with our previous 30-day study. However, the adhesion of the particles approximately 400 nm in diameter was only observed in the 3-day study. In the 30-day study, larger particles resembling platelets, usually ~5 μm in diameter, were observed frequently. Similar to the 400 nm particles in the 3-day study, these particles occurred less in the inlet and outlet ports of the 30-day sacs. Although no differences between anticoagulated and nonanticoagulated groups were observed at both the macroscale and microscale in the 3-day study, at 30 days, anticoagulation effects were observed. Specifically, in the 30-day study fewer platelet-like particles were present in the anticoagulated sacs compared to nonanticoagulated sacs. Differences in the results of the 3-day and 30-day studies suggest there is a time-dependent factor to the development of surface thrombosis in the blood sacs of LVAS. These differences as well
as similarities in surface topography between 3-day and 30-day explants, give new insight into surface thrombosis over time.

Although SEM, the microscopy technique used in this study, provided important topographical information on the explanted blood sacs, the information obtained was limited particularly with regards to the functionality of the features observed. SEM is a commonly used tool for evaluating surface thrombosis on explanted biomaterials but its drawbacks were encountered in this study. SEM image analysis is often limited to qualitative information. The small size and irregular shapes of the various structures observed made image analysis and quantification of surface density of the structures difficult. We addressed this issue with a semi-quantitative method of analysis by scoring the surface density from 0 to 3, reflecting negligible to high density of the structures. This scoring of the structures by level of surface density provided data for statistical analysis. Although the structures observed cannot be identified with absolute certainty, control samples prepared previously111 and information available on the morphology of platelets, fibrin, and other blood components were used to identify structures imaged by SEM. Siedlecki et al. imaged platelet-derived microparticles on biomaterial surfaces and have shown them to be approximately 125 nm in diameter,114 however the diameter of the particles on the blood sac surfaces in this study was approximately 400 nm. Although these particles might then seem to be larger than platelet-derived microparticles, microparticles of this size from platelets have also been reported. Hagerstrand et al. reported two groups of microparticles, one group of microparticles in the range of 150—200 nm diameter, and a second group of microparticles with a diameter range from 200—800 nm. Preliminary studies using immunofluorescent labeling provide some level of
confirmation of the structures adhered to the blood sac surfaces. SEM allowed simultaneous assessment of surface thrombosis and topography, and provided valuable information on the various structures present on the explanted blood sacs of the LVAS.

3.6 Conclusion

In this short-term 3-day in vivo study of surface thrombosis in LVAS, heparin anticoagulation appeared to be ineffective in prevention of surface thrombosis. For the duration of this short 3-day study, warfarin sodium was not an effective anticoagulant as assessed by PTs. Both macroscale and microscale thrombi were observed regardless of anticoagulation. At the microscale, surface thrombosis was found to be dependent on the region of the blood sac, coinciding with region-dependent shear stresses. Thus, surface thrombosis appears to be region-dependent in the blood sac, thereby indirectly implicating fluid flow as an important aspect of surface thrombosis. Comparison of this study to a parallel long-term 30-day in vivo study suggests differences in platelet response over time, with what are believed to be platelet-derived microparticles present at short time periods and fully spread platelets present at 30 days. These results demonstrate that there are both time-dependent and region/shear-dependent aspects of surface thrombosis in LVAS, and addressing these differences may be critical to future improvements in the hemocompatibility of LVAS.
Chapter 4

Platelet and Fibrin Adhesion on Retrieved Blood Sacs Imaged by Confocal Microscopy

4.1 Abstract

Fibrin and platelet adhesion on retrieved blood sacs were evaluated by indirect immunofluorescence and confocal microscopy for the LVAS implants retrieved after 3 and 30 days with and without anticoagulation. Confocal images were scored qualitatively for surface densities of platelet and fibrin features. Steps for image processing and filtering were established for quantitative analysis of platelet adhesion on the blood sacs.

Platelet and fibrin adhesion observed by confocal microscopy coincided with the SEM results, although important differences were also present. The fibrillar strands observed by SEM primarily in the inlets and outlets were rare in confocal images, although a fibrin strand with platelets of similar structure to the SEM structure was observed. Circular features observed by SEM, resembled the structure of the small fibrin clots seen in confocal images and often co-localized with platelets. These clots occurred at a higher surface density in the 30-day sacs, in agreement with SEM results. However, the reduced density of the platelet-like structures in the anticoagulated group observed by SEM was not obvious in confocal images. In the SEM analysis, particles approximately 400 nm in diameter were observed primarily in the 3-day study. Similar microparticles
were observed by confocal microscopy at a higher density in the 3-day study. No correlation between sac region-dependent fluid dynamics, and platelet and fibrin adhesion was observed.

4.2 Introduction

SEM analysis of surface thrombosis on blood sacs implanted for 3 and 30 days in a bovine model presented in previous chapters showed sac location-dependent surface topography. However, the technique used, SEM, was unable to distinguish polymeric from biologic features and assess the type of biologic material. Therefore, in this chapter indirect immunofluorescent labeling and confocal microscopy were used to identify, quantify, and analyze fibrin and platelet adhesion on the retrieved blood sacs.

Indirect immunofluorescent labeling involves first binding a primary antibody to the antigen of interest. A secondary polyclonal antibody tagged with fluorophores is used to bind to the primary antibody, thus indirectly fluorescently labeling the antigen of interest (Figure 4.1). A blocking agent, such as serum, is adsorbed to the sample surface to prevent nonspecific adsorption of the antibodies to the sample. This method is preferred over direct labeling involving fluorescently labeled primary antibodies because of its superior fluorescence signal. With indirect immunolabeling, multiple fluorescently-labeled secondary antibodies can bind to one primary antibody, thus amplifying by five to ten times the fluorescent signal that would be obtained with direct labeling.
Immunofluorescent labeling of platelets and fibrinogen will allow detection of thrombi on the surfaces of the retrieved blood sacs. Platelets can be identified by labeling for the $\alpha_{IIb}$ chain on the $\alpha_{IIb}\beta_3$ integrin present on the platelet membrane. Fibrin can be labeled with a monoclonal antibody solution that recognizes bovine fibrinogen. The anti-bovine fibrinogen antibody recognizes both fibrinogen and fibrin on the samples. Although fibrin can be labeled this way, using a primary antibody that recognizes fibrinogen, will not allow distinction of fibrinogen from fibrin. An alternative strategy would be to use an antibody that only recognizes an epitope of fibrin. Fibrinopeptides are cleaved from fibrinogen resulting in fibrin monomers that are polymerized to form fibrin. There are antibodies available that will only specifically recognize fibrin and not fibrinogen. However, for the bovine model, these antibodies are limited and costly.

Confocal microscopes have several advantages over fluorescence microscopes that warrant the use of the confocal microscope for this study. In preliminary work with

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**Figure 4.1. Schematic of indirect immunofluorescent labeling**, an assay which provides amplification of the fluorescent signal over direct labeling. An unlabeled primary antibody is bound to the antigen and fluorescently labeled secondary antibodies are bound to the primary antibody. Multiple secondary antibodies can bind to each primary antibody, thus amplifying the fluorescent signal that would be obtained from direct labeling of the primary antibody without using a secondary antibody.
the fluorescence microscope, autofluorescence of the blood sac polyurethane (PU) material was found to cause difficulty in imaging of platelets and fibrin on the surface (Figure 4.2). Eliminating such background out-of-focus light from planes above or below is the key feature of a confocal microscope. The laser scanning confocal microscope typically comes with multiple lasers providing a wide range of excitation wavelengths. This gives the user more options in selection of fluorophores compared to a typical fluorescence microscope where filter cubes for specific excitation and emission ranges would be required. More options of fluorophores allow selection of a fluorophore with an emission wavelength range where autofluorescence is weak. This helps minimize autofluorescence. 3-D imaging by collecting a z-series of optical sections is another advantage of the confocal microscope. In addition to being able to collect information on thickness of biologic material such as fibrinogen adsorption or fibrin layer, this capability will make imaging of the uneven PU samples easier. The PU sac samples are often of uneven thickness or curved, resulting in out-of-focus areas in images. By taking z-series, all areas can be imaged in focus and the series of images can be stacked or combined to get a final image with the entire area in focus. The confocal microscope’s many other settings that allow optimal imaging are mentioned in the following Materials and Methods section.
4.3 Materials and Methods

4.3.1 Indirect Immunofluorescence Assays

A sample approximately 1.4 cm by 0.7 cm was cut from each of the eight regions (Section 3.3 Figure 3.1) of the sixteen sacs retrieved in both the 3-day (n = 7) and 30-day (n = 9) studies discussed in the previous chapters. Samples were marked by placing cuts in the upper right-hand corner to distinguish the surface exposed to the blood \textit{in vivo} and the surface on the outside of the blood sac. The samples were placed in 12-well tissue culture polystyrene plates (VWR, Bridgeport, NJ) covering the samples with PBS until ready for immunolabeling. For each sac, a primary antibody solution was prepared by combining the following:

1. 8 ml of 6 % normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) as a blocking agent

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4_2.jpg}
\caption{High background autofluorescence from PU causes difficulty in imaging on blood sacs as seen in a comparison of a platelet-poor plasma clot on glass (A) and on PU (B).}
\end{figure}
2. 12 μl of mouse anti-bovine α<sub>IIb</sub>β<sub>3</sub> antibody (VMRD, Pullman, WA; 1mg/ml)
3. 8 μl of goat anti-bovine fibrinogen (American Diagnostica, Stamford, CT; 1 mg/ml)

For each sample, the PBS was replaced with 1 ml of primary antibody making sure samples were completely covered. Samples were incubated overnight at 4°C.

The following day, the wells were filled with PBS using an autopipette. The primary antibody solution was aspirated but the sample surfaces were kept submerged in solution at all times. The samples were rinsed two more times with PBS. The wells were filled with PBS and left for 10 min at RT to remove unbound antibodies. Samples were rinsed with PBS three more times. The following secondary antibody solution was prepared:

1. 8 ml of 6 % normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) as a blocking agent
2. 80 μl Cy3-donkey anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA; 1.4 mg/ml)
3. 40 μl Cy5-donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1.4 mg/ml)

The PBS was replaced with 1 ml of the secondary antibody solution for each sample. The samples were incubated in the dark for 60 min at RT. To rinse, the wells were filled with PBS using an autopipette. The secondary antibody solution was aspirated keeping the samples submerged at all times. The samples were rinsed two more times with PBS. The wells were filled with PBS and left for 10 min to remove unbound antibodies. Samples were rinsed with PBS three more times.
Due to the samples not always being flat and the thickness of the PU samples, the microscope slide coverslips were modified to hold the samples down flat for microscopy imaging. Ahead of time, cyanoacrylate (Crazy glue) was used to glue small pieces of blood sac PU (from unused sacs) to the four corners of each coverslip. Each sample was placed onto a microscope slide. Two drops of Gel/Mount® antifade solution (Biømeda, Foster City, CA) were placed onto each sample. Cyanoacrylate was applied to the top of the four pieces on each coverslip and the coverslip was placed over the sac sample and bound to the microscope slide.

Negative and positive control samples were prepared. The positive control PRP clot was prepared as described in Section 3.3 placing the labeled PRP clot on a PU sample cut from a clean PU sac. Two types of negative controls were prepared: A) No primary or secondary antibodies and B) No primary antibody, only secondary antibody. The negative controls were prepared using the following samples: samples from the back and center front regions of the sac from calf 2 of the anticoagulated 3-day group, and a clean PU sample from a blood sac that had not been in contact with biologic material. Wherever antibodies were excluded in the negative controls, the antibody solutions were replaced with 6% normal donkey serum.

4.3.2 Confocal Microscopy Imaging

Samples were imaged using the 543 nm and 633 nm lasers on a TCS SP2 AOBS confocal microscope (Leica, Deerfield, IL) with a 63x oil immersion objective (630x final magnification). Standard adjustments to instrument settings were made for optimal
imaging of samples for each fluorophore used: Cy3 (platelets) and Cy5 (fibrinogen). Parameter settings were set and saved for each fluorophore, and occasionally adjusted depending on fluorescence intensities of the samples. The emission ranges were set for each fluorophore and were not adjusted at any point. Emission wavelength ranges were set to 547-623 nm for Cy3 and to 660-780 nm for Cy5. Laser intensity were also optimized and set to 80.29% power for the 543 nm laser for Cy3 and to 50.7% power for the 633 nm laser for Cy5. Preliminary optimization of the image was achieved by adjusting the photomultiplier tube (PMT) gain and offset voltages, balancing between background noise and saturation, so as to image the dimmest to the brightest features of interest. The scanning settings of frame averaging and line averaging were set to 2 and 1 to reduce noise, and frame sequential scanning was used to avoid crosstalk caused by use of fluorophores with overlapping emission spectra. For each area imaged, a series of images over a selected z range was recorded. The focus was adjusted to bring the surface into focus from the top of the sample (set z-end point) and into the sample until features of interest were no longer visible. The z-start position was set. The z-series was recorded, using a step size of typically 0.5 µm to 1.02 µm, depending on the step size necessary to collect images covering the entire area in focus and on the total z-range to be covered. Z-series captured consisted of anywhere from one image to as high as over ten images stacked, and frequently consisted of four images with a step size of 1.02 µm, which covers a sample depth of approximately 4 µm. Five areas were imaged on each sample.
Negative controls were imaged to confirm minimal non-specific labeling and background fluorescence from the PU. The positive sample was imaged to confirm labeling. Calf sac samples labeled with primary and secondary antibodies were imaged.

4.3.3 Data Analysis

Confocal images were primarily analyzed qualitatively due to low contrast between background and features of interest, particularly for platelets, which caused difficulty in using image analysis software to obtain values such as particle counts and percent surface area. A set of images were used to develop a protocol for image processing to extract features of interest and exclude background for quantitative analysis of the confocal images.

4.3.3.1 Qualitative Analysis

The z-series of images for each area were combined into a maximum projection, which took the maximum fluorescence intensity in the series of images at each pixel location, to create a 2-D image. Images were examined to determine the primary platelet and fibrin structures on the surfaces. Analysis of the surface density of the main structures labeling for fibrinogen and platelets on the blood sac surfaces was performed for each region of the blood sac. Surface density of each type of structure was visually scored assigning one of four values: 0—None, 1—Low density, 2—Medium density, and
3—High density. All samples from the two study groups, 3-day and 30-day, were scored accordingly for each structure.

4.3.3.2 Quantitative Analysis

A sequence of steps was developed for image processing for quantitative analysis of platelet adhesion on the blood sac confocal images. Due to the autofluorescence of PU causing high fluorescence background, standard quantification of particles in fluorescence microscopy, including particle count and surface area were difficult and gave inaccurate results. Therefore, a set of images was used to establish an image processing protocol. The images used were: 1) Negative control of implanted blood sac sample, 2) Positive control of platelets on PRP, and 3) Retrieved sac sample from the inlet of the 3-day anticoagulated calf 1. Images were processed using ImageJ® 1.32J software (National Institutes of Health, Bethesda, MD). Images used for quantitative analysis were taken using the same confocal settings, except for the positive control.

Fluorescence intensity (FI) histograms of the negative and positive controls were compared to the retrieved sac sample to determine the FI ranges of PU autofluorescence and positive platelet labeling. This information was used to select a threshold for FI to eliminate background fluorescence as the first step of image processing for quantification of platelet adhesion. Once a threshold was determined, a count of particles and the area of each particle above the set threshold were calculated using the ‘Analyze Particles’ feature in ImageJ. The surface area of each particle was given in μm² after setting the scale for the 630x confocal images. A particle size limit was set to further reduce
background noise. A bovine unspread platelet could be as small as 1 μm in diameter so the particle size limit was set to a surface area of 0.44 μm² (0.75 μm diameter). The total and average surface areas and percent of platelet surface area were calculated for fluorescent particles above the threshold and greater than 0.75 μm in diameter. The established protocol summarized in the flowchart in Figure 4.3 was applied to the remaining four platelet images in the same region, the inlet, of the anticoagulated calf 1 from the 3-day study. An additional image with clearer platelets with brighter fluorescence and greater contrast from background, from the bottom region of the same sac, was also analyzed.
Figure 4.3. Sequence of steps for postprocessing of confocal images to quantify platelet adhesion involve setting a threshold for fluorescence intensity and applying a particle size filter to exclude small particles that are likely to be background noise.
4.4 Results

4.4.1 Qualitative Analysis

Three distinct structures that labeled for each the platelet integrin and fibrinogen were observed (Figure 4.4). Particles with specificity for the $\alpha_{IIb}$ chain of the platelet $\alpha_{IIb}\beta_3$ integrin were labeled with red Cy3. Three main structures were observed: 1) platelets, 2) microparticles, and 3) larger particles. Platelets approximately 2-3 $\mu$m in diameter were adhered throughout the blood sacs (diameter average of $2.70 \pm 0.29 \mu$m for 10 measurements on one image) (Figure 4.4A). Some had bright, strong fluorescent signals, with high contrast from the PU background, but in many areas the fluorescent Cy3 signal was weak and faint labeling of similar structures was observed.

Microparticles, although less frequent, were also observed (Figure 4.4B). Larger depositions, many greater than 10 $\mu$m in diameter, were often found co-localized with fibrinogen labeling (Figure 4.4C). These larger particles were likely thrombi with platelets and fibrin.

Both fibrinogen molecules adsorbed to the surfaces, as well as fibrin, were observed. Fibrinogen and fibrin were labeled with the blue Cy5 fluorophore. Fibrin structures of various sizes were observed and are shown in Figure 4.4(D-F). The three fibrin structures observed were: 1) long strands of fibrin, 2) small fibrin clots (typically circular, average diameter $3.86 \pm 0.62 \mu$m for 10 measurements on one image), and 3) larger thrombi with lengths as much as 18 $\mu$m. Specks of fibrinogen and layers of
fibrinogen or fibrin were also common. However, in evaluating thrombosis, fibrin was the focus in this study and were the only structures scored.
Figure 4.4. Structures observed by confocal microscopy with platelet integrin (red) or fibrinogen (blue) specificity. Platelets (A), platelet-derived microparticles (B), larger thrombi with platelets (C), fibrin strands (D), small fibrin clots (E), and larger fibrin strands (F) were observed at various densities throughout the blood sacs.
The surface densities of the six different structures evaluated, are shown in Figures 4.5 and 4.6 for those with platelet specificity and fibrinogen specificity, respectively. Relationships of fibrinogen and platelet surface densities with blood sac region, anticoagulation, and implant duration were observed. The top and bottom regions (Figure 2.1) differed most from the other six regions of the sac. Platelet adhesion was higher in the top and bottom regions in all groups: 3-day and 30-day, both anticoagulated and nonanticoagulated. Large particles with specificity for platelets and/or fibrinogen were observed most in the top region of the sac, between the ports. The 30-day blood sacs had a higher surface density of large particles, with platelet and/or fibrinogen specificity, compared to the 3-day sacs. Similarly, the 30-day blood sacs had a higher surface density of small fibrin clots compared to the 3-day sacs. On the other hand, the 3-day nonanticoagulated sacs had the highest density (average of 0.54 ± 0.06) of platelet-derived microparticles. The 3-day anticoagulated sacs, on the other hand, had few microparticles (average surface density of 0.11 ± 0.03). The small particles, microparticles, labeling for platelets (Figure 4.4B) were thought to be platelet-derived microparticles as discussed in the Discussion section 3.5 for the 400 nm diameter particles observed by SEM in the 3-day study.
Figure 4.5. Surface density scores of particles with platelet specificity for the two study groups, 3- and 30-day, anticoagulated and nonanticoagulated. Surface density of three platelet features (as estimated by size), platelets (A), microparticles (B), and large particles (C) were evaluated. Error bars = Standard error.

- 30-day anticoagulated
- 30-day nonanticoagulated
- 3-day anticoagulated
- 3-day nonanticoagulated
Figure 4.6. Surface density scores for fibrin structures were evaluated on sac samples from the 3- and 30-day, anticoagulated and nonanticoagulated studies. Three features were examined: fibrin strands (A), small fibrin clots (B), and large thrombi (C). Error bars = Standard error

- ■ 30-day anticoagulated
- □ 30-day nonanticoagulated
- ■ 3-day anticoagulated
- ■ 3-day nonanticoagulated
The capability to observe platelet and fibrin adhesion in the same sample area revealed frequent co-localization of the platelets and fibrin (Figure 4.7). Along with co-localization, in some images such as Figure 4.8, the circular regions of fibrin appeared darker in the center, suggesting a hole in the surface or less fibrin adhesion at the center. The sizes of these dark centers, averaging $5.59 \pm 1.02 \mu m$ in diameter and ranging from 3.91 to 7.45 $\mu m$ (for 10 measurements on one image), were similar to those of the pits seen in previous SEM images (Figure 2.7). A similar feature was observed previously by SEM as seen in Figure 4.9. Comparison of the SEM image and confocal image illustrates the advantage of confocal microscopy in which greater contrast is obtained between background and features of interest. In the confocal image, the locations of fibrin or fibrinogen and platelets are clear.
Figure 4.7. Co-localization of fibrin (A) and platelets (B) as seen in the overlay (C) was often observed as shown here on a sample from the bottom region of the 30-day anticoagulated calf 3 sac. Purple indicates co-localization in the overlay image.
Figure 4.8. Fibrin and platelet adhesion around what may be pits in sac PU. The dark centers to the circular regions of fibrin adhesion indicate decreased fibrin adhesion in the center, which may be indicative of pits previously observed by SEM on sac PU.
4.4.2 Quantitative Analysis

Images shown in Figures 4.10, 4.11, and 4.12 were used to establish a protocol for quantitative image analysis for confocal microscopy of retrieved blood sacs. The negative control in Figure 4.10 had dim fluorescence across the surface that results in background noise, reducing contrast between background and labeled features of interest. In the image of the positive control of labeled platelets on sac PU (Figure 4.11), the platelets are clearly visible and can be distinguished easily from the background. However, as shown in the image of the implanted sac sample from the 3-day anticoagulated calf 1 inlet region, the platelets and background were close in fluorescence.
intensity (Figure 4.12). This image was selected to use for developing the quantitative image analysis protocol because the low contrast between background and platelets makes it one of the more difficult confocal images to quantify.

Figure 4.10. This negative control of an implanted blood sac sample was prepared to test for non-specific labeling and used to measure PU autofluorescence. This sample from the 3-day anticoagulated calf 2 bottom region was only incubated with secondary antibody and donkey serum, no primary antibody. Scale bar = 20 μm

Figure 4.11. Positive control of platelets on sac PU sample was prepared in vitro with bovine platelet-rich plasma. Scale bar = 20 μm
Figure 4.12. Sample from implanted sac (3-day anticoagulated Calf 1 inlet region) was used to develop a set of conditions for processing confocal images. This is an example of low contrast between labeled platelets and PU background. Scale bar = 20 μm

FI histograms of the negative control of an implanted sac (unlabeled for platelets), the positive control of labeled platelets on sac PU, and the sample of an implanted sac labeled for platelets, are shown in Figure 4.13. Histograms of the negative control and implanted sac (Figures 4.13 A and C) had some similarities, with a large peak at 0 FI, a broad peak around 43 FI, and a smaller peak around 128 FI. The matching of these peaks suggests PU autofluorescence occurs at these FIs for the given confocal settings. The presence of a peak at 255 FI in the positive control and the implanted sample, both labeled for platelets, and its absence in the negative control, indicate that labeled platelets fluoresced at 255 FI. Figure 4.14, showing subtraction of the negative control histogram from that of the retrieved sac sample labeled for platelets, further illustrates that the lower range of FI is due to the sac PU background, and that the platelet fluorescence occurs at the higher FIs, primarily at 255. Images thresholded with minimum FI just below and above the smaller peak around 128 FI (127 and 138 FI), and at 255 FI were compared and
analyzed for particles. High particle counts, high number of particles with surface area of 1 pixel$^2$, and average particle surface areas below the size of platelets, were observed for images with thresholds of 127 and 138 FI min. This suggests background noise is still being included with these minimum thresholds. The histograms and particle analysis suggest the majority of platelet labeling is observed at 255 FI. During confocal imaging, differentiation of background features and platelets was difficult, and therefore confocal settings such as PMT gain and offset were set to include features that now appear to have been background. Therefore, platelet fluorescence signal was saturated and observed in the sharp peak at 255 FI. Suggestions for future improvement of platelet imaging are discussed in the Discussion section. Based on the histograms discussed and threshold settings examined, a threshold of 200 FI was set for platelet quantitative analysis of the confocal images.

With the threshold limit determined, the five images from the 3-day anticoagulated sac 1 inlet region were processed setting FI threshold to 200-255. The modified images were analyzed for particles by ImageJ. Surface area data for all particles was exported to Excel and a particle size exclusion was applied to remove background noise. Particles with surface area less than 0.44 μm$^2$ (diameter of 0.75 μm), smaller than unspread bovine platelets, were removed. Finally, particle count, average particle surface area, and percent of sample surface area with platelets were calculated on the final data set. The images before and after setting the FI threshold to 200-255 are shown in Figure 4.15. Particle analysis results before and after particle size filtering (minimum surface area of 0.44 μm$^2$) are shown in Table 4-1. Particle counts were greatly reduced, removing many 0.22 μm$^2$ (1 x 1 pixel) and 0.43 μm$^2$ (1 pixel x 2 pixel) particles.
(Figure 4.16), resulting in a higher average particle surface area. For the first five images from the same sample and same region of the sac, the average sizes before and after particle size filtering were 0.40 μm² and 1.57 μm², respectively. The average size of a platelet was expected to be approximately 1.77 μm² in surface area (assuming a diameter of 1.5 μm). The average particle size obtained before filtering was small for a platelet. On the other hand, after filtering by size the average particle surface area was 1.57 μm², which was close to that of a platelet.
Figure 4.13. Fluorescence intensity histograms for the negative control of an implanted sac (A), positive control of platelets on sac PU (B), and the sample of an implanted sac labeled for platelets (C). The implanted sac PU histograms have similar peaks, a broad peak around 43 FI and another small peak around 128 FI. The platelets fluoresced at the maximum FI of 255 (present in the positive control and retrieved sac sample, and absent in the negative control).
Figure 4.14. The histogram of the retrieved sac sample after subtraction of the background histograms shows that the majority of the labeled platelets occurred at FI 255. FI histograms of retrieved sacs: negative control not labeled for platelets, sample labeled for platelets, and subtraction of the two histograms.
Figure 4.15. Confocal images before (A) and after (B) setting minimum FI threshold to 200. Images are the five platelet images of the inlet region of the 3-day anticoagulated calf 1 and an image from the bottom region of the same sac, with platelets more clearly visible.
Table 4-1. Particle count, surface area, and % area before and after filtering by minimum particle size of surface area 0.44 µm² (diameter of 0.75 µm, slightly less than that of a platelet).

<table>
<thead>
<tr>
<th>Image</th>
<th>Particle Size Filter</th>
<th>Particle Data</th>
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<tbody>
<tr>
<td></td>
<td>Count</td>
<td>Surface Area (µm²)</td>
<td>Average ± Std Dev</td>
<td>% Area</td>
</tr>
<tr>
<td>1</td>
<td>Before 352</td>
<td>0.32 ± 0.47</td>
<td>0.20%</td>
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<tr>
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<td>After 27</td>
<td>1.30 ± 1.37</td>
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<tr>
<td></td>
<td>After 735</td>
<td>1.11 ± 0.86</td>
<td></td>
<td>1.45%</td>
</tr>
<tr>
<td>3</td>
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<td>0.54 ± 1.44</td>
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<td>2.23 ± 3.25</td>
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<tr>
<td>4</td>
<td>Before 1482</td>
<td>0.42 ± 0.52</td>
<td>1.10%</td>
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<tr>
<td></td>
<td>After 257</td>
<td>1.23 ± 0.85</td>
<td></td>
<td>0.56%</td>
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<tr>
<td>5</td>
<td>Before 10546</td>
<td>0.44 ± 1.16</td>
<td>8.30%</td>
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<tr>
<td></td>
<td>After 1282</td>
<td>1.82 ± 2.98</td>
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<td>4.14%</td>
</tr>
<tr>
<td>6</td>
<td>Before 1320</td>
<td>0.84 ± 1.53</td>
<td>2.00%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After 298</td>
<td>2.91 ± 2.21</td>
<td></td>
<td>1.54%</td>
</tr>
</tbody>
</table>
Discussion

Trends in platelet and fibrin adhesion observed by confocal microscopy have some similarities but important differences from the trends in features observed by SEM discussed previously. The fibrillar strands observed by SEM primarily in the inlets and outlets were rarely seen in confocal images, although a fibrin strand with platelets of similar structure to the SEM structure was observed. No strong trend in adhesion of fibrin strands was observed in the confocal images, although the average surface density of fibrin strands was higher in the anticoagulated group for both 3- and 30-day studies. The circular features observed by SEM in the 30-day sacs, which were considered

![Figure 4.16. Particle size filtering of confocal ‘image 3’ excluded a large number of small particles 0.22 μm² (1 pixel x 1 pixel) and 0.43 μm² (1 pixel x 2 pixels) that are likely to be background noise. Grey bars represent particles that were excluded and black bars represent particles that passed the particle size filter and were considered as platelets.](image)

4.5 Discussion

Trends in platelet and fibrin adhesion observed by confocal microscopy have some similarities but important differences from the trends in features observed by SEM discussed previously. The fibrillar strands observed by SEM primarily in the inlets and outlets were rarely seen in confocal images, although a fibrin strand with platelets of similar structure to the SEM structure was observed. No strong trend in adhesion of fibrin strands was observed in the confocal images, although the average surface density of fibrin strands was higher in the anticoagulated group for both 3- and 30-day studies. The circular features observed by SEM in the 30-day sacs, which were considered
platelets, resembled the structure of the small fibrin clots observed by confocal microscopy and often co-localized with platelets. These small fibrin clots were observed at a higher surface density in the 30-day anticoagulated and nonanticoagulated sacs, in agreement with SEM results. However, the trend of reduced density of the platelet-like structures in the anticoagulated group in SEM study was not as clear in the confocal images. In the SEM analysis, particles approximately 400 nm in diameter were observed in the 3-day study but not in the 30-day study. Similar microparticles were observed at a higher density in the 3-day study in the confocal images. Inconsistency between SEM and confocal results may be because some of the features observed by SEM are not fibrin or platelets, and therefore not observed by confocal microscopy. SEM analysis provided topographical information, both polymeric and biologic. Confocal microscopy provided functional information of fibrin and platelet adhesion. Therefore, discrepancies between SEM and confocal results may be used to differentiate polymeric and other biologic features in SEM images from fibrin and platelets.

Correlation of fluid mechanics with the fibrin and platelet adhesion was not as clear with the confocal microscopy measurements as they were with SEM surface topography discussed in Chapters 2 and 3. The primary difference in fibrin and platelet adhesion in the blood sacs was observed in the top and bottom regions (Figure 2.1) of the sac. Although no specific values for shear stress in these regions are readily available, Baldwin et al. did report good flow in these regions by a rotational flow through the entire main chamber of the sac. Baldwin et al. measured the Reynolds stresses in the pump, taking measurements at eight time points during the beat cycle from systole to diastole. Reynolds shear stresses were reported to be below 500 dynes/cm² over the
entire flow cycle within the main pumping chamber. The region of highest Reynolds
stresses in the pump was in the regurgitant jet of the aortic valve (specifically on the
minor orifice side) during diastole. The maximum Reynolds shear stress value recorded
in this region was 5500 dynes/cm² close to the valve ring. Reynolds shear stresses were
also high in the inlet region in the regurgitant jet of the mitral valve with a maximum
value of 3600 dynes/cm². However, one region in the inlet, below the minor orifice of
the mitral valve, which is not distinguished from the rest of the inlet in the SEM and
confocal analysis, had relatively low flow during some period of the cardiac cycle.
Similar fluid mechanics were reported by Hochareon for the same 70 cc pump. Wall
shear stresses can also be much lower, and were reported to be a maximum of 25
dynes/cm² in a similar pump by Tarbell et al. Although the fluid mechanics in the inlet
and outlet ports are clearly different from the rest of the sac, fibrin and platelet adhesion
was not distinct between these regions.

Although it was interesting to see fibrin adhered around what appeared to be pits
on the surface, the pits cannot be confirmed without looking at the same area for fibrin
and platelet adhesion with surface topography simultaneously. In addition, extensive
analysis would be necessary to determine whether the pits attract adhesion of fibrin and
platelet, and if so, to what extent. Such analysis was beyond the scope of this study and
was not performed.

It seemed that low contrast in FI between background and platelets would be a
problem on some retrieved sac samples, as seen in the image selected for analysis to
develop a image processing protocol for quantitative analysis. However, as seen in the
histograms in the quantitative analysis section, the majority of the platelet signal
appeared at FI 255. Therefore, although setting the threshold minimum to 200 may eliminate some platelets, the majority of the platelets should be included in the 200-255 threshold limit. Any background noise included in this range was further eliminated by using a particle size filter, using a minimum particle size limit of 0.44 μm².

During confocal imaging, differentiation of background features and platelets was difficult, and therefore confocal settings such as PMT gain and offset were set to include features that now appear to have been background. Therefore, platelet fluorescence signal was saturated and observed in the sharp peak at 255 FI. In the future, now knowing the features of interest, confocal settings can be adjusted to exclude more background, spread out the signal of the structures of interest, and avoid saturation. This will allow for greater detail of the platelet and fibrin structures.

In quantitative analysis of confocal images, confocal microscope parameter settings are important to consider. Changing settings between images that are to be compared can result in inaccurate measurements. Specifically, laser power and PMT gain and offset are settings that will affect the FI values. In this study, differences in FI were observed from sample to sample, some with high background FI. This required PMT gain and offset to be adjusted to avoid overloading and quenching or underloading of the entire area, both which would lead to losing features in the images. PMT gain and offset are linear to FI and therefore correction of FI of images could be made. However, laser power is not linear to FI and correcting for different laser powers would be difficult. Therefore, it is crucial to maintain the same laser power for images to be compared quantitatively and preferably, all imaging should be done maintaining the same PMT gain and offset as well.
Autofluorescence is a common problem with fluorescence microscopy and is one of the main advantages of using a confocal microscope. The pinhole eliminates collection of scattered fluorescence and limits the sampling depth. Therefore, autofluorescence of the PU should be minimal. With confocal microscopy, differences in thickness of the PU should not affect the background fluorescence, since the PU thickness is beyond the sampling depth of the microscope. Typically, sections of 1 μm depth were taken. However, as shown by the histogram of the sac PU, PU autofluorescence was observed and created difficulty in quantitative analysis of platelet and fibrin adhesion.

Autofluorescence observed in retrieved sacs appeared to be from adhered biologic material in addition to PU autofluorescence already discussed. The background fluorescence in the positive control of platelets on sac PU was lower making the platelets easier to distinguish from background. The histogram of the positive control platelets on PU lacked the peak around 128 FI, which was observed in the two histograms for the retrieved sac samples. It is therefore important to use a retrieved sac sample as a negative control to determine the autofluorescence range for biologic material deposited in vivo.

Confocal imaging of the retrieved blood sacs provided a qualitative assessment of the surface densities of fibrin and platelet. Trends in fibrin and platelet adhesion dependent on sac region, implantation duration, and anticoagulation were observed. A simple sequence of image and data processing were established for future quantification of platelet adhesion from confocal images.
Chapter 5

Platelet Adhesion on Polyurethanes of Varied Surface Chemistry at Low Shear Stresses Relevant to Reduced-Sized LVAS Pumps

5.1 Abstract

Hemocompatibility of polymers of different chemistry was evaluated as candidate polymers for a pediatric pump. Platelet adhesion *in vitro* studies were performed on candidate polymers, Biospan MS/0.4 (n = 3) and Biospan-P (n = 4), for the shear stress range of 0 to 10 dynes/cm². A rotating disk system was used to apply shear stresses linearly increasing with radial distance along the polymer sample immersed in platelet rich plasma for 2 hours at 37°C and platelet adhesion was measured by fluorescence microscopy. Platelet activation in the bulk suspension was measured by flow cytometry.

For both Biospan MS/0.4 and Biospan-P, low platelet adhesion was observed between shear stresses ranging from 0 to 10 dynes/cm², except for regions with possible disturbed flow due to edge effects from the experimental setup. There was no significant difference in platelet adhesion between Biospan MS/0.4 and Biospan-P at all shear stresses except at 5.5 and 8.9 dynes/cm².
5.2 Introduction

Selection of a polymer with minimal thrombogenicity is a vital criterion to the development of a pediatric LVAS in which significantly lower shear stresses have been suggested to promote thrombosis. Development of pediatric pumps has been limited due to the low number of patients in need of such devices. However, the benefits to the few patients and their families have been recognized and research and development of pediatric LVAS are under way.\textsuperscript{59} Surface thrombosis reported in preliminary \textit{in vivo} studies with the pediatric pump are thought to be the result of the lower shear stresses in the pump.\textsuperscript{88} Therefore, the shear-dependent thrombogenicity of the polymers was investigated in this study.

A hydrophilic PEO surface PU, Biospan-P, was compared to PDMS-capped Biospan MS/0.4 for platelet adhesion and bulk suspension platelet activation in a shear stress-dependent manner. The thrombogenicity of the polymers for the pediatric LVAS were evaluated at the lower range of shear stresses encountered in the current model of the Penn State Pediatric Ventricular Assist Device (PVAD) being designed by the Division of Artificial Organs in the Department of Surgery at The Pennsylvania State University Milton S. Hershey Medical Center.\textsuperscript{59} This study was intended to evaluate Biospan-P as a candidate polymer for the Penn State PVAD to reduce thrombosis or to provide an alternate polymer with similar thrombogenicity but superior mechanical properties. The hemocompatibility of Biospan MS/0.4 and BioSpan-P were evaluated by measurement of platelet adhesion and activation.
5.2.1 Candidate Polymers of Varied Surface Chemistry

**Biospan MS/0.4**

In this study, the SPEUU Biospan MS/0.4 (Polymer Technology Corporation) was tested as the baseline polymer. Biospan MS/0.4 (Polymer Technology Group) is capped with 0.4 wt% PDMS. This polymer is a SPEUU similar to Biomer, and consists of methylene bis(p-phenyl isocyanate) (MDI), approximately 2000 molecular weight poly(tetramethylene oxide) (PTMO) and mixed diamine chain extenders, ethylene diamine (ED) and 1,3-cyclohexanediamine (1,3-CHD). The chain ends are capped with approximately 2000 molecular weight PDMS lending to 0.4 wt% of the copolymer. A methacrol additive is also present in this polymer. Biospan has been successfully used in many blood-contacting medical devices, particularly in LVAS such as the LionHeart (Arrow International Corporation). Studies have shown this polymer to be chemically stable and was FDA-approved as a Biomer replacement.

**Biospan-P**

Biospan-P with PEO is designed to provide a hydrophilic surface that is reported to reduce protein adsorption. Biospan is a PU with a soft segment of PTMO, a hard segment of MDI, and mixed diamines. The lot used in this study also contains Tinuvin 328 and 770 UV stabilizers. Studies have suggested that surface PEO/PEG significantly improve the hemocompatibility of biomaterials and theories on why it is effective at reducing thrombosis have been discussed.
5.2.2 Shear Stress Variation by Rotating Disk System

Hemocompatibility of the above polymers were investigated over a range of shear stresses relevant to LVAS, particularly pediatric LVAS. Well-defined shear stresses is obtained using a rotating disk system (RDS)\textsuperscript{122,123} illustrated in Figure 5.1. The RDS consists of a shaft to which a circular stainless steel mounting disk is attached at the end. The biomaterial sample is cut into a circle with the same diameter as the disk and is mounted onto the disk with double-sided tape. A motor rotates the shaft and disk at controlled speeds. Insertion of the rotating disk with the attached biomaterial sample into solution creates a defined range of shear stresses over the surface of the biomaterial mounted on the disk.

![Rotating Disk System Diagram](image)

**Figure 5.1.** Rotating disk system (RDS) setup provides linear increase in shear stress at the biomaterial surface with increasing outward radial distance and a constant particle flux in the medium across the surface.
Obtaining these well-defined and consistent fluid dynamics requires that certain conditions and assumptions be met. The equation for the surface shear stress, \( \tau_s \), is given as

\[
\tau_s = 0.8\eta x \sqrt{\frac{\omega^3}{\nu}} \quad \text{(Eq. 5.1)}
\]

where \( \nu \) is the kinematic viscosity of the medium, \( \omega \) is the angular velocity of the disk, and \( \eta \) is the medium viscosity. \( \tau_s \) (dynes/cm\(^2\)) increases linearly with radial distance \( x \) (cm) from the disk center. The three conditions that must be met for Equation 5.1 to be valid are:

1. The disk is of infinite size and rotating in an infinite medium.
2. Laminar flow exists in the boundary layer at the biomaterial surface.
3. Steady-state conditions apply.

The first condition can be met by setting the disk radius and separation between disk and medium much greater than the boundary layer thickness. The second condition is achieved by maintaining the Reynold’s number of the system below \( 10^5 \).\(^{124} \) The equation for Reynold’s number for the RDS is

\[
\mathcal{R} = \frac{\omega r^2}{\nu} \quad \text{(Eq. 5.2)}
\]

where \( \nu \) is the kinematic viscosity of the medium, \( \omega \) is the angular velocity of the disk, and \( r \) is the mounting disk radius. Steady-state required in the third condition can be met by achieving stable angular velocity of the RDS.

When all three conditions are met, in addition to obtaining shear stress increasing linearly outward with radial distance, particle flux in the medium across the disk surface
is constant. The particle flux is calculated for diffusion-controlled conditions by Equation 5.3 as

\[
    j = \frac{0.62 D^{3/4} \omega^{3/4} C_\infty}{\nu^{1/4}}
\]

(Eq. 5.3)

where \( D \) is the diffusivity of particles in medium and \( C_\infty \) is the bulk particle concentration in solution.

Therefore, given that all three conditions mentioned are met, the RDS setup provides constant platelet flux and linear shear stress increase with disk diameter, making the system ideal for investigation of platelet adhesion at different shear stresses on various biomaterials.

### 5.2.3 Platelet Activation in Bulk Suspension

Activation of platelets may occur upon contact of platelets with a biomaterial surface. These platelets may adhere to the surface or they may remain in suspension. Activated platelets in suspension may adhere to surfaces in other locations of the bloodstream, then aggregate, and adhere to a vessel to form a thrombus or lead to an embolus. Activated platelets also release numerous activators for proteins in the coagulation cascade that will lead to coagulation and fibrin formation. Therefore, assessment of activation of platelets in bulk suspension due to contact with the polymer samples is important to consider. This is commonly quantified by flow cytometry.
5.3 Materials and Methods

5.3.1 Biomaterial Sample Fabrication

In order to assess the two polymers discussed, the polymer samples were prepared following the pediatric blood sac fabrication protocol developed by the Division of Artificial Organs in the Department of Surgery at The Pennsylvania State University. Sheets of Biospan MS/0.4 and Biospan-P, each 4¼" by 3", were prepared using a protocol similar to that used for blood sac fabrication, as illustrated in the flow chart in Figure 5.2. Glass slides were coated with low molecular weight polyethylene (LMWPE). To create a smooth surface, the slides were then coated with PDMS. Slides were then dipped in Biospan MS/0.4 or Biospan-P several times to achieve a polymer thickness of approximately 254 µm (corresponding to 10/1000 inches). Once dry, the polymer edge of the slide was cut and the LMWPE was melted out to release the Biospan samples. Once released, the Biospan samples were cured in the oven. From the Biospan rectangular samples, eight 2 cm diameter circular samples were obtained. The polymer samples were cut out using a sharpened stainless steel punch.

The surfaces of the Biospan MS/0.4 and Biospan-P samples prepared for RDS were imaged by atomic force microscopy (AFM) in tapping mode. The side removed from PDMS, the side to be exposed to the platelets was placed face up on an AFM stub with double-sided tape. One sample of each polymer was cleaned of loose debris with a stream of nitrogen gas prior to imaging. AFM imaging was performed on a Digital Instruments Nanoscope III Multimode AFM equipped with a ‘J’ scanner using silicon
probe tips. Several 100 μm x 100 μm and 50 μm x 50 μm images were taken. AFM images were processed using first order flattening and plane fitting.
Figure 5.2. Flow chart describing polymer fabrication process for disc samples used for RDS platelet adhesion studies.
5.3.2 PRP Preparation

In order to investigate platelet adhesion to the biomaterial surface and platelet activation of the bulk suspension, bovine platelet-rich plasma (PRP) was used. Fresh bovine blood was drawn from a healthy Holstein calf into a blood transfer bag with 3 U/ml of heparin anticoagulant. The anticoagulant acts to inhibit fibrin formation by inhibiting thrombin action and does not affect platelet activity. The blood was centrifuged at 600 x g for 20 min to obtain PRP supernatant. The PRP was transferred to a separate vial. The remaining blood was centrifuged at 1500 x g for 20 min to collect the platelet-poor plasma (PPP) supernatant. A sample of the PRP was sent to the clinical hematology lab at the Milton S. Hershey Medical Center for a platelet count. Once the platelet concentration of the PRP was obtained, PPP was used to dilute the PRP to a physiological concentration of 2.5 x 10^5 platelets/μl. 50 ml of the physiological concentration PRP was added to each of two 100 ml polytetrafluoroethylene (PTFE) beakers with internal diameter of 3.8 cm, resulting in a PRP medium of 2.76 cm depth. One beaker was used as a static control and the other was for the experimental RDS sample.

5.3.3 RDS Experiments

The PU sample prepared in Section 5.3.1 was mounted on the RDS stainless steel disk with tape also cut into a 2 cm diameter circle. The disk was screwed onto the Teflon rod of the RDS attached to the rotating shaft, and placed into deionized water overnight at room temperature to remove low molecular weight contaminants at the polymer surface.
The day of the RDS experiment, the sample was immersed in PBS at 37°C for 1 hr to equilibrate the sample. At the same time, the two beakers containing 50 ml PRP were incubated at 37°C for 1 hr.

To perform the experiment at shear stresses relevant to the PVAD, a shear stress range of 0-10 dynes/cm² was selected. In order to obtain this shear range and to satisfy the conditions discussed previously, the following experimental parameters were calculated. To develop a shear stress of 10 dynes/cm² at the 9 mm radius of the disk, an angular velocity of 238 rpm was required based on Equation 5.1. Detailed calculations are included in Appendix B. By using this experimental setup, the boundary layer thickness was calculated as 776 μm. This was almost 13 times less than the 1 cm radius of the rotating disk and was approximated as infinitely smaller than the dimensions of the 100 ml PTFE beakers and PRP medium. The first condition of an infinite system was therefore met. Under these parameters, the Reynold’s number was calculated to be 2150, far below the required 10⁵ for laminar flow in the boundary layer. In addition, the largest particles in the PRP, the platelets, have a diameter of approximately 2 μm, which was considerably less than the boundary layer thickness. This ensured that the laminar flow would not be disturbed by the particles in the medium. The third condition of steady state was achieved in this study by using the AFMSRX Analytical Rotator (Pine Instrument Company, Grove City, PA). This instrument provides full speeds up to 1000 rpm in 4 msec and has a velocity variation of ±1%. The duration of the experiments was 2 hrs allowing ample time for stable angular velocity to be achieved.

With experimental conditions calculated to meet the conditions necessary for steady state laminar flow for shear stresses from 0 to 10 dynes/cm², the polymer sample
mounted on the RDS was transferred from the PBS into the experimental RDS beaker of 50 ml PRP. The disk was lowered into the PRP until the polymer sample was immersed in PRP with minimal PRP contact with the surface of the stainless steel disk. The RDS motor was turned on to 238 rpm. After the desired time, the motor was stopped. The sample was rinsed by exchanging the PRP with PBS volume 5 times that of the PRP, aspirating the PRP while adding PBS with a 25 ml pipette. The PBS was then replaced in the same manner with 2 times volume of 1% PFA, and fixed for 1 hr at room temperature. The sample was then rinsed with 2 times volume of PBS.

5.3.4 Platelet Adhesion Measurement

Platelet adhesion to the RDS polymer samples was quantified using indirect immunofluorescent labeling. The antibodies selected for labeling platelets adhered to the biomaterial surface recognize the $\alpha_{\text{IIb}}$ chain of the $\alpha_{\text{IIb}}\beta_3$ integrin (also known as the glycoprotein GPIIb/IIIa) present in abundance on all platelets, and P-selectin, which is exposed to the outer surface of the platelet membrane upon platelet activation. These two antibodies were used together and labeled with the same fluorophore, phycoerythrin (PE) to ensure all platelets were identified and fluorescent signal was strong enough for visual detection.

The disk was unscrewed from the RDS shaft and placed in the well of a 6-well tissue culture polystyrene plate. A primary antibody solution was prepared with

1. 1 ml of 6% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) as blocking agent
2. 1.5 μl of mouse anti-bovine αIIBβ3 antibody (VMRD, Pullman, MA; 1mg/ml)

3. 10 μl of polyclonal rabbit anti-human CD62P antibody (Becton Dickinson, Franklin Lakes, NJ; 0.1mg/ml)

The PU sample on the stub was covered with 1 ml of the primary antibody solution and incubated overnight at 4°C. The sample was rinsed six times, filling the well with PBS each time. The sample was left covered with PBS for 10 min and the rinsing process was repeated. A secondary antibody solution was prepared with the following:

1. 1 ml of 6% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) as blocking agent

2. 10 μl of donkey anti-mouse-PE conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA; 0.5 mg/ml)

3. 10 μl of donkey anti-rabbit-PE conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA; 0.5 mg/ml PE)

The PU sample on the stub was covered with 1 ml of the secondary antibody solution and incubated in dark at room temperature for 1 hr. The sample was rinsed with PBS as described for the primary antibody solution. The sample was placed on a microscope slide. 50 μl of Gel/Mount® antifade solution (Biømeda, Foster City, CA) was placed on the sample and a coverslip was placed on the sample. The slides were stored at 4°C protected from light until imaged.

Imaging of platelet adhesion on PU samples was performed on a Nikon Optiphot 3 fluorescence microscope with 4x and 100x objectives. Black and white images were captured with the 100x objective (1000x total magnification) using a Diagnostic
Instruments Spot camera. Scribes of circles and arcs on the mounting disk below the PU sample were used to guide areas on the PU to be imaged for platelets. Using the 4x objective, an area in the center of the stub where the arcs intersect was selected for imaging. With the 100x objective, the area was imaged for platelets using the PE-filter on the microscope. In the center, six images, each next to the other, were collected. Images were also taken along each arc, at the intersection of the arc with each of the concentric circles, shown in Figure 5.3.

![Diagram](image_url)

**Figure 5.3.** Markings on the mounting disk were used in measurement of platelet adhesion to the mounted biomaterial sample. Platelet adhesion was measured at the intersection of the circles that were 1 mm apart and the lines that were 60° apart.

Analysis of the number of platelets was performed by counting the number of platelets in each image captured for the different radii of the PU sample. The number of platelets and the area of the field of each image were used with experimental parameters to calculate the adhesion coefficient (AC) as described by Equation 5.4. AC is the ratio of the number of platelets adhered to a unit area of PU surface to the number of platelets that are transported to the PU surface throughout the experiment. The number of platelets transported to the PU surface is given by the product of the platelet flux, \( j \) (platelets/sec/mm²) and the experiment duration, \( t \) (sec). \( N \) is the number of platelets/mm².
5.3.5 Flow Cytometry: Sample Collection, Immunolabeling, and Analysis

Flow cytometry samples were collected before and after running the RDS. Just prior to lowering the RDS polymer sample into the RDS beaker of PRP as discussed in Section 5.3.3, flow cytometry samples were collected for the time \( t = 0 \) from each the static and RDS beakers of PRP. To each of two 1.5 ml eppendorf tubes 0.5 ml of 1% PFA was added. To one tube, 0.5 ml of PRP from the static beaker was added and to the other tube, 0.5 ml of PRP from the RDS beaker was added. A positive control was also prepared by mixing 0.25 ml of PRP from the static beaker with 0.25 ml collagen (Becton Dickinson, Franklin Lakes, NJ) in a 1.5 ml eppendorf tube to activate the platelets. After at least 30 min up to 75 min 0.5 ml of 1% PFA was added to the positive control. Once the RDS was stopped after 2 hrs, 0.5 ml samples of PRP were taken from each static and RDS beakers and added to 0.5 ml of 1% PFA each in 1.5 ml tubes.

The assay for immunolabeling platelets for flow cytometry is shown as a flow chart in Figure 5.4. Each of the five 1.5 ml eppendorf tubes containing platelet samples collected during the RDS experiment were used to prepare 4 samples for flow cytometry: Control, Platelet, Activated Platelet, and Platelets and Activated State. Twenty 1.5 ml eppendorf tubes were labeled for each of these samples for each platelet sample: Static Before, Static After, RDS Before, RDS After, and Collagen 1 hr. Primary antibody solutions to label platelets and activated platelets were prepared as follows:

\[
AC(\%) = 100 \frac{N}{jt}
\]  
(Eq. 5.4)
Solution 1: Labels all platelets. 250 μl of 6% normal donkey serum and 0.75 μl of mouse anti-bovine α_{IIb}β_{3} antibody (VMRD, Pullman, MA; 1 mg/ml)

Solution 2: Labels activated platelets. 250 μl of 6% normal donkey serum and 5 μl of polyclonal rabbit anti-human CD62P antibody (Becton Dickinson, Franklin Lakes, NJ; 0.1 mg/ml)

A 25 μl aliquot of PRP from each PRP sample was placed in each of four tubes. A 25 μl aliquot of primary antibody solution was added to each appropriate tube of PRP. No antibodies were added to the control samples. To the 'Platelets and Activated State' samples, both primary antibody solutions 1 and 2 were added. Samples were incubated at room temperature for 1 hr. Secondary antibody solutions were prepared as follows:

Solution 3: Labels primary antibody for all platelets. 500 μl of 6% normal donkey serum and 10 μl of FITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 1.4 mg/ml)

Solution 4: Labels primary antibody for activated platelets. 500 μl of 6% normal donkey serum and 10 μl of PE-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 0.5 mg/ml PE)

Aliquots of 50 μl of secondary antibody solution were then added to the appropriate tubes of PRP. Solution 3 was added to tubes with solution 1 and solution 4 was added to tubes with solution 2. Samples were incubated in the dark for 30 min. Twenty flow cytometry tubes were labeled for the 20 samples of PRP prepared. 1.9 ml of PBS was added to each tube. To each eppendorf tube, 0.9 ml of PBS was added and 100
μl of sample was then transferred to the appropriately labeled flow cytometry tube. Flow cytometry data acquisition and analysis was performed.

Flow cytometry was performed on all 20 samples setting detectors to measure FITC and PE fluorophores, and to gate only platelets. 10,000 platelets were analyzed for each sample. The Control, Platelet, and Activated Platelet samples were used as controls to set gates for the 'Platelets and Activated State' sample. Flow cytometry analysis provided the percentage of platelets that were activated. The collagen-activated sample served as a positive control to confirm labeling and detection of activated platelets.

5.3.6 Statistical Analysis

RDS experiments were performed for each polymer, Biospan MS/0.4 (n = 3) and Biospan-P (n = 4). For each experiment, six measurements of platelet adhesion were made at each shear stress, resulting in 18 values of adhesion coefficient for each shear stress value. Values for all shear stress values for the different polymers were plotted. Flow cytometry was performed in each experiment to obtain a percent platelet activation of the bulk PRP for static and RDS samples. Statistical analysis was performed using the Mann-Whitney test for comparison of platelet AC% for the two polymers at each shear stress and ANOVA for platelet AC% at the different shear stresses for each polymer. A p-value less than 0.05 was taken as indicative of significant difference. For platelet activation of the bulk PRP, a paired t-test was used to compare the Static samples to the RDS samples with p < 0.05 indicating significant differences.
Figure 5.4. Flowchart of assay for indirect immunofluorescent labeling of bovine platelets and activated platelets for flow cytometry.
5.4 Results

5.4.1 Biomaterial Surface Topography

The surface topography of Biospan MS/0.4 and Biospan-P imaged by AFM was different for the two polymers (Figure 5.5). The polymers were prepared following the same blood sac fabrication protocol but the surfaces appeared distinct. Biospan MS/0.4 had pits of various sizes from 1 μm to over 10 μm in diameter. On the other hand, Biospan-P was free of pits but instead had some raised, elongated structures.
Figure 5.5. Surface topography of Biospan MS/0.4 (A) and Biospan-P (B) RDS samples. Pits of varied sizes were prevalent on Biospan MS/0.4 but absent on Biospan-P. Raised, elongated structures were present only on Biospan-P samples (100 μm x 100 μm images, 2500 nm height scales).
5.4.2 Platelet Adhesion

Platelet adhesion in vitro studies have been performed on candidate polymers, Biospan MS/0.4 and Biospan-P, for the shear stress range of 0 to 10 dynes/cm², using a rotating disk system. Platelet adhesion was minimal at the lower shear stresses up to at least 4.4 dynes/cm², with generally 0 – 4 platelets on each 98 μm by 73 μm image (Figure 5.6). Figure 5.7 shows the platelet adhesion coefficients (AC%) for Biospan MS/0.4 (n = 3) and Biospan-P (n = 4) samples, run on a rotating disk system for 2 hours at 37°C and imaged by immunofluorescence microscopy for platelets as we have described previously. For both Biospan MS/0.4 and Biospan-P, no significant difference in platelet adhesion was observed between the different shear stresses ranging from 0 to 10 dynes/cm². There was no significant difference in platelet adhesion between Biospan MS/0.4 and Biospan-P at all shear stresses except at 5.5 and 8.9 dynes/cm². The higher platelet AC% and larger error bars for Biospan-P arise from two of the four samples that had higher platelet AC%. The increase and variability in platelet adhesion at the higher shear stresses may be due to edge effects. Current results show low platelet adhesion on Biospan MS/0.4 and Biospan-P at low shear stresses, 0 to 10 dynes/cm² (except in regions of potential edge effect).
Figure 5.6. Low platelet adhesion to Biospan-P at 0 dynes/cm$^2$. Platelet adhesion was typically from 0-4 platelets in the 98 μm by 73 μm images at the lower shear stresses on both Biospan MS/0.4 and Biospan-P (10 μm scale bar).

Figure 5.7. Platelet adhesion (percentage of adhesion coefficient) on Biospan MS/0.4 (n = 3) and Biospan-P (n = 4) for the shear stress range 0 to 10 dynes/cm$^2$. The higher platelet AC% and larger error bars for Biospan-P arise from two of the four samples that had higher platelet AC%. A greater increase in AC% is observed above 5.5 dynes/cm$^2$ that we believe to be edge effects. Most samples for Biospan MS/0.4 and Biospan-P had similarly low values of platelet adhesion. □ Biospan MS/0.4  ● Biospan-P
Variation in AC% data at the higher shear stresses above 4.4 dynes/cm² for Biospan-P may be explained by high platelet adhesion on two Biospan-P samples shown in Figure 5.8. These same two samples, samples 3 and 4, are also responsible for the difference in the shear stress above which increased platelet adhesion is observed for the averages of each Biospan MS/0.4 and Biospan-P. Edge effects seem to occur above 7.8 dynes/cm² for Biospan MS/0.4 with 0.1% or less AC% for shear stresses from 0 to 7.8 dynes/cm². For the average of Biospan-P samples, 0.1% or less AC% was only measured from 0 to 4.4 dynes/cm². The increased platelet adhesion beyond 4.4 dynes/cm² may not be an edge effect but rather due to particularly high platelet adhesion on two Biospan-P samples. As seen in Figure 5.8 with the exception of Biospan-P samples 3 and 4, platelet adhesion does not increase until 7.8 dynes/cm² or higher, similar to Biospan MS/0.4 samples. Therefore, the increased platelet adhesion does not occur until the outer 2 mm of the 10 mm diameter sample at 7.8 dynes/cm² and above.
Platelet integrin binding to different proteins on the biomaterial surface is affected by shear stress as discussed in Section 1.4.4. Platelet adhesion to a biomaterial surface via binding to fibrinogen decreases with increase shear rate and is minimal at shear rates of 1500 s⁻¹. vWF binding to platelets on the other hand increases with increased shear rate. Although platelet binding to fibrinogen is minimal at 1500 s⁻¹ and platelet binding to vWF decreases with decreasing shear rates, at 50 s⁻¹ platelet adhesion via vWF is within the same range as adhesion via fibrinogen. The shear stresses applied in the RDS studies presented here were equal to shear rates from 0 s⁻¹ to 833 s⁻¹ (assuming viscosity of the PRP solution to be the same as that of plasma, 0.012 centistokes). Within the range of shear rates applied in these RDS experiments, based on the literature both platelet adhesion to the biomaterial surface may be mediated by fibrinogen and vWF.

Figure 5.8. Platelet AC% data for all experiments shows one or two Biospan-P samples with very different platelet AC% values. Platelet adhesion is particularly higher for Biospan-P samples 3 and 4 compared to all other Biospan MS/0.4 and Biospan-P samples above 4.4 dynes/cm². With the exception of these two samples, AC% for Biospan MS/0.4 and Biospan-P are very similar.
Savage et al. reported efficient platelet binding to fibrinogen up to 600-900 s\(^{-1}\).\(^{73}\) At the higher shear rates, at 667 s\(^{-1}\) (7.8 dynes/cm\(^2\)) and greater vWF may be the dominant protein mediating platelet adhesion. The increased platelet adhesion at these higher shear rates or shear stresses could be related to the protein-platelet interaction, although similar studies by Milner et al. at higher shear stresses up to 67 dynes/cm\(^2\) do not show increased platelet adhesion around 7.8 –10 dynes/cm\(^2\).\(^{44}\) Therefore, edge effects remains as the more likely cause of the increased platelet adhesion towards the outer edge of the samples.

### 5.4.3 Platelet Activation in Bulk Suspension

Platelet activation measured by flow cytometry was not significantly different between the control Static and the RDS samples for all experiments (Table 5-1). Percentage of platelet activation was calculated as the percentage of particles that labeled positive for platelets that also labeled for the integrin CD62P (only present on activated platelets). Differences between before and after running the RDS for 2 hrs was less than 1.36% in all cases, both Static and RDS, except for in the first experiment for Biospan MS/0.4. The Static Before sample had a false high reading due to an error in data acquisition. For Biospan-P, two RDS experiments were run simultaneously and therefore only one Static sample was prepared and used for Static flow cytometry samples, as seen in the repetition of Biospan-P Static values in Table 5-1. Neither Biospan MS/0.4 nor Biospan-P seemed to affect platelet activation of the bulk suspension.
5.5 Discussion

Differences in surface topography of Biospan MS/0.4 and Biospan-P may have affected platelet adhesion and activation. Both surfaces were expected to be smooth. The sources of the features observed are not clear so far. Abraham et al. also observed similar pitting on a series of Biospan and contribute the pits to leaching of an additive methacrol. The features on Biospan-P have not been observed previously and remain unidentified. Although the effects of these topographies are unknown, based on the

<table>
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<tr>
<th></th>
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<th>Platelet Activation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(% Total Platelets Sampled)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>1</td>
<td>Static</td>
<td>15.16%</td>
<td>1.83%</td>
</tr>
<tr>
<td>1</td>
<td>RDS</td>
<td>1.77%</td>
<td>2.31%</td>
</tr>
<tr>
<td>2</td>
<td>Static</td>
<td>2.03%</td>
<td>2.76%</td>
</tr>
<tr>
<td>2</td>
<td>RDS</td>
<td>2.12%</td>
<td>2.47%</td>
</tr>
<tr>
<td>3</td>
<td>Static</td>
<td>1.64%</td>
<td>1.64%</td>
</tr>
<tr>
<td>3</td>
<td>RDS</td>
<td>2.06%</td>
<td>2.15%</td>
</tr>
</tbody>
</table>

Table 5-1. Platelet activation in bulk PRP suspension due to biomaterial contact or shear stresses. *False high value due to data acquisition error
platelet adhesion and activation results, when following the described blood sac fabrication protocol, platelet adhesion and activation are similar.

The increase in platelet adhesion towards the outer edges of the RDS samples of both Biospan MS/0.4 and Biospan-P are thought to be edge effects. The polymer edges are not completely smooth and this is believed to create turbulence, altering the expected fluid dynamics. A similar trend of increased AC% towards the edges has been reported. Edge effects on AC% measurements at those shear stresses were obtained in this previous study by increasing the rotational speed of the RDS, thus increasing the range of shear stresses along the biomaterial sample. By doing this, the shear stresses that occurred at the outer edge will be closer in to the center of the sample, away from edge effects. Another set of experiments with a larger range of shear stresses was not performed for this study since the increased platelet adhesion occurred below approximately 5 dynes/cm² and remained constant above this up to 67 dynes/cm². A lower range of 0-10 dynes/cm² was necessary to obtain greater sensitivity to shear stress, with AC% measurements about every 1 dynes/cm².

The increased platelet adhesion towards the edges of the samples was greater for the average for Biospan-P samples than for Biospan MS/0.4. The Biospan-P samples were run in pairs when an additional RDS rotor and shaft became available. Thus the four samples run were done as two experiments. The increased platelet adhesion towards the outer half of the Biospan-P samples was seen mostly in two of the four samples, which were run together from the same blood. Although each sample is run separately in individual beakers, they are run with the same blood source. The data suggests additional
experiments would be beneficial in determining whether the increased platelet adhesion at higher shear stresses on Biospan-P are associated with edge effect or the polymer.

Measurements of platelet activation in the bulk PRP suspension showed no significant change with exposure to the shear stresses of the RDS. However, the number of platelets sampled by flow cytometry was a minute portion of the total platelets in the PRP in the RDS experiments. Therefore, if platelets were activated due to shear stresses from the RDS, it is not likely to be observed in the flow cytometry data. Increases in platelet activation measured by flow cytometry are more likely to be caused by platelets being in contact with the beakers over a time period of two hours during the RDS experiments. This is suggested based on the increased platelet activation in the samples after the 2 hours compared to before, in both static and RDS samples, observed in some experiments.

Although Biospan MS/0.4 and Biospan-P samples were compared in this study, the actual surface chemistry the platelets encountered may not have been different between the samples as was expected. The study was intended to compare two different chemistries, a hydrophobic silicone-based polyurethane and a hydrophilic PEO surface. However, due to the sac fabrication protocol used for preparing the RDS samples, it is possible that both Biospan MS/0.4 and Biospan-P were coated with a layer of PDMS, masking the chemical differences between the two polymers at the surface. Milner et al. performed a similar platelet study with Biospan MS/0.4 but reported higher AC% values than those in this study. A trend of increasing AC% with decreasing shear stresses < 5 dynes/cm² was also reported. One crucial difference in methods between the study by Milner et al. and this study is the protocol for sample fabrication. This may be the
reason for the discrepancies in platelet adhesion. Further studies would be required to confirm the surface chemistry of the same Biospan MS/0.4 prepared by different methods. For the purpose of this study, the sac fabrication protocol used to prepare RDS samples is current protocol for production of blood sacs, and therefore is the appropriate method of RDS sample preparation. If as suggested, after applying the sac fabrication protocol the surface chemistry is the same regardless of the polymer used, a different sac fabrication protocol should be selected to evaluate hemocompatibility of various surface chemistries. However, this is beyond the scope of this study.
Chapter 6
Discussion and Conclusions

Studies were conducted to evaluate the effects of implant duration, anticoagulation, blood sac region, and biomaterial chemistry on thrombosis at the biomaterial surface. *In vivo* studies involved implantation of LVAS into calves for 3 and 30 days, n = 3, 4 and 4, 5, with and without anticoagulation, respectively. Surface thrombosis was assessed on the PU blood sacs of LVAS by macroscale examination, SEM, and confocal microscopy. Platelet adhesion to two polymers of different chemistry and activation of bulk platelet suspension were assessed *in vitro* by immunofluorescence techniques over a range of low shear stresses relevant to pediatric cardiac assist pumps.

6.1 *In Vivo* Studies of Surface Thrombosis in LVAS

6.1.1 Summary of Macroscale and Microscale Surface Thrombosis Studies

6.1.1.1 Macroscale Surface Thrombosis

At the macroscale, each of the 3-day study sacs had numerous thrombi throughout the sac whereas in the 30-day study, only one sac from each of the anticoagulated and nonanticoagulated groups had numerous thrombi. This difference in macroscopic surface
thrombosis with implant duration is also reflected in the higher sac surface coverage by macroscopic thrombi in the 3-day study compared to the 30-day study (Table 6-1). These values also suggest that anticoagulation had no effect on surface thrombosis at the macroscale. The results suggested that no specific region of the blood sac was more prone to macroscale thrombosis in either 3-day or 30-day study (Figure 6.1).

Table 6-1. Average percentage of total sac surface covered by macroscale surface thrombi.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Average % Sac Surface Coverage by Macroscale Thrombi</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Anticoagulated</td>
</tr>
<tr>
<td>30-Day</td>
<td>0.23 ± 0.38%</td>
</tr>
<tr>
<td>3-Day</td>
<td>0.42 ± 0.24%</td>
</tr>
</tbody>
</table>
6.1.1.2 SEM Analysis of Microscale Topography and Surface Thrombosis

Microscale surface thrombosis was evaluated by imaging the sac topography by SEM and showed differences with implantation duration, anticoagulation, and blood sac region. The platelet-like structures observed on the sacs were different for the 3-day and 30-day sacs (Figure 6.2). The 30-day sacs had circular structures resembling platelets with diameter of approximately 3.5 to 4 μm, similar to that of a platelet, which has a diameter of 1.5 to 4 μm. The 3-day sac surfaces, on the other hand, had microparticles

Figure 6.1. Percentage of sac surface coverage by macroscopic surface thrombi in the 30-day and 3-day studies, anticoagulated and nonanticoagulated groups. 3-day sacs had higher surface coverage by macroscale thrombi than 30-day sacs. No sac region was particularly prone to surface thrombosis in both 3-day and 30-day studies.
approximately 400 nm in diameter, which is within the range of sizes of platelet-derived microparticles reported in the literature.\textsuperscript{113,114}

Figure 6.2. Different structures were observed for sacs implanted for 30 days (A) versus 3 days (B). Platelet-sized circular structures (approximately 4 μm in diameter) were adhered to 30-day sac surfaces whereas microparticles approximately 400 nm in diameter were present on the 3-day sacs.

Anticoagulation reduced adhesion of the platelet-like structures but not the fibrillar structures in the 30-day study. In the 3-day study, anticoagulation appeared to have no effect on any of the structures observed on the sacs. However, in both studies the inlets and outlets, known to have higher shear stresses than other regions in the sac, had fibrillar structures that were rarely observed in all other regions. The platelet-like structures in the 30-day and the 400 nm particles in the 3-day study were primarily observed in all regions except the inlet and outlet (although the inlet of the anticoagulated group had a high density of the 400 nm particles).
6.1.1.3 Confocal Microscopy Analysis of Platelet and Fibrin Adhesion

Confocal microscopy images analyzed for platelets and fibrin showed greater fibrin and platelet adhesion in the 30-day blood sacs. The 30-day blood sacs had a higher surface density of large particles and small fibrin clots compared to the 3-day sacs. On the other hand, the 3-day nonanticoagulated sacs had the highest density of platelet-derived microparticles. The anticoagulated 3-day sacs had few microparticles. Platelet and fibrin adhesion surface densities also appeared to be related to sac regions. The top and bottom regions differed most from the other six regions of the sac. Platelet adhesion was higher in the top and bottom regions of both 3-day and 30-day studies, both anticoagulated and nonanticoagulated. Large particles with platelets and fibrin were adhered at high densities in the top and bottom regions, particularly in the 30-day study.

6.1.2 Surface Features: SEM and Confocal Microscopy

The fibrillar structures seen in the inlets and outlets of blood sacs imaged by SEM were initially believed to be fibrin due to their resemblance in structure to positive controls of fibrin. This feature was not observed in the negative controls of samples from blood sacs that were never in contact with biologic material, with the exception of one area on one image out of 102 images taken from seventeen samples from the various regions of two blood sacs. The absence of the fibrillar feature in negative controls suggested that the feature was biologic. Another possibility is that it is a polymeric structure resulting from blood flow. While the negative controls were never in contact with blood, they also were not exposed to fluid flow, to which the implanted blood sacs
are exposed. Therefore, the effect of fluid flow on the blood sac surface in the different regions of the sac was not evaluated. The theory that the fibrillar feature observed by SEM is polymeric is supported by the absence of such a feature in the confocal samples labeled for fibrinogen and therefore for fibrin. SEM and confocal images suggest that this fibrillar structure is polymeric and does not affect thrombosis at the surface.

At the same time, the fibrin strand features observed by confocal microscopy were not seen in the SEM images. This is likely due to the low contrast in surface topography for SEM between the fibrin strands and the material below the fibrin strands (which may be a layer of protein or fibrin). Difficulty in assessment of fibrin adhesion was encountered in both SEM and confocal imaging. Limitations to topographical and structural differences for determining features in the SEM images have been discussed. In confocal images, there is no distinction between labeling of fibrinogen and fibrin. Therefore, structure was used to distinguish fibrinogen from fibrin. By relying on structure to assess fibrin adhesion layers of fibrin with no clear strands of fibrin may have been overlooked as fibrinogen layers. This would explain the higher density of fibrin strands in the anticoagulated blood sacs compared to the nonanticoagulated sacs. The anticoagulated sacs may have less fibrin adhesion and therefore strands of fibrin may be easier to observe compared to an entire layer of fibrin. Further z-scale analysis of the blood sacs may help understanding or clarification of fibrin adhesion in the blood sacs but this would require focused imaging of fibrin and carefully selected z-scale imaging, both of which are beyond the scope of this research.

Although the platelet-like structures were absent in the 3-day sacs imaged by SEM, platelets were present on these sacs at only a slightly reduced density than on the
30-day sacs when imaged by confocal microscopy. This difference may have resulted from some platelets being undetected in SEM analysis. The reason for platelets being undetected by SEM only in the 3-day sacs may be associated with the level of platelet spreading. Spread platelets will have less height and therefore would be more difficult to detect by SEM. Discrepancy between SEM and confocal microscopy results were also seen for fibrin adhesion.

Pits were frequently observed in SEM images and although they were not the focus of this research they must be addressed. The pits observed in SEM images were also present on negative control samples that were never implanted or in contact with biologic material. Therefore, the pits are a feature of the polymer that may be associated with the manufacturing process. The cause of these pits is unknown and may be any of the following: 1) degradation,\textsuperscript{107} 2) leaching of the methacrol additive in the polymer,\textsuperscript{125} or 3) air bubbles in the polymer. The biologic relevance of these pits, whether or not these pits encourage adhesion of platelets and thrombosis, is unclear and is beyond the scope of the research presented. Attraction of platelets or fibrin to the pits was not obvious from the SEM images, although occasional adhesion of what appeared to be a platelet or at times a thrombus occurred over pits. However, if the pits were covered by biologic deposition, it would not be possible to know this by looking at an SEM image. In the confocal images, fibrin or fibrinogen appeared to adhere around spread platelets within the range of the size of the pits observed by SEM. It is possible that these platelets are adhered over the pits and the fibrin or fibrinogen adheres around the pits but this has not been confirmed and further investigation would be necessary.
Future work of simultaneous imaging of the polymer topography and functional imaging of fibrin and platelets would help determine if there is any effect of the pits on thrombosis. Real-time imaging of labeled platelets and fibrinogen passing over pitted regions of the polymer may be a valuable experiment to address this question regarding the biologic relevance of the pits.

6.1.3 Comparison of Macroscale, SEM, and Confocal Microscopy Results in Relation to Implant Duration, Anticoagulation, and Blood Sac Region

6.1.3.1 Relationship between Implant Duration and Surface Thrombosis

The relationship between surface thrombosis and implant duration observed at the macroscale, by SEM, and confocal microscopy suggested more macroscale thrombi but lower microscale surface coverage by platelets and fibrin, after implantation for 3 days compared to after 30 days. The difference between macroscale and microscale surface thrombosis in relation to implant duration may be explained by embolization or thrombolysis. It is possible that the decreased macroscale surface thrombosis in the 30-day study is due to the large thrombi being released, embolized, from the biomaterial surface after long-term implantation. If this were the case, the emboli may obstruct vessels and cause tissue death and infarcts. The effects of emboli are often seen in the kidneys of the calves implanted with LVAS. However, the kidney infarct results shown in Appendix A do not show a higher score for kidney infarcts in the 30-day study compared to the 3-day study. This does not necessarily mean that macroscale thrombi recorded in the 3-day study were not released from the surface as emboli. It is possible
that the emboli were not large enough to occlude the renal arteries and arterioles or that
dissolution of the emboli occurred before entering the kidneys. Although it is the
macroscale thrombi that may embolize, occlude vessels, and cause infarcts, microscale
thrombosis can be equally important since increased activation of coagulation factors and
platelets at the microscale are what lead to macroscale thrombi.

Results supporting a time-dependent change in the platelet response in LVAS
include decreased platelet counts, thrombocytopenia, observed in the first few
postoperative days (Appendix A). Thrombocytopenia results from platelets being
removed from circulation faster than they are being replaced from bone marrow where
they are produced. Therefore, it is caused by either failure to produce platelets or
increased rate of removal of platelets from the blood. If platelet-derived microparticles
were formed from the platelets in suspension in blood during the first three days or so
postoperatively a decrease in platelet count would be expected. The platelet count data
showing decreased platelet concentrations at 3 days and normal platelet counts at 30 days
(Appendix A) does support the SEM and confocal microscopy results showing adhesion
of microparticles on the 3-day sac surfaces and not on the 30-day sacs. Platelet count
tests unfortunately do not detect or measure platelet-derived microparticles. As
mentioned previously, platelet-derived microparticles do not have to form in suspension
but may also form from platelets adhered to a surface as well.114

Surface density of small fibrin clots and large thrombi was greater in the 30-day
study than in the 3-day study in the confocal microscopy analysis. These results were not
comparable to the SEM results. The only fibrin-like structures observed by SEM were
observed in both 30- and 3-day studies with no difference in surface density. Small fibrin
clots and fibrin in large thrombi observed by confocal microscopy was undetected in SEM analysis. As discussed in Section 6.1.2, the fibrin features observed by confocal microscopy may have been considered platelets in the SEM analysis. Again, discussion of differences in features observed by SEM and confocal microscopy were discussed in further detail in Section 6.1.2.

6.1.3.2 Relationship between Anticoagulation and Surface Thrombosis

Anticoagulation had no effect on surface thrombosis at the macroscale. At the microscale, SEM results suggest a decrease in adhesion of platelet-like features and no difference in adhesion of microparticles with anticoagulation. Confocal microscopy qualitative analysis resulted in contradicting conclusions. Surface densities of platelets as well as small fibrin clots (which may be the same as the features referred to as ‘platelet-like’ features in SEM images) were not consistently lower for the average of the anticoagulated group in the 30- or the 3-day study compared to the nonanticoagulated group. These results suggest the anticoagulation treatment in these studies does not reduce fibrin or platelet adhesion at the biomaterial surface, with the exception of reduction of platelet-derived microparticles in the 3-day study with anticoagulation.

Each the SEM and confocal microscopy results can be explained by possible effects of anticoagulation on platelets but the inconsistency between the two microscopy techniques used to evaluate platelet adhesion is unexpected. The anticipated effect of heparin and warfarin sodium on platelets was two-fold. The anticoagulants used do not directly affect platelets. Therefore, it is reasonable to expect no change in platelet
adhesion with anticoagulation, as observed in the confocal results. The second possibility is that since platelets are closely associated with fibrinogen, fibrin, and the coagulation cascade, as shown in Figure 1.2, along with inhibition of fibrin adhesion platelet adhesion may be reduced with anticoagulation. SEM results support this possibility. The inconsistency between microscopy techniques may be the result of limited ability to detect features by SEM (without resorting to immunolabeling similar to that for confocal microscopy but using gold nanoparticles as a label).

An additional complication to SEM imaging is that the samples are in vivo samples implanted for days and may have complex biologic deposition. Blood components may be more clearly distinguishable and visible on in vitro samples compared to in vivo samples. The in vivo samples might have complex layers of blood components deposited over the days of implantation. This is not to say that it is not possible to clearly detect some biologic deposition on in vivo samples, since at times large thrombi, platelets, and cells have been clearly visible on retrieved sac samples and are frequently reported in the literature. It is possible however, to be unable to see some biologic deposition when imaging by SEM, if there is low contrast in height or surface topography. Since the primary interest in this work is platelet and fibrin adhesion, confocal microscopy is the preferred and more reliable method of analysis here.
6.1.3.3 Relationship between Blood Sac Region and Surface Thrombosis

No relationship between sac region and macroscale surface thrombosis but differences in surface thrombosis with blood sac region at the microscale were observed by both SEM and confocal microscopy. However, the higher density of fibrillar structures and lower platelet and microparticle adhesion in the inlet and outlet in SEM analysis was not observed in the confocal microscopy analysis. Surface density of platelets and fibrin were varied in the inlet and outlet for anticoagulated and nonanticoagulated groups of the 3- and 30-day studies, with no consistent trend. It is possible that platelet and microparticle adhesion appeared lower in the inlet and outlet because of the high density of the fibrillar feature, which might have overwhelmed other topography.

Although the inlet and outlet are regions of relatively higher shear stresses compared to the other regions of the sac and platelet adhesion decreases with increased shear stresses, the maximum shear stresses in both the ports and other regions of the sac are much greater than values above which minimal platelet adhesion is reported. These values above which minimal platelet adhesion occurs have been reported to range from shear rates of 1000 to 2000 s\(^{-1}\) corresponding to shear stresses from 40 to 80 dynes/cm\(^2\) in blood. Fibrin adhesion is also expected to decrease with increased shear stress. Based on such literature and the maximum shear stresses in the blood sac, minimal platelet and fibrin adhesion could be expected in all regions of the blood sac. However, the majority of wall shear stresses reported for the LVAS blood sac are lower than these
maximum shear stresses and are within the range in which platelets may adhere.

Bachmann et al. reported the majority of wall shear stresses to be 35 dynes/cm$^2$ for the LVAS.\textsuperscript{88} In the top and bottom regions of the blood sac where shear stresses are relatively low compared to the inlet and outlet ports and similar to the center of the sac, confocal images showed a high density of the larger thrombi and platelets (Figures 4.5 and 4.6). This trend was not observed in the SEM analysis. The large thrombi were clearly visible in confocal images and their absence in SEM images or the inability to recognize them in the SEM images indicates some detail of surface thrombosis on the \textit{in vivo} blood sacs may be neglected by use of SEM alone.

### 6.2 \textit{In Vitro} Platelet Studies at Low Shear Stresses on Polyurethanes of Varied Chemistry

Platelet adhesion was minimal, 0.1\% AC\% or lower for shear stresses from 0 to 7.8 dynes/cm$^2$ and 0 to 4.4 dynes/cm$^2$ for Biospan MS/0.4 and Biospan-P, respectively, after incubation of PRP with the biomaterial samples for two hours. Higher platelet adhesion at shear stresses above these values up to 10 dynes/cm$^2$ were thought to be edge effects. No significant platelet activation due to exposure to shear stress was observed.

The possibility of edge effects comes from rough edges of the polymer samples that may have been created when the samples were cut from larger sheets of polymer. This may have altered fluid flow and caused turbulent flow, which may have in turn affected platelet adhesion. Since there was concern of the edge effect occurring data on platelet adhesion at the higher shear stresses had to be considered inaccurate. In order to obtain platelet adhesion data at the higher shear stresses where the possible edge effects
were observed, future experiments applying higher shear stresses would be beneficial. Increasing rotating speed of the RDS or increasing the diameter of the sample will provide higher shear stresses along the sample. Although increasing the sample size will allow the sensitivity of platelet adhesion measurements at approximately 1 dyne/cm$^2$ increments, in order to meet the condition of the sample rotating in an infinite medium, the beaker diameter and PRP volume would have to be increased. This would require more blood in addition to the already large volume of blood required for each experiment. Therefore, although increasing the rotating speed will decrease shear stress sensitivity it will allow platelet adhesion measurements at higher shear stresses. If measurements are taken at smaller radius increments such as 0.5 mm instead of 1 mm increments, shear stress sensitivity could be maintained.

Platelet activation in the bulk suspension by contact with Biospan MS/0.4 and Biospan-P, measured by flow cytometry, was insignificant compared to negative controls of static PRP with no biomaterial contact. As discussed in Chapter 5, due to the small percentage of platelet sampling by flow cytometry, this method of measurement of platelet activation may not be able to detect smaller scale variations in activation. A more sensitive assay may be necessary for evaluation of smaller scale platelet activation in bulk suspension.

In order to accurately assess platelet response to the polymer chemistry at different shear stresses, all other variables must remain constant for all polymers. In the results presented, although both Biospan MS/0.4 and Biospan-P should have smooth surfaces as they are coated onto smooth PDMS, they are not. In fact, the topographies of the two polymer surfaces are vastly different. The effect topography had on platelet
adhesion to these two polymers is unclear. However, since the source or cause of the pits on Biospan MS/0.4 and the branching structures on Biospan-P are unknown, it may not be simple to produce RDS samples that are smooth or of the same topography. Careful assessment and modification of the fabrication process may be necessary to achieve the smooth surfaces.

Modification of the fabrication process, however, could alter the surface chemistry of the samples along with the topography. The effect of this was seen in the difference in platelet adhesion on Biospan MS/0.4 fabricated using a spincast method onto PDMS, compared to the blood sac fabrication protocol where Biospan MS/0.4 was coated onto a different PDMS by dipping the mold into the Biospan MS/0.4. The data reported comparing Biospan MS/0.4 and Biospan-P shows no significant difference in platelet adhesion for samples prepared from these two polymers using the same sac fabrication protocol. However, since the surface chemistries of the polymer samples were not assessed, the low platelet adhesion cannot be attributed to a PEO surface. The low and similar platelet adhesion on Biospan MS/0.4 and Biospan-P may be the result of similar surface chemistry of the polymers. Although the bulk chemistries of the two polymers are certainly distinct, there are preliminary studies (not included) suggesting that the surface chemistry of the side of the sample exposed to the platelets, which is the side coated onto PDMS during fabrication, may not be different for the two polymers. Surface chemistry analysis of the surface, if possible, of the hydrated surface, would allow greater understanding of the surface chemistry effects on platelet adhesion.

Several modifications of RDS experiments may allow more information to be extracted from the results. The results presented showed low platelet adhesion on both
Biospan MS/0.4 and Biospan-P, and no significant difference between the two polymers. Increasing platelet adhesion density on the polymer samples by increasing RDS running time may provide greater sensitivity to differences between polymers. For these experiments, the blood was anticoagulated with heparin to inhibit activation of the coagulation cascade, in order to assess platelet activity independently. However, there is interaction between coagulation factors and platelets, and are each affected by the other. The RDS system is therefore a very simplified system and assessment of platelet activity in the presence of normal function of the coagulation cascade would more closely depict the thrombotic response of blood to the biomaterials in vivo. In selecting a simple biologic system to test biomaterials, the system can be well controlled and the effect of a single factor can be assessed. However, a simple system is far from an accurate depiction of blood in a living system. Unfortunately, increasing the complexity of the experimental system to account for the various components of blood in a live body makes interpretation of the results difficult, in that the results reflect the individual as well as collective, direct and indirect effects of the biomaterial on the system.

6.3 Evaluation of Experimental Design

The experimental design for the in vivo calf studies performed to evaluate surface thrombosis in LVAS presented advantages over simplified in vitro studies and over the typical analyses of hemocompatibility of devices or biomaterials in vivo that are reported in the literature. The in vivo system is closer though not identical to the clinical system or environment in which the device biomaterial will ultimately be placed. The environment
in which surface thrombosis was assessed for the LVAS included the complex combination of variable blood flow, anticoagulation, circulating and replenished blood supply, and calf activity. Such real life variability is difficult, if not impossible, to duplicate \textit{in vitro}. The calf studies also allowed for long-term studies over days whereas \textit{in vitro} studies are limited to hours as was the case for the RDS experiments.

The combination of SEM and confocal microscopy supplied surface topography – polymeric and biologic, and functional studies of platelet and fibrin deposition. The SEM images revealed extensive pitting on the blood sac surface and fibrillar structures that appear to be polymeric but seem to have no effect on platelet or fibrin adhesion based on confocal images. These microscopy analyses provide such information not obtainable from quantitative macroscale assays such as measurement of lactate dehydrogenase (LDH) levels for platelet adhesion.\textsuperscript{126}

A key achievement in this \textit{in vivo} research, encompassing the points mentioned above, is a more extensive analysis of thrombosis at the biomaterial surface compared to that of many \textit{in vivo} studies in literature. In most studies reported in the literature hemocompatibility or thrombosis assessment consists of a few SEM images or gross examination of the device for large thrombi.

The \textit{in vivo} environment created, however, a challenge for correlation of surface thrombosis with specific parameters due to the complexity of the system and the uncontrolled fluctuations in variables. Transient low and high flow rates were recorded in all calves with standard deviations for each calf as high as 1.7 L/min. The additional pumping from the calf’s healthy ventricle, its activity, positions (side, prone or standing upright), and the position of the inflow cannula all alter blood flow. These uncontrollable
factors made it difficult to maintain the desired blood flow rates within a narrow range. The effects of the transient low and high flow rates on blood elements such as platelets and fibrin are unclear and their role in the surface thrombosis results of this work is undetermined. Savage et al. reported that over 80% of platelets adhered to glass by immobilized fibrinogen at lower shear rates remained adhered when shear rates were increased to 1500 s⁻¹, a shear level at which platelet binding to fibrinogen is minimal.⁷³ These results suggest that when low transient flow rates occur in the LVAS, it is possible for platelets to adhere to the blood sacs by binding to fibrinogen and remain adhered at higher flow rates. Platelet activation and aggregation occur at higher shear rates and may increase during the transient high flow rates. However, further investigation is necessary to understand the level of any activation or aggregation and whether or not it is significant.

Using a bovine model may have given different results from what would have been obtained in a human patient. The thrombotic response of bovine blood may differ from that of human blood. The calves used for the in vivo studies had healthy ventricles instead of the weakened ventricles in human patients with cardiovascular disease that would receive LVAS implants and as a result flow-through may have been a problem.

Transient low and high anticoagulation of the blood was noted in measurements of PTs and ACTs. Over anticoagulation could lead to bleeding but should not encourage thrombosis whereas low anticoagulation for short periods may be enough to allow significant thrombosis. The contributions of the many variables (some which cannot be controlled) to the experiment outcomes are not fully understood.
The main advantage of the *in vitro* platelet adhesion study performed on the RDS is the ability to control many factors. Unlike the *in vivo* experiments, fluid flow was well controlled (except at the edges of samples), anticoagulation dosage was consistent, and platelet count was adjusted to be consistent across experiments. Both platelet adhesion at the surface and platelet activation in bulk suspension were assessed. The controlled environment allows for stronger conclusions and understanding of the direct effect of the variable being tested since it is possible to maintain other parameters constant.

Unfortunately, for the RDS experimental setup there were still limitations to the experiments and parameters that were not fully characterized or controlled. Care was taken to be consistent with sample fabrication protocol to obtain consistently smooth sample surfaces and to coat the sample polymers onto cured PDMS for consistency in chemistry of the layer in contact with the sample polymer. However, topography of the two polymers tested was different from the expected smooth surface. Surface chemistry may not be the expected chemistry of the polymer but rather a surface coated with silicone contaminant in which case the expected difference in the polymer surface chemistries may be negated. Hence, topography became an unexpected variable and the expected difference in surface chemistry may not have been attained in the experiments.

In the RDS experiments, platelet adhesion and activation was tested with anticoagulated platelet-rich plasma, which is not representative of the biologic system encountered in the body. This simplified system ignored the roles of blood cells and the coagulation cascade in platelet adhesion and activation. As discussed previously, the coagulation cascade and platelets are closely related and assessment of thrombosis should consider both coagulation and platelet response although the two responses are
commonly examined separately. The absence of red cells can significantly change the interaction of platelets with a surface or other platelets.\textsuperscript{79}

The samples were not tested under the pulsatile flow pattern encountered with the natural heart or pulsatile LVAS. The effect of pulsatile flow on platelet adhesion and activation on the biomaterials tested should be assessed to better predict the performance of these polymers \textit{in vivo}. Additional research addressing the limitations of the platelet studies with the RDS would allow better prediction of the hemocompatibility of the polyurethanes in LVAS.

Future \textit{in vivo} studies may benefit from supplementary analyses including quantification of platelet adhesion, activation, and aggregation. There must be a combination of techniques for analysis of thrombosis consisting of both efficient quantification of platelet and fibrin adhesion as well as the performed qualitative assessment of the thrombotic response. Microscopy techniques are important in visualizing how platelets adhere in relation to polymer topography, or whether there are few large thrombi or a high density of individual platelets. If large thrombi detach and flow into small vessels, the risks of strokes and tissue infarcts may be high, whereas adhesion of numerous individual platelets to the surface may be of less risk for such results from emboli. However, quantifying platelet adhesion by such microscopy techniques can be difficult and time-consuming and should be supplemented by other quantitative assays.\textsuperscript{127,128}

Evaluation of clinical results for any correlations with the surface thrombosis observations is an important component to understanding the hemocompatibility of the LVAS. Thrombosis is closely related to embolization and kidney infarcts. Platelet,
fibrinogen, and fibrin responses are important both in suspension in blood and at the biomaterial surface. Kidney infarcts, thrombocytopenia, and similar results that are significant clinically, can be closely associated to the thrombotic response of blood at the biomaterial surface and deserve consideration. For future implant studies the clinical parameters should be carefully considered and those of interest should be recorded on a regular basis.

Bridging the gap between simplified in vitro and complex in vivo experiments is vital. The strengths of in vivo and in vitro studies must be combined to create an experimental system that is not oversimplified in order to represent a living system but not too complex that there are uncontrolled or unknown variables. Although the research performed had its strengths and advances over work presented in the literature, and represents one of the most detailed studies of the biologic response at the surfaces of these devices, there remain opportunities for improvement of the experimental design for future work.

6.4 Conclusions

This research demonstrated the effects of the variables: 1) implant duration, 2) anticoagulation, 3) fluid mechanics (in terms of blood sac region), and 4) biomaterial chemistry, on thrombosis at the biomaterial surface for specific application to LVAS. The thrombotic response in vivo is dynamic and evolves with time, and treatment of patients implanted with LVAS must include consideration of this. In agreement with clinical results showing that anticoagulation treatment is not fully effective in prevention
of thrombosis, this research illustrated presence of macroscale and microscale surface thrombosis despite anticoagulation. Specific regions of the blood sac with low and high shear stresses were prone to thrombosis. Such results demonstrate how better understanding the correlation between surface thrombosis and fluid mechanics is crucial to designing a more hemocompatible device. In assessment of the effect of polymer chemistry on platelet adhesion, the importance of fabrication method and final surface chemistry on platelet adhesion was recognized. The LVAS is a complex device, similar to many other medical devices, and involves numerous conditions that if changed, can significantly alter the hemocompatibility of the device.
Bibliography


Appendix A

Clinical Data on LVAS Implants in Bovine Models
## A.1 Implants Performed

Table A-1. Implant records including original calf numbers for all calves

### 30-Day Implants

<table>
<thead>
<tr>
<th>Calf #</th>
<th>Actual Calf #</th>
<th>Implant Date</th>
<th>Necropsy Date</th>
<th>Duration (days)</th>
<th>Anticoagulant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anticoagulated</td>
<td>Nonanticoagulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>111A</td>
<td>10/20/2003</td>
<td>11/19/2003</td>
<td>30</td>
<td>Heparin, Warfarin Sodium</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>1/19/2004</td>
<td>2/18/2004</td>
<td>30</td>
<td>Heparin, Warfarin Sodium</td>
</tr>
<tr>
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<td>80</td>
<td>4/17/2003</td>
<td>5/16/2003</td>
<td>30</td>
<td>Heparin, Warfarin Sodium</td>
</tr>
<tr>
<td></td>
<td>Nonanticoagulated</td>
<td>Anticoagulated</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>2/19/2002</td>
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</tr>
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<td>None</td>
</tr>
<tr>
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<td>1/19/2004</td>
<td>30</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>20C</td>
<td>2/9/2004</td>
<td>3/10/2004</td>
<td>30</td>
<td>None</td>
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### 3-Day Implants

<table>
<thead>
<tr>
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<th>Actual Calf #</th>
<th>Implant Date</th>
<th>Necropsy Date</th>
<th>Duration (days)</th>
<th>Anticoagulant</th>
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</thead>
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<td>Nonanticoagulated</td>
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<td></td>
</tr>
<tr>
<td>1</td>
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<td>11/11/2002</td>
<td>11/14/2002</td>
<td>3</td>
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</tr>
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<td>40</td>
<td>5/13/2004</td>
<td>5/16/2004</td>
<td>3</td>
<td>Heparin, Warfarin Sodium</td>
</tr>
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<td></td>
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<td>Anticoagulated</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>6/17/2004</td>
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A.2 Flow Rates

Table A-2. Average flow rates for 3- and 30-day studies

<table>
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<tr>
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<th>3-Day Study</th>
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<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Group</td>
<td>All</td>
<td>Calf 1</td>
<td>Calf 2</td>
<td>Calf 3</td>
<td>Calf 4</td>
</tr>
<tr>
<td></td>
<td>Anticoagulated</td>
<td>5.0 ± 1.5</td>
<td>5.1 ± 1.0</td>
<td>4.7 ± 1.5</td>
<td>5.6 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonanticoagulated</td>
<td>5.6 ± 1.5</td>
<td>4.7 ± 1.0</td>
<td>4.9 ± 1.2</td>
<td>6.6 ± 1.3</td>
<td>6.9 ± 1.5</td>
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</table>

<table>
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<tr>
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<th>30-Day Study</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>All</td>
<td>Calf 1</td>
<td>Calf 2</td>
<td>Calf 3</td>
<td>Calf 4</td>
<td>Calf 5</td>
</tr>
<tr>
<td></td>
<td>Anticoagulated</td>
<td>5.8 ± 1.4</td>
<td>5.5 ± 1.5</td>
<td>5.1 ± 1.6</td>
<td>6.5 ± 0.9</td>
<td>6.3 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonanticoagulated</td>
<td>5.8 ± 1.1</td>
<td>5.4 ± 1.0</td>
<td>5.5 ± 0.7</td>
<td>5.7 ± 0.9</td>
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<td>5.8 ± 1.3</td>
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A.3 Summaries of Anticoagulation ACTs, PTs, INRs, and Kidney Infarcts

Anticoagulation measurements of ACTs, PTs, and INRs were taken using blood samples from the calves during the studies. As part of the full necropsy, the kidneys were examined for infarcts, dead tissue resulting from deprivation of blood supply, which may be caused by a thrombus or embolus. Kidneys were examined and sectioned into several slices for infarcts, identified by discoloration of the tissue. Size, area, and/or number of the infarcts were recorded and photographs of kidney infarcts were taken. Kidney samples were also sent to Comparative Medicine (Milton S. Hershey Medical Center, Hershey, PA) for gross pathology. All of the above information was used to assess the level of infarcted kidney tissue in each calf and was scored from 0 to 4, 0 being none and 4 being extensive. The scoring of the kidney infarcts was performed by Dr. John Connell in the Department of Surgery.
All data presented here was collected and kindly provided by clinicians and staff of the Division of Artificial Organs in the Department of Surgery at the Milton S. Hershey Medical Center. Provided data was then summarized and analyzed to compare the 30-day and 3-day studies.

**Table A-3. Anticoagulation measurements and kidney infarct data for 3-day Study**

<table>
<thead>
<tr>
<th></th>
<th>Group 1: Anticoagulated Calves</th>
<th></th>
<th>Group 2: Nonanticoagulated Calves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calf</td>
<td>ACT (secs)</td>
<td>PT Ratio (Postop:Preop)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>243.9 ± 49.5</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>242.5 ± 35.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>254.4 ± 57.6</td>
<td>1.2 ± 0.1</td>
</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>186 ± 20.6</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>168 ± 7.1</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>183 ± 34.5</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>216.5 ± 55.9</td>
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<tr>
<td></td>
<td>All</td>
<td>188.4 ± 32.1</td>
<td>1.1 ± 0.2</td>
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</table>
Table A-4. Anticoagulation measurements and kidney infarct data for 30-day study

<table>
<thead>
<tr>
<th>Calf</th>
<th>ACT (secs)</th>
<th>PT Ratio (Postop:Preop)</th>
<th>INR</th>
<th>Kidney Infarct Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>246.1 ± 39.4</td>
<td>1.6 ± 0.4</td>
<td>2.4 ± 1.1</td>
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<tr>
<td>2</td>
<td>261.9 ± 71.6</td>
<td>2.0 ± 0.6</td>
<td>3.6 ± 1.8</td>
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<td>3</td>
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<td>4</td>
<td>257.7 ± 44.9</td>
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<td>3.3 ± 2.1</td>
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<td>All</td>
<td>252.9 ± 55.2</td>
<td>1.8 ± 0.5</td>
<td>3.1 ± 1.7</td>
<td>2.5 ± 0.6</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Calf</th>
<th>ACT (secs)</th>
<th>PT Ratio (Postop:Preop)</th>
<th>INR</th>
<th>Kidney Infarct Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>156.0</td>
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<td>1.2 ± 0.2</td>
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<td>1.1 ± 0.1</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
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<td>1.2 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>175.8 ± 21.8</td>
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<td>0.9 ± 0.3</td>
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</tr>
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<td>5</td>
<td>175.5 ± 24.7</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>3</td>
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<tr>
<td>All</td>
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<td>1.1 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>3.2 ± 0.4</td>
</tr>
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Figure A.1. Macroscopic thrombi compared to kidney infarct scores in the 3- and 30-day studies, anticoagulated and nonanticoagulated.
Figure A.2. ACTs for nonanticoagulated groups (Group 2) of 3- (A) and 30-day (B) studies were recorded only the day of operation but for all 3 PODs and close to POD 7, for the anticoagulated calves (Group 1) in the 3- and 30-day studies, respectively. Heparin was only given the first few postoperative days, so its effect was expected to end by POD 7.
Figure A.3. INRs for the 3-day study (A) were similar for the anticoagulated (Group 1) and nonanticoagulated (Group 2) groups since warfarin sodium was not expected to be effective in such a short time period. The effect of warfarin sodium anticoagulation was seen in the 30-day study (B).
Figure A.4. Coumadin (warfarin sodium) anticoagulation doses and measurements of PT and INR show warfarin sodium anticoagulation was ineffective over 3 days, with low PTs and INRs.
Figure A.5. Coumadin (warfarin sodium) anticoagulation doses and measurements of PT and INR show warfarin sodium anticoagulation was fairly consistent over the 30 days except for a few peaks in PTs and INRs, between POD 10-20, in all four calves.
Figure A.6. Heparin anticoagulation doses given kept ACTs fairly consistent for the 3-day study anticoagulated group.

- ACT
- Heparin Dose
Figure A.7. Heparin anticoagulation doses and ACTs for the 30-day study show consistent anticoagulation of all calves.

- ACT
- Heparin Dose
A.4 Platelet and Fibrinogen Blood Concentrations

![Graph showing platelet and fibrinogen concentrations for the 3-day study](image)

**Figure A.8.** Platelet and fibrinogen concentrations for the 3-day study
Figure A.9. Platelet and fibrinogen concentrations for the 30-day study
Appendix B

Calculations for RDS Experimental Setup

Calculations for the current RDS experimental setup were performed as shown in Figure B.1. The calculations show that the experimental setup conditions necessary for the shear stress equation (Equation 5.1) to be valid are met. The three conditions that must be met are: 1) The disk is of infinite size and rotating in an infinite medium, 2) Laminar flow exists in the boundary layer at the biomaterial surface, 3) Steady-state conditions apply. The first condition is met by setting the disk radius and separation between disk and medium much greater than the boundary layer thickness. The disk size and medium can be approximated as infinite, based on the calculations obtained for "infinite disk", and "infinite gap" and "infinite depth", respectively. The second condition is achieved by maintaining the Reynold’s number of the system below $10^5$. Steady-state required in the third condition can be met by achieving stable angular velocity of the RDS.
Figure B.1. Calculations for RDS experimental setup
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Yamanaka H and Siedlecki CA, "Evaluation of thrombosis at surfaces of cardiac assist devices: macroscale to microscale, podium presentation",

"Flow-dependent changes in topography of LVAD poly(urethane urea) blood sacs implanted in calves, podium presentation",