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ABSTRACT

Despite major advances in our understanding of the biochemical pathways of thrombus formation, our knowledge of the biophysical mechanisms that regulate thrombus growth remains limited. Explicitly, there is a paucity of data on how hemodynamics affects the physical properties of thrombi. The objective of this thesis is to measure and model the physical properties of thrombi as a function of the relative and absolute density of the blood cell platelets and the biopolymer fibrin.

A series of thrombi were made with known fibrin and platelet densities. The permeability was calculated by measuring the interstitial fluid velocity through the thrombi at a constant pressure gradient. Hindered diffusivity was measured using a diaphragm diffusion cell and fluorescence recovery after photobleaching. The elastic regimes of fibrin gels was measured as a function of strain in both shear and extensional geometries and compared to nonaffine and affine network models. The composition and structure of thrombi formed under flow using microfluidics was quantified using confocal and focused ion beam scanning electron microscopy.

The permeability of thrombi formed with a solid fraction of 0.02−0.61 ranged from $10^{-1}$ to $10^{-5}$ µm$^2$ and was best modeled as an array of disordered cylinders or as a Brinkman medium for low and high platelet densities, respectively. The diffusivity of coagulation zymogen-sized solutes was hindered by up to 93% over the same range and was best described by models accounting for both steric and hydrodynamic hindrance. Low density fibrin gels transition from a thermal network regime to a nonaffine mechanical to an affine mechanical regime with increasing strain. High density fibrin gels transition directly from a thermal to an affine mechanical regime. Within the thermal regime, the pore size can be predicted by the elastic modulus using semiflexible polymer theory providing a link between mechanics and
transport. Flow-formed thrombi were found be heterogeneous structures that contained up to 65% solids by volume.

Ultimately, these results enable the prediction of the properties of flow-formed thrombi. Importantly, all properties were found to be directly related to thrombus microstructure, thus providing a link between mechanics and transport that should prove useful in developing therapeutic approaches for thrombosis.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xiii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xiv</td>
</tr>
<tr>
<td>CHAPTER 1 - INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2 - BACKGROUND</td>
<td>8</td>
</tr>
<tr>
<td>2.1 Blood composition and rheology</td>
<td>8</td>
</tr>
<tr>
<td>2.2 Thrombosis and Hemostasis</td>
<td>9</td>
</tr>
<tr>
<td>2.2.1 Arterial thrombus formation</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2 Venous thrombus formation</td>
<td>14</td>
</tr>
<tr>
<td>2.2.3 Fibrinogen and fibrin network polymerization</td>
<td>14</td>
</tr>
<tr>
<td>2.2.4 Regulation</td>
<td>16</td>
</tr>
<tr>
<td>2.2.5 Fibrinolysis</td>
<td>16</td>
</tr>
<tr>
<td>2.3 Microfluidic devices for measuring clot formation under flow</td>
<td>17</td>
</tr>
<tr>
<td>2.4 Flow-formed clot structures</td>
<td>18</td>
</tr>
<tr>
<td>2.5 Overview of porous media</td>
<td>21</td>
</tr>
<tr>
<td>2.6 Transport in porous media</td>
<td>24</td>
</tr>
<tr>
<td>2.6.1 Permeability</td>
<td>24</td>
</tr>
<tr>
<td>2.6.2 Diffusion in porous media</td>
<td>27</td>
</tr>
<tr>
<td>2.7 Mechanical properties of biological networks</td>
<td>28</td>
</tr>
<tr>
<td>2.7.1 Network Categorization</td>
<td>29</td>
</tr>
<tr>
<td>2.7.2 Viscoelasticity and small strain dynamic mechanical analysis</td>
<td>30</td>
</tr>
</tbody>
</table>
CHAPTER 3 - HYDRAULIC PERMEABILITY OF BLOOD CLOTS AS A FUNCTION OF FIBRIN AND PLATELET DENSITY

3.1 Abstract ............................................................................................................................ 35

3.2 Introduction ...................................................................................................................... 36

3.3 Materials and methods ..................................................................................................... 39

3.3.1 Fibrinogen Solution .................................................................................................. 40

3.3.2 Fibrin Gel Preparation .............................................................................................. 40

3.3.3 Platelet Rich Clot Preparation .................................................................................. 41

3.3.4 Hydraulic permeability measurements ..................................................................... 41

3.3.5 Scanning electron microscope measurements of fiber diameter ......................... 42

3.3.6 Confocal microscopy of fibrin gels and platelet-fibrin thrombi ................................... 42

3.3.7 Hindered diffusivity measurements ......................................................................... 43

3.3.8 Statistical Analysis ................................................................................................. 44

3.4 Theory .............................................................................................................................. 45

3.4.1 Permeability of fibrin gels ........................................................................................ 45

3.4.2 Permeability of platelet rich clots ............................................................................. 47

3.4.3 Hindered diffusion .................................................................................................... 49

3.5 Results .............................................................................................................................. 51

3.5.1 Permeability of fibrin gels ........................................................................................ 51

3.5.2 Permeability of platelet rich clots ............................................................................. 55

3.5.3 Hindered diffusion in fibrin gels and platelet rich clots ........................................... 61

3.6 Discussion ........................................................................................................................ 62
CHAPTER 4 - ELASTIC BEHAVIOR AND PLATELET RETRACTION IN LOW AND HIGH DENSITY FIBRIN GELS ................................................................. 71

4.1 Abstract .......................................................................................................................... 71

4.2 Introduction ..................................................................................................................... 72

4.3 Experimental Methods ................................................................................................... 75

4.3.1 Materials ................................................................................................................... 75

4.3.2 Preparation of fibrin gels ........................................................................................ 76

4.3.3 Small strain dynamic shear rheology ....................................................................... 77

4.3.4 Shear and normal stresses under a strain ramp ........................................................ 77

4.3.5 Extensional rheology ................................................................................................ 78

4.3.6 Platelet retraction of fibrin gels ................................................................................ 78

4.4 Theory .............................................................................................................................. 79

4.4.1 Calculation of Mesh Size ......................................................................................... 79

4.4.2 Predicting mesh size from elasticity of thermal affine networks ............................. 80

4.4.3 Using negative normal stresses to categorize fibrin gels at moderate strain ............ 80

4.4.4 Distinguishing non-affine and affine mechanical networks ..................................... 81

4.5 Results .............................................................................................................................. 82

4.5.1 Verification of fibrinogen conversion to fibrin ........................................................ 82

4.5.2 Fibrin gels are linearly viscoelastic for all fibrinogen concentration at low strains 83

4.5.3 Strain hardening under shear is attenuated in high density fibrin gels..................... 85

4.5.4 Extensional rheology reveals two strain hardening regimes .................................... 86

4.5.5 High density fibrin gels act as thermal affine networks at small strains based on mesh size scaling ........................................................................................................... 88

4.5.6 Negative normal stresses show that fibrin gels act as mechanical networks at moderate strains ........................................................................................................... 88

4.5.7 Affine and non-affine mechanical networks as a function of fibrin density ............ 90
4.5.8 Platelet retraction of fibrin gels depends on both platelet and fibrin density .......... 92
4.5.9 Fibrin rheology state diagram .............................................................................. 93
4.6 Discussion .................................................................................................................. 96
4.7 Conclusions .............................................................................................................. 100

CHAPTER 5 - MEASURING THE PORE SPACE IN FLOW-FORMED CLOTS BY FOCUSED ION BEAM-SCANNING ELECTRON MICROSCOPY IMAGING .......... 102

5.1 Introduction ............................................................................................................ 102
5.2 Materials and Methods ........................................................................................ 103
  5.2.1 Materials ........................................................................................................... 103
  5.2.2 Whole blood clot formation .......................................................................... 104
  5.2.3 Confocal microscopy of flow-formed thrombi ............................................... 105
  5.2.4 Resin embedding ............................................................................................. 106
  5.2.5 FIB SEM imaging ............................................................................................. 107
  5.2.6 Area thresholding to determine solid fraction ................................................ 107
  5.2.7 Relief contrast microscopy of clots to determine clot area ............................... 108
5.3 Results .................................................................................................................. 108
  5.3.1 Thrombus heterogeneity ................................................................................. 108
  5.3.2 Flow-formed clots are comprised of mostly solids ....................................... 110
  5.3.3 The embedding procedure does not alter clot geometry ............................... 110
5.4 Discussion .............................................................................................................. 110
5.5 Conclusion ............................................................................................................. 114

CHAPTER 6 - GENERAL CONCLUSIONS .................................................................. 115

6.1 Summary of Results ............................................................................................ 115
6.2 Future Research .................................................................................................... 117
  6.2.1 Transport mechanisms of clot arrest ............................................................... 117
  6.2.2 Structures and mechanisms of thromboembolism ........................................ 119
LIST OF FIGURES

Figure 2.1. Model of vascular injury ................................................................. 10
Figure 2.2. Overview of clot formation ............................................................. 11
Figure 2.3. Platelet aggregation and coagulation schematics .......................... 13
Figure 2.4. Fibrin polymerization schematic .................................................... 15
Figure 2.5. Scanning electron micrographs of focused ion beam milled of blood clots .... 19
Figure 2.6. The solid components of the clot (platelets and fibrin) serve as a physical mechanism to inhibit clot growth. ................................................................. 21
Figure 2.7. Various types of propo media .......................................................... 22
Figure 2.8. The permeability of a regular porous medium with a well-defined geometry can be derived from the Navier-Stokes equations ........................................... 26
Figure 2.9. Hindered solute transport in pores ................................................... 28
Figure 2.10. Fiber type determines the behavior of the network ...................... 30
Figure 3.1. Fibrin gel morphology as a function of fibrin density ....................... 52
Figure 3.2. Fibrin fiber diameter as a function of fibrinogen concentration .......... 53
Figure 3.3. Comparison of fibrin gel permeability to fibrous media models ....... 55
Figure 3.4. Platelet rich clots morphology as function of platelet density .......... 56
Figure 3.5. Comparison of platelet rich clot permeability to mixed porous media models ... 58
Figure 3.6. Estimated permeability of blood clots as a function of platelet and fibrin volume fractions ...................................................................................... 60
Figure 3.7. Diffusion in fibrin gels and platelet rich clots .................................... 62
Figure 4.1. Laser scanning confocal microscopy images of fibrin gels ................ 83
Figure 4.2. Dynamic shear rheology of fibrin gels at small strains ................... 84
Figure 4.3. Strain hardening in low and high density fibrin gels ......................... 86
Figure 4.4. Biaxial extensional stress-strain data for fibrin gels ......................... 87
Figure 4.5. Mesh Size, $\xi$, of fibrin gels formed as calculated by measurements of $G'$ and permeability ...................................................................................... 89
Figure 4.6. Negative normal stress to shear stress ratio verses strain for fibrin gels .......... 90
Figure 4.7. The ratio of elastic moduli, $E(\gamma)$, to shear moduli, $G(\gamma)$, of fibrin gels.............. 91
Figure 4.8. Percent retraction of fibrin-platelet gels. ................................................................. 92
Figure 4.9. Proposed state diagram of the elastic regime of fibrin gels as a function of fibrinogen concentration and strain.. ................................................................. 94
Figure 5.1. Morphology of whole blood clots formed at 100 s$^{-1}$. .............................................. 109
Figure 5.2. Image process steps used to derive the solids fraction of whole blood clots...... 111
Figure 5.3. Images of flow-formed thrombi .............................................................................. 112
Figure A-1. Schematic of the space between two platelets .......................................................... 136
Figure A-2. Experimental set-up for measuring clot permeability.............................................. 140
Figure A-3. Image analysis for obtaining measured platelet area fractions ......................... 141
Figure A-4. Solutions for the normalized permeability of the Brinkman equation................. 142
Figure A-5. The effect of platelets on fibrin fiber morphology..................................................... 143
Figure B-1. Measurement of engineering stress and calculation of $E(\gamma)$ ......................... 150
Figure B-2. Absorbance of fibrinogen at 450 nm as function of time ................................. 151
Figure B-3. Reducing SDS-PAGE analysis of fibrin gels cross-linking................................. 152
Figure B-4. Representative $G'$ as a function time for a 100 mg/ml fibrin gel .................... 152
| Table 3.1. | Permeability of fibrin gels | 51 |
| Table 3.2. | Permeability of platelet rich clots | 57 |
| Table 4.1. | Dynamic shear storage (G’) and loss (G’’) moduli of fibrin gels at small strains | 85 |
| Table 4.2. | Rheological measurements of fibrin gels in extensional strain | 87 |
| Table 4.3. | Critical lengths (L_c) and filament length:critical length ratio (L_c/λ_{NA}) for fibrin gels | 96 |
| Table A-1. | Fractional Decrease in fluorescent signal as a function of z-distance | 139 |
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CHAPTER 1
INTRODUCTION

The circulatory system is the organ system by which cells transport the basic components needed for life throughout the body. Humans have a closed circulatory system in which the heart pumps blood through a system of arteries, arterioles, capillaries, venules and veins allowing for the efficient delivery of oxygen and nutrients, and removal of metabolic waste from all parts of the body. Vital to the function of the circulatory system is the process of hemostasis, which serves to limit the loss of blood from an injury. Hemostasis enables an organism to close off damaged blood vessels upon trauma, to keep the blood in a fluid state, and to remove blood clots after the restoration of vascular integrity (1). An imbalance in hemostasis can result in either excessive bleeding (hemorrhage) or excessive clotting (thrombosis), which can ultimately result in the obstruction of blood flow through vessels. Hemorrhage can be caused by trauma, deficiencies of coagulation factors, deficiencies in platelet adhesion or aggregation, or anticoagulant and antiplatelet drugs. Thrombosis can be caused by perturbations in blood composition that lead to a prothrombotic state (e.g. downregulation of anticoagulation factors), stasis in blood flow, or rupture of atherosclerotic plaques.

Hemostasis occurs through the intertwined processes of platelet plug formation and a series of biochemical reactions called coagulation, both of which are regulated by many biochemical and biophysical mechanisms. These reactions can reinforce or inhibit each other either directly or indirectly (2). Upon onset of injury endothelial cells and other blood vessel wall cells are removed exposing an extracellular matrix that promotes platelet adhesion and activation. Adhered and activated platelets aggregate with newly arriving platelets transported to
the injury by blood flow to form a platelet plug. Simultaneously, coagulation is initiated by subendothelial tissue factor and/or negatively charged surfaces (collagen, phosphatidylserine, polyphosphates) ultimately resulting in the generation of the serine protease thrombin, which serves a dual role as platelet agonist and catalyst for formation of biopolymer called fibrin. Once formed the hemostatic plug must stem the loss of fluid from the vessel. To accomplish this, the plug must be sufficiently impermeable to fluid flow and must possess the mechanical strength to withstand the forces imparted upon it by the flowing blood.

The composition of thrombi is highly dependent on both the local hemodynamics and the nature and extent of injury. Clots formed in areas of high flow tend to be platelet rich due to the platelet’s ability to adhere and aggregate at high shear stresses and are characteristic of myocardial infarctions that form at the site of an atherosclerotic plaque rupture. Clots formed where blood flow is slow tend to be fibrin and red blood cell rich because coagulation reactions and fibrin polymerization have a greater residence time in areas of recirculating flows such as in venous valve pockets. Flow dynamics contribute to a number of factors that determine the success of the clot including platelet activation, platelet attachment and detachment, embolus shedding, and thrombus heterogeneities (3). For instance, Nesbitt et al., used high-resolution intravital imaging techniques and hydrodynamic analysis to show that platelet activation is primarily driven by changes in blood flow parameters with soluble agonists having only a secondary role (4). During platelet aggregation, diminished $\alpha_{\text{IIb}}\beta_3$ activity results in instability and platelet detachment in flow-formed clots (3). Coagulation has been shown to contribute to thrombus formation and stabilization via the flow-rate dependent protease-activated receptors (PAR), flow-rate dependent activation of platelets, flow-rate dependent generation of pro-coagulant PS exposing platelets, and flow-rate dependent clot stabilization via fibrin
formation (3, 5-9). Finally, flow dynamics and the effects of flow dynamics on platelet
aggregation and coagulation have been shown to result in spatial heterogeneity for both in vitro
and in vivo formed clots (7). Multiscale simulations have shown increased flow rates during
thrombus growth leads to increased structural heterogeneity of the clot (10). These
heterogeneities and their associated physical properties, in turn, determine the size and frequency
of any emboli caused by blood-flow gradients and the likelihood of complications caused by
thrombosis (11-13).

Most of what is known about the physical properties of thrombi is based on clots formed
statically and at static protein and cell concentrations. Numerous studies have been conducted to
determine the effects of fibrin and thrombin concentration, platelet count, ionic strength, or other
drugs concentration on clot permeability and solute transport, but have focused on concentrations
of components less than or equal to concentrations found in plasma (14-21). Similarly, abundant
studies have been conducted on clot elasticity and with a focus on determining the effects of
thrombin concentration, ionic strength, and nonlinear elastic properties of clots formed at or near
plasma concentrations of proteins, and cells (14, 15, 22-30). As such there exists a gap in the
knowledge base of the physical properties of thrombi representative of those formed under flow,
which can contain significantly elevated levels of cells and proteins relative to plasma and have
heterogeneous structures (7, 31, 32). The focus of this thesis is on measuring and characterizing
the physical properties of thrombi as a function of their composition. In particular, I focus on the
hydraulic permeability, hindered transport, and mechanical properties of thrombi formed over a
wide range of platelet and fibrin concentrations to bound the possible values and present
relationships that can be used to determine the physical properties of clots found in vivo as a
function of clot composition.
Hydraulic permeability represents a material’s ability to transmit fluid. The primary use of permeability is in determining the average velocity at which a liquid of a known viscosity will flow through the porous material at a given pressure gradient. In hemostasis, measurements of permeability allow for the prediction of the rate at which fluid moves through the interstitial spaces of a clot. These predictions are important for estimating the rate at which coagulation enzymes/zymogens enter and leave a clot as well as for developing drug delivery strategies for disrupting clot formation or enhancing clot lysis. Measures of permeability also provide an estimate of the pore size of the material, which can in turn provide estimates of other physical properties such as estimates of hindered transport coefficients of molecules within the material, determination of transport limiting steps during the growth, arrest, and dissolution of a clot and estimates of mechanical properties based on the micro/nano structure of the material.

The mechanical properties that are relevant to clot stability include the shear and elastic moduli when subject to extensional, compressive, or shear stresses. The shear modulus and elastic modulus are measures of how a material deforms when exposed to a shear and extensional stresses, respectively. These moduli consist of an elastic component and a viscous component that describe if the material deforms elastically (stress proportional to strain), viscously (stress proportional to strain rate), or as a combination of the two. The breaking stress is a measure the stress required to cause catastrophic failure of the clot. These properties determine, in part, whether the stress imparted by blood flow will cause the hemostatic plug to deform reversibly or irreversibly, or embolize.

The specific aims of this thesis are: (1) Measure the volume fraction of the two major components of a clot, fibrin and platelets, in flow-formed clots. (2) Measure the effect of fibrin and platelet content on the hydraulic permeability and hindered diffusivity of clots. (3) Use
models of flow and diffusion through fibrous and porous media to identify predict the
permeability and hindered diffusivity of clots as a function of composition. (4) Measure the
effect of fibrin solid fraction and thrombin concentration on the mechanical properties on clots.
(5) Measure the effects of strain on the elastic regime of clots. (6) Use models to identify the
relevant variables that dictate solid mechanics of clots as a function of composition. (7) Connect
the physical properties of permeability, hindered diffusivity, and mechanics based on the
microstructure of representative blood clots.

CHAPTER 2 provides background information including analysis of the solid fraction of
in vivo formed clots and discussion of the current cell based models for clot formation that
utilize transport limited reactions and permeability models. Next, a review of microfluidic
models of thrombus formation is provided. Finally, background on previous measurements and
models of permeability and mechanics of blood clots and their components is discussed.

In CHAPTER 3 we measure the effects of fibrin and platelet solid fraction on clot
permeability and hindered diffusivity. Permeability and hindered diffusivity was shown to be a
strong function of both fibrin and platelet density. Fibrin gels formed with solids fractions of
0.02−0.54 have permeabilities of $10^{-1}−10^{-4} \mu m^2$. Platelet rich clots formed with solids fractions
of 0.02−0.61 have permeabilities of $10^{-1}−10^{-5} \mu m^2$. Fibrin gels formed with a fiber volume
fraction of 0.36 reduced the diffusion coefficients of 12, 29, and 54 nm solutes by 60%, 66% and
85%, respectively. Platelet rich clots with a platelet volume fraction 0.61 reduced the diffusion
coefficient of a 12 nm solute 93%. The permeability of fibrin gels were shown to be best
modeled as arrays of disordered cylinders with uniform diameters. The permeability of platelet
rich clots was shown to be best modeled as a Brinkman medium of coarse solids (platelets)
embedded in a mesh of fine fibers (fibrin). Taken together, our data shows that the permeability
of clots formed in vivo can vary by up to five orders of magnitude with pore sizes that range from 4 to 350 nm. These findings have important implications for the transport of coagulation zymogens/enzymes in the interstitial spaces during clot formation, as well as in the design of fibrinolytic drug strategies are discussed.

In CHAPTER 4 we measure and characterize the elastic regimes fibrin gels as a function of fibrin density and strain and show how the elasticity of fibrin effects clot retraction. At low strains, fibrin gels were shown to act as thermal networks independent of fibrinogen concentration. Within the low strain regime, the mesh size of fibrin gels can be predicted by the elastic modulus by semiflexible polymer theory, providing a link between gel mechanics and interstitial fluid flow. At moderate strains, we show that low density fibrin gels act as non-affine mechanical networks and transition to affine mechanical networks, while high density fibrin gels only act as affine mechanical networks. At high strains, individual fibrin fibers stretch independent of fibrin fiber density. Platelets can retract low density gels by greater that 80% of their initial volume, but retraction is attenuated in high fibrin gels and with decreasing platelet density. These results suggest that the nature of deformation of fibrin gels and thrombi is a strong function of fibrin density, which has ramifications for the embolization and lysis of thrombi.

In CHAPTER 5 we examine the solid fraction of flow-formed clots. Clots were formed in microfluidic channels under venous flow conditions and the solid fractions of platelets and fibrin was estimated using confocal microscopy and focused ion beam scanning electron microscopy (FIB-SEM). Our results show that clots formed under flow contain solid fractions much higher than clots formed statically. Consequently, we show a need for the analysis of the physical properties of clots representative of those formed in vivo; most previous studies have been conducted at low solids fractions not representative of flow-formed clots.
CHAPTER 6 summarizes the major findings and gives recommendations for future work. It is suggested that the effects of hindered transport on the growth, arrest, and dissolution of a clot be tested in a microfluidic channel, and the fracturing of clots tested in shear and extensional geometries.
CHAPTER 2
BACKGROUND

Blood serves many physiological functions. Among them are the transport of oxygen and removal of metabolic waste from tissues, identifying and removing foreign bodies, and delivering immune cells to the site of infection. The focus of this thesis is on hemostasis, also known as blood clotting, and specifically the transport and rheological properties of blood clots. This chapter provides a brief overview of the composition and physical properties of blood and the hemostasis.

2.1 Blood composition and rheology

Blood is a dense suspension of cells in a protein-rich liquid called plasma. The cell fraction is made up of red blood cells (also called erythrocytes or RBCs; diameter = 6–8 µm; 5–6 × 10⁶/µL), white blood cells (leukocytes or WBCs; d = 7–15 µm; 4–11 × 10³/µL; < 1% v/v) and platelets (d = 1–2 µm; 2–4 × 10⁵/µL; < 1% v/v). Plasma constitutes ~ 55% v/v of blood and is a solution of proteins, ions, and polysaccharides. RBC are by far the most numerous blood cell, comprising 35–50% of the volume of blood, and dictate the rheological properties of blood. Blood is a unique fluid in that it both shear thickens and shear thins. Shear thickening occurs at shear rates of less than 10 s⁻¹ and is caused by attractive RBC-RBC interactions that form rods of RBCs called rouleaux. Shear thinning occurs at shear rates greater than 1000 s⁻¹ and is caused by deformation of RBC. Platelet and leukocytes are forced towards the vessel wall by a process called margination that is caused by shear-induced diffusion due to interactions with RBC and the tank treading motion of the RBC membrane.
2.2 Thrombosis and Hemostasis

Excessive and deficient clot formation, thrombosis and bleeding, respectively, are the leading cause of death in the United States (33). The processes of thrombus, or blood clot, formation are often grouped into three categories: hypercoagulability, endothelial injury, stasis, collectively known as Virchow’s triad. Hypercoagulability is an increased concentrations of platelets, micro particles, and pro- and anticoagulation proteins that effect the bloods ability to form a thrombus (1). Endothelial injury causes blood to be exposed to procoagulant surfaces triggering platelet plug and fibrin formation. Stasis is abnormally slow or stopped blood flow and contributes to coagulation by the accumulation of clotting factors at a single location and through endothelial activation derived from hypoxic conditions in the stagnant blood.

Thrombus formation can vary greatly with location within the circulatory system. The composition of a blood clot depends on the local blood flow velocity, the nature and extent of the injury, and the vascular bed of the injury. In the large arteries where blood flow is fast, clots tend to be platelet rich because the platelets can adhere and aggregate at high shear stresses whereas coagulation products tend to be diluted. These ‘white clots’ are characteristic of myocardial infarctions that form at the site of an atherosclerotic plaque rupture. In the veins of the extremities where blood flow is slow and in areas of recirculating flows such as in venous valve pockets, clots tend to be fibrin and red blood cell rich because coagulation reactions and fibrin polymerization have a greater residence time. In these areas, the fibrin entrains red blood cells to give what are referred to as ‘red clots’. However, this white clot/red clot terminology oversimplifies the structure of the clot since both types of clots contain both platelets and fibrin, just in different relative densities. Clot composition is also highly heterogeneous. Clots formed in large vessels have a laminate structure consisting of a series of platelet-rich and fibrin-rich layers.
known as the lines of Zahn. Even at a smaller length scales clots form regions of platelet-rich and fibrin-rich areas, although the mechanism of assembly is unclear (Fig. 2.1).

2.2.1 Arterial thrombus formation

Arterial thrombi form at the site of vascular injury to stop the loss of blood out of the vessel in a process called hemostasis (Fig. 2.2) To efficiently extend to all reaches of the human body, blood flows through a series of arteries, veins, venules, and capillaries called the closed circulatory system. Lining this entire network is a layer of endothelial cells that maintain vessel patency by providing an inert surface that prevents blood cell adhesion. Endothelial cells also actively inhibit clot formation by secreting soluble chemicals (e.g. nitric oxide) that keep platelets, the primary blood cell responsible for clot formation, quiescent. At the site of a vascular injury, the endothelial cells are denuded or otherwise compromised revealing the subendothelial matrix. This matrix promotes clot formation by providing an adhesive surface for

Figure 2.1. Model of vascular injury. Human whole blood was perfused over a micropatterned spot containing collagen and the procoagulant protein tissue factor at a wall shear rate of 300 s⁻¹. Platelets are labeled red and fibrin(ogen) is labeled green. On the left is a top view of a clot and on the right a three-dimensional reconstruction. Note the heterogeneous distribution of platelet and fibrin over a homogenous procoagulant surface.
Figure 2.2. Overview of clot formation. Endothelial cells secrete platelet inhibitors that keep platelets in a quiescent state. This includes prostacyclin (PGI2), ADPases and nitric oxide (NO). At the site of an injury, the endothelial cells are removed and platelets adhere to and activate on the subendothelial matrix that contains various types of collagen and the adhesive protein von Willebrand factor (VWF). Once activated, platelets secrete soluble agonists such as adenosine diphosphate (ADP) and thromboxane A2 (TxA2). These agonists activate new platelets which then aggregate with existing platelets in the clot. Platelets can also aggregate in the absence of these agonists at high shear stresses. Simultaneously, the biochemical reaction pathway called coagulation takes place on procoagulant molecules in the subendothelial matrix and on the platelet surface. The end product of coagulation is an enzyme called thrombin that acts as a platelet agonist and a catalyst for the polymerization of the biopolymer fibrin.
platelet adhesion and by exposing procoagulant proteins that initiate a series of biochemical reactions called coagulation (Fig 2.3) (34).

Platelet adhesion occurs either directly through the platelet collagen receptors glycoprotein VI (GPVI) and the integrin $\alpha_2\beta_1$ or with the help of another ligand called von Willebrand factor (vWF) through glycoprotein Ib (GPIb) (35, 36). Adhesion causes platelets to become activated and to secrete platelet-activating chemicals into the surrounding fluid activating other platelets. Following adhesion, platelets can aggregate with each other via the ligands fibrin(ogen) or VWF through the integrin $\alpha_{\text{IIb}}\beta_3$ forming a mass called a platelet plug.

Coagulation is comprised of the extrinsic and intrinsic pathway. The extrinsic pathway is initiated by tissue factor (TF) present on the surface of cells within the vessel wall and also circulating in the blood on the surface of white blood cells, cancer cells, and on cell fragments called microparticles. TF serves as a co-factor for coagulation factor VII. The TF:VIIa complex activates two zymogens IX and X, which then must then make their way from the subendothelial surface through the fluid to the surface of an activated platelet to further propagate the cascade. Once on the platelet surface Xa binds with factor V to form the prothrombinase complex Va:Xa, which converts prothrombin (II) to the critical enzyme thrombin (IIa). Once produced, thrombin further activates platelets, acts in a feedback loop to increase the rate of its own production, and acts as a catalyst for the polymerization of a fibrous polymer called fibrin. The intrinsic pathway is initiated by a myriad of negatively charged proteins (kallikrein, histones), nucleic acids and inorganic polymers secreted from cells (polyphosphates) that activate coagulation factor XII. Once initiated, the intrinsic pathway proceeds through factor XI to eventually activate factors IX and X which results in a burst of thrombin and ultimately in the production of fibrin via the same prothrombinase complex as above.
Figure 2.3. Platelet aggregation and coagulation schematics. (left) Platelet adhesion receptors and their ligands. Each platelet’s surface bears ≈ 25,000 GPIb receptors that bind to surface-bound von Willebrand factor (vWF), ≈ 50,000 integrin αⅡbβ3 receptors that bind to fibrinogen and vWF, ≈ 4,000 GPVI receptors, and 1,000–4,000 integrin α2β1 receptors that bind to several types of collagen. The integrins must be activated to form strong long-lived bonds. Collagen is a major constituent of the subendothelial (SE) matrix; fibrinogen is an abundant plasma protein; and vWF is adsorbed to SE collagen, circulates in plasma, and is secreted by endothelial cells (ECs). (right) Schematic illustration of coagulation reactions. Most coagulation proteins are named using Roman numerals [e.g., factor IX (FIX)] and exist in an inactive (FVIII) and active form (FVIIIa). FIIa (also known as thrombin), FVIIa, FIXa, FXa, and FXIa are enzymes; their inactive precursors are called zymogens. FVa and FVIIIa are cofactors for the enzymes FXa and FIXa, respectively, and must be activated from their precursors FV and FVIII. Tissue factor (TF) is a cofactor for FVIIa. The coagulation enzyme-cofactor complexes form on SE and platelet surfaces and have enzymatic efficiencies $10^5–10^6$-fold that of the enzyme alone. The activation of a coagulation protein is by proteolysis (i.e., cutting) of the precursor by another enzyme. Thrombomodulin (TM) on ECs is a cofactor for thrombin in producing the inhibitor activated protein C (APC). Other major inhibitors are antithrombin (AT) and tissue factor pathway inhibitor (TFPI). Surface-bound enzyme complexes TF:VIIa, VIIa:IXa, Va:IXa, and TM:IIa and other surface-bound species are shown in boxes. Also shown are cell or chemical activation (purple lines), movement in fluid or along a surface (dark blue lines), enzyme action in a forward direction (solid gray lines), the feedback action of enzymes (dashed gray lines), binding to or unbinding from surface (light blue double-headed arrows), and chemical inhibitors (red circles).
2.2.2 Venous thrombus formation

Less is known about venous thrombus formation than about arterial thrombus formation. One major difference lies in the initiation of each. While arterial thrombus formation is initiated by endothelial disruption, a frequent mechanism leading to venous thrombosis is stasis in the absence of endothelial injury (37). Despite stasis being identified as a cause of venous thrombosis, the specific molecular and cellular events that translate depressed venous blood flow into thrombogenesis remain unclear. Some have linked inflammation to thrombosis (38, 39). Others have implicated leukocytes as a major player where reduced blood flow induces a proinflammatory endothelial phenotype initiating the recruitment of innate immune cells, particularly neutrophils and monocytes (40). Recruited leukocytes then start fibrin formation via blood cell-derived tissue factor, as they support leukocyte accumulation and promote neutrophil extracellular traps (NETs) formation. NETs formation, in turn, triggers thrombin production via the intrinsic pathway of the coagulation cascade and ultimately fibrin polymerization (41).

2.2.3 Fibrinogen and fibrin network polymerization

Fibrinogen is a 45 nm long, 340 kDa, trinodular molecule present in plasma at 2–4 mg/ml. It is made up of two outer globular domains (D domains) and an inner globular domain (E domain) connected by a flexible coiled region (Figure 2.4) (42, 43). Polymerization is initiated by cleavage of fibrinopeptides A (FpA) located on the E domain by thrombin. Removal of FpA produces a fibrin monomer by exposing binding sites called knobs ‘A’ which are complimentary to pockets ‘a’ located on each D-domain. Interactions between the A:a complementary binding sites produce aggregates of coupled, half-staggered monomers with the knob-hole interactions holding the two monomers together. As more monomers become activated, they add longitudinally to the dimer eventually forming large oligomers that are able
to laterally aggregate, called protofibrils. Because the process of protofibril formation involves two distinct binding steps a third molecule can interfere with the second step of the binding of the original two molecules leading to the formation of a branch (43). “Trimolecular junction”
branch points, which have three fibrin branches typically predominate, particularly in highly elastic fibrin gels (44, 45). Eventually a 3D spacefilling branched structure is formed where the pore sizes determined by the amount of branching and overlap of the fibers within the network. Finally, plasma transglutaminase, factor XIIIa, internally cross-links each fibrin strand by covalently connecting the D-domains within a fiber. These crosslinks can transversely connect D-domains of fibrin monomers that are already connected via A:a interactions or longitudinally connect D-domains of monomers that have no form of direct connection (46-48). This covalent crosslinking makes the polymerization process irreversible and stabilizes and strengthens the final network.

2.2.4 Regulation

Several mechanisms exist to inhibit coagulation preventing uncontrolled thrombus growth. Chemical inhibitors [antithrombin III (ATIII), tissue factor pathway inhibitor (TFPI), and activated protein C (APC)] present in the plasma act on various species in the network. ATIII is a serine protease inhibitor that chemically inactivates thrombin, Xa, and IXa in the fluid phase. TFPI is a plasma serine protease that modulates initiations of coagulation by inhibition of both Xa and the FVIIa:TF complex. APC proteolytically inactivates VIIIa and Va.

2.2.5 Fibrinolysis

After a clot has formed and the wound has healed the process of fibrinolysis dissolves the clot and the vessel returns to its original state. During fibrinolysis, the zymogen plasminogen, a serine protease in the plasma binds to fibrinogen. Plasminogen is converted to the enzyme plasmin by the action of either tissue plasminogen activator (tPA) or urokinase (uPA) that are secreted from the endothelial cells, the renal epithelium, or monocytes/macrophages. Once active, plasmin cleaves the connecting region between the D- and E- domains of a fibrinogen
molecule resulting in a solubilized fibrin degradation product called a D-dimer. Like during coagulation, there are a number of chemical inhibitors to this process. Most notable are thrombin activated fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor-1 (PAI-1) both of which circulate in the plasma. TAFI attenuates fibrin dissolution by decreasing fibrin-binding sites for fibrinolytic enzymes. PAI-1 inhibits both tPA and uPA (49).

2.3 Microfluidic devices for measuring clot formation under flow

Over the past decade, research has extended beyond identification of the key factors and reactions of thrombus formation and into the development of models that combine all three elements of Virchow’s triad with additional consideration being given to the effects of shear stress, shear rate effects, and transport mechanisms of flowing blood (2, 34, 43, 50-58). Shear stress [$\tau$ (force/area)] represents the force experienced at the endothelial wall and on the adherent blood cells because of the flowing blood. The shear rate [$\gamma$ (1/time)] represents the velocity gradient near the wall and is proportional to the flow rate through the channel. The shear stress is related to the shear rate through viscosity [$\tau = \mu \times \gamma$] which in the case of blood depends both on the fluid velocity and the size of the vessel. The shear rates typically encountered physiologically are lowest in veins (20 – 200 s$^{-1}$), highest in arterioles (50 – 1600 s$^{-1}$), and high in large arteries (300 - 800 s$^{-1}$).

One method of assessing flow-dependent clot formation and/or cell adhesion and aggregation is microfluidics. Falling into a subset of flow-based experimental techniques known as flow assays, microfluidics offer several advantages over traditional flow-assay techniques. Traditional flow-assays have geometries limited to straight channels that result in unidirectional laminar flow, require large volumes (10-100 mL), and typically only allow testing of one condition (shear rate and substrate) per assay. Consequently, these assays are not able to mimic
the fluid dynamics of complex geometries such as bifurcations, stenoses, and convoluted interconnected networks that contribute to important biophysical mechanisms, are inadequate when only limited sample quantities are available, and have limited throughput. Microfluidics address each of these limitations leading to the development of assays that have higher-throughput and better mimic the vasculature. First microfluidics enables complex geometries. One report used microfluidic devices to show that platelets adhere preferentially to low-shear regions downstream of stenoses (4). Additionally, microfluidics enables high-throughput and simultaneous testing of a range of shear conditions using minimal sample volumes. One such device reported the ability to test platelet function on type I collagen coated surfaces over a two orders-of-magnitude range of shear rates using only 15 µL of whole blood (59). Additionally, microfluidic devices can be combined with micropatterning techniques to define small focal patches of prothrombotic substances enabling high-throughput testing of thrombosis and hemostasis as a function of injury size and shape. Neeves et al. described a device consisting of a 100 µm strip of collagen patterned on a glass substrate perpendicular to an array of 13 microfluidic channels (80 µm × 100 µm) (60). This collagen ‘injury’ mimics the focal nature of animal models like the laser injury model, eliminated upstream issues regarding upstream activation of platelets, and enables real-time image quantification. These advantages make microfluidics ideal for the high-throughput study of flow-formed thrombi formation.

2.4 Flow-formed clot structures

One particularly important aspect of flow-based systems is the accumulation of clot components at the injury site that effect the growth, arrest, dissolution, and overall strength of a clot. Scanning electron micrographs of focused ion beam milled clots show both increased solid fractions (> 0.02 v/v) relative to statically formed clots and pore structures in both platelet-rich
and fibrin-rich regions of clots formed under flow in vitro that contain at least two length scales (Fig. 2.5). One length scale is defined by the blood cells, which can have diameters ranging from 1–25 µm and volume fractions ranging from 0.01–0.9. The space between cells can range from just few nanometers to hundreds of micrometers. The second length scale is defined by the fibrin matrix, which typically has diameters of 10-1000 nm. The fibrin matrix is mostly water by volume (>90%) and is extremely compressible and extensible. The space between fibers in the matrix can range from 1 nm to 100 µm. Taken together, these structures yield a mixed poroelastic medium where a set of roughly spherical objects (cells) is separated by a fibrous medium.

This pore structure affects a number of physical properties of a clot including the rate of solute and fluid transport and the mechanical strength of the clot. Solute movement in the clot is limited based on the relative diameters of the solutes and the pores and can result in the physical inhibition of coagulation factors within a growing or formed clot (21, 61-65). For instance, a
two-fold increase in solid fraction within in vivo formed clots was shown to result smaller pores and in up to a 16-fold reduction in the rate of solute transport (58, 66). Interstitial flows in the pores exist owing to the pressure gradient created by flowing blood along the periphery of a clot and by the pressure gradient that exists between the blood vessels and the lymphatic vessels. These interstitial flows are typically small ($< 1 \text{ cm s}^{-1}$), but are important for the exchange of coagulation factors, the delivery and retention of drugs, and the stability of the clot (34, 58, 67). Differences in introduction and removal rates due to flow conditions, pore structure, and platelet and protein binding determine the mass accumulation or loss at the injury site and may also serve as a physical mechanism for clot arrest. For small clots, flow-mediated dilution of reacting species is much more potent mechanism of clot arrest than chemical inhibition (50). In larger clots, adhesion of platelets to the subendothelium serves as a transport barrier preventing coagulation factors in the fluid from reaching surface-bound enzymes (Fig. 2.6). For instance, in vitro experiments have shown that the transport caused by clot solids can result in a 400-fold reduction of activation of X to Xa (68). After a clot has formed pressure-driven permeation is the main mode of transport within the clot, allowing for kinetically relevant thrombolysis. Without pressure driven permeation a fibrin clot can require over 1000 min to achieve 50% lysis with tPA; however, pressure-driven permeation was able to achieve 50% lysis in less than 20 min (20). Finally, the accumulation of components caused by transport determines, in part, the mechanical strength of a clot. Increased solid fractions within a clot generally leads to an increase in material strength and determines the susceptibility of the clot to embolism (12, 69). In particular, the concentration of fibrin in a clot is important as small changes in fiber volume fraction result in large changes in elastic modulus of fibrous materials. Overall, the relationship between flow-dynamics, pore structure and component accumulation, and physical properties
plays an integral role in the life cycle of a clot.

Despite their importance, there is a paucity of data on the physical properties of clots formed at solid fractions reflective of those formed under flow. The data that does exist has been collected at fibrin and platelet concentrations that are orders-of-magnitude less than what has been reported in clots formed in vivo (70, 71). The focus of this thesis is on the physical properties of clots formed with high platelet and fibrin concentrations that result from the advection of solids in clots formed under flow. Here, I provide an overview of the physical properties of permeability, hindered diffusivity, and viscoelasticity that determine the rate of interstitial flow, solute movement, and mechanical strength of thrombi, respectively.

2.5 Overview of porous media

This description of porous media is based on the description given in Chapter 8 of Truskey et al. (72). Porous media are solid materials with internal pore structures. The pore structure of a material varies significantly among different media and can be random or have a
well-defined structure. Random structures (Fig. 2.7 A & B) are often found in vivo, can have a range of pore size and spacing, and the porosity can vary throughout the material. Regular structures (Fig. 2.7 D & E) are often found in industrially fabricated materials such as women fabrics or processes such as heat exchangers and can have well defined pore sizing, spacing, and porosity.

Both random and regular structures can be fibrous, granular, or a combination of both. Fibrous structures (Fig. 2.7 A & D), such as hydrogels and woven fabrics, consist of many long thin solids and can have much of their overall volume occupied by the pore space. Granular structures
(Fig. 2.7 B & E), such as rock or sand, consist of solid particles and the pore space in between. Biological structures tend to be heterogeneous and can contain a mixture of both granular and fibrous structures simultaneously (Fig 2.7 C). In thrombi the solid structures are made primarily of platelets and fibrin but can also include red blood cells, white blood cells, and proteins. The cellular fraction is best described as granular and the fibrin as fibrous. The internal pores are filled with plasma that can contain free cells, or dissolved proteins, salts, and sugars.

Porous media are often characterized by their total porosity, $\varepsilon$, defined as:

$$\varepsilon = \frac{V_{\text{void}}}{V_{\text{total}}}$$

(2.1)

where $V_{\text{void}}$ is the void volume and $V_{\text{total}}$ is the total volume of the media. Though porosity provides a measure of the total void space of porous media, it does not account for the connectivity of pores or for which pores are available for transport. Pores can be isolated, nonpassing, or passing (Fig. 2.7 F). Isolated pores are not accessible to external solvents or solutes and do not contribute to the available pore space for transport. Nonpassing pores are accessible only at one external site. Solvents and solutes can travel in and out of nonpassable pores, but are unable to pass through the media. Passing pores either have two external access points or connect to other pores providing multiple external access points and enable solvents and solutes to pass through the media. The path length between the two endpoints of a passing pore is described by its tortuosity, $T$:

$$T = \frac{L_{\text{path}}}{L_{\text{straight}}}$$

(2.2)

where $L_{\text{path}}$ is the path length of the pore and $L_{\text{straight}}$ is the straight-line distance between the two ends of the pore. Taken together, porosity, tortuosity, as well as pore size can have a significant impact on transport of both solvents and solutes in the porous media.
2.6 Transport in porous media

Fluid and solute transport in porous media is of great importance in a wide range of applications including, but not limited to, transport of underground water and pollutants, hydrocarbon recovery, filter analysis, and thrombus formation. Permeability is a measure of the ability of fluids to pass through the pore space of a structure. It is among the most important properties that characterize a porous media and provides information of both pore size and of fluid and solute transport within a porous medium.

2.6.1 Permeability

Two methods exist to describe fluid flow in porous media, pore-level and continuum. The pore-level method requires solving the governing equations for fluid flow in the individual pores. The continuum method assumes the flow in individual pores can be represented by an averaged quantity at larger length scales and treats the porous media as a uniform material. For the continuum approach to be valid several length scale characteristics must hold true. First, the average pore size, \( d_{\text{pore}} \), must be much smaller than the characteristic length over which fluctuations in the porosity vary, \( L_{\text{REV}} \). The volume with dimensions \( L_{\text{REV}} \) is known as the representative elementary volume (REV). This volume is assumed to be representative of the entire media. If the length of the sample over which fluid flows is much larger than \( L_{\text{REV}} \) the details of the porous structure are ignored and any physical quantity in the material is defined as the volume average of the same quantity in a pure medium.

The equation for momentum balance in porous media is the phenomenologically derived Darcy’s law (73):

\[
\mathbf{v} = -\frac{k}{\mu} \nabla P
\]  

(2.3)
where $\bar{v}$ is the average velocity of the fluid, $\mu$ is the fluid viscosity, $P$ is the pressure and $k$ is a second order tensor and a material property called the permeability of the material that depends upon the microscopic structure of the material. In this work thrombi are assumed to be both isotropic and homogeneous on the length scales tested, reducing permeability to a scalar.

Though initially empirically derived, Darcy’s law has been theoretically derived from the Navier-Stokes equations using volume averaging techniques and assuming Stokes flow (74, 75). As such, Darcy’s law is only valid for Newtonian flows with low Reynolds numbers ($N_{Re} < 1$). Flows within thrombi can be considered to be Newtonian due to blood plasma being comprised largely of water. The small characteristic lengths associated with the components of thrombi dictate that the Reynolds number is sufficiently small ($N_{Re} << 1$) at physiological relevant flow rates. Thus the assumptions used in deriving Darcy’s law are valid in thrombi.

Conceptually, Darcy’s law can be derived assuming the porous medium is comprised of a well-defined geometry—a regular array of $n_A$ straight cylinder pores per unit cross-section each with a pore diameter, $d_{pore}$ (Fig. 2.8). Solving the Navier-Stokes equations results in the volumetric flow in each pore being equal to:

$$q_{pore} = -\frac{\pi d_{pore}^4}{128\mu} \frac{dp}{L}$$

(2.4)

where $q_{pore}$ is the volumetric flow rate in each pore, $dp/L$ is the pressure drop across the length of the pore, and $\mu$ is the viscosity of the fluid. The total flow across the entire porous medium, $Q$, is equal to the sum of the flow across each cylinder:

$$Q = An_A q_{pore}$$

(2.5)

where $A$ is the cross-sectional area of the porous medium. The velocity in the entire porous medium, $v_{Darcy}$, is then the sum of the velocities in each pore:
Comparison of Eqn. 2.3 and 2.6 reveal that for the specific geometry used in this example:

\[
k = \frac{n_A \pi d_{\text{pore}}^4}{128}
\]  \hspace{1cm} (2.7)

From this example it is easy to see that permeability, \( k \), is a material property directly controlled by the pore size within the material (\( d_{\text{pore}} \)) and porosity (\( n_A \)). Through the permeability is determined by the pore geometry of the porous medium, the average velocity through the medium, \( v_{\text{Darcy}} \), represents the average fluid velocity through the medium and neglects to specifically account for the microscopic geometry. Because permeability is determined by the solid fraction and the pore geometry of the medium it can be used to predict other pore-size
dependent phenomena such as solute diffusion or mechanical properties both of which are controlled at least in part by network size.

### 2.6.2 Diffusion in porous media

The governing equations of diffusion in porous media are the same as those in free solutions. Fick’s law still governs diffusion, however, diffusivity in porous media can be limited both by steric and hydrodynamic effects. Steric effects result when the diameter of a solute particle is larger than the diameter of a pore and the pore becomes impassable to the solute (Fig. 2.9 A). Hydrodynamic effects occur due to increased particle drag caused by the particle passing near a pore wall (Fig. 2.9 B). At low Reynolds numbers the disturbance produced by a moving particle extends well beyond the boundary of the particle to the pore wall. Thus the pore wall can have a significant effect on diffusion. These steric and hydrodynamic effects are collectively known as hindered diffusivity and depend on many factors including solute diameter, pore diameter, and tortuosity. Accounting for these effects, diffusion coefficients in porous media are expressed as an effective diffusion coefficient, $D_{\text{eff}}$;

$$D_{\text{eff}} = D_0 FS$$ (2.8)

where $D_0$ is the diffusion coefficient of the solute in free media, $F$ is a function accounting for increased drag on the solute due to near wall effects, and $S$ is a function accounting for steric effects and tortuosity. Because both permeability and hindered diffusivity are functions of pore size and tortuosity, effective diffusion coefficients are often expressed in terms of the permeability of the porous media. Therefore, we expect that measurements of permeability will allow for predictions of hindered diffusion in thrombi.
Mechanical properties of biological networks

Fibrin, one of the main components of a thrombus, is a fibrous network responsible for controlling a clot’s mechanical properties; determining if the thrombus will be able to withstand the stresses imparted on it by the flowing blood, if it will deform reversibly or irreversibly, or embolize. For instance, “acute” or newly formed thrombi with lower relative stiffness are more susceptible to embolism than “mature” thrombi with higher stiffness (76, 77). Categorizing the type of network (flexible, semi-flexible, or rigid) and the linear and non-linear elastic properties of the thrombus is important to determining mechanical integrity of a thrombus. Below is a discussion of network type, mechanical regimes, and a brief introduction of the methods used to describe each.

Figure 2.9. Hindered solute transport in pores. (A) Steric hindrance occurs when the solid component of the porous media physically blocks the solute particle. If the diameter of the pore is smaller than the particle diameter the particle will be sterically blocked. (B) Hydrodynamic hindrance occurs when viscous drag from the nearby pore cause the solute particle to move more slowly than in free media. Smaller pores result in increased effective drag relative to larger pores, even if the solute particle diameter is less than the pore diameter.
2.7.1 Network Categorization

Biological networks are a subset of a larger category of polymer networks, the mechanics of which can be sorted into one of three regimes, flexible, semi-flexible, or rigid, based on the ratio of the length scales, persistence length, $l_p$, and contour length, $L_c$, of the fibers within the network (Fig. 2.10) (78-80). Persistence length is the typical length scale for the decay of tangent-tangent correlations of a polymer chain (81). Contour length is the total length of the polymer chain between crosslinks. When $l_p << L_c$ the polymer chain is considered to be flexible. Flexible polymer mechanics are based on changes in the confirmation of the polymer chain and describe the elasticity of rubber-like polymers such as vulcanized natural rubber (82). At sufficiently large strains the chains of a flexible polymer maintain sufficient “slack” to form loops and maintain thermal fluctuations resulting in linear elastic behavior over wide range of strains. The elasticity of most biopolymer networks, including fibrin, is poorly described by flexible polymer mechanics. Semi-flexible thermal polymer elasticity is derived from the thermal fluctuations of the filaments between crosslinks and resist strain due to a decrease in entropic confirmations (83). When $l_c$ is comparable to $L_c$ the polymer chain is said to be semi-flexible. Semi-flexible filaments are not flexible enough to form loops within the polymer chain, yet are sufficiently flexible to undergo thermal bending fluctuations. Strains of only a few percent are sufficient to remove the “slack” from the network chains resulting in nonlinear behavior at moderate strains. Finally, when $l_p >> L_c$ the filaments are considered to be rigid and athermal. The filaments between crosslinks of athermal elastic-rod networks are sufficiently rigid to not undergo thermal fluctuations in the unstrained state. The elasticity of this type of network is derived from enthalpic bending and/or stretching of the filaments when strained and nonlinear behavior results from network properties (84).
Viscoelasticity and small strain dynamic mechanical analysis

This derivation of the Maxwell model of viscoelasticity and analysis through harmonic motion is presented in Chapter 8 of Rodriguez et al. (82). Viscoelasticity is the tendency of a material to exhibit both viscous and elastic behavior in response to deformation. Viscosity is a measure of the energy dissipated by a fluid in motion under the action of an applied force. Elasticity is a measure of energy stored by a material under the action of an applied force. When the amplitude of the strain is small a linear relationship between stress and strain is observed and the elasticity is considered to be linear. The strains at which linear behavior is observed and strain is considered small is material dependent and typically determined experimentally.

Figure 2.10. Fiber type determines the behavior of the network. Polymer networks are described as flexible if the persistence length, \( l_p \), of the fiber is much less than its contour length, \( L_c \). Even at large strains, thermal fluctuations still persist within the chain. If the persistence length, \( l_p \), of the fiber is roughly equal its contour length, \( L_c \), the network is described as semi-flexible. Stains of only a few percent are sufficient to eliminate the thermal fluctuations of the fiber. If the persistence length, \( l_p \), of the fiber is much less than its contour length, \( L_c \), the network is described as rigid and the filaments do not have significant thermal fluctuations. Increased strain results in the stretching of the fiber.
Viscoelasticity is most easily illustrated by Maxwell’s spring and dashpot model. A Hookean spring assumes:

$$\sigma_S = E\gamma_S$$  \hspace{1cm} (2.9)

where $\sigma_S$ is the stress due to elongation of the spring, $E$ is the Hooke’s law constant or elastic modulus of the material, and $\gamma_S$ is the elongation of the spring. The Newtonian viscous relationship is given by:

$$\sigma_D = \eta \frac{d\gamma_D}{dt}$$  \hspace{1cm} (2.10)

where $\sigma_D$ is is the viscous stress of the dashpot, $\eta$ is the fluid viscosity, and $d\gamma_D/dt$ is the rate of elongation (dashpot movement) over time. Assuming the total strain is equal to the sum of individual strains:

$$\gamma = \gamma_S + \gamma_D$$  \hspace{1cm} (2.11)

and that the stress is constant across the element:

$$\sigma = \sigma_S = \sigma_D$$  \hspace{1cm} (2.12)

allows for a combined expression (combination of Eqns. 2.10-2.12) of stress and strain as a function of time, and the physical constants $E$ and $\eta$:

$$\frac{d\gamma}{dt} = \frac{1}{E} \frac{d\sigma}{dt} + \frac{\sigma}{\eta}$$  \hspace{1cm} (2.13)

Eqn. 2.13 is the basis for the most popular technique used to quantify the viscoelasticity of a material known as dynamic mechanical analysis (DMA). DMA allows for the characterization of the viscoelastic properties of a material by imposing a continuous sinusoidal force on a material. The stress-strain relationship of a Maxwell element (Eqn. 2.13) in complex notation is given by:

$$\frac{d\gamma^*}{dt} = \frac{1}{E} \frac{d\sigma^*}{dt} + \frac{\sigma^*}{\eta}$$  \hspace{1cm} (2.14)
where $\gamma^*$ is the complex strain and $\sigma^*$ is the complex stress. Sinusoidal forces acting on a Maxwell element result in a strain with the same frequency as the force input, but out of phase. Therefore, substituting the derivative of a complex strain function with respect to time (the real part of which is the actual strain):

$$\gamma^* = \gamma_m e^{i\omega t}$$  \hspace{1cm} (2.15)

and its derivative:

$$\frac{d\gamma^*}{dt} = i\omega \gamma_m e^{i\omega t}$$  \hspace{1cm} (2.16)

where $\gamma_m$ is the maximum strain, $t$ is time, and $\omega$ is frequency into Eq. 2.14, results in measureable stresses being a function of the elastic modulus, $E$, the max elongation, $\gamma_m$, frequency and time.

$$\frac{d\sigma^*}{dt} + \frac{\sigma^*}{\eta} = i\omega \gamma_m E e^{i\omega t}$$  \hspace{1cm} (2.17)

Solving the linear differential equation (Eq. 2.17) yields the following result for complex stress and strain:

$$\frac{\sigma^*}{\gamma^*} = \frac{\omega^2 \theta^2 E}{1 + \omega^2 \theta^2} + \frac{i(\omega \theta E)}{1 + \omega^2 \theta^2}$$  \hspace{1cm} (2.18)

where $\theta$ is the ratio of viscosity to elastic modulus, $\eta/E$. The real term in Eq. 2.18 is known as the storage modulus, $E'$, and the imaginary term the loss modulus, $E''$. By convention of complex notation:

$$E^* = \left( E'^2 + E''^2 \right)^{\frac{1}{2}} e^{i\phi} = \frac{E \omega \theta}{\left(1 + \omega^2 \theta^2\right)^{\frac{1}{2}}}$$  \hspace{1cm} (2.19)

where
\[
\tan \delta = \frac{E''}{E'} = \frac{1}{\omega \theta} 
\]  

(2.20)

Substitution of Eq. 2.20 into Eq. 2.19 yields:

\[
\sigma^* = E^* \gamma^* = \frac{E \omega \theta \gamma^*}{(1 + \omega^2 \theta^2)^{\frac{1}{2}}} e^{i \delta} 
\]

(2.21)

The actual stress, \(\sigma\), is the real part of \(\sigma^*\) which depends on \(E\) and \(\gamma^*\) but leads by a phase angle \(\delta\). The max stress occurs at \(\omega t + \delta = 0\), giving

\[
\sigma_m = \frac{\omega^2 \theta^2 E \gamma_m}{(1 + \omega^2 \theta^2)^{\frac{1}{2}}} 
\]

(2.22)

which when substituted into Eqn. 2.21 yields:

\[
\sigma^* = \sigma_m e^{(i \omega t + i \delta)} 
\]

(2.23)

Thus, measuring the stress within a sample while imposing an oscillatory strain allows for identification of storage, \(E'\), and loss moduli, \(E''\), as a function of frequency, \(\omega\), max stress \(\sigma_m\), and time, \(t\).

### 2.7.3 Nonlinear elasticity

In addition to small deformation linear elastic properties, many biopolymer networks, including fibrin, exhibit nonlinear elastic properties that are currently not well understood. For instance, fibrin gels strain harden at moderate strains and generate negative normal stresses in response to shear stress. Stain hardening occurs in both fibrin gels and in individual fibrin fibers. The onset of hardening for an individual fibrin fiber begins at strains above 100\%, yet strain hardening in fibrin gels begins around strains of 10\% (81, 85). Further, strain hardening at low strains (below 75\%) is attenuated for fibrin gels formed at high fibrin concentrations and in the presence of platelets (27, 86). Fibrin gels also generate negative first normal stresses occur when
strained in a simple geometry. More generically, fibrin gels “pull” or “contract” in the normal
direction in response to stress in the shear direction. The magnitude of the stress generated is
dependent upon the nature of the fiber network and upon the strain. Both of these nonlinear
behaviors are not described by rubber theory. In particular, it is unclear how these properties are
affected by strain and fibrin concentration and if these nonlinearities are caused by the mechanics
of the network, by the mechanics of the individual fibrin fiber, or by a combination of both.

2.8 Summary

Taken together, the complex fluid dynamics of the circulatory system significantly effects
thrombus formation. Flow, in part, is responsible for determining the rate of accumulation of clot
components and plays a role in receptor-ligand dynamics. These effects result in thrombi with
complex porous structures which, in turn, affect the rate of fluid and solute transport in the
thrombi. Thus, fluid dynamics and clot structure exchange in a feedback loop that determines the
final structure of the clot. Finally, the final structure of the clot determines the mechanical
properties of a clot and ultimately determines if the clot will remain a physically stable
component of the hemostatic process, deform to result in excess bleeding, or embolize and
possibly result in a downstream blockage.
CHAPTER 3
THE HYDRAULIC PERMEABILITY OF BLOOD CLOTS AS A FUNCTION OF 
FIBRIN AND PLATELET DENSITY

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3.1 Abstract

Interstitial fluid flow and solute diffusion within blood clots are biophysical mechanisms that regulate clot growth and dissolution. Assuming that a clot can be modeled as a porous medium, the physical property that dictates interstitial fluid flow and solute diffusion are the hydraulic permeability and the hindered diffusivity. The objective of this study was to bound the possible values of these physical properties in clots formed in vivo and present relationships that can be used to estimate clot permeability and hindered diffusivity as a function of composition. A series of clots with known densities of fibrin and platelets, the two major components of a clot, were formed under static conditions. The permeability was calculated by measuring the interstitial fluid velocity through the clots at a constant pressure gradient. Hindered diffusivity was measured with a diaphragm diffusion cell and by fluorescence recovery after photobleaching. Fibrin gels formed with a fiber volume fraction of 0.02 – 0.54 had permeabilities of \(1.2 \times 10^{-1} – 1.5 \times 10^{-4} \, \mu\text{m}^2\). Platelet rich clots with a platelet volume fraction of 0.01 – 0.61 and a fibrin volume fraction of 0.03 had permeabilities over a range of

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1.1 \times 10^{-2} - 1.5 \times 10^{-5} \mu m^2. Fibrin gels formed with a fiber volume fraction of 0.36 reduced the diffusion coefficients of 12, 29, and 54 nm solutes by 60%, 66% and 85%, respectively. Platelet rich clots with a platelet volume fraction 0.61 reduced the diffusion coefficient of a 12 nm solute 93%. The permeability of fibrin gels and of clots with platelet volume fraction of < 0.2 were modeled as an array of disordered cylinders with uniform diameters. Clots with a platelet volume fraction of > 0.2 were modeled as a Brinkman medium of coarse solids (platelets) embedded in a mesh of fine fibers (fibrin). Hindered diffusivity of fibrin gels and platelet rich clots was predicted using permeability measurements and the effective medium model. Our data suggests that the permeability of clots formed in vivo can vary by up to five orders-of-magnitude, with pore sizes ranging from 4–350 nm that significantly restrict solute diffusion. These findings have important implications for the transport of coagulation zymogens/enzymes in the interstitial spaces during clot formation, as well as the design of fibrinolytic drug delivery strategies.

3.2 Introduction

Interstitial fluid flow through the extracellular space is an important phenomenon in the homeostasis, development, and pathology of tissues (67). Interstitial flows have been used to distribute drugs and drug delivery systems by either exploiting endogenous flows (87) or inducing flow by local infusion into a targeted tissue (88, 89). Blood clots are a tissue where interstitial flows exist owing to the pressure gradient created by flowing blood along the periphery of a clot. There is growing evidence that interstitial fluid and solute transport plays an important role in the growth, arrest, and dissolution of blood clots (34, 90). However, there are few measurements of the constitutive properties in clots that regulate interstitial transport. One of those properties is the permeability.
The permeability of a porous medium can be calculated from Darcy’s law:

\[ \nu = -\frac{k}{\mu} \nabla P \]  

(3.1)

where \( \nu \) is the interstitial fluid velocity, \( k \) is the permeability, \( \mu \) is the viscosity of the percolating fluid, and \( P \) is the pressure. The permeability is a function of the volume fraction of solids, pore size, and fiber or cell/cell aggregate size. A blood clot, like most tissues, is a material that has both fibrous and granular structures. We define fibrous structures as those defined by proteins and carbohydrates in the extracellular space and granular structures as those defined by cells. Fibrin fibers that form between and around platelets give the clot a fibrous structure. Platelets and platelet aggregates give the clot a granular structure. The relative density of these two components—fibrin and platelets—dictates the interstitial fluid transport. The diffusivity of a solute across a clot can be calculated from Fick’s law:

\[ j = -D \nabla c \]  

(3.2)

where \( j \) is the flux, \( c \) is the concentration and \( D \) is the diffusivity. The diffusivity across the membrane is a function of the solute size and the volume fraction of the solids, pore size, and fiber or cell/cell aggregate size. The objective of this study is to measure and model the hydraulic permeability and hindered diffusivity of clots as a function of platelet and fibrin density.

The hydraulic permeability of pure fibrin gels has been extensively measured dating back to Ferry and colleagues seminal studies in the 1970s (14, 15). To date, most reports of fibrin gel permeability have been measured at or near plasma fibrinogen concentrations (~ 3 mg/mL) (19). These permeability measurements are typically used to determine how biochemical conditions affect fibrin polymerization. For example, for a given fibrinogen concentration, fibrin gels formed at higher thrombin concentrations consist of densely packed thin fibers compared to gels formed at low thrombin concentrations (19). Permeability has also been used as a measure of
how a drug or certain disorders affect fibrin polymerization. Consumption of low doses of acetylsalicylic acid results in a 44% increase in fibrin permeability (18). Fibrin clots made from the plasma of individuals following ischemic stroke have lower permeability than healthy controls (16). Similarly, the hindered solute diffusion in hydrogels and other porous mediums has been extensively studied (61, 91). Most of these reports have focused on the effects of component size or charge in gels with very low solid fractions (<0.05) (62, 92-95). A major conclusion of these works was the degree of hindered diffusivity is directly related to the permeability of a medium (62). Permeability provides an estimate of the screening length or diameter of a porous medium; therefore, the degree of hindered diffusion can be predicted as a function of solute size and permeability of the medium.

There are few direct measurements of the permeability or hindered diffusivity of clots with significant cellular volume fractions. The volume fraction of platelets in clots formed from platelet rich plasma is ~0.005, too small to significantly influence the permeability or diffusion. Yet, the histology of clots retrieved from humans with atrial fibrillation and stroke suggest that platelets make up 20-80% of the solid volume (96, 97). Furthermore, the density of fibrin is likely much higher in clots than in gels formed at plasma concentration of fibrinogen. A recent electron microscopy study of aspirated clots from myocardial infarction patients found an average fibrin solids volume fraction of 56% (31).

In this study we measured how the volume fraction of fibrin and platelets affects fluid and solute transport within the interstitial spaces of clots. The focus here is on platelet rich clots that are characteristic of the initial stages of hemostatic and thrombotic clots. Our approach was to induce clotting with thrombin in suspension with known fibrin and platelet densities, and then measure the permeation of a buffer solution through the clots at defined pressure gradients and
the diffusion of dextran solutes through the clots in the absence of a pressure gradient. Clots were treated as porous media where the average interstitial fluid velocity at the macroscopic scale was governed by Darcy’s law. We measured the permeability and hindered diffusivity of fibrin gels with a fiber volume fraction of 0.02-0.54 and platelet rich clots (PRC) with a platelet volume fraction of 0.01-0.61. Measured permeability values were compared to models of fibrous, granular and mixed porous media to delineate the relative contributions of each component to the overall hydrodynamic resistance. Measured hindered diffusion coefficients were compared to values predicted by a hindered diffusivity model that accounts for both steric hydrodynamic hindrances (92). These data bound the range of clot permeability and hindered diffusivity and can be used for predicting clot growth and dissolution.

3.3 Materials and methods

Human fibrinogen (free of plasminogen, von Willebrand factor and fibronectin) in 20 mM sodium citrate, human \( \alpha \)-thrombin and bovine \( \alpha \)-thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Centrifuge filtration units with a 50 kDa molecular weight cut off were purchased from Millipore Corporation (Billerica, MA). Citrated bovine whole blood was purchased from Hemostat Laboratories (Dixon, CA). Trizma buffer, calcium chloride, sodium chloride and hexamethyldisilazane (HMDS) were purchased from Sigma-Aldrich (St. Louis, MO). Tris buffered saline (TBS; 50 mM Tris, 100 mM NaCl) was made in house at pH 7.4. AlexaFluor 488 labeling kit, DiOC6, Oregon Green 488 (70 kDa), tetramethylrhodamine labeled dextrans (500 kDa and 2,000 kDa), and fluorescein isothiocyanate (FITC) (70, 500, and 2,000 kDa) were purchased from Molecular Probes (Grand Island, NY).
3.3.1 Fibrinogen Solution

Fibrinogen stock solutions (14.2 mg/mL) in sodium citrate were concentrated to 130–160 mg/mL by centrifuge filtration. Two milliliters of the stock solution were centrifuged at 2200g for 10 h. After centrifugation the retentate was removed from the device and diluted for permeability measurements. A modified Clauss assay was performed to determine the fibrinogen concentration of the retentate (98). Briefly, 10 nM thrombin and 2.5 mM CaCl$_2$ was added to the diluted (100:1 in TBS) retentate fibrinogen solution into a well of a 96 well plate (200 µL final volume). After 20 min, the absorbance was measured in a plate reader (Victor X, PerkinElmer) and the concentration was calculated by comparing the absorbance to a standard curve.

3.3.2 Fibrin Gel Preparation

Permeation chambers were created by removing the top of the barrel of 3 mL plastic syringes (BD, Cat #309585) and sealing the adaptor of the syringe with a polydimethlysiloxane (PDMS) cork. Prior to forming a fibrin gel, 1.1 mL of a 3 mg/mL fibrinogen solution was incubated in the permeation chamber for 1 h and then rinsed with deionized water and dried with an airbrush. The adsorbed fibrinogen was necessary to provide an adhesive surface for the fibrin gels. Next, the fibrinogen solution used for gelation was added to the permeation chamber and 1M CaCl$_2$ was added to a final concentration of 2.5 mM CaCl$_2$. Finally, 200 nM human α-thrombin was added and quickly mixed with a Pasteur pipette to yield a final concentration of 10 nM thrombin. The chamber was then sealed with parafilm and allowed to gel for 24 hr. Fibrin gels were prepared for fluorescence recovery after photobleaching (FRAP) measurements by forming fibrin gels (20 µL) in dextran/buffer solutions (40 nM dextran) between a glass slide and a coverslip. Gels were allowed to form for 24 hours prior to FRAP measurements.
3.3.3 Platelet Rich Clot Preparation

Platelets were isolated from citrated bovine whole blood in a three-step process: (1) Whole blood was centrifuged at 40g for 2 h resulting in an erythrocyte rich bottom fraction and a platelet rich plasma (PRP) top fraction. (2) The PRP was pipetted off the top and then centrifuged at 2200g for 20 minutes. This step resulted in a platelet plug at the bottom of the centrifuge tube and platelet poor plasma (PPP) in the supernatant. (3) The platelet plug was resuspended in the PPP to the desired cell count. The PPP from (2) was added to the erythrocyte rich bottom fraction from (1), and the two-step centrifugation process was repeated again to extract the maximum number of platelets. From 1 L of whole blood, we recovered 2-4 mL of packed platelets. Packed platelets were diluted in plasma and the cell density was measured using a hemocytometer (DHC-N01, Neubauer Incyto, Chonan, South Korea). Once diluted to the desired platelet density, suspensions were prepared for permeation and diffusivity measurements. For permeability measurements, 500 µL of the resuspended platelets were transferred to the 3 mL permeation chambers and recalcified to 20 mM CaCl₂. Clot formation was induced with 10 nM bovine α-thrombin. Clots were allowed to form for 24 h to ensure complete conversion of fibrinogen to fibrin. For diffusivity measurements, clots were allowed to form on stainless steel mesh supports with a mesh size of 200 µm and allowed to form in a humid chamber for 24 h.

3.3.4 Hydraulic permeability measurements

After removal of the PDMS plug and the parafilm seal, a reservoir containing TBS was connected to the top of the permeation chamber (Fig. A-2). Small diameter silastic tubing (0.76 mm ID, Dow Corning, Midland, MI) was connected to the bottom of the chamber via a blunt 16 gauge needle. The volumetric flow rate of buffer through the fibrin gel or platelet rich clot was calculated by tracking the velocity of the air-buffer interface in the tubing. The
volumetric flow rate of the TBS was measured at 3–5 pressure gradients for 5 min. The large volume of the TBS reservoir ensured that the pressure gradient was constant over the course of the measurements. Measurements at different pressure gradients were taken in random order. We used a pressure gradient that was adequate to provide a measurable flow rate (1-10 µL/min) but did not possess enough force to rupture the gel. The flow through the clot was visualized by adding a food dye to the permeation buffer in a 1000:1 dilution. Ruptures or detachment from the wall were identified by fingers of dye protruding into the gel/clot.

3.3.5 Scanning electron microscope measurements of fiber diameter

Gel samples were prepared for scanning electron microscopy (SEM) by forming gels (3–156 mg/mL, 2.5 mM CaCl₂, 105 mM NaCl, 100 nM thrombin) in a Slide-A-Lyzer mini dialysis devices (ThermoScientific, Cat. No. 69560, Rockford, Il). After gelation, the clots were fixed in 2.5% glutaradehyde for 30 min. Next, the dialysis device was immersed in the following series of solutions for 10 min each to dehydrate the samples: deionized water in triplicate, 50%, 70%, 80%, 90%, 100%, 100% ethanol in DI water, 33%, 67%, 100%, 100% HMDS in ethanol. After the final HMDS treatment the clot was removed from the dialysis device and air dried before being sputtered with ~10 nm of gold. SEM images were taken at an accelerating voltage of 1.5 kV and 6 mm working distance. ImageJ software was used to manually measure the ~100 fiber diameters for each condition.

3.3.6 Confocal microscopy of fibrin gels and platelet-fibrin thrombi

Fibrin gels and PRC for confocal microscopy were prepared exactly as described above, except rather than forming the samples in a permeation chamber, the samples were formed between glass slide and glass coverslip. For fibrin gels, a fluorescently labeled fibrinogen (AlexaFluor 555) was added to each solution at a molar ratio of 1000:1 unlabeled:labeled
fibrinogen. For PRC, platelets were labeled with DiOC6 (10 µM mM) and fluorescently labeled fibrinogen was added at a molar ratio of 250:1 unlabeled:labeled fibrinogen. Images were captured on a laser scanning confocal microscope (Olympus Fluoview FV10i) using a 60X objective (NA = 1.2) and excitation/emission wavelengths of 556/573 nm and 495/521 nm for the AlexaFluor555 and DiOC6, respectively. Grayscale images were made binary in ImageJ software by subtracting the background using the Sternberg rolling ball method with a rolling ball radius that was at least 5 times larger than the largest group of platelets (~100 µm for higher platelet concentrations, ~10 µm for the 50x and below). After background subtraction a threshold value of 5 was applied to all images and the platelet volume fraction was calculated from binary images. The average area and diameter of platelet aggregates was also calculated from the binary images. Each step in the image analysis procedure is depicted in Fig. A-3.

3.3.7 Hindered diffusivity measurements

Fluorescence recovery after photobleaching (FRAP) is often used to measure diffusion coefficients in biological systems such as lipid bilayers or in intracellular compartments. The solute to be measured is labeled with a fluorophore that is known to easily photobleach with a suitable laser intensity. The diffusion of fluorescent molecules into the bleached region is measured over time and the transient recovery of fluorescence is used to calculate a diffusion coefficient. Here, we use FRAP to measure the diffusion of FITC-labeled dextran of 12, 29, and 54 nm in diameter in fibrin gels. Diffusion coefficients were calculated from the transient recovery profiles using the Matlab code described by Jönsson and colleagues (99). Owing to light scattering by platelets we could not use FRAP for platelet rich clots, and instead used a custom diaphragm diffusion cell as previously described (100). Briefly, TBS solution containing $C/C_0 = 1$ and $C/C_0 = 0$ solutions of dextran were pipetted into well-mixed reservoirs on opposite
sides of the formed clot. Dextran concentrations in each reservoir was measured over 5 days in a plate reader by removing a small sample from each reservoir. Samples were returned to their respective reservoirs after each measurement to maintain a constant volume. Effective diffusion coefficients in each clot were determined by using the MatLab function \textit{robustfit} to fit the data to the function, which was determined by a combination of Fick’s law and a total mass balance:

\[
\frac{C_{\text{low}} - C_{\text{up}}}{C_{\text{low},0} - C_{\text{up},0}} = e^{-\beta Dt}
\]  

(3.3)

where \(C_{\text{low}}\) and \(C_{\text{up}}\) are the dextran concentrations in the upper and lower reservoirs, respectively, \(C_{\text{low},0}\) and \(C_{\text{up},0}\) are the initial concentrations in each reservoir, \(D\) is the diffusion coefficient, \(t\) is time, and \(\beta\) is term accounting for the geometry of the system, where:

\[
\beta = \frac{AH}{L} \left( \frac{1}{V_{\text{low}}} + \frac{1}{V_{\text{up}}} \right)
\]  

(3.4)

where \(A\) is the area of the clot through which dextran diffusion occurs, \(H\) is the void fraction of the stainless steel mesh support, \(L\) is the diffusion length equal to the thickness of the clot, \(V_{\text{low}}\) is the volume of the lower reservoir, and \(V_{\text{up}}\) is the volume of the upper reservoir.

### 3.3.8 Statistical Analysis

A line was fit to the plot of the normalized flow rate (\(\mu Q/A\)) as function of the pressure gradient (\(\Delta P/L\)) using the MatLab routine \textit{robustfit}, which uses iteratively reweighted least squares with a bisquare weight function. The slope of that line was reported as the permeability according to Darcy’s law, Eq. 3.1. The uncertainly in the permeability is reported as the standard error of the fit. All other data is presented as the mean ± standard deviation. A weighted sum least squares parameter was used to compare model predictions of permeability to experimental data. The weighted residual was defined as:
where \( FE_i \) is the fractional error of the \( i \)th component, \( k_i \) is the \( i \)th measured permeability, \( \bar{k}_i \) is the theoretical permeability. The sum of squares of the fractional error is used to determine the goodness of fit parameter (\( \chi^2 \)):

\[
\chi^2 = \sum_{n=1}^{i} FE_i^2
\]

### 3.4 Theory

A series of theoretical models have been developed to predict both permeability and hindered diffusivity as a function of fibrin and platelet densities based on empirical relationships, the solutions to the equations of Stokes flow, and computer models.

#### 3.4.1 Permeability of fibrin gels

Numerous analytical, computational and empirical relationships have been developed to predict the permeability of fibrous media (101-108). Most of these relationships have the general form of:

\[
\frac{k_f}{a_f^2} = f(\phi_f)
\]

where \( k_f \) is the permeability, \( a_f \) is the fiber radius, and \( \phi_f \) is the fiber volume fraction. In this study we compare our experimental measurements of permeability for fibrin gels with three models. The first model is the Davies’ equation, which is an empirical relationship based on air flow through fibrous media at low Reynolds numbers (109):

\[
\frac{k_f}{a_f^2} = \left[16\phi_f^{1.5} \left(1 + 56\phi_f^3\right)\right]^{-1}
\]
Eq. 3.8 has been commonly used to estimate the permeability of fibrin gels and blood clots (19, 20, 56, 110). The second model by Jackson and James is the weighted average of the solution to Stokes equation for flow parallel to or normal to two-dimensional periodic square arrays of cylinders (103):

\[
\frac{k_f}{a_f^2} = \frac{3}{20\phi_f} \left[-\ln(\phi_f) - 0.931\right]
\]

(3.9)

The third model comes from Clague and colleagues who used the lattice Boltzman (LB) method to calculate the permeability of random fibrous media (111). We used Eq. 14 from reference (111) which is their solution for an array of disordered fibers:

\[
\frac{k_f}{a_f^2} = 0.50941 \left(\frac{\pi}{4\phi_f}\right)^{0.5} \left(\frac{\pi}{4\phi_f}\right)^2 e^{-1.8032\phi_f}
\]

(3.10)

Eq. 3.10 gives the appropriate scaling of the permeability over a wide range of fiber volume fraction (0.05–0.7). To compare these three models to our experimental data, it is necessary to estimate the fiber volume fraction, $\phi_f$, of the fibrin gels and the fiber radius, $a_f$, as a function of fibrinogen concentration, $c_{Fbg}$. The fiber volume fraction was calculated using a mass balance where we assumed that all the fibrinogen was incorporated into a fiber and that each individual fiber consists of an internal solid fraction, $\Phi_{int}$, and water fraction, $1-\Phi_{int}$. The internal solid fraction, $\Phi_{int}$, was estimated from neutron scattering data collected from fibrin gels formed at fibrinogen concentrations of 1–40 mg/mL (86). A fit of the data from Fig. 5 in reference (86) gives:

\[
\Phi_{int} = 0.015 \ln(c_{Fbg}) + 0.13
\]

(3.11)

where $c_{Fbg}$ is in mg/mL. We extrapolated Eq. 3.11 out to 155 mg/mL since, to the best of our knowledge, there is no data on the internal solid fraction beyond 40 mg/mL. It is possible that at
higher fibrinogen concentrations this trend may not hold, but given the weak dependence of internal solid fraction on fibrinogen concentration, any deviations should be small.

The overall fiber volume fraction ($\phi_f$) is calculated by:

$$\phi_f = \frac{c_{Fbg}}{\rho_m \Phi_{int}}$$  \hspace{1cm} (3.12)

The mass density of an individual fibrinogen protein ($\rho_m$) is 1.4 g/mL (112).

The dehydrated fiber radius was determined by analysis of SEM images as described in Section 3.3.5. The hydrated fiber radius ($a_f$) was calculated by:

$$a_f^2 = \frac{a_d^2}{\Phi_{int}}$$  \hspace{1cm} (3.13)

where $a_d$ is the dehydrated radius. We assume that there is no void space in the dehydrated fibers.

3.4.2 Permeability of platelet rich clots

In platelet rich clots, viscous losses on the platelet surface will contribute to the overall hydrodynamic resistance through the clot in conjunction with losses on fibrin fibers. Platelets also increase the superficial velocity within the fibrin gel and increase the tortuosity. Platelet rich clots have elements of both granular (platelets) and fibrous (fibrin) media. Similar to the treatment of fibrin gels described above, we compared our experimental data to three models that account for this type of mixed porous media.

In the first model, we treat the granular and fibrous media components as independent hydraulic resistances and add their contributions in parallel as an unweighted resistivity:

$$\frac{1}{k_t} = \frac{1}{k_p} + \frac{1}{k_f}$$  \hspace{1cm} (3.14)

where $k_t$ is the overall permeability, $k_p$ is the permeability of the platelets, and $k_f$ is the permeability of the fibrin. We refer to Eq. 3.14 as the resistors-in-parallel model.
unweighted resistivity was found to be the most accurate method of averaging permeabilities in bimodal mixtures of fibers of different radii (108). We measured \( k_f \) directly from bovine platelet poor plasma. We estimate \( k_p \) using the Kozeny-Carmen equation for packed beds (113):

\[
k_p = \frac{\Psi^2 (2a_p)^3 (1 - \phi_p)^3}{150 \phi_p^3}
\]

(3.15)

where, \( \Psi \) is the sphericity, \( \phi_p \) is the platelet volume fraction and \( a_p \) is the platelet radius. The diameter of a bovine platelet is \( \sim 1 \mu m \). Sphericity was calculated by assuming platelets are flat cylinders with a diameter of \( 1 \mu m \) and a height of 100 nm:

\[
\Psi = \frac{\pi^{1/3} (6V_p)^{2/3}}{A_p}
\]

(3.16)

where \( V_p \) is the platelet volume and \( A_p \) is the platelet surface area.

The second model we considered was the fractional packing model which was developed to describe porous media with a mixture of coarse and fine grains (114). In the fractional packing model a volume weighted fiber radius and volume fraction are used to modify the Kozeny-Carmen equation:

\[
k_t = \frac{\Psi^2 a_c^3 (1 - \phi_t)^3}{150 \phi_t^3}
\]

(3.17)

\[
a_c = \left(\frac{1}{a_p} + \frac{1}{a_f}\right)^{-1}
\]

(3.18)

where \( k_t \) is the total permeability, \( \phi_t \) is the total volume fraction of solids, \( \Psi \) is the sphericity, and \( a_c \) is the effective characteristic length.

The third model we considered was developed by Ethier and considers a two-component fibrous medium with highly dissimilar fiber radii (115). This model is particularly relevant for
blood clots because the radius of fibrin fibers (50–100 nm) is much smaller than the size of single platelet (1 µm). In the model, flow through the media is described using the higher-order Brinkman equation rather than Darcy’s law:

$$\mu \nabla^2 v - \frac{\mu}{K_f} v - \nabla P = 0$$  \hspace{1cm} (3.19)

where $\mu$ is the fluid viscosity, $v$ is the fluid velocity, $K_f$ is the fibrin permeability and $P$ is the pressure. In the Ethier model, the composite medium can be thought of as a matrix of fine fibers embedded with large solid inclusions. The no-slip boundary condition on these large inclusions is satisfied by the Laplacian term in Eq. 3.19. Experimental measurements of permeability of PRC were compared to the unit-cell matching in Fig. 3b of reference (115). Note that Eq. 3.19 is only applicable at length scales where the pressure gradient is balanced by the Laplacian of the velocity. This length is often referred to as the Brinkman screening length, and is equal to the square root of the permeability, $K_f$.

In all three of the mixed porous media models described above, the void fraction of the clot, $\varepsilon$, was calculated by:

$$\varepsilon = 1 - (\phi_p + \phi_f)$$  \hspace{1cm} (3.20)

where $\varepsilon$ is the void fraction of the clot, $\phi_p$ and $\phi_f$ are the volume fractions of the platelets and fibers, respectively. The platelet volume fraction was estimated based on calculations of the maximum packing of circular disks and verified by image analysis of confocal images of PRC as described in section 3.3.6. The volume fraction of the fibers was calculated using Eq. 3.12.

3.4.3 Hindered diffusion

One model used to describe the hindered diffusivity of solutes presented by Phillips accounts for both for both hydrodynamic and steric hindrance (92). This model combines the
effective medium model initially presented by Phillips and Brady with a refinement aimed
toward steric hindrance later made by Johnson et al. (62, 94). Phillips derived an expression for
an effective medium that relates the permeability of a porous medium to the hindered diffusion
coefficient:

$$\frac{D}{D_0} = FS$$  (3.21)

where $D$ is the diffusion coefficient of the solute in the clot, $D_0$ is the diffusion coefficient of the
solute in free solution, and $F$ and $S$ are terms accounting for hydrodynamic and steric hindrances,
respectively. The degree of hydrodynamic hindrance is determined by calculating the drag force
imparted by a sphere on a parallel array of fibers:

$$F = \left(1 + \frac{d^2}{4k} + \frac{d^2}{12k}\right)^{-1}$$  (3.22)

where $d$ is the diameter of the solute and, $k$, is the permeability of the medium (94). Steric
hindrance is determined based on computer simulations calculating the diffusivities of a
molecule moving through random, overlapping arrays of polymer chains assuming no
hydrodynamic interactions:

$$S = \exp\left\{-0.84\phi \left(1 + \frac{d}{d_f}\right)^{2.18}\right\}$$  (3.23)

where $\phi$ is the solids fraction of the medium, $d_f$ is the diameter of a fiber in the medium, and $d$ is
the diameter of the solute as above (116). Essentially, steric hindrances are accounted for by
adjusting the overall solid fraction to account for the finite size of the solute.
Results
Both the permeability and hindered diffusivity of fibrin gels and platelet rich clot were found to vary greatly with fibrin volume fraction. However, both properties were most sensitive to solid fraction in fibrin gels in the dilute limit whereas the greatest sensitivity in platelet rich clots occurred at higher solid fractions.

3.5.1 Permeability of fibrin gels

Fibrin gels were made from 3–156 mg/mL fibrinogen solutions with 10 nM thrombin (Table 3.1). The permeability of each gel was calculated using Darcy’s law, Eq. 3.1. Measurements were made at least three pressures gradients for each fibrinogen concentration. It was necessary to increase the magnitude of the pressure gradient concomitantly with fibrinogen concentration in order to achieve flow rates that could be measured over a reasonable time (Table 3.1). Experiments were conducted with food dye added to the permeation buffer in order to visualize the permeation front. For each gel, we measured the failure pressure which was defined

<table>
<thead>
<tr>
<th>( \phi_f )</th>
<th>( c_{Fbg} ) [mg/mL]</th>
<th>( a_f ) [nm]</th>
<th>( \Delta P/L ) [Pa/m]</th>
<th>( k_f ) (SE) ([\times 10^3 \mu m^2])</th>
<th>Effective Screening Length [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>.02</td>
<td>3</td>
<td>60</td>
<td>(3 \times 10^4 - 1 \times 10^5)</td>
<td>120 (15)</td>
<td>346</td>
</tr>
<tr>
<td>.03</td>
<td>7</td>
<td>58</td>
<td>(8 \times 10^4 - 1 \times 10^5)</td>
<td>52 (14)</td>
<td>228</td>
</tr>
<tr>
<td>.04</td>
<td>10</td>
<td>57</td>
<td>(7 \times 10^4 - 1 \times 10^5)</td>
<td>22 (3)</td>
<td>148</td>
</tr>
<tr>
<td>.08</td>
<td>20</td>
<td>55</td>
<td>(8 \times 10^4 - 5 \times 10^5)</td>
<td>7.0 (0.2)</td>
<td>84</td>
</tr>
<tr>
<td>.10</td>
<td>24</td>
<td>55</td>
<td>(3 \times 10^4 - 7 \times 10^5)</td>
<td>3.4 (0.6)</td>
<td>58</td>
</tr>
<tr>
<td>.18</td>
<td>48</td>
<td>53</td>
<td>(4 \times 10^5 - 2 \times 10^6)</td>
<td>0.73 (0.02)</td>
<td>27</td>
</tr>
<tr>
<td>.25</td>
<td>67</td>
<td>52</td>
<td>(4 \times 10^5 - 2 \times 10^6)</td>
<td>0.57 (0.07)</td>
<td>24</td>
</tr>
<tr>
<td>.27</td>
<td>72</td>
<td>52</td>
<td>(7 \times 10^5 - 2 \times 10^6)</td>
<td>0.51 (0.15)</td>
<td>23</td>
</tr>
<tr>
<td>.32</td>
<td>89</td>
<td>52</td>
<td>(4 \times 10^5 - 2 \times 10^6)</td>
<td>0.44 (0.04)</td>
<td>21</td>
</tr>
<tr>
<td>.35</td>
<td>96</td>
<td>52</td>
<td>(8 \times 10^5 - 2 \times 10^6)</td>
<td>0.33 (0.09)</td>
<td>18</td>
</tr>
<tr>
<td>.43</td>
<td>121</td>
<td>51</td>
<td>(8 \times 10^5 - 2 \times 10^6)</td>
<td>0.27 (0.12)</td>
<td>16</td>
</tr>
<tr>
<td>.54</td>
<td>156</td>
<td>51</td>
<td>(8 \times 10^5 - 3 \times 10^6)</td>
<td>0.15 (0.02)</td>
<td>12</td>
</tr>
</tbody>
</table>
Figure 3.1. Fibrin gel morphology as a function of fibrin density. Fibrin gels were made at various fibrinogen concentrations using 10 nM thrombin and imaged by confocal microscopy. Gels consisted of unlabeled fibrinogen and fluorescent labeled fibrinogen at a ratio 1000:1 unlabeled:labeled. Final fibrinogen concentration of gels: (A) 3 mg/ml; (B) 10 mg/ml; (C) 20 mg/ml; (D) 40 mg/ml; (E) 60 mg/ml; (F) 100 mg/ml. Scale bar = 20 µm.

as the pressure at which the gels either breaks apart or tears away from the tube wall. These failure events were clearly visible due to uneven distribution of the dye. For permeability measurement experiments the maximum pressure for each gel was 40%-60% of the failure pressure. The flow rate for each gel was measured at three pressures. The calculated permeability was independent of pressure over the range used in these measurements.

An increase in fiber volume fraction with increasing fibrinogen concentration was observed by confocal microscopy (Fig. 3.1). Gels made of 3–10 mg/mL fibrinogen consist of
fibers spaced as far apart as ~10 µm within a given plane. The effective hydrodynamic screening length for these gels, estimated as the square root of the permeability, is 148−346 nm (Table 3.1). Gels of 20–24 mg/mL fibrinogen consist of a dense mat of fibers with fibers spaced as far apart as ~1 µm within a given optical plane. These gels have an effective hydrodynamic screening length of 58−83 nm. Gels of 67 mg/mL and greater have no discernable porous structure at the resolution of the microscope and have an effective hydrodynamic screening length of 27 nm or less. The fiber volume fraction ranges from 0.02 (3 mg/mL) to 0.54 (156 mg/mL) as calculated by Eq. 3.12.

Dehydrated fiber radii were measured by SEM and were found to be relatively insensitive to fibrinogen concentration (Fig. 3.2). For example, the average fiber radius at 7 mg/mL fibrinogen was 21 ± 6 nm and the average fiber radius at 72 mg/mL was 24 ± 8 nm. Based on these measured values, we used 23 nm as an average dehydrated fiber radius for all fibrinogen

Figure 3.2. Fibrin fiber diameter as a function of fibrinogen concentration. Scanning electron micrographs were taken of fibrin gels as various fibrinogen concentrations to enable the measurement of fiber radius, \( a_d \): (A) 7 mg/ml; (B) 48 mg/ml; (C) 156 mg/ml; (D) Measured dehydrated radii of fibrin fibers. Scale Bar = 500 nm.
concentrations. The hydrated average fiber radius \((a_f)\) was calculated using Eq. 3.13 (Table 3.1). The hydrated fiber radius for each fibrinogen concentration was used for permeability calculations using the models described by Eqns. 3.8, 3.9, and 3.10.

The permeability of fibrin gels spans three orders-of-magnitude over a fiber volume fraction range of 0.02 to 0.54 (Table 3.1). The permeability was most sensitive to volume fraction over the range 0.02–0.18, which corresponded to permeabilities of \(1.2 \times 10^{-1} \mu m^2\) to \(7.3 \times 10^{-4} \mu m^2\). Over the volume fraction range of 0.18–0.54 the change in permeability was more modest, varying from \(7.3 \times 10^{-4}\) to \(1.5 \times 10^{-4} \mu m^2\). These observations are in agreement with models of fibrous media that show a linear sensitivity to volume fraction over the range of 0–0.05 compared to \(\ln(\phi_f)/\phi_f\) sensitivity to volume fraction over the range of 0.05–0.6 (104).

The measured dimensionless permeability \(k_f/a_f^2\) of fibrin gels was compared to predictions from three models of three-dimensional disordered fibrous media; the semiempirical Davies equation, Eq. 3.8, the Jackson and James expression which is a weighted average of analytical solutions for flow parallel and perpendicular to an array of cylinders, Eq. 3.9, and LB simulations for flow through disordered fibers, Eq. 3.10. Equations 3.9 and 3.10 are solutions to Stokes flow past arrays of periodic or randomly oriented cylinders, respectively. In the semi-dilute limit \((\phi_f < 0.2)\), all three models are in good agreement with the measured permeabilities (Fig. 3.3). The Jackson and James model asymptotically approaches zero as the volume fraction approaches 0.4. Both the Davies equation and LB simulations are in good agreement with experimental values over the entire range of volume fractions with \(\chi^2\) values of 15 and 5, respectively. Based on these comparisons, we conclude that fibrin gels over a fiber volume fraction of 0.02-0.54 are well described by models of disordered, cylinders with uniform diameters.
3.5.2 Permeability of platelet rich clots

Platelet rich clots (PRC) were formed by adding 10 nM thrombin to platelet suspensions in plasma with cell densities of $7 \times 10^5/\mu L$ to $5 \times 10^7/\mu L$. There was no visual evidence of platelet aggregation during the preparation of PRC prior to the addition of thrombin. The platelet volume fraction range was estimated based on analysis confocal microscopy images (Fig. 3.4) and spanned the range of 0.01 to 0.61 (Table 3.2). The permeability of PRC decreased by three orders-of-magnitude over this range of platelet volume fraction. The permeability of bovine plasma clotted with 10 nM thrombin in the absence of any platelets was $1.2 \times 10^{-2} \pm 0.6 \times 10^{-2} \mu m^2$. We did not observe a contribution of the platelets to the permeability for platelet volume fractions of less than 0.19 (Table 3.2). At a platelet volume fraction of 0.19–0.31 we observed a modest contribution of the platelets to permeability with ~50% decrease compared to a pure fibrin gel. At higher platelet volume fractions (>0.31) the
contribution of the platelets begins to dominate the overall hydraulic resistance. The permeability of a PRC with a platelet volume fraction of 0.37 was an order of magnitude less that that of a pure fibrin gel, decreasing from $1.5 \times 10^{-2}$ $\mu$m$^2$ to $1.1 \times 10^{-3}$ $\mu$m$^2$.

We compared experimental results to three mixed porous media models; a resistors-in-parallel model, Eq. 3.14, a fractional packing model, Eq. 3.17, and Ethier’s numerical calculations of the Brinkman equation for fibrous media with two dissimilar radii (Fig. 3.5). The resistors-in-parallel model treats the PRC as two independent and non-interacting media—a
Table 3.2. Permeability of platelet rich clots. Platelet rich clots (PRC) were made from solutions of platelets diluted into platelet poor plasma and clotted with 10 nM thrombin in the presence of 2.5 mM CaCl$_2$. Platelet volume fractions, $\phi_p$, were calculated from confocal microscopy images. Flow was induced through the thrombi by pressure gradients that range from $\Delta P/L$ [Pa/m]$_{\text{min}}$ to $\Delta P/L$ [Pa/m]$_{\text{max}}$. The radii of platelets and platelet aggregates, $a_p$, were estimated from confocal microscopy images of PRC. The permeability, $k_t$, is reported as a fit to Eq. 3.1 with uncertainty estimated by the standard error (SE) of the fit. The effective screening length is the square root of $k_t$.

| Measured Platelet Volume Fraction $\phi_p$ (SD) | Platelets Density [platelets/µL] | $a_p$ [µm (SD)] | $\Delta P/L$ [Pa/m] $| k_t$ (SE) [×10$^{-3}$ µm$^2$] | Effective Screening Length [nm] |
|-----------------------------------------------|---------------------------------|---------------|-----------------|--------------------------|
| 0.01 (0.004)                                  | 7×10$^5$                        | 1 (2)         | 3×10$^5$ - 8×10$^4$ | 11 (0.98)               | 105                      |
| 0.05 (0.01)                                   | 1×10$^6$                        | 1 (3)         | 3×10$^4$ - 8×10$^4$ | 12 (0.96)               | 110                      |
| 0.19 (0.02)                                   | 7×10$^6$                        | 4 (4)         | 3×10$^4$ - 8×10$^4$ | 6.7 (0.83)              | 82                       |
| 0.31 (0.04)                                   | 1×10$^7$                        | 8 (3)         | 3×10$^4$ - 8×10$^4$ | 6.1 (0.69)              | 78                       |
| 0.37 (0.05)                                   | 2×10$^7$                        | 16 (5)        | 6×10$^4$ - 2×10$^5$ | 1.1 (0.53)              | 33                       |
| 0.45 (0.02)                                   | 4×10$^7$                        | 17 (6)        | 1×10$^5$ - 4×10$^5$ | 0.20 (0.020)            | 14                       |
| 0.61 (0.06)                                   | 5×10$^7$                        | 26 (6)        | 1×10$^5$ - 2×10$^6$ | 0.015 (0.0019)          | 4                        |

Fibrous medium defined by fibrin and granular medium defined by platelets where the overall permeability is calculated by adding the resistances of each medium in parallel. We used the measured permeability of a fibrin gel from bovine plasma and estimated the platelet permeability using the Kozeny-Carmen equation, Eq. 3.15. The resistors-in-parallel model provides a good estimate of the permeability over most of the range of platelet volume fractions but begins to deviate as the platelet volume fraction increases beyond 0.3. The $\chi^2$ for this model is 0.95.

The fractional packing model assumes that the PRC can be treated as homogeneous porous medium based on a volume weighted volume fraction and a characteristic radius. A characteristic radius of 214 nm was calculated by taking the harmonic mean of the diameter of a fiber and the diameter of platelet. We calculated the characteristic radius based on the size of platelet aggregates (Table 3.2) as well, but there no appreciable difference between these values and those based on a single platelet. The permeability was then estimated using the Kozeny-Carmen equation Eq. 3.15. The fractional packing model predicts that the permeability is much lower than the measured values over nearly the entire range of platelet volume fractions.
At low platelet volume fraction (<0.1), the model overestimates the permeability as the PRC approaches a pure fibrin gel. Consequently, the fractional packing model is a poor fit with $\chi^2 = 500$. This suggests that PRC cannot be reduced to a porous medium with a single characteristic length scale over a large range of platelet volume fractions.

Comparison of the data with the Ethier model shows that a PRC is well described as a Brinkman medium. The best fit of Ethier’s calculation to the experimental data occurs when $k_f/a_{coarse}^2$ is close to unity (Fig. A-4), where $a_{coarse}$ is the radius of the coarse media (platelet aggregates). The $\chi^2$ value for this model equals 0.82. The calculated value of $k_f/a_{coarse}^2$ is ~0.01, based on the permeability of bovine plasma and the size of single platelet. This discrepancy suggests that $k_f$ may be larger in a PRC than in a fibrin gel in the absence of platelets. One explanation for a higher permeability is that some of the fibrinogen or fibrin monomers are bound to $\alpha_{IIb}\beta_3$ receptor on activated platelets, and thus are not available for polymerization into
fibers. There are ~80,000 $\alpha_{\text{IIb}}\beta_3$ receptors per platelet (117). At a platelet density of $10^7/\mu$L, the ratio of $\alpha_{\text{IIb}}\beta_3$ receptors to fibrinogen molecules is roughly 1:1, so the probability that a significant fraction of fibrin(ogen) is bound to platelet surfaces seems feasible. Comparison of confocal images of fibrin in the presence and absence platelets confirms that platelets affects fibrin polymerization (Fig. A-5). Fibers in the presence of platelets appear much shorter than in platelet poor plasma.

Because the Ethier model incorporates the volume fraction of fibrin and platelets as independent parameters, we can use these solutions to predict the total permeability of a clot based on the volume fractions of each component. Fig. 3.6 shows the predicted permeability of a clot as a function of platelet volume fraction at constant fibrin volume fractions. The fibrin permeability, $k_f$, was calculated using Eq. 3.10 since this gave the best fit to the experimental data. The measured platelet aggregate diameter, $a_{\text{coarse}}$, was used to determine the appropriate value for $k_f/a_{\text{coarse}}^2$. The total permeability, $k_t$, was then estimated from Fig. A-3.

The transition from a fibrin rich medium described by an array of uniform cylinders a to platelet rich medium described by Brinkman’s equation accounts for viscous losses on the surface of platelets. The criteria under which these viscous losses become significant can be estimated by considering the simple scenario of unidirectional flow through a fibrin gel between two parallel plates that represent two platelets spaced a distance $h$ apart is (see Appendix A.1 for derivation (72)). Assuming the flow is described by Eq. 3.19 the total permeability is:

$$k_t = k_f \left[ 1 - \frac{2\sqrt{k_f}}{h} \tanh \left( \frac{h}{2\sqrt{k_f}} \right) \right]$$

(3.24)

The first term in Eq. 3.19 represents Darcy’s law and the second term is the Brinkman correction. Note that as $\sqrt{k_f}/h \to 0$, the total permeability approaches the fibrin permeability
and Darcy’s law describes interstitial flow in the space between the two plates. The effect of the platelets on the interstitial velocity is only most significant for $\sqrt{k_f / h} < 0.05$. Thus, as the fibrin volume fraction increases the total permeability becomes less sensitive to platelet volume fraction. This is shown in Fig. 6 when comparing low and high fibrin volume fractions. For the case of $\phi_f = 0.03$ the permeability of a clot will decrease by over two orders-of-magnitude as $\phi_p$ ranges from 0.01 to 0.6. For the case of $\phi_f = 0.55$ the permeability of a clot will decrease only three-fold as $\phi_p$ ranges from 0.01 to 0.6.

Viscous losses alone do not account for the effect of the addition of platelets into a fibrin gel. Note that we begin to observe decrease in the permeability at a $\phi_p = 0.19$ (Table 3.2). The effective screening length of bovine plasma ($k_f^{0.5}$) is 109 nm, which based on the argument presented above suggests that platelets need to be ~2.2 $\mu$m apart to influence the permeability.
However, inspection of PRC images at $\phi_p = 0.19$ (Fig. 3.4D) show that gaps exist between platelets on the order of 10 $\mu$m. The influence of platelets at lower volume fractions than predicted by Eq. 3.24 is a result of an increase in tortuosity and an increase in superficial velocity within the fibrin gel. In the limit of low fibrin permeability or large platelet aggregates ($k_f/a_{coarse}^2 \rightarrow 0$), Ethier’s solution to the Brinkman equation reduces to:

$$k_t = k_f \left( \frac{1 - \phi_p}{1 + \phi_p} \right)$$ (3.25)

The numerator represents the area obstruction by platelets and platelet aggregates, and leads to an increase in superficial velocity in the fibrin gel. The denominator represents the increase in the tortuosity of the fluid path due to the platelets and platelet aggregates. Eq. 3.25 correctly predicts the influence of platelets over the range of $\phi_p$ from 0.01 to 0.31. For example, at $\phi_p = 0.19$, Eq. 3.25 predicts a permeability of $8.0 \times 10^{-3} \mu$m$^2$, which is close to the measure value of $6.7 \times 10^{-3} \mu$m$^2$. At $\phi_p \geq 0.37$, Eq. 3.25 overestimates the permeability, suggesting that viscous losses begin to play a role at these higher platelet volume fractions.

### 3.5.3 Hindered diffusion in fibrin gels and platelet rich clots

Fibrin gels and platelet rich clots identical to those formed for permeability measurements were formed for hindered diffusion measurements. The diffusion coefficients of 12, 29, and 54 nm solutes (70 kDa, 500 kDa, 2000 kDa dextrans) in fibrin gels and PRC were significantly hindered compared to free solution for all solutes. In 100 mg mL$^{-1}$ fibrin gels we observed 60%, 66% and 85% reductions of diffusion coefficients for 12, 29, and 54 nm solutes, respectively. Similar to the trend observed with the permeability, hindered diffusivity was most sensitive to volume fraction over the range 0.02–0.18 for all probes tested. This is expected, as the permeability is an indirect measure of pore size. The Phillips model (Eq. 3.21) was used to
predict the diffusion coefficient of the 12, 29, and 54 nm probes using the measured permeability as a model input (Fig. 3.7A). The Phillips model predicted the hindered diffusivity well for the 29 and 54 nm probes, but overestimated the diffusivity of the 12 nm probe at higher fibrinogen concentrations. The diffusion coefficient of a 70 kDa dextran in a PRC decreased by as much as 93% compared to free solution as platelet volume fraction was increased to 0.61. The Phillips model was used to accurately predict the degree of hindrance using the measured permeability as a model input (Fig 3.7B). Similar to the trend observed in permeability, the majority of the hindrance occurred above platelet volume fraction of 0.3.

3.6 Discussion

The objective of this study was to measure the permeability of fibrin gels and platelet rich clots over a wide range of fibrin and platelet densities. Fibrin gels were formed using a range of fiber volume fractions of 0.02–0.54, resulting in permeabilities of $0.12-1.5 \times 10^{-4} \mu m^2$. PRC were formed in plasma (~7 mg/mL fibrinogen) at platelet volume fractions of 0.01–0.61, resulting in
permeabilities of $1.1 \times 10^{-2} - 1.5 \times 10^{-5}$ $\mu$m$^2$. These data suggest that the interstitial flow within clots will vary widely depending on their structure. Fibrin gels were well described as a Darcy medium consisting of disordered fibers with a uniform diameter. PRC were best described as a Brinkman medium of platelets embedded in a fibrin gel. Diffusion coefficients were reduced in fibrin gels with 100 mg mL$^{-1}$ fibrin gels reducing diffusion coefficients by 60%, 66%, and 85% for 70 kDa, 50 kDa, and 2000 kDa probes, respectively. PRC also reduced solute diffusion; the diffusion coefficient of a 70 kDa dextran was reduced by as much as 93% compared to free solution as platelet volume fraction was increased to 0.61. Hindered diffusion in both fibrin gels and PRC was adequately described by the Phillips model using the measured permeability as a model input.

Permeability and solute diffusivity measurements of fibrin gels at low fibrinogen concentration ($3 - 10$ mg/mL) in this study are in good agreement (within 25%) of previously reported values of permeability and free solute diffusivity (19, 118). The volume fraction of fibers in gels made at plasma fibrinogen concentrations is less than 0.05. However, analysis of retracted clots from humans suggests that fibrin can make up a large fraction of the solids in arterial clots. For example, the solid fraction of emboli retrieved from patients with atrial fibrillation were reported to consist of 65% fibrin and the balance platelets (96). Similarly, electron microscopy analysis of the solid fraction of acute myocardial infarction clots show that these clots consist of 56% fibrin, 17% platelets and the balance other blood cells (31). These data are evidence that the volume fraction of interstitial fibrin within a clot is likely much higher than that of clot formed at plasma concentrations of fibrinogen, and in some types of clots, the fibrin fibers are likely the major transport barrier to fluid and solute transport. Based on these data from extracted clots we would predict that the permeability of fibrin rich clots such as these have
permeabilities in the range of $10^{-3} - 10^{-4} \, \mu m^2$, two to three orders-of-magnitude less than fibrin gels made at physiological fibrinogen concentration, and that solute diffusion within such clots would be hindered by over 50%.

Modeling the permeability and hindered diffusivity within fibrin gels requires the measurement of fibrin diameter and fiber volume fraction. Fibrin fiber diameter was relatively insensitive to fibrinogen concentration over the range of $3-156 \, mg \, mL^{-1}$. Neutron scattering studies of fibrin fibers formed from solutions of $2-40 \, mg/mL$ fibrinogen noted a similar insensitivity to fibrinogen concentration (86). The average hydrated fiber radius we measured over all fibrinogen concentrations was between 51–60 nm. This range is in good agreement with the maximum fiber radius of ~50 nm caused by the twisting of protofibrils (119). However, these radii are slightly lower than those measured by neutron scattering over a similar range of fibrinogen concentrations (60–70 nm), albeit with deuterated water. This could be a result of the assumption that there was no void space in the dehydrated fibers. If there were some voids within the fibers following fixation and dehydration preparation for SEM, then we would have underestimated the hydrated radius. It should be noted that this is the size of an individual fiber, but fibers can aggregate to yield effective fiber diameters of 100–300 nm (19). Thus, one potential source of error between the normalized permeability ($k_f/a_f^2$) and hindered diffusivity based on our measurements and those predicted in the fibrous media models is that we have assumed the appropriate characteristic length is the radius of an individual fiber. Nevertheless, it seems that transport through fibrin gels is well described by theories of flow and resistance through a network of three-dimensional disordered cylinders.

The permeability of fibrin gels over the entire range of fiber volume fractions were well described by several models of fibrous media. The semi-empirical Davies equation has been
commonly used to estimate the permeability of fibrin gels and blood clots in the literature (20, 56, 110). Here, we found the Davies equation had excellent agreement with experimental permeability at fiber volume fractions of 0.006 to 0.3, but underestimates the permeability at higher fiber volume fraction. The Jackson and James expression is another common expression used for three-dimensional fibrous media. Similar to the Davies equation, the Jackson and James expression accurately predicts permeability in the semi-dilute regime but approaches zero as the volume fraction approaches 0.4 due to the fact that this expression is based on asymptotic expansions in the dilute limit. The LB simulations presented by Clague and colleagues and found that there was good agreement over the entire range of fiber volume fractions measured experimentally. Therefore, we used Eq. 3.10 for calculating both fibrin gel permeability and the fibrin contribution to the overall permeability in PRC. Unlike the Jackson and James expression, Eq. 3.10 captures the appropriate scaling behavior in both the dilute and concentrated fiber volume fraction limits. In the dilute limit, the permeability scales as \( \ln(\phi_f) / \phi_f \). In the concentrated limit, the permeability scales according to the lubrication approximation (101, 104).

Hindered diffusivity over the entire range of fiber volume fractions was well described by the Phillips model. Because the Phillips model uses permeability as a direct model input the agreement between the model and the measured values indicates a direct relationship exists between permeability and hindered diffusivity and that measured values of permeability provide a good approximation of the pore size of a clot.

One potential limitation of the data in this study is that fibrin gels were formed under static conditions, resulting in an isotropic medium of randomly oriented fibers. However, there is evidence that flow may effect the orientation of fibrin fibers. For example, in a purified system consisting of fibrinogen and thrombin it was found that ability of fibrin fibers to form and
aggregate, as well as their orientation, is profoundly influenced by the shear rate and rate of thrombin generation (120). Similarly, flowing plasma over activated platelets or tissue factor presenting cells also showed that fibrin fibers tend to align in the direction of flow (120, 121). Finite element simulation of flow through networks of aligned fibers predict that the permeability in the direction parallel to fibers will be higher than in an isotropic network but that there is little difference in permeability for aligned and isotropic networks for flow perpendicular to fibers (107). The noted dependence of permeability upon alignment in these studies, however, was shown to be secondary compared to fiber diameter and fiber volume fraction. Random walk simulations of solute diffusion through fibrous matrices of various orientations and degrees of alignment indicate that, although the overall hindered diffusion coefficient is not affected by fiber orientation, diffusion anisotropy with preference for solute movement parallel to the fibers exists in aligned networks (122). The magnitude of anisotropy observed in these studies resulted in diffusion rates in the direction parallel to highly-aligned fibers being as much as 60% greater than in the transverse direction depending upon degree of alignment and ratio of solute diameter to fiber diameter. If these predictions held for fibrin rich clots, then the permeabilities presented in this study for isotropic fibrin gels could slightly underestimate the permeability and diffusion coefficients in the direction of flow.

Clots formed in large arteries or stenosis can consist of large volume fractions of platelets. For example, ~33% of thrombi formed on ruptured coronary plaques consist of a majority of platelets (123). Clots formed in vitro in a model of a stenotic artery consist of up to 80% platelets (124). Yet, there have been few measurements of permeability or hindered diffusivity of fibrin gels with a significant (>0.2) volume fraction of blood cells. Clots formed at a fibrin volume fraction of 0.03 had an exponential decrease in permeability and up to a 93%
reductions of diffusion coefficients with increasing platelet volume fraction over the range of $0.2 < \phi_p < 0.6$. A resistor-in-parallel model that takes into account the viscous losses on the platelets in parallel to those on the fibrin fibers provides a good first-order estimate of the permeability of PRC. However, the more rigorously derived Ethier model based on the Brinkman equation provides at least better qualitative treatment of the interplay between fibrin and platelets as platelet volume fraction increases. Specifically, increases in platelet volume fraction result not only in viscous losses on the platelet surface, but also results in an increase of the tortuosity of the flow path and in an increase superficial velocity of the fluid through the fibrin gel. Below a platelet volume fraction of 0.2, platelets play only a limited role in determining permeability and hindered diffusivity and the fibrin permeability provides a good estimate of the overall transport of the PRC. This is in agreement with the influence of erythrocytes in fibrin gels, which resulted in no significant difference in permeability up to a volume fraction of 0.2 (125).

A limitation of using the Ethier model to describe PRC is that it describes a medium consisting of two cylinders with dissimilar radii. Here, we have applied it to a medium consisting of fine fibers and larger disc or spherical particles. One difference between cylinders and spheres is that the drag force on a sphere is larger than on a cylinder with the same radius for low Reynolds number flows. Consequently, the effective coarse fiber radius in the Ethier model would likely be smaller than that of a platelet or platelet aggregate. We may have indirectly accounted for this difference because a larger value of $k_f a_{coarse}^2$ was needed to fit our experimentally measured permeability than would be predicted by the measured values of $k_f$ and $a_{coarse}$. The authors are unaware of any theoretical treatment of spherical or elliptical objects embedded in a fibrous medium with which we could compare our experimental results.
Similarly, the Phillips model of hindered diffusion assumes the medium to be comprised of an array of cylinders with a uniform diameter. This assumption is valid for fibrin gels, but breaks down for PRC and may be a source for the disagreement between the model and the measured diffusion coefficients. In particular, the steric hindrance terms of the Phillips model calculates effective solid volume based on the volume to length ratio of a long cylinder with a constant radius; however, platelets not fibers, make up a majority of the solid volume fraction of a PRC. This may lead to an over estimation of the effective solid volume and thus an underestimation of the diffusion coefficient. This discrepancy is most pronounces at low solid volume fractions where the steric term dominates. At high solid fraction (> 0.45) the hydrodynamic term of the Phillips model dominates; therefore, inaccuracies due to incorrect estimation of the steric hindrance is minimal.

Within a clot the distribution of platelets and fibrin can be heterogeneous. For example, histology of clots retrieved from individuals with ischemic stroke show thin layers of fibrin-platelet interspersed with erythrocytes and leukocytes (97). The nature of an injury also will affect the clot composition. Shallow injuries that expose the collagen rich extracellular matrix underneath endothelial cells will primarily promote platelet adhesion and aggregation. Deeper injuries can expose tissue factor (TF) bearing adventitial cells such as fibroblasts and smooth muscle cells, which can induce coagulation and thus produce more fibrin. Intravital microscopy in the murine laser injury model of thrombosis show that fibrin density tends to be greatest near the vessel wall and on the upstream edge of a clot during the initial stages of clot formation (126). Platelets within the interior of a clot form a densely packed core that may be impermeable to fluid and solute transport (127). Platelets along the periphery of the clot are less densely packed. This heterogeneity in platelet density seems to reflect platelet activation because
P-selectin positive platelets are observed in the core closest the site of injury (128). A morphology of a dense core of activated platelets surrounded by a more loosely packed inactivated platelets is also predicted in computational models (34, 56). These data suggest that there are regions of a clot, notably the outside shell of platelet and fibrin that are more permeable and hinder diffusion less, and thus subject to an evolving biochemical environment that includes the influx and removal of plasma and coagulation proteins. The data from this study can be used as a look-up-table of permeabilities and hindered diffusivities for different regions of clot within these computational models.

3.7 Conclusions

In this study we have examined a broad range of fibrin and platelet densities in an effort to bound the possible compositions of clots formed during the initial stages of hemostatic and thrombotic clots. We found that a combination of fibrous media models and mixed porous media models can be used to predict the permeability of clots over a wide range of fibrin and platelet volume fractions. Fibrin gels or platelet poor clots are well described as a medium of disordered fibers with a uniform diameter. Clots with significant volume fractions of platelets greater than 0.2 are best described as a Brinkman medium. Permeability models can, in turn, be used to predict hindered solute diffusivity in a clot. Using permeability as a model input, the Philips model, accounting for both steric and hydrodynamic hindrance, can be used to predict hindered diffusion within clots formed with a wide range of compositions. These models can be used to make predictions on the rate at which fluid and solutes can move through the interstitial spaces of a clot. Such predictions are important for estimating the rate of coagulation zymogens/enzymes entering and leaving a clot, as well as developing drug delivery strategies for disrupting clot formation or enhancing lysis.
3.8 Grants

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CHAPTER 4
ELASTIC BEHAVIOR AND PLATELET RETRACTION IN LOW AND HIGH DENSITY FIBRIN GELS

Modified from paper accepted for publication in Biophysical Journal*

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4.1 Abstract

Fibrin is a biopolymer that gives thrombi the mechanical strength to withstand the forces imparted on them by blood flow. Importantly, fibrin is highly extensible but strain hardens at low deformation rates. The density of fibrin in clots, especially arterial clots, is higher than in gels made at plasma concentrations of fibrinogen (3–10 mg/mL) where most rheology studies have been conducted. The objective of this study is to measure and characterize the elastic regimes of low (3–10 mg/mL) and high (30–100 mg/mL) density fibrin gels using shear and extensional rheology. Confocal microscopy of the gels shows that fiber density increases with fibrinogen concentration. At low strains fibrin gels act as thermal networks independent of fibrinogen concentration. Within the low strain regime, the mesh size of fibrin gels can be predicted by the elastic modulus using semiflexible polymer theory. Significantly, this provides a link between gel mechanics and interstitial fluid flow. At moderate strains, we find that low density fibrin gels

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act as non-affine mechanical networks and transition to affine mechanical networks with increasing strains within the moderate regime, while high density fibrin gels only act as affine mechanical networks. At high strains, the backbone of individual fibrin fibers stretch for all fibrin gels. Platelets can retract low density gels by greater than 80% their initial volumes, but retraction is attenuated in high density fibrin gels and with decreasing platelet density. Taken together, these results show that the nature of deformation of fibrin is a strong function of fibrin fiber density, which has ramifications for the growth, embolization and lysis of thrombi.

4.2 Introduction

Fibrin is one of the most extensively studied biopolymers in terms of rheological properties at both the single fiber and network scales (14, 15, 22-25, 29, 81, 85, 129, 130). Fibrin is extraordinarily extensible and has nonlinear rheological behavior such as strain hardening and negative normal stresses under shear strain (14, 15, 22-26, 29, 81, 85, 129-132). Most reports of fibrin gel rheology have been conducted on gels derived from fibrinogen concentrations at or near that of human plasma (~3 mg/mL) (14, 22, 29, 133-135). These low density (fibers/volume) fibrin gels have fiber volume fractions of less than 5%. Yet, thrombi formed in vivo, fibrin glues used for surgical wounds, and tissue engineering matrices can have fiber volume fractions of 10–50% (31, 32, 136, 137). The objective of this study is to measure the rheology of high density fibrin gels, defined as those derived from greater than 10 mg/mL fibrinogen, and to relate the mechanics of these gels to their network structure.

Thrombi formed under flow contain a higher density of fibrin than gels formed under static conditions at plasma concentrations of fibrinogen. For example, thrombi retrieved from patients with myocardial infarctions have a solid volume fraction of fibrin fibers of ~0.5 (31). This high density appears partially due to the effect of blood flow on fibrin deposition. Fibrin
gels formed under flow on procoagulant surfaces have fibrin density that are five-fold higher than gels formed at 3 mg/mL fibrinogen (32). Platelet retraction can reduce the volume of low density fibrin gels (2 mg/mL fibrinogen) by as much as 90%, thereby yielding a much denser network of fibers (138). The magnitude of platelet contractile forces imparted on a substrate increases with increasing stiffness of the substrate (139), however the ability of platelets to retract high density fibrin gels is unknown. Thus, the rheology of high density fibrin gels is important because it will determine, in part, whether the stresses imparted by blood flow will cause the clot to deform reversibly or irreversibly, or to embolize as well as the degree of platelet-mediated retraction.

In addition to its role in mechanics, the density of fibrin partly determines the fluid and solute transport into and out of a thrombus. In vitro (68), in vivo (58) and computational (34, 50, 56) studies suggest that the hindered transport of coagulation enzymes and platelet agonists in the interstitial space between platelets and fibrin fibers is a mechanism that contributes to thrombus arrest. Specifically the transport of coagulation enzymes from the injured vessel wall through the porous clot may regulate the late stages of clot growth (51). Moreover, the transport of fibrinolytic agents like tissue plasminogen activator into a thrombus regulates the rate of its dissolution (20). Measuring transport properties such as hydraulic permeability and diffusion coefficients in vivo is difficult compared to mechanical properties, which can be estimated by ultrasound and other imaging modalities (140). As such, providing a link between thrombi mechanics and transport should prove useful in developing therapeutic approaches for thrombosis.

Low density fibrin gels have a strain dependent rheology characterized by several elastic regimes. These regimes are categorized as networks undergoing either entropic, thermal
fluctuations or enthalpic, mechanical deformation. Mechanical deformation is further divided into affine and non-affine models. Affine models assume that fibers deform such that the strain of individual fibers is identical to the macroscopic strain; a Poisson’s ratio of one-half (i.e. incompressibility) is also assumed. Non-affine models account for deviations at the microscopic scale from the affine assumption (141). Nonlinearity in non-affine networks originates from network reorganization that leads to a transition from fiber bending/buckling to fiber stretching (84). For sufficiently large strains, fiber buckling may even lead to a decrease in shear modulus (141, 142). At low strains, low density fibrin gels have been shown behave as networks of semiflexible polymers where the elasticity is dominated by thermal fluctuations and entropic resistance to stretching; however, at high strains the nonlinear response of fibrin deviates from the entropic model (28). At higher strains low density fibrin gels undergo non-affine deformation (141, 143) and exhibit Poisson ratios above the incompressible value of 0.5 (i.e., they lose mass upon deformation) (135, 144).

In this report, we use shear and extensional rheology and platelet retraction to characterize the mechanical properties of low and high density fibrin gels (3–100 mg/ml) and determine how these properties relate to network structure. All gels act as thermal, affine networks at low strains. This allows for predictions of mesh size from elastic modulus, and thus provides a link between mechanics and fluid and solute transport. Non-affine behavior is apparent in low, but not high, density fibrin gels at moderate strains. Platelets retraction of fibrin gels is attenuated with increasing fibrin density.
4.3 Experimental Methods

Fibrin gels were made with at a range of fibrin concentrations and tested using both shear and extensional rheology over a range of strains. Platelet retraction was tested at physiological and low platelet counts.

4.3.1 Materials

Human fibrinogen (free of plasminogen, von Willebrand factor and fibronectin) in 20 mM sodium citrate and human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Factor XIII is present in the fibrinogen solution (145). Centrifuge filtration units with a 100 kDa molecular weight cut off were from Millipore Corp (Billerica, MA). Prostacylin (PGI2) was from Cayman Chemicals (Ann Arbor, MI). #88 T316 stainless steel mesh with 200µm openings was from TWP Inc. (Berkeley, CA). 10% (by weight) sodium dodecyl sulphate (SDS) solution, 40% Acrylamide (19:1 Acrylamide:Bis-acrylamide) solution, Ammonium persulphate (APS), tetramethylethylenediamine (TEMED), 2-mercaptoethanol (βME), 10x premixed electrophoresis buffer (containing 25 mM Tris, 192 mM glycine, pH 8.3), 4x Laemmli protein sample (containing 277.8 mM Tris-HCl, pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue), Tris base, Precision Plus Protein™ Kaleidoscope™ Standards (10-250 kDa) and Bio-safe Coomassie Blue stain were all purchased from Bio-Rad (Hercules, CA). Tris buffer was prepared by dissolving 1.5 M Tris base in deionized water and pH 8.8. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Tris buffered saline (TBS; 50 mM tris base, 100 mM NaCl, pH 7.4), modified Tyrode’s buffer (129 mM NaCl, 20 mM HEPES, 12 mM NaHCO3, 2.9 mM KCl, 1 mM MgCl2, 0.34 mM Na2HPO4·12H2O, pH 7.3), acid-citrate dextrose (ACD; 0.085 M sodium citrate 0.085 M, 0.11 M D-glucose, 0.071 M
citric acid monohydrate), and sodium citrate solutions (0.13 M, pH 9.1) were made in house. AlexaFluor 555 labeling kit was purchased from Life Technologies (Grand Island, NY).

4.3.2 Preparation of fibrin gels

Fibrinogen stock solutions were prepared as previously described (146). Briefly, fibrinogen stock solutions (12.9 mg/mL) were centrifuged at 4000 g at 37 °C for 2 h in filtration units. After centrifugation the retentate was removed from the device and diluted in TBS. The concentration of fibrinogen in the retentate was determined by using a modified Clauss assay to measure the concentration of a known dilution of retentate to achieve a ~1 mg/mL fibrinogen solution (see Appendix B.1); 10 nM thrombin and 2.5 mM CaCl₂ were added to 100X diluted retentate in a 96 well plate (200 µL final volume) (98, 147). After 20 min, the absorbance was measured in a plate reader (Victor X, PerkinElmer) and the concentration was determined from a standard curve. Both the concentrated fibrinogen stock solution and fibrinogen in sodium citrate were tested for the presence of aggregates by dynamic light scattering with a size range of 1 nm−10 µm (Zeta PALS, Brookhaven Instruments, Holtsville, NY). Fibrinogen solutions of 3, 10, 30, 50, and 100 mg/ml in TBS were mixed with 2.5 mM CaCl₂ (final concentration) and 10 or 100 nM thrombin (final concentration).

To determine the extent of conversion of fibrinogen to fibrin gels a test was conducted in which gels were allowed to form for 50 min after which excess buffer was added to the gels to capture any unincorporated fibrinogen for testing. Fibrin gels (0.5 mL) identical to those used for rheological testing were formed in 24-well plates for 50 min. After gelation, an additional 0.5 mL of TBS was pipetted onto the top of each gel. The gel-TBS mixtures were homogenized with a glass rod and incubated for 24 h to allow any unincorporated fibrinogen to diffuse out of the formed gel and into the TBS. After incubation the visible particles of the broken clot were
removed from the disrupted gel-TBS mixture by filtering the mixture through a screen with 200 µm × 200 µm pores. The remaining filtrate was tested for the presence of unreacted fibrinogen by polymerizing any unreacted fibrinogen present in the filtrate; CaCl₂ (2.5 mM) and thrombin (10 nM) were added to the filtrate to induce gelation and the modified Clauss assay (Appendix B.1) was used to determine the concentration of the fibrin in the gel.

4.3.3 Small strain dynamic shear rheology

Shear and normal stresses were measured with a DHR-3 rheometer from TA Instruments (New Castle, DE) using a parallel plate (d = 20 mm, stainless steel) and solvent trap to prevent evaporation. Recalcified fibrinogen (161.5 µL) was pipetted on the bottom plate. Thrombin (8.5 µL) was added to the fibrinogen solution and the top plate was quickly (~3 s) lowered to a gap height of 500 µm while being spun freely at approximately 60 RPM to induce mixing. Each sample was subjected to four tests: (1) shear storage (G’) and loss (G’’) moduli were measured at 1% strain and at 1 Hz for 3000 s to monitor gelation. Samples were assumed to have reached their steady state if G’ did not change by greater than ±5% over the time period of 2700–3000 s; (2) an oscillation sweep with strains of 0.01–1% at 1 Hz; (3) a frequency sweep from 0.01–3 Hz at 1% strain; (4) shear storage (G’) and loss (G’’) moduli were again measured at 1% strain and at 1 Hz but for 300 s to detect changes greater than ±10% in the gel induced by (2) or (3). If changes greater than ±10% occurred the sample was discarded.

4.3.4 Shear and normal stresses under a strain ramp

Using an identical sample preparation and gelation test (step 1 above), the gel was deformed at a strain rate of 0.01 s⁻¹ until each sample broke. The shear and normal stresses and the shear modulus (G) were recorded (79).
4.3.5 **Extensional rheology**

Glass slides (50 mm × 75 mm) were coated with 25% Triton X-100 in TBS to prevent fibrin from adhering to the glass. Fibrin gels (30, 50 and 100 mg/ml) were formed between two glass slides separated by a 450 µm (l = 50 mm, w = 10 mm, thickness = 0.45 mm) polypropylene spacer; 10 mg/ml fibrin gels were formed between two glass slides separated by three 450 µm spacers stacked on each other (total thickness = 1.35 mm). A thicker sample was needed for the low fibrinogen concentration to allow for handling without deforming the sample. Sample thickness varied by less than 1% across the sample. Gels were allowed to form in a humidified chamber for 50 min. Gel samples (l = 20 mm and w = 3–6 mm) were cut from each casting with a razor blade for tensile testing on a ARES-G2 rheometer (TA Instruments, New Castle, DE) with the Sentmanat Extensional Rheometer (SER) fixture (148) with rough textured drums. Samples were stretched at 10 mm/min (135). Elastic modulus, $E(\gamma)$, defined as slope of the stress-strain curve at each value of $\gamma$ was determined by fitting a fifth degree polynomial to the stress-strain data using the Matlab function `polyfit` and then taking the first derivative of the polynomial (Fig. B.1).

4.3.6 **Platelet retraction of fibrin gels**

Platelet mediated retraction of fibrin gels was performed using a modified version of previously reported protocols (149). Non-silicanized glass vials (Alltech Associates, Deerfield IL) were incubated with 5 mg/ml BSA in modified Tyrode’s buffer for 1 h at room temperature, rinsed with deionized water and dried with an air brush. Whole blood was collected by venipuncture into a syringe containing 3.8% sodium citrate. The first 5 mL of blood was discarded. ACD was then added to the blood in a 1:10 ratio. The anticoagulated blood was centrifuged at 200g for 20 min. Then supernatant platelet rich plasma (PRP) was removed, and
PGI$_2$ (1.3 µM) was added to inhibit platelet activation. The PRP was centrifuged at 1000g for 10 min and the platelet poor plasma (PPP) was decanted off the platelet plug, which was immediately resuspended in modified Tyrode’s buffer. After repeating the same wash step the resuspended platelets were enumerated (Z1 Coulter Counter, Beckman Coulter, Brea, CA). Platelets concentrations of $2 \times 10^4$ and $2 \times 10^5$ platelets/µL in 500 µL of 3, 10, and 30 mg/mL fibrinogen were placed in glass vials followed by the addition of MgCl$_2$ and CaCl$_2$ at 3.75 mM and 7.5 mM, respectively. Finally, clotting was induced with 10 nM $\alpha$-thrombin. Clots retraction was recorded for 5 h at 37 °C. Following retraction the entire system (clot + liquid + glass) was weighed. Next, the retracted clot was removed and the remaining liquid and glass were weighed. Finally, the mass of the dried glass vial was measured and the volume of the expelled liquid was calculated assuming a liquid density of 1.01 mg/ml. The percent retraction is reported as the volume of the expelled liquid relative to the initial volume of the clot.

\[
PR = \frac{V_{\text{tot}}}{\rho_l} \left( \frac{m_{g+l} - m_g}{m_{g+l}} \right)
\]

(4.1)

where $PR$ is percent retraction, $V_{\text{tot}}$ is the total clot volume, $m_{g+l}$ is the mass of the glass vial plus the liquid, $m_g$ is the mass of the glass vial, and $\rho_l$ is the density of the liquid. The calculated mass of the clot, $(m_{c+g+l} - m_{g+l})$ was used to confirm the total mass of the system did not change over the course of the experiment.

4.4 Theory

The following theoretical models of fibrous networks are used to interpret bulk rheology experimental data in the context of single fiber mechanics and network structure.

4.4.1 Calculation of Mesh Size

The mesh size, $\zeta$, of fibrin gels was determined by measuring the hydraulic permeability, $k$, by previously reported methods (146):
\[ \xi = k^{1/2} \]  

(4.2)

The square root of the permeability is a hydrodynamic screening length for flow through fibrous media that is used to estimate the distance between fibers in hydrogels (62, 150).

4.4.2 Predicting mesh size from elasticity of thermal affine networks

The shear modulus \( (G) \) of a thermal affine elastic network can be connected to mesh size of the network by:

\[ G = \frac{k_B^2}{k_B TL_c \xi^2} \]  

(4.3)

where \( \kappa_B \) is the bending modulus of a fibrin fiber (see Supporting Material for calculation), \( k_B \) is Boltzmann’s constant, \( T \) is the absolute temperature, and \( L_c \) is the length of a fiber in the network (151). The length of each fiber was taken to be 2 \( \mu \)m, within the range (2.86 ± 2.51 \( \mu \)m) as measured by Kim et al. (152). Ryan et al. reported that the fiber length was roughly independent of fibrinogen concentration from 0.5–6 mg/mL (153). The bending modulus, \( \kappa_B \), was estimated to be \( 4.5 \times 10^{-24} \) N m\(^2\) by assuming the fibrin fibers are bundles of coupled protofibrils (154) with each protofibril having a persistence length of 500 nm (81) (see Appendix B.5.2 for calculation).

4.4.3 Using negative normal stresses to categorize fibrin gels at moderate strain

Fibrin’s nonlinear elastic response has been described by two models: (1) The thermal network model that considers the gel fibers to have nonlinear force-elongations that arise from thermal fluctuations (155) and (2) the mechanical network model that considers the gel fibers to be mechanical rods with linear force-elongation relations where nonlinearity arises from nonaffine deformation and a shift from fiber bending to stretching at high strains (79).
The ratio of the negative first normal stress to shear stress ($-\sigma_N/\sigma_S$) as a function of strain can distinguish between the two models. Both models show a small-strain regime characterized by $\sigma_N \approx \gamma_S^2$ and $\sigma_S \approx \gamma_S$, where $\gamma_S$ is the shear strain. The range of strains that the small-strain regime scaling holds increases with increasing fiber density (number of fibers per unit volume) for the mechanical network model whereas in the thermal network the range decreases with increasing fiber density (26, 156). Accordingly, if the small-strain regime increases with increasing fiber density, the thermal network model is supported whereas if it decreases, the mechanical model is more appropriate. Additionally, within the mechanical model, a peak where the normal stresses exceed the shear stresses at small strains is indicative of fibers that are softer to bending rather than stretching (156). This behavior where the ratio of normal stress to shear stress is greater than unity is inconsistent with a purely thermal model (79).

4.4.4 Distinguishing non-affine and affine mechanical networks

Mechanical networks can be categorized as nonaffine or affine, depending upon their bending ($\kappa_B$) and stretching ($\mu$) moduli and fiber length (80, 157, 158). A distinguishing feature of these networks is the relationship between the shear modulus ($G$) and the elastic modulus ($E$). Purely affine networks are dominated by stretching modes, have a Poisson’s ratio ($\nu=E/2G-1$) of 0.5 (80) and are described by:

$$E_{\text{Affine}} = 3G_{\text{Affine}}$$  \hfill (4.4)

Nonaffine networks have elastic regimes dominated by bending modes and show deviations from Eq. 4.4 as predicted by elasticity theory (159). The transition from affine to nonaffine deformation is shown in a depression of $G$ (159, 160). As the affine linear modulus serves as the upper bound for $G$ at moderate strains, a depression in $G$ results in an increased ratio of $E/G$ for increasingly non-affine networks. These high values of $E/G$, by definition, result in an elevated
Poisson’s ratio ($\nu > 0.5$). Thus, volume is not conserved but rather decreases upon stretching, a phenomenon observed in fibrin gels as mass loss during extensional strain (135, 144).

### 4.5 Results

The elasticity of fibrin gels was found to be a strong function of fibrin concentration and strain. Fibrin gels transition from a thermal network regime to a nonaffine mechanical to an affine mechanical regime or directly from a thermal network to an affine mechanical regime depending upon fibrin concentration.

#### 4.5.1 Verification of fibrinogen conversion to fibrin

Fibrin gels were formed from 3, 10, 30, 50 and 100 mg/mL fibrinogen and 10 and 100 nM thrombin. The density of fibers increases with increasing fibrinogen concentration (Fig. 4.1). A stock solution of fibrinogen (12.9 mg/mL) was concentrated by centrifugation to 130–160 mg/mL. Absorbance measurements at 450 nm showed that the fibrinogen in the concentrated retentate remained soluble at room temperature (Appendix B.2 & Fig. B.2). This concentrated solution was found to contain no aggregates as measured by dynamic light scattering. The measured particle diameter of the concentrated fibrinogen solution was 30 nm with a dispersity of 0.2, identical to the values reported by the vendor. All fibrinogen appeared to be converted to fibrin. No fibrinogen was present in the excess TBS; the excess TBS solution did not form a gel but remained liquid upon testing the extent of reaction and no excess fibrinogen was detected by the modified Clauss assay. Factor XIII is present in the stock fibrinogen solution. SDS-PAGE analysis showed that 3–50 mg/mL fibrinogen gels were completely cross-linked as indicated by the absence of $\gamma$ monomers and presence of $\gamma$ dimers (Fig. B.3). 100 mg/mL fibrinogen gels were partially cross-linked as both $\gamma$ monomers and dimers were present.
4.5.2 Fibrin gels are linearly viscoelastic for all fibrinogen concentration at low strains

After monitoring gelation with step 1 for 3000 s (Fig. B.4), amplitude sweeps over strains of 0.01% to 1% at 1 Hz were conducted to confirm linear viscoelastic behavior. There was less than 5% change in G’ and G’’ for all gels over this range of strains suggesting linear viscoelastic behavior. At a strain of 1%, gelation was confirmed by a plateau in G’ during low frequency sweeps (0.01 Hz to 3 Hz) and linear viscoelasticity (Fig. 4.2) (161). G’ was roughly independent of frequency at all fibrinogen concentrations indicating the absence of relaxation mechanisms at these frequencies for a strain of 1%. G’ was more strongly dependent on fibrinogen concentration than thrombin concentration. G’’ showed a modest increase with increasing frequency at all fibrinogen concentrations, indicating a relaxation mechanism whose reciprocal time constant exists above the experimental range of the frequencies measured. This trend suggests that fibrin acts as a viscous liquid only on long time scales. For all gels tested the
Figure 4.2. Dynamic shear rheology of fibrin gels at small strains. G' (filled symbols) and G'' (open symbols) of fibrin gels formed at fibrinogen concentrations of (q) 3 mg/ml, (p) 10 mg/ml, (n) 30 mg/ml, (u) 50 mg/ml, and (l) 100 mg/ml as a function of frequency at a strain of 0.01. Gels formed with 10 nM (A) and 100 nM (B) thrombin. Data is presented as the mean and standard deviation (n=3).
dynamic shear storage modulus dominated the mechanical response; gels showed tan δ (G’’/G’) values of 0.05±0.01 independent of fibrinogen or thrombin concentrations. G’ and G’’ increased rapidly with fibrinogen concentration of 3–30 mg/mL, and were less sensitive from 30–100 mg/mL (Table 4.1).

### 4.5.3 Strain hardening under shear is attenuated in high density fibrin gels

The shear modulus (G) of fibrin gels formed with 10 nM thrombin was measured as a function of strain during a constantly increasing deformation test at a strain rate of 0.01 s⁻¹ over strains of $10^{-3}$ to 1 (Fig. 4.3). At low strains ($\gamma = 10^{-3}$) the G value was similar to the measured dynamic storage modulus (G’) value. This is expected as G’ was frequency independent and much greater than G’’ for all gels. G ranged from approximately 100–20,000 Pa as fibrinogen concentration was increased from 3–100 mg/ml, with most of the change occurring over the range of 3–30 mg/ml. Given the viscoelastic nature of the gels, it should be recognized that the strain hardening is expected to be ramp rate dependent. At the selected ramp rate, low density fibrin gels (3, 10 mg/mL) showed significantly greater strain hardening than high density fibrin gels (30, 50, 100 mg/mL) at strains of 0.1–1. Fibrin gels formed at 3 and 10 mg/ml had roughly one order-of-magnitude increase in G for $\gamma > 0.05$. Gels formed at 30–100 mg/ml either remained

<table>
<thead>
<tr>
<th>Thrombin [nM]</th>
<th>Fibrinogen [mg/mL]</th>
<th>G’ at 1 Hz [Pa] (SD)</th>
<th>G’’ at 1 Hz [Pa] (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3</td>
<td>158 (60)</td>
<td>5.6 (2.8)</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>964 (380)</td>
<td>75 (15)</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>8200 (1000)</td>
<td>4800 (130)</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>12,800 (5300)</td>
<td>700 (51)</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>16,500 (7700)</td>
<td>784 (173)</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>110 (16)</td>
<td>4.7 (0.6)</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>840 (65)</td>
<td>56 (3.0)</td>
</tr>
<tr>
<td>100</td>
<td>30</td>
<td>8200 (420)</td>
<td>320 (30)</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>19,100 (3100)</td>
<td>800 (100)</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>26,200 (3900)</td>
<td>1700 (367)</td>
</tr>
</tbody>
</table>

**Table 4.1.** Dynamic shear storage (G’) and loss (G’’) moduli of fibrin gels at small strains. Data is presented as the mean and standard deviation (SD) of n=3.
relatively constant at moderate strains or showed a very slight degree of strain softening below strains of 0.2 and a modest degree of strain hardening at higher strains.

4.5.4 Extensional rheology reveals two strain hardening regimes

Similar to shear rheology, the stress-strain relationship in biaxial extension of fibrin gels was weakly dependent on thrombin concentration compared to fibrinogen concentration (Table 4.2, Fig. 4.4 A and B). Note that 3 mg/mL gels were not tested because they were unable to support their own weight in the SER fixture. Stress-strain curves of all gels 10 mg/ml and greater showed two regions of strain hardening (Fig. 4.4 C and D). The first region occurred at strains of $0.01 < \gamma < 0.25$. This is similar to the strain hardening seen under shear stress in Fig. 4.3. The second region occurred at strains of $\gamma > 0.75$. At strains of $0.25 < \gamma < 0.75$, $E(\gamma)$
Table 4.2. Rheological measurements of fibrin gels in extensional strain.

<table>
<thead>
<tr>
<th>Thrombin [nM]</th>
<th>Fibrinogen [mg/mL]</th>
<th>$E(\gamma)$ at $\gamma = 0$ [Pa] $\times 10^4$</th>
<th>$E(\gamma)$ at $\gamma = 0.25$ [Pa] $\times 10^4$</th>
<th>Max $E(\gamma)$ [Pa]</th>
<th>Modulus Ratio</th>
<th>Stress at break [Pa] $\times 10^4$ (SD)</th>
<th>Strain at break (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
<td>0.9</td>
<td>3.4</td>
<td>23</td>
<td>6.7</td>
<td>5.1 (4.0)</td>
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<td>20.1</td>
<td>120</td>
<td>5.9</td>
<td>39 (6.8)</td>
<td>1.2 (0.07)</td>
</tr>
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</table>

Figure 4.4. Biaxial extensional stress-strain data for fibrin gels formed with 100 nM (A) and 10 nM (B) thrombin and 10 (magenta), 30 (blue), 50 (red), and 100 mg/ml (black) fibronogen. The shaded region represents the standard deviation (n=5-8). The corresponding elastic moduli were found by taking the first derivative of a fifth order polynomial fit to the stress-strain data for gels formed with 100 nM (C) and 10 nM thrombin (D).
remained relatively constant. The stress at break increased from $3.2 \times 10^4$ Pa to $39 \times 10^4$ Pa with increasing fibrinogen concentration. The strain at break was 1.0–1.6 for all gels, with no apparent trend for different fibrinogen concentrations. For reasons discussed below, the first strain hardening regime is likely caused by network rearrangement, and the second regime is likely caused by fiber stretching.

4.5.5 High density fibrin gels act as thermal affine networks at small strains based on mesh size scaling

The shear modulus ($G$) of thermal affine networks scales with mesh size according to Eq. 4.3. The mesh size of fibrin networks was independently estimated by Eq. 4.2 using measurements of hydraulic permeability (146) and by Eq. 4.3 using the measured $G$ values at low strains ($\gamma = 0.01$) for fibrin gels formed with 10 nM and 100 nM thrombin. There is good agreement between estimates of mesh size based on permeability and $G$ (Fig. 4.5 A and B). The predicted mesh size ranges from 300–400 nm in 3 mg/ml gels to 20–30 nm for 100 mg/mL gels. The largest change in mesh size occurred over the range of 3–30 mg/ml fibrinogen. There was only a modest change in mesh size at higher fibrinogen concentrations.

4.5.6 Negative normal stresses show that fibrin gels act as mechanical networks at moderate strains

The ratio between negative normal stress and shear stress ($-\sigma_N/\sigma_S$) at strains greater than 0.01 is characteristic of a network of mechanical elastic rods for all fibrinogen concentrations (Fig. 4.6 A–E). The $-\sigma_N/\sigma_S$ ratio shows an initial large increase with strain before reaching maximums between strains of 0.15–0.25, and then decreasing at higher strains. All gels showed a small strain regime with scaling of $\sigma_N \approx \gamma_S^2$ and $\sigma_S \approx \gamma_S$. This scaling held for larger strains with increasing fibrinogen concentration strongly suggesting a mechanical network. The flatter shape
Figure 4.5. Mesh Size, $\xi$, of fibrin gels formed with (A) 10 nM thrombin and (B) 100 nM thrombin as calculated by measurements of $G'$, Eq. 4.3 (white bars) and permeability, Eq. 4.2 (black bars) for fibrin gels formed with 3 – 100 mg/ml fibrinogen.
of the $-\sigma_N/\sigma_S$ curve and reduced peak with increasing fibrinogen concentrations suggests that gels with higher fiber density are more susceptible to fiber stretching relative to bending; a feature than might be expected as mesh size decreases and the length between strand supporting junctions decreases. These features are consistent with models of mechanical networks that predict fibers become less susceptible to bending as the maximum of the curve becomes smaller (79, 156).

4.5.7 **Affine and non-affine mechanical networks as a function of fibrin density**

The ratio of elastic modulus to shear modulus, $E(\gamma)/G(\gamma)$, can be used to characterize affine and non-affine mechanical deformation. Using data from shear and extensional rheology (Figs. 4.3 and 4.4), the ratio of the material functions $E(\gamma)/G(\gamma)$ as a function of strain was
compared for 10–100 mg/ml gels (Fig. 4.7). At low strain ($\gamma < 0.003$), $E(\gamma)/G(\gamma)$ was ~3, which is the affine prediction (Eq. 4.4). As strain was increased, $E(\gamma)/G(\gamma)$ of all gels increased, reaching local maximums at strains of 0.1–0.3 with the strain at maximum increasing with increasing fibrinogen concentration. As strain was further increased, $E(\gamma)/G(\gamma)$ of all gels decreased to local minimums at strains of 0.6–0.7. Finally, the $E(\gamma)/G(\gamma)$ ratio of all gels increased again as strain was further increased above 0.7. The $E(\gamma)/G(\gamma)$ ratio of the 10 mg/ml gel reached a maximum value of 48 at a strain of 0.15 before decreasing to a local minimum of 19 at a strain of 0.6. The $E(\gamma)/G(\gamma)$ ratio of the 30, 50, and 100 mg/ml gels were less sensitive to strain, all reaching local maxima of approximately 10 at strains of 0.2, 0.25, and 0.3, before decreasing to values of 3, 5, and 9 at strains of 0.7, 0.65, and 0.6, respectively. The decrease in $E(\gamma)/G(\gamma)$ toward the affine limit as strain is increased from 0.15 to 0.6 indicates that fibrin gels become more affine with increasing strain.

Figure 4.7. The ratio of elastic moduli, $E(\gamma)$, to shear moduli, $G(\gamma)$, of fibrin gels formed with 10 nM thrombin and 10 (magenta), 30 (blue), 50 (red), and 100 mg/ml fibrinogen (black) as a function of strain. The error bars represent the propagated error (shear n=3, extensional n = 5-8).
Platelet retraction of fibrin gels depends on both platelet and fibrin density

Fibrin gels derived from 3, 10, and 30 mg/ml fibrinogen and 10 nM thrombin were created with the inclusion of $2 \times 10^4$ and $2 \times 10^5$ platelets/µL and allowed to retract for 5 h at 37°C. The majority of the retraction occurred in the first hour and there was little change in gel volumes after two hours (Appendix C, Retraction Movie). Gels formed with $2 \times 10^5$ platelets/µL showed final retraction values 97.5% ± 0.1%, 89.3% ± 1.0%, 39.0% ± 2.9% for 3, 10, and 30 mg/ml gels, respectively (Fig. 4.8). Gels formed with 3 and 10 mg/ml fibrinogen and $2 \times 10^4$ platelets/µL retracted by 75.1% ± 10.5% and 23.4% ± 0.8%, respectively (Fig. 4.8). There was no measurable retraction for gels formed with 30 mg/ml fibrinogen and $2 \times 10^4$ platelets/µL. Due to the reduction in clot volume resulting from retraction, gels formed with $2 \times 10^5$ platelets/µL resulted in gels with final fibrin concentrations of 119 ± 0.1 mg/ml.
93 ± 1.1 mg/ml, 49 ± 3.7 mg/ml for gels with initial concentrations of 3, 10, and 30 mg/ml, respectively. Gels formed with $2 \times 10^4$ platelets/µL resulted in clots with fibrin concentrations of 12 ± 1.7 mg/ml and 13 ± 0.5 mg/ml for gels with initial concentrations of 3 and 10 mg/ml, respectively. The degree of retraction could be slightly overestimated due to collapse fibrin gels during removal from the tube. The contractile force of a platelet is 4 nN on a substrate with elasticity of ~10 kPa, which is comparable to the elasticity of a 30 mg/ml gel (Fig. 4.2) (139). Using this force, we estimated that a platelet could bend a fibrin fiber 2 nm and stretch a fiber 64 nm in a 30 mg/ml gel (see Appendix B.5.3 for calculation). The same force could bend a fiber 770 nm and stretch a fiber 64 nm in a 3 mg/mL gel. Although these calculations assume that fiber bending and stretching moduli are independent of fiber density, they do suggest a trend where platelet-mediated retraction in high density fibrin gels may be limited to fiber stretching, which is consistent with affine deformation.

4.5.9 Fibrin rheology state diagram

Results from shear and extensional rheology experiments show that low and high density fibrin gels are characterized by strain dependent network rearrangement and fiber stretching regimes. Fig. 4.9 shows a proposed state diagram of these different regimes. The evidence supporting this state diagram is summarized below.

At low strains ($\gamma < 0.01$), all fibrin gels exhibit an initial linear regime where elasticity is determined by thermal fluctuations of the fibrin fibers that undergo affine deformation regardless of fiber density. Fibrin elasticity in this low-strain linear regime is well described by the thermal affine model, Eq. 4.3; the mesh size for fibrin gels as measured by elasticity and permeability show good agreement over the entire range of concentrations tested. Furthermore, the E/G ratio is close to the affine limit for all gels at low strains. These results are in agreement with previous
Measurements of low density fibrin gels at low strains \((28)\). Here, we find that this model also holds for high density fibrin gels. At increasing strain, we find that all fibrin gels transition away from the linear regime at \(\gamma \sim 0.01\) as indicated by the deviation of \(E(\gamma)/G(\gamma)\) from the affine predictions (Fig. 4.7). This agrees with the results of Piechocka and colleagues who found that the ‘thermal slack’ is pulled out of fibrin fiber at strains of 0.01 for fibrin gels formed with between 3 – 8 mg/ml fibrinogen \((28)\).

At moderate strains \((0.01 < \gamma < 0.6)\) elasticity is determined by mechanical network deformations. The scaling in the small strain regime where \(\sigma_N \approx \gamma S^2\) and \(\sigma_S \approx \gamma S\) with fibrinogen concentration and the shape of the \(-\sigma_N/\sigma_S\) curve where the values of the ratio trends toward 0.5 with increasing strain with a possible peak at lower strains are characteristics of mechanical

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**Figure 4.9.** Proposed state diagram of the elastic regime of fibrin gels as a function of fibrinogen concentration and strain. At strains of less than a few percent, network elasticity is dominated by entropic restrictions of thermally fluctuating fibers (green) for all gel concentrations, this is the Affine Thermal (AT) regime. As strain is increased, fibrin gels transition into a regime where elasticity is mechanically dominated. High density (HD) gels transition directly to an affine mechanical stretching (AMS) regime where fibers are mechanically stretched (blue). Low density (LD) gels transition to a non-affine mechanical bending (NMB) regime where bent fibers (orange) allow for network rearrangement. Eventually, network rearrangement results in fibers that effectively span the network and stretch causing the LD gels to transition into the AMS regime. At strains greater than 60-70% the filament backbone of stretched due to protein unfolding or other mechanisms, this is the filament backbone stretching (FBS) regime.
networks. Whether network deformation is dominated by nonaffine fiber bending or affine fiber stretching depends on fiber density and strain.

Gels formed from 3 and 10 mg/ml fibrinogen appear to first transition into a nonaffine, bending-dominated deformation regime before ultimately reaching an affine, stretching-dominated regime at higher strains. The evidence of nonaffine mechanical bending is the high $E(\gamma)/G(\gamma)$ ratios indicating a strong deviation from the affine assumption. The peak in the $E(\gamma)/G(\gamma)$ ratio shows that the maximum nonaffine deformation occurs at a strain of ~0.15 before deformations become more affine $E(\gamma)/G(\gamma)$ decreasing) as strain is increased to 0.6. Furthermore, sharp peaks in the $-\sigma_N/\sigma_S$ ratios at strains of 0.1–0.2 are characteristic of the nonaffine, bending regime (79, 156). Microrheology measurements in low density fibrin gels show a peak at non-affine deformation at slightly lower strains (0.02), but show a similar trend toward affine deformation at increasing strain (36). At increased strain, the $-\sigma_N/\sigma_S$ ratio of all gels approach the same value of ~ 0.5, indicating a weak dependence on bending and a stretching dominated regime (79, 156).

Gels formed from 30, 50, and 100 mg/ml fibrinogen transition from affine thermal fluctuations directly into a predominantly affine, stretching-dominated regime. This conclusion is based on $E(\gamma)/G(\gamma)$ ratios that deviate only slightly from the affine prediction. High concentration gels also show a less pronounced peak in the $-\sigma_N/\sigma_S$ ratios before decreasing to approach the same value of ~ 0.5, indicating more stretching dominated deformations over the entire range of strains. Using criteria developed by Broedersz and colleagues, we can estimate the transition from non-affine to affine mechanical deformations using the ratio of fiber length ($L_c$) to critical length ($\lambda_{NA}$) that is a function of mesh size (see Appendix B.5.2). Gels formed with 30–100 mg/ml fibrinogen have $L_c/\lambda_{NA} >> 1$ suggesting that the mechanical deformation regime
of these gels is stretching dominated and is largely affine (Table 4.3). Gels formed at 3 and 10 mg/ml fibrinogen have $L_c/\lambda_{NA}$ on the order of unity, suggesting that these gels do not deform with purely affine modes, but instead have some degree of nonaffine bending.

At high strains ($\gamma > 0.6$), all fibrin gels again strain harden. We assume that the strain hardening in this regime is due to unfolding of fibrin molecules based on previous reports (28, 29, 133). However, other mechanisms may be simultaneously present.

### 4.6 Discussion

The objective of this study was to measure and characterize the mechanical properties of fibrin gels over a range of fibrinogen concentrations that are representative of arterial thrombi, fibrin surgical glues and tissue engineering scaffolds. Our results show that fibrin undergoes a variety of elastic deformations as a function of strain in agreement with previous reports (27, 81, 86, 131). Here, we show that there are unique features that distinguish low and high density fibrin gels. A notable finding was that low density fibrin gels appear to undergo both affine stretching and nonaffine mechanical bending at moderate strains, while high density fibrin gels undergo primarily affine mechanical stretching. This difference was also apparent in platelet retraction assays; retraction was significantly reduced in high density fibrin gels where fiber bending is presumably limited. The network mechanics of high density fibrin gels are only

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weakly dependent on fibrinogen concentration, suggesting that fibrin monomers contribute more
towards changes in the internal structure of fibers, rather than to the network structure above a
certain fibrinogen concentration.

The only previous rheological study of high density fibrin gels that the authors are aware
of comes from Weigandt and colleagues. They measured the mechanics and structure of high
density fibrin gels by neutron scattering coupled with shear rheology for fibrin gels derived from
fibrinogen solutions of 1 to 40 mg/ml (86). Our results from dynamic shear rheology and strain
ramp in shear deformation are in good agreement with their results. In particular, strain
hardening at moderate strains was attenuated with increasing fibrinogen concentration. The
degree of strain softening at strains ~0.1 that they observed for high density fibrin gels was more
pronounced than our observations. This difference could be explained by the differences in
solvent (water vs. deuterated water) and the relatively high salt concentration (0.5 M NaCl) used
in their study.

The observed trend of a transition from thermal semiflexible fibers to mechanical fibers
for all fibrin gels is consistent with the observations of by Piechocka et al. (28). They report that
fibrin gels from 0.06–8 mg/ml fibrinogen have linear mechanics described by thermal
fluctuations of fibers at very low strains, but that strains of only a few percent are adequate to
remove any thermal slack from the fibers and to force the fibers to mechanically deform. One
notable difference is our results suggest that low density fiber gels transition from a bending
controlled nonaffine regime into a mostly affine stretching dominated regime at higher strains, as
evident from the ratio of $E(\gamma)/G(\gamma)$ with increasing strains. This nonaffine regime is further
supported by measurements of the negative normal to shear stress ratio as a function of strain and
the direct observation of nonaffine deformations; Wen and colleagues used fluorescent
microscopy to track the displacement of beads embedded in a 2.5 mg/ml fibrin gel to show that fibrin gels deform nonaffinely with a sharp increase in nonaffine behavior up to a strain of 0.02 and then a decrease in nonaffine behavior at higher strains (143). The existence of a nonaffine regime caused by the fibers’ susceptibility to bending and buckling and resulting network ultracompresibility also explains the observed water loss of gels formed with lower fibrinogen concentrations (129). The affine deformations characteristic of high density fibrin gels may explain their strain softening due to compressive forces generated on fibers oriented 135° away from the horizontal axis during shear being great enough to yield fiber buckling where collapsed fibers no longer contribute to elasticity (84, 142). Moreover, the absence of a transition from bending to stretching likely reduces strain hardening at moderate strains (84).

Differences between nonaffine bending dominated elasticity in low density fibrin gels to affine stretching dominated elasticity in high density fibrin gels may have physiological significance in the volume and stability of thrombi formed in vivo. In particular, it appears that degree of platelet contraction depends on fiber density. The contractile force of an individual platelet increases with increasing substrate stiffness, suggesting that a platelet will pull on higher density fibrin gels with a force of up to ~79 nN (139). Despite this increased force, clot retraction was still attenuated in high concentration fibrin gels, presumably due to the affine stretching dominated deformation of the higher concentration gel. Platelets at concentrations representative of those found in blood were able to retract low density fibrin gels by over 90%, in agreement with previous reports (138). However, platelets were only able to retract a high density fibrin gel by 40%. Retraction of low density fibrin gels could be due to the ability of platelets to induce nonaffine, bending deformations of fibers and fiber rearrangement. Based on the estimated bending modulus and using the mesh size as a characteristic length, the force generated by a
single platelet is sufficient to cause bending deformations of almost a micrometer in 3 mg/ml gels, but only negligible bending deformation relative to stretching deformation in 30 mg/ml gels. A complementary or alternative explanation is that at mesh sizes of <100 nm it may be difficult for platelet to extend filopodia into the dense fibrous network during the retraction assays. These observations may be most relevant in venous thrombi that contain high solids fractions of fibrin and low solids fractions of platelets. It is possible that the increased platelet density associated with flow-formed arterial clots might produce sufficient force to retract even the most highly concentrated fibrin gels.

The physical properties of low and high density fibrin gels show a significant difference in the dependence on fibrinogen concentration. Both interstitial fluid flow, as measured by permeability, and elasticity, as measured by shear and extensional rheology, show a strong dependence on fibrinogen concentrations over the range of 3–30 mg/ml. At higher concentrations, the changes in these properties are modest up to 100 mg/ml. The strong dependence of elasticity on fibrinogen concentration at low fibrinogen concentrations can be attributed to the change in mesh size, and thus fiber density as previously reported (146, 153). This relationship is also evident in confocal microscopy images of low and high density fibrin gels. The weak dependence at higher fibrinogen concentrations suggests that the excess fibrin(ogen) may be leading to denser fibers. This reasoning is supported by neutron scattering studies that show solid volume fraction within fibers scales with increasing fibrinogen concentration (86). We hypothesize these denser fibers have different mechanical properties at high strains, although there are no single fiber measurements to date to support this hypothesis.

Regardless of fiber density, fibrin gels act as thermal affine networks at low strains. Here, we used this feature in combination with semiflexible polymer theory to estimate mesh size from
elasticity measurements. We found good agreement between mesh size as measured by two independent methods (permeability and shear elasticity) and agreement between our estimated mesh sizes at 3 mg/ml (~350 nm) with direct measurement of mesh size by scanning electron microscopy at 2.5 mg/ml (200–500 nm) (143). In these calculations we assumed a constant fiber length, which is consistent with measurements of low density fibrin gels (47, 48). However the fiber lengths in high density fibrin gels has not been measured to our knowledge. This structure-property relationship serves as a link that can be used to estimate difficult to measure, structure-related properties, such as permeability and hindered diffusion, from noninvasive, in vivo rheology measurements like ultrasound or in vitro measurements of clot mechanics like thromboelastogram (140, 162). Such measurements are potentially useful for guiding mechanical and fibrinolytic strategies.

4.7 Conclusions

In this study, we measured the mechanical properties of fibrin gels formed over the range of fibrinogen concentrations found in clots formed in vivo and used in fibrin glues and tissue engineering scaffolds. Shear and extensional rheology measurements show differences in the elastic regimes of gels formed at 3–10 mg/mL and 30–100 mg/mL fibrinogen. Interpretation of these results with deformation theories of fibrous networks suggests distinct network structures for these two types of fibrin gels.

4.8 Supporting Material

Materials and supplemental methods, four figures (Figs. B.1–B.4), one movie (Retraction Movie), and ten equations (Eqs. B.1–B.10) are available in the accompanying supporting material.
4.9 Acknowledgements

This work was supported by a Grant-in-Aid award from the American Heart Association, National Science Foundation CAREER Award (KBN), the National Institutes of Health (R21NS082933 and R01HL120728), and NSF Research Experience for Undergraduates Award EEC-1156745, and the Army Research Office DURIP W911NF-11-1-030.
CHAPTER 5
MEASURING THE PORE SPACE IN FLOW-FORMED CLOTS BY FOCUSED ION BEAM-SCANNING ELECTRON MICROSCOPY IMAGING

5.1 Introduction

Flow-formed clots have highly heterogeneous structures comprised of a dense mixture of cells and proteins that dictate the physical properties of a clot such as permeability, hindered diffusivity, and elasticity. Several studies have implicated hemodynamics as an important factor in regulating the role of clot growth and structure. For example, Onasoga-Jarvis et al. used microfluidic techniques to show that perturbations in a flow field are required to formed fibrin on tissue factor mimetic surfaces and that clot formation under flow results in a five-fold increase in fibrin concentration relative to plasma concentrations (32). Leiderman and Fogelson used numerical simulations for show that perturbations in the flow field due to clot growth results in thrombi with significant heterogeneity and that the reduced transport associated with the increase clot solid fraction limits clot growth to result in smaller, denser clots (34, 51). Further, a collection of studies utilizing a combination of computational fluid dynamic simulations and in vivo assays in the murine arterioles revealed that clot solid density varies radially within thrombi (58, 66, 163, 164). These studies identified dense “core” and less dense “shell” regions of a thrombus that limit the entry of plasma-borne molecules into the clot while simultaneously limiting the release of thrombin from within the clot.

Several studies have shown that in vivo clots can consist of a range of protein and cell fractions. Histology of clots retrieved from humans with arterial fibrillation and stroke suggest that platelets make up 20–80% of the solids volume of the clot (96, 97). A recent electron
microscopy study of aspirated clots from myocardial infarction patients found that the solids component of thrombi consisted of 56% fibrin, 17% platelets, 12% erythrocytes, 5% cholesterol crystals, and 1% leukocytes (31). These studies, however, are limited in that they do not account for the pore space of thrombi, but instead provide a by component breakdown of only the solid fraction of a clot. Therefore, there exist a need to precisely determine the composition of a clot while preserving a measurement of both solid fraction and void space of a clot.

In this chapter we describe a method to measure the solid fraction, porosity and pore structure of flow-formed clots. Microfluidic models of vascular injury were used to form whole blood clots on type I collagen at a wall shear rate of 100 s$^{-1}$. After clot formation, the fibrin and platelet density in the clot is determined by confocal images analysis. Finally, a detailed map of the nano-scale and micro-scale structures of the of the clot is generated using a focused ion beam-scanning electron microscope (FIB-SEM) to section and image the internal structure of the clot with the void space being preserved by polymer resin embedding. Using these techniques, we found that the solid fraction of a flow-formed clot was 0.61 ± 0.11. Further, this technique allows for direct measurement of the porosity of clots and size distribution and tortuosity of the pore structure of a clot.

5.2 Materials and Methods

Thrombi were formed by perfusing whole blood over a collagen “injury” in a microfluidic channel. The structure of the thrombi were then quantified by confocal microscopy and FIB-SEM.

5.2.1 Materials

Human type I fibrillar collagen was purchased from Chrono-Log Corp (Havertown, PA). Human fibrinogen (free of plasminogen, von Willebrand factor, and fibronectin) in 20 mM
sodium citrate and human α-thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). The AlexaFluor 555 labeling kit and DiOC6 (3,3’-dihexyloxacarbocyanine iodide) fluorescent dye were purchased from Molecular Probes (Grand Island, NY). Sodium cacodylate buffer (200 mM sodium cacodylate-HCl buffer, pH = 7.4), HEPES buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4), recalcification buffer (75 mM NaCl, 37.5 mM MgCl₂ in HBS ), bovine serum albumin buffer (BSA; 5 mg/ml BSA in HBS), and assay wash buffer (1 U/ml heparin, 7.5 mM NaCl, 3.75 mM MgCl₂ in HBS), were made in house. Uranyl acetate, propylene oxide (PO), and the SPI-Pon 812 Epoxy Embedding Kit (SPI-Pon 812 epoxy resin monomer, dodecenyl succinic anhydride (DDSA) hardener, nadic methyl anhydride (NMA), DMP-30 Epoxy Accelerator) were purchased from Structure Probe, Inc (West Chester, PA). Calcium chloride, sodium chloride, magnesium chloride, and HMDS (hexamethydisilazane), sodium cacodylate, fluormount aqueous mounting medium, and photo-grade gluteraldehyde were purchased from Sigma-Aldrich (St. Louis, MO).

5.2.2 Whole blood clot formation

Human type I fibrillar collagen was patterned as a 250 µm wide strip on a glass slide (75 mm × 25 mm) using a microfluidic channel (w = 250 µm, h = 25 µm). PDMS microfluidic devices for use in the flow assay were fabricated with each device having three independent channels (w = 500 µm, h = 50 µm, l = 20 mm). All surfaces were blocked with BSA buffer for 1 h at room temperature prior to use. Each channel was placed over the patterned collagen strip with the flow direction perpendicular to the patterned collagen direction.

Human whole blood was collected from healthy donors via venipuncture. Phlebotomy was conducted in accordance with the Declaration of Helsinki and under the University of Colorado, Boulder Institutional Review Board approval. Donors had not consumed alcohol
within 48 h prior to the draw, nor had they taken any prescription or over-the-counter drugs within the previous 10 days. Whole blood was drawn into sodium citrate (3.2 % citrate) anticoagulant. For confocal assays, a fluorescently labeled fibrinogen (AlexaFluor 555) was added to the whole blood at an approximate molar ratio of 250:1 unlabeled:labeled. Platelets were labeled with DiOC₆ (10 µM). After resentization for 10 min at 37°C, the whole blood was aliquotted for step-wise recalcification. The first aliquot of whole blood was recalcified to a final concentration of 7.5 mM NaCl and 3.75 mM MgCl₂ with recalcification buffer. The whole blood was perfused over the collagen test pattern for 200 s at a shear rate of 100 s⁻¹ in a PDMS microfluidic channel after which the a second aliquot was recalcified and perfused over the collagen. This process was repeated three times for a total assay time of 600 s (10 min).

5.2.3 Confocal microscopy of flow-formed thrombi

After clot formation, excess whole blood was removed from the microchannel by perfusion with wash buffer for 1 min. Clots were then fixed with 2% gluteraldehyde solution in wash buffer for 2 min. The microchannel was removed, clots were dried, fluoromount added, and the glass slide and clot capped with a coverslip. Images were captured on a laser scanning confocal microscope (Fluoview FV10i; Olympus, Melville, NY) using 10x (NA 0.4) and 60x (NA 1.2) objectives and excitation/emission wavelengths of 556/573 nm and 495/521 nm at laser intensities of 20% and 40% for the AlexaFluor555 and DiOC₆, respectively.

The platelet concentration and platelet volume fraction, \( \phi_p \), was calculated by counting the number of platelets within a sample volume, \( V_{\text{sample}} \), of a confocal image where sample volume is determined by:

\[
V_{\text{sample}} = A_{\text{sample}} l_{\text{plt}}
\]  

(5.1)
where $A_{\text{sample}}$ is the sample area of the confocal image and $l_{\text{plt}}$ is the linear distance occupied by a platelet. The linear distance occupied by a platelet is calculated by counting the number of platelets along a randomly oriented line in the image plane and assuming the in-plane platelet spacing is identical to the spacing normal to the image plane. Each platelet was assumed to have a mean volume of $8.48 \pm 0.71 \text{ fL}$ (165). The ratio of the total platelet volume (platelet number $\times$ platelet volume) to total sample volume is reported as the platelet volume fraction.

Fibrin concentration is a flow formed clot is calculated by comparing the fluorescence intensities of a region of interest of the flow form clot to a standard curve. The standard curve was created by forming fibrin gels with 3, 20, 50, and 80 mg/ml fibrin with a ratio of 250:1 unlabeled to labeled (Alexafluor 555) fibrinogen between a glass slide an a coverslip by the additions of 10 nM human $\alpha$-thrombin (final concentration). Images were taken using the same confocal microscope settings as used for the imaging of the flow-formed thrombi and the average image intensity recorded.

### 5.2.4 Resin embedding

After allowing the clots to form for 10 minutes, the whole blood was removed from the microchannels by perfusion with wash buffer for 1 min, after which the clots were preliminarily fixed with a 0.2% gluteraldehyde solution in wash buffer. After this initial fixing step, the PDMS microchannel was removed, replaced with a PDMS well, and the clot immerse in a series of solutions for 10 min each (unless otherwise specified) to prepare the clots for imaging with the focused-ion beam scanning electron microscope. Clots were immerse in: 100 mM sodium cacodylate-HCl buffer, pH = 7.4; 2% gluteraldehyde solution in 100 mM sodium cacodylate-HCl buffer (1 h); RO water ($5 \times 10$ min); 2% uranyl acetate solution in water (1 h); RO water ($5 \times 10$ min); 35%, 50%, 60%, 70%, 80%, 90%, 95% (2 $\times$ 10 min), 100%, 100% (20 min),
100% (30 min) ethanol in RO water; 50% PO in ethanol solution; 100% PO (2 × 15 min); 50% PO in SPI-Pon 812 resin mixture (SPI-Pon 812 45%, DDSA 28%, NMA 25%, DMP 2% v/v) (1 h); 100% SPI-Pon 812 resin mixture (48 hr) at 60˚C to allow resin to harden. After hardening of the resin the polymer block containing the clot was removed from the glass slide and sputter coated with a thin (~10 nm) layer of gold as the last step before imaging. Finally, the location of the clot in the resin was identified using a stereoscope and marked with a needle.

### 5.2.5 FIB-SEM imaging

Imaging was performed on a Helios NanoLab DualBeam focused ion beam-scanning electron microscope from FEI Company (Hillsboro OR). First, a sacrificial layer of platinum ~300 nm thick is deposited to protect the clot and resin to be imaged from the high current during milling. A trench (length = 15 µm, width = 5 µm, depth = 5 µm) was then cut into the resin embedded clot by milling the resin at an accelerating voltage of 30 kV and 21 nA of beam current. After milling the cross-section of the clot was imaged at 2.0 kV accelerating voltage and 0.69 nA beam current.

### 5.2.6 Area thresholding to determine solid fraction

The area occupied by the platelet clot was determined by performing a series of image processing steps using ImageJ software. The background was subtracted from the raw image using the Sternberg rolling ball method with a rolling ball radius that was at least five times larger than the largest feature visible (166). The contrast of the image was then enhanced by normalizing the image histogram after which the image was smoothed using a two sigma Gaussian blur. The perimeter of the clot was then manually selected as the region of interest and the image thresholded using the Default thresholding routine. To determine the area fraction of the thrombi while discounting the internal pore of a platelet, the area fraction is calculated by
discounting any pore smaller than 0.2 \( \mu \text{m}^2 \) in area, which is roughly the area of the pore of the open canalicular system (167).

5.2.7 Relief contrast microscopy of clots to determine clot area

Relief contrast images of the clot were taken by Hoffman modulation contrast (HMC) (20X, NA 0.45) on an IX81 inverted microscopy from Olympus (Melville, NY) at \( t = 600 \text{ s} \) (pre-fixed), after fixing with 0.2% gluteraldehyde but before removal of the microchannel (post-fixed), and after resin embedding (post-embedded) to quantify any clot structural changes during the embedding process. Clot area calculation was conducted using the same image processing method used for determining the solid fraction of the milled clots.

5.3 Results

Thrombi formed under flow resulted in thrombi with heterogeneous structures that were comprised of a majority of solids.

5.3.1 Thrombus heterogeneity

Thrombi were formed by perfusing recalcified whole blood over type I fibrillar collagen at a shear rate of 100 s\(^{-1}\). Analysis of confocal images of a flow-formed thrombi reveals clots formed of dense platelet aggregates containing heterogeneously dispersed pockets of dense fibrin (Fig. 5.1. A). The 495/521 nm (DiOC\(_6\)) channel of 300X magnification images (Fig 5.1. B) was used to determine both the linear distance occupied by a platelet, \( l_{\text{plt}} \), the platelet concentration, and the platelet volume fraction, \( \phi_p \), which were calculated to be \( 2.24 \pm 0.13 \mu \text{m/platelet} \), \( 7.7 \pm 1.2 \times 10^7 \text{Plt/\mu L} \), and \( 0.66 \pm 0.16 \), respectively, based on a minimum of five measurement taken at least three different locations in the thrombus. Three-dimensional reconstruction of a series of images taken at 1 \( \mu \text{m} \) height increments show the morphology of the clot and reveals an max thrombus height of 18 \( \mu \text{m} \) (Fig. 5.1. C). Confocal images at \( h = 7 \mu \text{m} \) allows for the
Figure 5.1. Morphology of whole blood clots formed at 100 s\(^{-1}\) containing labeled fibrinogen (red) at a 250:1 unlabeled:labeled ratio platelets labeled with DiOC\(_6\) (green). (A) The overall structure of a flow-formed clot shows both platelet-rich and fibrin-rich regions. (B) Individual platelet are resolved with the 495/521 nm channel. (C) Three dimensional reconstruction enables a details map of thrombus morphology. (D) Fibrin can accumulate in dense pockets or as strands in between platelet aggregates.

Quantification of fibrin density in different regions within the thrombus, with regions of interest 1, 2, 3, and 4 having fibrinogen concentrations of greater than 80 (above the range of the standard curve), 50 ± 4, 35 ± 3, and 6 ± 1 mg/ml fibrinogen, respectively (Fig. 5.1. D).
5.3.2 Flow-formed clots are comprised of mostly solids

Imaging of the cross-section of a clot formed by perfusing whole blood over fibrillar collagen at 100 s\(^{-1}\) using FIB-SEM showed that flow-formed clots consist of 61\% solids by volume. Figs. 5.2 A-D show the raw image of the sectioned clot, the background subtracted image, the contrast enhances image, and the thresholded image along with the region of interest used to determine the porosity of the clot. In addition, cross-section images reveal pore with high tortuosity as well as a nanometer to micrometer distribution of pore sizes. Analysis of the solid fraction of the cross-section in which pores smaller than 0.2 \(\mu\)m\(^2\) were discounted revealed the solid fraction of a flow formed clot when discounting the internal pores of a platelet is 0.65.

5.3.3 The embedding procedure does not alter clot geometry

Comparison of the areas of a flow-formed clot during different processing stages show that the size of the clot did not change during processing and therefore the size of the clot viewed with the FIB-SEM was representative of the clot size during the final stages of clot growth. The clot sectioned in Fig 5.2 is shown during different stages of processing along with the thresholded image at each stage (Fig. 5.3). During all stages of processing, the thrombus retained its overall shape (Fig. 5.3. A, C, E) and size with areas measured at 616, 632, and 674 \(\mu\)m\(^2\) for pre-fixed, post-fixed, and post-embedded clots, respectively (Fig. 5.3. B, D, F).

5.4 Discussion

The objective of this study was to develop a method to assess the structure and composition of flow-formed thrombi. Confocal image analysis was used to measure both the platelet and fibrin density as a function of position within a clot. FIB-SEM was used to precisely measure the total solid fraction and pore geometry of a clot. Combined, these data quantify the
Figure 5.2. Image process steps used to derive the solids fraction of whole blood clots formed at 100 s⁻¹. (A) Initial image, (B) Image with background subtracted using the rolling-ball method with a radius of 1000 pixels, (C) Contrast-enhanced and smoothed image after subtraction, and (D) Thresholded image and region of interest used to determine the area fraction outlined in yellow. Scale bar = 2 µm.
Figure 5.3. Images of flow-formed thrombi. (A) Thrombus at 10 min during flow with whole blood WB at 100 s⁻¹ (B) Thresholded image of thrombus in A. (C) Thrombus at after initial rinse with sodium cacodylate buffer but before the removal of the PDMS channel. (D) Thresholded image of thrombus in C. (E) Thrombus after resin embedding (F) Thresholded image of thrombus in E. Red rectangle highlights the thrombus selected for cross-sectioning and the dashed yellow line shows the approximate location of the cross-section. Scale bar = 20 µm
total accumulation and spatial variations of fibrin and platelets of the clot and provide detailed information about its the pore structure.

Confocal image analysis revealed that platelets occupy 66 ± 16% of the total volume of a flow-formed thrombi based a count of the platelets with an image plane and assuming a platelet volume. However, platelet volume can vary both from person to person and within an individual which may add error to our estimate (165). Measurement of fibrin concentration revealed pockets of fibrin within the thrombus with as little as 6 ± 1 mg/ml fibrin and with greater 80 mg/ml fibrin. These measurements of fibrin concentration in platelet dense thombi are made based on a comparison to a standard curve created in the absence of platelets which may scatter some of the light signal from the fibrin and result in an underestimation of the fibrin concentration. However, these estimates agree with measurements of the area fraction calculated by FIB-SEM imaging. Further, these estimates agree with in vivo measurements that determined the thrombus solid fraction of in vivo clots to be was roughly 0.5 by comparing the signal of fluorescently-labeled BSA in whole blood (with a porosity of 0.6 assuming a hematocrit of 0.4) to the signal within the clot during clot formation (66).

Images of the sectioned thrombus revealed a heterogeneous pore structure with pores on both micrometer and nanometer scales. It is possible that some of these areas, in particular the smaller pores, are actually isolated pores within the platelet that are part of either the open canalicular system or alpha-granules that may have reacted differently to the uranyl acetate stain. These pores would therefore not be part of void fraction available for transport within the clot. Estimates of the void fraction of a thrombus may potentially need to be adjusted to account for this discrepancy; however, these discrepancies would be less than ± 0.04 based on calculations of the solid fraction with pores smaller than 500 nm filled.
This method will prove useful in determining the morphology of clots formed over a range of physiological and pathological hemodynamic conditions. For instance, FIB-SEM analysis can be used in conjunction with microfluidic assays to form thrombi on collagen, tissue factor, and collagen and tissue factor surfaces, at low (100 s\(^{-1}\)), high (1000 s\(^{-1}\)), and pathological (10,000 s\(^{-1}\)) shear rates. Cross-sections can be cut into each thrombus both perpendicular and parallel to the direction of flow to determine the anisotropy of thrombus porosity, pore size distribution, and pore tortuosity as a function of prothrombotic surface and shear condition.

### 5.5 Conclusion

In this study, we have devised a method to quantify the structure and composition of flow-formed thrombi. We found that a combination of confocal image analysis and FIB SEM imagine allow to quantification of flow-formed clots on both the micrometer and nanometer scale, while also accounting for the pore space of the clot. Clots were found to be comprised of a greater than 60% solids with a high degree of variability in fibrin concentration. This method can be used to determine the precise morphology of clots formed at a large variety of hemodynamic conditions and will aid in delivering drug delivery strategies and in determining the mechanisms for uncontrolled clot growth.
CHAPTER 6

GENERAL CONCLUSIONS

6.1. Summary of Results

Thrombus formation is a complex process involving an intertwined series of events including platelet adhesion and aggregation, coagulation, and transport limitations that affect the final structure of the clot. In this thesis, the physical properties that affect the growth, structure, and mechanical strength of a clot were measured at fibrin and platelets densities relevant to those found in flow-formed clots, and constitutive models of each property dependence on fibrin and platelet density were developed.

In Chapter 3 the hydraulic permeability and hindered diffusivity of macromolecules were measured in fibrin gels and thrombi representative of the composition found in vivo. Fibrin gels formed with fiber volume fractions ranging from 0.02–0.54 had permeabilities ranging from $1.2 \times 10^{-1}$–$1.5 \times 10^{-4}$ $\mu$m$^2$. Platelet rich clots with platelet volume fractions of 0.01–0.61 and a fibrin volume fraction of 0.03 had permeabilities ranging from $1.1 \times 10^{-2}$ to $1.5 \times 10^{-5}$ $\mu$m$^2$. Fibrin gels formed with a fiber volume fraction of 0.36 reduced the diffusion coefficients of 12, 29, and 54 nm solutes by 60%, 66% and 85%, respectively. Platelet rich clots with a platelet volume fraction 0.61 reduced the diffusion coefficient of a 12 nm solute by 93%. The permeability of fibrin gels and of clots with platelet volume fraction less than 0.2 were modeled as an array of disordered cylinders with uniform diameters. Clots with a platelet volume fraction greater than 0.2 were modeled as a Brinkman medium of coarse solids (platelets) embedded in a mesh of fine fibers (fibrin). Hindered diffusivity of fibrin gels and platelet rich clots was predicted using permeability measurements and the effective medium model. Our data suggests
that the permeability of clots formed in vivo can vary by up to five orders-of-magnitude, with pore sizes ranging from 4−350 nm that significantly restrict solute diffusion. These findings have important implications for the transport of coagulation zymogens/enzymes in the interstitial spaces during clot formation, as well as the design of fibrinolytic drug delivery strategies.

In Chapter 4 the mechanical properties of fibrin gels formed at low (3−10 mg/mL) and high (30−100 mg/mL) concentrations of fibrin were measured and described at low, moderate, and high strains using shear and extensional rheology. Confocal microscopy of the gels shows that fiber density increases with fibrinogen concentration. At low strains fibrin gels act as thermal networks independent of fibrinogen concentration. Within the low strain regime, the mesh size of fibrin gels can be predicted by the elastic modulus using semiflexible polymer theory. Significantly, this provides a link between gel mechanics and interstitial fluid flow. At moderate strains, we find that low density fibrin gels act as nonaffine mechanical networks and transition to affine mechanical networks with increasing strains within the moderate regime, while high density fibrin gels only act as affine mechanical networks. At high strains, the backbone of individual fibrin fibers stretch for all fibrin gels. Platelets can retract low density gels by greater than 80% their initial volumes, but retraction is attenuated in high density fibrin gels and with decreasing platelet density. Taken together, these results showed that the nature of deformation of fibrin is a strong function of fibrin fiber density, which has ramifications for the growth, embolization and lysis of thrombi.

In Chapter 5 a method to assess the structure of flow-formed clots using microfluidic devices was described. Clots were formed by perfusing reconstituted whole blood over type I fibrillar collagen at a constant shear rate of 100 s⁻¹. The structure of each formed clot was measured using confocal microscopy and electron microscopy using a focused ion beam to
section the sample. Confocal microscopy revealed that flow-formed clots has platelet dense regions with approximately $7.7 \pm 1.2 \times 10^7$ Plt/µL corresponding to a total platelet solids fraction of $0.66 \pm 0.16$ v/v, assuming each platelet has a volume of $8.48 \pm 0.71$ fL (165). These values are in agreement with value of solid fraction obtained from electron microscopy measurements of ion beam-milled clots, which showed flow-formed clots contain minimum solid fractions of 0.61. These findings indicate that flow has a significant effect on clot structure and results in a much higher solid fraction compared to clots formed statically from platelet rich plasma.

6.2. Future Research

The models of the physical properties of thrombi established in this work allows for the predictions of properties relevant to thrombus growth and thromboembolism. Data of thrombi permeability and hindered diffusivity suggest that hindered transport is a mechanism of clot arrest. Data of thrombi elasticity suggest that fracture is a mechanism of thromboembolism.

6.2.1. Transport mechanisms of clot arrest

In this work, I have shown that fluid and solute transport through both fibrin gels and platelet rich clots is significantly hindered due to the decreased pore sizes caused by increased solid fractions. I hypothesize that this restricted transport serves as a mechanism to limit the extent of clot growth. As discussed in Chapter 2, important coagulation factors such as factor X and prothrombin must move from the plasma, to the interior of the clot to become activated, and then back to the exterior of the clot to convert additional fibrinogen to fibrin and activate additional platelets. The diameter of these coagulation factors are roughly 5 nm to 10 nm (thrombin and factor X, respectively), similar to the pore diameters of dense platelet rich clots and dense fibrin gels clots (4 nm and 12 nm, respectively) (168, 169). During the initial stages of formation, the solid fraction is small. The clot thus contains large pores that allow unrestricted
movement of solutes resulting in burst of coagulation and platelet activity and growth of the clot. As the solid fraction in the clot increases the pore size decreases to limit the introduction of zymogens and the exit of activated enzymes. This hypothesis has been tested and limitedly validated using a spatial-temporal mathematical model, with the limitations of experiments being the testing of only one injury size and the limitations of the model being the assumed function for permeability had not been validated by experimental measurements (34). Therefore, the need exists to test the restricted transport hypothesis in a more valid system, such as a microfluidic assay, where blood is perfused at a constant pressure over a prothrombotic substrate. Such measurements of hindered transport could then be used as a lookup table to improve computational methods.

Although arterial thrombosis occurs on times scales of minutes, other types of thrombi can take hours or days to form. These longer time scales are characteristic of venous thrombosis and intraluminal thrombosis in aortic abdominal aneurysms. To accomplish these times scales experimentally is a challenge. One potential strategy is to draw whole blood into sodium citrate and then recalcify it using a chaotic mixing device to achieve on-chip steady-state recalcification before being perfused over a patterned prothrombotic surface (170). This chaotic mixing device “mixes” two streams (whole blood and recalcification buffer) by utilizing a structured channel pattern to induce transverse flows within the microchannel to fold volumes of fluid over one another thus reducing the diffusion length within the fluid. Clot growth should be measured during the assay using both area analysis of microscope images and velocity measurements, and post-assay via confocal imaging. Velocity measurement should be made by measuring the streaklines of fluorescently labeled beads at a location upstream of the clot in a manner similar to previously described methods (171). Clots should be allowed to form at a series of shear rates
(150, 300, 750, 1500 s\(^{-1}\)) until either the channel becomes occluded or until the velocity in the channel remains constant, indicating no further change in the size of the thrombus. Post-assay, the total volume and solid fraction of each flow-formed thrombi can be measured using confocal and FIB-SEM imaging. From this set of experiments one would be able to identify the growth limits and corresponding solid density of clots as a function of shear rate, injury size, and channel size. Additionally, this experiment could be extended to test flow-regulated clot growth as a function of hematocrit, platelet count, and plasma concentrations of coagulation factors.

6.2.2. Structures and mechanisms of thromboembolism

Thromboembolism is a major concern in the area of thrombosis, and occurs when a thrombus or part of a thrombus becomes dislodged, travels downstream, and blocks a vessel at a new location. Thromboembolism can result in blockages of the main arteries to the heart, brain, or lungs causing myocardial infarction, stroke, or pulmonary embolism, respectively. In Chapter 4, the mechanical properties of fibrin, the biopolymer responsible reinforcing the strength of a thrombus, was measured and characterized. These experiments revealed that the strength of all fibrin gels tested far exceeded the stresses typically encountered in a vessel. Assuming a high physiological shear rate of 1600 s\(^{-1}\) and a blood viscosity of 0.003 Pa s, the highest stress typically encountered in a vessel can be calculated to be 5 Pa (\(\tau = \eta \times \gamma\)). This stress is far lower than the breaking strength of a 10 mg/ml fibrin gel which was found to be on the order of 10,000 Pa. Further, the elastic modulus of a 3 mg/ml at 1% strain was found to be on the order of 100 Pa, suggesting that the typical stresses encountered in a vessel should only minimally strain a thrombus, and should be incapable of causing thromboembolism. Nevertheless, thromboembolism continues to exist as a major problem.
I hypothesize that in order for a thrombus to embolize, the stress imparted on the clot by the flow must be concentrated into a small region of the clot. This stress concentration causes a fracture to develop, leading to an increased stress in the area immediately surrounding the initial break, which further propagates and ultimately leads to thromboembolism. This mechanism of failure is typical in materials where the elastic modulus exceeds the cohesive strength (stress at failure) of a material (172). Results from Chapter 4 revealed that the cohesive strength of a 10 mg/ml fibrin gel was similar to the elastic modulus of a 100 mg/ml fibrin gel. Therefore, spatial variations in the structure of a thrombus could result in a condition under which a small initial “crack” would propagate. Further, several studies have indicated that atherosclerotic plaque rupture is triggered by high-stress regions that develop into fractures due to spatial variations in the plaque structure (173, 174). Similarly, flow-formed thrombi often form with structural variations. Magnetic resonance imaging and immunohistochemistry consistently showed that ex vivo pulmonary emboli and thromboemboli were heterogeneous in structure and contained both a fibrin and RBC-rich region and a separate platelet-rich regions (11, 175). Due to the heterogeneous structure of a thrombus and the large range of possible mechanical properties associated with a variable structure, fracturing is a likely mechanism of failure of flow-formed thrombi.

The property that describes resistance to fracturing in elastic materials is known as fracture toughness or the material’s J-integral, $J_{lc}$, and is a measurement of the energy required to grow a thin fracture in a material. Fracture toughness can be measured in three differing shearing modes (I, II, and III) corresponding to tensile, shear, and tearing loads, respectively, with the tensile and shear modes being the most likely modes of thromboembolism.
To determine the feasibility of this hypothesis both the nonaffine behavior and the fracture toughness of model thrombi should be tested as a function of fibrin, platelet, and red blood cell concentration. In traditional composite material, stiffer regions embedded in a soft fiber matrix can generate a nonaffine strain field which could lead to stress concentrations in the material (159). Therefore, any nonaffine behavior would indicate the likelihood of a stress concentration leading to the initiation of a fracture. Nonaffine mechanical testing should be conducted using the methods described in chapter 4; the extend of nonaffine deformations should be mapped out as a function of platelet and RBC fractions by analyzing the ratio of shear to normal stress and $E(\gamma)/G(\gamma)$ to determine which concentrations of cell components help distribute or concentrate stress. The likelihood of fracture propagation should then be tested for each model thrombi according to the American Society for Testing and Materials (ASTM) test E1820-01 (176). Briefly, samples formed at each previously mentioned combination of components should be cast between two glass slide and cut into strips in a manner previously described in chapter 4. After creation of the test strips, a notch should be cut into the sample to a depth of one-half of the sample width with a razor blade. After notching the sample should be mounted to the ARES-G2 and extensional test performed on the Sentmanat fixture and a stress and fracture length measured as a function of strain to obtain a value for each thrombi’s fracture toughness. The value of fracture toughness obtains can then be used to estimate if a stress less than the failure stress of the thrombi itself will cause the thrombi to fracture.
REFERENCES CITED


126


APPENDIX A
SUPPLEMENTAL INFORMATION FOR CHAPTER 3

A.1 Derivation of permeability between two platelets embedded in a fibrin mesh

This derivation is presented in Example 8.7 on p. 417 of Truskey et. al and is shown here in terms of the variables defined in the manuscript text (72). Consider the case of two parallel plates separated by a distance $h$ that represent two platelets with a fibrin gel between them depicted above. There is an interstitial flow of fluid with a viscosity $\mu$ and a mean velocity of $U$ in the x-direction. The fibrin gel has a permeability $k_f$. The Brinkman equation for this problem reduces to:

$$\mu \frac{d^2 v_x}{dy^2} - \frac{\mu}{k_f} v_x - \frac{dP}{dx} = 0$$

(A-1)

where $v_x$ is the velocity in the x-direction and $P$ is the pressure. Note that $v_x$ is only a function of y and the pressure gradient is only a function of x. The boundary conditions are no-slip at the platelet surface and symmetry at the midline:

Figure A-1. Schematic of the space between two platelets. Platelets are modeled as two parallel plates with the space between each plate filled by a fibrin gel.
\( v_x = 0 \) at \( y = h/2 \)
\( \frac{dv_x}{dy} = 0 \) at \( y = 0 \) \hspace{1cm} (A-2)

The solution to Eq. A-1 for these boundary conditions is:

\[ v_x = \frac{k_f}{\mu} \frac{dP}{dx} \left[ 1 - \frac{\cosh \left( \frac{y}{\sqrt{k_f}} \right)}{\cosh \left( \frac{h}{2\sqrt{k_f}} \right)} \right] \hspace{1cm} (A-3) \]

The mean velocity can be calculated by:

\[ U = \frac{1}{h} \int_{-h/2}^{h/2} v_x dy = \frac{k_f}{\mu} \frac{dP}{dx} \left[ 1 - \frac{2\sqrt{k_f}}{h} \tanh \left( \frac{h}{2\sqrt{k_f}} \right) \right] \hspace{1cm} (A-4) \]

Finally, the total permeability between the two plates is defined by the ratio of the mean velocity and the pressure gradient \( (k_t = \mu U/(dP/dx)) \):

\[ k_t = k_f \left[ 1 - \frac{2\sqrt{k_f}}{h} \tanh \left( \frac{h}{2\sqrt{k_f}} \right) \right] \hspace{1cm} (A.5) \]

### A.2 Validation of 2D FRAP Approximation

The following study was conducted to determine if 2D analysis of fluorescence recovery after photobleaching (FRAP) is valid to analysis of the diffusion coefficients in 3D fibrin gels.

#### A.2.1 Introduction

Fluorescence recovery after photobleaching (FRAP) is often used to measure diffusion coefficients in lipid bilayers. The solute to be measured is tagged with a fluorophore. After an initial image is captured, the fluorophores within a region of the bilayer is quickly bleached with a high powered laser. After bleaching, the molecules within the bilayer are allowed to diffuse throughout the sample. The diffusion of fluorescent molecules into the bleached region is found by measurement of the fluorescent signal within the region over time. This technique has been so successful that programs have been written that analyze FRAP images and automatically
calculate diffusion coefficients even in non-ideal samples (convection, uneven illumination, diffusion while bleaching) (99). One drawback to this program is that it is designed to analyze 2D diffusion; however, if the extent of bleaching in the z-direction is much greater than the sample thickness the 3D situation behaves like the 2D situation and programs designed to analyze 2D diffusion can be used (177). The purpose of this work was to test if a 2D analysis can be used to analyze FRAP measurements in platelet rich thrombi and fibrin gels

A.2.2 Materials and Methods

Fibrin gels and platelets rich thrombi were formed with 0.02 mg/ml of 500 kDa FITC dextran included in the sample. Before bleaching, the sample thickness is measured by finding the focal planes at each end of the sample with a confocal microscope (Olympus Fluoview FV10i). After measurement a z-stack was taken of the pre-bleached sample with a 10x objective and a z-spacing of 10 µm. The sample was then bleached at 100% laser power and 3x optical zoom by setting the focal plan to the center of the sample with the confocal aperture wide open (5.0x) for 5 min. After bleaching a z-stack was taken of the entire sample. The extend of bleaching through the sample was determined by measuring the fractional decrease in fluorescent signal as a function of z-plane through the sample according to Eqn. A-6, where $FD$ is the fractional decrease of signal, $I_{post}$ is the post-bleach signal and $I_{pre}$ is the pre-bleached signal.

$$FD = \frac{I_{pre} - I_{post}}{I_{pre}}$$ (A-6)

A.2.3 Results

In all cases, sample thickness was between 40 µm and 50 µm. The measured fractional decrease in fluorescent signal for a 5 min bleach was roughly 0.33 in all cases. Table A-1 shows the factional decrease in signal for two different samples of platelet rich thrombi.
A.2.4 Discussion

According to Braga et al, if the sample thickness of sample is less than the finite axial size of the bleached volume then the 3D sample will behave like its 2D counterpart. Because the extent of bleaching in our samples roughly constant through the entire sample (doesn’t decrease in the z-direction over the entire sample thickness) 2D FRAP analysis can be used to measure diffusion coefficients in fibrin gels and platelet rich thrombi provided that the sample thickness is < 50 µm and that the numerical aperture is open to allow the laser to pass through the sample as a column as opposed to a cone. It should be noted that this 50 µm criterion is set only because that was the range that was tested. The actual value of extend of bleaching for this system my in fact be larger.

A.2.5 Conclusion

The 2D FRAP analysis and subsequent Matlab routine used in Jonsson et al. provides valid assessment of the diffusion coefficients in fibrin gels.

<table>
<thead>
<tr>
<th>Distance from center of sample [µm]</th>
<th>7×10^6 Plt/µL</th>
<th>2×10^7 Plt/µL</th>
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<tbody>
<tr>
<td>-20</td>
<td>0.30</td>
<td>0.36</td>
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<tr>
<td>-10</td>
<td>0.31</td>
<td>0.35</td>
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<tr>
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<td>0.31</td>
<td>0.34</td>
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<tr>
<td>10</td>
<td>0.32</td>
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<td>20</td>
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</table>
Figure A-2. Experimental set-up for measuring clot permeability. A reservoir containing TBS supported by a ring stand and clamp was connected to the top of the permeation chamber via an 8 mm ID Tygon® connecting tube. 0.76 mm ID silastic tubing was connected to the bottom of the chamber via a blunt 16 gauge needle. The volumetric flow rate of buffer through the fibrin gel or platelet rich clot was calculated by measuring the displacement of the air-buffer interface in the tubing at regular time intervals. (Insert) Image showing permeability measurements of 156 mg/ml fibrin gels.
Figure A-3. Image analysis for obtaining measured platelet area fractions. (A) Original 600x magnified confocal image showing only the platelets (green) of a PRC at a density of $4 \times 10^7$ platelet/µL. Scale bar = 20 µm. (B) Original image converted to 8-bit grayscale with ImageJ software. (C) Greyscale image after subtraction of the background using the Sternberg rolling ball method with a rolling ball radius of 10 µm. (D) Final image after thresholding with a threshold value of 5. Area fraction of platelets = 0.45.
Figure A-4. Solutions for the normalized permeability of the Brinkman equation from Ethier (115). The total permeability, $k_t$, is normalized by the permeability of the fine fraction, $k_f$. In our case, the fine fraction refers to the fibrin. Each line represents a different ratio between the fine fraction permeability and the square of the radius of the coarse objects ($k_f/a_{coarse}^2$): 10 (−−), 1 (−−), and 0.1 (⋯). The measured permeability (○) best fits the solution for $k_f/a_{coarse}^2 = 1$. 
Figure A-5. The effect of platelets on fibrin fiber morphology. The presence of platelet (A, C) leads to shorter fibrin fibers compared to platelet poor plasma (B, D). The volume fraction of platelets (A) is 0.61. Platelets are labeled green and fibrinogen is labeled red. Scale bar = 20 µm
APPENDIX B
SUPPLEMENTAL INFORMATION FOR CHAPTER 4

B.1 Modified Clauss Assay

Ten milliliters of stock fibrinogen solution at 12.9 mg/ml were dialyzed against TBS and then reduced to ~1 mL by centrifuging the solution at 37°C in a centrifugal concentrator device with a molecular weight cutoff of 100,000 Da. The concentration of the resulting retentate solution is ~129 mg/mL fibrinogen. A sample of this concentrated solution is diluted to ~1 mg/mL in TBS and 10 nm thrombin and 2.5 mM CaCl₂ (final concentrations) are added to induce gelation. The absorbance of this gel is measured at 405 nm and compared to a standard curve (dynamic range 0–1.5 mg/mL) to determine a more accurate concentration of the diluted sample. The initial estimate for the retentate solution concentration (129 mg/mL) is then refined based on the measured concentration of the diluted sample (e.g. if the diluted sample was determined to have 1.05 mg/ml fibrin instead of 1.0 mg/ml fibrin the initial estimate would be scaled by 1.05/1 to give a value of 135 mg/ml).

To validate this method, we compared value the concentration of fibrinogen as determined by the modified Clauss assay to the concentration of a 400X diluted retentate sample as determined by absorbance at 280 nm using a UV/VIS spectrophotometer with an extinction coefficient of 1.51 mL mg⁻¹ cm⁻¹ (178). The concentrations of the fibrinogen retentate as determined by the modified Clauss assay (129 ± 4 mg/ml) was found to be in good agreement with the value determined by UV/VIS (125 ± 2 mg/ml).
B.2 Solubility of fibrinogen solution

One hundred microliters of concentrated fibrinogen (130 mg/ml) and fibrinogen stock solution (12.9 mg/ml) were pipetted into the wells of a clear, flat-bottomed 96-well plate. Solutions were incubated at 4, 20, and 37 °C and the absorbance at 450 nm of was measured over a period of 45 h to determine solubility (179). Each condition was done in triplicate. The absorbance of both the concentrated fibrinogen and the stock fibrinogen incubated at 4°C increased with time indicating precipitation of fibrinogen (Fig. B-2). This precipitation was also observed by transition from a clear to cloudy solution. The absorbance of the same fibrinogen solutions incubated at 21°C and 37°C did not change of over the period of 45 hours indicating the fibrinogen remained in solution.

B.3 SDS-PAGE Analysis of Fibrin Cross-linking

Fibrin gels identical to those use in the mechanical testing experiments were formed with 3, 10, 30, 50, and 100 mg/ml fibrinogen and 10 nM thrombin (final concentrations) and allowed to gel for 50 minutes. Ligating reactions in the samples was quenched with 6 M urea, and 2% SDS at 37°C for 2 h. Gel electrophoresis (8% polyacrylamide) was prepared using standard protocols (180). Briefly, to make one gel, 1.6mL 40% acrylamide solution, 2 mL 1.5M Tris buffer, 80 µL each of 10% SDS solution and 10% (by weight) of APS solution and 8 µL of TEMED to 4.2mL of deionized water. The solution was mixed, cast into a mini gel 1 mm thick and allowed to polymerize for 45 minutes. Samples for electrophoresis were prepared by diluting proteins to ~12 µg of total protein in deionized water and adding 4x Laemmli loading buffer (1:3 loading buffer:sample). Total volume of ~25 µL of sample was loaded in each well. Proteins were fractionated into bands by electrophoresis running at 110 V for 90 minutes. Protein bands were visualized by staining with Coomasie blue stain for 1.5 h followed by washing in deionized
water. The gels were then imaged on an Alpha Innotech IS2200 digital UV-Vis imaging system (Santa Clara, CA). Fig. B-3 shows the results of the gel and demonstrates cross-linking for all fibrin gels.

B.4 Confocal microscopy of fibrin gels

Fibrin gels were prepared for confocal microscopy were prepared exactly as described above, except that samples were formed between glass slide and a #1 glass coverslip. Fibrinogen was labeled with AlexaFluor® 555 according to the manufacturer’s instructions and was added to each solution at a molar ratio of 500:1 unlabeled:labeled fibrinogen. Images were captured on a laser scanning confocal microscope (Olympus Fluoview FV10i) using a 60X objective (NA = 1.2) and excitation/emission wavelengths of 556/573 nm.

B.5 Supporting Calculations

The following calculations were used to calculate the bending and stretching moduli of fibrin and to estimate the nonaffine-affine transition.

B.5.1 Calculating the bending modulus of an individual fibrin filament

The persistence length of a bundle of fibers relative the persistence length of the sub-filaments within the bundle can be calculated using Eq. 2 from Bathe et al. (154):

$$\frac{\kappa_B}{\kappa_f} = N \left(1 + \frac{\chi^2(N-1)}{1 + c q_j N + \sqrt{N} \alpha} \right)$$  \hspace{1cm} (B-1)

where, $\kappa_B$ is the bending modulus of the bundle, $\kappa_f$ is the bending modulus of the sub-filament, N is the number of sub-filaments or protofibrils, $\alpha$, is a fiber coupling parameter, $c$, is the wave number, and $\chi$ is a parameter accounting for the finite thickness of the crosslinks. For the fully decoupled (no cross-linking) case, $\alpha = 0$ causing the bracketed term to be equal to unity. However, fibrin fibers are cross-linked by factor XIIIa in our rheology measurements (Fig. B-3).
The bending modulus of coupled (cross-linked) and decoupled (not cross-linked) fibrin filaments has been reported as 14.5 MPa and 1.7 MPa, respectively (134). Based on these values, we estimate the bending modulus of a fibrin filament to be 8.5 times greater (14.5/1.7) than the bending modulus of a bundle of fully uncoupled sub-filaments, reducing Eq. B-1 to:

$$\kappa_B = 8.5N\kappa_f$$  \hspace{1cm} (B-2)

For the protofibrils within our gels, $N$ is calculated using Eq. 6 from Weigandt et al. (86),

$$N = \frac{\Phi_{\text{int}}\rho_m R^2}{\mu_p}$$  \hspace{1cm} (B-3)

where $\Phi_{\text{int}}$ is the internal volume fraction of the fibrin filament, and $\rho_m$ and $\mu_p$ are the mass density and the mass to length ratio of a protofibril, equal to 1.4 mg/ml, and 340 kDa/22.5 nm, respectively (112). The value for $R$ used is the previously measured hydrated fiber radius (146). The internal volume fraction, $\Phi_{\text{int}}$, is a function of fibrinogen concentration and is based on a fit of the data presented in Fig. 5 in Weigandt et al.(86):

$$\Phi_{\text{int}} = 0.015\ln(c_{fbg}) + 0.13$$  \hspace{1cm} (B-4)

Using the above values, $N = 30$ for the filaments within our network.

Finally, we are able to estimate the bending modulus of a fibrin filament based on the persistence length of a protofibril. The bending modulus of a protofibril is related to persistence length of a protofibril by:

$$\kappa_f = L_{p,f}\kappa_T$$  \hspace{1cm} (B-5)

where $L_{p,f}$ is the persistence length of a protofibril previously measured to be 500 nm (81). We can then estimate the bending modulus of a fibrin filament to be $4.5 \times 10^{-25}$ Nm$^2$ by combining Eqns. B.2-B.5 to obtain
\[ \kappa_B = 8.5 L_{p,f} Nk_B T \]  

(B-6)

### B.5.2 Estimating the nonaffine-affine transition of fibrin gels as a function of fibrinogen concentration

The nonaffine-affine (NA-A) transition length for fibrous networks can be predicted based on the filament length, \( L_c \), mesh size, \( \xi \), filament stretching modulus, \( \mu \), and filament bending modulus, \( \kappa_B \) (181). Affine deformations become more favorable when filament length becomes larger than a critical length, \( \lambda_{NA} \), defined as:

\[ \lambda_{NA} = \frac{\xi^2}{L_b} \]  

(B-7)

and \( L_b \) is the characteristic bending length scale defined as a filament’s susceptibility to bending versus stretching:

\[ L_b = \sqrt{\frac{\kappa}{\mu}} \]  

(B-8)

Therefore, if \( L_c / \lambda_{NA} \gg 1 \) an affine, stretching dominated regime is expected whereas if \( L_c / \lambda_{NA} \ll 1 \) a nonaffine, bending dominated regime is expected. The stretching modulus, \( \mu \), is estimated to be 5 nN by assuming the stretch modulus of a filament is equal to the sum of the stretch modulus of the protofibrils within the bundle. This value is consistent with a previous estimate of \(~10\ nN\) for fibers with slightly larger diameters (29). The stretch modulus of an individual protofibril is estimated to be \( 1.70 \times 10^{-10} \) N with a corresponding length of 80 nm (28), assuming 30 protofibrils per bundle (\( N = 30 \)), giving \( \mu \) of \( 5 \times 10^{-9} \) N. Using this value and the value for bending modulus calculated previously, the characteristic bending length, \( L_b \), of a fibrin filament is calculated to be 30 nm. Combining \( L_b \) with estimates of mesh size as determined by permeability experiments allows us to calculate the NA-A transition length for each gel tested.
B.5.3 Estimating displacement of a fibrin caused by the platelet contractile force

The centerline bending displacement, $\delta_{\text{bend}}$, caused by a force imparted on a simply supported beam is given by:

$$\delta_{\text{bend}} = \frac{F_{\text{plt}} \xi^3}{48\kappa_B}$$

(B-9)

where $F_{\text{plt}}$ is the maximum contractile force of a platelet, $\kappa_B$ is the filament bending modulus, and length of bending is assumed to be the mesh size, $\xi$, of the network as calculated by permeability experiments (146). The stretching displacement, $\delta_{\text{stretch}}$, caused by a force pulling on the end of a beam is given by:

$$\delta_{\text{stretch}} = \frac{L_{\text{stretch}} F_{\text{plt}}}{\mu}$$

(B-10)

where $L_{\text{stretch}}$ (80 nm) and $\mu$ is the stretch modulus ($5 \times 10^{-9}$ N) as calculated in the previous section above. Using equations B.9 and B.10, we are able to estimate the displacement imparted upon a fibrin fiber by a platelet assuming a platelet contractile force of 4 nN on a substrate with elasticity of 10 kPa (139). An assumption in this calculation is that the bending and stretching moduli hold over the range of strains predicted.
Figure B-1. Measurement of engineering stress and calculation of $E(\gamma)$ of a 100 mg/ml fibrin gel formed with 10 nM thrombin. (A) The biaxial stress-strain data with the moving average (---), and 5th degree polynomial fit (—). The shaded region represents the standard deviation (n=5). (B) Bi-axial stress-strain data zoomed in to show the fit at low strain. (C) $E(\gamma)$ calculated as the first derivative of the 5th degree polynomial fit. (D) $E(\gamma)$ zoomed in to better show the modulus at low strain.
Figure B-2. Absorbance of fibrinogen at 450 nm as function of time of (A) Stock fibrinogen solution (12.9 mg/mL) from the supplier and (B) concentrated fibrinogen at 130 mg/ml for solutions incubated at 4 (u), 20 (n), and 37 °C (●). Increases in absorbance are indication of precipitation, which was evident at 4 °C, but no change was observed at 20 and 37 °C.
**Figure B-3.** Reducing SDS-PAGE analysis of fibrin gels cross-linking. *Lane 1:* molecular markers, *lane 3:* fibrinogen, *lane 4:* fibrin 3 mg/ml, *lane 5:* fibrinogen 30 mg/ml, *lane 6:* fibrinogen 50 mg/ml, *lane 7:* fibrinogen 100 mg/ml. Bands at 66, 53, and 47 kDa corresponding to the α, β, and γ chains of the fibrinogen monomer. The band at 100 kDa corresponding to the γ-γ dimer. All fibrin gels (3 – 100 mg/ml) showed the bands at 100 kDa corresponding to the γ-γ dimer indicating that all gels underwent γ-chain crosslinking. Gels formed with 3 – 50 mg/ml showed the absence of the 47 kDa (γ) band indicating that these gels were completely γ-chain crosslinked. Both the 100 kDa (γ-γ dimer) and the 47 kDa (γ-chain) were present for the 100 mg/ml gel indicating that this gel was crosslinked, but the extent of crosslinking is unknown. All gels showed bands at the very top of the well corresponding to αₙ polymer segments. The presence of bands at 66 kDa (α) in all gels show that α-chain crosslinking is incomplete in all gels.

**Figure B-4.** Representative G’ as a function time for a 100 mg/ml fibrin gel formed with 100 nM thrombin showing the lag, growth, and steady phases.
APPENDIX C

SUPPLEMENTAL ELECTRONIC FILES

Included is one video exhibiting the retraction of fibrin gels derived from 3, 10, and 30 mg/ml fibrinogen and 10 nM thrombin with the inclusion of $2 \times 10^4$ and $2 \times 10^5$ platelets/µL at 37 °C over the course of 5 h.

| Retraction Movie.mov | Quicktime file containing timelapse images of clot retraction |