THE MECHANICAL PROPERTIES OF INDIVIDUAL ELECTROSPUN FIBERS
AND FIBRIN FIBERS

BY

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LIST OF ABBREVIATIONS

AFM  Atomic Force Microscope
ECM  Extracellular Matrix
PDMS Polydimethylsiloxane
SEM  Scanning Electron Microscope
TEM  Transmission Electron Microscope
CD   Circular Dichroism
Ca   Calcium
Na   Sodium
Cl   Chlorine
HEPES 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
FXIII Coagulation Factor XIII
HFP  1,1,1,3,3,3-Hexafluoro-2-Propanol
MEM  Minimum Essential Medium
γ    Gamma
α    Alpha
β    Beta
Q    Glutamine
N    Asparagine
K    Lysine
R    Arginine
CVD  Cardiovascular Disease
cd   Controlled Diabetes
ud  Uncontrolled Diabetes

c  Control

CN/DAB  Chloronaphthol/3,3'-Diaminobenzidine
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Nanofibers play important mechanical roles in the body. Collagen, the most abundant protein in humans, forms fibers which comprise part of the extracellular matrix and tissues such as bone, cartilage and blood vessels, to name a few. Fibrinogen, a plasma protein, is converted into fibrin fibers which form the mechanical and structural support for blood clots. Biomimetic nanofibers can be synthesized outside of the body through the process of electrospinning. The mechanical properties of electrospun collagen and fibrinogen fibers are important because of their potential for tissue engineering, due to their biocompatibility. We studied the mechanical properties of individual electrospun collagen and fibrinogen fibers using a combined atomic force microscopy and optical microscopy technique. Using the same technique we also studied the mechanical properties of individual fibrin fibers, as the mechanical properties of blood clots have been shown to be related to diseases and disorders yet the mechanisms responsible for their mechanical properties are relatively unknown.

Electrospun type I collagen and electrospun fibrinogen fibers both showed viscoelastic properties. The extensibility of electrospun fibrinogen in buffer is 130% and its average modulus is 10 MPa. Electrospun collagen in ambient conditions has an
extensibility of 33% and a bending modulus of 7.5 GPa. Both fibers show strain
softening, however, collagen shows extreme strain softening and therefore only a bending
modulus is given.

Native crosslinked fibrin fibers have similar properties to electrospun fibrinogen
fibers. Crosslinked fibers have an extensibility of 130% and a modulus of 14 MPa. Uncrosslinked fibers are more extensible at 226% strain and softer with a modulus of 4 MPa. Crosslinked fibers rupture more often than crosslinked joints while uncrosslinked fibers rupture less often than uncrosslinked joints. Fibrin fibers with only alpha crosslinks are as extensible as uncrosslinked fibrin fibers but have a modulus in between crosslinked and uncrosslinked fibrin, of 10 MPa. Alpha crosslinked fibrin fibers have a larger elastic limit, 95% strain, than both uncrosslinked and crosslinked. Lastly, individual fibrin fibers from plasma samples were studied. Fibers from patients with type 2 diabetes show no difference in modulus or extensibility when compared with control patient samples.
CHAPTER I

INTRODUCTION
Nanoscience and nanotechnology have emerged as powerful fields throughout the past few decades, in part because of the unique physical properties and biological significance of nanoscale materials \(^1-3\). Physically their surface to volume ratio makes them ideal candidates for high-capacity binding applications. Biologically fibers such as collagen, fibronectin and other extracellular matrix (ECM) components occur naturally at the nanometer size range and inside the cell, microtubules and intermediate filaments range in diameter from 25 nm to 10 nm, respectively \(^4\). During blood coagulation fibrin, a main constituent of blood clots, form fibers in the range of 20 to 100 nm \(^5,6\). Nanofibers often play the role of a scaffold for cell adhesion, proliferation and differentiation, as well as, provide mechanical strength and support to the tissues and clots they help constitute. Because of the biological importance of nanofibers, here we report a study of the mechanical properties of individual nanofibers.

It is possible to study the mechanical properties of fibers in bulk and experiments have determined the creep, storage modulus, \(G'\), and loss modulus, \(G''\) of fibrin clots \(^7,8\) as well as network architecture, modulus and viscosity for collagen networks \(^9,10\), to name a few. However, the size of the individual fibers has made it difficult to study their mechanical properties at the individual fiber level. But it is exactly this information that is needed to understand and model the properties of the bulk fiber network. In modeling a fiber network one must know properties of the individual fibers composing the network, properties of the joints or branch points of the fibers and the overall architecture of the network \(^11-14\).

Recently much effort has been put forth to elucidate the mechanical properties of individual nanofibers. Studies on electrospun fibers have determined their bending
modulus and extensibility \(^{15-17}\); while studies on individual fibrin fibers have determined their modulus at low strains, extensibility and elastic limit \(^{18,19}\). We have worked in concert with these efforts to expand the knowledge of single fiber mechanical properties using a combined atomic force microscope (AFM) and inverted optical microscope. Our technique offers advantages over other single fiber methods, such as three point bending and optical tweezing, by offering a large range of applicable forces (\(10^2\) nN to \(10^4\) nN), by providing the ability to view the manipulation process and by allowing manipulations distances up to 100 μm. Using this combined microscopy technique, we have studied the mechanical properties of individual fibers electrospun from biological proteins, whose purpose is to mimic the ECM in tissue engineering; as well as, the properties of individual fibrin fibers in both native and variant forms, to advance the understanding of blood coagulation.

**ELECTROSPUN COLLAGEN AND FIBRINOGEN**

Electrospinning is a fiber production process capable of producing fibers in the length range of 10 nm to 10 μm. Patented in 1934 for textile use, electrospinning has recently received renewed interest, as electrospun nanofibers may be ideal candidates for applications such as biocatalyst\(^{20}\), high efficiency filtration\(^{21,22}\), composite materials\(^{23-25}\), and tissue engineering\(^{26-28}\). In electrospinning, fibers are formed from a solution consisting of a highly concentrated polymer and volatile solvent. The polymer solution is loaded into a syringe, charged to a high voltage and pumped from the syringe towards a grounded collector plate. The electric field, between the syringe and the collector, and the surface tension of the solution apply opposing forces to the liquid being expelled from
the syringe. When the force of the electric field exceeds the surface tension of the solution a Taylor cone is formed and the charged, volatile solvent is expelled from the tip in a very small stream\textsuperscript{29}. The electric field also acts to orient the individual molecules in the jet\textsuperscript{15}. As the jet approaches the grounding plate instabilities lead to whipping, bending and thinning of the jet\textsuperscript{30,31}, ultimately lowering the diameter of the fiber. For a schematic of the electrospinning setup see figure 1.

\begin{figure}[h]
  \centering
  \includegraphics[width=\textwidth]{electrospinning_setup.png}
  \caption{A schematic of the electrospinning setup. At the top a syringe pump is used to regulate the flow of the polymer solution (green) from the syringe. The needle of the syringe is charged to a high potential using a voltage supply. At the bottom left, the fibers being produced from the syringe are collected onto the grounded ridges.}
\end{figure}
The small size of the fibers leads to a high surface to volume ratio making electrospun fibers an ideal candidate for applications dependent on physical contact. In tissue engineering the surface area becomes important for cell seeding and proliferation while the diameter of the fibers mimics that of native ECM. We chose to study electrospun collagen and fibrinogen because of their potential for use in tissue engineering. The advantage of forming fibers from biological materials, such as fibrinogen, is that they have low immunogenicity, do not cause inflammation and do not result in toxic degradation products. Previous work seeding cells on electrospun collagen and fibrinogen scaffolds have shown fibroblast migration, fibroblast deposition of collagen and myotube formation.

While the biocompatibility of the material is of great importance in tissue engineering, the mechanical properties of the materials must also be considered. The mechanical properties of individual electrospun fibers and the scaffold they form are of great importance to cell differentiation and the mechanical response of the engineered tissue. The tissue must be able to withstand handling and implantation by the doctor as well as perform the desired mechanical task, such as the case of blood vessels which must hold up against blood pressure yet be compliant enough to bend around joints. As mentioned previously the mechanical properties of a network are dependent upon the individual fiber properties, the properties of the branch points and the network architecture. Matthews et al addressed the role of network architecture by showing the mechanical properties of electrospun mats are dependent on the alignment of the fibers. Because of the effect fiber alignment has on the mechanical properties of electrospun mats, much research has been conducted to control the alignment of electrospun fibers.
Our aim is to address the mechanical properties of individual electrospun fibers. Chapters 2 and 3 report the mechanical properties of individual electrospun collagen and fibrinogen fibers. In addition to their mechanical properties, Chapter 8 gives an assessment of their similarities and differences, as well as a comparison of the electrospun fibers to their native counterparts.

FIBRIN FIBERS

Fibrin fibers are a main constituent of blood clots, which perform the mechanical task of stemming the flow of blood. Fibrin monomers which assemble into fibers are formed from fibrinogen, a highly abundant plasma protein consisting of six polypeptide chains; 2 $\alpha$, 2 $\beta$ and 2 $\gamma$ chains which are bound together by 29 disulfide bonds. Fibrinogen has a tri-nodular shape, with two nodules (the D-domains) at either end and one nodule (the E-domain) in the center. The nodules are connected by two 17 nm long coiled coils consisting of three alpha helices coiled around one another. Fibrinogen is approximately 45 nm in length and 4.5 nm in diameter (Figure 2). In the event of injury to the vasculature, activated thrombin cleaves two fibrinopeptides A (16 residues) and two fibrinopeptides B (14 residues) from the amino termini of the $\alpha$ and $\beta$ chains, thus converting fibrinogen into fibrin. This cleavage exposes charge residues in the central E-domain of the monomer which interact with the D-domain of an adjacent monomer initiating aggregation.
Fibrin polymerizes spontaneously into a meshwork of fibers which comprises the structural component of a blood clot. Fibrin monomers arrange themselves in a half staggered orientation during fiber formation creating a 23nm banding pattern visible through transmission electron microscopy (TEM). Ryan et al proposed that the organization of fiber formation is dependent on the rate of fibrin production, monomer cleavage. She concluded, at high rates monomers join locally to from oligomers, an aggregate of only a few fibrin monomers, and protofibrils, many monomers formed in a double strand, which then interact laterally and longitudinally to form thin, highly branched fibers. At low rates of cleavage, Ryan argues monomers aggregate independently onto forming fibers and protofibrils, resulting in thicker less branched fibers. Weisel et al. showed the radial growth of fibrin fibers is limited by the inherent twist of the fibers, the diameter of fibrin continues to increase until the energetic cost of
stretching the protofibril to align with the twist is greater than that of the protofibril binding

Clotting Factor XIIIa, a transglutaminase, adds additional stabilization to fibers through covalent crosslinks between alpha chains and gamma chains of adjacent molecules. Crosslinking has been shown to stiffen clots and reduce the rate of fibrinolysis, the break down of a clot. Gamma-gamma and alpha-alpha crosslinks are dominant, however, gamma-alpha crosslinks are known to also form. Gamma dimers are the first to form followed by alpha multimers and lastly gamma trimers and tetramers. Gamma dimers are formed through the interaction of two adjoining D-domains, glutamine 398 or 399 and lysine 406 on the gamma chain are crosslinked in antiparallel fashion to the reciprocal molecule. Alpha crosslinks, in contrast, have many donors and acceptor sites on the alpha chain, of which some possibilities are lys 556, 580, 539, 508, 418, 448, 601, 606, 427, 429, 208, 224, 219 and gln 221, 237, 328, 366. One alpha chain can interact with multiple fibrin molecules, leading to alpha multimer formation through a partial or full complement of alpha crosslinks. Lastly, gamma tetramers and trimers form, possibly at fiber branch points, and have been shown to further decrease fibrinolysis.

The covalently crosslinked branched network of fibrin fibers supplies structural and mechanical support for the blood clot. The specificity of fibrin for its blood coagulation function is evident through diseases related to mutations and truncations of the fibrin monomer. Changes can lead to delayed aggregation as in Fibrinogen Genova, stiffening of clots as in Fibrinogen Dusart, or decreased clot lysis as in AαR16C (Fibrinogen Hershey III). The mechanical properties of blood clots have been

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related to clotting disorders and diseases such as hemorrhaging, thrombosis, myocardial infarction and stroke \cite{12,56}. For example, myocardial infarction can be linked to an increased storage modulus, $G'$ \cite{11,56-58}, and increased clot stiffness and the rate of fibrinolysis has been shown to differ in patients with coronary artery disease \cite{59}.

Macroscopic, mechanical properties of whole clots have been studied for decades and are fairly well known. Creep as well as the storage modulus, $G'$, and loss modulus, $G''$, have been measured under different conditions \cite{7,8}. It has also been shown that cross-linking affects the mechanical properties of the whole clot by increasing stiffness (i.e. $G'$) and reducing creep \cite{8}. Although macroscopic properties have been measured, the mechanisms responsible for the mechanical properties of blood clots are poorly understood \cite{60}. For example, calculations to determine branch point density of a fibrin clot from its stiffness are off by approximately 6 orders of magnitude \cite{61}. The mechanical properties of individual fibrin fibers, constituents of blood clots, have not been detailed. Our goal is to determine the properties on individual fibrin fibers. Through the study of individual fibrin fibers more insight into the mechanical properties of whole clots can be obtained as well as insight into the fibrin monomer.

Chapters 4 and 6 report the mechanical properties of individual crosslinked, uncrosslinked and crosslink variant fibrin fibers, formed from purified fibrinogen. Chapter 5 presents the properties of fibrin fiber branch points; and Chapter 7 presents the properties of fibrin fibers formed from plasma samples from patients with type 2 diabetes.
METHODS

A combined atomic force/optical microscopy technique was used to manipulate and extract the mechanical properties of the individual fibers. Fibers were prepared on a striated surface to eliminate frictional forces, an AFM cantilever was used for lateral manipulation of the fibers and an optical microscope was use to visualize the manipulation. Viscoelastic properties of the fibers were determined from stress-strain and stress-time manipulation curves. An overview of the methods and materials can be found below; extended details on the methods and materials particular to each fiber type can be found in the chapters to follow.

Sample Preparation

The surface was prepared using a soft lithography micromoulding technique, which creates a sample surface using a stamp and optical adhesive. Stamps were used to produce a striated pattern of 8 μm wide ridges and 12 μm wide and 6 μm deep grooves on a microscope cover slide. For the formation of electrospun fibers, the cover slide was placed in direct contact with the ground of the electrospinning apparatus and fibers were spun directly onto the prepared cover slide. For the formation of native, variant and plasma fibrin fibers, thrombin was added to the cover slide and the fiber polymerization reaction took place directly on top of the striated surface. The fibers were labeled by adhesion with fluorescent beads for visualization.
The sample was sandwiched between the AFM and the inverted optical microscope on a custom built stage that allowed independent movement of the objective, sample and the AFM cantilever. We used the AFM cantilever to manipulate, obtain force measurements and measure the radius of the fibers. We used the optical microscope to view and acquire movies and images of fiber manipulations and determine the method of fiber rupture.

For manipulation, we positioned the tip of the cantilever in a groove next to a fiber which suspended the groove onto the ridge and was aligned perpendicular to the ridges and grooves. The cantilever tip was raised in the z-direction until it was 1 μm below the top of the fiber. This gave a well defined cantilever moment arm and maximized the torque sensitivity of the cantilever while minimizing the occurrence of the fiber slipping off the tip. The cantilever was controlled by the NanoManipulator system (3rd Tech, Chapel Hill, NC), to laterally push and stretch the fiber (Figure 3). Optical images were used to determine the strain at which the fiber visibly deformed, that the fiber stayed anchored on the ridge and if the fiber ruptured along the length of the stretched fiber. Data acquired from fibers which did not stay anchored to the ridges were discarded.
AFM Lateral Force Acquisition and Calculation

During manipulation, the lateral force applied to the fiber caused a deflection in the left-right position of the laser in the photodiode (Figure 4). We converted the laser deflection, $I_l$, into lateral force, $F_l$, by multiplying it with the lateral force conversion factor $K_C$;

$$F_l = K_C \cdot I_l$$

$K_C$ can be determined via cantilever beam mechanics\textsuperscript{63-65},

$$K_C = \frac{Ew t^3}{6l^2 (h + t/2)} \cdot S_n$$

where $E$, $w$, $t$, and $l$ are the Young’s modulus, the width, the thickness and length of the cantilever, $h$ is the height of the tip. $S_n$ is the ‘normal sensor response’, i.e. the conversion of normal cantilever deflection to top – bottom photocurrent. The resonance frequency of the cantilever was used to determine the cantilever thickness via,
\[ f = 0.276 \cdot \frac{Ewt^3}{\sqrt{\rho (\pi \cdot h^3 \cdot l^3 + 0.2832wt^4)}} \]

where \( \rho = 2330 \text{ kg/m}^3 \) is the density of silicon. The error in \( K_C \), as determined from beam mechanics, can be estimated from the uncertainties in the quantities: l, 2%; w, 2%; t, 10%; h, 10%; and \( S_n \), 10%, yielding an overall error in \( K_C \) of about 56%. This method of determining \( K_C \) was double-checked with the glass fiber calibration method, in which \( K_C \) is determined by bending a small glass fiber with known dimensions and Young’s modulus. The error in \( K_C \) as calculated by the glass fiber calibration method was about 26%.

We used the AFM in topographical imaging mode to obtain the radius of the fiber on the ridge. Stress strain curves were obtained where stress is defined as

\[ \sigma = \frac{F}{A} \]

where \( F \) is the applied force and \( A \) is the cross-sectional area of the fiber. We used the cross-section of the initial, unstretched fiber in our calculations, i.e. engineering stress. We used engineering strain defined as

\[ \varepsilon = \frac{\Delta L}{L_{\text{init}}} \]

where \( \Delta L = L' - L_{\text{init}} \) is the change in fiber length; \( L' \) and \( L_{\text{init}} \) are the lengths of the stretched and unstretched fiber, respectively. Another strain measurement, true strain, could also be used and is defined as

\[ \varepsilon_{\text{true}} = \ln(1 + \varepsilon) \]
Figure 4 Schematic view of the AFM laser reflecting off the cantilever and hitting the photodiode. (A) The alignment of the laser on the photodiode before contacting the fiber. (B) The alignment of the laser on the photodiode after contact of the cantilever with the fiber. The force of the fiber causes the cantilever to twist resulting in a changed left-right photocurrent.
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CHAPTER II

THE MECHANICAL STRESS-STRAIN PROPERTIES OF SINGLE ELECTROSPUN COLLAGEN NANOFIBERS.

Christine R. Carlisle, Corentin Coulais, Martin Guthold

The following manuscript is in press in Acta Biomaterialia, 2010, and is reprinted with permission. Stylistic variations are due to the requirements of the journal. C.R. Carlisle performed all the experiments, analyzed the data and prepared the manuscript. C. Coulais helped determine the electrospinning parameters and collect the electrospun collagen samples. M. Guthold acted in an advisory and editorial capacity during data collection, analysis and manuscript preparation.
ABSTRACT

Knowledge of the mechanical properties of the electrospun fibers is important for their successful application to tissue engineering, material composites, filtration and drug delivery. In particular, electrospun collagen has great potential for biomedical applications due to its biocompatibility and promotion of cell growth and adhesion. Using a combined atomic force/optical microscopy technique, we determined the single fiber mechanical properties of electrospun collagen type I. The fibers were electrospun from a 80 mg/ml collagen solution in 1,1,1,3,3,3-hexafluoro-2-propanol and collected on a striated surface suitable for lateral force manipulation by the AFM. The small strain modulus, calculated from three point bending analysis, was 2.82 GPa. The modulus showed significant softening as strain increased. The average extensibility of the fibers was 33% of their initial length and the average maximum stress (rupture stress) was 25 MPa. The fibers displayed significant energy loss and permanent deformations above 2% strain.

INTRODUCTION

Collagen type I is a common element of the extracellular matrix (ECM) and plays an important structural role in the body providing strength and support to the aorta, skin, bones, ligaments and tendons, to name a few. The fundamental unit of collagen type I is composed of a triple helix consisting of three polypeptide chains, two $\alpha_1$(I) collagen chains and one $\alpha_2$(I) collagen chain, held together by hydrogen bonds and disulfide bonds. It has a length of approximately 300 nm and a diameter of 1.5 nm. These collagen triple helices aggregate to form collagen fibrils with an average diameter
between 50-200 nm and quarter stagger molecular arrangement resulting in a 67nm banding pattern or D-periodicity. The banding pattern can be seen under high resolution transmission electron microscopy \(^6\). Mechanical support, helping to resist tissue strain, is provided to adjacent collagen molecules by enzyme mediated cross-linking \(^7\). Natural collagen structures show a range of biomechanical properties from those of bone with a modulus of 17.2 GPa \(^8\) to that of skin with a modulus of 4 GPa \(^3\).

Aside from naturally formed collagen fibers, collagen fibers can also be produced through the process of electrostatic spinning. In this procedure, fibers are formed from a highly concentrated polymer in a volatile solvent. The solution is charged to a high voltage and pumped from a syringe towards a grounded collector plate. The electric field, between the syringe and the collector, and the surface tension apply opposing forces to the liquid as it is expelled from the needle. When the force of the electric field exceeds the surface tension a Taylor cone is formed and the charged, volatile solution is expelled from the tip in a very small stream \(^9\). As the jet approaches the grounding plate, instabilities in the jet lead to whipping, bending and thinning of the jet \(^10,11\), ultimately the diameter of the fiber is lowered to a value between 10 nm and 10 um. Experimental parameters, such as working distance, polymer concentration, voltage and flow rate, can be altered to produce fibers of desired diameters and porosity \(^12\).

The ease with which electrospun fibers can be produced makes them an ideal candidate for material composites and biomedical engineering applications, such as tissue engineering scaffolds, wound dressings, coatings, or drug delivery vehicles \(^12\). The diameter of electrospun fibers mimics that of the natural ECM and other biological fibers. Electrospun collagen, in particular, has great potential in scaffold engineering because
biomechanical structures formed from collagen have been shown to supply a substrate for cell adhesion improving cell growth and differentiation\textsuperscript{13}, while the helicity and rigidity of the collagen molecule supply strength\textsuperscript{14}. Scaffolds constructed from collagen blends have been studied in vitro for vascular graft applications\textsuperscript{15} and have shown potential as a treatment for skeletal muscle tissue defects\textsuperscript{16}.

The mechanical properties of biomedically engineered devices are very important to their function. First, the mechanical properties of the substrate affect cell differentiation\textsuperscript{17}. Second, the scaffold must have similar properties to the natural tissue it is replacing so that it can perform the function of the tissue. Finally, scaffolds must have the mechanical stability to handle manipulation by the physician during implantation as well as support tissue regeneration and structure degradation\textsuperscript{18}.

Characteristics such as orientation, density and mechanical properties of the constituent fibers determine whether the scaffold will have the desired mechanical properties. Techniques using rotating and split electrodes collectors have been used to orient fibers\textsuperscript{19-22}; and tests on oriented electrospun mats have shown that fiber orientation affects collagen matrix properties\textsuperscript{23}. Here we examine the properties of the constituent of the matrix, the individual electrospun fibers. Recently, the bending modulus of individual electrospun collagen type I fibers has been determined\textsuperscript{24}. In the present study we expand on this knowledge of the mechanical properties of individual electrospun collagen type I fibers by determining the strain softening behavior, extensibility, maximum stress, energy loss and deformation characteristics using lateral force atomic force microscopy (AFM).
MATERIALS AND METHODS

Substrate Preparation

The substrate was prepared using a soft lithography and MIMIC (micromoulding in capillaries) technique\textsuperscript{25}. A SU-8-silicon master grid with 12 \( \mu \)m wide and 6 \( \mu \)m deep channels and 8 \( \mu \)m wide ridges was used to create a PDMS (polydimethylsiloxane) stamp by pouring dimethylsiloxane plus catalyst (Sylgard, Dow Corning Corp., Midland, MI) onto the grid and curing the PDMS at 70\(^{\circ}\)C for one hour. A striated surface was formed on the top of a 60 x 24 mm, #1.5, microscope cover by pressing the PDMS stamp into a 10 \( \mu \)l drop of Norland Optical Adhesive-81 (NOA-81, Norland Products, Cranbury, NJ). The optical adhesive was cured for 70 seconds, with UV light (365 nm) (UVP 3UV transilluminator, Upland, CA) (Figure 5).

Electrospinning

A polymer solution, comprised of collagen type I, acid soluble from calf skin at 80 mg/ml (Elastin Products Company, Owensville, Missouri), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) was prepared. The solution was filled into a 1 ml volume, 4 mm diameter syringe. The syringe was outfitted with a 23 x ¾ -gauge butterfly and tubing infusion set needle and was placed in the syringe pump (NE-1000 Programmable Syringe Pump, New Era Pump System, Inc, Wantagh, New-York) and dispensed at a rate of 2 ml/hr. A voltage of 18 kV was applied to the syringe needle. The striated substrate was grounded with an alligator clip and placed at a distance of 25cm from the needle.
Combined Atomic Force Microscopy (AFM)/Inverted Optical Microscopy

The mechanical manipulations were performed as previously reported\textsuperscript{1}. Briefly, a combined AFM (Topometrix Explorer, Veeco Instruments, Woodbury, NY) and optical microscope (Zeiss Axiovert 200, Göttingen, Germany) instrument was used to manipulate and observe fiber manipulation\textsuperscript{26,27}. The dual microscopy system is set up so that the AFM rests on a custom made stage on top of the inverted microscope (Figure 5). The design allows for independent movement of the microscope objective, AFM cantilever and sample. For schematic see\textsuperscript{1}. Light was provided to the sample from the cantilever illumination bulb on the underside of the AFM. A Hamamatsu EM-CCD C9100 Camera (Hamamatsu Photonics KK, Japan) and IPLab software (Scanalytics, Fairfax, VA) were used to collect and analyze the bright field microscopy images and movies.

Fiber Manipulations and Force Calculations

Fiber manipulation and force acquisition was obtained as previously reported\textsuperscript{1}. Silicon cantilevers with a rectangular cross-section were used for AFM imaging, fiber manipulation and force acquisition (NSC12 without Al, force constant 14 N/m, length 90 µm, width 35 µm, tip height 15 µm; MikroMasch, Wilsonville, OR). Silicon cantilevers were used because of their commercial availability, large modulus ($E = 1.69 \times 10^{11}$ N m$^{-2}$) and, therefore, low compliance. The AFM cantilever was controlled by a nanoManipulator (3\textsuperscript{rd} Tech, Chapel Hill, NC). The AFM tip was placed next to the fiber in the center of the groove. It was then moved into the fiber, stretching the fiber laterally (Figure 5). The typical pulling rate was approximately 350 nm/s. Since the tip was
located in the groove of the surface frictional forces were eliminated. Stress-strain data was acquired by converting the left-right photodiode signal, \( I_l \), recorded by the nanoManipulator, into lateral force, \( F_l = K_C \cdot I_l \). The conversion requires the lateral force conversion factor \( K_C \), which can be determined from cantilever beam mechanics,

\[
K_C = \frac{E_w t^3}{6l^2 (h + t/2)} \cdot S_n,
\]

where \( E \), \( w \), \( t \), \( l \) and \( S_n \) are the Young’s modulus of silicon, \( 1.69 \times 10^{11} \) N m\(^{-2} \), the width, the thickness, the length and the normal sensor response of the cantilever, \( h \) is the height of the tip. The length, width and height of the tip were determined using the optical microscope and the thickness is calculated using the resonance frequency of the cantilever,

\[
f = 0.276 \sqrt{\frac{E_w t^3}{\rho (\pi h^3 t^3 + 2.832 w t l^3))}},
\]

where \( \rho = 2330 \) kg m\(^{-3} \) is the density of silicon.

To convert force to stress, \( (\sigma = F/A) \), the radius of the fiber was determined. The AFM was used in tapping mode to collect a topographical image of the manipulated fiber where it extended on top of the ridge. The diameter of the fibers was then determined from the z-axis topographical data. The stress was calculated assuming a constant fiber radius, engineering stress.
RESULTS

Fibers were electrospun from a solution of 80 mg/ml collagen type I dissolved in HFP and were collected on cover glass stamped with a striated surface. The striated surface had 12 μm wide and 6 μm deep grooves, and 8 μm wide ridges. The collected fibers had a uniform appearance and were randomly oriented on the surface (Fig. 6). The average fiber radius as determined by AFM topography was 302 ± 126 nm. Fibers were prepared in buffer as well as in ambient conditions, however, as previously reported the electrospun collagen fibers were partially soluble in buffer if they were not crosslinked \(^{28,29}\). Therefore, data was not taken on the hydrated fibers due to their loss of integrity.
The mechanical properties of the electrospun collagen type I fibers were tested using a combined AFM and inverted optical microscope system. Fibers oriented perpendicular to the ridge were chosen for manipulation because they provided an easy to analyze geometry (see materials). The fibers were stretched parallel to the ridges using the AFM tip and stress-strain curves were obtained (Fig. 7A-D). Figure 7E shows a typical electrospun collagen type I stress-strain curve. The slope of the stress-strain curve characterizes the stiffness of the material. The stress-strain curves of the electrospun collagen fibers showed considerable strain softening, i.e. a decrease in modulus (stiffness) as the strain increases. Because of the strain softening, we choose the commonly used three point bending model (with clamped ends) to calculate the small strain bending.
modulus, \( E = \frac{F \cdot l^3}{48 \cdot x \cdot \pi \cdot r^4} \) where, \( F \) is the force, \( l \) is the length of the fiber, \( x \) is the displacement and \( r \) is the radius. The three point bending modulus was determined for displacements up to 200 nm (\( \varepsilon \leq 0.056\% \)). The average three point bending modulus of electrospun collagen type I fibers was 2.8 ± 0.4 GPa (value ± standard error; \( n = 32 \)). The modulus of the fibers had a strong dependence on radius; as the radius of the fibers increased the modulus decreased (Figure 8A). The radii of the fibers tested ranged from 160 nm to 783 nm. The value of the modulus and modulus dependence on radius agrees with previously published data\(^{24}\).

The extensibility, or strain at which the fiber ruptures, was determined using the same fiber manipulations used to determine the modulus (Figure 7E). The extensibility of 24 fibers was measured and the average extensibility of electrospun collagen type I fibers was 33 ± 3\%. Extensibility of the fibers showed no dependence on fiber radius in the range tested, 160–783 nm.

The next property analyzed was the maximum stress or peak stress. The maximum stress is the highest stress value reached before the fiber ruptures (Figure 7E). In all manipulations, the softening of the fiber modulus lead to a plateau in the stress applied to the fiber. As the strain increased from zero to approximately 12% strain the stress increased. At 12% strain the stress applied to the fiber reached a plateau where it remained until the fiber ruptured. The maximum stress therefore occurred at 12% strain and at strains above 12% the fiber remained at the maximum stress until the fiber ruptured. The maximum stress for 15 fibers was measured and the average maximum stress was 25 ± 3 MPa. The maximum stress varied with fiber radius, similar to the modulus, as the radius increased the maximum stress decreased (Figure 8B).
calculating the peak stress the radius prior to manipulation was used, engineering stress was used to calculate the peak stress. While this assumption may not be accurate to describe the behavior of the fiber during manipulation, the Poisson’s ratio of electrospun collagen is not known and therefore cannot be used in the calculation. The engineering stress gives a lower boundary to the value of the peak stress. Another method of stress calculation used in absence of the Poisson ratio of the material is to assume the fiber maintains a constant volume during manipulation. The engineering stress can be converted to stress calculated for a constant volume using the following equation,

$$\sigma = \frac{F}{A}(\varepsilon + 1)$$

where F is the force, A is the cross-sectional area of the fiber before manipulation, $$\sigma$$ is the stress and $$\varepsilon$$ is the strain.
Figure 7 (A-D) Movie frames from an electrospun collagen fiber manipulation. The AFM cantilever is visible as a large, vertical shadow covering the right side of the image. The tip of the AFM can be seen to the left of the cantilever shadow due to optical parallax and the dark horizontal lines are the ridges of the patterned surface. In figure 7D the fiber has broken at the top ridge. (E) Typical stress-strain curve acquired during a fiber manipulation. The stress increases as the strain increases, however the rate at which the stress increases changes with strain and the fiber displays modulus softening with increasing strain. At the point when the fiber breaks, the stress drops back to zero. The strain value at which the fiber ruptures is the fiber’s extensibility. This fiber has an extensibility of 33%. The maximum stress can also be obtained from the graph; this fiber has a maximum stress of 21 MPa.
Figure 8 (A) A plot of modulus versus radius. The modulus decreases with increasing radius. (B) A plot of maximum stress versus radius. The maximum stress decreases with increasing fiber radius.
Next, stress-strain data were taken to probe energy storage and dissipation. In viscoelastic materials, a portion of the energy used to stretch the material is elastically stored while the rest of the energy is lost or dissipated to the surroundings through viscous processes. The energy dissipated or lost is proportional to the area between the forward and backward curve of the stress-strain plot (Figure 9A). The percentage of the input energy lost during a stretch cycle was strongly dependent on the maximum strain of the cycle. At low strains smaller percentages of energy loss occurred, and the material behavior was mostly elastic. However, at higher strains significant energy loss was seen. The energy loss per cycle increased linearly with increasing strain until a strain of 12%, at which point the energy loss saturated at 80% of the input energy. In other words, at strains above 12%, 80% of the energy required to stretch the fiber was not recovered as the fiber returned to the starting position. At low strain the energy loss followed a linear increase from 0% energy loss at 0% strain to 80% energy loss at 12% strain (Figure 9B).

When electrospun collagen type I fibers were stretched and returned to their initial position permanent deformation was detectable both visibly and through the stress-strain data. Visibly when the force stretching the fibers is released they do not return to their original shape, instead the fibers appear less taught and permanently deformed between the ridges. The stress-strain curve shows the permanent deformation in that the stress applied to the fiber, or the stress applied to the cantilever by the fiber, returns to zero before the fiber returns to its initial zero-strain position. The black curve in Figure 9C shows a manipulation of a fiber. On the return manipulation the stress returns to zero before the strain is zero indicating deformation in the fiber. As shown in Fig 9C, electrospun collagen type 1 fibers also show significant hysteresis. The slope of the
stress-strain curve for a second manipulation is less than that of the first manipulation (Figure 9C).

Figure 9 (A) Stress-strain curves depicting energy loss during a stretch cycle. The forward pull requires more force and therefore has a higher stress than the backward pull. The fiber indicated by the black curve was pulled to a strain of 2.3% and showed 20% energy loss; the gold fiber was pulled to a strain of 14.4% and the fiber had an energy loss of 82.5%. (B) Graph of energy loss versus strain. The gold dashed line shows the slope of the increasing energy loss as strain increases. At 12% strain the energy loss plateaus at 80%. (C) A stress-strain curve of two consecutive manipulations of the same fiber. The first manipulation is shown in black. The stress required to stretch the fiber during the first manipulation differs from the stress required for the second manipulation, shown in gold.
A combined atomic force/optical microscopy technique was used to probe the mechanical properties of nanometer sized dry electrospun collagen type I fibers through the collection of various stress-strain measurements. The extraction of mechanical properties through AFM lateral force manipulation has been previously used for measurements on natural fibrin fibers and electrospun fibrinogen fibers in buffer. Errors in the data acquired by the combined microscopy technique result from force and radius measurements, obtained by the AFM. The force measurements are calibrated against the cantilever beam method and the glass fiber method, see Liu, while the radius measurements are calibrated against a grid of known dimensions. The error in the force measurements is about 30% while the error in the radius measurement is about 20%. While the error in these measurements are larger than that of other nanomanipulation methods, such as scanning mode bending test with 12% error in the force measurement, this type of lateral force AFM data has shown agreement with various methods of nanomanipulation. Additionally the combined AFM/optical microscope measurements have the advantage that the fiber is visualized throughout the entire manipulation process, and that it allows for very large extensions, up to fiber failure.

Applying the combined microscopy technique to the electrospun collagen fibers we found that the fibers show clear viscoelastic behavior. The three point bending modulus, for deformations less than 200 nm ($\varepsilon \leq 0.056\%$), ranges from 0.2 to 8.0 GPa with an average of 2.8 GPa. This is in agreement with previously published data by Yang et al. on the modulus of individual electrospun collagen fibers determined by scanning mode bending. Yang also showed a decrease in the modulus of the fiber with an
increase in radius which was also clearly evident from our data \(^{24}\). The relationship between radius and modulus has also been seen in electrospun carbon nanofibers where heterogeneities in longitudinal and cross sectional area were deemed responsible for the complex association between radius and modulus \(^{36}\). Internal voids or molecular density may vary with respect to electrospun fiber size producing a larger fiber that is less dense or more porous than smaller fibers, this in turn would effect the modulus so that the larger fibers had a lower modulus than smaller fibers. However, Pai et al. found that while the void size varied with fiber radius the void to volume fraction remained relatively constant among electrospun fiber \(^{37}\). Another explanation for modulus dependence on radius is greater orientation in smaller fibers. Lim et al explained that the distribution of fiber diameters is formed from the random whipping of the jet as it approaches the collector plate. Sections of the jet become thinner, with greater molecular orientation and crystalline order as they undergo greater amounts of bending, elongation and solvent evaporation before reaching the surface. Lim et al also demonstrated that the thinner more crystalline fibers displayed greater stiffness and strength. It is likely that varying molecular orientation with fiber radius is also responsible for the modulus dependence on radius seen in electrospun collagen fibers \(^{38}\).

One advantage of lateral force AFM, over scanning mode bending and optical tweezing is that it allows for a large range of manipulation of individual electrospun fiber, including fiber failure. Continuous manipulation of the fibers until failure showed severe strain softening of the electrospun collagen type I fibers. The modulus of the fibers decreased drastically as the strain increased. At a strain of approximately 12% the modulus decreased to nearly zero and remained at that value until fiber rupture at an
average strain of 33%. This plateau in the stress-strain curve can be attributed to plastic
deformation of the fibers. Data on micron sized electrospun collagen fibers and mats also
displays similar significant strain softening \(^{39}\). At 12\% strain, a similar trend was seen in
the energy loss data. The energy loss in a manipulation cycle increased linearly from 0%
energy loss at 0\% strain to 80\% energy loss at 12\% strain; for strains above 12\% the
energy loss remained at 80\% of the input energy.

It appears, however, that plastic deformation begins within the fibers before the
modulus decreases to zero. The energy loss data suggest that electrospun collagen fibers
undergo plastic deformation beginning as low as 1\% strain and visible data suggest that
permanent deformation occurs above 2\% strain, that is fibers undergoing manipulations
greater than 2\% strain do not return to their original shape once they have been
manipulated. Aside from single manipulation deformations, electrospun collagen also
shows hysteresis, or memory of previous manipulations. The initial manipulation of the
fiber alters its properties; a second manipulation to a strain of equal or lesser value gives
an altered stress-strain behavior. However, subsequent strains, such as third or fourth
manipulations, will have identical stress-strain behavior as the second manipulation. This
suggests that in applications involving cyclic stress on electrospun collagen the original
response of the material as well as the material hysteresis should be considered.
Preparing electrospun collagen for use may require an initial manipulation of the material
to obtain reproducible mechanical properties.

Individual electrospun collagen type I fibers can be stretched to 1.33 times their
initial length before rupturing. In comparison to wet electrospun fibrinogen fibers,
another biocompatible protein, with an extensibility of 2.3x their original length \(^1\),
collagen is less extensible. However, the maximum stress of both dry electrospun collagen and wet fibrinogen is on the order of 20-30 MPa. It is important to consider fiber radius when considering maximum stress since the maximum stress displayed an inverse dependence on radius. Extensibility, however, did not depend on radius.

Previously Matthews has shown that the average modulus for longitudinally oriented electrospun collagen mats is $52.3 \pm 5.2$ MPa, with a peak stress of $1.5 \pm 0.2$ MPa. From these reports it is evident that the initial modulus and peak stress for individual fibers differs from the properties of the mats. The difference in modulus between mats and individual fibers is most likely due to the greater architectural complexity of the electrospun mats. For example, fiber orientation plays a major role in the mechanical behavior of the mats and it directly influences the modulus and peak stress of the mat. A second element of complexity is the effect of fiber radius on the modulus and peak stress of the mat. One simple way to change the modulus or peak stress of an electrospun mat might be to decrease or increase the average fiber radius. Therefore, collagen mats with a range of mechanical properties could be fabricated by controlling fiber diameter through solvent concentration as well as other spinning factors, as well as controlling the orientation of the fibers.

In addition to comparison with electrospun collagen type I mats and electrospun fibrinogen, the individual fibers can also be compared to their natural counterparts. Natural collagen type I fibers from rat-tail have a modulus between 5 and 11.5 GPa and collagen type I fibrils from bovine Achilles tendon have a dry bending modulus between 1 and 4 GPa. These values are similar to the initial modulus recorded for individual electrospun collagen fibers, 2.8 GPa. As mentioned previously, cell seeding and
differentiation the mechanical properties of the scaffold have a large effect on cell proliferation, therefore similarity between native fiber and electrospun fibers is beneficial to biomedical engineering\textsuperscript{17}. However, electrospun fibers cannot be directly compared to native fibers. Electrospun fibers are produced in HFP, a highly volatile buffer. HFP and similar fluorinated hydrocarbon buffers have been shown to promote $\alpha$-helix formation\textsuperscript{44}. CD spectra of electrospun collagen fibers have shown that 45\% of their proline helical content of collagen is denatured in HFP\textsuperscript{45} and therefore the individual monomers composing electrospun fibers are different from native collagen monomers. It has also been argued that collagen denatures into gelatin in fluorinated solvents such as HFP\textsuperscript{29}. However, tensile tests on collagen and gelatin mats have revealed different tensile moduli for the two molecules suggesting that while collagen is denatured in HFP it does display different behavior than gelatin electrospun in HFP\textsuperscript{46}. Despite the difference in the protein structure between native and electrospun collagen molecules, their fibers show somewhat similar mechanical behavior; compared to other biological fibers they are both relatively stiff and not very extensible\textsuperscript{47}.

The use of individual fiber properties in matrix modeling has been shown through combined microscopic and macroscopic modeling\textsuperscript{48-50}. In these studies whole matrix properties are modeled starting at the individual fiber and including fiber orientation. From these models ideal scaffolds could be designed with the knowledge and control of individual fiber properties and fiber orientation. Generally three elements are needed to explain and design matrix properties\textsuperscript{51,52}: 1) the properties of the individual constituents, 2) the properties of fiber interactions or branch points of the network and 3) the overall network architecture. Developments in controlling electrospun fiber orientation show
promise for control over network architecture, fiber branching is minimal in electrospinning, fiber interactions are based on friction between overlaying fibers and here we describe the individual fiber properties.

The use of electrospun protein fibers for medical application is inspired by the properties and components of the extracellular matrix (ECM) itself. By mimicking the size and mechanical properties of the ECM it is thought one may achieve good cellular adhesion and materials properties desired for tissue engineering. However, the application of electrospun fibers does not stop with tissue engineering, uses in drug delivery and dental composites have also gained recognition \(^{53-55}\). Through studies on the mechanical properties and response of single fibers we gain insight into improvements that could be made in fiber selection and formation for materials use.

**CONCLUSION**

In summary, we have shown that our combined microscopy technique is a good tool for extracting the mechanical properties of individual electrospun nanofibers. We have determined the mechanical properties of individual electrospun collagen type I fibers and have shown that electrospun collagen undergoes severe strain softening and the modulus and peak stress of the individual electrospun collagen type I fibers have a dependence on radius. We believe determining the properties of individual electrospun fibers will help to explain the properties of electrospun fiber mats from the ground up, potentially leading to the ability to assemble electrospun matrices with the desirable mechanical strength, mechanical properties and biocompatibility for their intended function, whether medical or textile.
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CHAPTER III
THE MECHANICAL PROPERTIES OF INDIVIDUAL, ELECTROSPUN FIBRINOGEN FIBRES

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ABSTRACT

We used a combined atomic force microscopic (AFM)/fluorescence microscopic technique to study the mechanical properties of individual, electrospun fibrinogen fibers in aqueous buffer. Fibers (average diameter 208 nm) were suspended over 12 μm-wide grooves in a striated, transparent substrate. The AFM, situated above the sample, was used to laterally stretch the fibers and to measure the applied force. The fluorescence microscope, situated below the sample, was used to visualize the stretching process. The fibers could be stretched to 2.3 times their original length before breaking; the breaking stress was $12 \cdot 10^6$ Pa. We collected incremental stress-strain curves to determine the viscoelastic behavior of these fibers. The total stretch modulus was $10 \cdot 10^6$ Pa and the relaxed elastic modulus was $5.2 \cdot 10^6$ Pa. When held at constant strain, electrospun fibrinogen fibers showed a fast and slow stress relaxation time of 3 and 55 s.

Our fibers were spun from the typically used 90% 1,1,1,3,3,3-hexafluoro-2-propanol (90-HFP) electrospinning solution and resuspended in aqueous buffer. Circular dichroism spectra indicate that alpha-helical content of fibrinogen is ~70% higher in 90-HFP than in aqueous solution.

These data are needed to understand the mechanical behavior of electrospun fibrinogen structures. Our technique is also applicable to study other, nanoscopic fibers.

INTRODUCTION

Fibrinogen is a highly abundant plasma protein with molecular mass 340,000 Da. It consists of six polypeptide chains; 2 Aα, 2 Bβ and 2 γ chains which are bound together by 29 disulfide bonds $^4$. Fibrinogen has a tri-nodular shape, with two nodules (the D-
domains) at either end and one nodule (the E-domain) in the center. The nodules are connected by two 17 nm long coiled coils consisting of three alpha helices. Fibrinogen is about 45 nm in length and 4.5 nm in diameter (Fig. 10A). In the event of injury to the vasculature, activated thrombin cleaves two fibrinopeptides A (16 residues) and two fibrinopeptides B (14 residues) from the amino termini of the Aα and Bβ chains (located at the central E-domain), thus converting fibrinogen into fibrin. Fibrin then polymerizes spontaneously into a meshwork of fibrin fibers which comprises the main structural component of a blood clot. Thrombin-activated factor XIII forms covalent γ–γ and α–α crosslinks between the γ– and α-chain of neighboring fibrin molecules, which further stabilizes the fibrin network. The fibrin fibers in this network have a wide range of diameters with an average of about 60 nm. The initial task of the fibrin fiber network is stemming the flow of blood to prevent blood loss; subsequently, it plays a significant role in controlling the enzymatic processes of wound healing.

Aside from these naturally formed fibrin fibers, fibrinogen can also be spun electrostatically into nanoscopic fibers. In this technique, termed “electrospinning”, fibers are formed from a thin stream of concentrated protein solution emerging from a syringe (Fig. 10B). The emerging protein solution forms fibers as it is subjected to a high electric field between the syringe needle and a grounding plate. The fibers are deposited on the grounding plate. Patented in 1934 for textile use, electrospinning has recently received renewed interest, as electrospun nanofibers may be ideal candidates for numerous, novel biomedical applications such as tissue engineering scaffolds, wound dressings, coatings, or drug delivery vehicles. In addition to biomedical applications, electrospun fibers may also find uses in novel polymer composites.
Electrospun fibrinogen fibers, in particular, might be attractive candidates for biomedical applications due to their biocompatibility. The immunogenicity of fibrinogen is low, as it does not cause inflammation and it does not result in toxic degradation products. Moreover, fibrinogen can be used autologously which nearly eliminates the risk of an immune response. Thus, fibrinogen fibers should be well-suited for applications in humans. Fibrinogen also is a natural choice to form artificial fibers, since one of its main physiological purposes is to form the fibrin fibers that comprise the structural scaffold of a blood clot. Fibrinogen regulates many processes in tissue repair and regeneration, and angiogenesis; electrospun fibrinogen fibers may, thus, be utilized to regulate some of these tissue-rebuilding processes, as well. Fibrinogen scaffolds have been shown to support cell proliferation and to promote cell interaction. Fibrinogen fibers can be dissolved again by the natural clot dissolution process (fibrinolysis), and are, therefore easily ‘degradable’ in the body. Scaffolds constructed from electrospun fibrinogen fibers have recently been tested successfully in urinary tract tissue engineering.

When designing structures or devices, such as tissue scaffolds, wound dressings or drug delivery particles, it is important that the mechanical properties of the device match the requirements of the task at hand, for example a device may have to be stiff, or compliant. The overall mechanical properties of any structure built from fibers depend on three distinct quantities: (i) the architecture of the structure, (ii) the properties of the single fibers comprising the structure and (iii) the junctions between the fibers comprising the structure. All three need to be known so that a structure with predictable mechanical properties can be designed.
Addressing item (ii), we developed a microscopy-based technique to determine the mechanical properties of individual electrospun fibrinogen fibers. This technique should also be widely applicable to investigate other nanoscopic fibers.

MATERIALS AND METHODS

Substrate Preparation

Preparation of the striated substrate is based on soft lithography and MIMIC (micromoulding in capillaries) \(^\text{15}\). Briefly, a PDMS (polydimethylsiloxane) stamp was prepared by pouring dimethylsiloxane plus catalyst (Sylgard, Dow Corning Corp, Midland, MI) onto a SU-8-silicon master grid (gift from Prof. Superfine, University of North Carolina, Chapel Hill) in a Petridish. The polymer was cured at 70°C for 1 h. The PDMS stamp was removed from the master and pressed into a 10 \(\mu\)l drop of Norland Optical Adhesive-81 (NOA-81, Norland Products, Cranbury, NJ) on top of a 60mm x 24mm, #1, microscope cover slide (VWR International, West Chester, PA). 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 we used had 12 \(\mu\)m wide and 6 \(\mu\)m deep channels and 8 \(\mu\)m wide ridges.

Formation of Electrospun Fibrinogen Fibers

Our procedures used for electrostatic spinning of fibrinogen fibers, schematically depicted in Fig. 10B, are based on those developed by Wnek et al. \(^\text{16}\). A polymer solution comprised of 100 mg/ml lyophilized bovine fibrinogen (Sigma-Aldrich Chemical Co), 9 parts 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich) and 1 part minimum
essential medium (MEM) (10x MEM, Gibco, Invitrogen cell culture) was prepared. SDS PAGE showed that this fibrinogen sample was about 79% pure, and that there was no detectable crosslinking under our experimental conditions (data not shown). The solution was filled into a 1 ml volume, 4 mm diameter syringe (Becton-Dickinson, Franklin Lakes, New Jersey). The syringe was outfitted with a 23x¾ -gauge butterfly and tubing infusion set needle (Abbott Laboratories, North Chicago, IL) and was placed in the syringe pump (NE-1000 Programmable Syringe Pump, New Era Pump System, Inc, Wantagh, New York) and dispensed at a rate of 1.9 ml/h. A voltage of 22 kV was applied across the needle and the substrate (grounding plate), which was placed at a distance of 12.5cm or 20cm from the needle. The grounding plate consisted of the cover glass (with the striated surface) connected to ground with an alligator clip. Between 5 μl and 15 μl of polymer solution was dispensed onto each striated cover glass surface.
Figure 10 (A) Crystal structure of chicken fibrinogen. The monomer is 45 nm long and composed of six polypeptide chains, two alpha chains labeled with gray, two beta chains labeled with black and two gamma chains labeled with gold. Bovine fibrinogen, used in our electrospinning experiments, is highly homologous and structurally very similar to chicken fibrinogen, except for the flexible α-chain C-terminal domain, which chicken fibrinogen lacks. (B) Schematic of the electrospinning setup. At the top, a syringe pump is used to regulate the flow of the polymer solution (green) from the syringe. The needle of the syringe is charged to a high potential using a voltage supply. At the bottom left, the fibers being produced from the syringe are collected on the substrate.
Labeling and Resuspension of Fibers

After fiber formation, the fibers were labeled with 24 nm diameter yellow-green carboxyl fluorescent beads (Invitrogen, Fluospheres, Carlsbad, CA) diluted 1/10,000 in fiber buffer-1 (10mM Hepes, 140mM NaCl, pH 7.4). A 100 µl drop of the diluted bead solution was placed on the electrospun fiber coated cover slide for 10 minutes; the slide was then rinsed with fiber buffer-2 (10mM Hepes, 140mM NaCl, 5 mM CaCl₂, pH 7.4) and stored in fiber buffer-2.

Combined Atomic Force Microscopy (AFM)/Fluorescence Microscopy

The mechanical manipulations of the electrospun fibers were performed with our combined AFM and optical microscope instrument (Fig.11) \(^{17,18}\). The AFM (Topometrix Explorer, Veeco Instruments, Woodbury, NY) fits our custom-built stage of the Zeiss Axiovert 200 inverted microscope (Zeiss, Göttingen, Germany). The stage is designed so that the AFM tip can be aligned with the objective lens and it also permits the sample to be moved independently of the AFM tip and the objective lens. This design allows us to position any area of the sample between the AFM tip and the objective lens. A Hamamatsu EM-CCD C9100 Camera (Hamamatsu Photonics KK, Japan) and IPLab software (Scanalytics, Fairfax, VA) were used to collect and analyze the fluorescence microscopic images and movies. For the AFM imaging and manipulation experiments, silicon cantilevers (Young’s modulus, \(E = 1.69 \times 10^{11}\) Pa) with a rectangular cross-section were used (NSC12 without Al, spring constant, \(k = 0.3\) N/m, length, \(l = 330 \mu m\); width, \(w = 34 \mu m\); thickness, \(t = 1.3 \mu m\); tip height, \(h = 15 \mu m\); MikroMasch, Wilsonville, OR).
Fiber Manipulations and Force Calculations

Stress is defined as $\sigma = \frac{F}{A}$, where $F$ is the force acting on the fiber (Fig. 11C) and $A$ is the cross-sectional area of the fiber. We used the cross-section of the initial, unstretched fiber in our calculations, i.e. we used the so-called engineering stress in this paper. Strain is defined as $\varepsilon = \frac{\Delta L}{L_{\text{init}}}$, where $\Delta L = L' - L_{\text{init}}$ is the change in fiber length; $L'$ and $L_{\text{init}}$ are the lengths of the stretched and unstretched fiber, respectively.

A nanoManipulator (3rd Tech, Chapel Hill, NC), was used to control the AFM tip movement in the x-, y- and z-direction. After aligning the AFM tip with the objective lens, the sample was moved until a fiber was centered in the field of view. The tip was then lowered onto a ridge of the striated substrate. Once the tip was on the surface, the nanoManipulator software was used to raise the tip in the z direction (away from the surface) and to move the tip next to the fiber in the center of a groove on the striated surface. The z-position of the tip was then further adjusted, so that the point of the tip was at the same height as the fiber. This last step was done to assure that the lever arm of the lateral force measurement would be equal to $h + t/2$, where $h$ is the tip height and $t$ is the thickness of the cantilever (see equation (2) for $K_C$ below).

The fiber was then stretched by laterally moving the tip, along the groove, into the fiber. Fibers suspended over such a surface provide an easy-to-analyze geometry for mechanical manipulations and for collecting stress-strain curves (Fig. 11C). Fig. 11D shows a movie sequence of a typical fiber stretching experiment.

The acquisition of the fiber stress-strain data involved a series of steps. The “left-right” photocurrent data (which corresponds to the twist of the cantilever) was recorded by the nanoManipulator during the manipulation. The left-right photocurrent signal, $I_l$,
was converted into lateral force, \( F_l \), (in newtons) by multiplying it with the lateral force conversion factor \( K_C \):

\[
F_l = K_C \cdot I_l
\]

\( K_C \) can be determined via cantilever beam mechanics,

\[
K_C = \frac{Ewt^3}{6l^2(h + t/2)} \cdot S_n
\]

where \( E \), \( w \), \( t \), and \( l \) are Young’s modulus, the width, the thickness and length of the cantilever; \( h \) is the height of the tip. \( S_n \) is the ‘normal sensor response’, i.e. the conversion of normal cantilever deflection to top-bottom photocurrent. The length and width of the cantilever and the height of the tip were determined for each cantilever individually with the optical microscope. The resonance frequency of the cantilever was used to determine the thickness of the cantilever via:

\[
f = 0.276 \cdot \sqrt{\frac{Ewt^3}{\rho(\pi^3 h^2 l^3 + 0.2832 wt^4)}}
\]

where \( \rho = 2330 \text{ kg/m}^3 \) is the density of silicon. The error in \( K_C \), as determined from the beam mechanics method, can be estimated from the uncertainties in the quantities: \( l \), 2%; \( w \), 2%; \( t \), 10%; \( h \), 10%; and \( S_n \), 10%, yielding an overall error in \( K_C \) of about 56%. This method of determining \( K_C \) (based on cantilever beam mechanics) was double-checked with the glass fiber calibration method, in which \( K_C \) is determined by bending a small glass fiber with known dimensions and Young’s modulus \(^{19}\). The error in the glass fiber calibration method is dependent upon the properties of the glass fiber (radius, length and Young’s modulus) and the cantilever deflection, \( \Delta y \), resulting in an overall error in \( K_C \) of about 26%. For details of both calibration methods, see Liu \(^{19}\). The normal sensor response, \( S_n \), was acquired before or after each manipulation experiment.
The cross-sectional area of the fibrinogen fibers, A, needed to calculate the stress applied to the fibers, \( \sigma = \frac{F}{A} \), was determined concurrently by imaging the fiber on the ridge with the AFM. The diameter, D, was determined from the height data and used to calculate the cross-sectional area \( A = \pi \left( \frac{D}{2} \right)^2 \). The error in D is about 10%.

The diameter of the fibers (~ 200 nm) could not be determined via optical microscopy, because it is below the resolution limit, \( d = \frac{0.61 \lambda}{N.A} \sim 430 \text{ nm} \), of our objective lens (40x, N.A. = 0.75, \( \lambda \sim 525 \text{ nm} \)).
Figure 11 (A) Photograph of the experimental setup, the AFM sitting on top of the inverted optical microscope. (B) Schematic of the experimental setup. The AFM tip is used to stretch fibers that are suspended over the grooves of a striated substrate. (C) Schematic top view. $L_{\text{init}}$ is the initial length and $L'$ the length of the stretched fiber. Using trigonometry, $L'$ can be calculated from the initial length and the tip travel, $s$. It can also be directly measured from the optical image. $F$ is the force of the AFM tip on the fiber and $F_1$ and $F_2$ are the forces felt by each leg of the fiber, respectively. The force on each leg of the fiber is dependent on the angle, $\alpha$, at which the fiber is being pulled. (D) Fluorescence microscopy movie frames of a stretching experiment. The fiber is anchored on two ridges (8 $\mu$m wide bars) and suspended over a groove (12 $\mu$m wide bar); the AFM cantilever appears as a faintly visible 35 $\mu$m shadow. The position of the AFM tip is marked with an asterisk (*).
Circular Dichroism (CD) Spectra

CD spectra were taken to compare the secondary structure of fibrinogen in aqueous solution, and in electrospinning solution. The CD spectra were taken for human fibrinogen solutions prepared in phosphate buffered saline (PBS, Sigma-Aldrich) and HFP. A total volume of 300 μl of fibrinogen solution was prepared in PBS at a concentration of 40.4 mg/ml fibrinogen (Enzyme Research Laboratories, South Bend, IN). 300 μl was also prepared in the HFP solution at a fibrinogen concentration of 32.7 mg/ml. Aliquots of each sample were diluted ~140-fold in their respective buffers prior to obtaining UV absorbance data in a BioRad SmartSpec Plus spectrophotometer and circular dichroic spectral data in a JASCO Model 720 spectropolarimeter. Both samples exhibited absorbance maxima near 280 nm, enabling calculation of the fibrinogen concentrations (0.292 mg/ml in PBS; 0.285 mg/ml in HFP) using a weight/volume extinction coefficient of 1.6 ml/mg-cm. CD spectra were taken with each sample from 195 nm to 360 nm for the HFP-fibrinogen solution and PBS-fibrinogen solution. Spectra were also taken for PBS and HFP solutions to be used as baseline measurements. Calculations of mean residue ellipticity, [Θ], versus wavelength were obtained using a mean residue weight of 114.7 for fibrinogen, calculated from its molecular weight (340,000) and total number of residues 20.

RESULTS

Fiber Morphology

Electrospun fibrinogen fibers were formed from a 100 mg/ml fibrinogen solution. Fibers were collected on either a regular cover glass substrate or a striated substrate with
12 μm wide and 6 μm deep grooves and 8 μm wide ridges prepared from optical adhesive.

The fibers, which were formed on the cover glass without ridges, were imaged with the AFM in air (Fig. 12). The fibers had a relatively smooth, uniform appearance and an average radius of 208 ± 18 nm (22 fibers, values are stated ± their standard error), in agreement with published data.⁵¹

**Figure 12 (A)** An AFM image of an electrospun fibrinogen fiber on a glass substrate. The color scale on the left corresponds to height. **(B)** A cross-section taken along the horizontal black line through the AFM image in A. The diameter is measured from the cross-section by looking at the z height difference between two points, one along the background and one at the top of the fiber. The diameter of this fiber is 120nm. **(C)** A 50 x 50 μm AFM scan of multiple electrospun fibrinogen fibers on glass.
Fiber Extensibility and Breaking Strain

We then tested the mechanical properties of single, electrospun fibrinogen fibers in aqueous solution. Fibrinogen fibers were electrospun on the striated substrate using the same 100 mg/ml fibrinogen solution as above, and re-suspended in fiber buffer-2. As seen in the fluorescence images in Fig. 13 A-D, taken from underneath the sample, the fibers are suspended over the 12 μm wide and 6 μm deep grooves and anchored on the 8 μm wide ridges. The AFM tip, situated above the sample, was used to stretch the fiber. Fig. 13 depicts the extensibility measurements of the fibers. Extensibility is defined as the maximum strain $\varepsilon_{\text{max}}$, at which a fiber will break. This is an important fiber property, as it determines the ultimate failure of a fiber. The extensibility of the fibers was tested by using the AFM tip to stretch the fibers parallel to the ridges until they broke (see Fig. 13A-D). The stretch rate for those experiments was between 250 and 425 nm/s. The fibers we studied were usually firmly (though non-specifically) anchored on the ridges, as we have seldomly seen them detach at the anchoring point when stretching them. Moreover, since we obtain images and movies of each manipulation, we can ascertain that the fibers are not slipping at the anchoring points. Fig. 13 E shows a histogram of the extensibility of 34 electrospun fibrinogen fibers. The average extensibility of the hydrated electrospun fibrinogen fibers was $\varepsilon_{\text{max}} = 1.3 \pm 0.1$; that is, electrospun fibrinogen fibers can be stretched to 2.3 times their initial length before breaking.

This extensibility is comparable to that of the high-extensibility natural fibers, and it is larger than that of the low-extensibility, natural fibers (see table 1). However, it is still less than the extremely large extensibility of hydrated, naturally formed, partially
crosslinked and non-crosslinked fibrin fibers \( (\varepsilon_{\text{max}} = 3.32 \text{ and } \varepsilon_{\text{max}} = 2.26, \text{ respectively}) \).

Naturally formed fibrin fibers are the most stretchable protein fibers in nature \(^{17}\).

Fig. 13 F shows a histogram of the maximum stress, \( \sigma_{\text{max}} \), at which the fibers ruptured. This quantity is important, because it is a measure of how much force a fiber can sustain, before breaking. The average maximum stress of the hydrated electrospun fibrinogen fibers formed at 100 mg/ml concentration was \( \sigma_{\text{max}} = 12.3 \pm 1.1 \text{ MPa} \).

---

**Figure 13 (A-D)** Fluorescent movie frames of an electrospun fibrinogen fiber manipulation. The fiber is suspended from two ridges over a groove. The AFM tip is labeled by a green circle. This fiber ruptured at the ridge in frame D. **(E)** Maximum extensibility of the hydrated electrospun fibrinogen fibers \( (34 \text{ data points}) \). The average maximum extensibility, \( \varepsilon_{\text{max}} \), was 1.3. **(F)** Maximum stress, \( \sigma_{\text{max}} \), at which hydrated electrospun fibrinogen fibers ruptured. The average maximum stress at which the fibers broke was 12 MPa. The range of the x axis in E and F is determined by the lowest and highest measured values.
Fiber Viscoelastic Properties

To determine the stiffness and several viscoelastic properties of electrospun fibrinogen fibers, we collected incremental stress-strain curves on 14 fibers. In this measurement, the fiber is first strained (stretched) a certain amount, and then held at that particular strain for a period of time. During this holding time, the fiber relaxes, which means that the force (stress) to hold the fiber at that position decreases, usually exponentially, to a certain value. The stress does not decay to zero, but rather approaches a non-zero stress value asymptotically. The fiber is then strained some more and held at that new strain for some time. During this holding time, the fiber again relaxes to a certain stress value. This incremental stretching process is repeated until the fiber ruptures. Several key insights about the mechanical behavior and performance can be gained from such incremental stress-strain curves. (1) Viscoelasticity: It can be determined if a fiber is mainly elastic, mainly viscous or viscoelastic. Totally elastic fibers (e.g. a perfect rubber band) would not relax at all; and totally viscous fibers (e.g. perfect play dough) would relax completely to zero stress during each holding period. Most polymeric fibers show viscoelastic behavior, i.e. a mixture of viscous and elastic behavior. Incremental stress-strain curves can be used to demonstrate that a polymer behaves viscoelastically and to separate the elastic and viscous behavior. (2) Relaxation time(s): Stress relaxation indicates viscous behavior and the rate(s) at which the stress decays at the holding points provides the relaxation times. The relaxation times may provide information about time scales of internal rearrangements of the fibers. (3) Elastic modulus: When plotting the relaxed stress values versus the strain, the slope is the elastic (or Young’s) modulus of the fiber. This corresponds to the elastic ‘stiffness’
of fibers, i.e. the stiffness for slow manipulations. (4) The total stretch modulus: When plotting the total (unrelaxed) stress versus the strain, the slope is the total stretch modulus. This modulus is speed (or frequency)-dependent. Because our experimental set-up currently has limited speed (frequency) range in the lateral force direction, we determined the total modulus at only one speed.

Fig. 14 shows a typical incremental stress-strain curve of electrospun fibrinogen fibers. Fig. 14A shows the graph of strain versus time; the fiber was first stretched to $\varepsilon = 0.27$ and held for 84s, the strain was then increased to $\varepsilon = 0.51$ and held for 74 s, the strain was further increased to $\varepsilon = 0.94$ and held for 80 s, the fiber then ruptured on the next incremental strain increase. Fig. 14B shows a plot of stress versus time of the same fiber manipulation. The time axis in Figs. 14A & B is the same. It can be seen that the stress relaxes during the time periods at which the fiber was held at constant strain; however, it does not relax to zero. This is indicative of viscoelastic behavior.

Fig. 14 C shows the complete stress versus strain curve of this manipulation (in gold) for this fiber. The top curve (solid black line) shows the total stress versus the strain, the three dips at $\varepsilon = 0.27$, 0.51 and 0.94 occur when the fiber is held at constant strain. The average slope of this curve corresponds to the average total modulus for hydrated, electrospun fibrinogen fibers (averaged over the entire strain region). It has a mean value of $(10 \pm 1) \cdot 10^6$ Pa (mean ± standard error for incremental stress-strain curves of N = 14 fibers). The dashed line corresponds to the relaxed, elastic component of the fiber. The slope of this curve corresponds to the elastic (Young’s) modulus and has a mean value of $(5.2 \pm 0.7) \cdot 10^6$ Pa.
Figure 14 (A) Strain versus times curve for an incremental fiber manipulation. During incremental manipulations the fibers were stretched forward and then held at a given strain (plateau regions of the curve) giving the fiber time to relax. (B) Stress versus time curve for an incremental fiber manipulation. The stress decays exponentially during the time period when the fiber is being held at a given strain. The decay indicates the electrospun fibrinogen fibers are viscoelastic. (C) Incremental stress versus strain for an electrospun fibrinogen fiber. The gold curve is the raw data for the fiber, the solid black line is the total modulus given by determining the slope between adjacent peaks, and the dashed black line is the elastic component of the total modulus. The elastic component is determined from fitting the exponential relaxations curves and again connecting adjacent relaxation minimums.
We also attempted to fit the clamped, cylindrical beam equation
\[ x = \frac{FL^3}{3\pi ED^4} \]
to our low strain data (\( \varepsilon \leq 0.1 \)), for which this equation is valid (\( x \) is the deflection, \( F \) is the applied force, \( L, E \) and \( D \) are the length, Young’s modulus and diameter of the fiber). However, the noise and uncertainty of our data in this small strain, small force regime (\( \varepsilon \leq 0.1, F \leq 10 \text{ nN} \)) were too large to obtain a reliable measurement of Young’s modulus by the clamped beam equation.

Aside from the total and elastic modulus, the incremental stress-time curves can also be used to extract the time scales for fiber relaxation. Fig. 15 shows a stress relaxation curve. Two exponentials, modeled by the equation
\[ y = a \cdot \exp(-t / \tau_1) + b \cdot \exp(-t / \tau_2) + c \]
fit the data extremely well (\( R > 0.98 \)), while a single exponential did not fit the data satisfactorily as it only fit the curve for either short or long times. The two relaxation times have mean values of 3.0 ± 0.3 and 55 ± 5 s.

Another function that may be used to fit the stress relaxation data is the stretched exponential function, also known as the Kohlrausch decay model,
\[ y = a \cdot \exp(-(t / \tau)^\beta) \]
This function also fits the data well, with no statistical difference in accuracy of fit between it and the double exponential curve. The stretched exponential model assumes a continuous distribution of relaxation rates, as opposed to the two rates given by the double exponential \(^2\)\(^3\). The average values for \( \tau \) and \( \beta \) are 98.1 ± 24.3 s and 0.43 ± 0.03 s.
Electrospinning fibrinogen fibers requires a very high protein concentration (80 mg/ml or higher), and it usually also requires a volatile, fluorinated hydrocarbon solvent, such as HFP. It is known that such solvents induce α-helical structure in proteins. We performed circular dichroism spectroscopy to determine the difference in secondary structures of fibrinogen in aqueous buffers and in 96.7% HFP (Fig.16). As expected, fibrinogen has a higher α-helical content in HFP (gold curve) than in aqueous buffer (black curve). Estimates of fibrinogen’s helix content in PBS (31-37%) and HFP (53-
63%) were obtained from the [Θ_{220}] values measured in each solvent, using calibration parameters of [Θ_{220}] approximately -37000 to -44000 deg cm^2 dmol^{-1} for a 100% helical polypeptide chain, as described by Bulheller et al. 2.

**DISCUSSION**

We have developed a combined atomic force microscope (AFM) /inverted optical microscope technique to study the mechanical and viscoelastic properties of nanoscopic fibers. The range of forces that could be applied with this technique, using a range of standard, commercially available cantilevers, covers six orders of magnitude from F = 10^{-2} nN to 10^4 nN. Many biological fibers range in radii from a few to several hundred nanometers and in elastic moduli from E = 10^6 Pa to 10^9 Pa (table 1). Thus, the forces

![Figure 16 (A) CD Spectra of fibrinogen in phosphate-buffered saline (PBS, solid black line) and spin solvent, hexafluoro-2-propanol (HFP, gold line). (B) CD spectra of pure alpha-helical, beta-I and beta-II configurations (Image from: Bulheller et al. 2 – reproduced by permission of the PCCP Owner Societies). It is apparent that fibrinogen remains largely folded in the spin solvent; and that its alpha-helical content increases upon transfer to the spin solvent.](image)
needed to investigate those fibers, \( F = E \cdot A \cdot \varepsilon \), where \( A \) is the cross-sectional area of the fibers and \( \varepsilon \) is the strain, are in the range of \( 10^{-2} \) to \( 10^4 \) nN, which is perfectly covered by this technique. Few other techniques will be able to cover this force range so well. The maximum force in laser tweezers is about 0.15 nN \(^{25}\) and in AFM normal force measurements the stretching process cannot be easily observed and the maximum force is limited by the substrate-sample and tip-sample bonds, which are typically on the order of a few nN \(^{26}\). Moreover, the set-up of our technique also provides a well-defined and easy-to-analyze geometry for longitudinal stress-strain measurements.

We have used this technique to determine several key mechanical properties of electrospun fibrinogen fibers. We studied these fibers, because they may form the basis for numerous medical and material science applications. Our data may be used to design fibrinogen fiber structures with predictable mechanical properties.

We found that electrospun fibrinogen fibers can be extended to 2.3 times their length (\( \varepsilon_{\text{max}} = 1.3 \)), and that the breaking stress is 12 MPa. Electrospun fibrinogen fibers also display clear viscoelastic properties, as they show stress relaxation when held at constant strain. The stress relaxation data of electrospun fibrinogen fibers can be fit very well with a double exponential model, yielding two relaxation times of 3 s and 55 s. The average total stretch modulus, at a pulling speed of 340 nm/s, is \( 10 \cdot 10^6 \) Pa; the elastic modulus (after relaxation) is \( 5.2 \cdot 10^6 \) Pa. Electrospun fibrinogen fibers show some strain softening as the total and elastic moduli decrease with increasing strain.
<table>
<thead>
<tr>
<th>Material</th>
<th>E (MPa)</th>
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<tbody>
<tr>
<td><strong>High extensibility, soft fibers</strong></td>
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<tr>
<td>Electrospun fibrinogen fibers</td>
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<td>1-2(^d)</td>
<td>1.9(^d), 3.1(^e)</td>
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<td>6-300(^f)</td>
<td>1.6(^f), 2.2(^g)</td>
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<td>Fibronectin</td>
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<td>Myofibrils (sarcomere), titin (connectin))</td>
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<tr>
<td>Microtubules</td>
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**Table 1** Adapted from Guthold \(^27\). Stiffness (Young’s modulus, E) and breaking strain (extensibility ε\(_{\text{max}}\)) of various protein fibers. There are two reasons for the range of values in the Young’s modulus of a given fiber: 1) Authors report a range of values. 2) Strain hardening/softening; the fibers become stiffer/softer as the strain increases.
It is interesting to compare the properties of electrospun fibrinogen fibers to the properties of other natural fibers (Table 1, more extensive Table, see Guthold 27). Biological fibers can generally be divided into two groups: (1) Soft, extensible fibers with Young’s modulus on the order of MPa and extensibility, $\varepsilon_{\text{max}}$, larger than 1 (can be stretched to at least twice their length). Members of this group are elastin fibers, resilin fibers, intermediate fibers, fibrillin fibers, fibronectin and the extremely stretchable, natural fibrin fibers. (2) Stiffer, less extensible fibers with an about 1000 times larger stiffness on the order of GPa and an extensibility smaller than 0.2. Members of this group are collagen fibers, actin filaments and microtubules. Electrospun fibrinogen fibers clearly fall into group 1 (soft and extensible).

It is also interesting to compare the properties of electrospun fibrinogen fibers more closely with the properties of naturally formed fibrin fibers, because they share the same building block. The alpha helical content of human fibrinogen in PBS, as determined here by CD spectroscopy, was 31-37%. This is consistent with crystallographic data indicating 30% helix content for chicken fibrinogen 1. Chicken fibrinogen displays strong sequence homology to the human and bovine proteins; however, it lacks the long, flexible $\alpha$-chain C-terminal domain 20. Most of the alpha-helical content of fibrinogen is located in the coiled coils spanning the E- and D-domain (see Fig. 10). The alpha helical content of human fibrinogen increased to 53-63% in HFP. The location of this additional, HFP-induced alpha-helical structure in fibrinogen is not known. We speculate that it may be in the large, mainly unstructured $\alpha$-chain C-terminal domain. Conversion of this domain would be minimally disruptive to the rest of the well-defined structure of fibrinogen, which is stabilized by 29 disulfide bonds.
The properties of fibrin fibers have been recently investigated \(^{17,28}\). Partially crosslinked and non-crosslinked fibrin fibers have a large extensibility (\(\varepsilon_{\text{max}} = 3.3\) and \(2.3\), respectively); crosslinked and non-crosslinked fibrin fibers have an elastic modulus of about \(15\ \text{MPa}\) and \(2\ \text{MPa}\), respectively. Thus, it appears that the mechanical properties of naturally formed fibrin fibers and electrospun fibrinogen fibers are somewhat similar, despite the fact that fiber formation is very different. Natural fibrin fibers are linked together by very specific bonds; in non-crosslinked fibers, these are the A:a knob-hole interactions. The two A knobs, which are exposed after thrombin cleavage of peptide A, located in the central domain of fibrin bind to the a-holes in the end domain of two adjacent fibrin molecule. This results in the regular, half-staggered arrangement of fibrin molecules to form a fibrin fiber. In crosslinked fibrin fibers, activated factor XIII forms longitudinal crosslinks (the \(\gamma-\gamma\) crosslinks) across the abutting end domains of two fibrin molecules and lateral crosslinks (\(\alpha-\alpha\) crosslinks) between adjacent fibrin molecules in the radial direction \(^{20}\).

It is unknown which specific molecular interactions hold electrospun fibrinogen molecules together, though it is clear that they are different from the A:a interactions and the crosslinking formed in natural fibrin fibers. The A knob is not available because no thrombin was added to remove fibrinopeptide A; and no crosslinks are formed under our electrospinning conditions (as analyzed by SDS PAGE, data not shown). Electrospun fibrinogen fibers are probably just held together by numerous non-specific interactions. Also, electrospun fibers are formed in dry conditions. The solvent must evaporate from the protein solution before the protein solution reaches the grounding plate during electrostatic spinning. The dried fibers are then rehydrated.
Since both electrospun fibrinogen and natural fibrin fibers show somewhat similar mechanical properties, but assemble in a very different way, it is likely that the underlying building block, fibrin(ogen), influences the mechanical properties of the whole fiber to a larger extent. This, then suggests that it should certainly be possible to electrospin fibers with different, and somewhat predictable, mechanical properties by using and mixing molecular building blocks with different properties.

Two underlying molecular mechanisms by which fibrin fibers can achieve their remarkably large extension and elasticity\textsuperscript{17} have recently been proposed: an extension of the alpha-helical coiled coils to beta strands, and a partial unfolding of the globular D-domains of the fibrin monomer\textsuperscript{27,29-31}. This two-step model is consistent with recent experimental findings. It is consistent with the observation that fibrinogen lengthens to twice its length at about 100 pN applied force. This lengthening is attributed to the alpha helical to beta strand conversion of the coiled coils\textsuperscript{30,31}. Partial unfolding of the D-domain was invoked to explain single molecule unfolding experiments on fibrinogen\textsuperscript{29}.

We propose that these two underlying molecular extension mechanism (alpha helical to beta strand conversion of coiled coils, and partial unfolding of D-domain of the underlying building block, fibrinogen) are also taking place in electrospun fibrinogen fibers. It would most straightforwardly explain the similar mechanical behavior of the two fibers.

McManus et al. tested the bulk mechanical properties of wet, non-woven mats made from electrospun fibrinogen fibers. The fibers in the mats were randomly oriented, i.e. not aligned in a particular direction. These mats had an extensibility, $\varepsilon_{\text{max}}$, of 1.0 – 1.4 and an elastic modulus of 0.4 – 0.6 MPa\textsuperscript{21}. Thus, the extensibilities of the mats and
the single fibers are similar; however, both the peak stress and the modulus of elasticity are about an order of magnitude lower for the mats, as compared to single fibers. These comparisons make it clear that the mechanical properties of the mats are a complex combination of the mechanical properties of the single fibers comprising the mats, and several other factors. These other factors include: (1) Void space: The mats contain a large amount of void space which will result in a smaller elastic modulus. (2) Fiber alignment: Since the fibers in the mats are initially not aligned, stretching of the mats may first align the fibers in the stretch direction before the individual fibers are stretched. Since aligning of the fibers likely requires less force than stretching them, this aligning mechanism would lower the elastic modulus for the mats. It would also increase the extensibility of the mats. (3) Fiber connections: The properties of the connections between fibers will influence the mechanical performance of the mats; for example, weak connections would result in a reduced elastic modulus, peak stress and extensibility for the mats. (4) Fiber entanglement: More entanglement would strengthen the mats and likely result in a larger elastic modulus for the mats.

These considerations indicate that the properties of macroscopic structures depend in a complex way on the properties of its individual constituents (fibers), the connections between its constituents and the underlying architecture. In future work we will attempt to unravel the contributions of each of these components to the properties of the overall macroscopic structure. This can be done by combining microscopic testing, as done in our paper, macroscopic testing, and finite element modeling to combine the microscopic and macroscopic properties. Such experiments should make it possible to design macroscopic structures with desirable properties, starting with the properties of the
microscopic fibers and the connections between them. One key application would be designing, modeling and testing scaffolds for cells in tissue engineering. Cells are extremely sensitive to the mechanical, architectural and chemical properties of the scaffolds and the underlying fibers. In the future we will also test fibers spun from different natural and synthetic materials (e.g. fibrinogen, collagen and mixtures) and evaluate their performance in tissue scaffolds.

CONCLUSIONS

We have developed a combined microscopic technique to study the mechanical properties of nanoscopic fibers and used it to investigate electrospun fibrinogen fibers. The mechanical properties of these fibers are important for numerous applications in tissue engineering as well as polymer composites and biomimetics. As compared to other biological protein fibers, electrospun fibrinogen fibers have a low modulus and a high extensibility. However, the electrospun fibrinogen fibers are not quite as soft and extensible as their naturally formed counterpart, fibrin fibers.

ACKNOWLEDGMENTS

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CHAPTER IV

THE MECHANICAL PROPERTIES OF SINGLE FIBRIN FIBERS

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The following manuscript is in press in The Journal of Thrombosis and Heamostasis, 2010, DOI: 10.1111/j.1538-7836.2010.03745.x. Stylistic variations are due to the requirements of the journal. C.R. Carlisle performed all experiments and analysis on crosslinked fibrin fibers. C.R. Carlisle, Dr. W. Liu and E.A. Sparks performed experiments and analysis on uncrosslinked and partially crosslinked fibrin fibers. The original manuscript draft was prepared by Dr. W. Liu and Dr. M. Guthold. Extensive redrafting and editing was performed by C.R. Carlisle and Dr. M. Guthold.
ABSTRACT

Background. Blood clots perform the mechanical task of stemming the flow of blood.

Objectives. To advance understanding and realistic modeling of blood clot behavior we determined the mechanical properties of the major structural component of blood clots, fibrin fibers.

Methods. We used a combined atomic force microscopy (AFM)/fluorescence microscopy technique to determine key mechanical properties of single crosslinked and uncrosslinked fibrin fibers.

Results and Conclusions. Overall, full crosslinking renders fibers less extensible, stiffer, and less elastic than their uncrosslinked counterparts. All fibers showed stress relaxation behavior (time-dependent weakening) with a fast and a slow relaxation time, 2 s and 52 s. In detail, crosslinked and uncrosslinked fibrin fibers can be stretched to 2.5 and 3.3 times their original length before rupturing. Crosslinking increased the stiffness of fibers by a factor of 2, as the total elastic modulus, $E_0$, increased from 3.9 MPa to 8.0 MPa and the relaxed, elastic modulus, $E_{\infty}$, increased from 1.9 MPa to 4.0 MPa upon crosslinking. Moreover, fibers stiffened with increasing strain (strain hardening), as $E_0$ increased by a factor of 1.9 (crosslinked) and 3.0 (uncrosslinked) at strains $\varepsilon > 110\%$. At low strains, the portion of dissipated energy per stretch cycle was small (<10%) for uncrosslinked fibers, but significant (~ 40%) for crosslinked fibers. At strains greater than 100%, all fiber types dissipated about 70% of the input energy.
We propose a molecular model to explain our data. Our single fiber data can now also be used to construct a realistic, mechanical model of a fibrin network.

INTRODUCTION

Blood clots have the essential mechanical task of stemming the flow of blood, and for the past six decades there has been continuing interest in resolving the mechanical properties of clots and their constituents. The importance of the mechanical properties of a clot is underscored by the fact that they can be related to clotting disorders and disease. Properties of the whole, macroscopic clot can be determined via rheometry. Moreover, single molecule experiments and the known X-ray structure of fibrinogen provide molecular information. A combination of rheometry, electron microscopy and small angle X-ray diffraction, has recently also been used to investigate the multiscale mechanical behavior of fibrin polymers, suggesting that protein unfolding plays an important role at the molecular scale, while fiber stretching and fiber alignment play key roles at the meso- and macroscopic scales. However, single fiber properties that connect underlying molecular mechanisms and macroscopic mechanical properties of a clot, have only recently started to emerge, and their understanding remains far from complete. This single fiber knowledge is critical for constructing mechanical models of clots and, thus, deeper understanding of clot behavior.

Blood clots are formed when soluble fibrinogen is converted to fibrin monomers that polymerize to form a network of fibrin fibers. The network is further stabilized by
the formation of covalent bonds (crosslinks) between specific glutamine and lysine residues of the fibrin monomers. It is this branched network of fibrin fibers that mostly determines the mechanical properties of a clot. Generally, the mechanical properties of such a network depend on three network properties: 1) the network architecture which describes the overall structural composition of the network; 2) the mechanical properties of the individual fibers that comprise the network; 3) the properties of the joints between fibers. The architecture of the fibrin network may be determined from microscopy images. However, beside the extensibility and elastic limit, the elastic modulus for small strains and the rupture force of dried fibrin fibers, the mechanical properties of single fibrin fibers are unknown. Yet, exactly this knowledge on the single fiber level is needed to construct and test mechanical models of clots and, thus, advance our understanding of clot mechanical behavior. The mechanical properties of fibrin fibers are among the crucial factors that determine if a clot will deform, rupture or embolize. Considering the fact that blood clots can embolize, it is clear that physiological blood flow can create a large enough force to deform and rupture fibrin fibers.

We have developed a combined AFM/fluorescence microscopy technique to determine the mechanical properties of individual nanoscopic fibers. We have collected stress-strain (force-extension) curves over the entire, extraordinarily large extensibility range of crosslinked and uncrosslinked fibrin fibers and report their distinct mechanical behavior. We have determined the i) extensibility (stretchability); ii)
elasticity; iii) strain hardening behavior (stiffening with increased stretching), iv) energetic behavior (dissipated and stored energy); v) total and vi) relaxed elastic modulus (viscoelastic stiffness), and vii) stress relaxation behavior (time-dependent behavior). We propose a molecular model, based on two major secondary structure transformations and an extension of the interacting αC regions of the fibrin monomer, to explain the observed mechanical behavior.

MATERIALS AND METHODS

Fibrin Fiber Formation

Uncrosslinked and crosslinked fluorescently labeled fibrin fibers were formed on a striated substrate made from cured optical adhesive (details, see supplement in appendix). All experiments were carried out in “fibrin buffer” (140 mM NaCl, 10 mM Hepes, 5 mM CaCl₂, pH 7.4). For each experiment, two samples were prepared in parallel. One sample was used for the AFM manipulation experiments, the other for SDS PAGE. Crosslinked samples showed complete γ−γ and α−α crosslinking (≥ 90%), while the samples without added Factor XIII showed no crosslinking.

Manipulation of Fibers

The mechanical manipulations on fibrin fibers were performed with a combined AFM/optical microscope instrument (details, supplement in appendix). The schematic set-up is shown in Figure 17A & B. The instrument is based on a Zeiss inverted optical
microscope (Zeiss Axiovert 200, Gottingen, Germany), a Hamamatsu, high sensitivity camera (EM-CCD C9100, Hamamatsu Photonics KK, Japan) and a Topometrix Explorer AFM (Veeco Instruments, Woodbury, NY). The microscope stage was designed so that the AFM tip, fiber sample and objective lens can move with respect to each other, which allows alignment of the fiber with the AFM tip and objective lens. The AFM manipulation experiments were done with a nanoManipulator, a software program that interfaces the AFM with a force feedback stylus and a graphics computer. The nanoManipulator provides control over the x-, y- and z-movement of the AFM tip. The AFM tip is used to stretch the fibrin fibers that are suspended across 12 μm wide channels in the striated, transparent substrate. The fluorescence microscope, situated underneath the substrate is used to collect movies of this stretching process. The extension of the fiber was determined by fluorescence microscopy and calculation using the distance traveled by the AFM tip (Fig. 17B). The applied force was determined from the twist of the cantilever. The force measurements were calibrated via the Sader method and the Liu method.

RESULTS

Stress-Strain Curves

For manipulations, we selected single fibers bridging the grooves in a straight line approximately perpendicular to the ridge edge. We have found that most fibers are well-anchored on the ridges of the striated substrate, even at extreme fiber extensions. We
excluded fibers that slipped on the ridges from our analysis. This experimental design yields a well-defined geometry to determine the mechanical properties of fibrin fibers (Fig. 17A & B).

The mechanical properties of polymers are generally determined via stress-strain (force-extension) curves in which stress, $\sigma$, is plotted as a function of strain (deformation), $\varepsilon$, under a variety of conditions. For longitudinal stretching experiments, as done here, stress is defined as $F/A$, where $F$ is the applied force and $A$ is the initial cross-sectional area of the stretched polymer. This is the definition of the commonly used engineering stress, which uses the initial cross-section and does not consider how the cross-sectional area changes as $\varepsilon$ increases. Engineering stress gives a lower limit for the stress applied to the polymer, since the cross-sectional area most likely decreases as the fiber is stretched. Strain is defined as $\Delta L/L_{\text{init}}$, or $(\Delta L/L_{\text{init}}) \cdot 100\%$, where $\Delta L$ is the change in length and $L_{\text{init}}$ is the initial length. A typical stress-strain curve of a fibrin fiber being stretched to 1.75 times its initial length, ($\varepsilon = 0.75$ or 75%), is shown in Figure 17C. The forward and return paths do not overlap; this means that energy (proportional to area under curves) is lost in this stretching cycle. However, although energy is lost, there is no permanent lengthening of the fiber ($\sigma$ on return path does not reach 0 until $\varepsilon = 0$). The energy loss is due to the viscous properties of fibrin fibers and it is an indication that fibrin fibers are viscoelastic polymers. The slope of this curve corresponds to the stiffness (modulus) of the fiber, here about 1.3 MPa. However, it is apparent that such a simple analysis of just the slope misses some key properties of the fiber, such as its
viscoelasticity, energy loss, and stiffening with increasing strain. In our experiments we
analyzed basic and viscoelastic mechanical properties. A typical movie sequence of a
fibrin fiber stretching experiment is shown in Figure 17D–F (additional data, see
supplement in appendix).

Figure 17 (A) Schematic of an atomic force microscope (AFM) sitting on top of an inverted
optical microscope. (B) Top view of a stretched fiber. The initial and stretched states are in
dotted gray and solid black, respectively. (C) Typical fibrin fiber stress-strain curve. (D-F)
Fluorescence microscopy movie frames of a stretching experiment. The fiber is anchored on
two ridges (brighter, horizontal, 8 μm wide bars) and suspended over a groove (darker,
horizontal, 12 μm wide bars); the AFM cantilever appears as a 35 μm wide, dark rectangle; the
AFM tip is indicated as a green dot.
Fibrin Extensibility and Elastic Limit

Previously, fibrin fiber extensibility, $\varepsilon_{\text{max}}$, defined as the strain (extension) at which fibers rupture, was determined to be 333% and 226% for partially crosslinked and uncrosslinked fibrin fibers, respectively $^{11}$. The elastic limit, $\varepsilon_{\text{elastic}}$, defined as the largest strain to which fibers can be stretched and recover to their original length without visible permanent deformation, was previously determined to be 180% and 120% for partially crosslinked and uncrosslinked fibrin fibers $^{11}$. It is important to note, that the fibers in this previous study were only partially crosslinked. Here, we determined the extensibility and elastic limit of fully crosslinked fibrin fibers. $\varepsilon_{\text{max}}$ was 147%, which is lower than both partially crosslinked and uncrosslinked fibrin fibers. We found the elastic limit of fully crosslinked fibers to be $\sim$ 50% strain. However, some fibers showed permanent deformation at strains as low as 10%. All mechanical properties of crosslinked and uncrosslinked fibers are summarized in table 2; table S1 in the supplement also includes all the data for partially crosslinked fibers.

Strain Hardening

Strain hardening refers to the phenomenon when the elastic modulus (stiffness, slope of the stress-strain curve) increases (hardens) with increasing strain. We observed strain hardening, occurring at approximately 100% strain, for all fibrin fibers. However, crosslinked and uncrosslinked fibers do display several differences: Uncrosslinked fibers are initially softer; they show larger extensibilities and significant strain hardening.
Crosslinked fibers are initially stiffer, show less extensibility and show less pronounced strain hardening. In Figure 18A, a stress-strain curve of an uncrosslinked fibrin fiber is plotted. In Figure 18B the total elastic modulus, $E_0$, i.e. the slope of the forward stress-strain curve in Figure 18A, is plotted as a function of strain. The curve displays a distinct sigmoidal shape; the total elastic modulus is relatively constant for the first 100% strain; it then increases by a factor of about 3 and remains at this higher value until the fiber ruptures. The strain hardening factor, $h$, i.e. the average ratio of the total elastic modulus at large strain ($\varepsilon > 110\%$) to that at small strain ($\varepsilon < 80\%$) are listed in table 2. On average, uncrosslinked fibers harden by a factor of 3.0 ($p \leq 0.0008$). A strain hardening factor of 1.9 ($p \leq 0.049$) was determined for crosslinked fibers. However, while crosslinked fibers did show significant strain hardening, as indicated by t-test analysis, hardening occurred with less consistency. Figure 18C shows a crosslinked fibrin fiber, stretched to 130%, which does not show strain hardening.
Stored and Dissipated Energy Per Stretch Cycle

As seen in Fig 17C, not all of the energy put into stretching a fibrin fiber (area under forward stress-strain curve) is stored. A fraction of this energy is lost (dissipated) due to viscous processes. The dissipated energy is proportional to the area inscribed by the forward and backward curves. We have determined the amount of dissipated energy per stretch cycle as a function of strain (Figure 19). Uncrosslinked fibers show little energy loss at low strains and 70% energy loss at high strains. Crosslinked fibers already show significant energy loss at low strains.

Figure 19A shows three pulling cycles on the same uncrosslinked fibrin fiber with strains of 48%, 85% and 125%, respectively. It can be seen that the fiber dissipated almost no energy (all energy is stored) in the first pull ($\varepsilon = 48\%$), as the area between the forward and backward curve is very small. However, for larger strains ($\varepsilon = 85\%$ and $\varepsilon = 125\%$) a significant fraction (28% and 43%, respectively) of the energy is dissipated. It
should be noted that even though large amounts of energy are dissipated, uncrosslinked fibers still return to their original length, meaning that they did not permanently lengthen, at strains less than 120% \(^\text{11}\).

In Figure 19B, the ratio (percentage) of dissipated energy to the total energy is plotted as a function of strain. The graph for uncrosslinked fibers shows a clear sigmoidal shape. The energy loss at low strains is very small; it then increases significantly at strains between 50%-100% and remains at a constant, higher energy loss level of around 70% for larger strain.

Conversely, crosslinked fibers showed significant energy loss at low strains (43% energy loss for small strains of \(\varepsilon < 40\%\)). At larger strains (\(\varepsilon > 40\%\)), the energy loss increased to a plateau of 70% at 100% strain. This increased energy loss (at low strains) for crosslinked fibers is consistent with the notion that crosslinked fibers undergo permanent deformation at lower strains (\(\varepsilon_{\text{elastic}} < 50\%\)) than uncrosslinked fibers.
Both uncrosslinked and crosslinked fibrin fibers show clear viscoelastic (time-dependent) behavior. We also found that crosslinking increases the stiffness (total and relaxed elastic modulus) by a factor of 2.

Incremental stress-strain curves are a technique to separate the elastic (energy stored) and viscous (energy lost) components in viscoelastic materials (Fig. 20). In this technique, the fiber is stretched to a certain strain, where it is held constant for some time.
Due to viscous processes, the fiber relaxes; that is, the force (stress) to hold it at that strain decreases. Subsequently, the fiber is stretched by another increment and held at constant strain. Again, the fiber relaxes and the stress decreases. This incremental straining is repeated several times. Figure 20A shows the strain vs. time curve of a fibrin fiber; the fiber was incrementally stretched to 23%, 46%, 75%, 104% and 138% strain and held constant at those strains for about 120 s. Figure 20B shows the corresponding stress vs. time curve of this fiber and it is readily apparent that the fiber relaxes (stress decreases) at each constant strain value. The x-axis (time) in Figure 20A and B are the same. The stress does not decay to zero, but to a constant value at each strain. When plotting each of the peak stress values vs. strain and the relaxed stress values vs. strain, the stress-strain curves in Figure 20C are obtained. The relaxed stress values, $\sigma_\infty$ for $t \rightarrow \infty$ were obtained by fitting two exponentials (details, see supplement in appendix). The slope of the higher curve in Figure 20C is the total elastic modulus, $E_0$, obtained before relaxation, and the slope of the lower curve is the relaxed, elastic modulus, $E_\infty$, of fibrin fibers, obtained for $t \rightarrow \infty$. For crosslinked fibers, $E_0$ was 8.0 MPa, and $E_\infty$ was 4.0 MPa. For uncrosslinked fibers, $E_0$ was 3.9 MPa and $E_\infty$ was 1.9 MPa. Somewhat simplistically speaking, $E_0$ corresponds to the stiffness of the fibers when they are pulled fast; and $E_\infty$ when they are pulled slowly. For comparison, other materials with stiffness in the MPa range are elastin fibers, spider silk, or a soft rubber band.\textsuperscript{19}
Stress relaxation, as seen in Fig. 20B, is indicative of viscous (time-dependent) processes. Stress relaxation curves are usually fitted with one or more exponential functions yielding stress relaxation times. We have done an analysis of the stress relaxation behavior of fibrin fibers using a generalized Kelvin model (see supplement in appendix). Both crosslinked and uncrosslinked fibers show two stress relaxation rates; a fast (2 s to 4 s) and a slow relaxation rate (49 s to 57 s). These two rates are indicative of

Figure 20 (A) Strain vs. time and (B) stress vs. time for an incremental stress-strain curve of a fibrin fiber. The x-axis (time) is the same for (A) and (B). The strain is held constant at 23%, 46%, 75%, 104%, and 138% for about 120 seconds. The stress decays exponentially at each strain value. (C) Total stress (red curve, squares) and relaxed stress (blue curve, dots) vs. strain as obtained from the data in (A) and (B).

Stress relaxation

Stress relaxation, as seen in Fig. 20B, is indicative of viscous (time-dependent) processes. Stress relaxation curves are usually fitted with one or more exponential functions yielding stress relaxation times. We have done an analysis of the stress relaxation behavior of fibrin fibers using a generalized Kelvin model (see supplement in appendix). Both crosslinked and uncrosslinked fibers show two stress relaxation rates; a fast (2 s to 4 s) and a slow relaxation rate (49 s to 57 s). These two rates are indicative of
two distinct molecular relaxation processes, such as a molecular unfolding or transformation event, occurring with these time scales.

Table 2
Crosslinked and Uncrosslinked Fibrin Properties

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Crosslinked</th>
<th>Uncrosslinked</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon_{\text{max}}$</td>
<td>147 % +/- 5%</td>
<td>226% +/- 8.7%*</td>
</tr>
<tr>
<td>$\varepsilon_{\text{elastic}}$</td>
<td>&lt; 50 %</td>
<td>120 % *</td>
</tr>
<tr>
<td>$E_0$ (MPa)</td>
<td>8.0 +/- 1.0</td>
<td>3.9 +/- 0.6</td>
</tr>
<tr>
<td>$E_\infty$ (MPa)</td>
<td>4.0 +/- 0.6</td>
<td>1.9 +/- 0.3</td>
</tr>
<tr>
<td>$\tau_1$ (s)</td>
<td>2.1 +/- 0.2</td>
<td>3.6 +/- 1.3</td>
</tr>
<tr>
<td>$\tau_2$ (s)</td>
<td>49 +/- 4</td>
<td>57 +/- 8</td>
</tr>
<tr>
<td>$h$</td>
<td>1.9 +/- 0.3</td>
<td>3.0 +/- 0.3</td>
</tr>
<tr>
<td>$E_{\text{loss}}$</td>
<td>$\leq$ 40-70%</td>
<td>0-70%</td>
</tr>
</tbody>
</table>

Table 2 Mechanical properties of fibrin fibers; the average values and standard errors are listed; see appendix for partially crosslinked fibers and statistics. Extensibility, $\varepsilon_{\text{max}}$; elastic limit, $\varepsilon_{\text{elastic}}$; $E_0$, total elastic modulus; $E_\infty$ relaxed elastic modulus; $\tau_1$ fast relaxation time; $\tau_2$ slow relaxation time; $h$, strain hardening factor (ratio of the total modulus at low strains (0-80%) to the total modulus at high strains (above 110%). Thus, the total elastic modulus for uncrosslinked fibers has a value of 3.9 MPa at low strains and 11.7 MPa at high strains. Crosslinked fibers did not show consistent strain hardening. $E_{\text{loss}}$, percent energy loss per stretch cycle (energy loss at low strains is close to 0% for uncrosslinked, and $\leq$ 40% for crosslinked fibers).

* Values from Liu 11.
DISCUSSION

We have investigated the mechanical properties of single crosslinked and uncrosslinked fibrin fibers. All our findings are summarized in table 2 (and table S1, which includes the data for partially crosslinked fibers).

Our values for the total elastic modulus, $E_0$, for uncrosslinked and crosslinked fibers (3.9 and 8.0 MPa) agree to within a factor of 2 with the small strain values obtained by Collet et al. (1.7 and 14.5 MPa) as determined by laser tweezers $^{12}$. The differences may be explained by the different experimental set-ups and inherent, instrumentation-related errors.

The force per fibrin monomer can also be estimated from our data and compared with single protein unfolding experiments (calculations, see supplement in appendix). From our data, the force per monomer at 100% strain is about 140 pN, which is consistent with the 100 pN force to stretch a single fibrin monomer by 100% found by Brown et al. $^8$. From our data, the rupture force per monomer is about 280 pN, which is similar to the 260 pN (2·130 pN) required to rupture the two A:a interactions between half-staggered monomers within a protofibril $^{20}$. Interestingly, these forces are smaller than some protein unfolding forces $^{21}$, suggesting that some regions of the fibrin monomer may unfold before the fiber ruptures.

The energy per monomer required to rupture the fiber, as obtained from the area under our stress-strain curves (2400 kJ/mol) is of the same order of magnitude as the
melting enthalpy of fibrin molecules (4650 kJ/mol) \(^{22}\) (calculations, see supplement in appendix), again indicating that fibrin may melt (denature), before the fiber ruptures.

Crosslinking had a significant and intriguing effect on several mechanical properties, and no effect on other properties. Uncrosslinked fibrin fibers are very extensible, are elastic at high strains, are relatively soft, show strain hardening by a factor of 3.0, and a sigmoidal energy loss curve going from 0% loss at low strains to 70% loss at high strains. Complete crosslinking makes fibers stiffer, less extensible, more susceptible to plastic deformation at a lower strain, and increased the low-strain energy loss. Being already stiffer, crosslinked fibrin fibers did not show the same amount of strain hardening and often did not extend to strains at which strain hardening would have occurred. \(\gamma-\gamma\) crosslinks form between two reciprocal sites on the abutting \(\gamma\)-nODULES of aligned fibrin monomers within a protofibril, and they are thought to be oriented along the longitudinal fiber axis. \(\alpha-\alpha\) crosslinks form between numerous sites on the extensive, flexible and partially unstructured \(\alpha\)C regions (\(\alpha221-610\)), and due to the length of this domain they may be oriented in the lateral (radial direction), and longitudinal direction. In previous experiments, partially crosslinked fibrin showed the largest extensibility (330%) \(^{11}\). Thus, crosslinking seems to have a bell-shaped effect on extensibility; from \(\varepsilon_{\text{max}} \sim 230\%\) (uncrosslinked) to \(\varepsilon_{\text{max}} \sim 330\%\) (partial crosslinking) to \(\varepsilon_{\text{max}} \sim 150\%\) (fully crosslinked). Full crosslinking appears to have a restricting effect on previously mobile regions (e.g. the \(\alpha\)C regions), it may prevent unfolding of domains and/or it might tighten the fibrin monomer and protofibril interactions.

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Clot rheology studies on whole clots showed that crosslinking induces a 2 to 3.5-fold increase of rigidity \(^4,^{23,24}\) of the whole clot, which is similar to the 2-fold increase in \(E_0\) of single fibers we observed.

To explain the observed mechanical behavior of fibrin fibers, we propose a model with the following three molecular mechanisms (Fig. 21). The mechanisms may occur in parallel as the fibers are stretched. 1) \(\alpha\)-helix to \(\beta\)-strand conversion of the two coiled coils of the fibrin monomer. This conversion can account for 90 – 100\% strain \(^8,^{19}\). 2) Deformation or partial unfolding of the \(\gamma\)-nodule of the fibrin monomer. This conversion may account for an additional 220\% strain \(^{19}\). These two mechanisms may dynamically fluctuate between each other (inter-convert). 3) Interaction and extension of the long, flexible and partially unstructured \(\alphaC\) region. The \(\alphaC\) region (\(\alpha221-610\)) consists of an unstructured connector region (\(\alpha221-391\)), and the terminal domain (\(\alpha392-610\)). There is evidence that interactions between the \(\alphaC\) regions may play a role in fiber assembly, especially lateral aggregation \(^{25-27}\). It was also found that a shorter length of the \(\alphaC\) connector (in different species) correlates with a lower extensibility of fibers \(^{26}\).
Fibrinogen monomer

Half-staggered assembly of three fibrin monomers into protofibril

A:a interactions

αC domain interactions promote protofibril lateral aggregation

αC chains, Bβ chains and γ chains are in blue, red and green, respectively. A cartoon depiction of the flexible αC region is added to the crystal structure as a blue line and blue square, αC regions may interact with each other within a protofibril, and across protofibrils. (B) Schematic model of half-staggered assembly of three fibrin monomers into protofibril, (β–nodule is no longer depicted). (C) An α-helix to β-strand conversion of the coiled coil and a slight straightening and alignment of the molecules could accommodate ~100% strain. Some of the αC regions are also extended at this point. (D) Higher strains, up to 320% could be accommodated by a partial unfolding of the globular γ-nodule; 230% strain is depicted. Further extension of the αC region could occur. (E) Interactions between αC regions promote lateral aggregation of protofibrils; they can be elastically extended.
This model may explain several experimental observations. 1) Two relaxation rates. If viscoelastic mechanism 1 and 2 occur at different rates and if they are interconvertible, two different relaxation rates would be observed. For example the $\alpha$-helical conversion may happen at a faster rate (and lower force) and the unfolding of the $\gamma_C$ region may happen at a slower rate (and higher force). 2) Strain hardening occurring at about 100% strain. The faster $\alpha$-helical conversion provides 100% strain, after that $\gamma$-nodule unfolding becomes dominant. 3) The dramatic and sigmoidal increase in energy loss with increasing strain. At larger strains the $\gamma$-nodule unfolds more extensively, which may not be totally reversible, thus resulting in large energy losses. 4) The elastic limit (no permanent lengthening) of uncrosslinked fibers is about 120% $^{11}$. The $\alpha$ to $\beta$ conversion (and initial unfolding of $\gamma$-nodule) may be largely reversible (despite some dissipative energy) and thus explain the observed 120% elastic limit of uncrosslinked fibrin fibers. 5) Brown et al. showed by force spectroscopy that a chain of fibrinogen monomers lengthens incrementally by about 90–100% upon the application of force $^8$. The $\alpha$-helical to $\beta$-strand conversion of the coiled coils is consistent with these measurements. This conversion was also observed in computational simulations $^{28}$. 6) Stiffening and decreased elasticity of the fiber upon crosslinking. The role of the two $\alpha_C$ regions in fiber assembly is still unclear; however, they are important for lateral aggregation $^{25-27}$. The $\alpha_C$-connector region alone ($\alpha_{221-391}$) can extend 61 nm (122 nm for both), and the whole $\alpha_C$ region ($\alpha_{221-610}$) can extend still farther. The $\alpha_C$ region could, therefore, elastically connect fibrin monomers within a protofibril, and they can
also easily reach across adjacent protofibrils. However, in order for the αC region to fully extend the molecule must unfold or the α:A interactions but rupture. The αC region interactions may be partly responsible for the elastic recoil forces. It would explain that fully crosslinked fibers become stiffer, less elastic and less extensible. There are numerous crosslinking sites and full crosslinking may, thus, limit the mobility of the αC-connector. More insights into the role of the α−α and γ−γ crosslinks could be gained by extending our single fiber experiments to crosslinking mutants.

It should now be possible to use our data on the single fibers, and the data from our companion paper on the strength of fibrin fiber joints, to build a realistic, mechanical model of a blood clot, by utilizing recently developed network modeling approaches. The model data could then be compared to whole clot measurements.

It would be equally interesting to build a model of a single fiber starting with an arrangement of single fibrin molecules, and testing the mechanical properties of such a model against our data.

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CHAPTER V

STRENGTH AND FAILURE OF FIBRIN FIBER BRANCH POINTS

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Blood clots form rapidly in the event of vascular injury to prevent blood loss. They may also form in undesired places causing heart attacks, strokes and other diseases. Blood clots can rupture, and fragments of the clot may lodge in distal blood vessels causing, for example, ischemic strokes or embolisms. Thus, there has been great interest to understand the mechanical behavior and failure mechanisms of blood clots and their constituents. To develop a mechanically realistic model of a blood clot, knowledge of the mechanical properties of its constituents is required. The major structural component providing mechanical strength to the clot is a mesh of fibrin fibers. Principally, three pieces of information are needed to develop realistic (fibrin fiber) network models: 1) The architecture of the network; 2) the properties of the single fibers; 3) the properties of the fiber branch points.

The architecture of the fibrin fiber network in a blood clot may be obtained from microscopy images. Recently, there has been significant activity in determining the mechanical properties of individual fibrin fibers. Here, we investigated the strength and failure of crosslinked and uncrosslinked fibrin fiber branch points (Figure 22). We report two distinct methods of failure; rupture at the joint, and rupture along a fiber. In fully crosslinked fibrin fibers, rupture occurred most often along the fiber. Conversely, in uncrosslinked fibers, failure occurred most often due to the detachment of a leg at the branch point. Perhaps the most unexpected finding was that neither crosslinked nor uncrosslinked joints showed failure due to extended unzipping of the joint, indicating that joints are rather resilient to failure. It appears that, in some cases, continuous unzipping may be prevented by a triangular branch point architecture, in which fibers (cross-struts) reach across all three legs in a Y-shaped junction. This architecture was revealed in many branchpoints as they were strained. This triangular architecture is consistent with a model in which polymerization occurs in all directions at the branchpoints, as proposed by Ryan et al. Along with triangular branchpoint architecture, twisting of protofibrils during aggregation may also prevent unzipping of branchpoints. Finally, we report the strength (stress at failure) of fibrin fiber branch points.

Fibrin is formed by thrombin-mediated cleavage of fibrinopeptides A and B from soluble fibrinogen. Fibrin monomers self-assemble into half staggered protofibrils, which associate to form fibers. During the formation of fibrin fibers, factor XIIIa,
covalently rigidifies molecule-molecule interactions through three types of crosslinks. (i) \(\gamma-\gamma\) crosslinks which form between the \(\gamma\)-nodule of abutting molecules; 2) \(\alpha-\alpha\) crosslinks which form multiple bonds between the \(\alpha\)C regions of adjacent molecules; and 3) the less frequent \(\alpha-\gamma\) crosslinks. Crosslinking of fibrin films and fibers increases the modulus (stiffness) while making fibrin fibers less extensible.\(^8\)\(^-\)\(^10\).

During clot formation the fibrin fibers branch and grow forming a network with interspersed platelets and red blood cells. The fiber joints, or branch points, in this network are mostly formed by the intersection of three fibers at a node.\(^6\)\(^,\)\(^11\). On a molecular level, there are two models as to how these joints form; the trimolecular joint model and the tetramolecular joint model.\(^12\)\(^,\)\(^13\).

To examine fibrin joints a striated substrate was prepared as previously reported\(^4\) using a micromolding technique\(^14\) and Norland Optical Adhesive-81 (Norland Products, Inc. Cranbury, NJ). Fibrin fibers were polymerized directly on the substrate with a human fibrinogen concentration between 0.5 and 1 mg/ml (American Diagnostica, Stamford, CT, FXIII depleted) and a thrombin concentration between 0.05 and 0.1 units/ml (Enzyme Research Laboratories, South Bend, IN). To form crosslinked fibers joints, 25 Loewy units/ml of FXIII were added during polymerization (Enzyme Research Laboratories, South Bend, IN). The fibrin fibers were labeled with 24 nm carboxyl, yellow-green fluospheres for visualization (Invitrogen, Carlsbad, CA). To test the extent of crosslinking, samples were prepared in parallel and subjected to SDS polyacrylamide gel electrophoresis. To within the sensitivity limits of the gel, crosslinking was complete (\(\geq\) 90 %).

Mechanical manipulation of joints was done with a combined atomic force microscope/fluorescence microscope (ThermoMicroscope Explorer AFM, Zeiss Axiovert 200, Hamamatsu EM-CCD C9100 camera, 3rd Tech NanoManipulator) as described by Liu\(^4\)\(^,\)\(^8\). The AFM tip applied a force to one leg of the joint. Fluorescence microscopy images were recorded and used to determine failure mechanism and measure initial length and strain at rupture of each leg of the joint (Figure 22 A, B).

Straining fibrin fiber joints resulted in two methods of joint failure; rupture along a fiber, and rupture at the node (Fig 23). Additionally we observed detachment of a fiber.
Uncrosslinked and crosslinked joints showed different rupture behaviors. Uncrosslinked joints ruptured 68.5% of the time at the node and 31.5% of the time along a fiber. Conversely, crosslinked joints ruptured 39.5% of the time at the node and 60.5% of the time along a fiber (Figure 22C, D). This suggests that individual crosslinked fibrin fibers are weaker than crosslinked joints, while the opposite is true for uncrosslinked fibrin fibers. Failure of crosslinked fibers before failure of joints may also be due to the lower extensibility of crosslinked fibers, 147% to 217% \cite{8,15}, as compared to uncrosslinked fibers, 226% \cite{4}. Also, the difference between crosslinked and uncrosslinked fibrin joints suggests that FXIIIa crosslinking provides a mechanism which fortifies and strengthens fiber branch points. Thus, natural, fully crosslinked clots may mainly fail by fiber rupture rather than rupture of branch points.

Perhaps most surprising is the finding that joints are so resistant to failure. We expected that especially uncrosslinked fiber joints would easily unzip, since bonds implicated in lateral aggregation are weak \cite{16}. However, we did not observe such unzipping events. A possible explanation might be that fibrin protofibrils aggregate in a helical manner around the fiber, limiting fiber size \cite{7} and preventing two dimensional unzipping.

Unzipping may be further prevented by a triangular architecture, in which three cross-struts prevent unzipping of the joint (Figure 22E-G). In this architecture, observed in 27% of both crosslinked and uncrosslinked joints, each fiber branches into the two additional fibers involved in the joint, resulting in a triangle at the branch point. The formation of this architecture suggests that fiber polymerization occurs in all directions, since all three fibers of the joint participate in the formation of the triangle. This is consistent with the model of fibrin branching proposed by Ryan et al. in which fibrin oligomers form and join together both lengthwise and laterally resulting in branching points and lateral aggregation \cite{6}. In about half of the joints showing triangular architecture, the shape is visible prior to manipulation. In the other half, it is not visible until manipulation has begun. Two possible explanations for the inconsistency of visible architecture are; 1) triangular architecture may be visible in the early stages of joint
formation and as the joint matures the triangle closes, 2) fibers with visible triangular architecture may have a larger joint which is therefore visible where as most joints may be too small to see. In cases of triangular architecture one cross-strut would often rupture without complete failure of the joint.

The results of joint manipulation suggest that crosslinking, twisting of protofibrils during aggregation and the method of lateral aggregation and branching in fibrin, which may result in triangular architecture, all help to strengthen fibrin fiber joints. Crosslinked and uncrosslinked joints display remarkable resilience to rupture. On average, the legs of crosslinked and uncrosslinked joints can be stretched to a strain of 132% and 146%, respectively, before rupture. Using a modulus of 8 MPa and 4 MPa for crosslinked and uncrosslinked fibers, this corresponds to a rupture stress (strength) of 11 MPa and 7 MPa, respectively. Aside from decreasing extensibility and increasing stiffness, crosslinking also decreases the likelihood of joint failure before fiber failure. The properties of the joints and as well as the single fibers can now be used to construct a realistic model of a blood clot.
**Figure 22** A) Schematic view of the experimental set-up. The AFM tip is located above the fibrin fiber joint which is formed on a striated substrate. The inverted fluorescence microscope allows visualization and movie collection of the manipulation from below. B) Movie frame of a fibrin fiber joint manipulation. The ridges of the striated surface are the bright horizontal bars. The location of the AFM tip is represented by a green asterisk. C) & D) Histograms of the maximum extensibility of the uncrosslinked and crosslinked joints. The data is color coded by failure mechanism. The maximum extensibility before detachment at the node is depicted in blue and the maximum extensibility before fiber rupture is depicted in red. The error in the strain is due to error in the measurement of the length of the fiber and is approximately 8%. All manipulations were done in fibrin buffer (140 mM NaCl, 10 mM HEPES, 5 mM CaCl₂, pH 7.4). E-G) Movie frames of a fibrin fiber joint manipulation in buffer showing triangular joint architecture. The triangular structure becomes visible as the joint is stretched. (A small piece of fiber from a previous manipulation can be seen stuck to the AFM tip.)
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Figure 23 Movie frames of a fibrin fiber joint manipulation in buffer (140 mM NaCl, 10 mM Hepes, 5 mM CaCl₂, pH 7.4) showing joint rupture at the node. The ridges of the striated surface are the bright horizontal bars. The AFM tip is depicted by a green asterisk. A-C) The AFM tip applies force to the lower right leg of the fibrin joint. D) The lower right leg has detached from the node of the joint. E-G) The AFM tip applies force to the upper right leg of the fibrin joint. H) The upper right leg has ruptured along the fiber. Part of the ruptured fiber is attached to the remainder of the joint. The other part of the ruptured fiber is seen attached to the tip.
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CHAPTER VI

THE MECHANICAL PROPERTIES OF INDIVIDUAL γQ398N/Q399N/K406R FIBRIN: AN INSIGHT INTO FIBRIN CROSSLINKING

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The following manuscript has been prepared for journal submission. All data was collected by C.R. Carlisle. Robert Ariens and Kristina Standeven prepared the recombinant γQ398N/Q399N/K406R fibrin. C.R Carlisle analyzed the data and drafted the manuscript. Dr. M. Guthold acted in an advisory and editorial capacity.
ABSTRACT

A combined fluorescence and atomic force microscope (AFM) was used to determine the modulus and extensibility of individual recombinant γQ398N/Q399N/K406R fibrin fibers. The γQ398N/Q399N/K406R mutations eliminate all gamma chain involved FXIIIa mediated crosslinks, while alpha chain crosslinks are able to form. Crosslinked γQ398N/Q399N/K406R fibrin fibers were 2.5x stiffer than their uncrosslinked counterpart while crosslinked wild type fibrin fibers were 3.75x stiffer than uncrosslinked wild type fibers. There was no significant difference in the extensibility of crosslinked and uncrosslinked γQ398N/Q399N/K406R fibers. However the crosslinking of wild type fibrin reduced its extensibility by 20%. The elastic limit of crosslinked γQ398N/Q399N/K406R fibers increased in comparison to uncrosslinked and wild type fibrin. These results suggest that alpha crosslinks are responsible for stiffening and increasing the elasticity of fibrin fibers while gamma crosslinks are responsible for stiffening, as well, and limiting fiber extensibility.

INTRODUCTION

Fibrinogen, an abundant plasma protein, is part of the hemostasis system. Enzymatically activated fibrinogen aggregates to form fibrin fibers, the mechanical and structural backbone of blood clots. The significance of fibrin for blood coagulation is evident through diseases related to mutations and truncations of the fibrin monomer. These changes in the monomer can lead to delayed aggregation as in Fibrinogen Genova, stiffening of clots as in Fibrinogen Dusart, or decrease in the rate of clot lysis as in Aα R16C (Fibrinogen Hershey III) 1-3.
Fibrin is a centrosymmetric protein, formed from a set of 3 unique polypeptide chains, alpha, beta and gamma. During blood coagulation fibrinogen is converted to fibrin through an enzymatic cleavage by thrombin. Fibrin then polymerizes to form protofibrils, which laterally aggregate into fibers forming a branched network. These fibers are further stabilized by FXIIIa which forms intermolecular ε-(γ-glutamyl)lysine covalent bonds \(^4,5\).

Both the alpha and gamma chains participate in fibrin crosslinking. Gamma-gamma and alpha-alpha crosslinks dominate. However, gamma-alpha crosslinks are known to also occur \(^6\). Gamma dimers are the first to form followed by alpha multimers and lastly gamma trimers and tetramers \(^7\). In gamma dimers, glutamine 398 or 399 and lysine 406 are crosslinked in antiparallel fashion to the reciprocal molecule on the adjacent gamma chain \(^8,9\). Alpha crosslinks, on the other hand, have many donors and acceptor sites. Some of the possible alpha crosslink donor and acceptor sites are lys 556, 580, 539, 508, 418, 448, 601, 606, 427,429, 208, 224, 219 and gln 221, 237, 328, 366 \(^10-13\). One alpha chain can interact with one or more adjacent fibrin molecules, leading to an alpha multimer and a partial or full complement of crosslinks on the alpha chain \(^13,14\). Gamma tetramers and trimers, the last stage of crosslinking, are hypothesized to form from branch points and have been shown to decrease fibrin lysis \(^7,15\).

In attempts to understand the role of gamma and alpha crosslinking, recombinant fibrin mutants have been prepared and studied. The first of these mutants, \(\alpha251\), truncates the alpha chain at amino acid 251, eliminating most of the alpha crosslink sites. Clots formed from this mutant were thinner, less stiff, and more susceptible to plastic deformation \(^16\). When crosslinked the \(\alpha251\) clots increase in stiffness by a factor of 1.6-
fold in comparison to 2.3-fold of normal fibrin. A second mutant, and the focus of our study, γQ398N/Q399N/K406R mutates all gamma crosslinking sites, eliminating all gamma chain involved crosslinking. Whole clot studies of the variant showed a 2.5-fold increase in crosslinked fiber modulus compared to a normal fibrin modulus increase of 3.5-fold. γQ398N/Q399N/K406R clots also showed no change in clot polymorphism, lysis or morphology.

In this study we seek to investigate γQ398N/Q399N/K406R properties on the single fiber level. At the single fiber level, changes in modulus can be assigned to the individual fiber, eliminating any effects due to clot morphology. Also, single fiber elastic limit and extensibility can be studied. Here we report the modulus, extensibility and elastic limit of individual γQ398N/Q399N/K406R fibrin fibers and speculate on the role of gamma and alpha crosslinking at the individual fiber level.

METHODS

Recombinant fibrinogen preparation

Methods for recombinant fibrinogen preparation were as previously described. Briefly, expression vector pMLP-γ, containing the entire gamma chain cDNA, was altered at codon 406 to create the γK406R mutation using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The resulting vector was used as a template for the Q398N and Q399N mutations. Mutations were confirmed by sequencing. Chinese hamster ovary cells were co-transfected to express the γ-variant vector as well as human Aα and Bβ fibrinogen chains. Protein was expressed and purified as described.
and dialyzed against 100 mmol l⁻¹ NaCl, 50 mmol l⁻¹ Tris at pH 7.4, and stored at –80°C.

Substrate/Sample Preparation

The striated surface was prepared as previously reported on a 60mm x 24mm, #1.5, microscope cover slide (VWR International, West Chester, PA) using a soft lithography and MIMIC (micromoulding in capillaries) technique. A PDMS stamp was prepared by mixing dimethylsiloxane plus catalyst (Sylgard, Dow Corning Corp, Midland, MI), pouring the mixture over a silicone master (7 um wide ridges, and 6 um deep by 13 um wide trenches) and curing at 70°C for 1 hour. The stamp was then removed from the master and stored in 2% SDS.

Tape was placed on a glass slide where the sample was to be prepared. Norland Optical Adhesive-81 (NOA-81, Norland Products, Cranbury, NJ) was placed around the tape and a large rectangle of PDMS was pressed into the glue. The optical glue was cured for 70 secs with UV light (365nm) (UVP 3UV transilluminator, Upland, CA). The PDMS was removed from the slide leaving a well formed from the glue. The tape was removed from the center of the well and the striated stamp was placed in a 10ml drop of Norland Optical Adhesive-81 where the tape was located. Again the optical glue was cured for 70 secs with UV light (365nm) (UVP 3UV transilluminator, Upland, CA). The stamp was then removed and stored in 2% SDS for subsequent use.

Fibers were formed on top of the striated surface in fibrin buffer (10mM Hepes at pH 7.4, 5mM CaCl₂ and140mM NaCl) with a final concentration of 0.5 mg/ml Fgn, 0.23 u/ml thrombin and 55 Loewy u/ml FXIII (crosslinked fibers only). The fibrin was
allowed to polymerize for 1 hour before being rinsed, labeled with fluorescent beads and stored in fibrin buffer.

Fibrin fibers were fluorescently labeled with 24 nm, yellow-green carboxyl fluorescent beads (Invitrogen, Fluospheres, Carlsbad, CA) diluted 1/10000 in calcium free fibrin buffer. To label the fibers, 200 μl of diluted beads were placed on the sample for 10 minutes, before being rinsed with fibrin buffer.

SDS Polyacrylamide Gel electrophoresis

Fibrin samples for electrophoresis were made in parallel with samples made for manipulation. Fibrinogen and FXIII were mixed together and separated, half on to the microscope slide for a manipulation sample and half into tubes for electrophoresis testing. Thrombin was then added and both were allowed to clot for 1 hr. After an hour reducing buffer and sampling buffer (Bio-Rad Lab Inc, Hercules, CA) were added to the tubes for electrophoresis and the samples were heated at 100ºC for 10 minutes. The tubes were then stored at -80ºC until used for SDS Page gel electrophoresis. SDS Page gel electrophoresis, 4% stacking and 7.5% resolving, was used to separate polypeptide chains by molecular weight and comassie blue staining was used to visualize the bands.

Optical/Lateral Force Microscopy

The manipulation and data acquisition was performed as previously reported. Briefly, the atomic force microscope (AFM) (Topometrix Explorer, Veeco Instruments, Woodbury, NY) was set on top of a custom made inverted optical microscope stage (Zeiss Axiovert 200, Göttingen, Germany). The stage allowed independent control of the
AFM cantilever, sample and microscope objective. A fiber, oriented perpendicular to the ridges, was chosen for manipulation by the cantilever (NSC12 without Al, force constant 14 N/m, length 90 µm, width 35 µm, tip height 15 µm; MikroMasch, Wilsonville, OR). The AFM cantilever was controlled by nanoManipulator software (3rd Tech, Chapel Hill, NC), allowing nanometer sized steps in all directions.

The nanoManipulator software recorded elapsed time, distance traveled by the AFM tip and lateral laser shift due to torsion of the cantilever. The force applied to the fiber, \( F_l \), can be calculated in a Hookean manner; \( F_l = K_c \cdot I_l \), where \( K_c \) is the force constant and \( I_l \) is the lateral shift of the laser. The force constant can be solved for by using the material properties and dimensions of the cantilever determined by optical microscopy; \( K_c = \frac{Ewl^3}{6l^2(h + t/2)} \cdot S_n \), where \( E \), \( w \), \( t \), \( l \) and \( S_n \) are the Young’s modulus of silicon 1.69x10\(^{11}\) N/m\(^2\), the width, the thickness, the length and the normal sensor response of the cantilever and \( h \) is the height of the tip. The overall error in \( K_C \) is about 56%. The radius of the fiber, measured on top of the ridge using the topographical function of the AFM, is then used to convert force into stress applied to the fiber.

The optical microscope is used to observe and record movies of the manipulation and to verify the fiber is firmly attached to the ridge. The optical image is also used to measure the initial length of the fiber and visually check the strain of the fiber in comparison to the stain calculated from the travel of the cantilever tip.
RESULTS

Crosslinking of fibrin fibers was tested by SDS Page gel electrophoresis, figure 24. Uncrosslinked wild type and γQ398N/Q399N/K406R fibrin showed three distinct bands; the alpha (63 kDa), beta (56 kDa) and gamma (47 kDa) chain. Crosslinked wild type fibrin showed the beta chain at 56 kDa, the gamma-dimer band around 94 kDa and a higher molecular weight band representing alpha polymers. The disappearance of the alpha and gamma bands and appearance of higher molecular weight bands indicates the crosslinking of these two polypeptide chains. The γQ398N/Q399N/K406R fibrin only shows alpha crosslinking, both the gamma and beta band remain at 47 kDa and 56 kDa, respectively; while the alpha band disappears and a high molecular weight alpha polymer band appears.
Figure 24 SDS polyacrylamide gel electrophoresis of fibrin. Lane I is the molecular weight standard. Lanes II and III are uncrosslinked γQ398N/Q399N/K406R fibrin; the alpha, beta and gamma chains are visible. Lanes IV and V are crosslinked γQ398N/Q399N/K406R fibrin. The beta and gamma chains remain while the alpha chain disappears due to crosslinking and appears at the top of the gel as a high molecular weight alpha multimer protein. Lanes VI and VII are crosslinked wild type fibrin. Both the alpha and gamma chains disappear and a gamma-dimer band appears at 94 kDa, as well as the alpha multimer band at the top of the gel. Lane VIII is uncrosslinked wild type fibrin showing the
The modulus of the fibers formed under crosslinked and uncrosslinked conditions was determined from stress-strain curves, figure 25. Uncrosslinked γQ398N/Q399N/K406R fibrin had a modulus of 4 ± 1 MPa (average ± standard error), while crosslinked γQ398N/Q399N/K406R fibrin had a modulus of 10 ± 3 MPa (average ± standard error). The modulus increased by 2.5-fold due to crosslinking by FXIIIa.

**Figure 25** Stress-Strain plot. The modulus of individual fibers can be determined from the slope of stress-strain graphs. As seen in the graph, the modulus of crosslinked wild type fibrin (red) is greater than that of crosslinked γQ398N/Q399N/K406R fibrin (blue) and uncrosslinked wild type fibrin (green).
The modulus of wild type fibrin was also determined for comparison. The modulus of wild type uncrosslinked fibrin was also 4 ± 1 MPa, in agreement with the γQ398N/Q399N/K406R modulus. However, the modulus of crosslinked wild type fibrin was 15 ± 2 MPa, differing from the crosslinked γQ398N/Q399N/K406R modulus (p=0.09). The modulus of wild type normal fibrin increased by 3.75-fold upon crosslinking.

The extensibility of individual fibers was determined by manipulating the fibers until they ruptured and recording the rupture strain, Figure 26. Fibers that detached or ruptured at the ridge were discarded. The extensibility of γQ398N/Q399N/K406R fibrin was not significantly affected by crosslinking. Crosslinked γQ398N/Q399N/K406R fibrin extended to 236 ± 10% while uncrosslinked extended to 243 ± 10% (p=0.623). Wild type fibrin, on the other hand, showed a significant decrease in extensibility due to crosslinking. Crosslinking wild type fibrin extended to 177 ± 9% while uncrosslinked wild type fibrin extended to 221 ± 7% (p<0.001).

Figure 26 Optical images of a γQ398N/Q399N/K406R fibrin extensibility manipulation. In the image the ridges appear as 7 μm wide, white, horizontal beams while the 13 μm wide wells appear dark. The AFM tip is indicated by a green asterisk and a fibrin fiber vertically extends across the well. (A-C) Snap shots of the manipulation of the fiber. (D) The fiber has ruptured along its bottom leg, the strain at which the fiber ruptures is recorded as the extensibility of the fiber. The extensibility of this fiber is 350%.
The elastic limit was evaluated through a series of manipulations. Each fiber was stretched forward and then back past the initial position until it detached from the tip. When the fiber detached from the tip, the fiber was visually inspected to determine whether the fiber snapped back to its original length or deformed. If the fiber was not deformed it was then stretched to a higher strain and tested for deformation again, this was repeated until the fiber deformed. The elastic limit was defined as the strain at which 50% of fibers would be deformed. Since not all fibers were stretched to the same strain a sliding average was used to determine the elastic limit. Above the elastic limit more than 50% of fibers stretched to a given strain deformed and below the elastic limit less than 50% of manipulated fibers deformed. A sliding average was used because of the large variation among fibers. Some fibers deformed at strains as low 45% while other stretched to 120% without deformation. Uncrosslinked γQ398N/Q399N/K406R and uncrosslinked wild type fibrin had an elastic limit of 63 ± 8% and 63 ± 4%, respectively. Crosslinked fibers had a larger elastic limit; crosslinked wild type fibers extended to 76 ± 4% before deforming, while crosslinked γQ398N/Q399N/K406R fiber had the largest elastic limit, with and extension of 95 ± 12% before deforming.

DISCUSSION

γQ398N/Q399N/K406R fibrin fibers are interesting to study because they eliminate all gamma chain involved crosslinking with minimal change to the structure of the fibrin monomer. They allow the effect of alpha crosslinking on fibrin to be studied independently from gamma crosslinking. The importance of alpha crosslinking can be seen in patients with clotting disorders related to the truncations or mutation of the αC
domain of the fibrinogen monomer such as Fibrinogen Dusart, Fibrinogen Perth, and Fibrinogen Marburg\textsuperscript{1,23,24}. 

In this study we examined the properties of individual fibrin fibers formed from $\gamma$Q398N/Q399N/K406R fibrin. Previous studies on fibrin fibers have focused on the entire fibrin clot, and have extracted data on modulus, elasticity, strain hardening and crosslinking\textsuperscript{16,17,25-27}. Specifically, $\gamma$Q398N/Q399N/K406R fibrin has been studied on the whole clot level to determine the modulus, clot morphology and polymerization and lysis rates\textsuperscript{17}. A comparison of the $\gamma$Q398N/Q399N/K406R fibrin clot studies to the individual fiber studies will determine if the changes seen in clot properties are due to changes in the individual fibers and/or network morphology. In the case of crosslinked $\gamma$Q398N/Q399N/K406R fibrin clots the modulus increased 2.5-fold in comparison to normal crosslinked clots with an increase of in modulus of 3.5-fold\textsuperscript{17}. The modulus change of the individual crosslinked $\gamma$Q398N/Q399N/K406R fibers was an increase 2.5-fold, with that of normal crosslinked fibers being 3.75-fold. The comparison of the fibrin clots to the individual fibers show good agreement indicating that the change in modulus of the clots is due almost entirely to changes in the individual fibers.

Through individual fiber studies we were also able to determine the elastic limit and extensibility of $\gamma$Q398N/Q399N/K406R fibers. The elastic limit of crosslinked $\gamma$Q398N/Q399N/K406R fibrin fibers was 95%. While the elastic limits for the wild type crosslinked fibrin was 76%. Both $\gamma$Q398N/Q399N/K406R and wild type uncrosslinked fibrin had an elastic limit of 63%. Interestingly, alpha crosslinking increased the elastic limit, while alpha and gamma crosslinks together had a lesser effect. The alpha crosslinks also contributed to the stiffening of the modulus of the fibers, as mentioned
above, however they did not affect the extensibility. The extensibility of crosslinked γQ398N/Q399N/K406R fibrin was 236% while the uncrosslinked extensibility was 243%. On the other hand, the extensibility of wild type fibrin, with both alpha and gamma crosslinking, was reduced from 221% for uncrosslinked to 177% for crosslinked fibrin. Previous studies have shown a positive correlation between increased extensibility and increased length of αC tandem repeats, suggesting the αC domain has a significant impact on extensibility. Our results suggest that crosslinking of the gamma chain also has a significant impact on extensibility \(^{28}\). The extensibility of fibrin is counterintuitive to its crystalline structure \(^{29-31}\), these single fiber studies of extensibility along with elastic limit and modulus give insight into the molecular workings of the fibers.

The results from the elastic limit, extensibility and modulus suggest that alpha crosslinks are responsible for partially stiffening the modulus and increasing the elastic limit; indicating the gamma crosslinks are responsible for increasing the stiffness of the fibers, as well, and decreasing the extensibility and elastic limit.

The alpha crosslinks alone do not account for the total modulus increase due to crosslinking of normal fibrin fibers. Alpha crosslinking in the γQ398N/Q399N/K406R increased the modulus by 2.5-fold, while normal fibrin increased by 3.75-fold. Studies on the αC domain of fibrin have involved whole clot experiments on α251 fibrin, which has an alpha chain truncated after the amino acid 251 eliminating the αC domain and leaving only gamma crosslinks. These results can be compared alongside γQ398N/Q399N/K406R fibrin as a gamma crosslinking only fibrin mutant, but, one must keep in mind that α251 fibrin is missing more than just the alpha crosslinking sites. However, results from modulus data on α251 also saw a partial increase in modulus in
comparison to uncrosslinked and crosslinked normal fibers. Therefore, both alpha and gamma crosslinks together are responsible for the total crosslinked fiber modulus increase. Data on uncrosslinked α251 also showed the presence of the αC domain significantly affected the uncrosslinked modulus. Uncrosslinked fibrin α251 was softer than normal fibrin by a factor of three. Therefore noncovalent intermolecular αC interactions also act to increase the modulus, which according to the results of Standeven et al and ourselves is then further increased by covalent crosslinking in the αC domains.

The same study on α251 fibrin also showed that fibrin lacking its αC domain had an increased plastic response to deformation, suggesting the αC domain may be responsible for the elastic response of fibrin. The increase in elastic limit of γQ398N/Q399N/K406R fibrin fibers, containing only alpha crosslinking supports this hypothesis. If this is the case, in uncrosslinked fibers the lack of covalent bonds in the αC domain may cause the intermolecular interactions of the αC domain to weaken and detach as stress increases leading to a point, the elastic limit, where the alpha chain can no longer restore the fiber to its original length. In alpha crosslinked fibers the covalent bonds in the αC domain may increase the strength of the αC intermolecular interactions and therefore increase the strain at which permanent deformation occurs. However, when both alpha and gamma crosslinks are present, as in normal crosslinked fibrin fibers, the elastic limit decreases to a lower strain value. We propose that the gamma crosslinks play a role in lowering the crosslinked elastic limit by changing the pattern of stress propagation through the fibrin monomers, leading to deformation. Located on the γC domain, gamma crosslinks may cause forces to propagate along the longitudinal direction of the monomer, channeling stress through the d-domain, coiled coil and n-domain,
We suggest that the result of the relocation of stress to these regions leads to unfolding, causing irreversible deformation.

**Figure 27** Schematic model of fibrin monomers under strain. (A) Representation of a fibrin monomer with both fibrinopeptide A and B, the arrow coming out of the central domain, still attached. (B) Three monomers arranged in the half staggered ordering of fibrin protofibrils. The gold line represents the location of gamma crosslinking between two adjacent monomers. (C) The extension of the fibrin oligomer under strain. The gamma crosslink hold the adjacent monomers together at the ends, potentially propagating the stress laterally through the monomer. The αC domains are excluded from the schematic because their locations and interactions are not well known.
The decrease in extensibility of normal crosslinked fibrin may be explained in the same manner. Again, the presence of gamma crosslinks leads to a lower extensibility, this decrease in extensibility may be due to the propagation of force longitudinally along the fibrin monomer. In γQ398N/Q399N/K406R fibrin, containing only alpha crosslinks the extensibility of the fibers is in agreement with uncrosslinked fibrin.

Force spectroscopy, computational simulations and individual fibrin fiber data has suggested the coiled coil region of the fibrin monomer is the first region of the molecule to extend due to stress \(^{32-34}\). The previous proposition does not disprove or disagree with this hypothesis, we are suggesting that the \(\alpha\)C domain is stretching in concert with the coiled coil region at low strain. Experiments and simulation involving the \(\alpha\)C region have been more difficult to study due to its multiple intermolecular interaction sites and its mobility \(^{10,13,14}\). Further studies on \(\alpha\)251 at the individual fiber level as well as studies on other fibrin variants at whole clot, single fiber and single molecule level will continue to improve our understanding of fibrin crosslinking, fiber formation and mechanical properties of blood clots.
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CHAPTER VII

THE INFLUENCE OF TYPE II DIABETES MELLITUS ON THE MECHANICAL PROPERTIES OF PLASMA FIBRIN FIBERS
ABSTRACT

Diabetes is a risk factor for cardiovascular disease (CVD), increasing one's risk 2-4 times. However, the relationship between diabetes and cardiovascular disease is not well understood. Alterations to the structure and mechanical properties of fibrin fiber clots are often found in CVD patients. Therefore, much work has gone into the study of the structural and mechanical properties of diabetic clots. Here we investigate the modulus and extensibility of fibrin fibers formed from patient plasma. We found no significant difference in the modulus or extensibility of diabetic versus control patients; indicating a change in modulus is not responsible for the increased risk of CVD in type 2 diabetes patients.

INTRODUCTION

According to the 1999-2002 National Health and Nutrition Examination Survey, the prevalence of diabetes in the US was 9.3%, or 19.3 million people. While diabetes within the US is greater than the global average, worldwide projections estimate the prevalence of diabetes to increase from 2.8% (171 million people) in 2000 to 4.8% (366 million people) in 2030. Diabetes increases the risk of cardiovascular disease (CVD) in patients by a factor of 2 to 4 with 70% of morbidity in diabetes patients being due to cardiovascular causes.

The connection between CVD and diabetes is not well defined. It is accepted that diabetes leads to microvascular complications and evidence also suggests a relationship between macrovascular disease and diabetic control. Hyperglycemia, increased blood sugar, has been shown to increase atherosclerotic plaque and has been associated with
CVD in some but not all studies\(^6,8\). However, there are many factors and risks that often occur simultaneously with diabetes that lead to increased CVD risk such as high blood pressure, lipid dysmetabolism, fibrinogen concentration and abnormalities of clotting factors\(^7\).

Clots from patients with CVD are denser, with increased modulus, decreased permeability and decreased lysis when compared to control clots\(^9\). In attempting to understand the mechanism responsible for increased risk of CVD in diabetic patients, research has been performed to measure the properties of fibrin clots with elevated glycation. In these studies clots are formed from purified fibrinogen and plasma fibrinogen from diabetic patients as well as fibrinogen incubated with glucose in the lab, invitro gylcation.

Studies have shown higher clotting activity\(^10\) as well as increased resistance to fibrinolysis in samples with increased glycation\(^3,11\). However, Lutjen et al found decreased resistance to fibrinolysis as glycation increased\(^12\). A shorter lag time for fibrin polymerization, forming clots with highly branched fibers and higher fiber density, as well as a decrease in lateral aggregation was reported in diabetic fibrin clots\(^10,11\). Alternately, no difference in fibrin polymerization, kinetics, clot porosity and clot compaction was also reported for diabetic samples\(^12,13\). In one study, crosslinking of the fibrinogen monomer by FXIII showed altered alpha multimer formation\(^12\) while another study reported no difference in crosslinking between uncontrolled and controlled diabetic samples\(^11\). Lastly, fibrin clots with uncontrolled diabetic patients have shown a larger proportion of elastic to inelastic component\(^11\).
To further investigate fibrin clots in diabetic patients we performed a preliminary study of the mechanical properties of individual fibrin fibers formed from plasma from uncontrolled diabetics, controlled diabetics and control individuals with no diabetes.

METHODS

Plasma Collection, Fibrinogen Concentration and Glycation

Diabetic and control patient plasma was collected by our collaborator Marlien Pieters. Citrated blood was collected to prevent clotting. Within 30 minutes of collection, the blood was centrifuged for 15 mins at 2,000g at 4°C. The plasma was extracted and stored at -80°C until fiber sample preparation. Test for fibrinogen concentration and fibrinogen glycation was also performed by the Pieters lab. Fibrinogen glycation was measured as previously described using a two-reagent enzymatic assay (GlyPro® assay, Genzyme Diagnostics, Cambridge, MA, USA; between-run CV = 5%).

Subjects

Subjects were selected and intervention was performed by the Pieters lab as previously described.

Diabetic Patients. Patients with a hemoglobin A1c (formed when glucose attaches to hemoglobin) greater than 9%, on a maximum dose of combination oral hypoglycaemic medications (Metformin and Sulphonylureas), with a body mass index (BMI) > 25 kg/m², between the ages of 40 and 65 with blood pressure sufficiently controlled (< 140/90 mmHg) were included in the study. Baseline blood samples were collected from patients before diabetic control intervention.
**Intervention.** Controlled diabetic patients samples were collected from the diabetic patients after intervention. Intervention was a three step process. First, patients were taught to self monitor glucose and coordinate insulin delivery with meals as well as manage hypoglyceamic event with glucagon. Secondly patients received 10 IU of insulin daily in addition to maximum dose of metformin. Sulphonylureas were stopped. Lastly short acting insulin was used to control post-prandial glucose levels. Once control was obtained, treatments continued for eight days before samples were taken.

**Control Patients.** Control patients were age and body mass index matched to the diabetic group and tested for diabetes by oral glucose.

**Samples preparation**

Coverslides were prepared for plasma samples preparation as previously described\(^14\). Optical adhesive (NOA-81, Norland Products, Cranbury, NJ) was placed on a coverslide and a polydimethylsiloxane (PDMS) stamp was used to create a 1.5 x 1.5 cm well for containing buffer. In the center of the well, optical glue and a second PDMS stamp was used to create a striated surface with 8 μm wide ridges and 12 μm wide grooves.

Fibrin fibers were then formed on top of the ridges. 14 μl of citrated plasma was combined with 0.1 U/ml thrombin and 20 mM of CaCl\(_2\). The reaction ran for 1 hr. After an hour the slide was rinsed with buffer, a pipet tip was used to manually remove excess fibrin fibers from the top of the sample and the sample was vigorously rinsed with buffer. Next the fibers were labeled with 24 nm carboxyl coated fluospheres (Invitrogen, Carlsbad, CA).
Manipulation

Fiber manipulation was performed as previously reported \(^ {14} \). Manipulations were performed by an atomic force microscope (AFM) (Topometrix Explorer, Veeco Instruments, Woodbury, NY) located on top of an inverted optical microscope (Axiovert 200, Zeiss, Gottingen, Germany). The fiber sample was placed between the AFM and optical microscope on our custom stage which allows independent alignment of the objective, fiber sample and AFM cantilever. Fibers were manipulated with the AFM cantilever (CSC-38, Mikromash, Willsonville, OR) and cantilever deflection, distance and time data were collected by NanoManipulator software (3rd Tech, Chapel Hill, NC). The conversion from cantilever deflection into force data was performed as previously reported and described by Liu et al\(^ {14,15} \).

RESULTS

Blood was drawn from 37 patients. For study of the mechanical properties of fibrin fibers formed from the plasma of diabetic patients, five samples representing members from each sample group; uncontrolled diabetics (ud), controlled diabetics (cd), and control (c) samples were chosen. Table 3 shows the fibrinogen concentration and fibrinogen glycation for each of the tested samples.
Table 3

Fibrinogen Concentration and Glycation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fibrinogen concentration (mg/ml)</th>
<th>Glycation (mol glucose/mol fibrinogen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ud (uncontrolled diabetes)</td>
<td>6.37</td>
<td>12.26</td>
</tr>
<tr>
<td>2 ud</td>
<td>3.38</td>
<td>8.41</td>
</tr>
<tr>
<td>1 cd (controlled diabetes)</td>
<td>3.60</td>
<td>5.00</td>
</tr>
<tr>
<td>3 c (control)</td>
<td>3.74</td>
<td>5.28</td>
</tr>
<tr>
<td>4 c</td>
<td>5.62</td>
<td>3.02</td>
</tr>
</tbody>
</table>

Sample 1ud had the largest fibrinogen glycation of all patient samples. Sample 1cd was plasma from the same patient as 1ud after intervention; therefore the diabetes was under control. Sample 3c was a control sample from a nondiabetic patient.

Modulus and extensibility were measured for each sample. The modulus for sample 1ud was $0.8 \pm 0.1$ MPa (average ± standard error) and extensibility was $180 \pm 8\%$. The modulus for 1cd was $2.1 \pm 0.5$ MPa and the extensibility was $159 \pm 10\%$. Lastly the modulus and extensibility for sample 3c was $2.8 \pm 0.6$ MPa and $167 \pm 18\%$, respectively. The difference in the modulus of sample 1ud and samples 1cd and 3c were significant ($p=0.02$ and $p=0.01$, respectively). However, there was no significant different in the extensibility between the samples.

The fibrinogen concentration and fibrinogen glycation of sample 1ud was higher than the other samples tested. To control for possible effects of fibrinogen concentration
and fibrinogen glycation on the modulus of fibrin fibers formed from the plasma samples, 2ud and 4c were tested. Sample 4c was a control patient sample with a similar fibrinogen concentration as sample 1ud, while 2ud was an uncontrolled diabetes sample with high fibrinogen glycation. The modulus of samples 2ud and 4c were $2.2 \pm 0.8$ MPa and $2.0 \pm 0.9$ MPa, respectively.

Fibrinogen concentration did not have a consistent effect on the modulus of individual fibrin fibers in the concentration range tested. Figure 28A is a histogram of the average modulus for each sample displayed from lowest fibrinogen concentration to highest fibrinogen concentration. As the graph depicts, there is no trend in the modulus with respect to fibrinogen concentration.

Similarly, figure 28B is a histogram of the modulus of the fibrin fibers versus the glycation of the fibrinogen molecule. Again there is no visible trend between the modulus of the fibrin fibers and the glycation of the fibrinogen.

**Figure 27 (A)** The fibrin modulus for each sample, sample indicated in bold inside the bar, plotted versus the fibrinogen concentration of each sample. The modulus does not show a trend versus concentration. (B) The fibrin modulus for each sample versus the glycation. Again there is no evident trend in modulus versus fibrinogen glycation.
DISCUSSION

Diabetes causes an increased risk of CVD, yet the mechanisms responsible for the increased risk are not well defined. In an effort to explore potential mechanisms for increased CVD risk we determined the modulus and extensibility of individual fibrin fibers. We saw a subtle increase in modulus between uncontrolled diabetes sample 1ud and plasma from the same patient after diabetes was under control, sample 1cd (p=0.02). The modulus of sample 1ud was also significantly smaller than sample 3c. Since sample 1ud had a large glycation, 12.26 mol glucose per mol fibrinogen, we further probed the effect of glycation on modulus by testing sample 2ud, which had a glycation of 8.41 mol glucose/mol fibrinogen. The modulus of 2ud was 2.2 MPa, similar to both controlled diabetes and control samples. This suggest that the decrease in modulus of sample 1ud was not due to fibrinogen glycation.

Along with increased glycation, sample 1ud also had a high fibrinogen concentration, 6.37 mg/ml. Fibrinogen concentration is known to affect the modulus of fibrin clots. Research has shown increased fibrinogen concentration leads to stiffer fibrin clots, though it is generally accepted that this increase in modulus is due to changes in network morphology, the effect of fibrinogen concentration on individual fiber properties has not been studied\textsuperscript{16,17}. Therefore, we compared the modulus of sample 4c, which had a similarly high fibrinogen concentration of 5.62 mg/ml, to the modulus of sample 1ud. The modulus of sample 4c, 2.0 MPa, was not significantly different samples 2ud, 1cd and 3c, suggesting the decrease in modulus of 1ud was not due to an increase in fibrinogen concentration. This result also suggests that fibrinogen concentration may not directly affect of the modulus of individual fibrin fibers. Therefore, fibrinogen concentration may
not change the modulus of individual fibrin fibers but instead act through network architecture to change the modulus of fibrin clots. A more thorough investigation of the influence of fibrinogen concentration on individual fibrin fiber properties is needed to confirm this assertion.

The difference in the modulus of 1ud is not easily explained by fibrinogen glycation or concentration. It is important to consider that these samples were formed from plasma which is a very complex mixture of proteins and molecules that may affect the properties of fibers. Whether the decrease in modulus of the 1ud sample resulted from a characteristic of uncontrolled diabetes or is a fluctuation around the average modulus of fibrin fibers would need further investigation. However, the preliminary results suggest the decrease is not the result of increase in fibrinogen concentration or glycation.

In conclusion, we formed fibrin fibers from plasma fibrinogen from uncontrolled diabetic, controlled diabetic and control patients. The stress-strain manipulation data was recorded and the modulus of the fibers was calculated. We determined uncontrolled type 2 diabetes mellitus does not result in a change in the modulus or extensibility of fibrin fibers.
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CHAPTER VIII

CONCLUSION
ELECTROSPINNING

The goal of tissue engineering is to develop biological substitutes that improve upon the limitation of organ transplant and biomaterial implantations \(^1\). Many techniques have been developed to obtain this goal such as, cell culturing, 3D printing, stereolithography, and acellularization. However, to date each method has its own set of problems to overcome before it can be put to use in tissue implantation \(^1,2\).

Electrospinning is a promising technique because biomaterials, which are biocompatible and biodegradable, can be used and the mechanical properties of the matrix can be controlled through orientation, fiber diameter and fiber density \(^2\)\(^-\)\(^5\). Choosing a material that promotes cell proliferation and adhesion while balancing the correct mechanical properties for function, cell differentiation and biodegradability is a challenge for tissue engineering. We have begun this challenge by determining the mechanical properties of individual electrospun fibers. We have focused on fibers produced from the biological polymers fibrinogen and collagen due to their intrinsic biocompatibility and biodegradability.

A Comparison of Electrospun Fibrinogen and Electrospun Collagen

Fibrinogen is an ideal material for tissue engineering due to its physiological roles as a mechanical and cell adhesion scaffold during blood coagulation, wound repair and tissue regrowth. A variety of cells, such as endothelial cell, smooth muscle cells, keratinocytes, fibroblasts, and leukocytes, have been shown to adhere to fibrinogen through its cell adhesion motifs, located along all three polypeptide chains (\(\alpha, \beta, \gamma\)) and including the cell adhesion motif Arg-Gly-Asp (RGD, A\(\alpha\)572–574) \(^6\)\(^-\)\(^8\). Fibrinogen
degradation products are also beneficial as they are known to attract monocytes and neutrophils to the region\textsuperscript{9}. Collagen, the most abundant protein in the ECM, likewise has similar physiological advantages. Many members of the integrin family, such as $\alpha_{1}\beta_1$ and $\alpha_2\beta_1$, as well as keratinocytes, fibroblast and endothelial cells, have been shown to directly bind collagen\textsuperscript{10,11}. Collagen also plays an important mechanical role in tissue strength as well as cellular mechanotransduction\textsuperscript{12}.

### Table 4

Comparison of Individual Electrospun Fibers

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Espun Fibrinogen (dry) (ref. Baker)</th>
<th>Espun Fibrinogen (wet)</th>
<th>Espun Collagen (dry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Modulus</td>
<td>$2 \pm 0.6 \text{ GPa}$</td>
<td>$14 \pm 0.9 \text{ MPa}$</td>
<td>$7.5 \pm 0.4 \text{ GPa}$*</td>
</tr>
<tr>
<td>Elastic Modulus</td>
<td>$3.7 \pm 0.5 \text{ GPa}$</td>
<td>$5.2 \pm 0.7 \text{ MPa}$</td>
<td>-</td>
</tr>
<tr>
<td>Max Stress</td>
<td>$1.2 \pm 0.3 \text{ GPa}$</td>
<td>$17 \pm 1.1 \text{ MPa}$</td>
<td>$48 \pm 3 \text{ MPa}$</td>
</tr>
<tr>
<td>Extensibility</td>
<td>$74 \pm 3%$</td>
<td>$130 \pm 10%$</td>
<td>$33 \pm 3%$</td>
</tr>
<tr>
<td>Relaxation fast</td>
<td>$1 \pm 0.1 \text{ s}$</td>
<td>$3 \pm 0.3 \text{ s}$</td>
<td>-</td>
</tr>
<tr>
<td>Relaxation slow</td>
<td>$11 \pm 1 \text{ s}$</td>
<td>$55 \pm 5 \text{ s}$</td>
<td>-</td>
</tr>
<tr>
<td>Deformation strain</td>
<td>$15%$</td>
<td>-</td>
<td>$\sim 1%$</td>
</tr>
<tr>
<td>Strain behavior</td>
<td>softening</td>
<td>Softening</td>
<td>Severe softening</td>
</tr>
</tbody>
</table>

* 3pt bending data
A comparison of the mechanical properties of electrospun fibrinogen and collagen can be found the Table 4. Fibrinogen was more extensible, more elastic and softer than collagen. The modulus and peak stress of electrospun collagen and fibrinogen showed an exponential dependence on radius. Smaller fibers had a larger modulus than fibers of larger radius; therefore, the values of modulus and peak stress have been reported for a fiber of diameter \( \approx 200 \text{ nm} \). This dependence on fiber radius is a beneficial feature because control of electrospun fiber diameter through processing parameters has been shown and; therefore, fine tuning the modulus of the fibers through diameter control can be achieved. Comparing the modulus of fibrinogen to collagen one finds that collagen produces stiffer fibers in ambient conditions. However, collagen fibers showed severe strain softening during manipulation. Strain softening is so severe that the maximum stress of collagen is almost 2 orders of magnitude smaller than that of fibrinogen. Due to the solubility of electrospun collagen fibers in aqueous environments, fibers must be cross-linked with gluteraldehyde for study. Yang et al. determined the modulus of electrospun collagen fibers in PBS to be 260 MPa (for a fiber \( \approx 200\text{nm} \)). As expected the modulus for electrospun collagen and fibrinogen decreased in buffer and collagen was stiffer than fibrinogen. Interestingly, both electrospun collagen and fibrinogen in buffer show similar moduli to their native fiber counterparts.

**A Comparison of Electrospun to Native Fibers**

A direct comparison of the mechanical properties of electrospun fibrinogen and native fibrin fibers in buffer can be made using the data presented in chapters three and four. Crosslinked fibrin fibers are chosen for comparison due to their physiologically
relevance. The activation of the enzyme FXIIIa, which crosslinks fibrin monomers within fibrin fibers, is one of the final steps in the clotting cascade. Patients who are FXIII deficient run the risk of impaired wound healing and hemorrhaging\textsuperscript{13}. Therefore, for biomimetic purposes a comparison will be made between electrospun fibrinogen and crosslinked native fibrin.

A couple of differences between the two fiber types can be inferred from their names. First, the basic subunit for electrospun fibrinogen fibers is fibrinogen. The monomers have not been cleaved by thrombin and therefore contain fibrinopeptides A and B (FpA and FpB) at the amino-terminus of the alpha and beta chains, respectively. Native fibrin fibers are formed from fibrin monomers which have had FpA and FpB cleaved by thrombin exposing the charged polymerization sites ‘A’ and ‘B’ necessary for fiber formation. Secondly, the method of fiber production is different. Electrospun fibers are formed from a process which requires a large electric field and a volatile solution, HFP in the case of the fibers in chapter 3. Native fibrin fibers are formed by spontaneous polymerization after activation by thrombin and they are formed in buffer with similar ion concentrations and pH to that of blood.

However, when looking at the mechanical properties of the two fiber types they show many similarities, see table 5. Electrospun fibrinogen and native crosslinked fibrin fibers have similar total modulus, 10 and 8 MPa, respectively, with no significant difference between the data (p = 0.2). They also have similar elastic moduli, extensibility and relaxation rates. Both electrospun and native fibrin also show a modulus dependence on diameter; the modulus decreases as the diameter increases. This suggests a fiber with
a dense core and looser packing of molecules as the radius increases. Electrospun collagen also shows similarities to native collagen, such as modulus and extensibility\textsuperscript{14,15}.

Table 5

Comparison of Electrospun Fibrinogen and Native Fibrin Fibers

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Electrospun Fibrinogen Fibers</th>
<th>Native Fibrin Fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Modulus (MPa)</td>
<td>10 ± 0.9</td>
<td>8.0 ± 1</td>
</tr>
<tr>
<td>Elastic Modulus (MPa)</td>
<td>5.2 ± 0.7</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>Extensibility</td>
<td>130 ± 10 %</td>
<td>147 ± 5 %</td>
</tr>
<tr>
<td>Peak Stress (MPa)</td>
<td>12.3 ± 1.1</td>
<td>15.1 *</td>
</tr>
<tr>
<td>Relaxation rate fast</td>
<td>3.0 ± 0.3</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Relaxation rate slow</td>
<td>55 ± 5</td>
<td>49 ± 4</td>
</tr>
</tbody>
</table>

* calculated from extensibility, modulus and strain hardening

The similarity between electrospun and native fibers is interesting because while the fibers are comprised of the same molecules, fibrinogen and collagen, during electrospinning the proteins are dissolved in HFP, a fluorinated hydrocarbon. Fluorinated hydrocarbon solvents promote the formation of alpha helices; therefore denaturing proteins\textsuperscript{16}. This denaturation can be seen in the CD spectra of both fibrinogen and collagen dissolved in HFP\textsuperscript{17,18}. Another distinct difference between the native and electrospun fibers is their molecular arrangement, the half staggered repeat of fibrin
fibers and the 64nm D-spacing of collagen fibrils are missing from electrospun fibrinogen and collagen fibers, respectively. Despite these differences the electrospun and native fibers show similar mechanical properties and electrospun fibers have also been shown to maintain the advantageous cell adhesion properties of their constituent molecules \(^{19,20}\).

This research provides an essential element, the mechanical properties of individual electrospun fibers, in the design and production of electrospun scaffolds. While fibrinogen and collagen have great potential in tissue engineering, further research on other biopolymers such as elastin, fibronectin, and laminin, as well as synthetic polymers should be carried out to expand the tools available for design of tissue scaffolds.

**FIBRIN**

The properties of fibrin are known to be important in the hemostatic balance. If a clot is mechanically weak it runs risk of hemorrhage; conversely clots that are too stiff increase the risk for cardiovascular disease. While the role fibrin mechanics play in hemostasis is understood the mechanisms determining the mechanical properties of clots are less understood. The study of individual fibrin fibers is one step in discovering the mechanisms behind the mechanical properties of blood clots.

**Fibrin Fiber Crosslinking**

Crosslinking of fibrin occurs through the enzymatic process of activated coagulation cascade Factor XIIIa, after fibrin fibers have formed. Gamma dimers form first between the \(\gamma\)-nodule of adjacent monomers and are thought to form in the
longitudinal direction\textsuperscript{21,22}. Alpha multimers form next between the αC regions of local monomers and are less well defined, as there are many potential alpha chain crosslinking sites.

Crosslinking of fibrin clots has been shown to increase storage modulus, $G'$, and loss modulus, $G''$ as well as reduce creep\textsuperscript{23,24}. In examining the properties of individual fibers we see a similar stiffening of crosslinked fibrin fibers, see table 6. Crosslinked fibers have an increased total and elastic modulus and a decreased extensibility over uncrosslinked fibers. However, both crosslinked and uncrosslinked fibers have similar relaxation rates suggesting crosslinking does not affect the mechanism responsible for the relaxation times. Another property independent of fibrin fiber crosslinking was the decrease in modulus of individual fibers as their diameter increased. This supports the theory that protofibrils twist and stretch as they aggregate limiting fiber diameter\textsuperscript{25}. As the diameter increases the profibrils must stretch further to maintain the crystalline pattern of fibrin decreasing the energetic advantage of aggregation and the density of the fiber.

There was some data disagreement between the modulus values for crosslinked fibrin collected during the native fibrin studies and the $\gamma Q398N/Q399N/K406R$ fibrin studies. The difference in fibrinogen production and purification between these samples could contribute to the mechanical differences seen in the fibers formed from these proteins. Where the data was inconsistent, data gathered from the wild type fibrin received from our collaborator Robert Ariens was used because it provides a common sample preparation for comparison of crosslinked, uncrosslinked and alpha crosslinked fibers. The elastic limit of fibrin also changed from previous data; however, we believe
data taken on wild type fibrin from the Ariens lab is the best representative data, as our technique and data analysis improved with continued data collection.

Table 6

The Mechanical Properties of Uncrosslinked, Crosslinked and Alpha Crosslinked Fibrin Fibers

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Uncrosslinked</th>
<th>Crosslinked</th>
<th>Alpha Crosslinked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Modulus (MPa)</td>
<td>3.9 +/- 0.3</td>
<td>14.7 +/- 2</td>
<td>10.2 +/- 3</td>
</tr>
<tr>
<td>Elastic Modulus (MPa)</td>
<td>1.9 +/- 0.3</td>
<td>5.6 +/- 1.0</td>
<td>5.5 +/- 2.4</td>
</tr>
<tr>
<td>Extensibility</td>
<td>226% +/- 7.4%</td>
<td>177% +/- 9.1%</td>
<td>236% +/- 9.6%</td>
</tr>
<tr>
<td>Elastic limit</td>
<td>63 %</td>
<td>75 %</td>
<td>95%</td>
</tr>
<tr>
<td>Relaxation rate fast (s)</td>
<td>2.9 +/- 0.5</td>
<td>2.1 +/- 0.2</td>
<td>3.1 +/- 0.4</td>
</tr>
<tr>
<td>Relaxation rate slow (s)</td>
<td>54 +/- 9</td>
<td>49 +/- 4</td>
<td>73 +/- 13</td>
</tr>
</tbody>
</table>

In our analysis of native crosslinked fibrin fibers we came across fibers with inconsistent and incomplete crosslinking, these fibers were labeled as partially crosslinked fibrin fibers and their properties were discussed in the supplement to chapter 4. Partially crosslinked fibers had a slight increase in modulus when compared to uncrosslinked fibrin, yet their extensibility was greater than that of both uncrosslinked and crosslinked fibrin. The unique mechanical properties of partially crosslinked fibrin fibers were not easily explained. In order to better understand the affect of crosslinking on mechanical properties of fibrin fibers we studied γQ398N/Q399N/K406R fibrin.
γQ398N/Q399N/K406R fibrin contains 3 point mutations which eliminated all Factor XIIIa mediated gamma crosslinking which enabled us to study the affect of alpha crosslinking on the mechanical properties of fibrin fibers, results summarized in table 6. Alpha crosslinking partially stiffened the fibers, suggesting that alpha and gamma crosslinks work in concert to gain the entire stiffening effect of crosslinked fibrin fibers. Surprisingly, alpha crosslinking had no effect on extensibility and increased the elastic limit of fibers. It has been suggested that sliding of protofibrils past one another may be responsible for the large extensibility of highly crystalline fibrin fibers. The increase in elastic limit due to alpha crosslinking may be the result of the ability of alpha chains to form inter-protofibril crosslinks, therefore providing a restorative mechanism to protofibrils that slide past one another. In the absence of alpha crosslinking, ie uncrosslinked fibrin fibers, the protofibrils are missing their restorative force.

While crosslinking played an important role in the mechanical properties of individual fibers it also affected the properties of fibrin fiber branch points. In designing and predicting the mechanical properties of a branched network, the constituent fiber properties, the network architecture and the branch point properties must be known. Therefore we studied the strength of crosslinked and uncrosslinked fibrin branch points. Crosslinked branch points are less likely to rupture than crosslinked fibers, while the opposite is true for uncrosslinked fibrin fibers. Therefore, crosslinking must fortify the joint, increasing its strength. A structural mechanism noticed through the manipulation of fibrin joints was a triangular joint architecture. This architecture supports the theory purposed by Ryan et al. in which fiber formation results from fibrin oligomers forming and joining together both lengthwise and laterally. 26.
Overall we determined the effect of crosslinking on the mechanical properties of fibrin fibers and fibrin joints. We found crosslinking to be responsible for stiffening and decreasing the extensibility of fibrin fibers, and also strengthening the fibrin branch points. Through the study of fibrin γQ398N/Q399N/K406R, we determined both alpha and gamma crosslinks were responsible for increasing the modulus of fibrin, while we speculate that gamma crosslinks alone play the role of reducing the extensibility and elastic limit of fibrin.

Fibrin Fibers from Plasma Samples

The study of individual fibrin fibers has lead to the investigation of fibers formed from plasma samples. Fiber formed from plasma samples offer different advantages and disadvantages over purified fibrinogen samples. In studying the molecular mechanisms of the fibrin molecule the simplified system using purified fibrinogen offers the best experimental control. Blood plasma contains many coagulation factors and molecules, therefore making it a complex, highly interactive system. A change in the mechanical properties of plasma fibrin fibers does not directly alert us to the mechanism responsible for the change but it does help in developing hypotheses and simplified systems for study.

The plasma samples we studied were from patients with diabetes. Patients with diabetes have an elevated risk for cardiovascular disease. We studied the modulus and extensibility of individual fibrin fibers formed from the plasma of patients with diabetes to determine if diabetes had an affect on these properties. Through the comparison of plasma fibrin fibers from a patient prior to and after glyceamic control the effect of glucose levels on the mechanical properties of fibrin fibers were probed. We found that
fibrin fibers formed from patients with diabetes did not consistently have a significant
effect on the stiffness and no effect on the extensibility over control patient samples.
Therefore we concluded that the elevated risk for cardiovascular disease results from
another mechanism.

Future work in the study of individual fibrins would expand the study of plasma
fibrin fibers to other groups. The next set of fibers to be tested in the Guthold lab is
plasma fibrin from 3 patient groups; young patients, old patients and old patients at risk
for cardiovascular disease. Many other natural fibrin mutations and diseases could be
tested in the same manner using plasma fibrin fibers. Aside from patient samples there
are still many purified fibrin fiber samples which could help elucidate the mechanisms of
fibrin mechanical properties. Lords and others are studying one such sample, α251, at
UNC Chapel Hill. This fibrin molecule is truncated at 251 amino acid on the alpha chain,
therefore shedding light on the significance of the αC region and eliminating alpha
crosslinking of fibrin of the monomer. A second variant for future study is the natural
variant γ/γ’. This fibrin variant binds Factor XIII and is thought to increase the stiffness
of clots.

APPLICATION TO MODELING

The results from the analysis of fibrin fibers and electrospun fibers are interesting
and important for gaining a better understanding of the mechanical properties of the
fibers as well as gaining insight into the molecular mechanisms relating to those
properties. However, manipulation of fiber joints was also studied in this work.
As previously mentioned three elements are necessary for understanding and modeling the mechanical properties of branching networks: network architecture, the properties of the constituent fibers and the properties of the branch points. Network architecture can be determined through scanning electron microscope (SEM) images and/or confocal microscope images, while this research has determined the mechanical properties of individual nanofibers, as well as probed the branch point between the fibers. Therefore, the requirements for network modeling have been achieved.

While efforts to model networks have been difficult and computationally demanding, modeling methods have emerged that show great promise. Collagen has been a popular fiber for network modeling since collagen fibers and gels have been extensively studied. The Barocas lab has developed a multiscale volume averaging method which uses the properties of individual fibers in creating a representative volume element, this volume element is repeated to form a macroscale representation of the network \(^{27,28}\). They next compared the computational predictions to experimental data and found good agreement \(^{29}\). The Szeri lab is currently using the properties of individual fibrin fibers and branchpoints to model the effect of microbubbles on the integrity of fibrin clots.

The research presented here determined the mechanical properties of a select group of nanofibers. We chose fibers based on physiological importance. We determined the mechanical properties of individual electrospun collagen type I fibers as well as electrospun fibrinogen fibers. We also determined the properties of fibrin fibers and fibrin joints and determined the effect of crosslinking on the mechanical properties of fibrin. Electrospun collagen and fibrinogen fiber properties provide a good start toward
creating a library of individual electrospun fiber properties; future work to expand this library would be beneficial for tissue engineering as well as other material applications. Work on the mechanical properties of individual fibrin fibers could continue in order to develop a better understanding of the molecular mechanisms of responsible for the mechanical properties of fibrin as well as the mechanical role fibrin fibers play in disease.
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APPENDIX

SUPPLEMENT: THE MECHANICAL PROPERTIES OF SINGLE FIBRIN FIBERS

W. Liu, C. R. Carlisle, E. A. Sparks, M. Guthold

The following supplement is to accompany the article in press in The Journal of Thrombosis and Hemostasis, 2010, DOI: 10.1111/j.1538-7836.2010.03745.x. Stylistic variations are due to the requirements of the journal. C.R. Carlisle performed all experimentation and analysis. Stress relaxation, generalized Kelvin model and detailed force per monomer and energy monomer were performed with the assistance of Dr. W. Liu and Dr. M. Guthold. The original manuscript draft was prepared by Dr. W. Liu, C.R. Carlisle and Dr. M. Guthold. Extensive redrafting and editing was performed by C.R. Carlisle and Dr. M. Guthold.
The Supplement Contains the Following Sections

Detailed Materials & Methods and SDS protein gel electrophoresis (to determine extent of crosslinking)

Additional Information on Fibrin Fiber Mechanical Properties

- Elastic modulus vs. radius.
- Stress relaxation and generalized Kelvin model
- Detailed force per monomer and energy per monomer calculations
- Mullins effect

Partially crosslinked fibrin fiber data

Table S1. All mechanical properties of uncrosslinked, crosslinked, and partially crosslinked fibrin fibers; including generalized Kelvin model properties and data statistics.

References

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MATERIALS AND METHODS

Preparation of Oregon-Green-Labeled Fibrinogen

Factor XIII-free fibrinogen (American Diagnostica, Inc. Greenwich, CT) was fluorescently labeled with about two Oregon Green 488 (OG) molecules as reported previously ¹.

Preparation of Striated Substrate
The striated substrate was prepared as reported previously \(^1\). It was obtained by pressing a PDMS (polydimethylsiloxane) stamp into a 10 μl drop of Norland Optical Adhesive-81 (NOA-81; Norland Products, Inc. Cranbury, NJ) on a clean, 60 mm x 22 mm, # 1.5 microscope cover slip (Fisher Scientific, Pittsburgh, PA). The NOA-81 was cured with UV light (365 nm) (UVP 3UV transilluminator, Upland CA) for about 70 seconds and the PDMS stamp was removed.

**Fibrin Fiber Formation**

Fibrin fibers were formed as reported previously \(^1\). To form uncrosslinked fibrin fibers, OG-labeled fibrinogen was diluted in “fibrin buffer” (140 mM NaCl, 10 mM Hepes, 5 mM CaCl\(_2\), pH 7.4); final concentration, 0.5 mg/ml. 18 μl of the fibrinogen solution were deposited onto the striated substrate and 2 μl of thrombin (final concentration: ~ 0.1 NIH unit/ml, Enzyme Research Lab, South Bend, IN) were mixed in to start fiber formation. After incubation for 1 hr in a saturated atmosphere, the sample was rinsed with and stored in fibrin buffer. To form crosslinked fibers, fibrinogen (American Diagnostica Inc, CT; final concentration 1 mg/ml) and Factor XIII (Enzyme Research Labs, South Bend, IN; final concentration, 25 Loewy units/ml) was combined before adding thrombin. A different fluorescence labeling method was used for crosslinked fibers. The fibers were labeled after formation through incubation for 10 minutes in calcium free fibrin buffer with carboxylate modified, 20 nm, yellow-green FluoSpheres (Molecular Probes, Invitrogen, USA).

**Combined AFM/Fluorescence Microscopy**
The mechanical manipulations on fibrin fibers were performed with a combined atomic force microscope/optical microscope instrument. The schematic set-up is shown in Figure 16 (main manuscript) and a more detailed description is given in $^{1,2}$. The instrument is based on an inverted Zeiss Axiovert 200 microscope (Zeiss, Thornwood NY), a Hamamatsu EM-CCD C9100 Camera (Hamamatsu Photonics KK, Japan), IPLab software (Scanalytics, Fairfax, VA) and a Topometrix Explorer AFM (Veeco Instruments, Woodbury, NY). The microscope stage was designed so that the AFM tip, fiber sample and objective lens can move with respect to each other, which allows alignment of the fiber with the AFM tip and objective lens. We used NT-MDT CSCS12 and NSC12 silicon cantilevers with a rectangular cross-section (Mikromasch, Wilsonville, OR).

Manipulating Fibrin Fibers

The AFM manipulation experiments were done with a nanoManipulator ($^{3rd}$ Tech, Chapel Hill, NC), a software program that interfaces the AFM with a force feedback stylus (PHANTOM, Sensable Technologies, Woburn, MA) and a graphics computer $^3$. The nanoManipulator provides control over the x-, y- and z-movement of the AFM tip. For these experiments, the AFM tip was held at a constant height, so that the apex of the AFM tip was about 1 μm under the fiber. As illustrated in Fig. 16, the tip of the atomic force microscope (AFM) is then moved laterally to stretch fibers that are suspended across channels on the striated substrate. The initial fiber length, $L_{init}$, was determined from optical images; the stretching displacement, $s$, was determined via AFM; the fiber strain $\varepsilon = (L’-L_{init})/L_{init} \cdot 100\%$ was determined using trigonometry ($L'^2 = s^2 + L_{init}^2$).
Determination of the stress involved several steps. The measured quantity is the “left – right” photocurrent of the AFM photodiode, $I_l$, which results from the deflection of the cantilever. This signal needs to be converted into force, $F$. We typically used the Sader method, based on cantilever geometry and resonance frequency (outlined below) and verified it with the Liu method, based on bending a glass fiber. For rectangular cantilevers,

$$F_l = \frac{Ewt^3}{6L^2(h + t/2)} \cdot S_n \cdot I_l \quad (\text{see } 5),$$

where $E = 1.69 \cdot 10^{11}$ N/m$^2$ is the Young’s modulus of the cantilever material (silicon), $w$, $t$ and $L$ are the width, thickness and length of the cantilever and $h$ is the tip height. $S_n = \Delta z/\Delta I_n$ is the sensitivity, where $\Delta z$ is normal (height) displacement of the cantilever and $I_n$ is the top – bottom photocurrent. We measured the cantilever length and width and tip height with the inverted optical microscope. The thickness of the cantilever was determined from

$$f_0 = \frac{1.02t}{2\pi L^2 \sqrt{E/\rho}},$$

where $f_0$ is the resonance frequency of the cantilever and $\rho = 2330$ kg/m$^3$ is the density of the silicon. The force applied to the fiber was then determined at each strain using the lateral force applied to the cantilever tip in conjunction with balancing of forces between the cantilever and the two halves of the fibers

$$F = \frac{F_l}{2 \cdot \sin \alpha},$$

where $\alpha$ is the angle between the initial position of the fiber and the current position, see Fig. 16B (main manuscript). The diameter of each fiber was determined by AFM-imaging the fiber near the anchoring points on top of the ridges. Stress is calculated via $\sigma = F/A$, where $F$ is force and $A$ is the initial fiber cross-section (we assumed a circular fiber cross-section).

Estimation of Instrumentation Error
We estimate that the errors in the force calibration, fiber diameter determination by AFM, and the strain determination by optical microscopy and AFM are about 30%, 20% and 5%, respectively.

**Strain Hardening**

Fig. 17B (main text) shows the slope of Fig. 17A (main text). Fig. 17B was obtained by determining $\Delta \sigma/\Delta \varepsilon$ for each data point of the stress-strain curve shown in a window of $\Delta \varepsilon = 20\%$.

**Energy Loss**

(Fig. 18, main text). The graph contains 24 and 61 data points for uncrosslinked and crosslinked fibers respectively. The data were smoothed by taking the sliding average of an $\varepsilon = 30\%$ window with an $\varepsilon = 15\%$ step size.

**SDS Protein Gel Electrophoresis**

Fibrin samples for manipulation were prepared in parallel with samples to be used for protein gel analysis of fibrin crosslinking. Just like the samples prepared for manipulation, the samples for gel analysis were allowed to clot for 1 hr before adding reducing buffer and sampling buffer (Bio-Rad Lab Inc, Hercules, CA). The tubes were heated at 100°C for 10 minutes and stored at -80°C until the gel was run. Polyacrylamide protein gel, 4% stacking and 7.5% resolving, electrophoresis in SDS was used for molecular weight separation and the proteins were stained with commassie blue staining. Crosslinking was determined through density comparison of the beta to gamma band
ratio or beta to alpha band ratio of noncrosslinked samples to partially crosslinked or crosslinked samples.

Figure S1 (A) Lane of an uncrosslinked fibrin sample. The three bands from top to bottom are the alpha, beta and gamma chains of the fibrin monomers. (B) Partially crosslinked fibrin lane. The beta chain does not crosslink and therefore remains dark, while the alpha and gamma bands become lighter. A band consistent with gamma-gamma crosslinking appears around 98 kDa. (C) Fully crosslinked fibrin lane. The alpha and gamma bands have disappeared and gamma-gamma and alpha multimer bands appear at higher molecular weights. The alpha multimers appear at the top of the gel.

ADDITIONAL INFORMATION ON FIBRIN FIBER MECHANICAL PROPERTIES

Elastic modulus vs. radius

The average diameter of the crosslinked fibers was 390 nm, with a range of 124 nm to 800 nm. The average diameter of noncrosslinked fibers was 180 nm, with a range of 94 nm to 708 nm. A plot of the total elastic modulus $E_0$ versus diameter, showed a trend of decreasing modulus with increasing diameter (Fig. S2). A similar trend was observed for the rupture force dependence of dry fibrin fibers on diameter $^2$. This suggests that the core of fibrin fibers is denser than the periphery. The decreasing
modulus can be fit with a power law of the form $E_o = cr^n$ where $r$ is the radius of the fiber. For crosslinked and uncrosslinked fibers fibrin fibers $n$ is -1.7 and -0.89, respectively (slope of log-log plot in Figure S2). There is no significant difference between the slopes of crosslinked and uncrosslinked data.

Figure S2  Log-log plot of the total elastic modulus for crosslinked fibrin fibers vs. radius of the fiber. Modulus values decrease as fiber radius increases. The data were fit by a power law; $E_o = cr^n$ where $r$ is the radius of the fiber and $n = -1.7$ and -0.89 for crosslinked and uncrosslinked fibers, respectively.
Stress Relaxation and Generalized Kelvin Model

Stress relaxation in incremental stress-strain curves is indicative of viscous (time-dependent) processes, and may be fitted by exponentials of the form

\[ \sigma(t) = \sigma_\infty + \sum_i \sigma_i \cdot e^{-t/i\tau_i} \]

Mechanistically this implies that upon stretching, elastic and viscous molecular processes occur within the fibrin fiber. In our model in Fig. 20 (main manuscript), we propose three different molecular mechanisms. \( \sigma(t) \) is the stress at any time \( t \) after relaxation started, \( \sigma_i \) is the stress due to time-dependent elements (see Kelvin model below), \( \tau_i \) is the relaxation time and \( \sigma_\infty \) is the stress left in the fully relaxed fiber \( (t \to \infty) \). We have analyzed the relaxation processes of fibrin fibers at different strains. Figure S3A shows a typical stress relaxation curve for a fibrin fiber (red curve). Initially, stress relaxation is dominated by a fast relaxation rate, then by a slower rate. A fitting curve, with two relaxation times, \( \tau_1 = 1.3 \text{ s} \) and \( \tau_2 = 22.9 \text{ s} \), is applied in Figure S3A (green line); it matches the data in the fast and slow relaxation regions very well. Using only a single relaxation time did not suffice to fit the data (blue line); it could only match either the slow or the fast relaxation region. Crosslinked partially crosslinked and uncrosslinked fibers required a minimum of two stress-relaxation rates for accurate fitting. As listed in Table S1, all have nearly the same fast rate (2 s to 4 s) and slow rate (49 s to 57 s). In Figure S3B, the relaxation rates are plotted as a function of strain and, although there is significant scatter, it appears that the relaxation times are independent of strain.

A simple 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 \( E_\infty \), in parallel with two Maxwell elements consisting of
a dashpot and a spring in series (Figure S3C). For this model, the equation for stress relaxation becomes

\[ \sigma(t) = \varepsilon_0 \left[ E_\infty + E_1 \cdot e^{-t/\tau_1} + E_2 \cdot e^{-t/\tau_2} \right] \quad (2). \]

\( E_\infty \) is the relaxed elastic modulus, \( E_0 \) is the total elastic modulus, \( E_0 = E_\infty + E_1 + E_2 \). The relaxation times, the elastic modulus of the springs, and the viscosity of the dashpot elements are related via \( \tau_1 = \mu_1/E_1 \) and \( \tau_2 = \mu_2/E_2 \). As described above, we have measured \( E_\infty, E_0, \tau_1, \tau_2 \), which are model-independent. Fitting the data to the generalized Kelvin model we have determined \( E_1, E_2, \mu_1, \) and \( \mu_2 \), which are model-dependent (see table S1).

The Kelvin model is intended as a macroscopic model that behaves mechanically equivalently to the fibers. Although the Kelvin model elements may not necessarily correspond to actual physical fiber elements, it is intriguing, and tempting to speculate that the Kelvin model elements may correspond to the three elements of our molecular model (Fig. 20, main text): 1) \( \alpha \)-helix to \( \beta \)-strand conversion of the two coiled coils of the fibrin monomer. 2) Deformation or partial unfolding of the \( \gamma \)-nodule of the fibrin monomer. 3) Interaction and extension of the long, flexible and partially unstructured \( \alpha \)C region.
Figure S3 (A) A fibrin fiber stress relaxation curve (red) fitted with a single exponential relaxation curve, \( \sigma_0(t) = \sigma_1 \cdot e^{-t/\tau_1} + \sigma_\infty \) (blue), and a double exponential relaxation curve, \( \sigma_0(t) = \sigma_1 \cdot e^{-t/\tau_1} + \sigma_2 \cdot e^{-t/\tau_2} + \sigma_\infty \) (green), where \( \tau \) are the relaxation times and \( \sigma_0 \) is the fully relaxed elastic stress. Pearson’s R value for the single relaxation fit is 0.91 and 0.98 for the double relaxation fit. The fitting coefficient for the double relaxation are \( \tau_1 = 1.3 \) s, \( \tau_2 = 22.9 \) s and \( \sigma_0 = 2.2 \) MPa. (B) Fast (F) and slow (S) relaxation times as a function of strain for crosslinked (X), uncrosslinked fibers (U). (C) Generalized Kelvin model consisting of a spring (elastic modulus \( E_\infty \)), in parallel with two Maxwell elements (each consisting of a dashpot (viscosity \( \eta_1, \eta_2 \); relaxation time \( \tau_1, \tau_2 \)) in series with a spring (elastic modulus \( E_1, E_2 \)).

Detailed Force Per Monomer and Energy Per Monomer Calculations

The force and energy per fibrin monomer can also be estimated from our data and compared with single protein unfolding experiments and melting experiments. For example, the fibrin fiber in Figure 17 contains about 1600 monomers per cross-section (fiber radius, 165 nm; fibrin monomer radius, 2.25 nm; 30% protein content of fiber) and a force of 220 nN is required to stretch it to 100% strain. Thus, the force per monomer at 100% strain is about 140 pN, which is consistent with the 100 pN force to stretch a single fibrin monomer by 100% found by Brown et al. Using similar
considerations, we estimate the rupture force per monomer to be about 280 pN ($\varepsilon_{\text{max}}=147\%$, strain hardening h=1.9). This value is similar to the 260 pN (2·130 pN) required to rupture the two A:a interactions between half-staggered monomers within a protofibril 9. For comparison, the unfolding forces of T4 lysozyme (mainly $\alpha$-helical) and the titin Ig-domain (mainly $\beta$-structure) are 64 pN 10 and 150-300 pN 11, respectively. Thus, judging by these force considerations, it is certainly possible that some regions of the fibrin monomer unfold before the fiber ruptures.

The energy required to rupture the fiber is of the same order of magnitude as the melting enthalpy of the fibrin molecules contained in the fiber. Again, using Figure 17, the energy to rupture the fiber (area under curve) is approximately $8 \cdot 10^{-13}$ J. Assuming that there are about 570,000 monomers in this fiber (fiber length and radius, 15,200 nm and 165 nm; fibrin monomer length and radius, 45 nm and 2.25 nm; 30% protein content of fiber), the rupture energy per monomer is about $4 \cdot 10^{-17}$ J ($2.4 \cdot 10^3$ kJ/mol). When taking melting curves of fibrinogen, two major peaks are observed 12. The low-temperature peak (56°C) likely corresponds to three separate melting events within the D region and the high-temperature (95°C) peak likely corresponds to a melting event within the E region and the melting of the coiled coils. The enthalpy of melting (area under curve) of the low-temperature peak is 3350 kJ/mol and of the high temperature peak 1300 kJ/mol. The total enthalpy of melting (4650 kJ/mol) is, thus, of the same order of magnitude as the rupture energy (2400 kJ/mole), again indicating that fibrin may melt (denature), before the fiber ruptures.

Mullins Effect

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Our data were all collected from ‘first pulls’ or ‘small pulls’; that is from fibers that had not been significantly stretched before. The “Mullins effect” refers to the phenomenon that the force required to stretch a polymer is smaller in the second loading cycle than it was in the first cycle. This effect has been observed in a number of materials such as rubber, filler-reinforced vulcanized rubber, rubber-like materials, and rat left ventricular myocardium tissue\textsuperscript{13}. We observed a Mullins effect in fibrin fibers when the strain is over 30\%, 100\% and 60\% for crosslinked, partially crosslinked (see supplement) and uncrosslinked fiber, respectively. Figure S4 shows consecutive stress-strain curves of a fiber that was stretched to the same strain three times. The loading curve for the second pull (purple curve) is significantly smaller than for the first pull (red curve), although the unloading curves are very similar for these two. For the third pull (green curve) and all the following pulls (if fiber is not stretched even farther), the loading and unloading curve will follow almost the same path as the second pull.

\textbf{Figure S4} Three consecutive loading cycles of 120\% strain were applied to same fibrin fiber. The forward force for the first pull is larger than for the second and third pull. The forward forces for the second and third cycle overlap and all return paths nearly overlap.
PARTIALLY CROSSLINKED FIBRIN FIBERS

Partially crosslinked fibers were formed as previously reported. Factor XIII (Enzyme Research Labs, South Bend, IN; final concentration, 100 Loewy units/ml) was added to the fibrinogen and buffer solution before thrombin was added. Other conditions and concentrations were the same as those used to form uncrosslinked fibers. SDS gel electrophoresis of the partially crosslinked samples showed 50 – 75% \( \gamma-\gamma \) crosslinking and 25 – 60% \( \alpha-\alpha \) crosslinking (Fig S1). We don’t completely understand why these fibers did not fully crosslink. Nevertheless, we feel that the results on the partially crosslinked fibers should be communicated for at least two reasons. First, they may be physiologically relevant as they represent an intermediate state between uncrosslinked and fully crosslinked fibers. Second, they show unique extraordinary mechanical properties. As previously reported, partially crosslinked fibers have an extensibility of 333% and an elastic limit of 180%, both values higher than those for uncrosslinked and crosslinked fibrin fibers. Partial crosslinking increased the extensibility and elastic limit, while the stiffness only slightly increased and the strain hardening and energy loss behavior remained similar to that of uncrosslinked fibers. We speculate that the more fully formed \( \gamma-\gamma \) crosslinks are responsible for the large extensibility, as these crosslinks may stabilize the fiber in the longitudinal direction.

Partially Crosslinked Fiber Viscoelastic Properties

Identical experiments to the ones reported in the main paper were done for partially crosslinked fibers. The results are summarized in table S1. Engineering stress and incremental stress-strain curves were used to determine the total elastic modulus (4.6
MPa), the relaxed elastic modulus (2.6 Mpa) and the relaxation times (2s and 54s) of the partially crosslinked fibers. The generalized Kelvin model was once again used to fit the relaxation, such that elastic moduli of the springs, $E_1$ and $E_2$, and viscosities of the dashpot elements, $\mu_1$ and $\mu_2$, were determined. Partially crosslinked fibers showed similar strain hardening, $h = 3.5 \ (p \leq 0.0015)$, and energy loss characteristics to uncrosslinked fibers. Energy loss increases sigmoidally, with a low energy loss at strains below 50% which then increases to a higher total energy loss at strains above 100%. Although energy loss increases as strain increases, as previously reported, partially crosslinked fibers can be strained up to about $\varepsilon = 180\%$ and still visibly return to their initial length $^1$.

In our partially crosslinked fibers, $\gamma-\gamma$ crosslinks were predominant. While in the crosslinked fibers $\gamma-\gamma$ and $\alpha-\alpha$ crosslinking was complete (within the detection limit of the SDS PAGE). Our data suggest that the fully formed $\alpha-\alpha$ crosslinks may be largely responsible for not only reducing fiber extensibility but also stiffening the fiber. Physiologically, $\gamma-\gamma$ crosslinks form more rapidly than $\alpha-\alpha$ crosslinks $^{14}$. Therefore, the properties of partially crosslinked fibers may be representative of fibers found in newly forming clots which then progress with time to fully crosslinked fiber properties.
All mechanical properties of uncrosslinked, crosslinked, and partially crosslinked fibrin fibers; including generalized Kelvin model properties, and data statistics.

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Crosslinked</th>
<th>Partially Crosslinked</th>
<th>Uncrosslinked</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\epsilon_{\text{max}}$</td>
<td>147 % +/- 5%</td>
<td>333 % +/- 13%*</td>
<td>226% +/- 8.7%*</td>
</tr>
<tr>
<td>$\epsilon_{\text{elastic}}$</td>
<td>&lt; 50 %</td>
<td>180 % *</td>
<td>120 % *</td>
</tr>
<tr>
<td>$E_0$ (Mpa)</td>
<td>8.0 +/- 1.0</td>
<td>4.6 +/- 0.7</td>
<td>3.9 +/- 0.3</td>
</tr>
<tr>
<td>$E_\infty$ (Mpa)</td>
<td>4.0 +/- 0.6</td>
<td>2.6 +/- 0.3</td>
<td>2.0 +/- 0.2</td>
</tr>
<tr>
<td>$\tau_1$ (s)</td>
<td>2.1 +/- 0.2</td>
<td>2.0 +/- 0.2</td>
<td>2.9 +/- 0.5</td>
</tr>
<tr>
<td>$\tau_2$ (s)</td>
<td>49 +/- 4</td>
<td>54 +/- 7</td>
<td>54 +/- 9</td>
</tr>
<tr>
<td>$h$</td>
<td>1.9 +/- 0.3</td>
<td>3.5 +/- 0.8</td>
<td>3.2 +/- 0.4</td>
</tr>
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<td>$E_{\text{loss}}$</td>
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<td>0-70%</td>
<td>£40-70%</td>
</tr>
<tr>
<td>$E_1$ (Mpa)</td>
<td>1.3 +/- 0.2</td>
<td>1.1 +/- 0.3</td>
<td>1.6 +/- 0.8</td>
</tr>
<tr>
<td>$E_2$ (Mpa)</td>
<td>2.0 +/- 0.2</td>
<td>0.8 +/- 0.12</td>
<td>0.6 +/- 0.2</td>
</tr>
<tr>
<td>$\mu_1$ (Mpa*s)</td>
<td>3.2 +/- 0.6</td>
<td>1.0 +/- 0.4</td>
<td>2.5 +/- 0.6</td>
</tr>
<tr>
<td>$\mu_2$ (Mpa*s)</td>
<td>155 +/- 47</td>
<td>33 +/- 6.9</td>
<td>37 +/- 17</td>
</tr>
</tbody>
</table>

Table S1 The average values and standard errors are listed. Extensibility, $\epsilon_{\text{max}}$, (89 data points, crosslinked fibers), elastic limit, $\epsilon_{\text{elastic}}$, (55 data points, crosslinked fibers), $E_0$, total elastic modulus; $E_\infty$, relaxed elastic modulus (moduli obtained from 86 crosslinked and 38 uncrosslinked data points collected over the entire strain range). $\tau_1$ fast relaxation time; $\tau_2$ slow relaxation time (average values of 86 data points for crosslinked fibers and 71 data points for uncrosslinked fibers). $h$, strain hardening factor; this is the ratio of the total modulus at low strains (0-80%) to the total modulus at high strains (above 110%) (average values of 26 uncrosslinked fibers). Thus, the total elastic modulus for uncrosslinked fibers has a value of 3.9 MPa at low strains and 11.7 MPa at high strains. Crosslinked fibers did not show consistent strain hardening. $E_{\text{loss}}$, energy loss at high strains (energy loss at low strains is close to 0% for uncrosslinked, and £40% for crosslinked fibers). $\epsilon_{\text{max}}$, $\epsilon_{\text{elastic}}$, $E_0$, $E_\infty$, $\tau_1$, $\tau_2$, $h$, are measured values; $E_1$, $E_2$, $\mu_1$, $\mu_2$ are model-based values obtained by fitting our relaxation data to the generalized Kelvin model depicted in Figure S3C. * Values were obtained from 1.
REFERENCES


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C.R. Carlisle, Robert Ariens, Kristina Standeven, Martin Guthold. “Mechanical properties of Individual γQ398N/Q399N/K406R fibrin: An insight into fibrin crosslinking.”