SINGLE FIBRIN FIBER MECHANICAL PROPERTIES AND LYSIS

BY

WEI LI

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# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ v

LIST OF TABLES .......................................................................................................... viii

LIST OF ABBREVIATIONS ........................................................................................ ix

ABSTRACT .................................................................................................................. xii

Chapter

I. INTRODUCTION ...................................................................................................... 1

II. THE EFFECT OF CARDIOVASCULAR DISEASE AND AGE ON FIBRIN FIBER MECHANICAL PROPERTIES ................................................................. 18

III. FIBRIN FIBER STIFFNESS IS STRONGLY AFFECTED BY FIBER DIAMETER, BUT NOT BY FIBRINOGEN GLYCATION ........................................ 43

IV. INVESTIGATION OF FIBRIN FIBER INTERNAL STRUCTURE: PROTEIN DENSITY AND BOND DENSITY DECREASE WITH INCREASING DIAMETER .................................................................................................................. 72

V. HIGHLY STRETCHABLE, BIOCOMPATIBLE, STRIATED SUBSTRATE MADE FROM FUGITIVE GLUE ............................................................................. 95

Published in *Materials*, June 2015

VI. STRETCHING SINGLE FIBRIN FIBERS SLOWS DOWN LYSIS MORE THAN FXIII CROSSLINKING ................................................................. 115

VII. CONCLUSION ...................................................................................................... 138

APPENDIX

EFFECT OF STRENuous EXERCISE ON THE MECHANICAL PROPERTIES OF FIBRIN FIBER AND HEMOSTATIC SYSTEM ........ 150
Chapter II Supplementary Information ..................................................166
Chapter III Supplementary Information..................................................169
Chapter IV Supplementary Information ..................................................180
SCHOLASTIC VITAE.................................................................................183
LIST OF FIGURES

1. Schematic plot of fibrinogen molecule ................................................................. 3
2. Schematic diagram of fibrin polymerization ......................................................... 4
3. Schematic plot of laser deflection of atomic force microscope (AFM) ................. 9
4. Experimental setup of combined AFM / fluorescence microscope .................. 11
5. Schematic overview of fibrinolysis ................................................................. 13
6. Single fiber AFM/fluorescence microscopy technique ........................................ 25
7. Extensibility of fibers from three different groups .............................................. 27
8. Elastic limit with respect to Age and CVD ...................................................... 29
9. Incremental stress and strain curves for single fibrin fibers ............................. 31
10. Total Modulus with respect to Age and CVD .................................................. 32
11. Total Modulus as a function of Diameter with respect to Age and CVD ....... 33
12. Fibrin fiber manipulation ............................................................................. 52
13. Fibrin fiber mechanical properties as a function of glycation ....................... 56
14. Fibrin fiber properties as a function of fibrinogen concentration .................. 57
15. Fibrin fiber modulus as a function of fiber diameter ....................................... 59
16. Fibrin fiber models and their corresponding Young’s modulus ..................... 61
17. Experimental setup for light intensity measurement ....................................... 80
18. Experimental setup for force measurement ..................................................... 81
19. Light intensity of electrospun fibrinogen as function of diameter .................. 84
20. Light intensity of wet fibrin fiber cross section as function of diameter ......... 85
21. Fibrin fiber molecule as function of diameter ............................................... 87
22. Schematic image of internal fibrin fiber structure ................................................................. 90
23. Setup of stretchable substrate ................................................................................................. 100
24. pH tolerance test ..................................................................................................................... 105
25. Salt solution tolerance test ..................................................................................................... 106
26. Human mammary epithelial cells grown on fugitive glue substrate ..................................... 107
27. Fibrin fibers on stretched substrate ....................................................................................... 108
28. Stretchable substrate ............................................................................................................... 124
29. Time-dependent activation of plasminogen .......................................................................... 125
30. Receding clot experiments ..................................................................................................... 126
31. Lysis of stretched single fibrin fibers .................................................................................... 127
32. Confocal images of fibrin fibers after one hour of lysis ......................................................... 128
33. Program fitting of lysed fiber ................................................................................................ 129
34. Relationship of fiber elongation and fiber stretching ............................................................ 130
35. Schematic plot of internal fibrin fiber .................................................................................... 142
36. Fibrin fiber manipulation ........................................................................................................ 156
37. Extensibility data for healthy young people ......................................................................... 158
38. Extensibility data for old CVD patients ............................................................................... 158
39. Diameter distribution of three groups .................................................................................... 166
40. The plot of diameter vs. strain (extensibility) ....................................................................... 167
41. Incremental stress–strain curves and relaxation ................................................................. 170
42. The relaxed modulus as a function of fibrinogen glycation and diameter ......................... 172
43. Fiber diameter, as determined by SEM ................................................................................ 174
44. Fiber diameter distributions for each group .......................................................................... 174
45. Mechanical properties as a function of glycation ........................................... 177

46. Mechanical properties as a function of fibrinogen concentration ..................... 178

47. Total Modulus as a function of fiber diameter ................................................. 179

48. Light intensity of electrospun fibrinogen as function of diameter (other two) ... 180

49. Light intensity of wet fibrin as function of diameter (other two) ....................... 181

50. Light intensity of dry fibrin fiber cross section as function of diameter ............ 182
LIST OF TABLES

I. Mechanical properties of single fibrin fibers from different groups .................... 28
II. Significant difference among different groups (P-value from T-TEST) ............... 28
III. Significant difference among healthy people and CVD Patient Groups .......... 141
IV. Demographic data for each of the groups ....................................................... 154
V. Average extensibility data for each of the groups .......................................... 159
VI. Statistical analysis (t-test) for each of the groups ......................................... 159
VII. The mean blood coagulation values .............................................................. 159
VIII. Fibrinogen concentration for each study group ............................................ 167
IX. Statistical analysis among differing groups for CVD Study ............................ 167
X. The medication taken by five old cardiovascular patients ................................ 168
XI. Statistical analysis of fibrin mechanical properties with fibrinogen glycation... 175
XII. Statistical analysis of fibrin fiber diameter and modulus .............................. 175
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$A$</td>
<td>Area</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<td>ANOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>aPTT</td>
<td>Activated Partial Thromboplastin Time</td>
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<tr>
<td>ASA</td>
<td>Acetylsalicylic Acid</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BPE</td>
<td>Bovine Pituitary Extract</td>
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<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
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<td>CRP</td>
<td>C-reactive Protein</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>$D$</td>
<td>Diameter</td>
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<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
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<tr>
<td>EDAD</td>
<td>Energy Dispersive x-ray Diffraction</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FpA</td>
<td>Fibrinopeptide A</td>
</tr>
<tr>
<td>FpB</td>
<td>Fibrinopeptide B</td>
</tr>
<tr>
<td>FV</td>
<td>Factor V</td>
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<tr>
<td>FX</td>
<td>Factor X</td>
</tr>
<tr>
<td>FXIII</td>
<td>Factor XIII</td>
</tr>
<tr>
<td>$G'$</td>
<td>Storage Modulus</td>
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<td>$G''$</td>
<td>Loss Modulus</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>GHR</td>
<td>Glycine-Histidine-Arginine</td>
</tr>
<tr>
<td>GPR</td>
<td>Glycine-Proline-Arginine</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HFP</td>
<td>Hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human Mammary Epithelial Cell</td>
</tr>
<tr>
<td>I</td>
<td>Intensity</td>
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<tr>
<td>MEGM</td>
<td>Mammary Epithelial Cell Growth Medium</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MEMS</td>
<td>Micro-electro-mechanical Systems</td>
</tr>
<tr>
<td>NOA-81</td>
<td>Norland Optical Adhesive-81</td>
</tr>
<tr>
<td>OH</td>
<td>Old Healthy</td>
</tr>
<tr>
<td>OR</td>
<td>Old at risk for cardiovascular disease</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen Activator Inhibitor-2</td>
</tr>
<tr>
<td>PAOD</td>
<td>Peripheral Arterial Occlusive Disease</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene-terephthalate</td>
</tr>
<tr>
<td>PI</td>
<td>Polyimide</td>
</tr>
<tr>
<td>R</td>
<td>Radius</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small Angle x-ray Scattering</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue-type Plasminogen Activator</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>u-PA</td>
<td>Urokinase-type Plasminogen Activator</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>$Y$</td>
<td>Young’s Modulus</td>
</tr>
<tr>
<td>$Y_0$</td>
<td>Relaxed or Elastic Modulus</td>
</tr>
<tr>
<td>$Y_{\infty}$</td>
<td>Total Modulus</td>
</tr>
<tr>
<td>YH</td>
<td>Young Healthy</td>
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<tr>
<td>$\varepsilon_{\text{elastic}}$</td>
<td>Elastic Limit</td>
</tr>
<tr>
<td>$\varepsilon_{\text{eng}}$</td>
<td>Engineering Strain</td>
</tr>
<tr>
<td>$\varepsilon_{\text{max}}$</td>
<td>Extensibility or Maximum Extension</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Bond Density</td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>Fast Relaxation Time</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>Slow Relaxation Time</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Stress</td>
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Fibrinogen, one of the most abundant proteins in blood plasma, plays a central role in hemostasis and thrombotic disease. In the final step of the coagulation cascade, thrombin proteolytically converts fibrinogen to fibrin, which then forms a mesh of fibrin fibers. This mesh is the major structural component of a blood clot. Over the last few years the mechanical properties of fibrin fibers, such as their modulus, elasticity and extensibility, have been determined using samples formed from purified fibrinogen. In my work, presented in this dissertation, I initiated study on the more complex and more physiologically relevant fibrin fibers formed from plasma samples, in an effort to find relationships between single fibrin fiber mechanical properties and disease states.

Cardiovascular disease (CVD) and diabetes are among the leading causes of death and disability globally. We determined the mechanical properties of single fibrin fibers from individuals who suffered from these diseases by using a combined atomic force microscope/fluorescence microscope. We found that fibrin fibers from healthy individuals are less stretchable and elastic than those from CVD patients (who also took aspirin). This indicates that there is a structural change in fibrin fiber for CVD patients (who also taking aspirin). In contrast, diabetes did not affect any fibrin fiber mechanical
properties. Moreover, we found that the modulus, Y, decreases exponentially with fiber
diameter, D, typically as Y \sim D^{-1.5}, except for CVD samples for which Y \sim D^{-1.0}. To
explain this phenomena, we propose a novel model for the internal structure of fibrin
fibers, in which they have a densely packed core and less dense periphery.

Another important system in blood, fibrinolysis (the dissolution of blood clots), is
influenced by many factors. Since fibrin fibers (clots) experience strain (shear force) by
blood flow naturally, we also investigated the effect of external tension on fibrinolysis.
We created a novel biocompatible stretchable substrate made from fugitive glue to stretch
single fibrin fiber. We found that fiber stretching slows down lysis of single fibrin fibers
and this effect is more pronounced than the slowing influence of FXIII mediated fibrin
crosslinking on lysis.
CHAPTER I

INTRODUCTION
There is a great deal of research being performed on cardiovascular disease (CVD) and diabetes, as they are the leading cause of death globally. Since the properties of fibrin clots play an important role in these diseases, most of my projects are focused on fibrin fibers. The first half of my dissertation describes the mechanical properties of fibrin fiber formed from the plasma of individuals with CVD and diabetes. In the second part I present the work on the treatment of these diseases, fibrin clot dissolution. Together, these studies provide a better understanding of fibrin-related disease systems and provides insights into possible future diagnostics.

FIBRIN FIBERS

Fibrin is a key and abundant protein in blood, and it is very crucial in blood coagulation and fibrinolysis. It plays an important role in stemming blood flow, wound healing, hemostasis, inflammation and thrombosis [1]. Fibrinogen, a precursor of fibrin, is a 340-kDa glycoprotein. It is 45 nm long, 4.5 nm in diameter with a trinodular shape, consisting of two distal D domains and one central E domain that are connected by coiled coils. There are two sets of three polypeptide chains (Aα, Bβ and γ), which are joined together within its N-terminal E domain by disulfide bonds. The distal D domain consists of βC and γC domains which contain polymerization sites, termed a- and b-pockets or a- and b-holes [2-4]. αC regions are located at the C-terminal ends of Aα-chains, and consist of compact globular αC domain and a flexible αC connector [5] (Figure 1).
**Figure 1.** Schematic plot of a fibrinogen molecule. The central E domain with fibrinopeptides (FpA and FpB) is connected to two distal D domain consisting of βC and γC domains via the coiled coils. The coiled coils are composed of three chains, Aα (in red), Bβ (in green) and γ (in blue) chain. Figure adapted from [6].

Fibrin formation is initiated by a serine protease called thrombin. Thrombin cleaves two fibrinopeptides A (FpA) from Aα-chains in E region, exposing a binding site (GPR) (A-knob) which interacts with a specific binding pocket (a-pocket) in the D region. This involves one fibrinogen molecule interacting with two others, then this molecule set binding to nearby sets, with the resulting polymer appearing as a half-staggered protofibril [7-9]. Fibrinopeptide B (FpB) is cut at a relatively slower rate compared with the cleavage of fibrinopeptide A by thrombin. The cleavage of fibrinopeptide B from Bβ-chains exposes another binding site (GHR) (B-knob) in the E region for a specific binding pocket (b-pocket) in the D region [8]. This process is also associated with the release of the αC region which is available for intermolecular interaction [10, 11]. These protofibrils then aggregate laterally to create multi-stranded fibers. (Figure 2)
Figure 2. Schematic diagram of fibrin polymerization. Thrombin cleaves fibrinopeptide A first, resulting in fibrin monomers, oligomers and protofibrils. Secondly, fibrinopeptide B is cleaved preceding the release of αC regions from central E domain to let protofibrils aggregate laterally. Figure adapted from [9].

Thrombin also cleaves Factor XIII (FXIII) which plays an important role in fibrin cross-linking. FXIII is a tetramer composed of two A- and two B-subunits. The active site of the enzyme is located in subunit A, while the B-subunit stabilizes and transports the hydrophobic A-subunit [12]. Thrombin cuts the activation peptide in the FXIII A-subunit, followed by the calcium induced dissociation of the A- and B-subunit. This allows the
exposure of the active enzyme FXIIIa [13]. FXIIIa catalyzes the formation of the γ-glutamyl-ε-lysine bond between glutamine and lysine residues within γ-γ and α-α chains [13, 14]. These cross-linking covalent bonds stabilize the protofibrils and lateral aggregation.

FIBRIN FIBER FROM CARDIOVASCULAR AND DIABETES DISEASE PATIENTS

Besides normal clot formation, fibrin fibers are also crucial in blood related diseases, for instance: cardiovascular disease and diabetes. Cardiovascular disease (CVD) is a leading cause of death globally. It encompasses a class of diseases that involves changes of function in the heart or blood vessels. It includes many different types, such as: ischemic heart disease, rheumatic heart disease, peripheral artery disease, stroke, hypertensive heart disease and others. Some of them involve atherosclerosis, or hardening of the arteries. This may be caused by high blood pressure, smoking, diabetes, lack of exercise, obesity or high blood cholesterol. These heart diseases are typically conditions that develop when plaque builds up in the walls of arteries. This buildup narrows the arteries, making it harder for blood to flow through [15]. If a large blood clot forms, it can halt blood flow, and can cause a heart attack or stroke.

Abnormally high fibrinogen blood concentration is usually associated with a risk of thrombosis [16-18] (Thrombosis is the formation of a blood clot inside a blood vessel, obstructing the flow of blood). However, it remains unclear whether the higher fibrinogen level itself is the cause or the effect of CVD. What is clear, is that CVD profoundly correlates to changes in fibrin structure. Fibrin clots formed from CVD patients have decreased permeability, faster fibrin polymerization, higher fiber density and they have
smaller pores and thinner fibers [19-21]. These changes in fibrin fiber size and structure make them less susceptible to lysis and may also result in changes of the internal structure and mechanical properties of fibrin fibers in a clot. In Chapter II, we use a novel technique to investigate this change in mechanical properties of fibrin fiber from the plasma of individuals with CVD.

Diabetes is a group of metabolic diseases in which high blood sugar levels exist over a prolonged period. It is a risk factor for cardiovascular disease (CVD), increasing the risk by 2-4 times [22]. Among the Diabetes class diseases, the major cause of mortality and morbidity is due to CVD. Similar to CVD patients, increased fibrinogen level was also found in Diabetes patients in some studies [23]. Furthermore, blood clots from Diabetes patients are denser, with thinner fibers and are less porous than those from healthy individuals [24-26]. One possible explanation for these structural changes is glycation of fibrinogen. Most glycation happens on the lysine residues of the fibrinogen molecule [22, 27], since the lysine residue is also involved in fibrin cross-linking by FXIII, and it is the binding site for plasminogen, tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1). Therefore, the binding of glucose at this site may influence the fibrin fiber structure, its functionality, and its mechanical properties. We will talk about diabetes induced changes in fibrin fiber mechanical properties in Chapter III.

From the studies of CVD disease and Diabetes, it is apparent that there are structural changes of fibrin clots and single fibers. This may raise questions regarding what the internal structure of fibrin is and if it also changes in the disease. For decades, people have believed that a single fibrin fiber has homogeneous structure [8, 28].
However, more recent studies demonstrate that a single fibrin fiber doesn’t have a uniform cross-sectional density [6, 29], instead it has a fractal lateral structure. Protein density is a good indicator of the fiber structure. It can be determined by neutron scattering and light scattering. The result from these techniques shows that fiber density is only 30% (70% solvent) [30, 31]. Moreover, with the help of high-resolution Atomic Force Microscope (AFM), it can be observed that molecular packing inside the single fiber is more dense and tighter than that on the surface [6, 32], and the fibrin cross section has a fractal dimension of 1.3 [33]. (We know that the cross section of a uniform cylindrical structure should have a dimension of 2 ($N \propto R^2$, where $N$ is number of molecules)). These findings are exciting initial research findings that are starting to reveal the internal structure of a single fibrin fiber and some diseases-induced structural changes. In Chapter IV, we use microscopy-based methods to investigate and give a better understanding of these structural concerns.

MEASUREMENT OF MECHANICAL PROPERTIES OF FIBRIN FIBER

Fibrin fibers, which are essential in regulating thrombosis and severe bleeding, have viscoelastic properties [1, 34]. It was observed that the mechanical properties of fibrin fibers are altered in diseased individuals; for instance, fibrin clots from CVD patients have a higher modulus (stiffer) [35, 36]. Therefore, it is important to investigate and understand the mechanical properties of fibrin fibers and clots from healthy and unhealthy individuals. Some experiments have already been performed on whole blood clots. Rheology techniques have been used to test the storage modulus ($G'$) and loss modulus ($G''$) [37, 38]. However, very few experiments have been applied to investigate
the mechanical properties at the single fibrin level, due to its small size and densely packed branched meshes. Single fiber properties are important, since they form the basic structure of blood clots and these properties in combination with clot architecture determine the functional behavior of clots. Also, there are some limitation of the current techniques for measuring single fibers, like optical tweezers and three point bending that can only test forces at the pN level. To fill this gap, our group invented a combined atomic force microscope (AFM)/fluorescence microscope technique to investigate mechanical properties of single fibrin fibers. In this technique, AFM force data are collected from above, while a fluorescence microscope provides images beneath (See Figure 4). This is very beneficial in single fiber measurement efforts, as the AFM offers a large force range from $10^{-2}$ nN to $10^4$ nN, much larger than optical tweezers. In addition, this technique makes it possible to test the properties of different kinds of bio- or non-bio-nanofibers, most importantly fibrin fiber for my experiment (from purified fibrinogen and from plasma).
Figure 3. Schematic plot of laser deflection of an atomic force microscope (AFM). Lateral force measurement is shown on the left, when a tip is pulling a fiber causing a torque laterally, the laser is then deflected in the x-direction on the photodiode. Normal force measurement is shown on the right, while the tip is pushing the surface causing a laser deflection in the y-direction on the photodiode.

Atomic force microscope (AFM) is a very high-resolution type of scanning probe microscope, with resolution on the order of fractions nano-scale, more than 1000 times better than the optical diffraction limit. It is widely used in materials science and biology (e.g. to determine the stiffness of biological samples [39], get the roughness of surface of materials [40]), since it gives high resolution scanning images and can provide force data (e.g. measure stiffness) for (non-) bio-materials. The AFM cantilever tip is a very important part in scanning and force measurement, and exists in a variety of shapes, sizes, stiffnesses and materials according to various inquires and necessary applications. The smaller the tip diameter the higher resolution images can be obtained. Recently, the αC region of the fibrinogen molecule was revealed by a tip with a diameter lower than 1 nm [32]. This opens a new method of investigation, revealing the sub-molecular structure
of fibrinogen. The AFM has two principal working modes, one is imaging mode, and the other is the force measurement mode. Its basic working principle is that when the tip touches the sample surface, the cantilever bends causing a deflection of the laser which is reflected off the cantilever. The deflection is detected by a four quadrant photodiode, then processed by a feedback system to adjust the sample-to-tip position. This distance change will be recorded and used to show the topology of the sample or to calculate the force exerted on the sample. For normal force measurements (normal to the substrate surface), the deflection of laser is in the y-dimension on the photodiode; while in our experiment, we use the lateral force measurement (parallel to the substrate surface), the deflection of laser shows the changes in x-direction on photodiode (see Figure 3). In our experiment, fibrin fibers (fibrin fibers from purified fibrinogen and from plasma of healthy people and patients) are formed on top of a striated substrate located in between the cantilever tip of AFM and fluorescence microscope (Figure 4). The tip pulls the fiber laterally at a rate around 300-400 nm/s while the force data is recorded, the fluorescence microscope provides images to visualize the pulling process. From the force data, we can calculate the mechanical properties (extensibility, elasticity and modulus) of fibrin fibers.
Figure 4. Schematic representation of the experimental setup of combined AFM/fluorescence microscope. Fibrin fiber are formed on the striated substrate, the AFM cantilever tip pulls the fiber from above while the fluorescence microscope provides images from underneath. (A) is a side view of setup, (B) is the top view.

FIBRIN DISSOLUTION

The majority of my experiments are focused on fibrin fibers from patients with cardiovascular disease (CVD) and Diabetes. Since there is evidence that fibrin mechanical properties are altered in diseased individuals, it is crucial to concentrate on ways to improve treatment options for these individuals. There is evidence that whole clots from these people have tight and rigid network structures, as well as decreased permeability [19-21]. Therefore, we are interested in the fibrinolytic process in which a fibrin clot becomes dissolved.

Fibrinolysis plays an important role in hemostasis, it prevents and diminishes blockage of blood vessels and maintains blood flow. It is as important as a blood coagulation. These two process need to exist in balance, or thrombosis or severe bleeding could result [41, 42]. A number of enzymes and their specific inhibitors exist to regulate
the fibrinolytic system (Figure 5). The central protein that cleaves fibrin fibers into degradation products is plasmin. It is formed from its precursor plasminogen under the activation of tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA) [43]. Plasminogen is a single chain glycoprotein consisting of five sequential homologous kringle domains and a serine protease module [44]. It is converted into plasmin by t-PA that is released from endothelial cells [45]. This t-PA enzyme consists of a finger domain, a growth factor domain, two kringles and a serine protease [46]. There are some lysine binding sites in several plasminogen kringles and the second t-PA kringle which play a key role in the interaction and recognition of fibrin, α2-antiplasmin and cell surfaces [47-50]. These interactions with fibrin provide sufficient activated plasmin levels to cleave fibrin fibers into degradation products. The initial cleavage by plasmin happens at the αC domains followed by several cuts in the coiled-coil region between the D and E domains [51], resulting in degradation products of different sizes of D-D:E complex, cross-linked D-D dimer, E fragment and αC domain remnants [52].

Clot dissolution needs to be tightly regulated to prevent severe bleeding by the inhibitors α2-antiplasmin and plasminogen activator inhibitor-1, and -2 (PAI-1, PAI-2). α2-antiplasmin is the major inhibitor of plasmin, it contains a secondary binding site which interacts with the lysine binding sites on the 1-3 kringles of plasmin and plasminogen [53, 54]. PAI-1 and PAI-2 are inhibitors for t-PA and u-PA while PAI-1 is considered to be the major inhibitor. It inactivates t-PA or u-PA by forming a stable complex with them and also inhibits plasmin generation [9, 43, 55].
Figure 5. Schematic overview of fibrinolysis. A fibrin clot is dissolved by plasmin formed from its precursor plasminogen under the activation of tissue (or urokinase)-type plasminogen activator (t-PA or u-Pa). The enzymes in red indicate the inhibitors of this fibrinolytic pathway (plasminogen activator inhibitor-1 and -2 (PAI-1, -2), and $\alpha_2$-antiplasmin).

There are many factors in blood that can influence clot dissolution. For instance, FXIII slows down fibrinolytic rate [56], high prothrombin levels result in clots that resist fibrinolysis [57], clots containing fibrin, DNA and histones delays lysis [58]. Recently, studies have shown that fibrinolysis also varies on specific fibrin structure and can be altered under external tension; fibrin clots with a dense network of thinner fibrin fibers lyse slower than coarse clots with thicker fibers [59]. Stretched fibrin clots lyse slower than unstretched clots [60, 61]. These are very interesting findings in fibrinolysis, however, they are all done at the fibrin clot level; very few studies have been performed at the single fiber level. In Chapter VI our group utilized a newly created stretchable substrate (described in Chapter V) to investigate lysis of a single fibrin fiber under tension. This provides a better understanding at the microscopic level of fibrinolysis.


CHAPTER II

THE EFFECT OF CARDIOVASCULAR DISEASE AND AGE ON FIBRIN FIBER MECHANICAL PROPERTIES

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The following manuscript has been prepared for journal submission. Wei Li and Stephen Baker collected data and prepared the manuscript. Wei Li analyzed the data. Peter Brubaker provided patient plasma samples. Martin Guthold acted in an advisory capacity and edited the manuscript.
ABSTRACT

Many factors relating to lifestyle or diet are known to be associated with cardiovascular disease. However, the underlying molecular connections between CVD, coagulation proteins and fibrin fibers remain poorly understood. Using a combined atomic force microscope (AFM)/fluorescent microscope technique, we studied the mechanical properties of fibrin fibers formed from the blood plasma of individuals from three different categories: Middle-aged healthy males, older healthy males, and older males with CVD. We found that fibrin fibers from older males with CVD are 30% more stretchable and 50% more elastic than those from the other two groups. Age did not affect single fibrin fiber mechanical properties. Fibers from all three groups had the same average Young’s modulus (stiffness), \( Y \). \( Y \) strongly varied with radius, \( R \); for healthy individuals the modulus varied as \( Y \sim R^{-1.5} \), whereas for individuals with CVD it varied as \( Y \sim R^{-1.0} \). This radius dependence of the modulus is consistent with a new fiber model in which fiber density strongly varies with radius. Fibers have a denser core and a less dense periphery; this radius dependence is less pronounced in fibers from individuals with CVD.

INTRODUCTION

Fibrin, the protein that provides the structural network for a blood clot, is formed from the precursor fibrinogen [1]. This 340 kDa protein consists of two distal D-domains and one central E-domain connected by two identical sets of triple alpha-helical coiled-coils. The N-terminal of the E-domain consists of two pairs of fibrinopeptides denoted as FpA and FpB. The activated serine protease Thombin cleaves the fibrinopeptides,
exposing the A and B-knobs, and thus converting soluble Fibrinogen to Fibrin. The knobs are now able to bind to the respective a and b pockets in the D-domains, which causes Fibrin to polymerize and form protofibrils. These protofibrils are then able to laterally aggregate to form fibrin fibers and eventually an entire clot.

Blood clots and, and therefore also the fibrin fiber network making up the blood clot, perform the mechanical task of preventing the flow of blood. After acute trauma, platelets and fibrin aggregate around the trauma site. During normal healthy coagulation, platelets aggregate first, and then fibrin will form a network, which strengthens the clot, and the flow of blood will be stopped. What is unclear is how various parameters are affected by the various parameters needed to get to the point of a normal blood clot. What is clear is that the strength and mechanical response of this clot is important for coagulation. Without a subsequent mechanical response as a result of trauma equal to the trauma presented, blood will continue to flow and more and more life threatening problems may result. Understanding how this mechanical task works and what variations may be present allows for a better understanding of the clot as a whole and what can be done to prevent further complications.

Previous studies have determined the viscoelastic properties of fibrin fibers using Atomic Force Microscopy and optical tweezers. Much of the previous work that was done was with purified fibrinogen to study these mechanical properties for uncrosslinked, crosslinked, and partial crosslinked fibrin samples [2, 3]. Collet et al. used plasma samples to determine clot elasticity both in the presence and absence of clot stabilizing Factor XIII (FXIII) [2-6]. Liu et al showed that crosslinked fibers were stiffer, less elastic, and less extensible than uncrosslinked fibers while Carlisle et al showed that the
mechanical properties of partially crosslinked fibers fell somewhere in between [2, 3, 5]. Collet et al showed similar results for uncrosslinked and crosslinked clots made from plasma samples [4].

Cardiovascular disease (CVD) was shown to be correlated with various clot related factors including: increased fibrinogen concentration, denser clot structure, smaller fibrin fiber size, and increased resistance to fibrinolysis [7-10]. Studies have shown that higher than normal fibrinogen concentrations in blood are associated with increased risk for CVD though it is unclear whether this is a cause or effect [11]. Recent studies have focused on the change in clot structure in patients that have CVD or are at risk for CVD. These findings indicate that blood from patients having CVD or at risk for CVD form clots with smaller fibers that are more densely packed than normal clots [8]. As a result of this modified clot structure, clots from CVD patients are also more difficult to lyse resulting in a higher risk for thrombosis. Though the effect of pharmaceuticals such as Acetylsalicylic Acid (ASA) have been shown to modify the clot structure allowing for less dense clots that are easier to lyse [12-15], it is unclear how the rigidity and overall mechanical properties of the clot are effected selectively by CVD and/or ASA.

Here we determined single fibrin fiber mechanical properties as a function of age and CVD. All individuals with CVD also took ASA. We found that there was no significant difference in single fibrin fiber mechanical properties (extensibility, elasticity and modulus) between the middle aged individuals and the older, healthy individuals. Thus, fibrin fiber properties do not seem to change as a function of age. In contrast, there was a marked difference between the fibers from individuals with CVD as compared to
the healthy middle-aged and healthy old group. Fibers from individuals with CVD were significantly more extensible and more elastic; though fibers from all three groups had the same modulus. We also found that the modulus is strongly dependent on fiber diameter; this is unexpected because the modulus is a material constant and should not depend on fiber dimensions. We found that the modulus is proportional to $R^{-1.5}$ for healthy individuals, regardless of age, and it is proportional to $R^{-1.0}$ for older individuals with CVD. One would expect an $R^0$ dependence for fibers with a solid cross-section or $R^{-1.0}$ for fibers with a spoke-like cross-section similar to a bicycle wheel. This is an important finding as it may help to distinguish the effect the CVD has on the physical and mechanical properties of fibrin fibers in the presence of ASA.

MATERIALS AND METHODS

Plasma Collection

5 ml of whole blood samples was taken from male patients in three different categories: 5 young healthy (YH, <50 years old), 5 old healthy (OH, >50 years old), and 5 old at risk for cardiovascular disease (OR, >50 years) and stored in citrated tubes. All patients were nonsmokers. OR patients were controlled with various medications including various does of ASA. Other medication history can be found in the supplemental information. Whole blood samples were then centrifuged at 3700 rpm and large particles (red blood cells) were discarded.
Substrate Preparation

Preparation of the striated substrate is based on soft lithography and micromoulding in capillaries [16]. We have described this technique previously [17, 18]. Briefly, a PDMS stamp is pressed into a 10 µl drop of Norland Optical Adhesive-81 (NOA-81, Norland Products, Cranbury, NJ) on top of a 60 mm x 24 mm, #1.5 microscope cover slide (Thomas Scientific, Swedesboro, NJ). The NOA-81 was cured for 70 s with UV light (365 nm setting, UVP 3UV transilluminator, Upland, CA) and the stamp was removed. The substrate pattern had 6.5 µm wide ridges separated by 13.5 µm wide and 6 µm deep channels.

Formation of Fibrin Fibers from Plasma

Plasma samples were then aliquoted into 28µl samples and stored at -80°C. Prior to clot formation in vitro, individual 28 µl samples were thawed at room temperature for 5 minutes. 8 µl of 100 mM CaCl$_2$ was added to the plasma sample to reach a final CaCl$_2$ concentration of 20 mM. 18 µl of this mixture was added to the striated substrate followed by 2µl of human alpha thrombin (final concentration 0.1 NIH units/ml, Enzyme Research Laboratories, South Bend, IN). Reactions were allowed to run for ~ 60 minutes. Then a pipet tip was used to carefully remove the top layer of the clot and the slide was rinsed with Fibrin Buffer-1 (pH 7.4, 10mM Hepes, 140mM NaCl). 24 nm fluorescent beads (Invitrogen, Fluospheres, Carlsbad, CA) diluted 1/100 with Fibrin Buffer-1 were added to the slide and the whole sample was allowed to incubate for 10 minutes. Samples
were then rinsed and stored in Fibrin Buffer-2 (pH 7.4, 10 mM Hepes, 140 mM NaCl, 5 mM CaCl$_2$).

*Fibrin Fiber Manipulation*

Fibrin fiber mechanical properties were found using a combined atomic force microscopy (AFM)/ fluorescence microscopy technique as described previously. Below we will briefly describe this technique. Fiber samples are prepared as described above and stored in a buffer solution. Samples were placed on an inverted optical microscope (Zeiss Axiovert 200, Göttingen, Germany) and the AFM (Topometrix Explorer, Veeco Instruments, Woodbury, NY) was positioned on top of the microscope with the sample between the two. This set-up allows for manipulations to be viewed with the optical microscope while fibers are manipulated with the AFM as can be seen in Figure 6A.

Single fibers suspended over the ridges of the striated substrate are laterally pulled and the AFM cantilever is torqued. NanoManipulator software (3rd Tech, Chapel Hill, NC) provides precise control of the AFM tip and collects force and position data during fiber manipulations. Fiber diameters are measured on the ridges of the striated substrate using tapping mode. From these data the actual force on the fiber is calculated; stress and strain are determined as $\sigma = F/A$ and $\varepsilon = \Delta L/L_{\text{init}}$, where $A$ is the fiber cross-sectional area, $L_{\text{init}}$ is the initial length of the fiber and $\Delta L$ is the change in fiber length.
**Statistical Analysis**

Statistical analysis was done using SOFA statistics software (Paton-Simpson & Associates Ltd). Significant different of two groups is determined by a two-tailed t-test. Significant different of two slopes is calculated by analysis of covariance (ANCOVA).

![Diagram](image)

**Figure 6.** Schematic of Single Fiber AFM/Fluorescence Microscopy Technique. (A) Fibrin fiber sample cover slides with wells and ridges are sandwiched between an AFM (top) and an inverted microscope (bottom). This allows for single fibers spanning the well to be mechanically manipulated by the AFM cantilever tip from above. (B) Top down view of a single fiber manipulation. (C) Snap shots of a single fiber manipulation during an extensibility test. Scale bar is 10 µm.

**RESULTS**

Fibrin fibers are formed on the ridges as shown in Figure 6. They are very sticky and remain attached to the ridges very well without any further treatment. Occasionally slippage occurs between the fibers and the substrate; these data were excluded from analysis. We used only fibers that were vertically straight across the ridges (for easy to analyze geometry). We tested three main mechanical properties of single fibrin fibers.
(extensibility, elasticity, and modulus) from blood plasma of healthy individuals and individuals with cardiovascular disease. For each experiment, we took at least 20 measurements per sample.

Fiber extensibility

Initially we determined the extensibility, $\varepsilon_{\text{max}}$, of single fibrin fiber. It is defined as the extension at which the fiber breaks during a pull. As shown in Figure 7C, the lateral force increases as the fiber is stretched, then suddenly drops to zero as the fiber breaks. Extensibility is defined as the maximum strain a fiber can sustain before it breaks

$$\varepsilon_{\text{max}} = (\Delta L_{\text{max}} / L_{\text{ind}}) \times 100\%; \text{ where } \Delta L = L_{\text{final}} - L_{\text{ind}}, \ L_{\text{final}} \text{ is the final length of the fiber. In all samples, we see the elastic modulus (slope of stress-strain curve) increases as strain increases (As shown in Figure 7C). This is known as strain hardening.}

Averaging the data from each group, we found the average extensibility for healthy middle-aged people was $118\% \pm 29\%$ (average ± standard deviation), for the healthy old people it was $129\% \pm 24\%$, while for the old CVD people it was $177\% \pm 12\%$ (Table I). These data are similar to the result from the previous purified fibrinogen [2], in that fibrin fibers are very extensible. For quantitative analysis (P-value from T-TEST in Table II), it was found out that old CVD group is significantly different from healthy middle age ($P << 0.01$) and healthy old group ($P << 0.01$), but there is no significant difference in extensibility within the healthy groups ($P = 0.51$) ($P$ value $< 0.05$ for two groups to be considered significantly different). In summary, fibrin fibers from old individuals with CVD (who all took ASA) are more stretchable (~1.5 times) than
those from healthy people, but age doesn’t have a significant effect on the mechanical properties of single fibrin fiber.

**Figure 7.** Extensibility of Fibrin Fibers with respect to Age and CVD. (A) Extensibility of each individual sample. (B) Average extensibility of each sample. (C) Plot of the strain vs. lateral force. Lateral force increases until at a strain of 227.5% where the fiber breaks and the lateral force drops to zero. Middle aged healthy people in pink (213 data points). Healthy older people in orange (266 data points). Older CVD people in blue (279 data points). Error bar is the standard error.
Table I: Mechanical properties of single fibrin fibers from different groups.

<table>
<thead>
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<th>Healthy Middle Age</th>
<th>Healthy Old</th>
<th>Old CVD</th>
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<tbody>
<tr>
<td>Extensibility (%)</td>
<td>118 ± 29</td>
<td>129 ± 24</td>
<td>177 ± 12</td>
</tr>
<tr>
<td>Elasticity (%)</td>
<td>43 ± 6</td>
<td>40 ± 5</td>
<td>57 ± 4</td>
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<tr>
<td>Total Modulus (Pa)</td>
<td>1.4E7 ± 5.8E6</td>
<td>1.6E7 ± 5.6E6</td>
<td>1.7E7 ± 3.8E6</td>
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<tr>
<td>Normalized Total Modulus (Pa)</td>
<td>8.6E6 ± 2.3E6</td>
<td>8.3E6 ± 2.4E6</td>
<td>1.1E7 ± 4.8E6</td>
</tr>
</tbody>
</table>

Table II: Significant difference among different groups (P-value from T-TEST).

<table>
<thead>
<tr>
<th></th>
<th>Healthy Middle Age</th>
<th>Healthy Middle Age</th>
<th>Healthy Old</th>
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</thead>
<tbody>
<tr>
<td><strong>T-TEST(P value)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extensibility</td>
<td>0.51</td>
<td>1.19E-18</td>
<td>1.03E-16</td>
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<tr>
<td>Elasticity</td>
<td>0.15</td>
<td>2.23E-24</td>
<td>1.03E-32</td>
</tr>
<tr>
<td>Total Modulus</td>
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<td>0.68</td>
<td>0.28</td>
</tr>
<tr>
<td>Normalized Total Modulus</td>
<td>0.84</td>
<td>0.09</td>
<td>0.14</td>
</tr>
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**Fiber viscoelastic properties**

The second mechanical property, the elastic limit, $\varepsilon_{\text{elastic}}$, is the maximum strain a fiber can sustain and still return to its original length without any permanent elongation. To test the elastic limit, we first pulled the fiber to a small strain, then released the fiber, after it reached zero strain we pulled the fiber to a larger strain, then released again to zero strain. This process was repeated until it the fiber was permanently deformed, that is, it reached zero stress (force), before the fiber returned to its original length. This whole process can be seen in Figure 8C, the first three pulls haven’t reach the elastic limit, since strain goes to zero as the lateral force reach zero. However, the fourth pull was beyond the elastic limit, because the strain is not zero as the lateral force reaches zero.
Since with our method, we cannot tell the exact position at which the fiber gets deformed, we define a lower limit (fiber hasn’t deformed) and upper limit (deformed). The elastic limit is then within these two limits. In Figure 8C, the elastic limit is within the third and fourth pull, which is between 30% and 47%.

**Figure 8.** Elastic limit with respect to Age and CVD. (A) The elastic limit of each individual in each sample. (B) Average elastic limit of each sample. (C) A representative force-strain curve (the stress would be obtained by dividing the force by the fiber cross-sectional area). We tested a total of 144 fibers for the healthy middle aged people (pink), 143 for healthy old people (orange), 139 for old CVD people (blue). Error bar is the standard error.
Since the real elastic limit falls into a certain range of the lower and upper limit, we found the elastic limit of three different groups are as following: healthy middle age 35% - 47%, healthy old 34% - 46%, old CVD 49% - 64%. For statistical analysis, we get the average elastic limit for each group: healthy middle age 43% ± 6%, healthy old group 40% ± 5%, old CVD group 57% ± 4% (average ± standard deviation).

It can be seen that there is a significant difference between diseased and healthy people from Figure 8 and the P value of the T-TEST in Table II. It shows that fibrin fibers from old individuals with CVD (and taking ASA) are more elastic (~1.4 times) (P << 0.01) than those from healthy people, but age still does not play a role in changing the mechanical properties of single fibrin fibers (P = 0.15).

Total and relaxed elastic moduli (fiber stiffness)

The third mechanical property, the elastic modulus, Y, is defined as the ratio of the stress to the strain (slope of a stress-strain curve) at small strains. To determine the modulus, we perform and incremental stress-strain experiment: We stretch the fiber to a certain strain, hold it there without further stretching for some time. During this time the fiber relaxes (strain decreases). The fiber is then stretched again by another increment, again held at the strain for a certain amount of time, during which stress relaxes. This process is repeated several times.

Stress relaxation is indicative of viscous property. For the stress-time curve, we use double exponential to fit the curve, since we found two relaxation time (fast and slow).
Figure 9. Incremental Stress and Strain Curves for Single Fibrin Fibers. (A) Strain versus time. (B) Stress versus Strain. (C) Stress versus time. (D) Individual stress versus time relaxation curve showing a double exponential curve fit (black) to the raw data.
Figure 10. Total Modulus with respect to Age and CVD. (A) Total modulus of each sample. (B) Average total modulus of each sample. (C) Normalized total modulus of a standard fiber of 130 nm. (D) Average normalized total modulus. Middle aged healthy people in pink (110 data points). Healthy old people in orange (104 data points). Old CVD people in blue (116 data points). Error bar is shown as standard error.

We calculated the modulus for each sample, got the average value for healthy middle aged group is 13.5 MPa, healthy old group is 18.6 MPa, old CVD is 16.6 MPa. From the plot (Figure 10A and 10B) and statistical analysis (T-TEST in Table II), we did not see a significant difference among these three groups, no difference between healthy and diseased people (P = 0.68, 0.28), and no difference between the different age groups (P = 0.31).
**Relationship of diameter and modulus**

We did not see any difference in the moduli of the three different groups. However, closer inspection of the data revealed a clear and very strong relationship between the diameter of the fiber and the total modulus (Figure 11). The total modulus decreases as the diameter of the fiber increases. They have a power law relationship, which is seen as a linear relationship in the log-log plot of Figure 11. The slope (exponent of the power law) is -1.48 ± 0.19 (healthy old samples, $R^2 = 0.36$), -1.37 ± 0.15 (healthy middle age samples, $R^2 = 0.45$), -0.91 ± 0.16 (old CVD samples, $R^2 = 0.22$).

**Figure 11.** Total Modulus as a function of Diameter with respect to Age and CVD. (A) Healthy middle age people with 110 data points. (B) Healthy old people with 104 data points. (C) Old CVD people with 116 data points.
Since we observed that modulus strongly depends on fiber diameter, we calculated the normalized modulus of a standard (average) fiber diameter from each of the three different groups to see if there is a difference between them. The average fiber diameter from each of the three different groups is close to 130 nm (Seen in Figure 39). Thus, we calculated the normalized total modulus as follows: Since we found the relationship between modulus $Y$ and diameter $D$ of each group $Y \sim D^{\text{slope}}$, we first multiplied each modulus data point by $D^{\text{slope}}$ to eliminate the diameter dependence. Then we multiplied this value by $130^{\text{slope}}$ to get the normalized total modulus. When examining the data for the normalized modulus, there is still no significant difference between the three groups (as shown in Figure 10C and 10D, Table I and II).

Even though the modulus shows a strong diameter-dependence, the extensibility showed no diameter-dependence (details shows in Figure 40).

DISCUSSION

Cardiovascular disease has been shown to correlate with various clot related factors and structural properties in addition to the rate at which the clot breaks down (fibrinolysis). Although many of the risk factors associated CVD are well known (such as high cholesterol, high blood pressure, and overall clot structure) and have been well studied it is still unclear if the mechanical properties of blood clots and their building blocks, mainly fibrin fibers, are correlated by these diseases. In the present study, we aimed to determine how the mechanical properties of single fibrin fibers made from plasma clots were affected by CVD and age as well as how the radius-dependence of the fiber modulus changes between healthy and CVD patients. We have to keep in mind that
all CVD patients took Acetylsalicylic Acid (ASA), which may have an effect on fibrin fiber mechanical properties. Given our samples, we could not determine whether the changes in mechanical properties were associated with CVD or ASA.

Fibrinogen concentration has been shown to be an important indicator for cardiovascular disease risk [7, 19, 20], though it is not clear whether fibrinogen concentration plays a causal role or if it is a result of CVD. For the present study, it is also important to note that fibrinogen concentration somewhat depends on age; younger patients typically have lower plasma fibrinogen concentrations than older patients [21, 22]. Thus, in our samples, one might expect the plasma fibrinogen concentration to be lower in the young healthy (YH) samples than the old healthy (OH) and old cardiovascular disease (OR). We saw a slight increase in modulus from YH to OH and from OH to OR, though the difference is not statistically significant (See Supplement). Fibrinogen concentration may be responsible for the small (but insignificant difference) in modulus, but it very likely is not the very large, and statistically significant difference in extensibility, elasticity and diameter dependence of the modulus.

Patients with CVD showed a significant difference in some mechanical properties when compared to healthy patients (young and old). OR patients had more extensible single fibers and more elastic fibers than healthy patient, both YH and OH. These findings indicate that patients with CVD (taking ASA) have fibrin fibers that can be stretched further without breaking or permanently deforming. Our previous studies with purified fibrin showed that fibrin fibers have differing mechanical properties depending on whether they are crosslinked or uncrosslinked [2]. Crosslinked fibrin fibers had a maximum extension of 147%, while uncrosslinked fibers had a maximum extension of
Our current findings show an extensibility of 118%, 129%, and 177% for YH, OH, and OR samples respectively. Since these samples use plasma and not purified fibrinogen it is expected that there is FXIII present in each of the samples and, thus, for extensibilities would be expected to be in the range of crosslinked fibers and not uncrosslinked fibers. Liu et al showed that crosslinked fibers had elastic properties up to 50% while uncrosslinked fibers are elastic up to 120% [2,5]. In the current study we measured an elasticity of 40-57% for all groups which again falls within the region for crosslinked fibers and outside the region of uncrosslinked fibers.

Looking at Figure 11, the slope of the modulus vs. diameter plot (i.e., the diameter dependence of the modulus) is different for each sample group, despite the fact that the overall average modulus of each group was the statistically the same). This diameter dependence of the modulus means the internal structure of the individual fibrin fibers is different for the CVD groups as compared to the two healthy groups. The shallower slope seen in the CVD sample set (-0.91) indicates that the fibers are more uniform or not as porous at the periphery as those found in both healthy groups (-1.48 for YH and -1.37 for OH). Early electron microscopy studies showed that fibrin fibers likely have a dense inner core with a less dense outer diameter [23]. We have previously shown similar diameter dependence for purified fibrin fibers using AFM [24] while more recent studies have looked at the internal structure of individual fibrin fibers using light scattering [25]. Taken together our current findings indicate that fiber assembly may be a little different for patients with CVD; though, it should be noted that we are unable to determine whether this effect is due to CVD or due to the presence of ASA in the CVD sample set patients plasma.
The clots of cardiovascular disease patients typically have a denser fibrin network with thinner fibers than normal healthy individuals [4, 26, 27]. A common test to determine risk for CVD is to test a patient’s plasma fibrinogen concentration. As Kotze et al asserts, final clot structure and fibrinogen concentration are not correlated in a simple fashion, and other risk factors such as age, metabolic syndrome, C-reactive protein (CRP), high density lipoprotein (HDL)-cholesterol and homocysteine also have a significant effect on clot structure, independent of fibrin concentration [27]. We did not show an increase in plasma fibrin concentration between either older and younger patients (YH and OH) or healthy patients and patients with CVD (YH and OH versus OR). Our findings did not show a significant change in the mechanical properties of single fibrin fibers with age, but did show a change in the extensibility and elasticity of fibers from patients that were healthy when compared to patients with CVD. These results indicate that although fibrin concentration and age did not play a significant role in determining the single fiber mechanical properties of fibrin fibers made from fibrin clots, patients that are being treated for CVD have fibers that are more elastic and more extensible than healthy patients. Even though the patients from old cardiovascular disease group have different risk for CVD, however, they have the same mechanical properties, we think that because 1) Different types CVD may have same fibrin fiber. 2) This same effect may be due to the effect of Aspirin.

Antovic et al. showed that fibrin clot permeability was changed in a dose dependent manner in the presence of ASA [13]. They showed that low doses of ASA (37.5 mg/day) had a more drastic effect on the permeability of the clot, causing larger pores and larger fibers, than high doses of ASA (either 320 mg/day or 320mg/day
followed by one time 640mg dose) while not effecting the concentration of fibrin found in any of the tested patients [13]. Similarly, other studies have noted that clot structure is changed causing clots made from smaller, more densely packed fibers to have larger, more porous clots that are more susceptible to lysis in the presence of ASA [8,12,14,15]. It should be noted that patients with coronary artery disease or patients with cardiovascular events while on aspirin where still shown to have clots with thinner, more densely packed fibers that were less susceptible to lysis [8]. Further studies are needed, as a result, to better understand the mechanism for this ASA resistance in these diseases.

We note that we are unable to determine the cause and effect of the change in mechanical properties, and CVD. Untreated CVD patients are uncommon in the United States and as a result a high percentage of CVD patients will also be subject to ASA treatment. Healthy, untreated patients typically have more porous clots with larger fibers that are more susceptible to lysis than CVD patients with less porous, smaller fiber clots. Aspirin treatment in CVD patients causes the clot to become more porous, with larger fibers. Individual fibrin fibers also show a change with aspirin treatment in CVD patients. Here we see a change in the internal structure of individual fibrin fibers with CVD and aspirin. As noted previously, fibrin fibers are commonly found with a dense inner core following by a less dense outer core with the density being diameter dependent. A recent study by Svensson et al. found the acetylated lysine residues on fibrinogen using mass spectroscopy [28]. Similarly, Sobel et al. found twelve different lysine residues on the α-chain involved in crosslinking [29]. The glutamine acceptor crosslinking sites for the lysine donor cites have also been identified [30]. At least one, but more likely four, of these acetylated lysine sites on the α-chain are also involved in crosslinking with
glutamine acceptors. Considering that crosslinking effects the structure of not only the clot itself, but also the structure of the single fibers that make up the clot, we propose that there may be a link between our diameter density dependence and the acetylation cites found on the fibrin(ogen) molecules. Further studies with larger patient samples are be needed to confirm this hypothesis as well as a control with acetylated fibrin(ogen) from patients that do not have CVD.

CONCLUSIONS

Cardiovascular disease is associated with many of the factors involved in blood coagulation, including fibrinogen concentration, clot structure, fibrin fiber size, and clot lysis. We have determined that while patient age does not affect the mechanical properties of single fibrin fibers made from blood plasma, patients with CVD (and taking ASA) have more stretchable and more elastic fibrin fibers. This finding, combined with previous studies showing an increases lysis time, could help explain why patients with CVD risk have more difficulty breaking down unwanted thrombi. Interestingly, while we did not find an effect on modulus with either age or CVD risk or treatment, we did see that the internal structure or diameter density dependence changed between healthy patients and those being treated for CVD. We hypothesize that this diameter density change may be related to the acetylation sites found on fibrinogen that are also involved in normal crosslinking with Factor XIII.
ACKNOWLEDGEMENTS

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CHAPTER III

FIBRIN FIBER STIFFNESS IS STRONGLY AFFECTED BY FIBER DIAMETER,
BUT NOT BY FIBRINOGEN GLYCATION

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The following manuscript has been prepared for journal submission. Wei Li and Justin Sigley collected all the data, all analysis, and drafted the manuscript. Christine Helms helped with initial preparation of the manuscript. Marlien Pieters supplied the plasma samples and edited the manuscript. Martin Guthold acted in an advisory and editorial role during data collection and manuscript preparation.
ABSTRACT

We used a combined atomic force microscope (AFM)/fluorescence microscope technique to determine the mechanical properties of individual fibrin fibers formed from blood plasma. Blood samples were taken from uncontrolled diabetic patients, as well as age, gender, and BMI-matched healthy individuals. The patients then underwent treatment to control blood glucose levels before end blood samples were taken. The fibrinogen glycation of the diabetic patients was reduced from 8.8 to 5.0 mol glucose/mol fibrinogen. The healthy individuals had a mean fibrinogen glycation of 4.0 mol glucose/mol fibrinogen. We measured fiber stiffness (stretch modulus), extensibility and relaxation times of individual fibrin fibers. We found fibrinogen glycation had no significant systematic effect on modulus, extensibility, or relaxation times. However, we did find that the fiber modulus, Y, strongly decreases with increasing fiber diameter, D, as \( Y \propto D^{-1.6} \). This unusual diameter dependence of the modulus suggests that fibrin fibers have an inhomogeneous cross-section with a dense core of well-connected protofibrils; connections between protofibril then strongly decrease as the fiber diameter increases. Our data provide an explanation why blood clots comprised of a meshwork of thin fibers are stronger and harder to dissolve, than clots comprised of thick fibers.

INTRODUCTION

The main physiological function of blood clots, which are comprised of platelets and a meshwork of microscopic fibrin fibers, is stemming the flow of blood. Since this is a mechanical task there has been a continuing effort over the past 60 years to study the mechanical properties of clots. The vast majority of these studies were done on whole
clots. For example, rheometry techniques were used to determine the loss and storage modulus, and other mechanical properties of clots [1, 2]; it was found that these properties can be associated with thrombotic disease [3-7]. In a clinical setting, thromboelastography is used by clinicians to uncover clotting abnormalities and to reduce risk in surgeries [8, 9]. In an effort to deepen understanding of clot behavior, new techniques have been developed in recent years to determine the mechanical properties of single fibrin fibers within a fibrin clot [10-12]. Atomic force microscopy (AFM) and optical traps have been used to determine the stiffness of single fibers and other key properties, such as their extraordinary extensibility and elasticity [10-12]. Single fiber experiments bridge the gap in scale between whole clot experiments and molecular experiments, and the data from single fiber experiments are a key building block in constructing realistic models of fibrin clots [13]. Single fiber data are also used to test the predictions of molecular dynamics simulations at the molecular level [14-16].

Most single fiber investigations were done on clots formed from purified fibrinogen, with little data available on the mechanical properties of individual fibrin fibers in plasma clots, which are more physiological and quite different in structure and properties [12]. The use of plasma clots would make it possible to directly measure the properties of pathophysiological clots from patients, who suffer from specific clotting disorders and diseases. The present study is the first single fiber study using patient plasma clots.

Diabetes is a risk factor for cardiovascular disease (CVD), increasing CVD risk by 2-4 times [17] with 68% of morbidity in diabetic patients being due to CVD [18]. According to the Center for Disease Control, diabetes affects 25.8 million people or 8.3%
of the population in the United States [18]. The relationship between CVD and diabetes is not well understood, although alterations to the properties of fibrin clots have been reported for diabetic patients. Several studies examined clots formed from plasma or fibrinogen isolated from diabetic patients, as well as fibrinogen glycated in the lab, in an attempt to understand the connections between CVD and diabetes. However, many of these studies have conflicting findings, likely as a result of differences in study design (plasma vs. purified fibrinogen; use of diabetic vs. normal plasma with added glucose), study population and analytical techniques and methods. An important plausible mechanism for altered fibrin networks in diabetic patients is the non-enzymatic glycation of fibrinogen in the presence of uncontrolled blood glucose levels. A few studies have reported increased resistance to fibrinolysis in samples with increased glycation [17, 19]. Another study, however, found decreased resistance to fibrinolysis as glycation increased [20]. Some studies showed a shorter lag phase in polymerization and decreased permeability in diabetic clots [19, 21], while a few other studies showed no difference in polymerization, kinetics, clot porosity, and clot density in diabetic samples compared to control samples [20, 22]. No studies investigating single fiber properties in diabetes clots have been reported until now.

In the present investigation, we measured the mechanical properties of individual fibrin fibers formed from diabetic patients and healthy controls. Our goal was to investigate whether altered fibrin clot properties in diabetic patients were related to effects of protein glycation on single fiber properties. Our results show that increased glycation does not alter the modulus or extensibility of single fibrin fibers in a predictable way. We, thus, conclude that glycation does not have a direct effect on single fiber
mechanical properties; the negative effects of diabetes on cardiovascular health likely has a different origin than altered single fiber mechanical properties.

Because of the large variation in fiber size in these experiments, we also investigated the effect of fiber diameter. We found that the stiffness (modulus) of fibrin fibers, $Y$, strongly decreases with increasing fiber diameter, $D$, in all samples. $Y$ was proportional to $D^{-1.6}$ over a tested diameter range from 19 nm to 408 nm. This means that thin fibers (e.g., $D = 30$ nm) are 40 times stiffer than thick fibers (e.g., $D = 300$ nm). This is very unusual behavior since the modulus is a material constant and does not depend on the dimensions (diameter) of material, assuming the material has a regular, homogeneous cross-section. Our finding, therefore, implies that fibrin fibers do not have a homogeneous cross-section of uniformly connected protofibrils, as is commonly thought. Instead, the density of protofibril connections, $\rho$, strongly decreases with increasing diameter, as $\rho \propto D^{-1.6}$. Thin fibers are much denser, or have more densely or strongly connected protofibrils, than thick fibers. We propose a new fibrin fiber model in which fibers are less densely connected at increasing diameters, resulting in a lower modulus. We corroborate this model with measurements made on individual fibers in plasma fibrin clots as well as purified fibrin clots.

This finding has several important implications. While the assembly of monomers into protofibrils, via the A:a, B:b and D:D interactions, is relatively well understood, the lateral assembly of protofibrils into mature fibers is poorly understood, and models for the lateral cross-section of a fiber are only speculative. This lateral assembly is one of the critical pieces still missing from a full understanding of clot formation. Our data provide information about the lateral, cross-sectional organization of fibrin fibers, and therefore,
contribute to our understanding of fibrin fiber formation. Models for fibrin fiber lateral assembly need to be modified to account for this decreasing lateral density. It is also important to note that any parameter that affects the radius of a fiber, such as fibrinogen or thrombin concentrations, will strongly affect the modulus of the fiber and thus, the entire clot. Thin fibers are denser than thick fibers and thick fibers have a denser core and a less dense periphery. Thus, thin fibers, and the core of thick fibers are likely harder to dissolve. This key finding may explain the clinically found relationship between thin fibers and increased thrombotic diseases [3].

METHODS

Plasma Collection, Determination of Fibrinogen Concentration and Glycation

To determine if glycation had an effect on the mechanical properties of single fibrin fibers, twenty Type 2 diabetic and eighteen non-diabetic individuals (control) were recruited [19, 22], of which we used a subset for the single fiber studies. Patients had to be uncontrolled (HbA1C > 9%) on maximum-dose combination oral hypoglycemic medication, BMI > 25 kg/m², 40-65 years of age, and blood pressure sufficiently controlled (< 140/90 mmHg) to be included in the study. Baseline blood samples were collected, after which patients underwent a three-step intervention program. First, patients were taught how to monitor glucose, coordinate insulin use with meals, and manage hypoglycemic events with glucagon. Secondly, patients received 10 IU (equivalent to 0.347 mg) of insulin daily in addition to the current treatment of maximum dose oral hypoglycemic treatment. Metformin use was unchanged, sulphonylureas were stopped, and insulin use was adjusted individually until 4 out of 5 subsequent fasting
blood glucose values were less than 7.2 mM. Lastly, short acting insulin was used to control post-prandial glucose levels (<10 mM). Once both fasting and post-prandial glycemic control were achieved, the subjects remained on treatment for eight days before end blood samples were collected. Non-diabetic control subjects with matching age, gender, and BMI were included. Baseline oral glucose tolerance tests were done to rule out diabetes in the controls.

Citrated blood was collected from patients. Within 30 minutes of collection, the blood was centrifuged for 15 minutes at 2000 g at 4°C. The plasma was extracted, snap-frozen and stored at -80°C until fiber sample preparation. Fibrinogen concentration was measured using a modified Clauss method (Automated Coagulation Laboratory, Instrumentation Laboratories, Milan, Italy). Fibrinogen glycation was measured as previously described [22] using a two-reagent enzymatic assay (GlyPro® assay, Genzyme Diagnostics, Cambridge, MA, USA; between-run CV = 5%).

**Substrate Preparation**

Striated cover slides were prepared for plasma samples as previously described [11, 23, 24]. Briefly, optical adhesive (NOA-81, Norland Products, Cranbury, NJ) was placed on a cover slide. A rectangular polydimethylsiloxane (PDMS) stamp was pressed into the adhesive to create a 1.5 cm x 1.5 cm wide and few mm deep well for holding buffer. In the center of the well, a drop of optical glue was placed and a second PDMS stamp was used to create a striated surface with 6.5 µm wide ridges and 13.5 µm wide grooves. The optical glue was then cured under 365 nm UV light (UVP 3UV transilluminator, Upland CA) for 1.5 minutes.
Fibrin Sample Preparation

All chemicals were from Sigma-Aldrich unless otherwise noted. To form fibrin fibers, an 18 µl aliquot of plasma solution (14 µl of citrated plasma and 4 µl of 0.1 M CaCl$_2$) was combined with 2 µl of thrombin (Enzyme Research Laboratories, South Bend, IN, final concentration 0.1 NIH units/ml) and pipetted onto the striated cover slide. The clotting reaction ran for 1 hour in a moist atmosphere at room temperature. This time period was chosen to allow completion of fiber formation including stabilization of fibrin by factor XIIIa. After an hour, the slide was rinsed with calcium free buffer (140 mM NaCl, 10 mM Hepes, pH 7.4). A pipette tip was used to manually remove excess fibers from the top of the sample. The sample was then rinsed with fibrin buffer (140 mM NaCl, 10 mM Hepes, 5 mM CaCl$_2$, pH 7.4). The fibers were labeled with 20 nm carboxyl coated fluorospheres (Invitrogen, Carlsbad, CA) and rinsed with fibrin buffer once again.

Purified fibrin samples were formed similar to the plasma fibrin samples. In this case, a 2 µl mixture of thrombin (final concentration 0.3 NIH units/ml) and FXIII (Enzyme Research Laboratories, South Bend, IN, final concentration 9 Loewy units/ml) was added to 18 µl of purified fibrinogen in concentrations varying from 0.8 mg/ml to 6 mg/ml. After clotting, the mixtures were rinsed with calcium free buffer and labeled with 20 nm fluorospheres.
Manipulations

Fibrin clots were visualized on an inverted fluorescence microscope (Typically we use 40X lens with NA 0.7) (Figure 12). Straight fibers that spanned a groove and had no branch points within that groove were chosen for manipulation. Groove width corresponds approximately to the length between branch points in a plasma fibrin clot [6, 12]. Fiber modulus, extensibility, and relaxation behavior were determined as previously described [10]; for details, see supplement. Manipulations were performed using a combined atomic force microscope (AFM) (Topometrix Explorer, Veeco Instruments, Woodbury, NY) and inverted fluorescence microscope (Axiovert 200 or Observer D, Zeiss, Göttingen, Germany). The fiber sample was placed between the AFM and optical microscope using a customized stage which allows the sample to be moved independently of either microscope. Illumination of the sample is provided by a camera light in the AFM. Fibers were stretched with the AFM probe (CSC-38, MikroMash, Willsonville, OR) at a rate of ~320 nm/s. Cantilever deflection, distance and time data were collected by NanoManipulator software (3rd Tech, Chapel Hill, NC). Images were collected during manipulation by a Zeiss AxioCam and Zeiss Axiovert software, or a Hamamatsu EM-CCD C9100 camera (Hamamatsu Photonics, KK, Japan) with IPLab software (Scanalytics, Fairfax, VA). Fiber diameter, D, was determined by AFM imaging the fiber on top of the ridge adjacent to where the fiber was manipulated. The fiber cross-section was calculated assuming a cylindrical cross-section.
**Figure 12.** Fibrin fiber manipulation. (A) Schematic of fibrin fiber manipulation. The fiber is suspended over the grooves in a striated substrate. The AFM tip, located above the sample, pulls on the fiber while the optical microscope, located below the sample, acquires images and movies of the manipulation. (B) Top view schematic of fiber manipulation. $L_{\text{initial}}$ is half the initial length of the fiber, $L'$ is half the length of the stretched fiber and $s$ is the distance the tip has traveled. $L'$ can be found trigonometrically from $L_{\text{initial}}$ and $s$, and the strain can be calculated from these quantities (see text). Schematic (A) and (B) adapted from [24]. (C) Optical microscopy movie frames of a fiber being stretched and broken. The large dark object is the AFM cantilever and the AFM tip is marked by an asterisk). The fiber broke at strain of 200%.

**Statistical Analysis**

Means and standard deviations were calculated using standard equations. To determine statistical significance between samples, a two tailed t-test was used with an $\alpha$ level set at 0.05. Linear and monotonic relationships between two variables were tested by Pearson’s correlation and Spearman’s correlation.
RESULTS

Fiber viscoelastic properties

Fibrin fibers of plasma clots from 14 different plasma samples were investigated. We took about 15 measurements per sample for all experiments. The samples included 4 control (non-diabetic) individuals, 5 controlled and 5 non-controlled diabetic patients. Blood was collected from the diabetic patients both before intervention (uncontrolled diabetics) and after intervention (controlled diabetics). Several clots were made from the same plasma samples over the duration of the study. We did not observe any significant difference in the viscoelastic properties of fibers from the same plasma sample over the course of the investigation. The samples were all collected from females between the ages of 44 and 65 with a median age of 58. We did not include males to limit the number of variables. Blood fibrinogen concentration of the 4 control and 5 diabetic patients ranged from 3.5 to 5.6 mg/ml. Fibrinogen glycation ranged from 3.0 to 11.8 mol glucose/mol fibrinogen. Prior to intervention, uncontrolled diabetic patients had an average fibrinogen glycation of $8.8 \pm 3.4 \text{ mol gluc/mol fibrinogen}$, which decreased to an average of $5.0 \pm 2.4$ after intervention. The glycation of all diabetic patients decreased after intervention. The non-diabetic control group had an average fibrinogen glycation of $4.0 \pm 1.0$.

We determined several key mechanical properties – extensibility, the total and elastic modulus, and stress relaxation times – for plasma fibrin fibers as a function of glycation, fibrinogen concentration and fiber diameter.
**Extensibility.** In an extensibility measurement, the fiber is simply stretched until it breaks, and the strain at the breaking point, $\varepsilon_{\text{max}} = \Delta L_{\text{max}} / L_{\text{init}}$, is termed extensibility. For instance, an extensibility of 1 means the fiber breaks at twice its initial length.

**Stiffness (stretch moduli) and relaxation times.** We used incremental stress-strain (force-extension) curves to determine the stiffness (moduli) and stress relaxation times of fibrin fibers. In a simple stress-strain curve a force, $F$, is applied longitudinally to an elastic fiber with cross-sectional area, $A$. The fiber extends by a distance $\Delta L = L - L_{\text{init}}$, where $L_{\text{init}}$ is the initial length of the fiber, and $L$ is the extended length of the fiber. The stretch modulus, $Y$, is the proportionally constant between the applied stress, $\sigma = F/A$, and the resulting strain, $\varepsilon = \Delta L / L_{\text{init}}$; $\sigma = Y \cdot \varepsilon$. An example of an incremental stress-strain curve is shown in supplementary Figure 41. In these measurements, the fiber is stretched, then held at a constant strain for a period of time, before being stretched again. The process is repeated at higher and higher strains. The slope of the unrelaxed stress-strain curve corresponds to the total stretch modulus (total stiffness) and the slope of the relaxed stress-strain curve corresponds to the elastic component of the total modulus. We found that the elastic modulus is always about a factor of 0.6 lower than the total modulus and, for simplicity, we will only report the total modulus here (elastic modulus, see supplement). When the fiber is held at constant strain, the stress decays (see supplement). This is indicative of viscoelastic behavior. The simplest mechanical model that can account for these observations (the two relaxation rates; stress does not decay to zero) is a generalized Kelvin model, consisting of an elastic spring with modulus $Y_{\infty}$, in parallel with two Maxwell elements consisting of a dashpot and a spring in series [10]. For this model, the equation for stress relaxation becomes
\[ \sigma(t) = \varepsilon_0 \left[ Y_\infty + Y_1 \cdot e^{-t/\tau_1} + Y_2 \cdot e^{-t/\tau_2} \right] \] 

(1)

\( Y_\infty \) is the relaxed elastic modulus, \( Y_0 \) is the total elastic modulus, \( Y_0 = Y_\infty + Y_1 + Y_2 \). By fitting an exponential curve to the stress decay, we can determine key mechanical properties – the total modulus, the elastic modulus, and relaxation times of the fibers, detail see in [10].

**Glycation.** Fiber extensibility for the various plasma samples from diabetic and control subjects varied from 1.2 to 2.7, largely in agreement with previously reported values for purified fibrinogen samples [10, 11]. There was no clear trend in a plot of extensibility vs. glycation (Figure 13A). The total modulus of the fibrin fibers varied from 1.0 MPa to 28 MPa (Figure 13C) and, analogously, the elastic modulus of the fibers varied from 0.6 MPa to 17 MPa, always approximately a factor of 0.6 lower (supplement). The modulus of fibrin fibers strongly varies with fiber diameter as \( Y(D) = Y_0 \cdot D^{-1.6} \) (see below, Figure 15A). Therefore, in order to properly compare the moduli of the different fiber samples with each other, the modulus needs to be adjusted for this diameter dependence. We, therefore, calculated a diameter-normalized modulus, \( Y_{130}^n \), for each fiber sample. We first multiplied each individual fiber data point by \( D^{+1.6} \) to determine \( Y_0 \), and then we multiplied this value by \( 130^{-1.6} \), because the average fiber in each group was 130 nm (Supplemental Figure 44). We averaged these values to obtain the diameter-normalized, average modulus for a standard 130 nm fiber, \( Y_{130}^{130} \), for each sample. When plotting the normalized, modulus, \( Y_{130}^n \), versus glycation, again no apparent trend is seen. When measuring stress decay, we found the fibers have an average fast relaxation time of \( \tau_1 = 2.3 \) s and an average slow relaxation time of \( \tau_2 = 34 \) s (Figure 13B), and it appears that \( \tau_1 \) and \( \tau_2 \) also do not show a dependence on glycation. In related
published work, it was shown that glycation also did not seem to affect the diameter distribution of fibrin fibers [19], other than a slightly higher fraction of larger fibers in the endpoint diabetic sample (after glycemic control). Therefore, it is also unlikely that glycation will indirectly affect single fibrin fiber mechanical properties through a change in diameter.

In summary, glycation does not seem to have an effect on single fibrin fiber mechanical properties (Figure 13).

![Figure 13](image)

**Figure 13.** Fibrin fiber mechanical properties as a function of glycation. (A) Extensibility; (B) Fast and slow stress relaxation time; (C) Total modulus; (D) Diameter-normalized total modulus for average 130 nm fibrin fiber. No significant, systematic trend is observed between any mechanical properties and glycation. Error bars are standard error of the mean. We focused on the mechanical properties as changing of glycation, not on the differences among three different groups. Color-coded plot with different groups can be found in Supplement Figure 45.
**Fibrinogen concentration.** Numerous clinical and epidemiological studies indicate that elevated fibrinogen concentration may be a risk factor for cardiovascular disease [25]. Using our plasma samples, we also tested if fibrinogen concentration – in our available range from 3.5 mg/ml to 5.6 mg/ml – had an effect on single fibrin fiber properties. All of our samples had relatively high fibrinogen concentrations, consistent with reports finding higher fibrinogen levels in African and African-American individuals as compared to a range of 1.5 mg/ml to 3 mg/ml typically found in healthy, white individuals [26, 27]. Fibrinogen concentration did not have a consistent, systematic effect on fibrin fiber extensibility, relaxation time, modulus or diameter-normalized modulus. (Figure 14A-D), at the concentrations we tested.

**Figure 14.** Fibrin fiber properties as a function of fibrinogen concentration. (A) Extensibility; (B) Fast and slow stress relaxation time; (C) Total modulus; (D) Diameter-
normalized total modulus for average 130 nm fibrin fiber. No significant, systematic
trend is observed between these mechanical properties and fibrinogen concentration.
Error bars are standard error of the mean. We focused on the mechanical properties as
changing of fibrinogen concentration, not on the differences among three different
groups. Color-coded plot with different groups can be found in Supplement Figure 46.

Fiber diameter. Experimental and clinical evidence indicates that fibrin clots
comprised of highly branched networks with thin fibers are associated with thrombotic
disease [3-6]. However, the underlying reason for this association is not known. We,
therefore, decided to also investigate the direct effect of fiber diameter on fiber modulus.
Figure 15 shows the results of this analysis – fiber modulus strongly decreases with
increasing radius for all plasma samples, and for samples formed from purified
fibrinogen. There was a significant (p < 0.01) negative power law relationship between
fiber modulus and fiber diameter (notice log scaling). The exponent for plasma
fibrinogen fibers is -1.6 (N = 213, R² = 0.26) while the exponent for purified fibrin fibers
is -1.4 (N = 116, R² = 0.17); that is, the modulus Y, depends on the diameter, D, as
\( Y \propto D^{-1.6} \) (plasma) and as \( Y \propto D^{-1.4} \) (purified fibrinogen). Thus, two plasma fibers with
different diameters, \( D_1 \) and \( D_2 \), would have a different modulus by a factor of \( (D_1/D_2)^{-1.6} \).
This is very unusual, since the modulus is a material property and should not depend on
fiber dimensions. As explained in more detail in the discussion, our data imply that fibrin
fibers do not have a homogeneous cross-section, since for a homogenous cross-section
the modulus would be independent of the diameter.
DISCUSSIONS

We set out to measure the mechanical properties of single fibrin fibers in plasma clots formed from diabetic patients to determine if fibrinogen glycation had an effect on fiber mechanical properties. Our work resulted in three major overall findings. 1) Our combined AFM/inverted optical microscope technique is suitable to test complex plasma samples, in addition to the samples formed from purified fibrinogen that have been tested before. This opens up the possibility to investigate many different patient samples. 2) We found that there was no significant direct correlation between fibrinogen glycation and fibrin fiber extensibility, modulus, and stress relaxation, as tested by Pearson’s correlation and Spearman’s correlation, details, see supplement) Thus, the known clinical correlation between diabetes and cardiovascular disease likely is not due to altered mechanical properties of fibrin fibers as a result of hyperglycated fibrinogen. 3) Fibrin...
fibers diameter ranged from about 20 nm to 400 nm, and we, therefore also investigated the effect of diameter on single fibrin fiber mechanical properties. We observed a strong negative power law relationship between fiber modulus, Y, and fiber diameter, D; Y scales as $D^{-1.6}$ (plasma samples) and as $D^{-1.4}$ (purified fibrinogen). The strong dependence of the modulus on fiber diameter is very unusual and has interesting and significant consequences for whole clot properties, and especially for the internal structure and lateral assembly of fibrin fibers, as discussed in the next paragraphs.

*Whole clot modulus.* Because thin fibers are stiffer, whole clots comprised of many thin fibers would have a higher modulus than clots comprised of fewer thick fibers, at similar fibrinogen concentrations. For near physiological fibrinogen concentrations (ranging from about 1 mg/ml to 8 mg/ml), clots formed at higher thrombin concentrations or higher fibrinogen concentrations had thinner fibers (and more branch points) [2]. And, indeed, the whole clot modulus has also been shown to increase in these clots with thinner fibers [2]. This increase has generally been attributed to the increased density of branch points observed in clots with small diameter fibers. However, in the current study, we also observed an increased fiber modulus, as fiber diameter decreased, in the absence of branch points. Thus, the increased modulus of clots formed from thinner fibers might be due to an increased modulus of the single fibers that comprise the clot, as well as due to increased branch points.

*Internal fibrin fiber structure and lateral fiber assembly.* The stretch modulus is a material property that is used to define the stiffness of materials under tensile stress, as outlined in the Results section. The modulus does not depend on the dimensions of a material (e.g. does not depend on fiber diameter), provided the material composition is
homogenous. Homogeneous in our fiber context means that the fiber would have a uniform density of equally connected protofibrils in the radial direction (Figure 16A). Our observation that the modulus of fibrin fibers depends on the diameter implies that fibrin fibers do not have a homogenous cross-sectional composition. Thus, we propose a new model, in which the density of protofibrils and/or the density of protofibril connections within a fiber varies with diameter (Figure 16). It is important to note that, since we measure the strength of a fiber, we can draw conclusions about the density of bonds that connect protofibrils together, not just the density of protofibrils.

Figure 16. Fibrin fiber models and their corresponding Young’s modulus. (A) A fiber with a uniformly connected cross-section of protofibrils would have a Young’s modulus that is independent of diameter, D. (B) A fiber with a bicycle spokes-like cross-section would have a Young’s modulus with that decreases with fiber diameter as $D^{-1}$. (C) In our experiments the Young’s modulus of the fiber varies as $D^{-1.6}$; the modulus strongly decreases with increasing diameter, indicative of a fractal cross-section.
From a materials mechanics point of view, the Young’s modulus is proportional to the density (and strength) of bonds that connect the material subunits in the longitudinal direction. For fibrin fibers these are the bonds that connect protofibrils with each other. Since fibers grow simultaneously in the longitudinal and lateral direction to form a mature fiber, protofibrils need to assemble in a staggered fashion to form a mature fiber [28]. This means that the lateral bonds between protofibrils also provide longitudinal strength.

We assume that fibrin fibers consist of an array of longitudinally arranged, ribbon-shaped protofibrils [28]. In such an array we further assume that protofibrils are connected with each other such that the force to stretch a fibrin fiber is directly proportional to the number of bonds connecting the protofibrils. For a cross-section in which the protofibrils are evenly distributed, the number of protofibril bonds, $N_p$, increases proportionally to the cross-sectional area ($A = (\pi/4)D^2$ for circular cross-section) and, thus, proportional to $D^2$. The force to stretch a fiber $F(D)$ increases as $D^5$, and the Young’s modulus would be independent of $D$ (Figure 16A). The cross-sectional protofibril bond density $\rho_p = N_p/A$ would be constant, i.e. independent of $D$. Using similar arguments, if we assume a fibrin fiber forms a bicycle spokes-like structure of protofibrils, the number of protofibril bonds per cross-section, $N_p$, increases linearly with diameter. Thus, the stretching force required to reach a specific strain would also increase linearly as a function of the radius, $F(D) \propto D$, and, the protofibril bond density, $N_p$ would vary as $1/D$, and the Young’s modulus would also vary as $1/D$ (Figure 16B). In our experiments, the Young’s modulus decreases even stronger with diameter – as $D^{-1.6}$. To explain these data, we propose a model in which the protofibril bond density, $\rho_p$, also
varies as \( D^{-1.6} \) (Figure 16C). In this model, the fiber has a dense core of well-connected protofibrils that becomes less dense as more protofibrils aggregate onto the outside of the fiber.

We will now discuss this model in the context of Yang et al.’s multi-bundle model [28], which is based on protein-protein contacts as seen in various fibrinogen crystals, and some recent diffraction, scattering and imaging experiments that can probe the internal structure of fibrin fibers. Yang et al. proposed that fibrin monomers assemble into wavy protofibrils ribbons via the known and well-accepted A:a, B:b and D:D interactions. Protofibrils then assemble via lateral associations between \( \gamma \)-chains and \( \beta \)-chains into mature fibers. It is likely that the largely unstructured \( \alpha \)-C regions also play a critical role in the lateral assembly of protofibrils [29, 30]. The protofibrils are staggered in the x and y (lateral), and z (longitudinal) direction; this stagger along all three axes is required so that fibers can simultaneously grow in the lateral and longitudinal direction. The multi-bundle model results in a regular, crystalline fiber assembly with unit cell dimensions of 19 nm x 19 nm in the lateral (radial) dimension, and 46 nm in the longitudinal dimension. Peaks corresponding to these unit cell dimensions have been observed in energy dispersive x-ray diffraction (EDAD) [31], and small angle x-ray scattering (SAXS) [32]. However, the peaks corresponding to the lateral periodicity (19 nm) were broad and weak [32], indicating only weak ordering in the lateral (radial) direction. Moreover, AFM images suggest that the 22.5 nm periodicity (corresponding to the half-staggered arrangement of the 45 nm fibrin monomer in the longitudinal direction) disappears as fibers increase in diameter [33]. SAXS and light scattering data point to a fiber with a protein content of only 15% and a very porous cross-section, that
becomes increasingly porous as the diameter increases [32]. AFM rupture force experiments on dry fibers also suggest a fiber cross section that becomes increasingly porous with increasing diameter [34]. All these experiments that probe the internal structure of fibrin fibers suggest a loose, open and only weakly crystalline structure. Some studies suggest that the density of protofibrils might decrease with increasing radius [32-34].

Our experiments probe the density of bonds between protofibrils, i.e. the strength of the connections between protofibrils, and constitute the first direct evidence that the connections between protofibrils become fewer and/or weaker as the fiber diameter increases. In all plasma fibers, the bond density decreases as approximately $D^{-1.6}$ (Figure 16C).

**Implications for fiber assembly.** It is not yet clear what mechanisms might restrict bond formation between protofibrils and restrict protofibril aggregation as fibers grow thicker. One possible mechanism might be twisting, increasing path length and increased stretching of protofibrils in thicker fibers, i.e., protofibrils loose register due a changed binding geometry as the fiber diameter increases [35]. Another, different mechanism, that would also be consistent with our data, is activation-limited or diffusion-limited aggregation [36] of fibrin fibers from protofibrils. Recent work on whole clots found that incipient clots have a fractal dimension of 1.7 [37], and the authors suggest that clots may assemble via activation-limited aggregation of clusters of rod-like particles [38]. When applied to the assembly of single fibers, these activation-limited or diffusion-limited aggregation mechanisms would result in a fractal fiber cross-section, in which the bond density (or protofibril density) would decreases with increasing diameter, as we have
observed experimentally. In the context of a fiber cross-section, the fractal dimension, $F$, is the exponent in the relationship between the number of bonds (or protofibrils), $N$, and the diameter of the fiber, $D$; $N = D^{F}$. For a solid, homogeneous cross-section (Figure 16A), $F$ would be 2. For purely diffusion-limited aggregation, $F$ would between 1.5 and 1.71, i.e. the fiber has less protein content as its diameter increases. For activation-limited and diffusion-limited aggregation, $F$ would be less than that. In our data (Figure 16C), the bond density, $\rho$, scales as $D^{-1.6}$, and thus the number of bonds per cross-section, $N$, would scale as $D^{-1.6+2} = D^{0.4}$. $N = \rho \cdot A$, where $A$ is the cross-sectional area of the fiber, $A = \pi(D/2)^2$. Thus, our data suggest that fibrin fibers have a very low fractal dimension of 0.4 for the number of bonds in a fibrin fiber cross-section. The fractal dimension for the number of protofibrils would be higher, if the number of bonds per protofibril decreases as the fiber diameter increases. Our data suggest that fibrin fibers start out with a well-connected semi-crystalline core of protofibrils, but then the fiber becomes increasingly porous and disorganized as more protofibrils aggregate. This increased disorganization may be consistent with the out-of-registry assembly model [35], and recently proposed early branching model [39].

**Clinical implications and fibrinolysis.** There is increasing experimental and clinical evidence that fibrin clots comprised of highly branched networks with thin fibers are associated with venous or arterial thrombosis, in particular myocardial infarction, ischemic stroke and venous thromboembolism [3-6]. There is also experimental evidence that clots with thinner fibers lyse slower than clots with thick fibers, even when normalized for total fibrinogen concentration [40]. Our model provides a simple
explanation for the slower lysis of clots comprised of thin fibers – thin fibers have a higher bond density than thick fibers and are, thus, harder to lyse.

**Blood clot modeling.** While many studies of the mechanical properties of whole fibrin clots can be found in the literature, relatively few studies have been done on the individual components making up these clots. Proper modeling of whole clots is needed for accurate predictions of the effect of diseases on the mechanical properties of clots. It should be possible to calculate the bulk mechanical properties of a whole fibrin clot by knowing the properties of the individual components. Our bottom-up approach provides the foundation for determining initial parameters for modeling whole fibrin clots. A distinct advantage of this approach is the ability to determine the differences in the mechanical properties of networks with very dissimilar structures. The results here demonstrate why the properties of different types of fibers must be measured and not make assumptions about the properties of fibers with different structures.

**CONCLUSION**

Using plasma samples from uncontrolled and controlled diabetic individuals and a non-diabetic control group, we determined that glycation does not seem to have an effect on single fibrin fiber mechanical properties. This implies that the observed epidemiological correlation between diabetes and CVD likely does not have a molecular origin at the single fibrin fiber level.

Using these plasma samples and samples prepared from purified fibrinogen, we observed a strong dependence of fibrin fiber modulus on fiber diameter, D; the modulus decreases
as $D^{-1.6}$. This observation can be best explained with a new fibrin fiber model in which the cross-sectional density of bonds within fibrin fibers decreases with increasing diameter. That is, fibrin fibers become less densely connected as their diameter increases. Such a model is not consistent with a crystalline, homogenous cross-section of equally connected protofibrils, for which the modulus would be independent of $D$.

Our findings imply that any parameter that affects the radius of fibrin fibers, such as fibrinogen concentration or thrombin concentration, will have a strong effect on the modulus of single fibers. This, in turn, will have a strong effect on whole clot mechanical properties, and, presumably, the \textit{in vivo} behavior of blood clots. Clinically, our model provides a simple explanation why clots comprised of thin fibers are harder to lyse – thin fibers have a higher bond density than thick fibers. In addition, in clot dissolution, lytic factors, such as plasminogen, and plasminogen activators, tPA, urokinase and streptokinase, need to reach the inside of clots and the inside of fibrin fibers. Our model, in which thinner fibers are denser than thick fibers, implies that it should be easier to dissolve clots that consist of fewer thick fibers than those that consist of many thin fibers, which is consistent with experimental \cite{40} and clinical observations \cite{3}.
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CHAPTER IV

INVESTIGATION OF FIBRIN FIBER INTERAL STRUCTURE: PROTEIN DENSITY AND BOND DENSITY DECREASE WITH INCREASING DIAMETER

Wei Li, Justin Sigley, and Martin Guthold

Wei Li performed the experiment, analyzed the data and wrote the manuscript. Justin Sigley collected modulus data from the plasma sample of black female. Martin Guthold gave kind advice and edited the manuscript.
The major structural component of a blood clot is a meshwork of fibrin fibers. The longitudinal assembly of fibrin monomers into protofibrils is well understood; however, the radial (lateral) assembly of protofibrils into mature fibers is poorly understood. It has long been thought that the internal structure of fibrin fibers is regular and homogenous; that is, the protein density and the bond density between protofibrils are uniform. We performed two types of experiments to investigate the internal structure of fibrin fibers. We formed fibrin fibers with fluorescently labeled fibrinogen and determined the light intensity of a fiber, I, which is proportional to the number of monomers, as a function of fiber diameter, D. We found that the intensity, I, scales as \( I \sim D^{1.43 \pm 0.03} \). This implies that the cross-sectional monomer density also scales as \( D^{1.4} \), and not as \( D^2 \), as would be expected for fibers with a solid, homogeneous cross-section. We also determined the Young's modulus, E, as a function of fibrin fiber diameter, and found that E strongly decreases with increasing D, as \( E \sim D^{-1.5} \). The modulus data suggest that the number of bonds per cross-section scales as \( D^{0.5} \). Our data are consistent with a fiber model that has a dense core and a very loosely connected periphery. In contrast, electrospun fibrinogen fibers, used as a control, do seem to have a homogenous cross-section.
INTRODUCTION

_Fibrinogen (general)._ Fibrinogen is a key protein in blood, which upon activation by thrombin polymerizes into a meshwork of fibrin fibers. Fibrin fibers form the major structural component of a blood clots. In hemostasis, they stem blood flow in the event of injury and trauma, and they are involved in the initiation of wound healing [1]. In thrombotic disease, unintended formation of blood clots cause such diseases as myocardial infarction, ischemic strokes, deep vein thrombosis and pulmonary embolisms.

Fibrinogen is a 340-kDa glycoprotein, with an elongated, trinodular shape, it is 45 nm in length and 4.5 nm in diameter. It is composed of two distal D domains and one central E domain, which are connected by triple-helical coiled coils [2-4].

_Protofibril formation._ The first step in fibrin fiber formation is the formation of protofibrils. It is initiated as thrombin proteolytically removes fibrinopeptide A from the central E-domain of fibrinogen, thus converting fibrinogen to DesA fibrin. Knob A, which remains after cleavage of fibrinopeptide A, fits into ‘hole a’, located in the terminal D domain. Fibrin monomers assemble via these A:a interactions in a half-staggered fashion into double-stranded protofibrils. The interactions between abutting D interfaces, called D:D interactions, are also important for protofibril assembly. It is well-accepted in the field that the key interactions for protofibril assembly are the A:a and D:D interactions. If these interactions are blocked, protofibril formation is severely impaired, which is not the case for any other interactions.

_Lateral aggregation._ The two-stranded, protofibrils then assemble laterally (radially) into mature fibers [5-7]. However, the interactions for this lateral assembly are much less understood, and the internal arrangement of protofibrils within a mature fiber
is not clear. Some studies suggest a relatively regular internal structure, in which the protofibril is packed in a semi-crystalline fashion [6, 8] while others suggest that it is packed randomly [9, 10]. A commonly used method/technique investigating the properties and the structure of fibrin clots is turbidimetry and light scattering, which provide information about fiber size [11, 12] and fiber (protein) density [13, 14]. There are numerous papers about the mechanical properties and structures of a whole blood clot [15, 16]. However, there are only a few papers about single fibrin fibers [17-19]. Moreover, scattering techniques which draw conclusions about single fibers, take data that are averaged over many fibers, and then information about single fibers is extracted, in other words, the information about single fibers is indirect [20]. If there are significant differences in single fibers, that difference will be lost in averaging methods. We directly measure each single fiber, which allows us to resolve differences between single fibers.

Fibrin Fiber Lateral Structure. The lateral aggregation of fibrin fibers is poorly understood. In the past, most models assumed a uniform cross-sectional density [6, 8, 21]. Recently there has been some evidence that the internal structure is not homogenous [22, 23]. Protein density is a good indicator to the fiber structure. Neutron scattering and light scattering are most frequently used to indirectly determine protein density. These techniques have been used to show that protein content inside a fibrin density is only 20% (80% solvent) (some theoretical studies tried to explain this phenomenon [24, 25]) and the lateral structure of fibrin fiber appears to be fractal [23]. Similar results were found with the help of high resolution atomic force microscope (AFM), it is observed that molecule packing inside the fiber might denser and tighter than that on the surface [22,
Early rupture force measurements on dry fibrin fibers suggested that the cross section of fibrin fibers may have a fractal dimension of 1.3 [9].

In the presented work, we used fluorescently labeled fibrinogen to obtain a relationship between fiber light intensity $I$, which is proportional to the number of monomers in a fiber cross-section, and fiber diameter, $D$. We found that $I \propto D^{1.4}$, suggesting a denser core and a less dense periphery. For a fiber with uniform density, the number of fibrinogen molecules would increase as the square of the diameter, $D^2$.

In addition to the fibrin(ogen) and protofibril packing inside a fiber, the bonds involved in lateral aggregation are also not well understood. Bonds are important because they hold the fiber together. In the past, there has been no method to determine bond density inside a fiber. The stretching force of a single fiber is proportional to the number of bonds inside the fiber. For a fiber with homogeneous bond density, the stretching force would increase as $D^2$ (since the cross-sectional area of a fiber increases as $D^2$). However, our data show that the stretching force, $F \propto D^{0.6}$, i.e., with a significantly lower power than $D^2$. This agrees with our proposed model that fibrin fiber is not homogenous.

Combining our light intensity and force data, we propose a model for the internal structure of fibrin fiber. It has a dense core of well-connected protofibrils that becomes less dense as more protofibrils aggregate onto the outside of the fiber.

Possible physiological implications. Moreover, in fibrinolysis process, bonds connecting protofibrils together need to be lysed. More the bonds there are, the harder it will be to lyse the fibers. So the phenomenon that thin fibers lyse slower than thick fibers [27, 28] is a good indicator that fibrin fiber is not homogenous.
MATERIALS AND METHODS

Plasma Collection

Blood samples were from several different population groups. Blood was collected from healthy males from two different subgroups: five healthy middle-aged individuals (40 – 50 years old) and five healthy old individuals (>50 years old). Blood samples were into citrated tubes and then centrifuged at 3700 rpm to remove cells and large particles; the remaining plasma was stored at -80 °C until further use.

Blood was also collected from black female Africans from three different subgroups: five controlled diabetic patients (Type II Diabetes) (receiving insulin treatment), five uncontrolled diabetic patients (Type II Diabetes) and a control group of five healthy (non-diabetic) subjects. Blood from these groups was centrifuged at 2000 g at 4 °C for 15mins. The remaining plasma was stored at -80 °C until further use.

Striated substrate preparation

A drop of optical adhesive (NOA-81, Norland Products, Cranbury, NJ) was placed on top of a coverslip. Then a rectangular polydimethylsiloxane (PDMS) stamp was pressed into the optical glue to form a striated substrate with 6.5 µm wide ridges and 13.5 µm wide grooves. The optical glue was then cured under 365 nm UV light (UVP 3UV transilluminator, Upland CA) for 1.5 minutes, at which point it was ready to use.
Formation of Fibrin Fibers

Electrospun fibrinogen fiber

100 mg/ml lyophilized bovine fibrinogen (Sigma-Aldrich Chemical Co.) is mixed with a solution of 9 part 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma Aldrich) and 1 part minimum essential medium (MEM, 10× MEM, Gibco, Invitrogen cell culture). Then 3.17 mg Rhodamine 6G fluorophore (Eastman Kodak, Rochester, NY) was added into the mixture to get the final ratio of 15 fluorophores per fibrinogen molecule. The electrospun fibrinogen fiber was formed on top of striated substrate as described in [29].

Fibrin fiber from plasma

All chemicals were from Sigma-Aldrich unless otherwise noted. Fibrin fibers are formed from human plasma described above. 2 µl of 0.1 NIH units/ml thrombin and a 18 µl mixture of plasma and 20 mM CaCl₂ were pipetted onto the striated substrate. The sample was kept in wet atmosphere at room temperature for one hour. After that the top layer of fibrin clot was rinsed off by using calcium free fibrin buffer (140 mM NaCl, 10 mM, Hepes, pH 7.4) and 20 nm carboxyl coated fluorospheres (Invitrogen, Carlsbad, CA) was applied onto the substrate and incubated in fibrin buffer before use.

Fibrin fiber from purified fluorescently labeled fibrinogen

Fluorescence intensity measurement. Fibrin fibers are formed from purified fibrinogen that was labeled with Alexa 546 fluorophore (Life technologies, Grand Island, NY) with a ratio of 15 fluorophores per fibrinogen monomer. 18 µl fibrinogen solution (13.5 µl fluorescently labeled and 4.5 µl non-labeled fibrinogen (Enzyme Research
Laboratories, South Bend, IN) both at 1.5 mg/ml) along with 2 µl 0.1 NIH units/ml thrombin was pipetted onto a coverslip. The sample was kept in wet atmosphere at room temperature for one hour. After this period, a skin of fibrin clot was gently rinsed off and incubated in fibrin buffer (140 mM NaCl, 10 mM, Hepes, 5 mM CaCl₂, pH 7.4).

**Force measurement.** Fibrin fibers are formed as described above, except that it was formed from non-fluorescently labeled, purified fibrinogen on a striated substrate.

Fluorescence Microscopy (Optical Microscopy)

We used the inverted fluorescence microscopy (Axiovert 200 or Observer D, Zeiss, Göttingen, Germany) in this experiment. Fluorescence images were taken under 40X lens (NA of 0.7), with an excitation source from Osram Short Arc Mercury lamp (Osram HBO 103W/2, Atlanta Light Bulbs Co., Atlanta, GA). In order to get the same photobleaching for all the fibers in a particular image, fluorescence images of each area were taken only once, under the same conditions.

**Fibrin Fiber Manipulation**

**Fluorescence intensity measurement.** Fibrin fibers were formed on a coverslip and fluorescence images of the fibers were taken immediately after illuminating the fibers to avoid photo-bleaching as much as possible. Since the diameter of fibrin fibers is below the resolution limit of light microscopy, the AFM was used to obtain images of the same location as the fluorescence image (Figure 17). Fiber height as determined from AFM topography images was taken as the fiber diameter (fiber width in AFM images is
significantly exaggerated due the tip broadening effect). ImageJ software was used to determine the light intensity of the cross section of single fibers.

![Figure 17](image)

**Figure 17.** Experimental setup of fluorescence intensity measurement. (A) Schematic lateral view of setup. (B), (C) Fluorescence images of fibrin fibers. (D) AFM topography image of the same region of fibrin fibers as in (C). (E) Fluorescence intensity distribution of the cross section (along the red line) of a single fiber; total fluorescence intensity of this fiber corresponds to the area under this curve. (F) Height distribution of the cross section (along the red line) of the fiber in (D), which is the same fiber as in (C). Even though (C) looks really bright, it didn’t reach the gray value limit (the limit is 16384), so the figure is not saturated.
**Force data measurement.** Fibrin fiber mechanical properties were determined using a combined fluorescence microscope and atomic force microscopy (AFM) technique, as described before [18, 29, 30]. Briefly, as shown in Figure 18, fibers were formed on the striated substrate. The AFM (Topometrix Explorer, Veeco Instruments, Woodbury, NY) tip is located above the sample for manipulation, while the inverted fluorescence microscopy (Axiovert 200 or Observer D, Zeiss, Göttingen, Germany) provides images from below.

![Figure 18](image_url)

**Figure 18.** Experimental setup for force measurement. (A) Schematic lateral view of setup. Adapted from [30]. (B) Single fibrin fiber stress vs. strain plot. The modulus corresponds to the slope of this plot. (C) Fluorescence images of a fiber being stretched and broken. The large dark object is the AFM cantilever and the AFM tip is marked by an asterisk. The fiber broke at strain of 200%.
Statistical Analysis

The significant difference between two groups is determined by using a two-tailed t-test. The significant difference between two slopes is calculated by analysis of covariance (ANCOVA).

RESULTS

The goal of our paper is to investigate the internal structure of fibrin fibers; in particular we aim to determine how the number of fibrin monomers, and the number of bonds inside a fibrin fiber vary as a function of fiber diameter. These data will provide insights into the structural arrangement of fibrin monomers and bonds inside a fibrin fiber. We used two types of experiments to determine these quantities. 1) Fluorescence intensity measurements of single fibers formed from fluorescently labeled fibrinogen – these data provide information on the number of fibrin monomers. 2) Stretching force measurements on single fibers – these data provide information on the number of bonds in a fibrin fiber.

Fluorescence Intensity as a Function of Fiber Diameter

In these experiments, fibrin fibers were formed on a flat coverslip from fibrinogen that was labeled with Alexa-546. The fiber diameter, which is beyond the resolution limit of light microscopy, was determined from an AFM topography image of the same area (Figure 17). Photobleaching was reduced by having the shortest possible exposure times and by taking images as rapidly as possible. Fluorescence images of each area were taken only once, under the same conditions, so that residual photobleaching was the same for
all fibers in a particular image. Moreover, to average out small fluctuations, we analyzed four closely spaced cross-sections of each fiber (see Figure 17) and took the average. We did the same four-measurement averaging procedure to determine the diameter of each fiber from the AFM scans.

Dry Electrospun fibrinogen fiber (mixed with Rhodamine 6G)

As a control experiment, we first determined the fluorescence intensity of electrospun fibers. Dye was mixed into the spinning solution. For a homogenous cross-section with an evenly distributed dye, the fluorescence intensity should increase as $D^2$, since the cross-sectional area of a fiber (assuming it is cylindrical) is $\pi(D/2)^2$. We aimed to have about the same number of fluorophores in the electrospun fibers as in the fibrin fibers (15 fluorophores per monomer). So, the molar ratio of fluorophore to fibrinogen monomer in the spinning solution was 15:1.

In this control experiment, we found out that the total light intensity, $I$, increases with increasing diameter as $I \propto D^{1.9\pm0.1}$, which means that the number of fluorophores, $N_f$, also increases as $N_f \propto D^{1.9\pm0.1}$ (Figure 19A). This experiment was performed in triplicate (additional data, see supplement Figure 48). If we assume a circular cross-section of the electrospun fiber, $A = \pi(D/2)^2$, the fluorophore density $\rho_F = N_f/A$ is constant, as would be expected for a homogeneous fiber with an even distribution of fluorophores (Figure 19B).
Figure 19. Fluorescence intensity of electrospun fibrinogen fiber cross section as function of diameter. (A) The slope of the relationship between light intensity and fiber diameter is 1.9 on a log-log scale. (B) Fluorophore density \( \rho_F = \frac{N_f}{A} \) is independent of fiber diameter, as would be expected for a homogeneous fiber. Each data point represents four measurements.

Wet Fibrin fiber labeled with Alexa 546 fluorophore.

Fibrinogen, labeled with 15 Alexa 546 fluorophores per fibrinogen monomer was used to form fibrin fibers on a flat glass substrate. For these fibrin fibers, fluorescence intensity also increased with increasing diameter, however it only increases as \( I \propto D^{1.4\pm0.2} \) (Figure 20A). Since the total fluorescence intensity, \( I \), of a fiber cross is proportional to the number of monomers in a cross-section, \( N_m \), this implies that \( N_m \propto D^{1.4\pm0.2} \). This is a much lower exponential power than 2, which was seen in the electrospun control fibers, and which would be expected for homogenous fibers. (These experiments were carried out in triplicate, other plots, see supplement Figure 49). To obtain the fibrinogen monomer density (protein density), we divide by the cross-sectional area (assuming cylindrical cross-section, \( A = \pi(D/2)^2 \)). The protein density, \( \rho_p \propto D^{-0.6} \) decreases with increasing diameter (Figure 20B). This means that thinner fibers have a higher density.
than thicker fibers. It suggests that the cross section of fibrin fiber is not homogeneous, they have a higher density in the core, and lower density at the periphery.

Dry fluorescently labeled fibrin fiber, showed a similar relationship, as the intensity, I, varied as $I \propto D^{1.21 \pm 0.14}$ (Details in supplement Figure 50).

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**Figure 20.** Fluorescence intensity of fibrin fibers (in buffer) as a function of diameter. (A) The slope of the relationship between light intensity and fiber diameter is 1.4 on a log-log scale. (B) The slope of the relationship between protein density and fiber diameter is -0.6 on a log-log scale. Each data point represents four measurements.

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**Fiber Modulus as a Function of Fiber Diameter**

The number (and strength) of bonds inside a fiber is proportional to the force that is required to stretch the fiber. Therefore, determining the stretching force as a function of diameter will provide information on the bond density of a fiber cross-section. Before stretching a fiber, we first used the AFM in imaging mode to determine the diameter of the fiber (on the anchoring ridge). We then used the AFM tip to pull the same fiber to obtain a stress-strain curve. This procedure is described in detail in [18, 30]. The modulus, (stiffness) of the fiber corresponds to the slope of the stress-strain curve. The fibrin fibers in these force measurement were all formed from plasma from the various different groups, and also from purified fibrinogen.
In all of our force experiment, for every sample, we took at least 20 measurements, so there are at least 200 data points in each group. The three different groups are: plasma samples from white males, black females, and from purified fibrinogen. We saw very similar relationships between modulus and fiber diameter for fibers from all groups. The modulus, $Y$, decreased strongly with as fiber diameter increases, with an exponential power of about -1.5; that is, of $Y \propto D^{-1.5}$.

Specifically, for plasma samples from while male, the slope of this relationship was $-1.4 \pm 0.1$ in log-log scale, while the slope for plasma samples from black female was $-1.6 \pm 0.2$. The slope for samples from purified fibrinogen was $-1.4 \pm 0.3$ (Figure 21).

From the exponential relationship (power of -1.5) between modulus and fiber diameter we got here ($Y \propto D^{-1.5}$), we can know that stretching force, $F$, increases exponentially (power of 0.5) as fiber diameter increases, $F \propto D^{0.5}$ (Modulus is proportional to stretching force, $Y=F/A$, assuming cylindrical cross-section, $A=\pi(D/2)^2$). Because stretching force is proportional to the number of bonds, $N_b$, connecting protofibrils together, this means $N_b \propto D^{0.5}$. This indicates that the bond density in the cross-section of single fibrin fiber is not uniform, since for a uniform cylindrical cross-section, the bond density should increase as fiber diameter squared, as $N_b \propto D^2$. So the result we got here ($Y \propto D^{-1.5}$) demonstrates that bond density decreases as fiber growing.
Figure 21. Fibrin fiber modulus as a function of diameter. (A) For plasma samples from white males, the slope was $-1.4 \pm 0.1$ in log-log scale. (B) For plasma samples from black females, the slope was $-1.6 \pm 0.2$. (C) For samples from purified fibrinogen, the slope was $-1.4 \pm 0.3$.

DISCUSSION

In our study, we found that the light intensity, $I$, which is proportional to the number of fibrinogen molecules, $N_{f}$, in a fiber depends on fiber diameter, $D$, $I \propto D^{1.4}$ for wet fibrin fibers. This implies that the protein density decreases as $\rho_{p} \propto D^{-0.6}$. In electrospun fibrinogen fiber, which was used as a control fiber with a homogeneous cross-section, the light intensity increased with fiber diameter $I \propto D^{1.9}$, close to the expected $D^{2}$ (and constant density). In the force measurement, for different plasma and purified fibrinogen samples, fiber modulus $Y$ decreased as fiber diameter $D$ increased power exponentially of $Y \propto D^{-1.5}$. This implies that the bond density (bonds per cross-
section) as strongly decreases as $\rho_B \propto D^{-1.5}$, significantly stronger than the protein density as $\rho_p \propto D^{-0.6}$, and far from a homogeneous density for which $\rho$ would be independent of $D$.

**Model of fibrin fiber internal structure.** There is some evidence that the internal structure of fibrin fibers has some regularity and some crystallinity. In SEM images, often a clear banding pattern with spacing of 22.5 nm – half the length of a fibrin monomer – can be seen [16, 31]. This fits well with the regular, half-staggered arrangement of the fibrin monomers in protofibrils and suggests a somewhat regular arrangement of protofibrils inside a fibrin fiber, as proposed in Yang et al.’s multi-bundle model [21]. However, this seemingly regular arrangement of protofibrils might have been partly induced by the vacuum conditions inside the SEM chamber. A similar, though less pronounced banding pattern with $\sim 22.5$ nm spacing, was also seen in some AFM images; the pattern seemed to disappear for larger fibers [22, 26].

Energy dispersive x-ray diffraction (EDAD) [8] and small angle x-ray scattering (SAXS) [32] was used to probe the internal structure of fibrin fibers. Peaks corresponding to lateral periodicity of 19 nm were broad and weak [32], indicating only weak ordering in the lateral (radial) direction. SAXS and light scattering data point to a fiber with a protein content of only 15% and a very porous cross-section that becomes increasingly porous as the diameter increases [32].

Even though currently there are some evidences about the internal fibrin structure, most of these methods and techniques only give the indirect information like neutron scattering and light scattering [33, 34]. So our experiment fills this gap and provides more direct evidence to the internal structure.
Our data a consistent with following model: We proposed a possible model that fibrin fiber does not have a homogeneous structure but rather a dense and well-connected core and less dense, and very poorly connected periphery.

To explain our model, we should briefly discuss our data in the context of two simpler models. First, for a homogeneous cross-section of uniformly connected molecules, the intensity (number of molecules) and number of bonds would increase quadratically with diameter; the protein and bond density, and the Young’s modulus, would not depend on diameter. Second, for a model in which the cross-section looks like the spokes of a bicycle wheel, the intensity (number of molecules) and number of bonds would increase linearly with diameter; the protein and bond density, and the Young’s modulus, would decrease as $D^{-1}$.

We observed that the relationship between the number of molecules and fiber diameter is $N_f \propto D^{1.4}$, thus, somewhere between a homogeneous fiber and the bicycle spokes model.

The situation for the bonds (connections that hold a fiber together) is somewhat different. We observed that the number of bonds only increases as $N_b \propto D^{0.5}$, and, thus, less than for the bicycle spokes model. This could be interpreted that fibrin fibers have a well-connected core, but that connections drop off strongly toward the outside of the fiber.

Recently, as new technology developed, more detailed information about the internal structure of fibrin fiber came out. Yermonahos et al [23] found out that fibrin fiber appeared to be fractal meshed by using spectrometry and small angle x-ray scattering measurements. With high-resolution AFM, it is shown that thinner fiber is denser than thicker one, molecule packing decreases as fiber becomes thicker [22, 26].
What’s more, our data is consistent with the early paper that fiber cross section has a fractal dimension 1.3 [9].

This result may be important for fibrinolysis, since the number of bonds should affect lysis—more bonds means slower lysis. Numerous papers found that thinner fibers lyse slower than thicker ones [27, 35]. Combining our light intensity and modulus data, we proposed a possible internal structure model of fibrin fiber that it has a densely packed, well-connected core and less dense, loosely connected periphery (Figure 22).

![Figure 22. Schematic image of internal fibrin fiber structure. Fibrin fiber has a densely packed, well-connected core and less dense, loosely connected periphery.](image)

**CONCLUSION**

The internal structure of fibrin fibers, and especially the packing of protofibrils has been unclear. In this paper, we tried to gain insights into this structure by using fluorescence intensity and force (modulus) measurement. In the fluorescence intensity measurement, we found that light intensity of fiber cross section is dependent on fiber diameter as $I \propto D^{1.43\pm0.20}$ ($N \propto D^{1.43\pm0.20}$). In the force data (modulus), we got a relationship between modulus and fiber diameter as $Y \propto D^{-1.5}$, indicating that the number
of bonds connecting fibrin subunits (protofibril) together decreases dramatically as fiber diameter increases. These relationship demonstrates that protofibrils are densely packed in the center, it becomes loosely packed as to the periphery. So based on all the results, we proposed a possible model for fibrin fiber internal structure that it has a densely packed, well-connected core and less dense, loosely connected periphery.

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Wei Li and Tomas Lucioni performed all experiments, except the cell growth experiments, and wrote the manuscript. Xinyi Guo, Amanda Smelser and Wei Li performed the cell experiments and cell viability assay. Martin Guthold supervised the experiments and edited the manuscript.
ABSTRACT

We developed a novel substrate made from fugitive glue (styrenic block copolymer) that can be used to analyze the effects of large strains on biological samples. The substrate has the following attributes: (1) It is easy to make from inexpensive components; (2) It is transparent and can be used in optical microscopy; (3) It is extremely stretchable as it can be stretched up to 750% strain; (4) It can be micro-molded, for example we created micro-ridges that are 6 µm high and 13 µm wide; (5) It is adhesive to biological fibers (we tested fibrin fibers), and can be used to uniformly stretch those fibers; (6) It is non-toxic to cells (we tested human mammary epithelial cells); (7) It can tolerate various salt concentrations up to 5 M NaCl and low (pH 0) and high (pH 14) pH values. Stretching of this extraordinary stretchable substrate is relatively uniform and thus, can be used to test multiple cells or fibers in parallel under the same conditions.

INTRODUCTION

Cells and other biological samples are often exposed to stresses and strains in their natural environment, and these stresses and strains can have a significant biological effect. For example, the growth of smooth muscle cells and the import of nuclear proteins are stimulated by strains [1], and bone formation is stimulated in the presence of mechanical stimuli [2]. In the last few years, strong evidence has emerged that cells are sensitive to the mechanical properties (e.g., stiffness) of their environment. In a seminal paper, Engler et al. showed that the differentiation of stem cells is influenced by substrate mechanical properties [3]. These researchers observed that stem cells will differentiate
into bone-like cells when grown on stiff substrates or into neuron-like cells when grown on soft substrates. Moreover, mechanical strain plays an important role in stem cell differentiation and function: global gene expression changes (for example, smooth muscle markers increase, cartilage matrix decrease) when cells are aligned parallel to the strain axis [4]. Furthermore, intracellular calcium oscillation in human mesenchymal stem cells is governed by mechanical tension [5] and mesenchymal stem cell differentiation into vascular smooth muscle cells may be promoted by uniaxial strain [6].

Similarly, biological fibers in the body are often exposed to stresses and strains. For example, blood flow exerts stress on fibrin fibers during blood coagulation, and blood flow can affect the structure of blood clots [7, 8] and the interaction of fibrin fibers with platelets [9]. Elastin fibers [10], which are found in the skin and lungs, experience strain during respiration and are very extensible; collagen fibers, found in cartilage and connective tissue, experience strain during movement, but are not as extensible [11].

Therefore, when investigating the behavior of biological samples in the lab, a stretchable substrate is required in many situations. There are numerous devices and techniques to apply and test the effect of stresses and strains on cells. One example is traction force microscopy, which uses a stretchable substrate. The migration of normal 3T3 (3-day transfer, inoculum 3 x 10^5 cells) cells can be detected by using traction force microscopy, where the cell body was pulled forward by the dynamic traction forces at the leading edge [12]. Shao et al. described a homemade cell stretching device to demonstrate that external mechanical stretch plays a key role in regulating subcellular molecular dynamics with the F-actin cytoskeleton [13]. Moore’s group used their stretching device to show that phenotype modulation (alignment and altered mRNA
expression) can be induced by stretching 10T1/2 (from Embryonic mesenchymal cell line) cells [14].

Nano- and microfiber properties are often measured by suspending fibers over microridges in a substrate and then the fibers are manipulated with an AFM (Atomic Force Microscope) [15]. The mechanical properties of different nanofibers have been determined by this method, such as fibrin fibers [15, 16], electrospun collagen fibers [17] and electrospun fibrinogen fibers [18]. This sophisticated AFM technique allows for precise mechanical manipulations of nanofibers, and numerous mechanical properties can be extracted with this technique. However, individual fibers are pulled one at a time, which is tedious, time-consuming and not efficient. Varju et al. showed that a strained blood clot lyses more slowly than an unstrained blood clot [19]. So, to investigate the effect of strain on single fibrin fibers, an AFM could be used, but a technique that allows the investigation of multiple fibers in parallel would be more efficient. With our novel, highly stretchable substrate, it is possible to manipulate an array of single fibers simultaneously, instead of only one single fiber. This facilitates investigations of the effect of strain on single biological nanofibers, such as collagen and fibrin, and other nanofibers, such as electrospun nanofibers [18], that are used in biomedical engineering applications.

Besides investigating biological samples, stretchable substrates may also be used in flexible electronics. Stretchable materials can serve as substrates, onto which circuits and electronics are engineered [20, 21]. Other times, conducting materials are injected into the substrate [22, 23]. Often these materials have a rather low stretch limit, just a little above 100% [24]. However, some applications may require significantly higher
elongations. Another drawback of current stretchable devices, like bio-
Microelectromechanical systems (bioMEMS) [25, 26] and traction force microscopy [12], is the high cost of these devices.

In this paper, we report tests on fugitive glue (a styrenic block copolymer), which is extraordinarily stretchable (up to a 750% strain). It also is very inexpensive, easy to obtain and easy to handle. Moreover, it can be molded into microstructures, and presumably many other shapes. Furthermore, it is transparent (for use in optical microscopy), it can withstand extreme environments, like strong acids/bases (between pH 0 and 14), it is compatible with salt solutions and biological samples (fibrin fibers), and it is non-toxic to cells.

MATERIALS AND METHODS

*Stretchable Substrate Preparation*

The stretchable substrate was made from fugitive pressure sensitive adhesive (Surebonder AT-10154 Hot Melt, Hotmelt.com, Edina, MN 55439, www.hotmelt.com); this type of glue is also called hot melt pressure sensitive adhesive or, colloquially, credit card glue. Chemically, this adhesive is a styrenic block copolymer. A drop of hot fugitive glue was placed onto the surface of a microscope cover glass slide (No. 1.5, 24 mm × 60 mm) (Fisherbrand, Pittsburgh, PA, USA) from a Surebonder PRO100 Hot Melt Gun (Hotmelt.com, Edina, MN 55439, www.hotmelt.com). Immediately afterward, a rectangular PDMS (Polydimethylsiloxane) (6 mm × 8 mm) stamp with imprinted grooves and ridges was pressed into the glue. After it cooled down and dried (4 min), the PDMS
stamp was peeled off, leaving ridges and grooves in the fugitive glue (width and height of the ridges was 6.5 µm, width of the grooves was 13.5 µm, measured by scanning electron microscope (SEM, Amray 1810 scanning electron microscope, AMRAY, Bedford, MA) in a previous publication [18]. Next, the imprinted fugitive glue substrate was manually stretched to the desired length, as follows. The imprinted substrate was carefully peeled off the glass cover slide, then manually stretched to a specific length, and anchored back down again. For anchoring we used Adhesive Squares (1/2 inch x 1/2 inch Adhesive Squares™ RS Industrial, Inc., Buford, GA) as follows. The squares, which are about 1 cm × 1 cm where stretched into a string of about 15 cm. This string was then used to tie down the two sides by wrapping them around the cover glass as shown in Figure 23.

![Figure 23](image)

**Figure 23.** Setup of stretchable substrate. (A) Schematic of forming fugitive glue with ridges and grooves; (B) Photograph of fugitive glue substrate with ridges and grooves; (C) Photograph of stretched substrate.
**pH and Salt Solution Tolerance Test**

400 µL solutions with different pH values and salt concentrations were deposited onto the surface of the stretchable substrate and left for 1.5 h at room temperature. The following solutions were used (all solutions from Fisher Scientific, Pittsburgh, PA, USA): 1 N HCl (pH 0), pH 4 buffer solution (pH-meter calibration solution, Potassium Acid Phthalate), pH 10 buffer solution (pH-meter calibration solution, Boric Acid-Potassium Chloride-Sodium Hydroxide buffer), 1 N NaOH (pH 14), 5 M NaCl (Sigma-Aldrich, St. Louis, MO, USA), 5 M MgCl₂ (Sigma-Aldrich). Microscope images were taken before and after the tests with an inverted optical microscope (Axio Observer D1, Zeiss, Thornwood, NY, USA) with a 40x objective lens.

**Fibrin Fibers**

An 18 µL solution of purified human fibrinogen (Enzyme Research Laboratories, South Bend, IN, final concentration 1 mg/mL) was placed onto the surface of the stretchable substrate. Then 2 µL of thrombin (Enzyme Research Laboratories, South Bend, IN, final concentration 0.1 NIH (National Institute of Health) units/mL) were added into it and kept in a wet environment at room temperature for 1 h. After that, a skin (part of the clot) on this solution was peeled off with a pipette tip to reduce the density of the clot before imaging. Fibrin fibers on the stretchable substrate were kept continuously in fibrin buffer (140 mM NaCl, 10 mM, Hapes, 5 mM CaCl₂, pH 7.4).
Cell Growth

For the cell growth experiments, we used a flat (instead of a striated) substrate made from fugitive glue. Glass bottom dishes (Willcowells, Amsterdam, NL) of size 35 mm $\times$ 2 mm were purchased and assembled in the lab. A drop of hot fugitive glue was placed onto the surface of a petri dish, then a PDMS stamp with flat surface was pressed into the glue and removed after 4 min.

Human mammary epithelial cells (HMECs) were purchased from Lonza (Lonza Group Ltd, Walkersville, MD, USA) and used within 6 passages from their original state from Lonza. HMECs were cultured in Mammary Epithelial Cell Growth Medium–MEGM (Lonza) with 0.4% bovine pituitary extract (BPE) (Lonza), according to the distributor’s recommendations. Cells were cultured and maintained in a culture incubator at 37 ºC with 5% CO$_2$. Pictures were taken 48 h after the cells were seeded (Figure 26).

Cell Viability Assay

The cell viability assay was done by using LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies, Grand Island, NY, USA). 400 µL of 1 µM Calcein AM (acetomethoxy derivate of calcein) (used to stain live cells, ex/em~495 nm/515 nm) and 400 µL of 10 µM Ethidium homodimer-1 (used to stain dead cells, ex/em~495 nm/635 nm) were added into the petri dish with cells prepared as described above and incubated for 15–20 min. Green fluorescence indicated the activity of intracellular esterases present in live cells, and red fluorescence indicated the loss of cell membrane integrity in dead cells. Fluorescence images of live and dead cells and Differential Interference Contrast (DIC) images of cells were taken with a Nikon Eclipse Ti, 20x objective, NA 0.75.
RESULTS

Stability

Mechanical Stability and Stretchability

We found the substrate to be mechanically stable (it kept its original shape and length) even when stretched to about 250%. In our 24-hour test, it kept its original shape in air at room temperature. Therefore, we kept the stretching percentage at around 250% in the following experiments. In the most extreme case we tested, the substrate could be stretched to around 750%, but it was only mechanically stable for 10 min. These experiments demonstrate that substrates formed from fugitive glue are extremely extensible and hold their shapes for long enough time periods to do many biological and other experiments. The extensibility far exceeds that of other common materials used in recently described stretching devices, such as Polydimethylsiloxane (PDMS) [20], Polyethylene-terephthalate (PET) [27], Polyimide (PI) [28] and silicone [29]. A concentrated strain of 107% has been reported on a soft, thin PDMS film area in microsupercapacitor arrays [20]. PET substrates coated with an acrylic primer can be stretched to over 70% without breaking [27]. None of these materials can be stretched to several times their original length. In separate experiments, we also tested PDMS (Sylgard 184 Silicone Elastomer Kit, 10:1 mix ratio, Dow Corning, Midland, MI, USA), Norland optical adhesive 81 (Norland Products Incorporated, Cranbury, NJ, USA) and silicone (GE Silicone II, Kitchen and Bath Caulk, clear color, purchased at Home Depot, Atlanta, GA, USA); they were all significantly stiffer than fugitive glue and significantly less extensible.
A familiar example of fugitive glue is its common use to attach credit cards to paper (credit card glue), and it can be easily stretched to several times its original length.

Fugitive glue is a styrenic block copolymer, a type of thermoplastic elastomer. Their mechanical properties, which are similar to rubber, stem from their microstructure. Microscopically, styrenic block copolymers consist of two hard polystyrene end blocks that are connected by a soft, elastomeric midblock (linker), typically made of polybutadiene or polyisoprene.

pH Tolerance Test

Some experiments require extreme pH values, so the ability of a substrate to tolerate highly acidic and basic surroundings can become important. We, therefore, also tested the pH tolerance of our fugitive glue substrate from pH 0 to pH 14. Some polymers may degrade at these extreme environments, e.g., polyanhydrides degrades at high pH values [30], poly(dl-lactide-co-glycolic acid)-methoxypoly (ethyleneglycol) (PLGA-mPEG) microparticles show degradation in strong acid and base [31]. However, our stretchable substrate made from fugitive glue maintained its mechanical and chemical stability under both highly acidic and basic environments for up to at least 1.5 h. There was no discernable degradation (by visual inspection under a microscope with a 40x objective lens) and the shape of the grooves and ridges was not affected (Figure 24).
Figure 24. pH Tolerance Test. (A) Initial image right after adding pH. (B), (C), (D), (E) Images taken after 1.5 h incubation at pH 0, 4, 10, 14. The images before and after adding solutions are at different locations of the same sample. Some crystals formed in the pH 14 solution, probably due to the high Na\(^+\) concentration, but the substrate appears unaffected by the solution.

Salt Solution Tolerance Test

For biological and non-biological experiments, different salt solutions may be applied to the substrate. So, it is also important to test if this material can withstand different salt solutions. We selected two commonly used salts, NaCl and MgCl\(_2\), at very high concentrations (near their solubility limit), reasoning that if fugitive glue can withstand such extremely high salt concentrations, it should also be able to withstand lower salt concentrations. In this experiment, a 5 M NaCl solution and a (2.5 M NaCl + 2.5 M MgCl\(_2\)) solution were applied to the stretchable substrate. After 4 h, no salt
deposits and no deformation or degradation of the ridges and grooves were observed (Figure 25).

Figure 25. Salt Solution Tolerance Test. (A) Initial image that was taken right after adding salt solution to the substrate. (B) and (C) Images of the substrate after a 4 hour-incubation with 5 M NaCl and (2.5M NaCl + 2.5M MgCl₂) solutions. The images before and after adding salt solution are at different locations of the same sample.

In summary, the stretchable substrate made from fugitive glue can accommodate a broad range of solution conditions that may be found in many experiments.

**Bio-Compatibility**

**Cell Growth on Fugitive Glue Substrate**

Since many biological samples are exposed to stress and strains in their environment, we tested if our stretchable fugitive glue substrate is suitable for biological samples. We tested cells and biological fibers. First, we tested if this material is toxic to cells. Human Mammary Epithelial Cells (HMECs) were grown on fugitive glue for 48 h (Figure 26A,B). Cells were well attached and grew well on the fugitive glue substrate.
Thus, it appears that fugitive glue is suitable as a cell substrate. About 85% of cells are alive (stained green), whereas about 15% of cells are dead (stained red).

![Image](A)

![Image](B)

**Figure 26.** Human mammary epithelial cells grown on fugitive glue substrate. (A) Differential Interference Contrast (DIC) images of cells. (B) Fluorescence images (20x objective lens) of live cells (stained green) and dead cells (stained red) of same field of view as in (A).

**Fibrin Fiber Formed on Fugitive Glue Substrate**

Besides cells, we also tested if our fugitive glue substrate is compatible with biological fibers. Fibrin fibers are the major structural and mechanical component of a blood clot. They have an average diameter of about 130 nm. Fibrin fibers form from fibrin monomers, the activated form of the blood protein, fibrinogen. Fibrinogen gets converted to fibrin by thrombin in the last step of the coagulation cascade. Fibrin fibers can be easily formed in the lab, by adding thrombin to fibrinogen. In previous work, we have determined various mechanical properties of single fibrin fibers, such as their stiffness, extensibility and elasticity [15, 16]. As shown in Figure 27A, fibrin fibers form well on this substrate, and they strongly adhere to the substrate. We did not observe any slipping or detachment, even at over two-fold extensions (Figure 27B). Since fibrin fibers
experience stress during blood circulation [7, 8], there is a strong interest in investigating fibrin fiber mechanical properties. Our stretchable substrate provides a novel approach for these investigations.

Figure 27. Fibrin fibers on the unstretched substrate (A), and stretched substrate (268%) (B). The substrate has imprinted ridges and grooves. The width of the groove is 13.5 µm (before stretching), and 36.2 µm (after stretching).

DISCUSSION

We have described and tested a highly stretchable substrate made from fugitive glue, a styrenic block copolymer. It is moldable, transparent to visible light (usable as a substrate in optical microscopy), tolerant to high and low pH values and salt concentrations, and compatible with biological cells and fiber samples. Compared to other devices that are used to apply strain to biological samples on the microscale, it is among the least expensive and easiest to handle and manufacture. Other devices include bio-Microelectromechanical systems (BioMEMS) and some home-made stretching devices.
BioMEMS are MEMS devices for biological applications, which are manufactured using similar microfabrication techniques as those used to create integrated circuits. They are usually used in biosensors, pacemakers, immunoisolation capsules, and drug delivery systems [32]. BioMEMS have been used to apply strain to adherent fibroblasts and detect the de-adhesion force [25], and to test cell force responses: strongly linear, reversible, and repeatable under large stretches [26].

Some home-made devices have also been used to apply external strain to biological systems. For example, Heo et al. applied input pressure (air input) from underneath to a PDMS layer, so that the cells on the layer can be stretched [33]. Another novel stretching device is based on the movement of computer-controlled, piezoelectrically actuated pins of a refreshable Braille display underneath a sample to generate strain on a elastometric PDMS membrane’s top surface. The Braille pins could provide 20%–25% maximal strain in the radial direction [34]. Yang’s group used a force sensor probe coated with biomolecules to stretch cells [35]. Wipff et al. used PDMS as the elastic membrane, mixed with tracking particles to monitor the degree of substrate expansion under stretch [36].

All these are examples of well-suited devices for biological stretch experiments on a micrometer scale. Many have a limited stretching range (around 20%–30%), since they use PDMS. Our stretchable substrate is a good substrate choice when large strains are required, since it is extremely extensible (about 750%). It is also less stiff than PDMS, and can be stretched manually.
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REFERENCES


CHAPTER VI

STRETCHING SINGLE FIBRIN FIBER SLOWS DOWN LYSIS MORE THAN FXIII CROSSLINKING

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The following manuscript has been prepared for journal submission. Wei Li and Tommy Lucioni designed the experiment, Wei Li performed the experiment, analyzed the data and wrote the manuscript. Rongzhong Li programeed the Mathematica code. Martin Guthold provided all the kind advice and edited the manuscript.
ABSTRACT

Blood clots experience strain from blood flow, clot retraction and from interactions with platelets and other cells. This strain might be utilized to control blood clot dissolution (fibrinolysis). Blood dissolution (fibrinolysis) is as equally important as blood coagulation. We developed a transparent, striated and highly stretchable substrate made from fugitive glue (a styrenic block copolymer) to investigate how strain affects lysis of single, suspended fibrin fibers. In this assay lysis was manifested by fiber elongation and collapse. We found that stretching slowed lysis by up to a factor of 5 in uncrosslinked fibers, up to a factor of 3 in crosslinked fibers. Crosslinking by itself (of unstretched fibers) only slowed lysis by a factor of 1.7, meaning that stretching has a bigger effect on lysis than crosslinking. Our data suggest that stretching may distort unfolding of plasmin binding sites, or distort the alignment of plasmin binding sites with respect to the plasmin cleavage site.

INTRODUCTION

Blood coagulation – the process by which blood clots are formed, and fibrinolysis – the process by which blood clots are broken down, are regulated by multiple, often interconnected factors. In the injury-free and inflammation-free state, the coagulation cascade, which triggers blood coagulation, is not activated, and any residual pro-coagulant factors, such as thrombin, are further suppressed by suppressors, like antithrombin. In case of trauma or injury to a blood vessel, the coagulation cascade is activated and a clot is formed locally [1, 2]. The final step in the coagulation cascade is the proteolytic cleavage of fibinopeptides A & B from fibrinogen by thrombin, thus
converting fibrinogen to fibrin. Fibrin then polymerizes into a mesh of fibrin fibers, which constitutes the major structural component of a blood clot. Clot formation in case of vascular injury is beneficial, as it prevents the loss of blood. However, clots can also be harmful, for example, when they form as a result of arteriosclerosis in the arteries around the heart, triggering myocardial infarction, when they form in the veins of the leg (deep vein thrombosis) or when they rupture and travel in the vasculature resulting in embolisms and ischemic strokes.

The fibrinolysis system breaks down clots to prevent permanent occlusion of blood vessels. It is controlled by numerous factors, including activators, inhibitors, cofactors and receptors. The key enzyme in fibrinolysis is plasmin, a serine protease that cleaves fibrin and, thus, dissolves a clot. Plasmin is the activated form of plasminogen; and most activation occurs when tissue plasminogen activator (t-PA) cleaves plasminogen at the Arg561 - Val562 peptide bond. This activation is significantly enhanced (~100-fold), when both plasminogen and t-PA are bound to fibrin, though it can still occur for the unbound molecules. This activation is inhibited by plasminogen activator inhibitor-1 (PAI-1). In blood, any free plasmin is rapidly inactivated by α2-antiplasmin. In a clot, plasmin is shielded from α2-antiplasmin.

Plasmin mainly binds to two specific sites on a fibrin(ogen) molecule, in particular it binds to a region at α148-160 near the D-domain of fibrin(ogen), and to α392-610 in the αC-region. Plasmin cleaves all three strands in the coiled coils between residues β42-43 and β53-54, which is near the middle of the coiled coils [3]. Plasmin can also cut at secondary sites, yielding different fibrin degradation products [4-6]. Fibrinolysis is not only controlled by enzymes, but also by additional factors, such as the
network structure of a clot. For example, high prothrombin levels increase thrombin and abnormal fibrin network generation, which resist fibrinolysis [7]. Clots with a dense network of thinner fibrin fibers lyse more slowly than coarse clots with thicker fibers [8], and lysis is delayed by clots containing DNA and histones [9]. Factor XI (FXI)-deficient plasma sample exhibited a shortened lysis time [10]. Moreover, changes at the molecular level of fibrin can influence lysis, as crosslinking of fibrin fibers by factor XIII (FXIII) increases lysis time in both static and flow systems [11].

As outlined above, regulation in fibrinolysis can be accomplished by different means, such as cleavage of a protein (plasminogen), simply binding to a factor and blocking it, or by influencing the clot network structure and molecular structure. In addition to these elements, mechanical modifications, often termed ‘mechanobiological signaling’, may be another factor that controls fibrinolysis. Recently, Varju et al. and Adhikari et al. found that strained fibrin clots lyse more slowly than clots under unstretched conditions [12, 13]. This was a counterintuitive finding since, from a macroscopic point of view, strained specimens would be expected to break faster. This finding may imply that structural changes in the fibrin fibers at the molecular level prevent plasmin from cutting or from being activated. The notion that strain could be utilized to affect lysis would make sense from a physiological point of view, since fibrin fibers do experience strain in blood, especially in the early stages of clot formation. Fibers are stretched when a clot retracts [14], when platelets interact with fibrin fibrins [15], and when blood flow exerts mechanical forces on fibrin fibers [16, 17] – and this strain may be a novel factor that regulates fiber lysis [18].
Such mechanobiological signaling has been observed in other systems. For example, stretching fibronectin III acts as a mechanosensitive control of ligand recognition [19]. In coagulation, von Willebrand factor reveals additional binding sites after being stretched under blood flow (von Willebrand factor binds to platelet to stop bleeding in small vessels) [20, 21].

We developed an assay that uses a novel, highly stretchable substrate made from fugitive glue (a styrenic block copolymer) [22] to investigate the effect of stretching on the lysis of single fibrin fibers. We found that stretching single fibrin fibers slows down fibrin lysis more than FXIII crosslinking, which is one of the key stabilizing factors of a blood clot. Thus, stretching fibrin fibers may be one of the key elements that protects fibers from lysis. Using our assay, we found that FXIII slowed lysis by a factor of 1.7, whereas stretching slowed lysis by a factor of 5 times (uncross-linked fiber at a 2.5 fold stretch). We propose that stretching may destroy plasmin binding sites, or distort the alignment of the plasmin binding site and the fibrin cleavage site.

MATERIALS AND METHODS

Substrate Preparation:

A drop (~1 cm² in diameter, ~2 mm in height) of hot fugitive glue, a styrenic block copolymer (Surebonder AT-10154 Hot Melt, Hotmelt.com, Edina, MN 55439, www.hotmelt.com), was deposited onto a microscope cover glass slide (No. 1.5, 24mm × 60mm, Fisherbrand, Pittsburgh, PA) from the Surebonder PRO100 Hot Melt Gun (Hotmelt.com, Edina, MN 55439, www.hotmelt.com). Immediately afterward, a rectangular PDMS (Polydimethylsiloxane) stamp with imprinted grooves and ridges was
pressed into the glue, and let sit for 4 minutes to completely cool down, before being peeled off. To stretch the fugitive substrate, it was peeled off the substrate, stretched manually to the desired length, and then anchored again to the substrate by wrapping the ends with an about 15 cm long, flexible adhesive string. The adhesive string was created by stretching Adhesive Squares (1/2 inch × 1.2 inch Adhesive Square™ RS Industrial, Inc., Buford, GA) to 15 cm long string (Figure 28).

**Fibrin Sample Preparation:**

A 20 µl mixture of 18 µl purified fibrinogen (1 mg/ml, Enzyme Research Laboratories, South Bend, IN) and 2 µl of thrombin (final concentration: 0.1 NIH units/ml, Enzyme Research Laboratories, South Bend, IN) was placed into the fugitive substrate well and incubated in a wet atmosphere at room temperature for 1 hour. After that, the substrate was rinsed with fibrin buffer (140 mM NaCl, 10 mM Hepes, 5 mM CaCl₂, pH 7.4) and then 2 µl 20 nm carboxyl coated fluorospheres (100 times dilution) (Invitrogen, Carlsbad, CA) were added to the solution. After 10 minutes of incubation, the sample was rinsed and kept in fibrin buffer before adding t-PA (tissue plasminogen activator) and plasminogen.

**Lysis of Suspended Fibrin Fibers:**

5 µl recombinant human t-PA (10 µg/ml, Aniara, West Chester, OH) and 5 µl plasminogen (3.28 mg/ml, Athens Research & Technology, Athens, GA) were mixed and incubated for 1 hour before applying to the fibers. Figure 29 shows that plasminogen is fully activated by t-PA after this incubation. The 10 µl mixture was then applied to the
sample. (t-PA, final concentration of 0.48 µg/ml and plasminogen, final concentration of 0.16 mg/ml). Fluorescence images were taken every 5 minutes at the same location with an inverted optical microscope (Axiovert 200, Zeiss, Thornwood, NY, USA).

*Colorimetric Plasmin Activation Assay:*

For the plasmin activitation assay, 157.5 µl dimethyl sulfoxide (DMSO) was added to 5 mg N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (Sigma-Aldrich, St. Louis, MO) to get a 50 mM substrate solution. Plasmin was activated by mixing t-PA (10 µg/ml) and plasminogen (3.28 mg/ml) at a volume ratio of 3:1 for a certain time period. At each time point, a 1 µl aliquot was mixed with 97 µl of assay buffer (0.1M NaPO₄, pH 7.8, made from Na₂HPO₄ and NaH₂PO₄) (Sigma-Aldrich, St. Louis, MO) and 2 µl of substrate solution, and the absorption at 410 nm was recorded as a function of time (time is after plasminogen and t-PA was mixed).

*Plasmin Activity Assay (Receding Clot Assay):*

Fibrin clot formation: A 18 µl mixture was made from non-fluorescence labeled purified fibrinogen (concentration 1.5 mg/ml) and Alexa 546 fluorophore labeled fibrinogen (concentration 1.5 mg/ml) (Life Technologies Corporation, Carlsbad, CA) with a volume ratio of 3:1. For the crosslinked fibrin sample, 2 µl of the mixture of thrombin (final concentration 0.1 NIH units/ml) and FXIII (final concentration 9 Loewy units/ml, Enzyme Research Laboratories, South Bend, IN) were added into the 18 µl fibrinogen mixture. For the uncrosslinked fibrin sample, 2 µl of thrombin (final concentration 0.1 NIH units/ml) was added into the 18 µl fibrinogen mixture.
After one hour incubation in a wet atmosphere at room temperature, a small square glass cover slip (18 mm × 18 mm, Fisher Scientific, Pittsburgh, PA) was placed on top of the clot and two layers of double-sided tape (Scotch, 3M Inc., St. Paul, MN) was used on both sides of the cover slip to anchor it. A 10 µl mixture of t-PA (10 µg/ml) and plasminogen (3.28 mg/ml) was injected from one side of the clot, and fluorescence images were taken every 4 minutes at the same place under 10X lens.

Confocal Microscopy:

Fibrin fibers were formed on the unstretched substrate (described in Fibrin Sample Preparation part). Additionally, 200 nm carboxylate coated fluorospheres (100 times dilution) (Life Technologies Corporation, Carlsbad, CA) were added to the substrate, so that the top of the ridges and the bottom of the groove could be distinguished. The sample was imaged with a Zeiss LSM 710 confocal microscope (Zeiss, Thornwood, NY, USA) one hour after of adding the activated plasmin. The fibers were imaged with a 63x oil immersion lens (1.4NA), a series of vertical stack images (z-stacks) were collected every 0.5 µm over 13 µm (from below the bottom of the groove to above the top of the ridge).

Fibrin Fiber Length Analysis:

A custom written Wolfram Mathematica 10 (Champaign, IL) program was used to accurately and unbiasedly determine fibrin fiber length before and after lysis. The program reads in the experimental image and converts every pixel’s brightness to grayscale ranging from 0 to 255 (black to white). After applying a certain threshold, only
the bright pixels for fibers will remain in the image. We specify one fiber by clicking at its starting and ending point. The program will find the central points along the fiber automatically. The raw sequence of the central points is then fitted by polynomials of the form $a + b \cdot x^{-1} + c \cdot x^{-0.5} + d \cdot x + e \cdot x^2 + f \cdot x^3 + g \cdot x^4$. Note, that unlike the case in general fitting purposes, we do not need to predict the curve outside the sample points’ range. As long as we connect the sample points with a smooth curve, we can calculate its length using the fitting function.

RESULTS

The overall goal of our work was to test the hypothesis that stretching has a significant effect on single fibrin fiber lysis. An affirmative outcome would imply that fiber stretching may be an important factor that controls clot lysis via mechanobiological effects. To test this hypothesis, we first developed a transparent, slightly adhesive and highly stretchable substrate with micrometer-sized grooves in it, made from fugitive glue (styrenic block copolymer) [22]. This special substrate allowed us to simultaneously stretch multiple, single fibers by the same amount. Upon addition of activated plasmin we could observe what effect stretching had on lysis. Our set-up is a further development of a set-up by Bucay et al., which was used to investigate the lysis of single fibrin fibers that were suspended over fixed (not stretchable) microridges in a substrate. These authors found that thin, single fibrin fibers lyse quicker than thick, single fibers [23]. The schematic and photos of our stretchable, striated substrate is shown in Figure 28.
**Figure 28.** Stretchable substrate. (A) Schematic of the process for making the substrate. Photograph of unstretched substrate (B) and anchored, stretched substrate (C).

**Plasmin activation**

Before we investigated lysis of suspended single fibers, we confirmed in a colorimetric assay that the used plasmin was fully activated by t-PA. Figure 29 shows the results of the colorimetric plasmin activation assay. In this assay, t-PA and plasminogen were first mixed and added to a solution containing N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide, a chromogenic substrate for plasmin. Upon cleavage by plasmin, this substrate absorbs light at 410 nm. From Figure 29, it can be seen that plasminogen was fully activated by t-PA after 40 minutes. In all subsequent lysis experiments, we only used fully activated plasmin; that is, plasmin that was t-PA activated for at least 40 minutes (typically one hour).
Figure 29. Time-dependent activation of plasminogen. The activation rate of plasminogen by t-PA was determined by using N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate as substrate. Full activation (100%) was achieved at 40 min, and the percentage of activation at other time points were normalized to the percentage at 40 min. All assays were performed in triplicates.

Clot receding

In addition to the colorimetric plasmin activation assay, we also performed a receding clot assay to confirm normal plasmin activity in crosslinked and uncrosslinked clots (Figure 30). Plasminogen was activated by t-PA for 1 hr, and then added to a clot that was formed between two glass cover slips. Plasmin digested the clot, as can be seen by the receding clot/plasmin interface (Figure 30A – F). Clots that were crosslinked by FXIII were digested at a slower rate. The lytic rate for clots crosslinked with FXIII was 3.1 µm/min, whereas it was 4.8 µm/min for uncrosslinked clots.
Figure 30. Receding clot experiments. (A) – (C) lysis of clot with FXIII, (D) – (F) lysis of clot without FXIII. (A), (D) are before adding the activated plasmin; (B), (E) are 14 minutes and (C), (F) are 28 minutes after adding the activated plasmin. The scale bar is 50 µm. The images were taken with a 10x lens. (G) Plot of dissolved clot height vs. time; the slope represents the lytic rate, which was 3.1 µm/min for crosslinked clots and 4.8 µm/min for uncrosslinked clots.
After having confirmed plasmin activity, we investigated if stretching fibrin fibers had an effect on their lysis. We formed fibrin fibers on the stretchable, striated substrate (Figure 28), and added the activated plasmin for one hour. In this suspended fiber assay, lysis was manifested as fiber elongation, rather than a complete transverse cut (Figure 31). This is somewhat unusual since it has been observed that fibers do get completely cut in whole clot assays. However, this lengthening has also been observed by Bucay et al. in their lysis assay of suspended fibers [23].

In addition to plasminogen activated by t-PA, we also tested plasminogen that was activated by uPA, and by Streptokinase, and we tested commercially available (already activated) plasmin. All of these enzymes gave the same result; that is, we saw similar elongation of fibrin fibers upon lysis, and very few, discernable transverse cuts.

**Figure 31.** Lysis of stretched single fibrin fibers (stretched 268%, width of the groove is 36.2 µm). (A) is before adding the activated plasmin. (B) is at one hour after.
Z-stack images of lysed fibers

Z-stack images from a confocal microscope show that, upon lysis, fibrin fibers drop to the bottom of the groove, and remain there (Figure 32). Therefore, the likely sequence of events upon lysis is that fibers get weakened, and then they drop to the bottom of the groove. Since fibrin is very sticky, they likely get stuck there. And even though lysis proceeds, it is not noticeable any longer, since excised pieces will remain stuck to the bottom of the groove.

Figure 32. Confocal images of fibrin fibers after one hour of lysis. The images of the same fiber are taken with focus on the top of ridge (A), on the bottom of groove (B). The images are taken under 63X oil immersion lens. Scale bar is 5 µm.

Fiber elongation vs. stretching

We will, therefore, take apparent fiber elongation as a measure of lysis; all unlysed fibers appear straight, whereas lysed fibers appear elongated. Here the elongation of fiber is in 3D, but in the analysis, we used 2D projection of this 3D elongated fiber. To quantify fiber lysis, we simply determined fiber elongation due to lysis. We used a custom-written Mathematica program to fit a polynomial to the unlysed and lysed
(elongated) fiber to accurately and unbiasedly determine their lengths, $L_{\text{initial}}$, and $L_{\text{lysed}}$ (Figure 33B). The percent elongation, and our measure of lysis, is $\Delta L_{\text{lysis}} = \left( \frac{L_{\text{lysed}} - L_{\text{initial}}}{L_{\text{initial}}} \right) \cdot 100\%$.

Figure 33. Program fitting of lysed fiber (width of the groove is 17 µm). (A) is at one hour after fiber dissolving. (B) is the measurement by the Wolfram Mathematica program.

We determined this measure of lysis, $\Delta L_{\text{lysis}}$ (fiber elongation upon lysis) as a function of fiber stretching. Fiber stretching ranged from 0% (unstretched fibers) to 250%. For each stretching percentage, we determined $\Delta L_{\text{lysis}}$ of numerous fibers, resulting in a distribution of $\Delta L_{\text{lysis}}$ values. In Figure 34 we plotted the peak values of the $\Delta L_{\text{lysis}}$ distributions vs. fiber stretching.

Three main conclusions can be drawn from these plots (Figure 34). First, lysis ($\Delta L_{\text{lysis}}$) strongly decreases with fiber stretching for both, uncrosslinked fibers (Figure 34A) and FXIII crosslinked fibers (Figure 34B). Second, this effect is more pronounced for uncrosslinked fibers, where lysis is slowed down by a factor of 5 (at a 2.5 fold
stretch), than for crosslinked fibers, where lysis is slowed down by a factor of 3 (at a 2.1 fold stretch). Third, stretching slows down lysis more than FXIII crosslinking. For example, comparing the unstretched data points (0% stretch on x-axis in Figure 34A and 34B), slows down lysis by 1.7 times.

Figure 34. Relationship of fiber elongation percentage and fiber stretching percentage. (A) is forming fibrin fiber without FXIII, (B) is with FXIII. Red spot is unstretched state. N represents the number of fibers.
DISCUSSION

_Placeholder text for a discussion section._

*Stretchable substrate and suspended fibrin fiber assay.* We developed a highly stretchable, striated, transparent substrate to investigate the effect of stretching on single fibrin fiber lysis. The stretchable substrate had the advantages that many individual fibers could be investigated _simultaneously_, that the amount of stretching could be easily varied and controlled, and that the amount of stretching on each fiber was well defined. Studying single fibers, rather than a ‘bulk clot’ is significant, because it eliminates the effects of clot network structure and architecture. A caveat of our suspended fiber assay is that lysis has to be defined as fiber elongation, $\Delta L_{\text{lysis}}$, rather than fiber severance, as is typically observed in whole clots [24, 25]. Elongated fiber that collapsed to the bottom of the grooves was the final state, not an intermediate state, in our experiments. We tried different combinations of different concentrations of t-PA with plasminogen, uPA with plasminogen, streptokinase with plasminogen and already activated plasmin, as well as different concentrations of thrombin. We, furthermore, tried much longer time periods (overnight, whole day). All of these gave the same result – elongated, rather than severed fibers. We showed by confocal microscopy that lysed fibers drop to the bottom of the groove. Lysis may stop once a fiber is attached to a surface, or, if lysis continues, the resulting fiber fragments may stick to the surface and appear like a whole (elongated) fiber. This fiber elongation upon lysis was also observed in Bucay’s suspended fibrin fiber assay; though, this group also observed severed fibers. A possible explanation might be that their grooves were deeper, so some elongated fibers did not reach and stick to the bottom of the grooves [23].
**Key findings.** The key findings from our experiments are: 1) Stretching strongly slows lysis; 2) this effect is more pronounced for uncrosslinked fibers; and 3) stretching has a much larger slowing effect than crosslinking fibers.

**Possible physiological relevance.** Blood clot formation and lysis are tightly controlled processes in the body that are regulated by numerous factors. Too much or too little of either clot formation or clot lysis can be life-threatening or lead to debilitating diseases. Mechanobiological signaling – a process, in which a mechanical stimulus leads to a biological effect, may be a novel factor to control clot lysis. Blood clots experience strain from different sources, for example from blood flow, from platelets, and when a clot retracts. It has also been suggested that fibrin fibers are prestrained as they formed across the ridges [23]. From a physiological point of view, clots lysis should be inhibited at the beginning of clot formation, right after an injury occurred, since that is when the clot is needed. In this situation, blood is still flowing, and the exerted strain from blood flow might prevent lysis. Once a clot is fully established and stabilized, wound healing commences, strain is reduced and at that point a clot may be broken down. Our findings on single fibers lysis agrees with the observation that stretched whole clots also lyse more slowly [12, 13].

**Molecular mechanism.** Fibrin fibers have high extensibility, meaning they can be stretched far before they break, and high elasticity, meaning they can be stretched far, and still return to their original shape [26, 27]. The molecular mechanisms for the high extensibility and elasticity are still not fully understood, but computer modeling experiments suggest that protein unfolding, such as the conversion of the α-helical coiled coils into β-strands may play a role [28, 29]. There are several molecular binding sites for
t-PA, plasminogen and plasmin on fibrin(ogen) molecules. There is a binding site for t-PA at γ312-324 and a plasminogen binding site at α148-160 in the D region of fibrin(ogen), and another set of t-PA and plasminogen binding sites in the αC-region at Aα392-610. Plasmin cleaves in the coiled coil, between residues β42-43 and β53-54 which is around the middle of the coiled coils [3]. There is evidence that during stretching, some of these binding sites unfold, which makes it harder for t-PA and plasmin to bind and cleave. Zhmurov et al. found that force-induced molecular elongation is followed by unfolding of the α-helical coiled coils and extension of γ-chain nodules (specifically residues γ309-326, which is the binding site for t-PA) [28]. Lim’s group showed that under stretching, fibrin(ogen) unfolding in the triple helical region, starts in the middle of the coiled coils, which is the region for plasmin cleavage [30]. Our data suggest that during stretching, binding sites for t-PA and plasmin(ogen) are destroyed (unfolded), which will slow down lysis.

_Cross-linking vs. stretching._ Factor XIII catalyzes the formation of γ-glutamyl-ε-lysine bonds (crosslinks) between the γ- and α-chains of adjacent fibrin molecules. Reciprocal γ–γ crosslinks form across abutting D-domains between γ-Gln 398 or 399 and γ-Lys 406. α–α crosslinks can form between several different glutamine donors in the flexible, unstructured α-C connector (α-Gln 221, 237, 328, 366) and several lysine acceptors in the globular α-C domain (α-Lys 418, 448, 508, 539, 556, 580, 601). Thus, one α-chain can interact with one or more adjacent fibrin molecules, leading to α-multimers [31-33]. These covalent bonds increase the stiffness of fibrin fibers and clots. In addition, they slow lysis. This phenomenon was observed at the whole clot level [11, 34] and also here at the single fiber level. Lysis (as measured by ΔL_lysis_) at the single
fibrin level decreases by a factor of 1.7 due to FXIII crosslinking. However, stretching has a significantly larger effect as it slows lysis by a factor of 5 times (uncrosslinked fiber, 2.5 fold stretch), and a factor of 3 (crosslinked fiber 2.1 fold stretch).

*Fiber internal structure and diffusion.* Fibrin fibers have a very open internal structure consisting of about 80% solvent (water) and only 20% protein. The pore size in fibrin fibers may be large enough for proteins, such as factor XIII, t-PA or plasmin, to move and diffuse inside a fiber [35, 36]. It may simply be the case that the pore size decreases as fibers are stretched, thus, slowing down movement of these proteins and lysis.

CONCLUSION

In this study, we used a stretchable substrate made from fugitive glue (stryrenic block copolymer) and a suspended fibrin fiber lysis assay to investigate the effect of stretching on single fibrin fibers lysis. Stretching significantly slows down lysis, this effect is more pronounced in uncrosslinked fibers than for crosslinked fiber. Moreover, stretching has a stronger effect than FXIII cross-linking. These results may due to a distorted alignment of plasmin binding sites and cleavage sites. Our results suggest that strain may be a novel mechanobiological factor that regulates fibrinolysis.

ACKNOWLEDGEMENT

We thank Dr. Zhong Fang for the help in colorimetric assay, thank Dr. Keith Bonin for the help in confocal microscope experiment.
REFERENCE


CHAPTER VII

CONCLUSION
For decades, much research attention has been directed towards cardiovascular disease (CVD) and diabetes, since they are the leading cause of death globally. When people have CVD, their blood vessels may be narrowed by plaques as cells, lipids and other material builds up in the vessel walls. This dramatically slows and alters the flow of blood, and when plaque ruptures or gets inflamed a blood clot may form at this site and may cause heart attack or stroke [1]. Fibrin fibers are the primary component of a blood clot, so the investigation of its properties is urgent and crucial. One goal of this dissertation is to reveal the changes in the mechanical properties of fibrin fibers from plasma of individuals with CVD or diabetes. The long term goal of this research is to provide possible future diagnostics for these diseases.

In healthy individuals, the two important processes, blood coagulation and blood dissolution, are at dynamic equilibrium. When a blood clot forms, it only forms locally and the fibrinolytic system is also activated to prevent the clot from uncontrolled growth, which maintains a proper rate of blood flow [2, 3]. In addition to the blood coagulation in this study, it is also equally vital to consider fibrinolysis. It is known that many factors affect lysis in blood [4-7], another goal of this dissertation is to demonstrate the effect of some factors on fibrinolysis. This will provide further understanding of the dissolution system.

FIBRIN FIBERS FROM CARDIOVASCULAR DISEASE (CVD)

Cardiovascular disease is associated with many factors involved in blood coagulation. Higher than normal fibrinogen concentration has been found in CVD patients [8-10], as well as altered fibrin clot structure. Fibrin clots from CVD patients
consist of smaller pores, higher fiber density, thinner fibers and have decreased permeability, as well as faster fibrin polymerization [11-13]. Besides structural information, we focused on mechanical properties of fibrin fibers from plasma of CVD patients, specifically extensibility, elasticity and modulus. To my knowledge, no other studies have been performed using single fibrin fiber from plasma, as other investigations utilize fibrin fiber from purified fibrinogen. Even though using purified fibrinogen gives the best experimental control, it lacks many native coagulation factors and molecules, since blood plasma is a complex and interactive system. This study involved three different groups, healthy middle age, healthy old people and old cardiovascular disease (CVD) patients (who also took aspirin). It is interesting note that fibrin fiber from older CVD individuals are 1.5 times more stretchable, and 1.4 times more elastic than those from both healthy middle aged and older people (Table III). Age did not have an effect on either extensibility or elasticity. No statistical difference in modulus was found among these three groups. However, we did discover that the modulus Y depends on fiber diameter D, and that modulus decreases as fiber diameter increases. For healthy groups, they have a similar slope of dependence of about -1.4 (Y ~ D^{-1.4}) on a log-log scale. While for the CVD group, it was surprising to see a different slope of -1.0. This slope difference demonstrates that there is a change in the internal structure of the fibrin fiber. All patients from the older CVD group were treated with Acetylsalicylic Acid (ASA), and ASA itself has an influence on blood clotting, resulting in larger porous clots with thicker fibers that are more susceptible to fibrinolysis [14-17]. In addition, the acetylation site interferes with normal cross-linking [18] causing structural change. So the cause of
the slope difference needs further study to separate CVD disease state from ASA presence, perhaps by utilizing a healthy subject group taking ASA.

<table>
<thead>
<tr>
<th>T-TEST(P value)</th>
<th>Healthy Middle Age</th>
<th>Healthy Middle Age</th>
<th>Healthy Old</th>
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</thead>
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<td>Healthy Old</td>
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<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Old CVD</td>
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<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Total Modulus</td>
<td>0.31</td>
<td>0.68</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table III: Significant difference among healthy people and CVD patient groups (P-value from t-test).

FIBRIN FIBERS FROM DIABETES DISEASE

Diabetes is a risk factor of CVD, with some studies showing that fibrin clots of diabetics have altered structural characteristics [19-22]. There are also conflicting studies showing no difference in structural and mechanical properties of clots in diabetic individuals [23]. Given this discrepancy, we set out to further investigate this relationship. In Chapter III, we used the same methodology for testing (combined atomic force microscope (AFM) / fluorescence microscope) as in the Chapter II CVD study. Fibrin fibers are formed from the plasma of female South African diabetes patients and fibrinogen glycation was measured as glycemic control. Extensibility, elasticity, relaxation time and modulus were measured in the study, there was no significant dependence on fibrinogen glycation for any of the tested physical properties of fibrin. However, we did observe a clear dependence between modulus and fiber diameter, with modulus decreasing exponentially (-1.6 power) as fiber diameter increased. Besides these fibrin fibers from plasma of diabetic patients, we also tested fibrin fiber from purified
fibrinogen. The same relationship was found between modulus and fiber diameter with an exponential power of -1.5. This finding serves as collaborative data of the non-uniform fibrin fiber internal structure, suggesting that fibrin fiber is not homogeneous. Consistent with this result, we see that the density of protofibrils and/or the density of protofibril connections within a fiber might decrease with increasing radius, thus we propose a model that fibrin fiber has a very densely packed core and less dense periphery due to the mechanism of twisting or diffusion-limited aggregation (Figure 35). This provides a new insight and model for fibrin fiber internal structure.

**Figure 35:** Schematic plot of internal fibrin fiber structure. (A) A fiber with a uniformly connected cross-section of protofibrils. (B) A fiber with a bicycle spokes-like cross-section. (C) A fiber with a dense core and less dense periphery (our proposed fibrin model).
INTERNAL STRUCTURE OF FIBRIN FIBER

For decades, researchers treated fibrin fiber as a homogeneous structure [24, 25]. However, more recent studies revealed that fibrin fibers doesn’t have a uniform cross-section density [26, 27]. Considering our findings from Chapter II and III, that the modulus is not constant with varying fiber diameter, we were intrigued by the internal structure of fibrin fiber. In Chapter IV, we used fluorescently labeled fibrinogen (with a specified ratio of fluorophore / fibrinogen molecules) to form fibrin fibers, so that the total intensity of the fiber should be proportional to the number of fibrinogen molecules. AFM is used to collect a scanning image providing an accurate fiber diameter, while intensity of the cross-section of the fiber is obtained from fluorescence signal. Interestingly, we found that the intensity (I) of the cross-section of the fiber increases as the fiber diameter (D) increases with a slope of 1.4 in log-log scale. As a homogeneous cylindrical structure should have a slope of 2 (I ~ π*D^2) (Figure 35), this suggests that the protofibril density decreases as fiber diameter increases. As the stretching force is proportional to the number of bonds connecting protofibrils together, from the results of CVD and diabetes studies in Chapter II and III (Y ~ D^{-1.5}), we see that the protofibril bonds decrease with increased fiber diameter. Taken together, we propose a model that fibrin fibers have a dense core of well-connected protofibrils that become less dense as more protofibrils aggregate onto the outside of the fiber. This finding provides quantitative and direct evidence of a heterogeneous fibrin fiber internal structure.
STRETCHING INFLUENCES FIBRINOLYSIS

Fibrinolysis is as important as coagulation in blood. It is a process that controls the rate and extent to which blood clots get dissolved. In previous CVD and diabetes studies, we observed that blood vessels of these patients were blocked by blood clots, so it is crucial to get a better understanding of the fibrinolytic system. It is known that many factors affect the fibrinolytic rate [4-7], and external tension may be one of them. Recently, studies showed that external force could directly hinder fibrinolysis [7, 28]. However, these experiments were all performed at the fibrin clot level, and nothing was known about the lytic rate of single fibrin fiber under tension (which is important, since a single fibrin fiber is the basic structure of fibrin clots). Due to its small size (diameter about a couple of hundred nm) and its tight packing into branchy meshes, few extant substrates can be used for stretching single fibrin fiber. In Chapter V, we invented a novel stretchable substrate made from fugitive glue. This kind of fugitive glue is very extensible (above 750% elastic limit), can be molded into different shapes, and is compatible with biological samples (cells and fibrin fibers). In our experiment (in Chapter VI), single fibrin fibers are formed on top of a striated stretchable substrate (molded with ridges and grooves). This substrate can be stretched to any desired length within 750%, meaning single fibrin fibers can be stretched to the same extent (since fibrin fibers are well attached on the two sides of ridges in the substrate). Interestingly, it was found that stretched single fibrin fibers lyse at a slower rate than unstretched fibers. Since FXIII effects fibrinolytic rate as well [4], we did a comparison experiment, observing that stretching single fibrin fibers slows down lysis more than FXIII crosslinking. This slower lytic rate may due to the destruction of binding sites for t-PA.
and plasminogen (enzymes for fibrin dissolution) during fibrin unfolding under stretch [29, 30]. Therefore, external tension is a crucial factor in fibrinolysis and needs to be well studied, as fibrin fibers (clots) naturally experience strain (shear force) by blood flow [31, 32]. This study provides a good foundation and novel result in this field (fibrinolysis).

FUTURE STUDY

There are some future studies can be done in consideration of these projects. In the CVD study, we demonstrated that fibrin fibers from CVD individuals (also taking ASA) are more extensible and elastic than healthy subjects and that they have altered fibrin structure. However, we could not separate changes due to CVD or ASA, as it has been established that acetylation sites interfere with normal fibrin cross-linking sites which may cause structural change. Further studies can be done on CVD patients without ASA treatment as well as healthy people taking ASA to determine how CVD/ASA alone affect fibrin structure, mechanical properties, and which might have greater influence. This will provide additional power for interpretation of our current study.

For both CVD and diabetes disease studies, we can perform additional experiments to strengthen statistical measures. It is also better to expand the sample type to different sexes and races, since we only tested white males from the United States in CVD, black female from South African in diabetes. This will give us a better and more general understanding of how these diseases affect fibrin fibers.

For the internal structure of fibrin fiber project, we can add more direct evidence regarding our proposed model that fibrin fiber has a dense core and less dense periphery. Turbidity (and other) measurements can be used to test the number of protofibrils across
different time points in fibrin fiber formation. This will provide clues regarding the relationship between the protofibril density and diameter, which we can use to fit to our proposed model that protofibril density decreases as fiber diameter increases. All these experiments will make our fibrin model more concrete and provide a better understanding of fibrin fiber structure.

For the fibrinolysis experiment, it was very interesting to discover that stretching slows down the lytic rate. We want to further understand the underlying mechanism of this phenomenon and test our speculation (the slower lytic rate is due to the denaturing of t-PA and/or plasminogen binding sites). It is known that fibrin fiber is extraordinary extensible and elastic [33], and fibrin starts to unfold in different regions under different extension [34]. Single fibrin fibers can first be stretched to different extents and then allowed to relax. Then, addition of t-PA and plasminogen to the system will test the lytic rate. This study will provide more information and further our knowledge about the fibrinolytic system.
REFERENCES


APPENDIX

EFFECT OF STRENUOUS EXERCISE ON THE MECHANICAL PROPERTIES OF FIBRIN FIBER AND HEMOSTATIC SYSTEM

Wei Li, Peter Brubaker, and Martin Guthold

Wei Li performed the experiment, analyzed the data and wrote the manuscript. Peter Brubaker provided plasma samples. Martin Guthold gave advisory and edited the manuscript.
ABSTRACT

Fibrin fiber is an important protein in blood, it plays a key role in stemming the blood flow and wound healing. Since it is also very crucial in blood coagulation and fibrinolysis pathway and since we have known that strenuous exercise induces the changes in hemostatic system, so we are really interested to see how fibrin fiber mechanical properties changes after acute exercise. That’s the first focus of this paper. Another attraction for us is that we would like to see the changes for the old cardiovascular disease (CVD) patients after strenuous exercise. We recruited five healthy young people and five old CVD patients, their blood were drawn right before and after 30-45 minute bout of aerobic exercise followed by 15-20 minutes of strength training exercises. It was revealed that extensibility of healthy young people decreased after acute exercise, while no consistent changes for old CVD patients. This may due to the elevated fibrinogen concentration and the changes of internal fibrin fiber structure. For the healthy people, their hemostatic system is in hypercoagulable state with coagulation and fibrinolysis both actively working (Activated partial thromboplastin time (aPTT) decreased significantly, Factor VIII and plasminogen were pronounced elevated after acute exercise). While for the old CVD patients, an activated coagulation pathway (Factor V and Factor VIII were elevated) and a less active fibrinolytic activity was observed after exercise. These results indicated that strenuous exercise may increase the risk for acute thrombotic events and CVD patients.
INTRODUCTION

It is known that hemostatic system is very important, since it regulate the coagulation and fibrinolysis, to keep the body balanced between antithrombotic and thrombotic status. Unbalanced system may lead to severe bleeding or thrombosis (risk of cardiovascular disease) [1, 2]. It is also important to notice that physical exercise leads to the activation of blood coagulation and fibrinolysis, and these changes depend on different exercise models, timelines and intensity [3-5]. Strenuous acute exercise creates a hyper-coagulable state in the blood [6]. This process might result in exercise-induced coronary ischemia. Among the healthy subjects, there will be a transient increase in blood coagulation after acute exercise, demonstrated as a shortened activated partial thromboplastin time (aPTT) [7, 8] and elevated level of Factor VIII [9, 10]. Strenuous exercise also induced the enhancement of fibrinolysis, shown as a rise in tissue-type plasminogen activator (t-PA) [11], decrease in plasminogen activator inhibitor (PAI) [12] and increase in clot degradation product [13].

For patients with coronary artery disease (CAD), several studies reveled that fibrinolysis activity was impaired, due to the increase of plasminogen activator inhibitor-1 (PAI-1) [14, 15]. Rehabilitative exercise for CAD patients has no detrimental effects on fibrin and plasmin formation (coagulation and fibrinolysis) [16]. While a submaximal exercise on a treadmill elevated thrombin formation in patients with peripheral arterial occlusive disease (PAOD) [17]. However, fewer experiments were done for the strenuous acute exercise for the cardiovascular disease (CVD) patients. So in this paper, we would like to reveal the changes of the hemostatic system for CVD patients.
Fibrin fiber is a key protein in blood, it plays an important role in intrinsic pathway. It is formed by its precursor fibrinogen under the activation of thrombin and cross-linked under the effect of Factor XIII [18]. Patients with CVD are associated with high level of fibrinogen [19], and the accumulated fibrin fibers entrap platelets to stem the flow of blood which eventually lead to coronary blockages and ischemic events. It is very extensible, for the purified uncross-linked fibrin fibers, they have an average breaking strain of 226 ± 52%, while 332 ± 71% for the purified cross-linked fibers [20]. It can be break down by an enzyme plasmin which is formed from its precursor plasminogen under the activation of t-PA. Since fibrin fibers play an important role in blood coagulation and fibrinolysis, it is interesting to see how their mechanical properties changes after acute exercise. In this paper, we used a combined atomic force microscope (AFM) and fluorescence microscope to test the extensibility of fibrin fibers before and after strenuous bout of exercise. This is the first time to combine mechanical changes in fibrin fiber properties and the underlying pathophysiology of blood coagulation pathway together, this gave a more thoroughly view. Further, it is of great interest to determine how factors such as age, disease status, and physical activity affect fibrin and blood coagulation.

MATERIALS AND METHOD

Participants

In this experiment, there involved two different groups: Healthy young group (YH) and Old cardiovascular disease group (OD). For YH group, five healthy young males (age 18-25) without any risk factors for cardiovascular disease were recruited. For
OD group, there are five older males (age 60 or older) with multiple risk factors for cardiovascular disease which include hypertension (3 participants), hyperlipidemia (2 participants), a first degree atrioventricular block (1 participant), an acute aortic dissection surgery (1 participant), and Type 2 diabetes mellitus (1 participant). Demographic data for these groups are shown in Table IV. None of these 10 participants exercised “regularly” (less than twice a week) and were not taking any abnormal medication that would affect blood clotting.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Age range (years)</th>
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<td>20-23</td>
<td>24.26</td>
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<td>Old, diseased (n=4)</td>
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<td>65-81</td>
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Table IV. Demographic data for each of the groups.

Exercise Procedure and Blood Processing

All participants performed a 30-45 minute bout of aerobic exercise, including treadmill jogging, walking, and biking. Followed by 15-20 minutes of strength training exercises of the upper and lower body using free weights and/or machines.

Right before and after exercise, blood from all the participants were drawn and stored in the tubes containing EDTA, then the blood was centrifuged in a Beckman model Tj-6 centrifuge at 3700 RPM for 15 minutes at room temperature. Some of the plasma was sent to Lab Corp. to test blood coagulation cofactors through Disseminated Intravascular Coagulation (DIC) Profile, Comprehensive Plus.
Samples Preparation

Substrate Preparation: Striated substrate for plasma samples was described previously [21]. A drop of optical adhesive (NOA-81, Norland Products, Cranbury, NJ) was placed onto the cover glass. A rectangular polydimethylsiloxane (PDMS) stamp (1cm x 1cm) was pressed into the adhesive to create a striated surface with 6.5 µm wide ridges and 13.5 µm wide grooves. Then it was cured under 365 nm UV light (UVP 3UV transilluminator, Upland CA) for 1.5 minutes.

Fibrin Fiber Formation: 2 µl of thrombin (Enzyme Research Laboratories, South Bend, IN, final concentration 0.1 NIH units/ml) was added to the mixture of 14 µl of plasma and 4 µl of 0.1 M CaCl$_2$ on the top of the striated substrate. After 1 hour reaction in a moist atmosphere at room temperature, the top skin of fibrin clot was removed by pipette tip, followed by rinsing with calcium free fibrin buffer (140 mM NaCl, 10 mM Hepes, pH 7.4), then the fibers were labeled with 20 nm carboxyl coated fluorospheres (Invitrogen, Carlsbad, CA), rinsed and kept with fibrin buffer (140 mM NaCl, 10 mM Hepes, 5 mM CaCl$_2$, pH 7.4).

Manipulation

We use a combined Atomic Force Microscope (AFM) and Fluorescence Microscope to test properties of fibrin fibers (Figure 36). The AFM (Topometrix Explorer, Veeco Instruments, Woodbury, NY) tip is located above the sample for manipulation, while the inverted fluorescence microscope (Axiovert 200 or Observer D, Zeiss, Göttingen, Germany) provides images from below. NanoManipulator software (3rd
Tech, Chapel Hill, NC) provides precise control of the AFM tip and collects force and position data during fiber manipulations.

**Figure 36.** Fibrin fiber manipulation. (A) Schematic of fibrin fiber manipulation. (B) Top view schematic of fiber manipulation. Schematic (A) and (B) adapted from [21]. (C) Optical microscopy pictures of a fiber being stretched and broken. The large dark object is the AFM cantilever and the AFM tip is marked by an asterisk). The fiber broke at strain of 180%.

**Statistical Analysis**

To determine statistical significance between samples, a two tailed t-test was used with an $\alpha$ level set at 0.05.
RESULTS AND DISCUSSION

Mechanical property of fibrin fiber is very important, since fibrin fibers play a key role in stemming blood flow and wound healing. It is known that exercise affect fibrinogen concentration, so in this paper we would like to see if mechanical property of fibrin fiber would be affected by exercise or not. We used a combined AFM and fluorescence microscope (described in Materials and Method) to investigate the extensibility of fibrin fibers formed from two different groups (Healthy young people and Old CVD patients).

There are five individuals in each groups, more than 20 measurements were taken for each sample. The average extensibility of healthy young people before and after taking acute exercise are 176% ± 8% and 151% ± 10% (Figure 37); Average extensibility of old CVD patients before and after acute exercise are 190% ± 26% and 199% ± 19%. (See in Table V) (Figure 38) For statistical analysis, we run t-test for all of these samples, it is shown that acute exercise has a significant effect on fibrin fiber mechanical properties of healthy young people (P=0.003); fibrin fiber extensibility decreases significantly after exercise by an average of 14%. However, we didn’t observe a significant difference among old CVD people (See in Table VI), the breaking strain of some old CVD patients decrease while some increase after acute exercise. This may due to the elevated fibrinogen level in CVD patients and the change of internal structure of fibrin fibers for CVD disease [22].
Figure 37. Extensibility data for five healthy young people. Blue refer to pre-exercise, red refer to post-exercise. Error bars are standard deviation.

Figure 38. Extensibility data for five old CVD patients. Blue refer to pre-exercise, red refer to post-exercise. Error bars are standard deviation.
### Extensibility

<table>
<thead>
<tr>
<th>Extensibility</th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Exercise (Healthy Young)</td>
<td>176% ± 8%</td>
<td>151% ± 10%</td>
</tr>
<tr>
<td>Acute Exercise (Old Disease)</td>
<td>190% ± 26%</td>
<td>199% ± 19%</td>
</tr>
</tbody>
</table>

*Table V.* Average extensibility data for each of the groups.

<table>
<thead>
<tr>
<th>T-TEST</th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Exercise (Healthy Young)</td>
<td></td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Acute Exercise (Old Disease)</td>
<td></td>
<td>0.315</td>
<td></td>
</tr>
</tbody>
</table>

*Table VI.* Statistical analysis (t-test) for each of the groups.

<table>
<thead>
<tr>
<th>Coagulation Factor</th>
<th>HY Pre</th>
<th>HY Post</th>
<th>Change (in %)</th>
<th>OD Pre</th>
<th>OD post</th>
<th>Change (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin</td>
<td>111</td>
<td>112</td>
<td>0.90</td>
<td>90.2</td>
<td>75.8</td>
<td>-15.96</td>
</tr>
<tr>
<td>Alpha 2 Antiplasmin</td>
<td>107.4</td>
<td>103</td>
<td>-4.10</td>
<td>84.8</td>
<td>85</td>
<td>0.24</td>
</tr>
<tr>
<td>Fibrinogen (in mg/dL)</td>
<td>248</td>
<td>243.8</td>
<td>-1.69</td>
<td>283.6</td>
<td>298.8</td>
<td>5.36</td>
</tr>
<tr>
<td>Platelets (in x10E3/dL)</td>
<td>308</td>
<td>389.75</td>
<td>26.54</td>
<td>246.8</td>
<td>248.6</td>
<td>0.73</td>
</tr>
<tr>
<td>INR</td>
<td>1.06</td>
<td>1.1</td>
<td>3.77</td>
<td>1.05</td>
<td>1.025</td>
<td>-2.38</td>
</tr>
<tr>
<td>Prothrombin Time (in s)</td>
<td>11.32</td>
<td>11.35</td>
<td>0.27</td>
<td>11.325</td>
<td>11.225</td>
<td>-0.88</td>
</tr>
<tr>
<td>aPTT (in s)</td>
<td>29.2</td>
<td>21</td>
<td>-28.08*</td>
<td>27.75</td>
<td>27.75</td>
<td>0.00</td>
</tr>
<tr>
<td>D Dimer (in ug FEU/mL)</td>
<td>0.28</td>
<td>0.28</td>
<td>0.00</td>
<td>0.6</td>
<td>0.64</td>
<td>6.67</td>
</tr>
<tr>
<td>Factor V</td>
<td>94</td>
<td>94</td>
<td>0.00</td>
<td>104.2</td>
<td>115.6</td>
<td>10.94*</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>121.4</td>
<td>360.8</td>
<td>197.2*</td>
<td>157.2</td>
<td>178.4</td>
<td>13.49</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>105.2</td>
<td>111.2</td>
<td>5.70</td>
<td>97.2</td>
<td>92.2</td>
<td>-5.14</td>
</tr>
</tbody>
</table>

*Table VII.* The mean blood coagulation values (from Dissiminated Intravascular Coagulation profile) for the healthy young (HY) male participants and the older, diseased (OD) before (pre) and after (post) exercise. Change, expressed in percentage, was calculated between pre and post values for both groups. Italicized values are lower than the normal range, and bolded values are above the normal range. Values with an asterisk (*) denotes significance (p<0.05)
Besides measuring mechanical property of fibrin fiber, we also tested the change in blood coagulation factors pre- and post-exercise. For healthy young (HY) subjects, activated partial thromboplastin time (aPTT) was found decreased significantly (P=0.003) after acute exercise. APTT is a maker commonly used in coagulation test, and is also a performance indicator measuring the efficacy of both the intrinsic and the common coagulation pathways [23]. In this test, aPTT decreased by an average of 2.95 s post exercise, these demonstrate that exercise-induced elevation of coagulation activity is more pronounced after strenuous exercise. Similar result (aPTT decreased) was also observed in healthy males after strenuous exercise [7, 22]. Factor VIII which is involved in intrinsic coagulation pathway was pronounced elevated by 197.2% (p = 0.01) post-acute exercise. It is a glycoprotein cofactor that serves as a critical component in the intrinsic blood coagulation pathway [24]. The level of factor VIII is also observed elevated up after strenuous short-term exercise in other literature [8]. These significant changes of these two factors demonstrate that the intrinsic coagulation pathway was at an increased level post-acute exercise.

For the HY group, the activation of the fibrinolytic system was also elevated after acute exercise, since plasminogen was observed increased. Plasminogen is a precursor of plasmin which initiates fibrinolysis, it is activated by tissue-typed plasminogen activator (t-PA) and then dissolve blood clots into degradation products [25]. So the increase of plasminogen signifies that fibrinolytic system was working to dissolve clots. The whole system is hypercoagulable state with both mechanisms (coagulation and fibrinolysis) working.
For the old diseased (OD) group, we saw that there is a great increase (10.94%) (P=0.01) in Factor V. Factor V in its activated form, FVa, is a critical cofactor for the protease FXa and accelerator of thrombin generation during fibrin clot formation [26]. Similar to HY group, the level of Factor VIII also increased after acute exercise. For the elevated level of Factor V and Factor VIII in the old CVD group indicate that the coagulation pathway is more active after strenuous exercise.

Different from HY group, the plasminogen of OD group decreased after acute exercise, and its level is lower than in the HY group. This reveals that fibrinolytic pathway is less active for the old diseased people. There is also an interesting change in OD group, antithrombin level decreased after acute exercise. It is the inhibitor of thrombin (thrombin initiates blood clotting), so the decrease of antithrombin elevated coagulation activity and also increased the level of fibrinogen, as we observed. It is known that the fibrinogen level are higher for the CVD patients than for the healthy people [19], as we can see in Table VII, but differently from HY group, fibrinogen level increased after strenuous exercise. This change along with the decrease of plasminogen indicate that after acute exercise OD group was in a hypercoagulation state, but a less active fibrinolytic activity, this makes acute exercise a risk factor for old CVD patients.
CONCLUSION

This work provided two new views of exercise-induced hemostatic changes. Firstly, it gave the result of how the hemostatic system changes for the old CVD patients after strenuous exercise. Secondly, this is the first time to combine mechanical properties of fibrin fiber and pathophysiology of blood coagulation pathway together. It makes the story more thoroughly. According to the interesting result, we can tell that acute exercise has effect on healthy young people, their fibrin fiber extensibility decreased by an average of 14% post-exercise. Activated partial thromboplastin time (aPTT) decreased significantly, Factor VIII was pronounced elevated and plasminogen was observed increased after acute exercise. These indicated the hemostatic system is in hypercoagulable state with coagulation and fibrinolysis both actively working. While for the old CVD patients, this is not a consistent change of fibrin fiber extensibility. After acute exercise, we saw an activated coagulation pathway (elevated level of Factor V and Factor VIII) after exercise, but less active fibrinolytic activity. So strenuous exercise may elevated the risk for acute thrombotic events and CVD patients.
REFERENCE


Diameter distribution of three groups

Figure 39. Diameter distribution with respect to Age and CVD. Average diameter of healthy middle age is 130 nm, healthy old is 140 nm, old CVD is 120 nm. We use 130 nm as the standard fiber diameter to get normalized total modulus for three groups.
Relationship of diameter and extensibility

![Graph showing the relationship between strain and diameter](image)

**Figure 40.** Maximum Strain versus Diameter. (88 data points) Since we knew that the modulus is dependent on the diameter of the fiber, here we want to know whether the extensibility depends on the diameter. We measure both the diameter of the fiber and the strain for extensibility. However, from the plot we see no significant relationship between diameter and extensibility.

**Fibrinogen concentration of each sample**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Healthy Middle Age (g/l)</th>
<th>Healthy Old (g/l)</th>
<th>Old CVD (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0.71</td>
<td>Sample 2</td>
<td>Sample 11</td>
</tr>
<tr>
<td>Sample 4</td>
<td>0.80</td>
<td>Sample 3</td>
<td>Sample 12</td>
</tr>
<tr>
<td>Sample 6</td>
<td>0.55</td>
<td>Sample 5</td>
<td>Sample 13</td>
</tr>
<tr>
<td>Sample 7</td>
<td>0.53</td>
<td>Sample 9</td>
<td>Sample 14</td>
</tr>
<tr>
<td>Sample 8</td>
<td>0.66</td>
<td>Sample 10</td>
<td>Sample 15</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.65</strong></td>
<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
</tr>
<tr>
<td>Sample 11</td>
<td>0.95</td>
<td>Sample 12</td>
<td>Sample 13</td>
</tr>
<tr>
<td>Sample 12</td>
<td>0.78</td>
<td>Sample 13</td>
<td>Sample 14</td>
</tr>
<tr>
<td>Sample 13</td>
<td>0.74</td>
<td>Sample 14</td>
<td>Sample 15</td>
</tr>
<tr>
<td>Sample 14</td>
<td>0.65</td>
<td>Sample 15</td>
<td>Sample 15</td>
</tr>
<tr>
<td>Sample 15</td>
<td>0.38</td>
<td>Sample 16</td>
<td>Sample 15</td>
</tr>
</tbody>
</table>

**Table VIII.** Fibrinogen concentration for each study group.

<table>
<thead>
<tr>
<th>T-TEST</th>
<th>Healthy Middle Age</th>
<th>Healthy Middle Age</th>
<th>Healthy Old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Old</td>
<td>Old CVD</td>
<td>Old CVD</td>
</tr>
<tr>
<td>T-TEST</td>
<td>0.63</td>
<td>0.23</td>
<td>0.43</td>
</tr>
</tbody>
</table>

**Table IX.** Statistical analysis among differing groups for CVD Study.
<table>
<thead>
<tr>
<th>Sample #11</th>
<th>Medication</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atenolol (50 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Benazepril (10 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Lipitor (40 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Aspirin (81 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Nitroglycerin (0.4 mg)</td>
<td>As needed</td>
</tr>
<tr>
<td></td>
<td>Prilosec</td>
<td>As needed</td>
</tr>
<tr>
<td></td>
<td>Celebrex (100 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td>Sample #12</td>
<td>Lipitor (10 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Aspirin (81 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Lisinopril (10 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td>Sample #13</td>
<td>Enalapril (5 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Toprol-XL (25 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Metformin (1000 mg)</td>
<td>Twice daily</td>
</tr>
<tr>
<td></td>
<td>Lipitor (40 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Glipizide (10 mg)</td>
<td>Twice daily</td>
</tr>
<tr>
<td></td>
<td>Aspirin (325 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td>Sample #14</td>
<td>Pravastatin (40 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>MTV</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Fish oil (1200 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Iron (65 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Aspirin (81 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Lisinopril (5 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Cosamin DS (1500/1200)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>St. John’s Wort (300 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Avodart (0.5 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Ginkgo biloba (60 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Ginseng (200 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td>Sample #15</td>
<td>Lipitor (10 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Toprol-XL (12.5 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Aspirin (325 mg)</td>
<td>Once daily</td>
</tr>
<tr>
<td></td>
<td>Synthroid (75 mg)</td>
<td>Once daily</td>
</tr>
</tbody>
</table>

Table X. The medication taken by five old cardiovascular patients.
Supplement

Incremental stress-strain curves

Figure 41 shows an example of an incremental stress-strain curve to determine the moduli and stress relaxation times. The fiber is stretched then held at a constant strain for a period of time before being pulled again. The process is repeated at higher and higher strains. The plots show that as a fiber is held at constant strain, the stress decays. This is indicative of viscoelastic behavior. The simplest mechanical model that can account for these observations (the two relaxation rates; stress does not decay to zero) is a generalized Kelvin model, consisting of an elastic spring with modulus $Y_\infty$, in parallel with two Maxwell elements consisting of a dashpot and a spring in series. For this model, the equation for stress relaxation becomes

$$\sigma(t) = \varepsilon_0 \left[ Y_\infty + Y_1 \cdot e^{-t/\tau_1} + Y_2 \cdot e^{-t/\tau_2} \right]$$

(1).

$Y_\infty$ is the relaxed elastic modulus, $Y_0$ is the total elastic modulus, $Y_0 = Y_\infty + Y_1 + Y_2$, and $\varepsilon_0$ is the strain at which the fiber is held. By fitting an exponential curve to the stress decay, we can determine key mechanical properties – the total modulus, the elastic modulus, and relaxation times of the fibers.
Figure 41. Incremental stress–strain curves and relaxation. (A) Strain versus time for an incrementally stretched fiber. The fiber was stretched to a given length and held at a constant strain while allowing the fiber to relax. The plateaus on the graph indicate where the AFM tip was stopped and the fiber was held at constant strain during the manipulation. (B) Stress versus strain for fibrin fibers that were pulled incrementally. The red curve is the raw data for the fiber, the black solid line approximates the total modulus determined by the stress before relaxation, and the black dashed line approximates the elastic modulus component of the total modulus. Exact values were determined by fitting the data with equation 1. (C) Stress versus time for an incrementally stretched fiber. Stress decays exponentially when the fiber is held at a constant strain, indicating viscoelastic behavior. (D) Example relaxation curve for fibrin fiber. The curve was fit with a double exponential function (equation 1) which produced two relaxation times. The fast relaxation time for this fiber was $\tau_1 = 2.1$ s and the slow relaxation time was $\tau_2 = 17.8$ s.
Relaxed Elastic Modulus

In addition to the total stretch modulus $Y_0$, which we report in the main manuscript, we also determined the relaxed, elastic modulus $Y_\infty$, from the fits of the incremental stress strain curves.

For all experiments, we found that $Y_\infty$ is typically about a factor of 0.6 smaller than $Y_0$, and that it shows the same behavior as $Y_0$. In particular, $Y_\infty$ does not depend on glycation and fibrinogen concentration diameter dependence as $Y_0$. $Y_\infty$ also decreases with increasing diameter, as $Y_\infty \propto D^{-1.5}$. The data for $Y_\infty$ are summarized in supplemental figure 42.

Glycation vs. Elastic modulus (Relaxed Modulus)
**Fibrinogen vs. Elastic modulus (Relaxed Modulus)**

![Graphs showing Fibrinogen vs. Elastic modulus](image)

**Relationship of diameter and elastic modulus (relaxed modulus)**

**Plasma sample**

![Plasma sample graph](image)

**Purified Fibrinogen sample**

![Purified Fibrinogen sample graph](image)

**Figure 42.** The relaxed, elastic modulus $Y_\infty$ as a function of glycation (no dependence), fibrinogen concentration (no dependence), and diameter (strong $D^{-1.5}$ dependence).
Scanning Electron Microscopy

Scanning electron microscopy was used to determine the fibrin structure of clots formed from purified fibrinogen of diabetic and control subjects. Clots were formed by addition of 0.5U/ml α-human thrombin and 3mM CaCl₂ to 1mg/ml fibrinogen in 0.15M NaCl, 0.05M Tris-HCl, pH 7.4 (final concentrations). Samples were prepared as described previously. Clots were observed and photographed digitally in many different areas, using a scanning electron microscope (XL 20, FEI, Hillsboro, Oregon, USA). Fiber diameters were measured from micrographs at 10,000 x magnification using ImageJ software (National Institutes of Health, USA). The thicknesses of at least 100 different fibers were measured per micrograph, with at least 6 micrographs imaged for each patient. Using scanning electron microscopy (SEM) on the plasma samples, we found that the average fibrin fiber diameter slightly decreases from about 105 nm to 85 nm (from fitted line) as the original plasma fibrinogen concentration increases from about 3.5 mg/ml to 5.5 mg/ml (Supplementary Figure 43). However the samples investigated by SEM were all formed from purified fibrinogen and investigated at 1 mg/ml.
Figure 43. Fiber diameter, as determined by SEM (N = 21, $R^2 = 0.1731$), slightly decreases with increasing original plasma fibrinogen concentration. However, all SEM measurements were done with fibrinogen at 1 mg/ml that was purified from the original plasma samples.

Diameter distribution

Figure 44. Fiber diameter distributions for each group. The red dot represents the average diameter, $D_{avg}$, for each group; uncontrolled diabetic, $D_{avg} = 130 \pm 4$ nm (N = 60); control group, $D_{avg} = 130 \pm 1$ nm (N = 56), controlled diabetic, $D_{avg} = 130 \pm 3$ nm (N = 45).
Statistical Analysis

We use Pearson’s correlation (testing linear relation) and Spearman’s correlation (testing monotonic relation) to test if there is relationship between two variables. Details are in the following table.

<table>
<thead>
<tr>
<th></th>
<th>Pearson’s correlation</th>
<th>Spearman’s correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycation vs. Total Modulus</td>
<td>-0.028</td>
<td>-0.007</td>
</tr>
<tr>
<td>Glycation vs. Normalized Total Modulus</td>
<td>-0.133</td>
<td>-0.174</td>
</tr>
<tr>
<td>Fibrinogen Concentration vs. Total Modulus</td>
<td>0.065</td>
<td>0.046</td>
</tr>
<tr>
<td>Fibrinogen Concentration vs. Normalized Total Modulus</td>
<td>0.018</td>
<td>0.103</td>
</tr>
<tr>
<td>Glycation vs. Extensibility</td>
<td>-0.072</td>
<td>-0.095</td>
</tr>
<tr>
<td>Fibrinogen Concentration vs. Extensibility</td>
<td>0.161</td>
<td>0.143</td>
</tr>
<tr>
<td>Glycation vs. Fast Relaxation Time</td>
<td>0.258</td>
<td>0.018</td>
</tr>
<tr>
<td>Glycation vs. Slow Relaxation Time</td>
<td>-0.065</td>
<td>-0.042</td>
</tr>
<tr>
<td>Fibrinogen Concentration vs. Fast Relaxation Time</td>
<td>-0.229</td>
<td>-0.418$^*$</td>
</tr>
<tr>
<td>Fibrinogen Concentration vs. Slow Relaxation Time</td>
<td>-0.251</td>
<td>-0.151</td>
</tr>
</tbody>
</table>

**Table XI.** Statistical analysis of fibrin mechanical properties with fibrinogen glycation.

<table>
<thead>
<tr>
<th></th>
<th>Pearson’s correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter vs. Total Modulus (Plasma sample)</td>
<td>-0.513</td>
</tr>
<tr>
<td>Diameter vs. Total Modulus (Purified Fibrinogen)</td>
<td>-0.405</td>
</tr>
</tbody>
</table>

**Table XII.** Statistical analysis of fibrin fiber diameter and modulus.
Pearson’s correlation is testing linear relationship. 0.00 to 0.19 (-0.00 to -0.19) “No or negligible relationship”. -0.4 to -0.69 “Strong negative relationship”

Spearman’s correlation is testing if one variable is monotonic function of the other. 0.00 to 0.19 “very weak relation”

*Spearman’s correlation R value of “Fibrinogen concentrate vs. Fast Relaxation Time” is not a small value, maybe can use a monotonic function to fit it. However, from Pearson’s correlation and from the real plot (the trend), we consider there is no significant relationship between fibrinogen concentrate and fast relaxation time.
Mechanical properties as a function of glycation

**Figure 45.** Color-coded mechanical properties as a function of glycation. Yellow – uncontrolled diabetic; purple – controlled diabetic; red – control group.
Mechanical properties as a function of fibrinogen concentration

**Figure 46.** Color-coded mechanical properties as a function of fibrinogen concentration. Yellow – uncontrolled diabetic; purple – controlled diabetic; red – control group.
Total Modulus as a function of fiber diameter

\[ y = -1.6179x + 3.8116 \]
\[ R^2 = 0.26279 \]

**Figure 47.** Color-coded total modulus as a function of fiber diameter. Yellow – uncontrolled diabetic; purple – controlled diabetic; red – control group.
Supplement

Dry Electrospun fibrinogen fiber (with Rhodamine 6G)

**Figure 48.** Light intensity of electrospun fibrinogen fiber cross section as function of diameter. (A) and (C) show the slope of the relationship between light intensity and fiber diameter in log-log scale. (B) and (D) show that fibrinogen molecule density is independent of fiber diameter. Each data point represents four measurements.
Figure 49. Light intensity of fibrin fiber (in buffer) cross section as function of diameter. (A) and (C) show the slope of the relationship between light intensity and fiber diameter in log-log scale. (B) and (D) show the slope of the relationship between protein density and fiber diameter in log-log scale. Each data point represents four measurements.
Dry fibrin fiber (with Alexa 546)

For the dry fibrin fiber (dried with Nitrogen), the averaged slope of plot fiber diameter vs. light intensity is 1.21±0.14 (1.24, 1.17, 1.23).

**Figure 50.** Light intensity of dry fibrin fiber cross section as function of diameter. (A), (B) and (C) show the slope of the relationship between light intensity and fiber diameter in log-log scale. Each data point represents four measurements.
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PUBLICATIONS:

“Highly Stretchable, Biocompatible, Striated Substrate Made from Fugitive Glue”.
Materials, 8, p3508-3518
Manuscripts Submitted:

Li, W., Sigley, J., Pieters, M., Helms, C., Nagaswami, C., Weisel, J., Guthold, M. “Fibrin Fiber Stiffness Is Strongly Affected by Fiber Diameter, but not by Fibrinogen Glycation”

Li, W., Baker, S., Brubaker, P., Guthold, M. “The Role of Cardiovascular Disease in Fibrin Fiber Mechanical Properties.”

Li, W., Lucioni, T., Li, R., Guthold, M. “Stretching Single Fibrin Fiber Slows Down Lysis More Than FXIII Crosslinking”

Manuscripts in Preparation:

Li, W., Sigley, J., Guthold, M. “Investigation of Fibrin Fiber Internal Structure: Protein Density and Bond Density Decrease with Increasing Diameter”

Li, W., Brubaker, P., Guthold, M. “Effect of Strenuous Exercise on the Mechanical Properties of Fibrin Fiber and Hemostatic System”