Project Summary

Overview

Blood clots are a mixture of red blood cells, platelets, and a polymeric fibrin matrix that prevent the loss of blood during wound healing. During blood clot formation, fibrin polymerizes into a 3-D gel wherein the fibers have <u>inherent tension</u>. After polymerization, clots are subject to <u>applied tension</u>, which originates from numerous sources including platelet contraction, blood hemodynamics, and the motility of cells trapped within the fibrin gel. The fibrin network must withstand vascular forces during wound healing but then be dissolved afterwards, to avoid blood vessel occlusion, in a process called fibrinolysis.

A key knowledge gap lies in understanding the extent to which, and the mechanisms by which, inherent and applied tension regulate the fibrinolytic process. Demonstrating the existence and importance of tension in clots during lysis has been a foundational part of the research programs of all three PIs over the past 4-5 years, and they anticipate this research continuing over the next decade. The PIs have previously demonstrated that inherent tension helps to accelerate lysis, while applied tension hinders lysis. This proposal extends these initial findings to perform the first multiscale study correlating amounts of fiber and clot tension with fibrinolytic rates. Each aim of the proposal centers on testing one hypothesized mechanism of how tension affects lysis: 1) causing structural rearrangements in the gel prior to lysis, shrinking the volume and expelling lytic enzymes; 2) altering the binding kinetics of enzymes to fibrin; and 3) causing structural rearrangements within the fibers and network structure during lysis, thereby clearing the fibrin more rapidly from the clot volume. Each hypothesis will be tested using a multidisciplinary research approach involving tensile testing, time-resolved microscopy, and mathematical modeling.

In addition to the important research questions, topics of tension and lysis provide important instructional models for students from diverse academic backgrounds and educational levels. At the elementary school level, the PIs will expand/improve a summer camp that discusses tension, lysis, and other STEM topics using LEGO®. At the collegiate level, the PIs have published numerous papers with undergraduate first authors focused on tension and lysis and actively engage in REU mentoring programs. Interdisciplinary lysis studies also have resulted in training Masters and PhD students. This grant proposes to continue these successful training programs and adds an exchange student component, where students will visit the labs of the other PIs on the grant to obtain cross-disciplinary training.

Intellectual Merit

<u>A comprehensive understanding of lysis requires a determination of how vascular forces influence the digestion of blood clots across the molecular, fiber, and network scales.</u> The PIs' collaborative, multidisciplinary approach combines multiscale experiments and mathematical models to interrogate these processes and enables the isolation of different mechanisms by which tension regulates fibrinolysis. The Bannish group has developed the most biochemically- and structurally-detailed mathematical model of lysis to date, which connects micro- and macroscale lysis. The Hudson Lab has pioneered the microscale studies of fibrinolysis, emphasizing the importance of fiber tension. The Tutwiler Lab has expertise in macroscale experiments related to clot structure, mechanics, and fibrinolysis. To date, models of fibrinolysis completely ignore tension and often focus on a limited spatial scale. This work will result in a transformative combinatorial multiscale mathematical and experimental model that can predict lytic outcomes as a function of mechanical forces. A significant advance forward from the current state of the art, this work will lay a foundation for others to study lysis biomechanics.

Broader Impacts

Diseases of the cardiovascular and circulatory systems are leading causes of death and disability worldwide, and all result, in part, from a failure of the fibrinolytic system. This work will provide the first framework for understanding the importance of fibrin tension in regulating the fibrinolytic process, which ultimately could contribute to improved therapeutics for cardiovascular diseases. It will also broaden participation in STEM of undergraduate students, recruited from underrepresented groups, who have less exposure to research opportunities. Elementary school children will be exposed to STEM principles through weeklong summer programs (piloted in summer 2022) that teach engineering and design principles, focused on tension, through Lego®. Graduate and undergraduate students funded by this grant will help develop materials for the summer programs that can be broadly distributed by the PIs.

Title: Collaborative Research: The Role of Tension in the Digestion of Blood Clots

PROJECT DESCRIPTION

<u>BACKGROUND</u>
Hemostasis, or the cessation of bleeding following injury, occurs through a balance of clot formation (coagulation) and clot degradation (fibrinolysis). As a clot forms several key processes occur. First, platelets become exposed to activation factors in the subendothelial layer that leads to the rolling and tethering of the platelets to the surface. In parallel, the activation of the coagulation cascade culminates in the formation of a polymeric fibrin network. Fibrin provides the main structural and mechanical stability to blood clots. Platelets and fibrin interact through αIIbβ3 integrins on the platelet surface. Collectively, platelets, fibrin, and entrapped red blood cells create a hemostatic seal. We propose to study the interplay between clot mechanics and clot degradation from the micro through macroscale levels. For the purposes of this grant, we define "microscale" as the molecular/protofibril/fiber length scale (defined in the next paragraph) and "macroscale" as the entire clot.
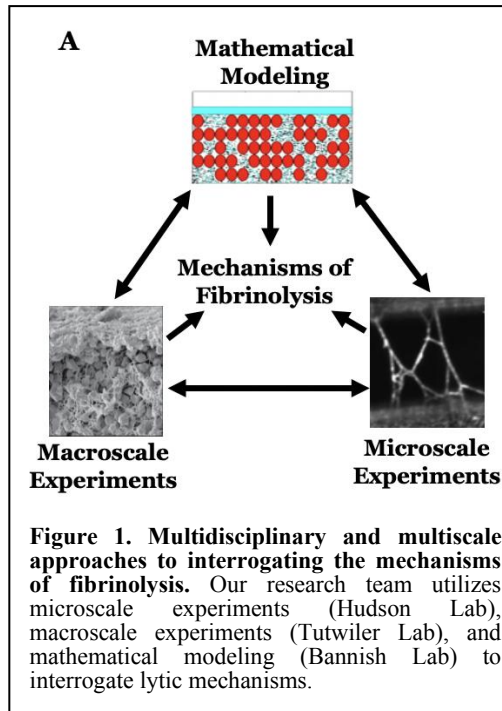
Fibrin polymerization results in <u>inherent tension</u> on the fibrin network. Fibrin is created when thrombin cleaves fibrinopeptides from fibrinogen monomers. Fibrin forms a multiscale, hierarchical structure where monomeric fibrin polymerizes into double stranded protofibrils. The protofibrils are made up of half staggered, overlapping fibrin monomers that are linked through noncovalent bonds. Protofibrils then bundle laterally into fibers, branch into a three-dimensional network, and are stabilized by factor XIIIa (FXIIIa) crosslinking (1, 2). We have previously shown through both microscale experiments (3) and macroscale experiments (4) that during the polymerization process fibrin fibers generate an <u>inherent tension</u>. We observe ~5 Pa of stress generated within the fibrin network during polymerization (4). This generation of inherent tension is not fully understood but it is hypothesized to occur due to axial twisting during the polymerization process that is needed to form the characteristic half staggered protofibril.

Clot contraction results in <u>applied tension</u> on the fibrin network. Following their activation, platelets can generate contractile forces through their acto-myosin cytoskeleton. These contractile forces drive the volume shrinkage (clot contraction) of the blood clots as the forces are propagated through the clot volume due to platelet-fibrin interactions. We and others have shown that platelets are able to generate up to 70 nN/platelet or up to 250 Pa of stress in the network (5, 6), suggesting that the <u>applied tension</u> on the fibrin network during clot contraction is much larger than the tension generated during polymerization. Moreover, platelets drive the densification of fibrin during the contraction process, which alters the fibrin network structure. Fibrin network structure influences both the mechanical and enzymatic stability of the blood clot (7-11).

Fibrinolysis is subject to enzymatic and mechanical regulation. Fibrinolysis is the proteolytic degradation of fibrin fibers by plasmin. During lysis, plasmin cuts fibrin molecules at specific sites, resulting in the eventual transectional cleavage of fibers (12). For clarity in this proposal, "cleavage rate" will be defined as the rate at which a single fibrin fiber is transected, while "lysis rate" will be defined as the rate at which a fibrin network is cleared from a region. This process is regulated by numerous enzymes and inhibitors, as well as by mechanical forces. Since this proposal focuses on the role of tension, only the most prominent enzymes and inhibitors will be discussed. At the surface of fibrin, plasmin is predominantly activated from its inactive precursor, plasminogen, by tissue-type plasminogen activator (tPA), which noncovalently binds to fibrin. Plasminogen, tPA, plasminogen activator inhibitor-1 (PAI-1, an inhibitor of free tPA), and α2-antiplasmin (α2-AP, a potent inhibitor of plasmin) are all present during fibrin polymerization, and activated platelets release additional pro/anti-fibrinolytic factors in the early stages of activation. Therefore, the relative concentrations of both pro- and anti-fibrinolytic molecules regulate fibrinolysis and change throughout the process. Additionally, fibrinolysis is regulated by mechanical forces. We have shown that the inherent tension on fibrin fibers propels lysis by causing rearrangements in clot

structure (3, 13). In contrast, the application of applied tension has been shown to delay fibrinolysis, resulting in an up to 3-fold reduction of plasminogen activation in stretched vs unstretched fibers (14, 15). The mechanisms underlying the differential influence of inherent vs applied tension during lysis have not been characterized and addressing this knowledge gap forms the intellectual framework of this proposal.

Mathematical models of fibrinolysis. Mathematical models can be used in conjunction with laboratory experimentation to gain a deeper understanding of biological processes. Previous fibrinolysis modeling and experimental efforts have focused primarily on a singular temporal or spatial scale (12, 13, 16-19), and none have explicitly included tension of fibrin fibers. Here, we aim to combine experiments and mathematical modeling across multiple scales, and develop the first fibrinolysis model that includes tension, which will advance knowledge of the mechanisms underlying fibrinolysis. Mathematical modeling can aggregate a variety of molecular factors, time scales, and spatial scales to isolate the effects of individual factors, integrate knowledge gained from experiments, make predictions, and understand new data, in a manner that is often impossible with experiments alone. Previous mathematical models have been limited in their scope and cannot be used to model complicated blood clots because they are unable to account for the differences in lysis in clots of varying structure (18-24). We previously developed a heterogenous, 3-D, stochastic, multiscale model of fibrinolysis that will be modified and extended for the current proposal (25, 26). Additionally, we will develop a complementary model that will capture the network-level structural rearrangements that occur following the lysis of fibers under tension. Here, experiments and modeling will be explored in tandem; experiments will inform the further development and validation of our models and the models will be used to predict and understand mechanisms of fibrinolysis (Figure 1). This combination of modeling and experiments establishes the necessary foundation for a translational framework which can lead to a deeper understanding of the tension-dependent mechanisms of fibrinolysis.
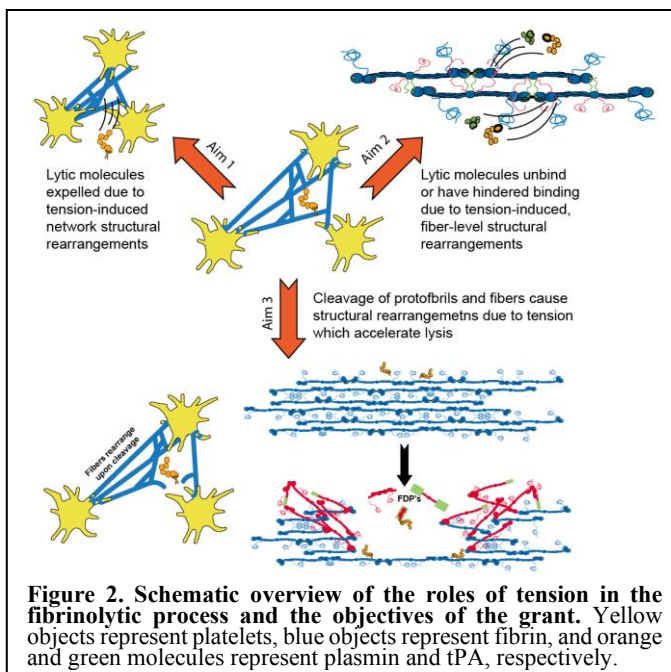

Figure 1. Multidisciplinary and multiscale approaches to interrogating the mechanisms of fibrinolysis. Our research team utilizes microscale experiments (Hudson Lab), macroscale experiments (Tutwiler Lab), and mathematical modeling (Bannish Lab) to interrogate lytic mechanisms.

Feasibility. A comprehensive understanding of lysis requires a determination of how vascular forces influence the digestion of blood clots across the molecular, fiber, and network scales. By bringing together research teams that have performed detailed microscale measurements of fiber lysis emphasizing the importance of fiber tension (Hudson), macroscale lysis experiments (Tutwiler), and structurally- and biochemically detailed mathematical models, including both micro- and macroscale lysis features (Bannish), we are uniquely poised to do this work. The PIs' collaborative, multidisciplinary approach combines multiscale experiments and mathematical models and enables the isolation of different mechanisms by which tension regulates fibrinolysis.

OBJECTIVES AND RESEARCH PLAN

The goals of this proposal are focused on elucidating the fibrinolytic effects of the mechanical changes that occur during clot formation, contraction, and lysis. We will 1) determine the effect of tension on network rearrangements and the expulsion of lytic enzymes, 2) establish how tension regulates the exposure of lytic binding sites, and 3) ascertain the role of tension in the structural rearrangements that occur during lysis (Figure 2). Previous work has demonstrated that inherent tension accelerates lysis while applied tension hinders lysis. The mechanisms for this behavior are largely unexplored and the tension threshold where

force begins hindering rather than accelerating lysis is unknown. Fundamentally, this threshold is important since fibrinolysis occurs during the time course of clot formation and beyond; tension thresholds can connect lysis mechanisms to the evolution of fiber tension as a function of time. Translationally, the competing effects of tension may shed light on the limited efficacy of current fibrinolytic treatments for thrombotic diseases such as stroke.

Hypothesis. We hypothesize that fiber tension primarily regulates fibrinolysis through three mechanisms. We propose that 1) Applied tension shrinks the spacing between fibers, causing the expulsion of fluid and altering both the local fibrin and lytic enzyme concentrations and the clot permeability; 2) Tension (both inherent and applied) regulates the binding sites of lytic enzymes such tPA and plasmin; 3) Tension (both inherent and applied) pulls fibers apart during digestion and causes network rearrangements after every fiber cleavage event. The objective of this proposal is to test these hypotheses and determine their relative contribution in regulating fibrinolysis through a combination of macroscale and microscale *in vitro* experiments and mathematical modeling.

Figure 2. Schematic overview of the roles of tension in the fibrinolytic process and the objectives of the grant. Yellow objects represent platelets, blue objects represent fibrin, and orange and green molecules represent plasmin and tPA, respectively.

Results from prior NSF support. *V. Tutwiler*: CBET #2207577; 9/2022-8/2025 (PI: J. Freeman); "Injectable nanoparticles for soft tissue recovery and strength enhancement." The work is ongoing and publications/research products are in progress. V.Tutwiler has mentored two NSF-sponsored REU students, one of these students (Hajer Ali) presented her research at the BMES annual meeting and has a manuscript under review.
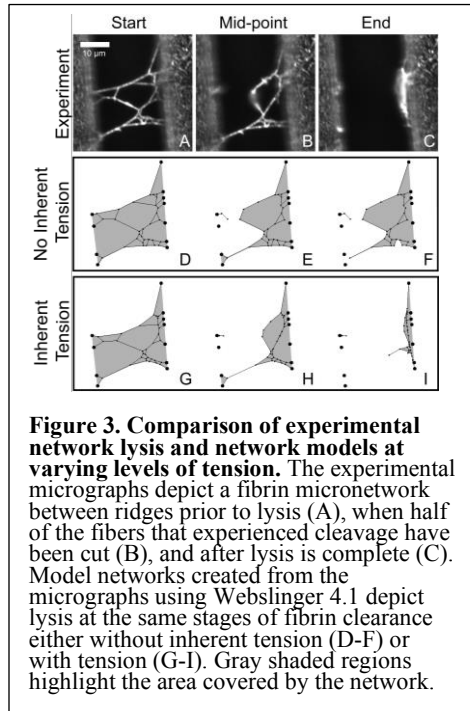
N. Hudson has mentored two NSF-sponsored REU students who presented their work at BMES annual meetings; one of these students (Kristine Peterson) is a co-author on the recent publication:
Popovic, G., Kirby, N.C., Dement, T.C., Peterson, K.M., Daub, C.E., Belcher, H.A., Guthold, M., Offenbacher, A.R., Hudson N.E. Development of Transient Recombinant Expression and Affinity Chromatography Systems for Human Fibrinogen. *IJMS*. 2022 Jan 19;23(3). PMID: 35162976. PMCID: PMC8835685.
B. Bannish: N/A

Rationale and preliminary data. We have previously shown that fibrin polymerization results in inherent tension at both the single fiber and network scale (3, 4, 12, 27). As platelets contract the network and hemodynamic forces exert stress on the network, the quantity of applied tension increases. Understanding how this progression of tension alters the mechanisms of fibrinolysis is the focus of this proposal.

We hypothesize that multiscale structural changes are the primary method through which tension modulates lytic mechanisms. *Before* and/or concurrent with the inception of lysis, applied and inherent tension cause changes to the fibrin network geometry and environment that affect lytic outcomes (14, 28, 29). For example, the application of applied tension has been shown to delay fibrinolysis in fibrin gels and result in an up to 3-fold reduction of plasminogen activation in stretched vs. unstretched fibers (14). However, the mechanisms for this reduction have been debated. One hypothesis is that smaller spacing between the fibers in stretched gels leads to slower diffusive transport of enzymes into the networks (28). Alternatively, enzymes may be expelled from a clot due to the application of tension during clot contraction, altering the fibrinolytic environment (30). In Objective 1, we will test these competing hypotheses to determine how tension-induced structural rearrangements of the network alter the lytic process.

Figure 3. Comparison of experimental network lysis and network models at varying levels of tension. The experimental micrographs depict a fibrin micronetwork between ridges prior to lysis (A), when half of the fibers that experienced cleavage have been cut (B), and after lysis is complete (C). Model networks created from the micrographs using Webslinger 4.1 depict lysis at the same stages of fibrin clearance either without inherent tension (D-F) or with tension (G-I). Gray shaded regions highlight the area covered by the network.

In addition to affecting the concentrations of enzymes in the network (Objective 1), applied tension results in decreased plasmin generation (14) and reduced tPA binding (29). We hypothesize that both inherent and applied tension alter binding affinities of lytic enzymes to fibrin, which would explain, in part, these alterations in plasmin generation. The fact that fibrin binding enhances the conversion of plasminogen to plasmin suggests that the inherent tension involved in the polymerization process may drive a conformation change exposing tPA and/or plasminogen binding sites. Applied tension causes molecular unfolding of the coiled coils and γ-nodules of fibrin, which exposes potential plasmin(ogen) binding and cleavage sites in these regions. Collectively, these observations suggest that molecular structural changes may play a role in the tension mediated regulation of fibrinolysis. We propose to directly test the effect of tension on enzyme binding and activation and elucidate how these tension-induced enzymatic changes influence lytic outcomes (Objective 2).

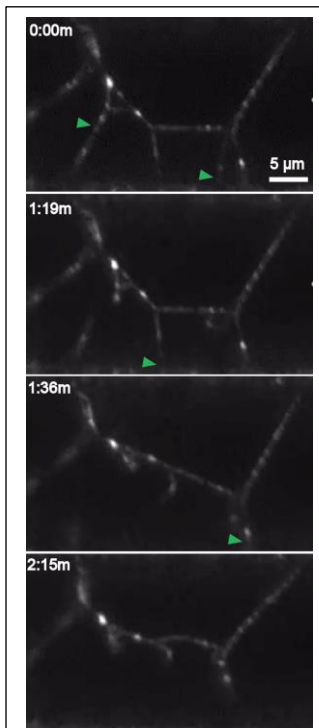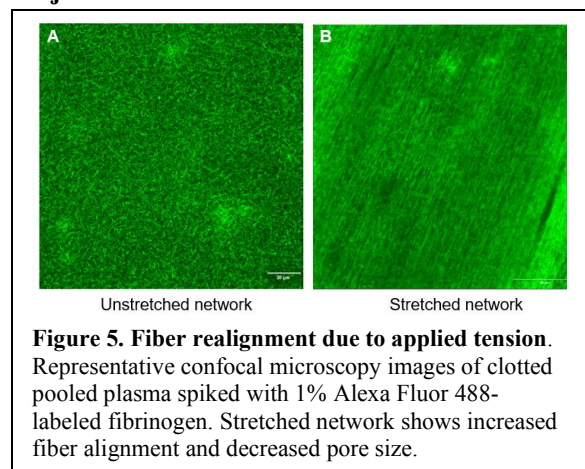*During* the lysis process, tension also affects the clot environment. Our recent results (12) show that inherent tension may help to drive the lysis of individual fibers by pulling the fiber apart at sites of digestion, as evidenced by the sliding of fibrin away from the cleavage site. We have also shown that as a result of inherent tension, every fiber cleavage event results in a redistribution of the tension in surrounding fibers, causing changes in network architecture, fiber bundling, recoil, and collapse (3). Through these effects, only 50% of fibers in a region need to be cleaved for the region to be cleared of fibrin. A preliminary mathematical model of small fibrin networks under tension supports these results (Figure 3). Based on this work, we hypothesize that inherent fiber tension plays important roles both in regulating the microscale fibrinolytic susceptibility of individual fibers and in driving fiber macroscale network *clearance* (the removal of all fibrin fibers from an area/volume). Preliminary data (Figure 4) suggest that the inherent tension in fibers is reduced during the lysis process, indicating that polymerization stretches fibers beyond their equilibrium length.

It is unclear whether changes in network architecture during the lytic process due to inherent-tension play a significant role in the lysis of fibrin under applied tension. As previously discussed, networks under applied tension typically lyse more slowly (14, 28), while our own data (3, 12) demonstrate that inherent tension accelerates lysis. Therefore, there must be a threshold tension at which tension transitions from accelerating to hindering lysis. We hypothesize that the same structural changes that accelerate lysis through inherent tension (described in the paragraph above) will also be present under applied tension, but that other processes, such as decreased enzyme binding (investigated in Objective 2) have a greater effect on cleavage and lysis rates and therefore lead to a net overall reduction in lysis rates. We propose to quantify the threshold value where tension transitions from increasing to decreasing lysis rates and investigate the effects of inherent- and applied-tension-induced structural changes during lysis (Objective 3).

Figure 4. Experimental image series demonstrating the subsequent loss of inherent tension in a fibrin network during fibrinolysis. Green arrows demonstrate the location of a cleavage event that will occur in a subsequent frame.

Taken together, our research approach seeks <u>to disentangle the relative roles of competing, tension-induced effects on lysis rates, all of which are related to structural changes in the fibrin spatial hierarchy. These effects include: 1) enzyme expulsion from the network and hindered effective diffusion into the network (Objective 1), 2) altered enzyme binding (Objective 2), and 3) physical alterations in the fiber and gel structure during digestion (Objective 3).</u> Collectively we have extensive and interdisciplinary expertise, combining laboratory experimentation and mathematical modeling to tackle this challenge. Experimental results will be complemented and informed by <u>the first mathematical model to incorporate fiber tension in predicting fibrinolytic outcomes.</u> A preliminary model (Sec. O3.d) that includes tension confirms that a small fibrin network can be cleared if only a fraction of the fibers are cleaved by plasmin. By building the full model in close conjunction with the laboratory experiments we will be able to: cheaply and relatively quickly simulate physical experiments to test experimental hypotheses; test a wide range of parameter values that would be prohibitively time-consuming or costly to do in the lab; aggregate a variety of molecular factors, time scales, and spatial scales to isolate the effects of individual lytic factors in a way that is impossible experimentally; and integrate knowledge gained from experiments in order to make predictions and new hypotheses. To date, models of fibrinolysis completely ignore tension and often focus on a limited spatial scale. Our work will result in the first validated mathematical model that can predict lytic outcomes as a function of mechanical forces in the vasculature, which will be a significant advance forward from the current state of the art.
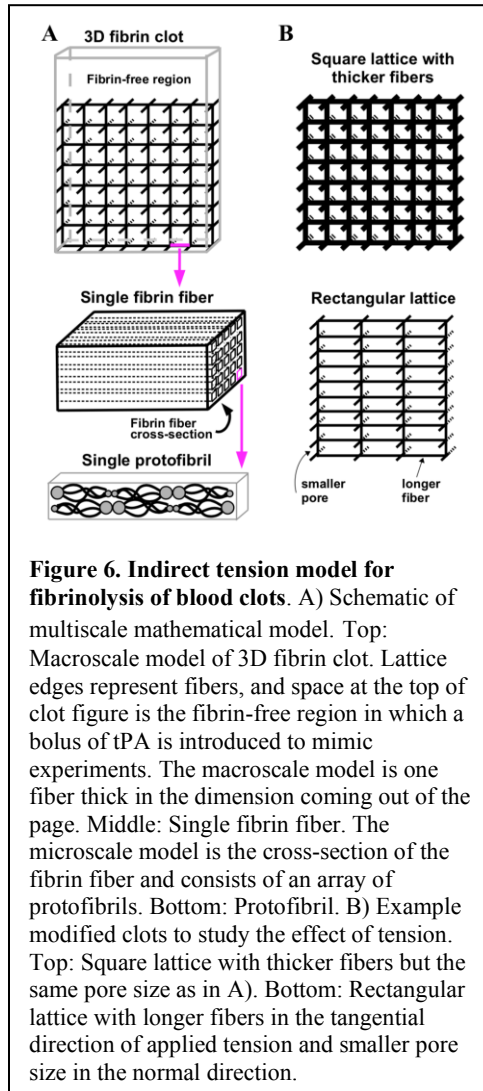
Objective 1: Assess the influence of tension on network rearrangements and expulsion of lytic enzymes. Our preliminary data shows that applied tension on the fibrin network results in network scale structural rearrangements (Figure 5). We hypothesize that these structural changes, such as a decrease in pore size and/or fiber bundling directly alter the lysis rates by impeding the diffusion of tPA into and through the clot. Our preliminary results show that a decrease in pore size results in a slower rate of fibrinolysis (31). However, the structural rearrangements that occur due to applied tension also result in the expulsion of serum, which may contain fibrinolytic enzymes and contribute to the impairment of fibrinolysis. Here, we will utilize multiscale experiments and mathematical modeling to disentangle the effects of network structural changes

Unstretched network   Stretched network

Figure 5. Fiber realignment due to applied tension. Representative confocal microscopy images of clotted pooled plasma spiked with 1% Alexa Fluor 488-labeled fibrinogen. Stretched network shows increased fiber alignment and decreased pore size.

from the expulsion of serum in regulating fibrinolysis.

O1.a Quantify the tension induced structural rearrangements of the fibrin network. We and others have shown that applied tension induces structural rearrangements in fibrin networks (14, 29, 32-35); however, a rigorous structural characterization that can be used to obtain correlations between structural rearrangements (O1.a) and fibrinolytic parameters (O1.b-O3) is lacking. We will utilize a combination of confocal, transmission electron (TEM) and scanning electron (SEM) microscopy to determine how the application of tension alters the microstructure of the fibrin network. Plasma samples pooled from 25+ donors (Cone Biologics) will be activated through the addition of 25 mM CaCl2 and 75 pM tissue factor and allowed to polymerize for at least 1 hour to ensure full crosslinking. Samples will be stretched (25, 50, 100 and 200% original length) and fixed at that extension prior to imaging. For confocal microscopy, samples will be spiked with 1% Alexa Fluor 488 labeled fibrinogen to allow for visualization of the fibrin network; fibrin network density, pore size, branching, protofibril packing density, and fiber length will be quantified using ImageJ and Matlab. For SEM, samples will be dehydrated, and sputter coated; images will be analyzed for fibrin fiber density, fiber diameter and branching. For TEM, samples will be thinly

Figure 6. Indirect tension model for fibrinolysis of blood clots. A) Schematic of multiscale mathematical model. Top: Macroscale model of 3D fibrin clot. Lattice edges represent fibers, and space at the top of clot figure is the fibrin-free region in which a bolus of tPA is introduced to mimic experiments. The macroscale model is one fiber thick in the dimension coming out of the page. Middle: Single fibrin fiber. The microscale model is the cross-section of the fibrin fiber and consists of an array of protofibrils. Bottom: Protofibril. B) Example modified clots to study the effect of tension. Top: Square lattice with thicker fibers but the same pore size as in A). Bottom: Rectangular lattice with longer fibers in the tangential direction of applied tension and smaller pore size in the normal direction.

sectioned transversely to allow for the analysis of bundling/bundle diameter and fiber diameter through the cross-section of the fiber. We will assess each of these network and fiber parameters as a function of network strain to determine the extent to which each parameter is altered under strain (Figure 5). Structural changes will be used to inform the modification of the mathematical model to ensure that it reflects the application of strain (O1.c).

O1.b Quantify the effect of altered network structure on lysis.
Samples will be polymerized in the same manner described in O1.a in a 10mm x 10mm x 1mm mold and following polymerization they will be transferred to a tensile tester (Biomomentum) at the same, fixed length to preserve their inherent tension. Samples will then be stretched (25, 50, 100, and 200% strain) or relaxed (0.5% strain) to examine applied tension or evaluate the effect of inherent tension, respectively. Samples will be held at the fixed strain and 75 ng/ml tPA will be added to the center front face of the plasma clot. We will collect high resolution video recordings of the lysis process using a mounted camera to visually track the progression of fibrinolysis (33). In addition, the rate of fibrinolysis will be quantified by measuring the loss in force at a fixed strain over the course of time following initiation of fibrinolysis. A spearman correlation matrix will be conducted between the changes in the fibrin network structure parameters following stretching such as diameter, alignment, and packing density (collected in O1.a) and fibrinolysis data collected in this objective to determine the dependence of fibrinolysis on structural changes. In addition, we will vary the network structure through the addition of different amounts of tissue factor (6-600 pM) to assess how changes in the pore size and fiber diameter influence the rate of fibrinolysis.

O1.c Mathematically model the lysis of structurally altered fibrin clots due to tension. To better understand the structural mechanisms responsible for varied lysis due to tension, we will use our previously published multiscale mathematical model of fibrinolysis (25, 26), henceforth referred to as the indirect tension model. The model utilizes a stochastic, kinetic algorithm to account for microscale biochemical reactions and translate them into macroscale rates of fibrinolysis of a 3-dimensional (3D) fibrin clot (Figure 6A). Briefly, a stochastic microscale model of a single fiber cross-section is run at least 50,000 times to obtain data about single fiber lysis times and the length of time tPA stays bound to a fiber. These data are then used in a macroscale model of a 3D square lattice representing a fibrin clot, from which we obtain fibrin degradation rates and spatiotemporal tPA distributions. This model is a powerful tool and has already successfully resolved conflicts in experimental literature about the lysis rates of clots of different structure (25). Here, we propose to adjust this indirect tension model – based on knowledge gained from O1.a and O1.b – to untangle the structural effects of tension on fibrinolysis. Specifically, we will study the effects of tension-induced fiber spacing and fiber alignment and bundling (Figures 5&6B) on lysis rates.
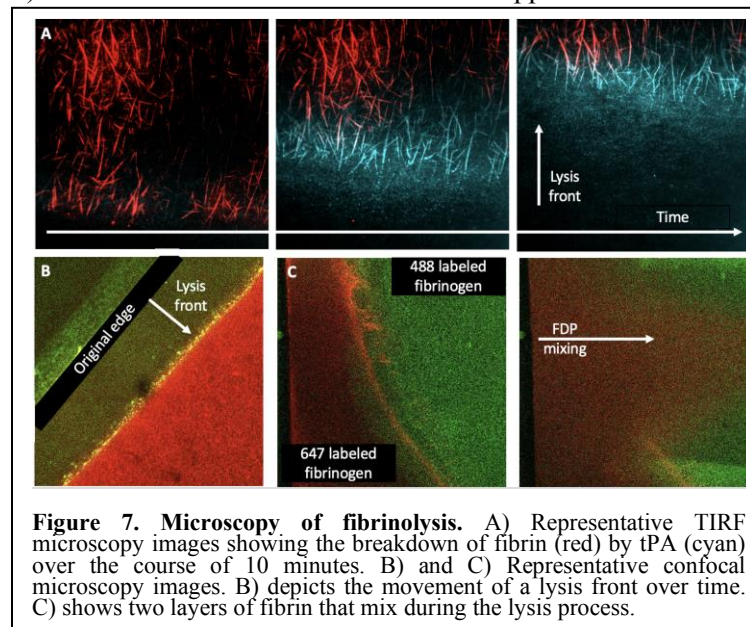
Our and other's experiments have shown that stretched clots have fibers that align along the direction of strain, with much smaller pore sizes than unperturbed clots (14, 32, 34). To mimic stretched clot conditions, we will adjust the macroscale model to be a rectangular (rather than square) lattice with longer fibers and smaller pores in the direction of the applied tension (Figure 6B). We will run the macroscale model with a particular focus on the mean time for tPA to diffuse through the clot. We will

perform multiple simulations using a range of fiber lengths and pore sizes obtained from the experiments in O1.a. As a result of these simulations, we will determine the underlying mechanisms responsible for the experimental observations about the effect of applied tension on lysis rate and effective tPA diffusion. To understand the relative role of tension-induced *fiber spacing* independent of fiber alignment or bundling, we will revert to the square lattice in the macroscale model and vary the pore size between fibers while keeping other parameters fixed. To investigate the relative role of tension-induced *alignment and bundling* independent of fiber spacing, we will: 1) keep pore size fixed in the square lattice macroscale model, but use thicker fibers (to mimic bundling), and 2) use the rectangular lattice macroscale model with the original fiber diameter and pore size in the normal direction to the applied tension, and the necessarily-bigger pore size (due to the longer fibers) in the tangential direction to the applied tension (to mimic fiber alignment). A one-way ANOVA (α=0.05) will be performed on the lysis rates to validate the model results by comparing them to matched experimental data.

O1.d Measure and model enzyme expulsion under varying degrees of tension. We will collect expelled serum during the stretching process and measure the expulsion of tPA, PAI-1, a2-AP, plasmin, and plasminogen using immunoassays to assess if tension induced expulsion of enzymes plays a critical role in regulating fibrinolysis. We will compare this to levels in unclotted plasma to determine total concentrations in the plasma and calculate a relative amount of enzyme expulsion as a function of applied strain. Moreover, we will control the composition of the supernatant by removing and/or adding additional enzymes to determine if the expelled serum contributes to fibrinolysis. Lysis rates will be normalized to the relative enzyme expulsion if needed. A spearman correlation will be performed between the percent of enzyme expelled and the extent of fibrinolysis to determine the driving factors of fibrinolysis. The model described in O1.c will be rerun using different enzyme concentrations that mimic the measured experimental conditions in order to quantify how the combination of altered network structure and lower enzyme concentration affects lysis.

Expected outcomes. Successful completion of this objective will result in several measurable outcomes: 1) a direct correlation between amount of applied tension and resulting clot structural features such as fiber

density, diameter, branching, and pore size; 2) direct correlations between tension-induced clot structure and the fibrinolysis rate; 3) measurements of the amounts of fibrinolytic enzymes expelled as a function of clot stretching; 4) development of a multiscale model that will help differentiate the mechanisms responsible for varied lysis rates in stretched clots.

Figure 7. Microscopy of fibrinolysis. A) Representative TIRF microscopy images showing the breakdown of fibrin (red) by tPA (cyan) over the course of 10 minutes. B) and C) Representative confocal microscopy images. B) depicts the movement of a lysis front over time. C) shows two layers of fibrin that mix during the lysis process.

Objective 2: Determine how tension regulates the exposure of lytic enzyme binding sites. The application of tension to the fibrin network and individual fibrin fibers results in molecular unfolding in addition to the macroscopic structural arrangements (27, 29, 36-38). We hypothesize that the molecular unfolding will lead to the exposure and/or blocking of lytic enzyme binding sites, and therefore affect single fiber and full clot lysis.

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O2.a Assess the binding/unbinding of tPA and/or plasmin in stretched and unstretched fibers and networks. Through the use of AlexaFluor-488 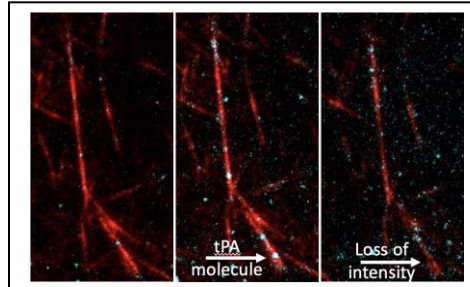labeled tPA and plasma spiked with 1% (v:v) AlexaFluor-647 labeled fibrinogen in total internal reflection fluorescence (TIRF) microscopy we will track molecular events during microscale lysis (Figure 7). By titrating the tPA or plasminogen concentration to reach single molecule binding and unbinding events (Figure 8), we will track the dwell time of tPA on fibrin. Dwell times will be measured on unstretched as well as stretched clots. For stretched clots, gels will be polymerized as described in O1.a, stretched to a fixed strain (25%-200%), then affixed (28) to a 1.5 coverslip for imaging. Fiber density will be varied by altering the clotting conditions to reflect the tension induced changes determined in O1.a and the interplay between density and enzyme dynamics will be calculated from single molecule experiments and small network lysis. Fiber diameters and tPA dwell times will be used as model parameters for the microscale mathematical models.

Figure 8. Single molecule microscopy of tPA (cyan) interacting with fibrin (red). Image captured using a Nikon Ti-E TIRF microscope. tPA labeled with Alexa Fluor 488 and fibrin labeled with Alexa Fluor 647.

O2.b Measure cross-sectional digestion rates of fibers in stretched vs non-stretched networks. While experiments in O2.a aim to interrogate binding and unbinding rates of tPA/plasminogen, because the fibers are attached to a surface, fiber cleavage rates would be artificially skewed. In this sub-objective we will quantify the local fiber digestion rates caused by these enzymes as a function of tension in single, suspended fibers. We will polymerize fibers on microprinted ridges that have been formed in an optically transparent glue (15). The glue will either be pre-stretched (allowing for relaxation and the loss of inherent tension) or stretched after polymerization (creating applied tension) using a homebuilt stretching device. tPA and/or plasmin will be added to the top of the liquid droplet containing the fibrin samples to initiate lysis, and time-series images of the fibers during digestion will be collected using epifluorescent microscopy. Figure 9 shows preliminary data of the plasmin-mediated digestion of one fibrin fiber, where digestion corresponds to loss in average cross-sectional fluorescence intensity. Control experiments testing for photobleaching will be performed to ensure that intensity loss corresponds to lysis. Like our recently published results (12), we will quantify the digestion rate (Figure 9) and the number of digestion sites in each fiber. Moreover, we will use fluorescence intensity to determine the fraction of the fiber cross-section that must be digested prior to the fiber snapping apart, which will be utilized in the model described
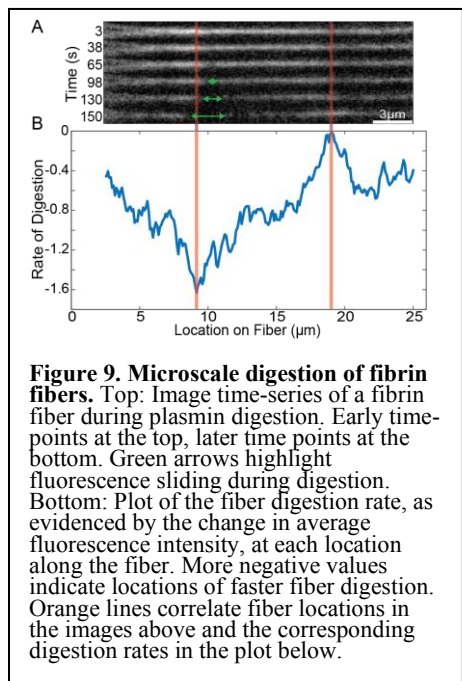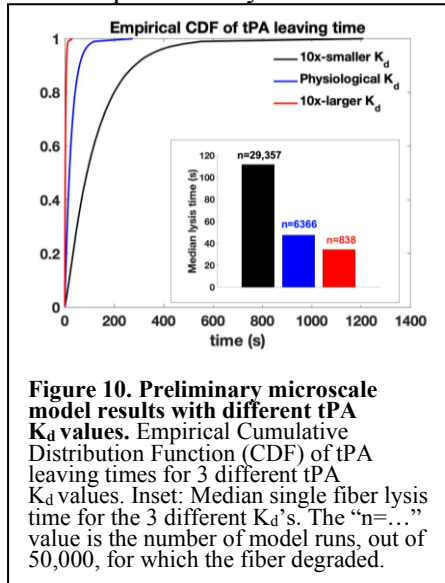
Figure 9. Microscale digestion of fibrin fibers. Top: Image time-series of a fibrin fiber during plasmin digestion. Early time-points at the top, later time points at the bottom. Green arrows highlight fluorescence sliding during digestion. Bottom: Plot of the fiber digestion rate, as evidenced by the change in average fluorescence intensity, at each location along the fiber. More negative values indicate locations of faster fiber digestion. Orange lines correlate fiber locations in the images above and the corresponding digestion rates in the plot below.

in O2.c. To test lysis rates in the absence of inherent tension, pre-stretched fibers will be relaxed until they are no longer taut, prior to the addition of digestive enzymes. We have shown that fibers polymerize in a state that is strained (stretched) 23% beyond their equilibrium length (3); by polymerizing fibers on pre-stretched glue, relaxing the glue will also relax the fibers, and thus reduce or eliminate inherent fiber tension (Figure 4). Similar experiments will be performed at different levels of applied tension, where the fibers will be stretched after polymerization to a fixed extension (extension values of 0, 25, 50, 100 and 200% beyond equilibrium will be utilized) and held at that extension during lysis. Note that at 0% applied strain the fibers still have inherent tension. For all microscale experimental conditions described, at least 100 fibers will be analyzed to ensure statistical robustness.

2332976

O2.c Vary enzyme binding/unbinding rates in presence of different levels of tension in the mathematical model. Our multiscale indirect tension model (described in O1) will be used to elucidate specific tension-induced enzymatic mechanisms responsible for the experimental results obtained in O2.a and O2.b. In particular, we will systematically change the binding/unbinding rates of tPA and plasmin, as well as the plasmin-mediated cleavage rate of fibrin, to learn how tension-induced enzymatic changes affect lysis. The model is an important tool because it allows us to quickly and precisely vary parameters in a way that is experimentally intractable or impossible.

Figure 10. Preliminary microscale model results with different tPA K_d values. Empirical Cumulative Distribution Function (CDF) of tPA leaving times for 3 different tPA K_d values. Inset: Median single fiber lysis time for the 3 different K_d's. The "n=…" value is the number of model runs, out of 50,000, for which the fiber degraded.

To start, we will investigate lysis of single fibers (as in O2.b) using the microscale model of a fiber cross-section. Based on experimental data, the microscale model will be modified in both of the following ways to account for applied tension: 1) the fraction of fibrin in the fiber cross-section that must be degraded for the fiber to be considered cleaved will be decreased as tension increases (the baseline model assumes that 2/3 of the fibrin in a cross-section must be degraded and then the fiber snaps apart due to tension); and 2) kinetics of the lytic enzymes (binding/unbinding rates, as well as the plasmin-mediated cleavage rate of fibrin) will be systematically varied to model the potential effect of tension on decreasing enzyme activity. We will run simulations using modifications 1 and 2 above both separately and together to delineate the individual and combined effects of these changes. Modification 1 will be achieved by running 50,000 simulations of the microscale model for 4 different fractions of cross-section that must degraded for cleavage to occur (1/2, 1/3, 1/4, and 1/8), and for any fractions obtained experimentally in O2.b. The digestion rate will be calculated for each simulation and compared to experimental rates from O2.b. Modification 2 will be achieved by doing a parametric sweep of many different tPA and plasmin binding/unbinding rates (while keeping the dissociation constants, K_d, fixed), many different dissociation constants (while keeping either the binding or binding rates fixed), and many different plasmin-mediated cleavage rates. For each set of parameters, we will generate empirical distributions of the length of time tPA stays bound, the number of plasmin molecules created, and the single fiber lysis time, as well as calculate the digestion rate (Figure 10). We will run statistical analyses to determine which sets of parameters give significantly different results, which will allow us to identify the kinetic conditions that have similar effects on lysis. We will also use statistics to compare the model digestion rate results to experimental data; we hypothesize that the model kinetic conditions that best fit the experimental data best represent the physiological conditions. Finally, modifications 1 and 2 will be combined, and the simulations and analysis outlined above repeated, to understand how the fraction of fibrin that must be degraded for cleavage to occur, and the kinetic parameters, work together to influence single fiber lysis.

Information gathered from the microscale simulations will be used in macroscale simulations to identify the enzymatic effects of tension on lysis of fibrin networks (as in O2.a). Each microscale scenario (e.g., a specific percentage of cross-sectional fibrin needed for single fiber cleavage along with the specific enzymatic rates) will be repeated in the macroscale model to quantify lysis patterns and rates and to calculate the lysis times to 50% and 90% clot digestion. Thus, we will obtain relationships between all of these quantities and the amount of tension applied to a clot. These model results, coupled with the experimental results, will help identify the specific tension-induced mechanisms that have the largest effect on lysis of fibrin clots.

Expected outcomes. Successful completion of this objective will result in the following outcomes: 1) a correlation between tPA binding/unbinding rates and the extent to which a fiber/clot is stretched; 2) measurements of the cross-sectional degradation rate of a single fiber as a function of applied strain/tension;

3) identification of the tension-induced enzymatic mechanism(s) responsible for the different degradation rates under different levels of stretching.

Objective 3: Determine the role of tension in regulating inter-fiber space and causing structural rearrangements during lysis. Polymers, such as fibrin and rubber, that are under tension will snap backwards when they are cut. Our labs have previously shown that through this mechanism inherent fiber tension may play a role in both enhancing the digestion of individual fibers (12) and clearing networks of fibers more rapidly (3). However, the role of tension has only been inferred through simple modeling and has not been tested directly. Moreover, it is unclear whether these mechanisms play a significant role in the case of fibrin under applied tension. Additionally, because networks under applied tension typically lyse more slowly, there must be a threshold amount of tension where it transitions from accelerating to hindering lysis (Figure 11). Identifying this threshold will help to determine how the susceptibility to lysis evolves over time, as clots become more tensed.

O3.a Determine the threshold tension where tension goes from accelerating to hindering fibrinolysis.
We will perform a set of experiments both at the microscale and the macroscale to determine the threshold tension. For macroscale experiments, samples will be prepared as described in O1. Samples will be polymerized in a 10mm x 10mm x 1mm mold and following polymerization they will be transferred to a tensile tester (Biomomentum) at the same, fixed length to preserve their inherent tension. To test lysis rates under applied tension, the clot will be stretched to a fixed extension (extension values of 25%-200% beyond equilibrium will be utilized) and held at that extension during lysis. 75 ng/ml tPA will be added to the center front face of the clot immediately after loading in the horizontal direction into the tensile tester, and the rate of fibrinolysis will be measured by the reduction in that load over time. Inherent tension will be tested in the same way, however instead of stretching the clot further, tension will be reduced by relaxing the fibrin clots by 10 - 25% strain. Each macroscale tension experiment will be run 3+ times for a given strain, to ensure robust statistical results.

Similar experiments will be carried out at the microscale to investigate the tension threshold of individual fibers. Experiments will be performed using the same approach as described in O2.b. To test lysis under applied tension, the fibers will be stretched after polymerization to a fixed extension (extension values of 25%-200% beyond equilibrium will be utilized) and held at that extension during lysis. Inherent tension will be tested in the same way, however instead of stretching the clot further, tension will be reduced by relaxing the stretched glue by 10 - 25% strain. tPA and/or plasmin will be added to the samples and fibrinolysis will be assessed by quantifying the time to first cleavage event (the cleavage time). We predict that the removal of inherent tension will hinder cleavage times (Figure 11). For each amount of fiber strain, 100 fibers will be analyzed for cleavage times, ensuring robust statistical results.

Figure 11. Hypothesized tension threshold where fiber tension transitions from enhancing lysis to decreasing lysis.

These combined experiments will quantify the cleavage times of individual fibers and the lysis rates of networks under both inherent and applied tension. In so doing, we will be able to determine the threshold tension (Figure 11). Because the acceleration and hindering of lysis due to tension likely stem from different mechanisms, determining the threshold tension will help to inform which molecular mechanisms may be impacting these tension-dependent processes.

O3.b Measure fluorescence sliding to determine the role of inherent fiber tension in regulating the digestion of individual fibers. We recently showed that when observing fibrin fibers during fibrinolysis, florescence signal "slides" away from digestion sites (12). We hypothesized that this sliding was a result of the inherent tension in the fibers, which accelerated lysis by pulling the fibrin protofibrils apart within the
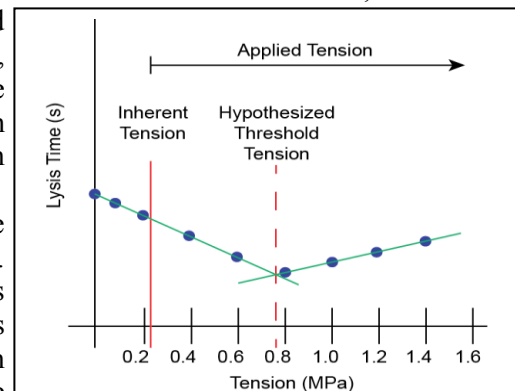
fiber (Figure 12). Using mathematical models, we more recently hypothesized that inherent tension arises from twisting of protofibrils as the fiber grows in diameter, and thus thicker fibers will have more inherent tension (4). Herein we will directly test this hypothesis by quantifying the extent of fluorescence sliding
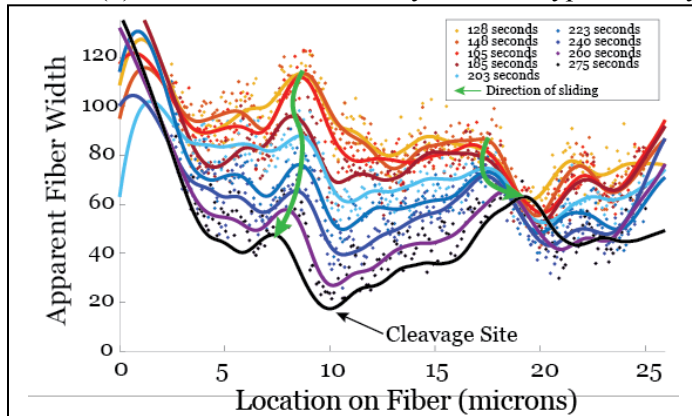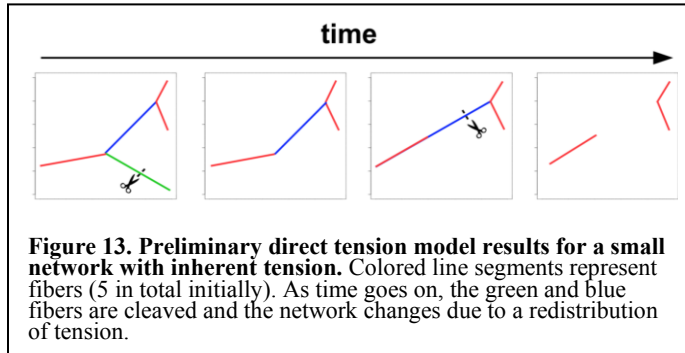

Figure 12. Sliding of fluorescent peak intensity away from sites of digestion. Intensity (apparent fiber width) can be seen sliding longitudinally away from the cleavage site (located at 10 μm), to both the left and right. Longitudinal sliding indicates that the inherent tension in fibers pulls the fiber during digestion.

under specific strains and fiber diameters. Fibrin fibers will be polymerized between microprinted ridges by mixing fibrinogen, plasminogen, and thrombin (and FXIIIa). The relative ratios of fibrinogen and thrombin will be varied to generate thicker or thinner fibers (13). AlexaFluor488 or 647 labeled fibrinogen will be added at a 1:65 (labeled:unlabeled) ratio, which provides the brightest fibers without hindering polymerization or altering lysis (12). Fiber diameters will be quantified using super-resolution STORM imaging (39). tPA will be added to the top of the liquid droplet containing the fibrin samples to initiate lysis, and time-series images of the fibers during digestion will be collected using epifluorescent microscopy. Figure 9 shows preliminary data of the plasmin-mediated digestion of one fibrin fiber, where digestion corresponds to loss in average cross-sectional fluorescence intensity. Similar to our recently published results (12), we will quantify the extent of fluorescence sliding during digestion (Figure 12) and correlate this with fiber thickness and/or the amount of tension. For all microscale experimental conditions described, at least 100 fibers will be analyzed to ensure statistical robustness. Control experiments testing for photobleaching will be performed to ensure that intensity loss corresponds to lysis. We will measure fluorescence sliding in the case where we pre-stretch the glue, polymerize the fibers, and then relax the glue, thereby removing inherent tension, and in the case where we apply additional tension from strains ranging from 25% - 200% (as in O3.a). This combination of microscale experiments will quantify the extent to which inherent and applied tension affect the observed fluorescence sliding and will directly test the hypothesis that sliding is a function of fiber diameter and/or amount of tension.

O3.c Determine the role of network rearrangements after cleavage events on lysis rates. We previously showed that in 2D networks the inherent tension enables fibrin to be cleared more rapidly due to network rearrangements after each fiber cleavage event (3). We hypothesized that this mechanism would be even more prevalent in 3D clots and especially 3D clots under applied tension. In this aim, we propose to test this hypothesis directly. Fibrin clots will be polymerized as described in O1. Using either high frame rate deconvolution epifluorescent microscopy or spinning disk confocal super resolution microscopy, we will rapidly capture 3D volumes of fibrin clots as they are digested. Cleavage events will be identified by the cutting of an individual fiber into two or more fragments between subsequent images. Using the area fraction tool in ImageJ we will quantify the local area/volume cleared of fibrin after each individual cleavage event and will calculate the volumetric rate of clearance per cleavage event. 100 cleavage events will be quantified for each condition tested. Lysis rates will be determined for networks with only inherent tension and with applied tension ranging from 25 – 200% strain.

O3.d Develop a new mathematical model that directly includes tension and use it along with the existing indirect tension model to more fully understand the mechanisms governing the tension threshold. To more directly probe the tension threshold, a new 2D agent-based mathematical model of a small fibrin network with inherent tension will be developed from an existing unpublished preliminary model. Since this model will explicitly include tension, we refer to it as the direct tension model. The direct tension model networks will be generated in Matlab using images of experimental clots like those in Figure

3. Each fiber in the experimental clot will be represented by a line segment with an associated fibrin density,

Figure 13. Preliminary direct tension model results for a small network with inherent tension. Colored line segments represent fibers (5 in total initially). As time goes on, the green and blue fibers are cleaved and the network changes due to a redistribution of tension.

determined by the pixel density in the original image. To account for tension, the fibers will be treated as springs stretched beyond their equilibrium length and connected to masses at fiber junctions (3, 40). We will model different amounts of tension by changing the initial extent to which the springs are stretched beyond their equilibrium length, from 25% to 200%. tPA and plasminogen will be added and then at each time step, with specified probabilities: the enzymes can diffuse to an adjacent location, bind to fibrin (if in close enough proximity), and unbind from fibrin; tPA can convert plasminogen to plasmin; and the fibrin can be degraded by plasmin. During a given time step, if a fiber is cleaved (i.e., if it has been degraded by plasmin "enough" times - as determined by the fibrin density), then that fiber will be removed from the network and the new equilibrium shape of the network will be calculated (Figure 13). This process will be iterated until all fibers are either cleaved or collapse due to the redistribution of tension after each fiber cleavage. Data gathered from the model will include the physical location of fibers and enzymes at each time step, the density of fibrin along each fiber at each time step, the time at which each fiber was cleaved or collapsed, and the location along the fiber at which the cleavage occurred. We will run the model at least 10 times for each network. The order in which fibers are cleaved will vary between simulations, which will allow us to investigate the interplay between network geometry and inherent tension in regulating fibrinolytic outcomes.

The probabilities governing enzyme diffusion, binding/unbinding, conversion, and cleavage are based on the physiological kinetic rates. Hence, we will investigate the effects of varying kinetic rates in the presence of tension by changing the appropriate probabilities in the direct tension model. We hypothesize that it is this combination of both tension and altered kinetics that causes a threshold tension beyond which tension hinders, rather than aids, lysis. By computing small network lysis times at different levels of tension and with different kinetic rates, we will identify which combinations of tension and kinetics accelerate or decelerate lysis.

Expected outcomes. Successful completion of this objective will result in the following outcomes: 1) identification of the threshold tension where lysis switches from being aided by to being hindered by tension; 2) a correlation between single fiber cleavage time and amount of inherent or applied tension; 3) quantification of fluorescence sliding (i.e. the springing back of fibrin protofibrils) in single fiber digestion as a function of tension and of fiber diameter; 4) identification of the relationship between network lysis rates and individual fiber cleavage events under applied tension; 5) development of the first mathematical model of fibrinolysis to directly account for fibrin fiber and fibrin network tension.

INTELLECTUAL MERIT

This work will directly test hypotheses regarding the role of inherent and applied tension in regulating the digestion of blood clots (fibrinolysis). Specifically, we hypothesize that tension regulates clot digestion through three specific mechanisms: 1) expulsion of lytic molecules and/or hindering enzyme diffusion by rearranging the clot architecture, 2) altering the lytic molecule binding sites on fibrin, and 3) pulling the fibers during digestion. This interdisciplinary research program, consisting of multiscale experiments and mathematical modeling will directly test each of these hypotheses and determine the extent to which each mechanism contributes to the decreased rates of fibrinolysis of stretched clots. The Bannish group has developed the most biochemically- and structurally-detailed mathematical model of lysis to date, which connects micro- and macroscale lysis. The Hudson Lab has pioneered the microscale studies of fibrinolysis, emphasizing the importance of fiber tension. The Tutwiler Lab has expertise in macroscale experiments

2332976

related to clot structure, mechanics, and fibrinolysis. This work will result in the first combinatorial multiscale mathematical and experimental models that can predict lytic outcomes as a function of mechanical forces in the vasculature. This will be a significant advance forward from the current state of the art, as currently no mathematical models of fibrinolysis consider the role of tension in any capacity.

BROADER IMPACTS

Opportunities of a multidisciplinary training environment. The proposed research will enhance the scientific environments at Rutgers University, the University of Central Oklahoma, and East Carolina University. Students at each institution will benefit from cross-disciplinary training involving engineering, mathematics, biochemistry, and physics, which will prepare them for a career in a workforce that increasingly values an interdisciplinary skill set. This training will be accomplished by providing undergraduate students from each laboratory the opportunity to participate in an existing REU program at Rutgers, and by facilitating a summer exchange program between the laboratories. Dr. Tutwiler is actively involved in the Rutgers University Cellular Bioengineering REU program, where she coordinates weekly Innovation and Entrepreneurship lessons for all students in the program. This culminates in a business plan developed around students' summer projects and a pitch in the form of both a presentation to guest "investors" and a poster to their 100+ REU peers across Rutgers University. Additionally, students from each university will have the opportunity to train for a week in the summer at each/either of the other labs. They will receive direct interdisciplinary training from the PIs, learn new experimental or modeling skills, interact with their counterparts from the other universities, and share their knowledge with visitors to their university. Exchange students will be given a pre- and post-survey with learning objectives arranged by the PIs to determine their knowledge gained while visiting the other institutions.

Inclusion of underrepresented groups. All three institutions have large and diverse student bodies (>25% of students are NSF-defined underrepresented groups and >54% of all students are women) that make them ideal training grounds for fulfilling the inclusive mission of the NSF. The PIs have mentored numerous students from underrepresented groups: Dr. Tutwiler has mentored 13 of 18 female high school through PhD level students including 4 current female PhD students and 9 NSF-defined underrepresented minorities; 15 of 23 undergraduate and master's students Dr. Bannish has mentored were women, 6 were first generation college students, and 6 were NSF-defined underrepresented minorities; Dr. Hudson has mentored 10 women out of 26 students and mentors undergraduate students as part of ECU's Louis Stokes Alliance for Minority Participation (LSAMP) program. The PIs will actively recruit and train students from diverse backgrounds to participate in the proposed research. Advertisements about the research opportunities will be made to campus groups such as Latinos in STEM, Society of Women Engineers, and Native American Student Association. The Rutgers NSF REU, which Dr. Tutwiler is involved with and will be used as part of the exchange program for this project, targets recruitment of underrepresented minorities and first-generation students to broaden participation and inclusion of underrepresented groups. Moreover, all three PIs serve on or chair the graduate admissions committees in their respective departments, reflecting that they can work to recruit and admit diverse student populations.

Promotion of experiential learning across education levels. All PIs are actively involved in developing innovative curriculum. For example, Dr. Tutwiler revamped the sophomore level Introduction to BME course to include 3 hands on lab experiences that directly related to the lecture material and the subject matter of this proposal. The students learned about the mechanics of blood clots during the lectures on soft tissue mechanics, where able watch a demo of the tensile testing of blood clots in the Tutwiler laboratory, and then performed a hands-on lab with the mechanical testing of rubbers bands as proxies for blood clots. This lab was modified and demonstrated as part of the high school level lecture series that Dr. Tutwiler and several of her students gave at Piscataway Highschool.

Structure and mechanics themed summer program based on Lego®. Dr. Hudson coaches a state championship qualifying Lego® robotics team as part of his community service. Through this process he has developed an experiential learning program for introducing students to core ideas in this research proposal through Lego®. A pilot Lego® camp was run in the summer of 2022, which included 28 elementary school aged children ranging from K-5. As part of the camp, students were introduced to themes of forces and tension through Lego®. In addition, student engagement was assessed using the MUSIC model of motivation. In the MUSIC model, people's motivations to engage in activities are affected by five perceptions: eMpowerment, Usefulness, Success, Interest, and Caring. We assessed motivation using the MUSIC inventory. Results of the pilot study showed that students displayed high motivation in each of these five perceptions, especially Success 3.5/4, Interest 3.6/4, and Caring 3.5/4. As part of the broader impacts of this proposal, we will build on the success of the pilot camp and run a Lego®-based camp every summer during the duration of the project. A diverse student group will be recruited using the summer camp program of a local elementary school near the ECU campus. Each summer 20-30 students will be enrolled in the program and will have their motivation assessed using the MUSIC inventory. Undergraduate and graduate students funded by this grant will assist in development of program materials. During one day of the camp, PIs Bannish and Tutwiler will do a virtual call with the students to describe how the principles they are learning through Lego® apply to real world examples. The summer program materials will be organized and broadly distributed by the PIs, so that similar programs can be run throughout the country.

Nucleating and promoting multidisciplinary research. The PIs are involved with international and national organizations such as serving as Councilors for the International Fibrinogen Research Society (Tutwiler and Hudson), Public Policy Committee member for the American Society of Cell Biology (Tutwiler), and Early Career Committee member for the Biophysical Society (Hudson). Dr. Bannish serves on 3 UCO committees that all promote interdisciplinary research collaborations between faculty and students at UCO and in the broader Oklahoma community and is an associate editor for the undergraduate research journal SIURO. Dr. Tutwiler is a Provost Research Leadership Fellow where she spearheads events such as Ideation Forums that bring together novel multidisciplinary teams for the opportunity to present their research ideas and receive seed funding.
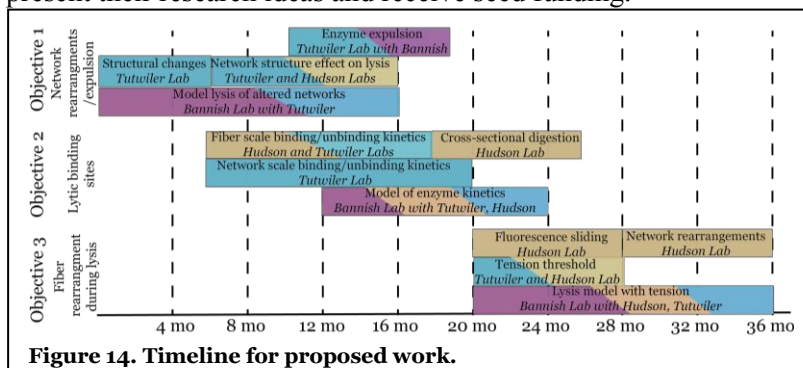

Figure 14. Timeline for proposed work.

PROJECT SCHEDULE AND EVALUATION OF SUCCESS
A timeline of our tasks is shown in Figure 14. Our success will be appraised based on the milestones listed below.
Milestone 1: Develop a multiscale understanding of tension's role in regulating the fibrinolytic process.
Milestone 2: Ascertain the critical strain at which tension transitions from accelerating to hindering fibrinolysis.
Milestone 3: Develop and validate the first mechanically coupled model of fibrinolysis to identify the tension-dependent mechanisms driving single fiber and fibrin clot lysis.

MANAGEMENT PLAN

Roles and integration of skills. PI Hudson will serve as the project lead and point person for microscale experiments. PI Tutwiler will supervise the macroscale experiments. PI Bannish will coordinate the mathematical modeling efforts. These efforts will be integrated across all three proposed objectives.

Management and coordination. The research will be overseen in a collaborative way by each of the individual PIs. To facilitate knowledge exchange, the team will continue to meet monthly via video

2332976

conference and, if possible, hold annual in person meetings. Team communication will be coordinated via Slack^{TM}, email, and Zoom^{TM}. Box^{TM} cloud storage will be utilized for data sharing. During monthly meetings, the PIs will: review progress of research, education, and outreach activities; plan preparation of manuscripts and conference presentations; strategize future research undertakings; and identify risks and alternative approaches as needed. Manuscript specific video meetings will be held with student lab members on an as needed basis. The PIs have been working together for 3+ years. They have successfully published 5 collaborative manuscripts (4 with student lead authors) (12, 31, 41-43) and 15 conference/symposium abstracts with 2+ of the PIs involved. Three additional manuscripts are in preparation. This exemplifies the fact that the PIs work together successfully and efficiently, and that they are effective mentors to students.

Risk mitigation and alternative strategies. For all objectives, to promote rigor and reproducibility we will: i) regularly screen literature for new studies related to fibrinolysis and tension and critically assess their methods and results, ii) establish written methods before executing experiments, iii) include controls, determine inclusion/exclusion criteria, and perform power analyses, iv) analyze results using statistical tests, and v) constantly back up data on multiple formats. Mathematical models will employ existing accepted methods and algorithms, and new modeling techniques will be affirmed through peer-reviewed publications. Control simulations will be run for new models to ensure results give the expected outcome, before moving on to more sophisticated simulations. Even with these safeguards, it is possible that we will encounter problems; our plans for mitigating and overcoming potential obstacles are described here.

Fluorescence microscale lysis and stretching experiments will be performed in a homemade stretching device. While preliminary data suggests that the device is adequate for the described experiments, other devices have also recently been described (29), which could also be built if our homemade device proves inadequate. The TIRF imaging described in O2.a requires the fibrin to be fixed to a glass surface. If imaging conditions require it, additional fixing agents/approaches (15) could be tested to optimize fluorescence signal. In our experimental design, we commonly propose to test strains of 0, 25, 50, 100 and 200%. If the determination of mechanisms requires a finer gradation of strains, experiments could be performed in 10% strain increments. In most microscale experiments, we apply tension through the application of strain to the fibrin, but do not directly measure the tension. If experimental results suggest that direct measurements of fibrin fiber tension would be beneficial, approaches such as FRET-based tension sensors (44) or traction force microscopy (45, 46) could be pursued.

The indirect tension model is a modified version of a well-established, experimentally verified model (25, 26) so the biggest modeling risk is the novel direct tension model (O3.d). A preliminary version of the model is already operational, but agent-based models like the proposed direct tension model can be computationally intensive, especially over larger spatial or temporal scales. If the new direct tension model is unable to probe questions on the spatiotemporal scale of interest, we can still attempt to determine the mechanisms behind the tension threshold by combining the indirect tension modeling approaches of O1.c and O2.c. We will investigate the combination of tension-induced altered network structure and altered chemical kinetics using the experimentally verified indirect tension model. We will obtain lysis data for various combinations of network structures and kinetic parameters. By identifying which scenarios result in accelerated or decelerated lysis, and through comparison with experimental data, we will determine the relative influence of different tension-induced changes on lytic outcomes.

CONCLUSIONS

The combination of multiscale experiments and modeling described in this proposal will determine mechanisms by which tension regulates fibrinolysis and provide a fertile training ground for students ranging from elementary school to doctoral. Our compelling preliminary data demonstrate the underappreciated importance of tension in fibrinolysis, and the proposed research will lay a foundation for an entire field of biomechanical fibrinolysis studies. The proposed work can be expanded to investigate tension due to blood flow and/or pathological conditions. Our interdisciplinary research team is uniquely poised to do this research and train the next generation of scientists.

Page 25 of 95

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