MECHANICAL CHARACTERIZATION AND SIMULATION OF MURINE THROMBI

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by

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Abstract

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Deep vein thrombosis, pulmonary embolism, and abdominal aortic aneurysms are blood-related diseases that represent a major public health problem. These diseases are characterized by the formation of a thrombus (i.e., blood clot) that either blocks a major artery or causes an aortic rupture. Identifying the mechanical properties of thrombi can help determine when these incidents will occur. In this investigation, a murine thrombus, formed from platelet-rich plasma, calcium, and thrombin, was nanoindented and the elastic modulus was determined via elastic contact theory. This information was used as input to an inverse finite element simulation, which synthesized optimal values for the elastic modulus and viscosity of the thrombus using a viscoelastic material model. A sensitivity analysis was also performed to determine which material parameters have the greatest affect on the simulation. Results from this investigation demonstrate the feasibility of the mechanical characterization of a murine thrombus using nanoindentation.
Dedicated to Mom, Dad, Paul, Brian, and Charlie.

Your love, support, and encouragement have enabled me to achieve my goal of obtaining this degree.
3.2.1 Rheometry ............................................. 16
3.2.2 Ultrasound .......................................... 17
3.2.3 Other Mechanical Methods ....................... 18
3.3 Nanoindentation ....................................... 20
  3.3.1 Tip Shape and Size .................................. 21
  3.3.2 Angle Offset ......................................... 22
  3.3.3 Adhesion ............................................. 23
3.4 Sample Preparation .................................... 23
3.5 Indentation Tests ..................................... 25
3.6 Elastic Contact Theory ............................... 29
3.7 Results .................................................. 30
3.8 Discussion .............................................. 31
3.9 Limitations ............................................. 32
3.10 Conclusions ........................................... 33

CHAPTER 4: FINITE ELEMENT MODELING .................... 37
  4.1 Overview ................................................ 37
  4.2 Background ............................................ 37
  4.3 Significance .......................................... 39
  4.4 Finite Element (FE) Model ......................... 40
  4.5 Biphasic Theory ...................................... 42
  4.6 Permeability .......................................... 42
  4.7 Optimization-based Inverse FE Technique ....... 43
  4.8 Kelvin-Voigt (KV) Model ............................ 44
  4.9 KV Results and Discussion ......................... 46
  4.10 Standard Linear Solid (SLS) Model ............... 46
  4.11 SLS Results and Discussion ....................... 48
  4.12 Sensitivity Analysis .................................. 51
  4.13 Sensitivity Analysis Results and Discussion .... 52
  4.14 Limitations .......................................... 56
  4.15 Conclusions ........................................... 56

CHAPTER 5: CONCLUSIONS .................................. 60
  5.1 Summary of Original Contributions ............... 60
  5.2 Recommended Future Work .......................... 61
    5.2.1 Nanoindentation Tear Testing .................. 61
    5.2.2 Live-animal in vivo Micro-Computed Tomography Scans .... 62
    5.2.3 Nanoindentation Testing of Harvested in vivo Thrombi .... 63
    5.2.4 Fluid-Structure Finite Element Simulation .......... 63
    5.2.5 Flow Chamber Simulation ....................... 65

BIBLIOGRAPHY ............................................. 67
FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic of deep vein thrombosis (DVT)</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic of pulmonary embolism (PE)</td>
<td>4</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic of abdominal aortic aneurysm (AAA)</td>
<td>6</td>
</tr>
<tr>
<td>2.1</td>
<td>Conversion of fibrinogen to fibrin</td>
<td>10</td>
</tr>
<tr>
<td>2.2</td>
<td>Electron micrograph of a fibrin fiber</td>
<td>10</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematic of fibrin assembly</td>
<td>11</td>
</tr>
<tr>
<td>2.4</td>
<td>SEM of a thrombus with thick fibers and few branch points</td>
<td>12</td>
</tr>
<tr>
<td>2.5</td>
<td>SEM of a thrombus with thin fibers and many branch points</td>
<td>12</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic of dynamic mechanical analysis as presented by Hinnen et al. (2007)</td>
<td>19</td>
</tr>
<tr>
<td>3.2</td>
<td>Schematic of contact area during sample deformation as depicted by Xie et al. (2005)</td>
<td>20</td>
</tr>
<tr>
<td>3.3</td>
<td>Schematic of cylindrical flat punch indenter tip in full contact with a specimen</td>
<td>22</td>
</tr>
<tr>
<td>3.4</td>
<td>PRP clot on glass microscope slide</td>
<td>24</td>
</tr>
<tr>
<td>3.5</td>
<td>Photograph of the TI 950 Triboindenter™</td>
<td>26</td>
</tr>
<tr>
<td>3.6</td>
<td>Experimental setup of the inside of the nanoindenter</td>
<td>27</td>
</tr>
<tr>
<td>3.7</td>
<td>Experimental setup of the nanoindenter stage</td>
<td>27</td>
</tr>
<tr>
<td>3.8</td>
<td>Schematic of indentation positions for one PRP specimen</td>
<td>28</td>
</tr>
<tr>
<td>3.9</td>
<td>Plot of the indentation loading functions</td>
<td>28</td>
</tr>
<tr>
<td>3.10</td>
<td>Representative plot of the mean load-displacement curve for one specimen</td>
<td>30</td>
</tr>
<tr>
<td>4.1</td>
<td>FE mesh of thrombus</td>
<td>41</td>
</tr>
<tr>
<td>4.2</td>
<td>Coordinate system for FE simulation</td>
<td>41</td>
</tr>
<tr>
<td>4.3</td>
<td>Schematic of optimization loop</td>
<td>44</td>
</tr>
</tbody>
</table>
4.4 Schematic of Kelvin-Voigt (KV) model .................................. 45
4.5 Optimized KV data ..................................................... 47
4.6 Schematic of standard linear solid (SLS) model ...................... 48
4.7 Optimized SLS data .................................................... 49
4.8 Four key characteristics for sensitivity analysis ....................... 53
4.9 Initial indentation depth (IID) plot .................................. 53
4.10 Total creep displacement (TCD) plot ................................ 54
4.11 Final indentation depth (FID) plot .................................. 54
4.12 Initial loading slope (ILS) plot ...................................... 55
5.1 Flow chamber schematic .............................................. 65
TABLES

3.1 SUMMARY OF NANOINDENTATION TESTS . . . . . . . . . . . . 25
3.2 THROMBUS ELASTIC MODULUS VALUES CALCULATED VIA
ELASTIC CONTACT THEORY FOR VARYING LOADS . . . . . . . 31
3.3 REPORTED ELASTIC MODULUS VALUES FOR THROMBI . . . . 34
4.1 CALCULATED PERMEABILITY VALUES . . . . . . . . . . . . . . . 43
4.2 SUMMARY OF MODELING . . . . . . . . . . . . . . . . . . . . . . 51
4.3 OPTIMIZED MATERIAL PARAMETERS . . . . . . . . . . . . . . 52
4.4 REPORTED VISCOSITY VALUES FOR THROMBI . . . . . . . . 58
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>Abdominal aortic aneurysm</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>FE</td>
<td>Finite element</td>
</tr>
<tr>
<td>FID</td>
<td>Final indentation depth</td>
</tr>
<tr>
<td>FLSC</td>
<td>Freimann life science center</td>
</tr>
<tr>
<td>IID</td>
<td>Initial indentation depth</td>
</tr>
<tr>
<td>ILS</td>
<td>Initial loading slope</td>
</tr>
<tr>
<td>KV</td>
<td>Kelvin-Voigt</td>
</tr>
<tr>
<td>PE</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>SLS</td>
<td>Standard linear solid</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
</tr>
<tr>
<td>TCD</td>
<td>Total creep displacement</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
</tr>
<tr>
<td>UMAT</td>
<td>User-defined material</td>
</tr>
</tbody>
</table>
SYMBOLS

\( d \) Diameter of elastomeric o-ring

\( D \) Diameter of flat punch nanoindenter tip

\( e \) Void ratio

\( E \) Elastic modulus

\( E_1 \) Elastic modulus (SLS model)

\( E_2 \) Elastic spring constant (SLS model)

\( h \) Nanoindentation depth

\( k \) Kozeny’s constant

\( K \) Permeability

\( N \) Number of specimens

\( P \) Nanoindentation load

\( R \) Radius of flat punch nanoindenter tip

\( R_f \) Corner radius of flat punch nanoindenter tip

\( S_p \) Surface area of elementary particle \( p \)

\( V_p \) Volume of elementary particle \( p \)

\( \epsilon \) Porosity
\[ \eta \quad \text{Viscosity} \]
\[ \nu \quad \text{Poisson’s ratio} \]
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CHAPTER 1

BLOOD-RELATED DISEASES

1.1 Overview

A thrombus, or blood clot, may form in a vein or artery, often without warning, and result in sudden death due to either: 1) dissociation and relocation of the thrombus (which may obstruct a small vein) or 2) aortic rupture. In order to better characterize thrombi interactions in the body, many attempts have been made to simulate thrombus formation (AlMomani et al., 2007; Dai et al., 1999; Huang et al., 2005; Pivkin et al., 2006), aging of a thrombus (Brighton et al., 2007; Geier et al., 2005; Karpiouk et al., 2008; Parsons et al., 1993; Siebers et al., 2004; Xie et al., 2005), and aortic rupture (Arab-Ghanbari et al., 2009; Borghi et al., 2006; Di Martino et al., 1998; Khanafer et al., 2006; Luo et al., 2009; Ravhon et al., 2001; Schei et al., 2003; Wolters et al., 2005). While a number of these simulations include hemodynamic variations, many of them fail to include a characterization of thrombus mechanical properties such as the elastic modulus $E$ and viscosity $\eta$. The effects of these properties on thrombi dissociation and aortic rupture are not well characterized. This lack of knowledge has hampered improvements in preventative treatment and management of many blood-related diseases, as well as the development of novel drug therapies. Accurately determining the mechanical properties of thrombi can be utilized as inputs to computational simulations of thrombus-related disorders. This will provide a means to unravel the key factors that
result in the dissociation of a thrombus or to more accurately identify an individual’s risk of aortic rupture.

The long-term goal of this study is to better understand the mechanical properties of thrombi to provide for the development of new treatment protocols for diseases such as abdominal aortic aneurysms (AAA) and deep vein thrombosis (DVT). For example, understanding the factors that facilitate the dissociation of a thrombus from the vessel wall can be utilized to drive the development of thrombus-targeted drug therapies. The objective of this research, which is one step in pursuit of that goal, is to characterize the mechanical properties of a thrombus. The central hypothesis is that of all the mechanical properties of thrombi, the elastic modulus $E$ and viscosity $\eta$ have the greatest affect on clot deformation and are therefore the main causes of aortic rupture and thrombi separation from a vessel wall. This hypothesis has been formulated based on data which show that the mechanical properties of thrombi change over time, suggesting that as the properties change the risk of dissociation increases (Aglyamov et al., 2004).

The rationale for the proposed research is that knowledge of the mechanics of thrombi dissociation and rupture is expected to improve the understanding of blood-related diseases, their progression, and any potentially-fatal thrombus disease state configurations in representative human vascular geometries. The goal of this investigation is to measure the mechanical behavior of murine (rat) thrombi under physiologic conditions via nanoindentation and to computationally optimize the mechanical properties via a finite element (FE) simulation. From these experiments, the elastic modulus of thrombi will be estimated via elastic contact theory. Then, the force-displacement response of the nanoindentation study will be simulated using rheological models, where the elastic modulus and viscosity will be computationally optimized.
1.2 Deep Vein Thrombosis (DVT)

Deep vein thrombosis is a common blood-related disease, which occurs when a thrombus forms deep within a vein, usually located in one leg (Fig. 1.1). This disease is characterized by partial or complete blockage of blood circulation in the vein. Approximately two million incidences of DVT occur annually. Roughly 600,000 people are hospitalized annually in the U.S. and 300,000 people die due to developing a pulmonary embolism (PE).\(^1\) Causes of DVT include immobilization, hospitalization, surgery, trauma to the lower leg, pregnancy, obesity, and hypercoagulation. Although possible symptoms include pain, swelling, tenderness, discoloration, redness of the affected area, and skin that is warm to the touch, approximately half of all DVT cases are asymptomatic (Piazza and Goldhaber, 2006). Current treatments consist of anticoagulants (i.e., heparin and warfarin), thrombolytics, filters, and compression stockings.\(^2\)

\(^1\)The Coalition to Prevent Deep Vein Thrombosis, http://www.preventdvt.org
1.3 Pulmonary Embolism (PE)

Over one-third of DVT patients encounter a PE\(^3\), which occurs when the thrombus dissociates from its surroundings in the leg, travels through the blood stream, through the heart, and finally resides in the lungs, where it partially or completely blocks a pulmonary artery or one of its branches (Fig. 1.2). This critical disease is the leading cause of hospital deaths\(^2\), as there are approximately 650,000 incidences occurring annually in the United States\(^4\). Of the 300,000 people who die each year from PE, roughly 60,000 individuals die within 30-60 minutes after the symptoms begin\(^5\). Causes of PE include immobilization, travel, recent surgery, heart disease, and obesity. Like DVT, PE can also be asymptomatic; however, symptoms that do occur include permanent lung damage, chest pain, shortness of breath, anxiety, coughing, sweating, and passing

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\(^3\) Statistics about pulmonary embolism, http://www.wrongdiagnosis.com

out\textsuperscript{6,7}. If a PE is detected before hospitalization, treatments consist of anticoagulants heparin and warfarin\textsuperscript{2}. Treatments during hospitalization include oxygen, anticoagulant heparin, blood pressure elevators, and thrombolytics\textsuperscript{2,8}. The diseases DVT and PE are commonly grouped together and often referred to as a venous thromboembolism (VTE).

1.4 Venous Thromboembolism (VTE)

Venous thromboembolism represents a major public health problem, with more than 1.25 million Americans affected each year and more than 300,000 deaths\textsuperscript{1,4}. Approximately 20\% (one in five) of individuals die almost immediately from PE, and 40\% die within three months (Heit, 2006, 2008). Roughly 30\% of DVT survivors will experience a recurrent episode within the next 10 years\textsuperscript{3}. Treatments for the survivors include compression stockings, oral warfarin, and heparin injections (Farley et al., 2009). Surgical treatments include catheter embolectomy, vena cava filter insertion, and surgical embolectomy (Shaughnessy, 2007; Sims, 2007). It is expected that the number of cases of VTE grows at the same rate as overall population growth; however, the incidence of VTE increases with age (Silverstein et al., 1998). Therefore, as the average age of the U.S. population increases, it is possible that the incidence of VTE will outpace population growth.

1.5 Abdominal Aortic Aneurysm (AAA)

Abdominal aortic aneurysm is another life-threatening disease involving thrombi. Layers of thrombi form and build up on the inside wall of the abdominal aorta, causing

\textsuperscript{7}MedicineNet, Inc., http://www.medicinenet.com
\textsuperscript{8}eMedicine Health, http://www.emedicinehealth.com
significant widening of the artery (Fig. 1.3) and, in some cases, rupture. This disease causes approximately 15,000 deaths in the U.S. annually\(^5\), and it typically affects individuals aged 55 years and older. Concern for this disease is rising due to increased life expectancy. Causes of AAA consist of genetics, post-trauma, arteritis, and mycotic infection. Most abdominal aortic aneurysms are asymptomatic and are often found during tests for other conditions; however, the most common symptom is pain. Treatments for AAA include endovascular surgery and removal of the aneurysm if the aorta diameter is 5.5 cm or larger\(^6\).

1.6 Summary

Together, these diseases have a global impact on the lives of over 2.5 million people each year. Determining the mechanical properties of thrombi for use in future computer simulations can help diagnose blood-related diseases (i.e., DVT, PE, VTE, and AAA) at an earlier stage in their progression, thereby providing medical doctors time to prescribe...
treatments. As motivated throughout this chapter, time is of the essence in providing immediate care to these patients to improve their chances of survival. In addition, increased knowledge of thrombi properties can lead to the development of preventative medicine. Both of these outcomes have the potential to decrease the number of deaths from the aforementioned diseases.
CHAPTER 2
BIOLOGY OF THROMBI

2.1 Overview

In this study, the elastic modulus $E$ of a thrombus is determined via nanoindentation, which requires the use of ex vivo thrombi. Accurately creating ex vivo thrombi is a challenge, as the formation of a thrombus is a complicated biological process involving many factors including fibrinogen, platelets, calcium ions, and thrombin, amongst other constituents (i.e., tissue factor, proaccelerin, activated $V$, etc.). The accurate development of the desired thrombus configuration requires an understanding of both: 1) the basic biology of the thrombus formation process and 2) how changes in the concentration of the constituents impact the structure of the thrombus.

2.2 Conversion of Fibrinogen to Fibrin

The thrombus formation process is initiated by converting fibrinogen to fibrin. Fibrinogen is an elongated plasma glycoprotein that has a molecular mass of 340,000 $g/mol$ (Sidelmann et al., 2000), a plasma concentration of 2-4 $g/l$ (Sidelmann et al., 2000), and a length of approximately 45 $nm$ (Kaneider et al., 2010). It is also characterized by two domains; the $D$-domains are the two outer parts of the molecule while the $E$-domain is the central part of the molecule.
A simplified schematic of the thrombin-induced conversion of fibrinogen to fibrin is depicted in Fig. 2.1. Bonding thrombin to fibrinogen is mediated through the fibrinogen recognition site in thrombin, termed exosite 1. This binding activates fibrinogen and cleaves two small peptides designated fibrinopeptide A (FPA) and fibrinopeptide B (FPB). The newly-activated fibrinogen molecules form soluble complexes called fibrin monomers. When the concentration of fibrin monomers increases due to persistent thrombin activity, long double-stranded protofibrils, which have a periodicity of 22.5 nm (Mosesson, 2005; Weisel, 2007), are formed. Cross-linked fibrin is then developed from several factors. First, sufficiently long protofibrils aggregate laterally. Then, the combination of calcium ions from blood and the activation of coagulation factor XIII (FXIII) to FXIIIa by thrombin, initiates the cross-linking process. Cross-linked fibrin forms fibers of varying diameters that branch out to create a thrombus.

2.3 Protofibrils

Fibrin monomers cross link together to create strands called protofibrils. These protofibrils twist around each other to form a fiber. To maintain a periodicity of 22.5 nm, the protofibrils stretch as they wrap around the path length to form fibers of varying diameters (Mosesson, 2005; Weisel, 2007). Therefore, protofibrils which are under tension account for the observation that fibers making up a thrombus are very straight (Fig. 2.2). Fibers stop growing when the energy required to stretch an added protofibril exceeds the energy of bonding.

Fiber branching produces a three-dimensional network (Fig. 2.3). In fact, fiber ends are rarely seen in an undamaged, normal fibrin clot. Most branch points consist of three fiber segments of nearly equal diameters (not like that of a tree) that join together with band patterns aligned. Branch points make fibers into a stable network. Depending on
Figure 2.1. Schematic of conversion of fibrinogen to fibrin as presented by Sidelmann et al. (2000).

Figure 2.2. Electron micrograph of a fibrin fiber ($D \approx 0.1 \, \mu m$) as illustrated by Weisel (2007).
2.3 Schematic diagram of fibrin assembly as depicted by Mosesson (2005). Both orange and light orange are used to depict fibrin molecules and clarify exactly how the molecules attach to each other to form a fibrin assembly.

The concentration of thrombin is one of the most important factors with respect to fibrin structure; it can change the structure of the thrombus. In fact, Ryan et al. (1999) found that no clotting was observed in the absence of thrombin. Low thrombin concentration is roughly 0.05 NIHU/ml (National institute of health unit per ml), which produces thick fibrin with few branch points (Fig. 2.4). High thrombin concentration is roughly 5 NIHU/ml, which produces thin fibrin with many branch points (Fig. 2.5). Fibrin constitutes 0.25% of the volume in a clot, while the remaining 99.75% of the volume of the clot is liquid occupying the space between polymers.
Figure 2.4. Scanning electron micrograph of a thrombus with thick fibers and few branch points as illustrated by Weisel (2004). These were made from recalcified plasma with a low thrombin concentration. Same scale as that in Fig. 2.5.

Figure 2.5. Scanning electron micrograph of a thrombus with thin fibers and many branch points as illustrated by Weisel (2004). These were made from recalcified plasma with a high thrombin concentration. Scale bar = 5 µm.
2.5 Plasma Clots

Platelet-rich plasma (PRP) clots are widely used in literature to study properties of thrombi because their structure and mechanical properties are similar to whole blood clots (Bale et al., 1985; Brown et al., 2009; Carr and Carr, 1995; Carr et al., 2002; Collet et al., 2002, 2000, 2005; Dempfle et al., 2008; Fukada et al., 1984; Gennisson et al., 2006; Huang et al., 2007; Janmey et al., 2009; Li et al., 2007; Riha et al., 1999; Semeraro et al., 2007; Vicario et al., 2009; Weisel, 2004, 2007). The process used to create PRP clots (i.e., centrifugation) results in a high concentration of activated platelets that bind clotting factors and protect them from being inhibited by antithrombin-dependent anticoagulants. Thus, coagulation interferences are minimized, which facilitates clot creation. For this reason, PRP clots were used in this study.

2.6 Platelets and Plasma Clots

Platelets are cells that normally circulate in blood. Once specific receptors on platelet membranes interact with their ligands, a reaction is initiated that leads to platelet activation. Platelet activation is well characterized and includes activation of membrane receptors (one of which is fibrinogen), shape change, granular secretion, cytoskeletal reassembly, platelet cohesion, and aggregation. It is important to note that platelets begin the coagulation process. This is evidenced by fibrinogen, which does not assemble into fibrin until platelets are activated. Also, thrombi cannot form until fibrin is formed.

2.7 Plasma Clots Related to Diseases

The mechanical properties of thrombi as well as the mechanical factors regulating thrombi formation are poorly understood; however, these are believed to regulate
thrombus formation, propagation, and embolization (Anand et al., 2006). Determining these properties (i.e., elastic modulus \( E \), viscosity \( \eta \)) can help perfect computer simulations of thrombi dissociation and aortic rupture and can also be used to analyze thrombi behavior and progression throughout various disease-state configurations.

2.8 Scope of Investigation

This investigation is the first to utilize nanoindentation to determine the mechanical properties of thrombi (Slaboch and Ovaert, 2009). Nanoindentation has been successfully applied to estimate mechanical properties of other soft viscoelastic materials (elastic modulus on the order of \( 10 \; kPa \)), such as hydrogels (Liu et al., 2009). Thus, nanoindentation is a promising method for characterizing this class of materials.

The purpose of this study is three-fold. First, the mechanical behavior of murine (rat) thrombi will be measured via nanoindentation testing. Second, elastic contact theory will be used to calculate an approximate elastic modulus value. Third, the approximate elastic modulus value will be used as an input into a finite element (FE) optimization program, where the elastic modulus will be optimized and a corresponding viscosity value will be computed and optimized as well.
CHAPTER 3

NANOINDENTATION OF MURINE THROMBI

3.1 Overview

Nanoindentation is a novel technique for determining the mechanical properties of soft materials. This testing method penetrates a hard indentation tip into a sample while recording the load and displacement. From these measurements, the mechanical properties of the sample can be computed. Nanoindentation has several advantages over other testing methods, including a small scale of measurements (i.e., loads and displacements in the $nN$ and $nm$ range, respectively), indenter tips that can be customized (i.e., size, shape, material, surface coating, etc.), and high resolution sensitivity for both load and displacement.

Nanoindentation has been used to successfully determine the mechanical properties of biological structures similar to thrombi, such as cartilage (Simha et al., 2007). Based on the use of nanoindentation for other soft materials, this technique is suitable for determining the mechanical properties of thrombi. Furthermore, a comprehensive literature review of the evaluation of thrombi mechanical properties was conducted, and nanoindentation testing has not been utilized as a candidate methodology.
3.2 Mechanical Characterization of Thrombi

As previously mentioned, the formation of thrombi is a complicated biological process. While researchers have studied many aspects of this process, there is still a great deal to be learned. It has been suggested that material properties may be used to describe all thrombi (van Dam et al., 2008). However, as our knowledge increases, new testing methods must be validated to ensure that the measured properties are accurate. Ultimately, the goal of this work is to minimize the pain and suffering of those with the thrombus-related diseases by improving diagnostic techniques and treatment. This work has the potential to lead to more effective and less expensive drug therapies. Current methods for determining the mechanical properties of thrombi include rheometry (Brooks and Easthope, 1981; Evans et al., 2008; Fukada et al., 1984; Kaibara, 1996; Riha et al., 1999; van Dam et al., 2006), ultrasound (Aglyamov et al., 2004; Huang et al., 2005; Ravhon et al., 2001; Wolters et al., 2005; Xie et al., 2004, 2005), and other techniques that utilize custom-made instruments (Hinnen et al., 2007; Wang et al., 2001). A review of these testing methods, along with their disadvantages compared to nanoindentation, will be presented in the following sections.

3.2.1 Rheometry

Rheometry is often used to characterize thrombi due to its ability to easily measure properties of viscoelastic and liquid materials. An advantage of this method is the ability to measure mechanical properties of the entire thrombus at the continuum-level. van Dam et al. (2006) used a shear rheometer with parallel plate geometry to calculate the storage modulus \( G' \) and loss modulus \( G'' \) of luminal and medial thrombi. Thrombi were harvested from human patients undergoing surgical abdominal aortic aneurysm (AAA) repair, where the thrombus diameter ranged from 10 – 14 mm with a wall
thickness of 2 mm. Each thrombus was cut from the surrounding tissue and cored to the appropriate diameter. One advantage of nanoindentation is the use of specimens as small as 3 mm in diameter and 1 mm thick, due to the small size of indenter tips. Both parallel plate shear rheometry and nanoindentation require flat specimens to obtain accurate results. Rheometry compresses specimens using two flat plates with a diameter range of 10 – 14 mm. Therefore, rheometry studies require a flat specimen that has a diameter of 10 mm or greater. Finding a naturally formed flat thrombus in the body with a 10 mm diameter is very difficult, and removing both the top and bottom surfaces of a naturally formed thrombus to make it flat could result in inaccurate or altered measurements of the properties. For nanoindentation, the flat cylindrical indenter tip typically has a diameter of 2 mm or less. Therefore, accurate measurements can be obtained via nanoindentation while testing a flat specimen having a diameter as small as 3 mm. In addition, to reduce the external error in the calculation of the mechanical properties, related to harvesting and cutting the thrombi from the surrounding tissue, the nanoindentation studies for this investigation were designed such that the thrombi were not harvested and transferred to a testing environment; rather they were created in the same container in which testing took place.

3.2.2 Ultrasound

Ultrasound is another common method used to noninvasively determine the mechanical properties of thrombi (Aglyamov et al., 2004; Huang et al., 2005; Raghavan et al., 1996; Xie et al., 2004, 2005). Advantages to ultrasound are that it is noninvasive and can be performed in vivo, thereby minimizing the error from harvesting the tissue; however, measurement error is introduced when reconstructing images and applying a constitutive model to the specimen to calculate the desired properties. Many researchers
who use ultrasound are interested in the change in mechanical properties over time and believe that the ability to measure properties over time has greater significance than the error accrued during the analysis. Aglyamov et al. (2004) have found the elastic modulus of rat thrombi by combining conventional ultrasound with elasticity imaging. Each specimen was scanned one time per day over a period of 10 days. Following this methodology, nanoindentation can also be performed over a period of several days. Between tests, the thrombus would be preserved in an incubator. This is a more direct way to calculate the elastic modulus of the thrombus and removes the additional steps of reconstructing strain images and creating a constitutive model, as commonly used in ultrasound. In addition, ultrasound testing can be more costly than nanoindentation testing.

3.2.3 Other Mechanical Methods

Since it is challenging to test soft viscoelastic materials, such as thrombi, many other custom-made devices have been designed, built, and used. Hinnen et al. (2007) used an automatic dynamic mechanical analyzer to deform a thrombus using a pair of equal and opposite forces (Fig. 3.1). The forces are used to calculate the mechanical properties, specifically the shear modulus, elastic modulus, storage modulus, and loss modulus (Hinnen et al., 2007). This method of determining mechanical properties requires the thrombi to be cut to a uniform shape and glued between two sheets of glass. Gluing both sides to glass may alter the mechanical property measurement. As the specimens are sufficiently thin (i.e., $2.5 - 4.0 \text{ mm}$), it is possible that the results may include the strength of the glue and the thrombus, obscuring the data. Indenting does not require the thrombus to be glued in any way; however, the specimen must be fastened along all outer edges to restrict movement. This does not affect the results due to
Figure 3.1. Schematic of equal and opposite forces ($F$) applied to the specimen during dynamic mechanical analysis as presented by Hinnen et al. (2007). Points $A$ and $B$ move to points $a$ and $b$ while points $E$ and $F$ move to points $e$ and $f$.

the fact that the indent is near the middle of the specimen, far away from the edges.

Wang et al. (2001) used uniaxial tensile testing to determine the mechanical properties of a thrombus. In their study, a computer-controlled microstepping motor pulled two tees apart. Each tee had a clamp attached to it, which was used to hold the specimen. A load cell with a 1 kg capacity was mounted to one tee to measure the applied force. Mechanical properties were then calculated using the measured force. This tensile testing device may have creep and stress-relaxation capabilities, as does the nanoindenter, but each test would require a new sample. Several nanoindentation tests can be performed on the same specimen because the tip diameter can be much smaller than the thrombus diameter. As long as the contact area from the tip does not overlap between tests, multiple indents can be performed on the same specimen.

Xie et al. (2005) developed a mechanical measurement device that compressed the entire sample while measuring force and displacement. A strain gage measured the
compression force while a rectangular pressing stamp compressed the specimen (Fig. 3.2). Using this device, each test requires a new specimen because the entire diameter is compressed. It also assumes that the specimen is initially uniform and will deform uniformly. Actual thrombi are not perfectly round and will most likely have non-uniform deformations, leading to measurement errors. As previously mentioned, nanoindentation minimizes contact length errors by utilizing a flat cylindrical punch with a diameter less than 2.0 mm. When using the mechanical measurement device by Xie et al. (2005), the entire thrombus must align properly with the pressing stamp to avoid tilting of the stamp; however, the nanoindenter only requires an alignment the size of the tip diameter, which is sufficiently smaller than the diameter of the thrombus.

3.3 Nanoindentation

Nanoindentation is a high-precision technique capable of characterizing the mechanical properties of both hard and soft materials. In a typical nanoindentation test, a hard indentation tip, much stiffer than the sample, with known mechanical properties is pressed into a small volume of sample whose mechanical properties are unknown.
Nanoindentation frequently utilizes very small loads (i.e., on the order of \(nN\)), tip sizes (i.e., diameter on the order of \(nm\)), and displacement of the tip into the specimen (i.e., on the order of \(nm\)). This investigation utilizes loads in the \(nN\) range and displacements in the \(nm\) range. The accuracy of nanoindentation depends on the hardness of the material tested and the indenter tip shape. Point indenters with a small contact area are more appropriate for indenting hard materials, while flat punch indenter tips with a large contact area are appropriate for indenting soft materials. During the nanoindentation testing, the load and displacement are recorded. These values are commonly plotted to create a load-displacement curve, which can be used to extract mechanical properties of the sample.

The determination of the elastic modulus via nanoindentation requires isolation of thrombi from their native surrounding tissue; however, the ability to execute several indents on the same specimen and the ability to perform creep and stress-relaxation tests gives rise to nanoindentation as a more versatile option than the aforementioned testing methods. The small, flat tip \(D = 1.75\ mm\) can be used to indent multiple areas of the specimen \(d = 18.7\ mm\) without overlapping indents or disturbing the rest of the specimen.

### 3.3.1 Tip Shape and Size

Nanoindentation can be performed using various indentation tips including Berkovich, spherical, and flat punch tips. Tip selection is crucial, as choosing the wrong tip can result in erroneous measurements. Spherical and Berkovich tips are commonly used in other nanoindentation tests, but are impractical for thrombi because they are classified as point indenters and will pierce through the surface of the specimen (Muto and Sakai, 2004). These tips encounter difficulties identifying the surface and are far less sensitive
Figure 3.3. Schematic of a cylindrical flat punch indenter tip, with radius $R$ and corner radius $R_f$, shown in full contact with a specimen.

to changes between moving through air and moving through soft, viscoelastic materials. A custom-made cylindrical flat punch tip can more readily detect these changes and locate the specimen’s surface due to the constant contact area. This is important, as the contact surface area must be sufficiently larger than any of the constituents of the thrombus. Therefore, the properties of the entire thrombus structure are being measured by an indent. For this investigation, a custom-made Garolite LE cylindrical flat punch tip ($D = 1.75 \, mm$) was used for all tests (Fig. 3.3).

3.3.2 Angle Offset

Previous methods of measuring the mechanical properties of thrombi have the potential to introduce measurement error in the form of either an angle offset or incomplete contact between the thrombus and the compression device. This occurs when the specimen is not completely flat and a portion of the indenter tip contacts the surface before the rest of the tip (nonuniform contact); this is observed when the unloading curve from the indentation test is nonlinear. In nanoindentation, the angle offset is minimized by the scale of the measurement. For a cylindrical flat punch (Fig. 3.3), only a $1.75 \, mm$ diameter contact area must be in full contact, consequently allowing more
measurements per specimen. Other measuring methods require as much as 14 mm of flat contact area to ensure zero angle offset (van Dam et al., 2006).

3.3.3 Adhesion

Another potential difficulty to overcome is molecular adhesion of the thrombus to the Garolite LE indenter tip. The custom-made cylindrical flat punch has a relatively large diameter ($D = 1.75 \text{ mm}$) for nanoindentation, as many tips have a diameter of less than 1 mm, often in the µm range. This results in a large surface area, which can create electro-static forces across the surface. To overcome these forces, a thin coating of human hair-extracted gamma Keratose was applied to the tip. Obtained from Wake Forest University, Keratose adheres for one week without causing corrosion. Since the coating is sterile, no adverse effects between the coating and the thrombi were seen.

3.4 Sample Preparation

The murine (rat) model was chosen due to availability of thrombi and the fact that rats are low on the phylogenetic scale. A research proposal for exsanguination was approved (protocol # 12 – 125) by the Institutional Animal Care and Use Committee (IACUC) at the University of Notre Dame. The animals were housed in the Freimann animal care facility where Freimann Life Science Center (FLSC) personnel cared for them. In case of illness, FLSC personnel followed standard procedures to initiate treatment. Murine blood was obtained by trained FLSC personnel. To obtain enough blood for the study, rats were sacrificed in accordance with the FLSC standard operating procedure (SOP) for euthanasia. Briefly, euthanasia consisted of carbon dioxide followed by exsanguination. The rats did not suffer from chronic pain or distress.

To prepare the thrombus, a 3.8% sodium citrate solution was utilized as the antico-
agulant. It was mixed in a ratio of 9 parts blood to 1 part citrate (pH 7.4). The sodium citrate was drawn in by a sterile 12 ml syringe prior to obtaining blood. Approximately 5 ml of whole blood was then drawn from each rat by cardiac puncture according to the FLSC SOP for cardiac exsanguination of rats. The citrated blood was injected into a 15 ml sterile centrifuge tube and inverted by hand for 2 minutes to ensure that complete mixing occurred. Citrated whole blood was then centrifuged at 100 x g for 5 minutes. The platelet-rich plasma (PRP) (i.e., the top layer of the supernatant) was collected with a micropipette.

In preparation for containing the thrombus, an elastomeric o-ring (d = 18.7 mm) was cemented to a standard glass microscope slide. The PRP was then brought to 37°C using a water bath. A thrombus was formed by adding 45 µl of 2% calcium chloride to 300 µl of PRP. This was hand mixed by inversion of the microcentrifuge tube for 30 s, and pipetted onto the microscope slide inside the o-ring (Fig. 3.4). Immediately, 3 µl (3 NIHU/ml) of 37°C rat thrombin (Enzyme Research Laboratories, South Bend, IN) was pipetted into the PRP/calcium solution to complete the coagulation process. This PRP clot sat at ambient temperature for approximately 2 hours until it became a matte
opaque color and was fully coagulated. For this study, 12 PRP clots were successfully created and each was tested within 12 hours of harvesting the blood. A total of 11 rats (9 female, 2 males) were used.

3.5 Indentation Tests

All nanoindentation tests were performed using a TI 950 TriboIndenter™ (Hysitron, Inc., Minneapolis, MN) as shown in Fig. 3.5. The testing area inside of the nanoindenter is displayed in Fig. 3.6. An indentation test is initiated by first securing the thrombus to the stage. Next, the stage is translated beneath the optics in order to view the thrombus. Once the indentation site is selected, the stage automatically moves back under the flat punch indenter tip for testing. The glass microscope slide was secured on the stage of the indenter using plastic screws (Fig. 3.7). Tests were performed at ambient temperature (25°C) and at atmospheric pressure. Twelve thrombi were created, each being considered as a single specimen (i.e., \( N = 12 \)) and indented at least 7 times, as shown in Fig. 3.8, for a total of 91 indents (Table 3.1). No appreciable differences were observed in the indentation curves between the center and outer indents.

All indentation tests were creep tests with a 1 s loading period, a 5 s holding period, and a 1 s unloading period included in the load function (Fig. 3.9). During the hold-

| TABLE 3.1 |
| SUMMARIZED OF NANOINDENTATION TESTS |

<table>
<thead>
<tr>
<th>Load</th>
<th>30 (( \mu N ))</th>
<th>70 (( \mu N ))</th>
<th>150 (( \mu N ))</th>
<th>250 (( \mu N ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Specimens</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Number of Indents per Specimen</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Total Number of Indents</td>
<td>10</td>
<td>18</td>
<td>7</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 3.5. Photograph of the TI 950 Triboindenter™ (Hysitron, Inc., Minneapolis, MN).
Figure 3.6. Experimental setup of the inside of the nanoindenter. The stage with the thrombus attached moves underneath the optics for viewing. It then moves back underneath the flat punch indenter tip for testing.

Figure 3.7. Experimental setup of the nanoindenter stage with the glass microscope slide secured by 6 plastic screws.
Figure 3.8. Schematic of potential indentation positions for one PRP specimen that was indented 7 times. Each “X” represents an indent.

Figure 3.9. Plot of the loading functions used during nanoindentation testing.

During the holding period of a creep test on a soft material, the indenter tip will further penetrate the specimen in order to maintain the constant maximum load. If the travel of the indenter tip exceeds the maximum allowable penetration of the indenter (5 μm), then an error occurs and the test is aborted. Determining the maximum allowable load for a creep test...
without a testing error is important to ensure that the tests are accurate and repeatable. It also ensures that complete contact occurs between the thrombus and flat punch tip. The maximum loads were tested incrementally at 30 $\mu N$, 70 $\mu N$, 150 $\mu N$, and 250 $\mu N$ (Table 3.1). The (un)loading rate was 30 $\mu N/s$, 70 $\mu N/s$, 150 $\mu N/s$, and 250 $\mu N/s$ for the 30 $\mu N$, 70 $\mu N$, 150 $\mu N$, and 250 $\mu N$ tests, respectively. Tests were also performed at loads higher than 250 $\mu N$; however, all tests at these loads encountered errors and those data are not presented. When comparing the results from the aforementioned tests, it was found that the measurement noise was minimized at a maximum load of 250 $\mu N$. Therefore, 8 specimens were tested at the maximum allowable load of 250 $\mu N$ and the data analysis was primarily focused on this group of tests. Note that the tests performed here are considered nanoindentation tests because no other testing device has the ability to perform tests in the nN range for both load and displacement (i.e., 250 $\mu N = 250,000$ nN and 5 $\mu m = 5,000$ nm).

3.6 Elastic Contact Theory

Elastic contact theory was used to estimate the elastic modulus. According to this theory, the elastic modulus is calculated as

$$ E = \left( \frac{3}{4D} \right) \left( \frac{P}{h} \right), $$

(3.1)

where $D$ is the punch diameter, $P$ is the load, and $h$ is the indentation depth. This equation assumes that Poisson’s ratio $\nu = 0.5$, which is reasonable for thrombi (Maier et al., 2010). It also assumes that the nanoindenter cylindrical flat punch tip is perfectly rigid, the PRP clot is isotropic, and the elastic portion of the stress-strain curve fully recovers (Juliano et al., 2006). The quantity $P/h$ represents the stiffness, and is also the slope of a (un)loading curve of a creep test (Fig. 3.10). The loading curve was used in
Figure 3.10. Representative plot of the mean load-displacement curve for one specimen, tested under a maximum load of 250 $\mu$N.

this investigation to calculate the slope because it was linear and adhesion was observed during unloading, resulting in a nonlinear unloading curve.

3.7 Results

Mechanical properties of a rat thrombus have been obtained to demonstrate feasibility of the characterization of this material via nanoindentation. The indentation tests performed on a specimen ($N \geq 7$) were averaged to create the mean load-displacement curve for that specimen (i.e., one curve was obtained for each specimen). A representative plot of the mean load-displacement curve for one specimen tested under a maximum load of 250 $\mu$N is displayed in Fig. 3.10. The slope ($P/h$) of the entire loading curve from each mean load-displacement plot was used to calculate the elastic
TABLE 3.2

THROMBUS ELASTIC MODULUS VALUES CALCULATED VIA
ELASTIC CONTACT THEORY FOR VARYING LOADS

<table>
<thead>
<tr>
<th>Max Load (µN)</th>
<th>Number of Specimens</th>
<th>Elastic Modulus $E$ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1</td>
<td>43.7</td>
</tr>
<tr>
<td>70</td>
<td>2</td>
<td>26.3, 30.1</td>
</tr>
<tr>
<td>150</td>
<td>1</td>
<td>71.9</td>
</tr>
<tr>
<td>250</td>
<td>8</td>
<td>55.1 ± 12.1</td>
</tr>
</tbody>
</table>

modulus for each specimen via the elastic contact theory (Eq. 3.1). Values of the elastic modulus are reported in Table 3.2. A standard deviation was calculated for the specimens that were tested at the maximum load (Table 3.1). The modulus values are in the range of soft materials (on the order of kPa), as expected.

3.8 Discussion

Elastic modulus values of thrombi reported in the literature vary widely from $1.7 \cdot 10^{-6}$ kPa to 5,134 kPa (Table 3.3). As one would expect, the testing method greatly affects the modulus values. Using a rheometer or viscometer results in modulus values less than 10 kPa (Henderson and Thurston, 1993; Riha et al., 1999; Ryan et al., 1999; van Dam et al., 2006). Custom-made instruments and compression testing yield modulus values between $10 - 1000$ kPa (Hinnen et al., 2007; Xie et al., 2005). Finally, tensile testing results in modulus values between $100 - 5000$ kPa (Di Martino et al., 1998; Raghavan et al., 1996; Xiong et al., 2008). The compression tests performed by Xie et al. (2005) most closely resemble the nanoindentation testing presented in this investigation. Xie et al. (2005) created a deep vein thrombosis in rats, harvested the thrombus, and tested it in compression using a pressing stamp (Fig. 3.2). During
their tests, the force and displacement were measured. The differences between Xie et al. (2005) tests and the results presented in this study are: 1) the composition of the thrombi and 2) the actual testing apparatus. After 14 days, the mean elastic modulus for the Xie et al. (2005) experiment was $82.83 \pm 37.78 \text{kPa}$. While the average value of the elastic modulus for this investigation ($55.1 \pm 12.1 \text{kPa}$) is lower than Xie et al. (2005), it is within the same order of magnitude. The average elastic modulus is also well within the observed range of elastic moduli values (Table 3.3). Therefore, it is reasonable to conclude that the calculated average elastic modulus value is consistent with values reported in the literature.

It can be seen that the unloading curve from the indentation test is nonlinear (Fig. 3.10). Adhesion and incomplete indenter tip contact with the specimen are potentially the main contributors to the nonlinear behavior observed. While adhesion was minimized by coating the tip with Keratose, it was not fully eliminated. Incomplete indenter tip contact could also be a possible contributor to the nonlinear unloading curve, as this would cause an angle offset between the tip and thrombus, which may have occurred during unloading of the specimen.

Typically, nanoindentation is used on hard materials (elastic modulus on the order of $\text{GPa}$), such as cortical and trabecular bone (Wang et al., 2006). Potential difficulties exist with using nanoindentation on soft materials (elastic modulus on the order of $\text{kPa}$); however, this technique has successfully been used to indent soft hydrogels (average elastic modulus on the order of $10 \text{kPa}$) (Liu et al., 2009) and now thrombi.

3.9 Limitations

In the vascular system, the blood clot and its immediate environment are at a temperature of $37^\circ C$; however, the testing stage of the indenter is not temperature controlled.
Therefore, the actual temperature of the testing environment of the thrombus was $25^\circ C$ (i.e., room temperature). No noticeable problems were observed during testing (such as adhesion, drying, or cracking of the thrombus).

3.10 Conclusions

Nanoindenting thrombi is a novel method used in this investigation to determine the elastic modulus $E$ of thrombi. This was accomplished by using the mean load-displacement nanoindentation curve for each specimen and elastic contact theory. The maximum testing load ($250 \mu N$) was determined via a sequential search method. Indentation tests were successfully performed on 8 specimens at the maximum load, with 7 indents per specimen. The mean elastic modulus of the thrombi, calculated by elastic contact theory, was $55.1 \pm 12.1 \, kPa$, which is within the range of values reported in the literature.
<table>
<thead>
<tr>
<th>Author</th>
<th>Blood Type</th>
<th>Description of Thrombus</th>
<th>Test Method</th>
<th>Elastic Modulus ($kPa$)</th>
</tr>
</thead>
</table>
| Slaboch (current investigation) | Murine     | Created PRP clots by adding calcium and thrombin                                        | Nanoindention                | ECT: $E = 55.1 \pm 12.1$
|                               |            |                                                                                       |                              | SLS: $E_1 = 5.62 \pm 1.37$, $E_2 = 21.34 \pm 4.31$
|                               |            |                                                                                       |                              | KV: $E = 24.5$                                               |
| Henderson and Thurston (1993)  | Human      | Created whole blood and plasma clots by combining whole blood or plasma with bovine thrombin | Viscometer                   | $1.7 \cdot 10^{-6}$                                         |
| McCarty et al. (2011)         | Bovine     | Created thrombi by adding calcium.                                                      | Confined compression         | Bovine: $0.05 - 0.4$
|                               | Rabbit     |                                                                                       |                              | Rabbit: $0.1 - 0.75$                                         |
| Riha et al. (1999)            | Human      | Created human thrombi by combining PRP with calcium chloride.                           | Rheometer                    | $0.58$                                                      |
| Schmitt et al. (2011)         | Porcine    | Created whole blood clots by adding calcium.                                            | Dynamic ultrasound elastography | SLS: $E_1 = 0.718 \pm 0.122$, $E_2 = 0.521 \pm 0.246$
<p>|                               |            |                                                                                       |                              | KV: $E = 0.998 \pm 0.117$                                   |</p>
<table>
<thead>
<tr>
<th>Author</th>
<th>Blood Type</th>
<th>Description of Thrombus</th>
<th>Test Method</th>
<th>Elastic Modulus (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ryan et al. (1999)</td>
<td>Human</td>
<td>Created human thrombi by combining varying amounts of fibrinogen, thrombin, calcium chloride, and FXIIIa.</td>
<td>Rheometer</td>
<td>1.5</td>
</tr>
<tr>
<td>van Dam et al. (2006)</td>
<td>Human</td>
<td>Surgically removed thrombi from AAA patients.</td>
<td>Rheometer</td>
<td>5.8</td>
</tr>
<tr>
<td>Hinnen et al. (2007)</td>
<td>Human</td>
<td>Surgically removed thrombi from AAA patients.</td>
<td>Dynamic mechanical analysis</td>
<td>45</td>
</tr>
<tr>
<td>Xie et al. (2005)</td>
<td>Rat</td>
<td>Created DVT in rats and performed <em>ex vivo</em> mechanical measurements.</td>
<td>Compression</td>
<td>83</td>
</tr>
<tr>
<td>Author</td>
<td>Blood Type</td>
<td>Description of Thrombus</td>
<td>Test Method</td>
<td>Elastic Modulus (kPa)</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------</td>
<td>-------------------------------------------------------------</td>
<td>---------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Di Martino et al. (1998)</td>
<td>Human</td>
<td>Surgically removed thrombi from AAA patients and tested them using an instron.</td>
<td>Tensile test</td>
<td>131</td>
</tr>
<tr>
<td>Xiong et al. (2008)</td>
<td>Human</td>
<td>Surgically removed thrombi from AAA patients.</td>
<td>Tensile test</td>
<td>5000</td>
</tr>
<tr>
<td>Raghavan et al. (1996)</td>
<td>Human</td>
<td>Surgically removed thrombi from AAA patients.</td>
<td>Tensile test</td>
<td>5134</td>
</tr>
</tbody>
</table>
CHAPTER 4

FINITE ELEMENT MODELING

4.1 Overview

The creep data from the nanoindentation tests were analyzed with an inverse finite element (FE) simulation tool using rheological models (i.e., Kelvin-Voigt (KV) and Standard Linear Solid (SLS)). These models were coded as user-defined subroutines (UMAT) in ABAQUS® (ABAQUS Inc., Providence, RI). An optimization routine in MATLAB® (The MathWorks, Inc., Natick, MA) was used in combination with the ABAQUS® FE code to automatically fit the experimental data. Values of the mechanical properties from the two-parameter KV model ($E$, $\eta$) and three-parameter SLS model ($E_1$, $E_2$, $\eta$) were obtained from these simulations. In addition, a sensitivity analysis was then performed on the SLS data.

4.2 Background

The FE method is a useful tool for analyzing complex materials with complicated fluid-structure interactions. In the context of thrombi, a fluid structure interaction occurs between the thrombus and the arterial wall. Some studies are focused on the blood flow around the thrombus (Dai et al., 1999; Khanafer et al., 2006), whereas other studies are focused on the thrombus itself and model its geometry (Borghi et al., 2006) as well as changes in the shear mechanical properties throughout the thickness of the
thrombus (van Dam et al., 2008). Several FE models involving thrombi are concerned with the vessel wall stress and investigate aortic rupture associated with abdominal aortic aneurysms (AAA) (Arab-Ghanbari et al., 2009; Vande Geest et al., 2006; Wolters et al., 2005). Vande Geest et al. (2006) included the intra-luminal thrombus in the simulation of an AAA to determine the affects it had on the rupture of the aorta. Wolters et al. (2005) used a fluid/solid FE mesh to simulate the interaction between AAA blood dynamics and AAA wall mechanics.

Other researchers have developed FE models that are related to deep vein thrombosis (DVT), focusing on treatment and preventative therapies. Swaminathan and Hu (2006) modeled the flow impedance and the clot-capturing efficiency of a vena cava filter. Narracott et al. (2009) used a FE simulation to model calf compression with an external pressure cuff. Arab-Ghanbari et al. (2009) modeled turbulent flow in an artery and determined its relationship to wall stress and thrombus rupture leading to pulmonary embolisms (PE). However, their work focused solely on the fluid-structure interactions and did not consider the affects of the mechanical properties of the thrombus (i.e., elastic modulus, viscosity).

While the aforementioned FE simulations are related to thrombi, none of them fit the clotting behavior to rheological models. Schmitt et al. (2011) characterized thrombi using several viscoelastic models including Maxwell, Kelvin-Voigt, Jeffrey, Zener (i.e., SLS), and generalized Maxwell. Incidentally, the study performed by Schmitt et al. (2011) is very similar to the current investigation. There are three main differences between these two studies: 1) Schmitt et al. (2011) used porcine blood, whereas this investigation used murine blood, 2) Schmitt et al. (2011) created thrombi with whole blood and calcium, whereas this investigation created thrombi with platelet-rich plasma (PRP), calcium, and thrombin, and 3) Schmitt et al. (2011) used dynamic ultrasound
elastography for testing, whereas this investigation utilized nanoindentation testing.

4.3 Significance

A thrombus is a time-dependent viscoelastic structure and modeling it accurately presents many challenges. Most researchers who develop models to study thrombi are interested in fluid-flow interactions. However, this research is focused on the mechanical properties of thrombi. To the best of the author’s knowledge, this is the first investigation to utilize nanoindentation to determine the mechanical properties of thrombi (Slaboch and Ovaert, 2009).

For this investigation, the loading curve and holding curve components are simulated and used to calculate the mechanical properties of thrombi. This is due to the adhesion and incomplete contact affects that occurred during the unloading period. In this case, adhesion in the form of electrostatic forces contributed to the nonlinearity of the unloading curve. While adhesion was minimized by coating the indenter tip, it was not eliminated in this study. As previously mentioned, the indenter flat punch tip must be in complete contact with the sample to minimize measurement errors. This is evidenced by a linear unloading curve; however, since the indenter tip used in this investigation was large for a nanoindentation test ($D = 1.75 \, mm$), complete contact was not observed during the unloading period. The inability to maintain complete contact was in part due to the highly viscoelastic nature of the thrombus. If complete contact would have been observed, the loading and unloading curves would have been almost parallel to each other and have approximately the same slope. For this investigation, it was assumed that the slope of the loading curve characterized the elastic modulus of the thrombus. Therefore, for this portion of the investigation, the loading curve and holding curve components of the indentation results are simulated via FE and used to calculate
the mechanical properties of interest. Another reason for simulating the loading and holding curves is that all of the mechanical properties (elastic modulus, elastic spring constant, and viscosity) can be evaluated using these portions of the experimental data.

As a proof-of-concept model, a simple two-parameter KV model was used to calculate and optimize the elastic modulus and viscosity. To further minimize the error between the experimental and computational results, a three-element SLS model was then used. The three-element viscoelastic SLS rheological finite element model has been used to estimate mechanical properties of other soft viscoelastic materials (elastic modulus on the order of $10 \, kPa$), such as hydrogels (Liu et al., 2009). Based on its previous use, the SLS model was adapted for studying thrombi, to more accurately characterize and optimize the elastic modulus, elastic spring constant, and viscosity.

4.4 Finite Element (FE) Model

A 2D axisymmetric, poroviscoelastic FE model was developed using the SOILS analysis in ABAQUS®. The model consisted of a PRP clot and the flat punch indenter tip. Two-node axisymmetric rigid elements were used for the rigid indenter tip (RAX2) and four-node axisymmetric hybrid quadrilateral elements with pore pressure (CAX4P) were used for the thrombus. Frictionless contact was assumed and a zero pore pressure condition was set on the thrombus surface.

One difficulty in the FE simulation is accurately modeling around the corner (edge) of the indenter tip, which has a radius of $R_f = 20 \, \mu m$. The mesh near the corner of the indenter was refined to ensure accuracy of the results (Fig. 4.1). A total of 4,516 elements were used for the thrombus. The centerline of the thrombus was constrained against movement in the $x$-direction, while the bottom was constrained against movement in the $y$-direction, as defined by the coordinate system (Fig. 4.2). The upper and
right edges of the thrombus were defined as permeable. The indenter tip was con-
strained against movement in the $x$-direction and also constrained against all rotations.
Loading conditions for the input file were the same as the nanoindentation tests, with
a maximum load of 250 $\mu N$, a (un)loading time of 1 s and a holding time of 5 s. The
input file for these modeling configurations was run in conjunction with a user-defined
material (UMAT) model for either the KV or SLS model.

Figure 4.1. Axisymmetric finite element mesh with flat punch indenter.

Figure 4.2. Coordinate system used in ABAQUS® for the FE simulation.
4.5 Biphasic Theory

The thrombus is modeled as a biphasic material consisting of a porous solid medium and a fluid. Development of the constitutive model can be found in Liu (2009). For a generic biphasic material, the solid phase can be described by any solid continuum theory such as elasticity or viscoelasticity. In this case, the solid phase of the thrombus is viscoelastic. Driven by a pore pressure gradient, fluid can flow through the porous solid medium, as described by Darcy’s Law. Both the solid and the fluid phases bear the total load on the biphasic material.

4.6 Permeability

Permeability of the solid medium is an important parameter in the fluid flow behavior. As the permeability increases, it is easier for fluid to flow through the solid medium. A range of permeability values were input into the FE simulation. These values were calculated from the Carman-Kozeny equation

\[ K = \frac{\epsilon^2}{(1 - \epsilon)^2} \left( \frac{1}{k \left( \frac{S_p}{V_p} \right)^2} \right), \quad (4.1) \]

where \( \epsilon \) is the porosity, \( S_p \) and \( V_p \) are the surface area and volume of the elementary particles, and \( k \) is Kozeny’s constant as tabulated in Happel and Brenner (1965). This relation can be rewritten in terms of the radius of the elementary particles \( R \), such that

\[ K' = \frac{\epsilon^3}{(1 - \epsilon)^2} \left( \frac{R^2}{9k} \right), \quad (4.2) \]

It is assumed that the radius \( R = 10^{-12} m \) (Basmadjian, 1984). Using Happel and Brenner’s (1965) tabulated porosity and Kozeny constant values, a range of permeabil-
TABLE 4.1
CALCULATED PERMEABILITY VALUES

<table>
<thead>
<tr>
<th>Permeability $K$ ($mm^2$)</th>
<th>Porosity $\epsilon$</th>
<th>Kozeny constant $k$</th>
<th>Void ratio $e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.5051 \cdot 10^{-5}$</td>
<td>0.99</td>
<td>71.63</td>
<td>99</td>
</tr>
<tr>
<td>$7.1429 \cdot 10^{-7}$</td>
<td>0.9</td>
<td>11.34</td>
<td>9</td>
</tr>
<tr>
<td>$1.9698 \cdot 10^{-7}$</td>
<td>0.8</td>
<td>7.22</td>
<td>4</td>
</tr>
<tr>
<td>$7.3136 \cdot 10^{-8}$</td>
<td>0.7</td>
<td>5.79</td>
<td>2.33</td>
</tr>
<tr>
<td>$2.9354 \cdot 10^{-8}$</td>
<td>0.6</td>
<td>5.11</td>
<td>1.5</td>
</tr>
<tr>
<td>$1.1721 \cdot 10^{-8}$</td>
<td>0.5</td>
<td>4.74</td>
<td>1</td>
</tr>
<tr>
<td>$4.3509 \cdot 10^{-9}$</td>
<td>0.4</td>
<td>4.54</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Permeability values were calculated (Table 4.1).

The ABAQUS® input file requests permeability and void ratio values. Since no fluid flow was seen leaking out from underneath the indenter tip during testing, the initial permeability value was assumed to be the lowest possible value, $4.3509 \cdot 10^{-9} mm^2$, with the least fluid flow. The void ratio (Table 4.1) for each permeability value was calculated using

$$e = \frac{\epsilon}{(1 - \epsilon)^2}, \quad (4.3)$$

where $e$ is the void ratio and $\epsilon$ is the porosity.

4.7 Optimization-based Inverse FE Technique

MATLAB® was used to optimize the material property inputs to the ABAQUS® simulation, to minimize the error between the simulated and experimental results. An inverse FE technique identifies the parameters of the constitutive model. The minimized objective function is the squared error between the experimental and simulated load vs.
Figure 4.3. MATLAB®-based finite element optimization loop as depicted by Liu (2009).

displacement curves. The MATLAB® optimization toolbox provides the function fmincon, which determines the minimum of a constrained nonlinear multivariable function using a sequential quadratic programming algorithm. Thus, the optimization code is used to drive the parameter changes for the thrombus simulations. Lower and upper bound estimates of the material parameters are provided to the optimization routine as constraints. The optimization loop is an ensemble of subroutines coded in MATLAB® that calls (iterates) the ABAQUS® FE creep model (Fig. 4.3). Using an n-dimensional parameter space, the code iterates until a satisfactory correspondence between the experiment and the simulation is achieved. The result of this MATLAB®/ABAQUS® program provides the optimized material parameters.

4.8 Kelvin-Voigt (KV) Model

As a proof-of-concept study, a two-element KV viscoelastic model was developed, which consists of a linear spring in parallel with a dashpot (Fig. 4.4). The KV material
behavior is simulated in ABAQUS® using a UMAT model. There are four initial parameters to set ($E$, $\eta$, $\nu$, $K$). The elastic modulus value calculated from elastic contact theory is set as the initial elastic modulus in the inverse FE simulation. The initial viscosity was assigned an initial value of $\eta = 2.25 \text{ kPa-s}$. Poisson’s ratio is assumed to be $\nu = 0.49$ (Maier et al., 2010), and the permeability $K$ ranged from $1.5051 \cdot 10^{-5} \text{ mm}^2$ to $4.3509 \cdot 10^{-9} \text{ mm}^2$ (Basmadjian, 1984). Two material parameters (elastic modulus $E$ and viscosity $\eta$) are optimized and become input values to the FE simulation.

As previously mentioned, only the loading and holding curves were analyzed due to the thrombi adhesion, incomplete contact of the indenter tip, and the fact that the mechanical properties can be optimized using these portions of the curve. To decrease the computational cost and increase the stability of the simulations, an average curve for each specimen was created and used to demonstrate feasibility of the combined indentation-finite element characterization method. Since this method is a proof-of-concept study, only one average specimen curve was analyzed using the KV model.
4.9 KV Results and Discussion

A representative plot of the experimental data and final (optimized) FE simulation result using a two-parameter KV model is shown in Fig. 4.5. As illustrated, this material model was unable to fully capture the loading behavior. The optimized elastic modulus was $E = 24.5 \text{ kPa}$ and the optimized viscosity was $\eta = 9.00 \text{ kPa}\cdot\text{s}$. Note that the average elastic modulus value determined from the FE simulation ($24.5 \text{ kPa}$) is on the same order of magnitude to that obtained via elastic contact theory ($55.1 \pm 12.1 \text{ kPa}$). Both of these elastic modulus values are also within the observed range of elastic modulus values (Table 3.3), and are the same order of magnitude as other compression tests (Hinnen et al., 2007; Xie et al., 2005). Therefore, the results of this investigation are consistent with reported values from literature. In addition, the difference between the viscosity and elastic modulus is one order of magnitude, which is comparable to differences observed by others (Henderson and Thurston, 1993; Schmitt et al., 2011).

4.10 Standard Linear Solid (SLS) Model

To minimize the error between the computational and experimental data, a three-element viscoelastic rheological model (i.e., SLS model) was developed, which consists of a linear spring in parallel with a Maxell element (Fig. 4.6). The SLS material behavior is simulated in ABAQUS® using a UMAT model. There are five initial parameters to set ($E_1$, $E_2$, $\eta$, $\nu$, $K$). The elastic modulus value calculated from elastic contact theory was set to the initial elastic modulus $E_1$ and the elastic spring constant $E_2$ in the inverse FE simulation. The initial viscosity was assigned an initial value of $\eta = 2.25 \text{ kPa}\cdot\text{s}$. Poisson’s ratio was assumed to be $\nu = 0.49$ (Maier et al., 2010), and the permeability ranged from $1.5051 \cdot 10^{-5} \text{ mm}^2$ to $4.3509 \cdot 10^{-9} \text{ mm}^2$ (Basmadjian, 1984). Three material parameters (elastic modulus $E_1$, elastic spring constant $E_2$, and
Figure 4.5. FE simulation (black line with circles) corresponds to indentation results of a thrombus (solid blue line) for the two-parameter KV model. Note that the parameters listed at the top of the figure are [elastic modulus $E$ (MPa) and viscosity $\eta$ (MPa⋅s)].
Figure 4.6. Schematic of the standard linear solid (SLS) three-element viscoelastic model consisting of a Maxwell element in parallel with a linear spring. $E_1$ is the elastic modulus, $E_2$ is the elastic spring constant, and $\eta$ is the viscosity.

viscosity $\eta$) are optimized and become input values to the fluid-structure FE simulation. Again, only the loading and holding curves were analyzed due to the thrombi adhesion, incomplete contact of the indenter tip, and the fact that the mechanical properties can be optimized using only these portions of the curve. The average curves from each of the eight specimens, tested at a maximum load of 250 $\mu N$, were simulated using ABAQUS® with the aforementioned UMAT for describing the behavior of the SLS model.

4.11 SLS Results and Discussion

A representative plot of the experimental data and final (optimized) FE simulation result using a three-parameter SLS model is shown in Fig. 4.7. Average values of optimized elastic modulus ($E_1 = 5.62 \pm 1.37$ kPa), elastic spring constant ($E_2 = 21.34 \pm 4.31$ kPa), and viscosity ($\eta = 209.84 \pm 70.25$ kPa·s) from the SLS viscoelastic model were obtained from these simulations. Note that the average elastic modulus value and the elastic spring constant determined from the FE simulation are well within
Figure 4.7. FE simulation (black line with circles) corresponds to indentation results of a thrombus (solid blue line) for the three-parameter SLS model. Note that the parameters listed at the top of the figure are [elastic modulus $E_1$ (MPa), elastic spring constant $E_2$ (MPa), viscosity $\eta$ (MPa·s), and Poisson’s ratio].
the observed range (Table 3.3) and are within one order of magnitude of the value obtained using elastic contact theory \( E = 55.1 \pm 12.1 \text{ kPa} \). In addition, the difference between the viscosity and elastic modulus for the SLS model is two orders of magnitude, which is comparable to differences observed by others (Henderson and Thurston, 1993). The viscosity values reported in this investigation are several orders of magnitude higher than the values reported in the literature (Table 4.4). There are several reasons for this difference. Few studies report viscosity values due to the difficulty in performing this measurement. Most studies determine the viscosity of a PRP clot that is considered to be a viscoelastic fluid; however, the PRP clots used in this investigation are considered to be viscoelastic solids. All of the prior research that determined viscosity values have utilized testing methods that either have vibrations (elastography) or fluid flow (viscometer). The thrombi in this investigation were not agitated in any way. Therefore, this investigation is the first report of viscosity values of static thrombi.

A summary of the comparable data between this investigation and the study by Schmitt et al. (2011) is given in Table 4.2. Note that the SLS model is commonly called the Zener model, which is the notation used by Schmitt et al. (2011). The differences between the elastic moduli \( E, E_1, \) and \( E_2 \) for the two studies are one to two orders of magnitude. Since the variability in elastic modulus values can encompass over six orders of magnitude (Table 3.3), these differences are reasonable. There are six orders of magnitude difference between the viscosity values reported by Schmitt et al. (2011) and the viscosity values reported in this investigation. Reasons for this difference are stated in the preceding paragraph. Also, fewer investigations report viscosity values than those that report elastic modulus values. There have been data reported with six orders of magnitude difference between elastic modulus values. The difference between these modulus values is acceptable because thrombi are time-dependent,
<table>
<thead>
<tr>
<th>Model</th>
<th>Parameter</th>
<th>This Investigation</th>
<th>Schmitt et al. (2011) Investigation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastic Contact Theory</td>
<td>$E (kPa)$</td>
<td>$55.1 \pm 12.1$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\eta (kPa \cdot s)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kelvin-Voigt</td>
<td>$E (kPa)$</td>
<td>$24.5$</td>
<td>$0.998 \pm 0.117$</td>
</tr>
<tr>
<td></td>
<td>$\eta (kPa \cdot s)$</td>
<td>$9.00$</td>
<td>$3.3 \cdot 10^{-4} \pm 1.2 \cdot 10^{-4}$</td>
</tr>
<tr>
<td>Standard Linear Solid</td>
<td>$E1 (kPa)$</td>
<td>$5.62 \pm 1.37$</td>
<td>$0.718 \pm 0.122$</td>
</tr>
<tr>
<td></td>
<td>$E2 (kPa)$</td>
<td>$21.34 \pm 4.31$</td>
<td>$0.521 \pm 0.246$</td>
</tr>
<tr>
<td></td>
<td>$\eta (kPa \cdot s)$</td>
<td>$209.84 \pm 70.25$</td>
<td>$1.01 \cdot 10^{-3} \pm 9.1 \cdot 10^{-4}$</td>
</tr>
</tbody>
</table>

viscoelastic materials. Likewise, it is possible that viscosity values can differ by six orders of magnitude depending on the blood type used, the type of thrombus created, and the testing method.

4.1.2 Sensitivity Analysis

The optimized material parameters are obtained by minimizing the difference between the FE simulation and the nanoindentation test data. In order to determine which material parameters had the greatest affect on the nanoindentation simulation, a sensitivity analysis was conducted.

Different approaches to conducting a sensitivity analysis exist. For example, the entire simulation curve can be compared with the entire nanoindentation curve and an overall error can be calculated (i.e., $R^2$ value). However, two simulations could potentially display very different overall behaviors, yet have the same $R^2$ value. For this reason, the sensitivity analysis compared material parameters while looking at four key characteristics of typical creep curves: the initial indentation depth at the maximum
TABLE 4.3

OPTIMIZED MATERIAL PARAMETERS

<table>
<thead>
<tr>
<th>Elastic Modulus</th>
<th>Elastic Spring Constant</th>
<th>Viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_1 ,(kPa)$</td>
<td>$E_2 ,(kPa)$</td>
<td>$\eta ,(kPa\cdot s)$</td>
</tr>
<tr>
<td>5.875</td>
<td>28.75</td>
<td>148.75</td>
</tr>
</tbody>
</table>

load ($IID$), the total creep displacement during the holding period ($TCD$), the final indentation depth at the maximum load ($FID$), and the initial loading slope ($ILS$) (Fig. 4.8). The IID analysis examined the location of the point where the loading and holding curves meet. The TCD analysis examined the length of the holding curve. The FID analysis examined the location of the ending point of the holding curve. Finally, the ILS analysis examined the slope of the loading curve for the simulation. Each of the three material parameters (elastic modulus $E_1$, elastic spring constant $E_2$, and viscosity $\eta$) was varied $\pm$ 50%, independently, from the optimized values given in Table 4.3. The percent change in the curve characteristics ($IID$, $TCD$, $FID$, $ILS$) was computed as a function of the percent change in the individual parameters ($E_1$, $E_2$, $\eta$).

4.13 Sensitivity Analysis Results and Discussion

Results from the sensitivity analysis can be seen in Fig. 4.9 (IID), Fig. 4.10 (TCD), Fig. 4.11 (FID), and Fig. 4.12 (ILS). The elastic spring constant ($E_2$) had the greatest affect (up to 60%) on the initial indentation depth at the maximum load (IID). Changes in the elastic modulus and viscosity values altered the IID by $\pm$ 10%. Changes in the total creep displacement (TCD) were greatly influenced by the initial viscosity value (up to 20%). Minimal changes in creep displacement were observed when either the
Figure 4.8. Identification and location of four key characteristics used in the sensitivity analysis.

Figure 4.9. Sensitivity results for the SLS model showing the percent change in the initial indentation depth (IID) at the maximum load as a function of the percent change in the material parameters. Note that $E_1 =$ elastic modulus (blue line with circles), $E_2 =$ elastic spring constant (red line with squares), and $ETA =$ viscosity (green line with traingles).
Figure 4.10. Sensitivity analysis for the SLS model showing the percent change in the total creep displacement (TCD) as a function of the percent change in the material parameters. Note that $E_1 =$ elastic modulus (blue line with circles), $E_2 =$ elastic spring constant (red line with squares), and $ETA =$ viscosity (green line with triangles).

Figure 4.11. Sensitivity analysis for the SLS model showing the percent change in the final indentation depth (FID) at the maximum load as a function of the percent change in the material parameters. Note that $E_1 =$ elastic modulus (blue line with circles), $E_2 =$ elastic spring constant (red line with squares), and $ETA =$ viscosity (green line with triangles).
Figure 4.12. Sensitivity analysis for the SLS model showing the percent change in the initial loading slope (ILS) as a function of the percent change in the material parameters. Note that $E_1 =$ elastic modulus (blue line with circles), $E_2 =$ elastic spring constant (red line with squares), and $ETA =$ viscosity (green line with triangles).

According to this sensitivity analysis, the elastic spring constant had the greatest affect on the percent change of the curve characteristics, which is reasonable because this term was the dominant stiffness term in the SLS model. Recall that the elastic spring constant represents the stiffness of the viscoelastic behavior, and thrombi are highly viscoelastic materials. The viscosity had the second greatest affect and the elastic modulus had a minimal affect on predicted material behavior of the simulation.
4.14 Limitations

Thrombi are extremely difficult to model accurately due to their viscoelastic nature. The SLS model is able to better model the experimental data than the KV model because the additional spring allows the model to follow the initial loading curve. A higher-order model, having at least three springs and two dashpots, could potentially improve the curve fit; however, this was not incorporated into the model presented here due to computational time restrictions.

4.15 Conclusions

Determination of murine thrombus properties has been accomplished using a combined technique of experimental nanoindentation and inverse FE modeling. Close agreement between the finite element model and the experimental data was attained (Fig. 4.7). The elastic modulus data and elastic spring constant are in good agreement between a simplified elastic solution ($E = 55.1 \pm 12.1 \, kPa$) and the inverse FE results for the SLS model ($E1 = 5.62 \pm 1.37 \, kPa$, $E2 = 21.34 \pm 4.31 \, kPa$). They are also in agreement with values reported in the literature (Table 3.3). In addition, the order of magnitude differences observed between the elastic modulus and viscosity values are in agreement with observations from the literature (Henderson and Thurston, 1993). The viscosity values reported in this investigation (KV: $\eta = 9.00 \, kPa\cdot s$, SLS: $\eta = 209.84 \pm 70.25 \, kPa\cdot s$) are 4 – 6 orders of magnitude higher than reported values (Table 4.4). This is reasonable because highly viscoelastic materials are difficult to characterize. The reported elastic modulus values (Table 3.3) range six orders of magnitude; in the same manner, it is feasible for viscosity values to range six orders of magnitude. In addition, the thrombi tested in this investigation are viscoelastic solids (not viscoelastic fluids, as reported in previous studies) and were not agitated in any way during testing.
The differences in thrombi creation methods and testing methods also explain the high viscosity values. Since it is difficult to measure the viscosity of the thrombus, not as many investigations report viscosity values as those who report elastic modulus values. The magnitude difference in elastic modulus values changes based on testing methods. Rheometers and viscometers report low elastic modulus values \( E = 1.7 \cdot 10^{-6} - 5.8 \, kPa \), compression devices report higher values \( E = 45 - 83 \, kPa \), and tensile tests report the highest values \( E = 131 - 5134 \, kPa \). In the same manner, the range of viscosity values can differ based on the testing method. All of the values reported in the literature are derived from a viscometer-type testing method (Table 4.4). This investigation is the first report of a thrombus viscosity value using a compression test. It is important to note that compression test elastic modulus values were higher than viscometer test elastic modulus values (Table 3.3). In the same manner, compression test viscosity values are higher than viscometer test viscosity values. Thus, the mechano-rheological properties derived from this method may be used in more advanced thrombus formation, fluid-structure interaction, and thrombus dissociation simulations.
<table>
<thead>
<tr>
<th>Author</th>
<th>Blood Type</th>
<th>Description of Thrombus</th>
<th>Test Method</th>
<th>Time (hrs)</th>
<th>Viscosity ($kPa\cdot s$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slaboch (current investigation)</td>
<td>Murine</td>
<td>Created PRP clots by adding calcium and thrombin.</td>
<td>Nanoindentation</td>
<td>2-3</td>
<td>SLS: 209.84 ± 70.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KV: 9.00</td>
</tr>
<tr>
<td>Williams (1987)</td>
<td>Human</td>
<td>Stated the viscosity of whole blood as related to the shear rate. This is not a thrombus.</td>
<td>N/A</td>
<td>N/A</td>
<td>$3.0 \cdot 10^{-6} - 4.0 \cdot 10^{-6}$</td>
</tr>
<tr>
<td>Henderson and Thurston (1993)</td>
<td>Human</td>
<td>Combined bovine thrombin with either whole blood or plasma.</td>
<td>Viscometer</td>
<td>0-0.5</td>
<td>$4.0 \cdot 10^{-5} - 1.0 \cdot 10^{-4}$</td>
</tr>
<tr>
<td>Schmitt et al. (2007)</td>
<td>Porcine</td>
<td>Created whole blood clots by adding calcium.</td>
<td>2D dynamic elastography</td>
<td>0-3.3</td>
<td>$1.5 \cdot 10^{-4} - 3.0 \cdot 10^{-4}$</td>
</tr>
<tr>
<td>Schmitt et al. (2011)</td>
<td>Porcine</td>
<td>Created whole blood clots by adding calcium.</td>
<td>Dynamic ultrasound elastography</td>
<td>0-2</td>
<td>SLS: $1.01 \cdot 10^{-3} \pm 9.1 \cdot 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KV: $3.3 \cdot 10^{-4} \pm 1.2 \cdot 10^{-4}$</td>
</tr>
<tr>
<td>Author</td>
<td>Blood Type</td>
<td>Description of Thrombus</td>
<td>Test Method</td>
<td>Time (hrs)</td>
<td>Viscosity (kPa·s)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------</td>
<td>------------------------------------------</td>
<td>------------------------</td>
<td>------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Gennisson et al. (2006)</td>
<td>Porcine</td>
<td>Created whole blood clots by adding calcium.</td>
<td>Transient elastography</td>
<td>0-3.3</td>
<td>$5.0 \cdot 10^{-4} - 1.25 \cdot 10^{-2}$</td>
</tr>
</tbody>
</table>
CHAPTER 5

CONCLUSIONS

5.1 Summary of Original Contributions

This investigation successfully captured thrombus mechanical properties using a novel nanoindentation technique. A computational model was created to replicate the experimental test results, where it was found that the SLS model had the better agreement between the experimental data and simulations than the KV model.

The original contributions from this work include:

• Determining a repeatable testing method for nanoindentation of a PRP thrombus.

• Using elastic contact theory to calculate the average elastic modulus as $E = 55.1 \pm 12.1 \, kPa$.

• Developing a 2D axisymmetric, poroviscoelastic FE model for the thrombus and nanoindentation punch.

• Applying a KV FE model as a proof-of-concept computational simulation, and determining that the elastic modulus is $E = 24.5 \, kPa$ and the viscosity is $\eta = 9.00 \, kPa \cdot s$.

• Applying a SLS FE model to improve the fit between the simulated and experimental data, and determining that the elastic modulus $E_1 = 5.62 \pm 1.37 \, kPa$, the
elastic spring constant is $E_2 = 21.34 \pm 4.31 \text{kPa}$, and the viscosity is $\eta = 209.84 \pm 70.25 \text{kPa} \cdot \text{s}$.

- Performing a sensitivity analysis on the SLS FE model data.
- Identifying the elastic spring constant $E_2$ as the parameter with the greatest affect on the change in curve characteristics.
- Identifying the viscosity $\eta$ as the parameter with the second greatest affect on the change in curve characteristics.
- Identifying the elastic modulus $E_1$ as the parameter with the least affect on the change in curve characteristics.

5.2 Recommended Future Work

Three different types of thrombi are frequently used in experiments: whole blood thrombi (plasma, cells, platelets, and thrombin), platelet-rich plasma (PRP) thrombi, and platelet-poor plasma (PPP) thrombi. The next step to this research is to nanoindent whole thrombi and PPP thrombi. All three types of thrombi can be compared and contrasted with each other, and the PPP thrombi can act as the control. The elastic modulus, elastic spring constant, and viscosity of whole thrombi and PPP thrombi can be determined via the SLS FE model presented here.

5.2.1 Nanoindentation Tear Testing

As previously mentioned, thrombi can dissociate from the wall of a blood vessel and cause harm in the body. The force required to dislocate the thrombus is not widely studied. Therefore, the next goal of this research is to identify the tear strength of a thrombus. The nanoindenter has multiple functions and performing tear tests is one of
them. These tests can be performed using a spherical tip ($D = 100 \, \mu m$). The initial load on the thrombus can start out at $5 \, \mu N$ and can be increased by $5 \, \mu N$ until tears are seen on the surface via a microscope.

The TriboIndenter™ software can record the normal force, normal displacement, lateral force, and lateral displacement. Using the normal force and the lateral force, a coefficient of friction is automatically computed in the software. The maximum stress (tear strength) of the thrombus can be calculated using the coefficient of friction and the equations developed by Sackfield and Hills (1983a,b,c).

5.2.2 Live-animal in vivo Micro-Computed Tomography Scans

The results produced in this investigation are a stepping stone to an in vivo analysis of thrombi in rats. Live-animal in vivo micro-computed tomography (µ-CT) scans can be performed via a live-animal µ-CT scanner (Bioscan, Washington, DC) before any cutting, harvesting, or nanoindentation testing. In order to see the thrombus in the scanner, a contrast agent must be used. If an appropriate contrast agent is used, the same rat can be imaged over a period of days. One possibility for identifying thrombi is antibody-targeted gold nanoparticles (Nanoprobes, Inc., Yaphank, NY). Since they are biocompatible, multiple scans can be performed on each animal. Therefore, the number of rats used in future studies can be minimized. This novel method of µ-CT in vivo detection of thrombi with gold nanoparticles can be tested in vitro using whole thrombi before injecting the rats with the gold nanoparticles.

Once the gold nanoparticles are tested in vitro, they can be injected in vivo. Prior to injection, a thrombus can be induced in one leg of the rats via slow release of AngII (Luo et al., 2009). During the µ-CT scanning, the leg without the thrombus can act as the control.
Each rat can be anesthetized and placed in a tube for scanning. Data from the 3D $\mu$-CT scans can be taken for the same rat over a period of 10 days. Since the rats can survive with antibody-targeted gold nanoparticles inside them, only a few rats are needed for this test. An accurate and consistent measurement of the thrombus structure over a period of time can also be determined.

5.2.3 Nanoindentation Testing of Harvested \textit{in vivo} Thrombi

To verify the elastic modulus and tear data for all three types of \textit{in vitro} thrombi (whole thrombi, PRP, PPP) as determined from the nanoindentation tests, the rats can be sacrificed (after all of the $\mu$-CT scanning is complete) and the \textit{in vivo} thrombus can be harvested. The same indentation and tear tests can be performed using the harvested thrombi and the TriboIndenter\textsuperscript{TM}. The harvested blood vessels can be stored at $4^\circ C$ and brought to $37^\circ C$ prior to testing. To obtain a flat indentation and tear surface, the vessel can be sliced in half using a wire. In order to obtain the largest possible surface area to indent, the thrombus can be sliced directly down the middle, parallel with the vessel wall.

Slicing soft tissue can be troublesome as it can bunch up easily, resulting in irregular surfaces. If needed, the blood vessel can be frozen and then sliced with a hot wire. However, should the wire fail to produce a sufficiently flat surface, the vessel can be embedded in optimal cutting temperature (OCT) compound and frozen. It can then be sliced with a microtome.

5.2.4 Fluid-Structure Finite Element Simulation

It appears that the FE method has never been used to determine the relationship between the mechanical property affects of thrombi and thrombi dissociation from the
vessel wall. Only one of the aforementioned studies was concerned with dissociation, Arab-Ghanbari et al. (2009), and the affects of the mechanical properties were not modeled. One of the aforementioned studies was concerned with the mechanical properties of thrombi (van Dam et al., 2008); however the focus of the study was on rupture of the aorta, not on dissociation of the thrombus from the wall. Thrombi have the ability to form, interact with, and dissociate from their surroundings (Xu et al., 2008); clot morphology can significantly affect their progression. Thrombi exhibit a complex fluid-structure interaction behavior; one that can ultimately lead to a potentially-fatal dissociation, migration, and obstruction condition in the vascular system.

The 3D images obtained from the μ-CT scans can be used as input for a fluid-structure FE simulation to create a geometrically accurate thrombus. The simulation can be developed to include the thrombus, the blood vessel interface and the surrounding fluid. Then, a design-of-experiments (DOE) approach can be used to determine which mechanical properties (elastic modulus and tear strength) have the greatest affect on thrombi dissociation from the vessel wall. Due to complex fluid-structure interactions, other parameters (i.e., thrombi density, thrombi shape, thrombi wall adhesion strength, fluid viscosity (a simplified way to simulate two-phase flow), and fluid flow rate) may also be involved in the dissociation of the thrombus. Therefore, these parameters can also be modeled in the fluid-structure FE simulation to determine which one(s) have the greatest affect(s) on thrombi dissociation.

Initially, two-dimensional simulations can be run to validate the modeling procedure. Then, three-dimensional geometries can be simulated on a High-Performance Computing Cluster (HPCC), which can enable the simulation of more physiological thrombi shapes. The FE mesh for the thrombus can be created by modeling the shape and structure of the thrombus from the μ-CT images obtained in vivo. This simulation
can account for changes in the geometry over a period of 10 days, the mechanical properties of the thrombus (elastic modulus, viscosity, tear strength), and the flow pattern around it.

5.2.5 Flow Chamber Simulation

To verify the results from the fluid-structure FE simulations, an in vitro simulation of fluid flow over a thrombus can be performed using a custom-built flow chamber (Fig. 5.1). This would consist of the chamber, a recirculating fluid bath, and a pump. The square tunnel can be created from plexiglass and the thrombi (i.e., whole thrombi, PRP, or PPP) can be created as before. The fluid flow rate can be varied, and the pressure and velocity can be calculated for each flow rate using Bernoulli’s equation. A microscope slide can be used to hold the thrombus. This slide can be set into the base of the chamber so that the top of the slide is even with the floor of the chamber, as illustrated, exposing the thrombus to the fluid flow.

Since approximately half of the cases of VTE currently go undiagnosed\(^1\), an accurate FE simulation has the potential to aid doctors in correctly diagnosing VTE. Using the experimentally-determined thrombi mechano-rheological and strength properties

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as inputs to confined, arterial fluid-structure finite element simulations can drive the development of probabilistic models for potentially-fatal thrombus disease-state configurations in representative human vascular geometries. Flow chamber simulations can validate the FE simulation results. Future studies can establish ways of monitoring mechanical properties \textit{in vivo} in rats and eventually in humans. After obtaining the mechanical properties and the 3D image of the human blood vessel containing the thrombus, the entire anatomical structure (i.e., thrombus and vessel) can be input into a FE simulation to properly diagnose VTE, evaluate the risk of thrombus dissociation and relocation (i.e., embolism), and the probability of recurrent episodes.


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