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REVIEW ARTICLE

Why fibrin biomechanical properties matter for hemostasis and thrombosis

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Abstract

Polymeric fibrin displays unique structural and biomechanical properties that contribute to its essential role of generating blood clots that stem bleeds. The aim of this review is to discuss how the fibrin clot is formed, how protofibrils make up individual fibrin fibers, what the relationship is between the molecular structure and fibrin biomechanical properties, and how fibrin biomechanical properties relate to the risk of thromboembolic disease. Fibrin polymerization is driven by different types of bonds, including knob-hole interactions displaying catch-slip characteristics, and covalent crosslinking of fibrin polypeptides by activated factor XIII. Key biophysical properties of fibrin polymer are its visco-elasticity, extensibility and resistance to rupture. The internal packing of protofibrils within fibers changes fibrin biomechanical behavior. There are several methods to analyze fibrin biomechanical properties at different scales, including AFM force spectroscopy, magnetic or optical tweezers and rheometry, amongst others. Clinically, fibrin biomechanical characteristics are key for the prevention of thromboembolic disorders such as pulmonary embolism. Future studies are needed to address unanswered questions regarding internal molecular structure of the fibrin polymer, the structural and molecular basis of its remarkable mechanical properties and the relationship of fibrin biomechanical characteristics with thromboembolism in patients with deep vein thrombosis and ischemic stroke.

KEYWORDS
biomechanics, clot structure, fibrin, thromboembolism, thrombosis

1 | INTRODUCTION

One of the key functions of blood coagulation is to produce a fibrin clot to stem bleeding after injury. The protease responsible for fibrin production is thrombin, which has many other functions including the activation of platelets but also the activation of anticoagulant systems (protein C) and inhibition of fibrinolysis (via the thrombin activatable fibrinolysis inhibitor).[1] Fibrin is produced in a matter of minutes by thrombin-mediated cleavage of fibrinogen, a protein with a very high concentration in the blood, second only to albumin and the immunoglobulins. Once converted from highly soluble fibrinogen to fibrin, the protein rapidly generates a network that contains

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pores for the accommodation of cells, and fibers that branch and anchor this matrix to the injury site.[2,3] Thus, blood contains a system that can trigger instant formation of a temporary matrix to stem bleeding and initiate tissue repair responses.

The fibrous matrix generated by fibrin has important biomechanical characteristics. These properties, which include elastic and viscous characteristics as well as extensibility, stiffness, and resistance to rupture, are important for its interaction with cells during wound healing.[4] Furthermore, fibrin biomechanics are crucial for the ability of blood clots to withstand disruption due to movement and other mechanical stresses. However, the relevance of fibrin biomechanical properties for thrombosis and thromboembolic disorders is less well understood.

In this review, we discuss how the fibrin matrix is formed from fibrin monomers including the types of bonds and forces that control this process. Then we consider what is known regarding the internal structure of fibrin fibers, and the implications of this structure for fibrin biomechanical characteristics. Next specific rheological properties are reviewed in detail, including strength of fibrin and its fibers, their elasticity and viscous properties, the extensibility of fibrin fibers and a process called strain hardening that is a typical characteristic of fibrin. Finally, we discuss rupture of fibrin fibers or clots, and the clinical relevance of fibrin biomechanical characteristics for hemostasis and thrombosis, including thromboembolic disorders.

2 | FORMATION OF PROTOFIBRILS AND PROTOFIBRIL PACKING IN FIBRIN FIBERS

Thrombin, which is generated by the activated coagulation pathway, cleaves fibrinopeptide A and B from fibrinogen to initiate fibrin clot formation. As a result, knobs 'A' and 'B' on the N-termini of the α- and β-chains, respectively, become exposed and bind the 'a' and 'b' holes in the C-terminal domains of the γ- and β-chains respectively in adjacent fibrin(ogen) molecules via noncovalent knob-hole interactions (Figure 1A–C).[5] Thus, the so far soluble fibrinogen turns into fibrin, which aggregates and forms the fibrin network. Much is known about the molecular detail of bond formation, yet the exact process of how these interactions govern the internal structure of fibers, and develop into a 3D network, is still not fully understood.

2.1 | Bonds in fibrin

The noncovalent knob-hole bonds are essential for fibrin polymerization since a fibrin mutant with non-functional A and B knobs showed no signs of polymerization whatsoever.[6] Among the knob-hole bonds, the 'A:a' knob hole interaction is the most significant and shows the largest binding strength.[7] Absence of fibrinopeptide A cleavage in fibrin from a patient with homozygous dysfibrinogenemia impaired polymer formation,[8] while polymerization was completely abolished in a murine model with mutations in the fibrinopeptide A cleavage site,[9] indicating the importance of the A:a interaction in driving fibrin polymerization. The strength of A:a knob-hole interactions change with tensile force, showing 'catch-slip' bond behavior. First the bond strengthens with increasing tensile strain, as a moveable flap on the γ-nodule closes in on hole 'a'.[10] This 'catch' characteristic may play an important role in early protofibril formation. The bond next turns into a 'slip' state above approximately 40 pN tensile force resulting in the dissociation of the knob-hole bond.[11] 'B:b' knob-hole bonds are 6–8 times weaker than 'A:a'.[12] Fibrinopeptide B cleavage is delayed in time compared with fibrinopeptide A and has been reported to enhance lateral assembly of fibrin fibers.[13]

During protofibril formation, γ-γ interactions occur across the D:D interface of adjacent fibrin monomers. Once activated by thrombin, factor XIII crosslinks this interface forming two antiparallel γ-γ bonds in the first few minutes of clotting.[14] There has been some debate if γ-γ crosslinks form in longitudinal[15,16] or transverse[17] direction. Recent AFM studies together with molecular simulation support the longitudinal theory.[18]

The C-terminal part of the α-chain extends significantly from the D-region forming the αC-region (Figure 1A). Bonds between the αC-regions connect protofibrils laterally, providing a zipper or Velcro effect along the length of the fibrin fiber (Figure 1D). The αC-region consists of an unstructured flexible αC-connector (between residues α221-391) and a compact globular αC-domain (α392-610).[19,20] In fibrinogen, the αC-region shows a compact conformation,[21] and either folds back, binding the E-region in the presence of fibrinopeptide B,[22] or detaches from the E-region and shows a string-like form with a globule at the end.[21] Following the cleavage of fibrinopeptides, the αC-region detaches, extends, becomes more flexible (Figure 1B),[21] and forms intramolecular interactions with αC-regions of adjacent fibrin molecules.[22] These interactions are thermodynamically driven[23] between β-sheet structures via β-hairpin swapping.[24]

A fibrin network still forms from fibrinogen with an αC-region partially truncated at residue 251,[25] however this α251 truncated variant results in a denser network containing thinner fibers and more branchpoints.[26] Of note, physiological low molecular weight fibrinogen metabolites are present in vivo,[27] where either one (LMW) or both (LMW') Aα-chains are truncated at various places.[28] The presence of the αC-region results in thicker fibers by reducing the threshold length of protofibrils for lateral aggregation but it does not change the basic structure of protofibrils, thus the αC-region likely has a kinetic effect on the lateral assembly of protofibrils.[21] A recent study from our lab found that the absence of the αC-domain (α390 truncation) leads to thinner, highly branched fibers resulting in reduced clot firmness, whereas abolishing the complete αC-region including the globular domain and the connector region (α220 truncation) impairs longitudinal growth of protofibrils and results in clots with even lower firmness and a highly abnormal network structure with stunted fibers and a severely reduced resistance to fibrinolysis.[29] Thus, besides the kinetic effect on fibrin assembly and lateral aggregation, the αC-region has important roles in clot formation including longitudinal fibrin fiber growth and clot stability.

Like for γ-γ interactions, activated factor XIII crosslinks the αC interactions too, yet these crosslinks form in a later phase of

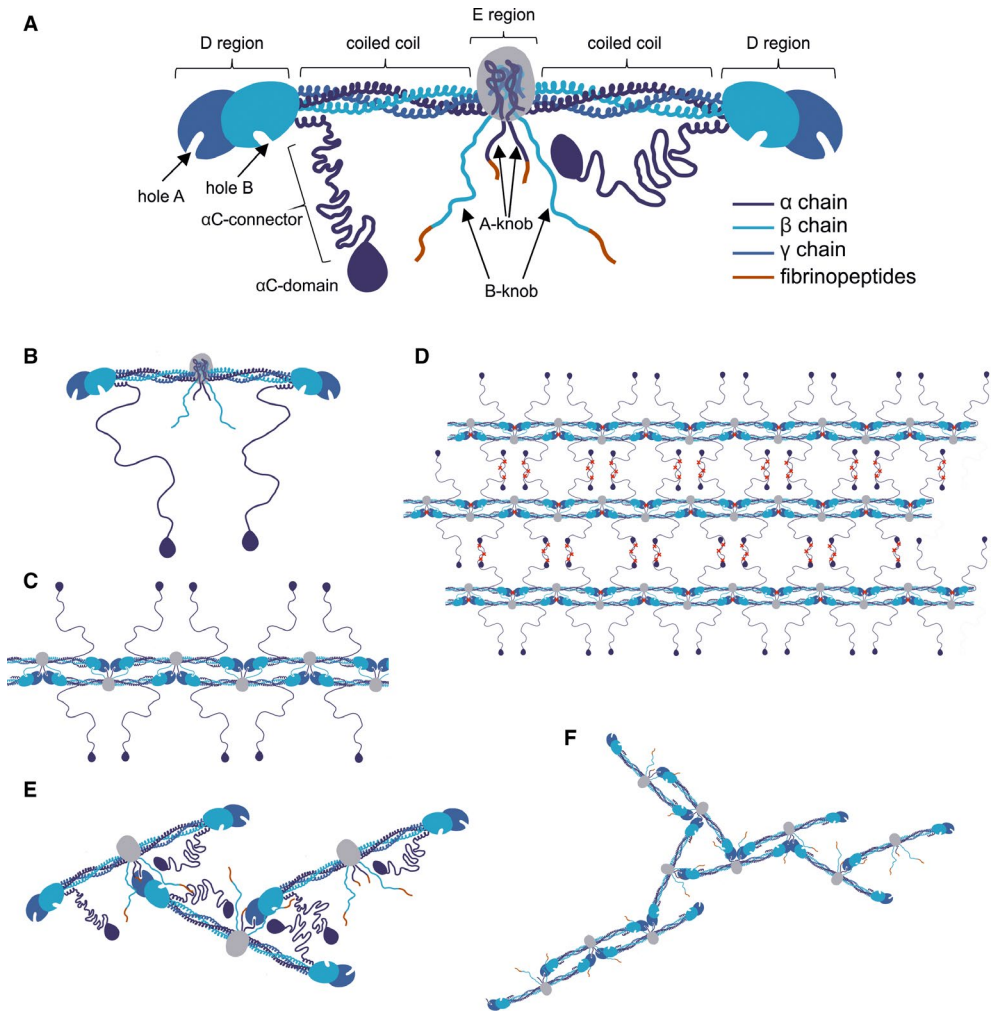

FIGURE 1 Early formation of fibrin. (A) Schematic representation of the fibrinogen monomer. The N-terminal of the pairs of polypeptide chains (α, β, and γ) are connected via disulfide bonds in the central E-region and twist around each other to form a coiled coil. The C-terminal region of the β- and γ-chains forms the globular D-region at the side of the molecule, which includes 'hole A' and 'hole B'. The C-terminal of the α-chain folds back to the coiled coil and forms the αC-region, composed of an αC-connector and αC-domain. (B) Fibrin monomer. Once fibrinopeptides are cleaved, knobs A and B becomes exposed, and the αC-region extends. (C) Double stranded protofibrils form, as fibrin monomers bind to each other via knob-hole interactions. (D) Interconnecting protofibrils. Red crosses represent covalent crosslinks made by FXIII on the D:D interface, and multiple crosslinks between the αC-regions. (E) Y-ladder model: fibrin(ogen) monomers can form intermediate Y-ladder structures when only one fibrinopeptide-A is cleaved. (F) Highly branched structure, containing both Y-ladder and double stranded structures. αC-regions are not included here for clarity

clotting.[14] Crosslinking of the αC-region results in more rapid fiber formation and thicker, straighter fibers.[30] Thus, even though α-chain crosslinks mainly form in later phases of clotting, they likely have a complementary role in protofibril assembly. Possibly αC crosslinks pull the protofibrils closer to one another, increasing the density of the fiber and allowing more protofibrils to laterally assemble into thicker fibers.

2.2 | Protofibril structure

Structural transitions during the formation of fibrin fibers and network were first visualized by electron microscopy (EM) which demonstrated two stages of assembly. The prominent process in the first phase involves aggregation of fibrin monomers into half-staggered double stranded structures called protofibrils via knob-hole bonds (Figure 1C).[31] In the second phase, protofibrils align in parallel and aggregate laterally forming fibrin fibers (Figure 1D) coinciding with the gelation and generation of a fiber network.[32]

EM provides details about the structure of the (proto)fibrils, but may miss intermediate structures with short lifetime during early stages of polymerisation.[33] One such structure is suggested in the Y-ladder model: fibrin(ogen) monomers can bind via only one A:a knob-hole bond, resulting in Y-ladder like structures (Figure 1E). This is an intermediate state before both A:a knob-hole bonds form, generating straight, double stranded protofibrils. In this intermediate state an extensive network of thin, frequently branching long polymers can form (Figure 1F), which collapse into a main fiber via lateral

interactions. This collapse increases fiber thickness rapidly, reduces the frequent branching and leads to a lower number of fibers in the network.[33] However, there is yet no direct structural evidence for the Y-ladder model. Recent AFM images showed early stages of polymerization[34] and protofibrils were interpreted later as frequently branching structures possibly resembling the structures proposed by the Y-ladder model.[35] Although speculative, intra-protofibril branching can be an important consequence of this model, as protofibrils within the fibers can also be interconnected via such branching thus via A:a knob-hole bonds and not just via lateral interactions.[35]

2.3 | Internal structure of fibrin fibers

As a result of the clotting process, fibrin fibers and the network are formed. The fibrin 3D network builds at very early stages of clotting (even before the formation of mature fibers),[35] thus individual fibers are hard to isolate and less is known about the inner structure of fibrin fibers: how protofibrils are connected or packed within a fiber, which bonds bear the load upon mechanical perturbance and which events lead to fiber rupture.

We know that the internal structure of fibrin fibers is porous and protofibrils within the fibers are not tightly packed.[36] The internal structure of fibers can be revealed by scattering techniques using visible light (turbidimetry and fibrinography), neutrons (small angle neutron scattering (SANS)) or X-ray (small-angle X-ray scattering (SAXS)) for even smaller details. SAXS shows a long range axial (longitudinal) packing order along fibrin fibers around 22.5 nm distance,[37] corresponding to the distance between the globular D- and E-regions of fibrin(ogen) monomers. These 22.5 nm repeats are also present as a band pattern on fibrin fibers on electron microscopy images.[38] However, only a shortrange and weak lateral (or radial) order of around 13 nm[39] and 18 nm[40] was found, indicating that fibrin fibers have less ordered packing laterally than axially. Turbidimetry and fibrinography are multiwavelength scattering methods optimized to reveal detail about the protein density and protofibril content of fibrin fibers.[37,41,42] Different parameters may impact on protein density of the fibers: increasing thrombin concentrations decrease protein density, and clots of the γA/γ' splice variant of fibrinogen also result in less dense fibers.[43] Functionally, reduced protofibril packing in fibers results in a decreased clot stiffness.[43] In addition to the scattering methods, lateral atomic force microscopy experiments showed fiber stiffness to decrease with increasing fiber diameter, suggesting that protein density of fibers decreases with increasing fiber diameter.[44]

3 | FIBER STRETCHING AND RHEOLOGY

The mechanical properties of fibrin evolved so that fibrin fulfils its main physiological role, i.e. to be strong enough to seal wounds and flexible to prevent rupture. As fibrin is the scaffold of blood clots, it plays an important role maintaining this structural and mechanical integrity. Clot mechanical behavior involves interplay between the structure of the network including the arrangement of fibrin fibers and the fiber branchpoints, and mechanical behavior of fibrin itself.

3.1 | Measures of fibrin biomechanical properties

Even though fibrin is a mesh of interconnected fibers, and thus individual fibers are hard to isolate, the mechanical properties of individual fibrin fibers can be measured using an atomic force microscopy (AFM) and optical fluorescence based method.[45] Fibers can be deposited across microwells of a striated substrate, then pulled sideways with an AFM cantilever. The stress exerted on the fiber is calculated from the lateral deflection of the cantilever and the stress-strain behavior of the fiber analysis (Figure 2A). The slope of the stress-strain curve is the stiffness, or elastic modulus of the fiber at a given strain. The higher the stiffness, the more stress is required for a given elongation. Further parameters can be measured, such as extensibility of the fiber (maximal amount of strain before rupture), maximal force or maximal stress needed for rupture. Viscoelastic properties can be analyzed by following fiber behavior upon release: if the stress-strain curve returns on the same trajectory upon release as when it was pulled and no hysteresis is present between the pull and release curves, the behavior is elastic, the fiber structure is fully recovered, and no energy is lost. However, if the curve follows a different path upon release and a hysteresis, or lag, appears between the pull and release curves, the behavior is partially viscous, and some of the energy was dissipated by structural transitions (Figure 2A). In some cases, the structure does not fully recover. The elastic limit is the maximal strain where no energy is dissipated, and the mechanical response is still purely elastic.

Oscillatory tests can be used in rheology to describe the viscoelastic behavior of a material. Storage (or elastic) modulus G', in phase with the applied shear deformation is a measure of the purely elastic behavior, while the component out of phase, the loss (or viscous) modulus G" is a measure of viscous behavior (Figure 2B). Storage modulus is used as a measure of clot stiffness.[46] This parameter is related and convertible to stiffness (or Young's modulus) Y and bulk modulus K, but while G' is measured in shear deformation (force causing deformation parallel to the applied stress), Y is measured at normal deformation (tensile/compressive force causing deformation perpendicular to the applied stress) and uniaxally (in one direction only). The elastic and viscous moduli can also be analyzed with micro-rheometer devices, where passive thermal fluctuations of the embedded beads,[47] or optical[48] or magnetic forces induce controlled motion of the bead, informs us about viscoelastic behavior (G' and G")[49,50] (Figure 2C). An AFM-based nanorheology method provides another useful approach for the analysis of fibrin elastic and viscous properties at the micrometer scale.[51]

TEG and ROTEM are rheometry-based assays that are widely used in clinical practice.[52] These oscillatory tests measure numerous parameters (Figure 2D), from which the maximal amplitude reached during the clotting process (also called maximal clot firmness) is

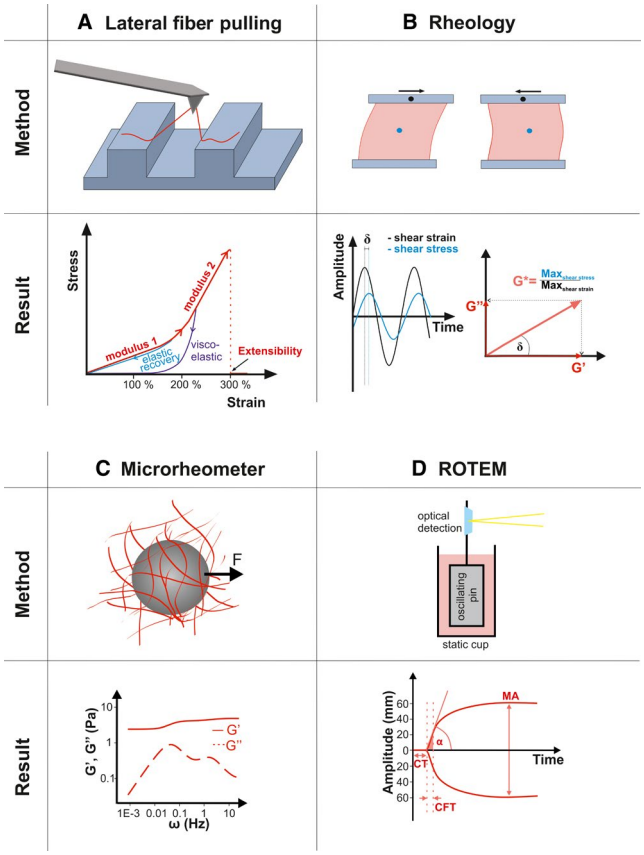

FIGURE 2 Methods used to measure fibrin biomechanical properties and resultant parameters. Red color represents fibrin sample (in method) and the parameters measured (in result). (A) Lateral fiber pulling using atomic force microscopy (AFM). Individual fibrin fibers form across microwells of a striated substrate and are pulled with the AFM cantilever. The stress-strain behavior of the fiber is followed, where stress is calculated from the lateral deflection of the cantilever and strain from the Y-position. Low and high strain stiffness (modulus 1 and 2) are estimated as the slope of the linear part of the curve. Extensibility is the maximal strain a fiber can be elongated to before rupture. The clearly elastic behavior at low strains (elastic recovery, light blue) becomes viscoelastic at higher strains (viscoelastic, purple curve) as hysteresis appears between the pull and release curves. (B) Oscillatory test in rheology. Sample is placed between two plates and oscillatory strain is applied on one of the plates. If viscous behavior is present, a phase shift (δ) can be detected between the applied shear strain (black curve) and the resultant shear stress (blue curve). Complex shear modulus (G*) is calculated as the ratio of the maximal amplitude of the shear stress and maximal amplitude of shear strain. G', which is 'in phase' with the applied shear stress is the storage or elastic modulus, while G", which is 'out of phase' is the loss or viscous modulus. (C) Microheometer. During passive rheology measurements, constant or intermittent force (F) is applied on the microbead, embedded in the fibrin network. The motion of the bead is tracked and used to calculate storage modulus (G') and loss modulus (G") based on the method of Evans et al.[98] These moduli can be calculated for a frequency range (ω), limited by measurement time (lower limit) and time resolution of the detection (upper limit). Oscillation mode works on the same principle as shown for rheology: oscillatory force is applied and G' and G" are calculated from G*. (D) ROTEM. An oscillating pin is immersed in a cup filled with the sample. As the sample clots, it mechanically couples the pin and the cup, thus altering the movement of the oscillating pin. The measure of the coupling is detected as amplitude signal and followed along the clotting (and lysis) process. Parameters measured: *CT* (clotting time, time from activation until 2 mm amplitude is reached), *CFT* (clot formation time, timeframe between 2 and 20 mm amplitudes) *α angle* (tangential curve at 2 mm amplitude point, measure of the kinetics of clotting) *MA* (maximal amplitude, measure of the firmness of the formed clot)

closely related to stiffness. However, the data are presented in relative arbitrary units (amplitude of movement as the result of mechanical coupling) and are thus hard to relate to quantitative parameters of stiffness such as Young's (Y) or storage moduli (G').

3.2 | Fibrin extensibility and strain stiffening

Probably the most surprising mechanical property of fibrin is its high extensibility even though fibers are straight structures: fibrin networks only break at approximately 250% strain.[53,54] Straight fibers are visible using confocal microscopy.[48,55] A recent study showed that fibrin fibers have inherent tension as a result of further polymerization once the initial network structure has formed, and are extended approximately 23% over their equilibrium length they would have if isolated,[56] suggesting that fibers are not only straight, but also prestrained. However, bulk measurements show that fibers are not completely straight but straightened by up to 25% shear strain of the whole clot, and fibers only start to align in the direction of stress above 25% shear strain.[54,57] Fibers appear less straight on electron microscopy images than confocal microscopy[29] and fibers appear less straight in uncrosslinked clots.[30]

Strain stiffening is a typical behavior of filamentous biological polymers[58] and involves complex interplay of entropic and enthalpic elasticity: low strain mechanical behavior is dominated by entropic behavior, when thermal undulations are pulled out decreasing the degree of disorder, the entropy,[58] while enthalpic behavior dominates at high strains when the filament backbone is stretched leading to actual bonds being pulled, thus increasing enthalpy, the internal energy.[59] Strain stiffening is an increase in stiffness of a (bio) material upon transition from the flexible entropic to the more rigid enthalpic regime, improving the mechanical integrity of the material. In bulk measurements, fibrin networks show two strain stiffening regimes: one under 25% strain, and one above 25%.[54,60] Initial strain stiffening, occurring at network level under 25% strain may originate from minor straightening of fibrin fibers, as the small thermal fluctuations of fibers are pulled out (decreasing the entropy) and fibers start to be pulled (decreasing the enthalpy). Yet, strain stiffening between 25% and 250% strain cannot arise from fiber straightening as by then fibrin fibers are already straight, thus it likely originates from the mechanical properties and internal structure of individual fibrin fibers. Altogether, the mechanical behavior of the network is largely determined by the mechanical behavior of individual fibrin fibers, yet changes at network level, especially at low (<25%) strains also

play a role. Besides, fibrin clot stiffness is orders of magnitude lower than expected using a model of rubberlike elasticity, considering the branchpoint density and the straight conformation of fibers,[61] and possibly has intrafiber origin.

Single fiber pulling experiments show large extensibilities, similar to or slightly higher than that at network level: fibers can be elongated up to 300%–400% of their original length before rupture.[45] The elastic modulus of fibrin fibers is typically below 10 MPa,[62,63] similar to a silicone rubber, or elastin or resilin fibers, where unfolding of an amorphous domain results in rubberlike elasticity, but much lower than for actin or collagen for example, where highly regular structures are extended.[64] Furthermore, single fibrin fibers show strain-stiffening.[63,65,66]

Several studies investigated structural origins of high fibrin fiber extensibility and strain stiffening. A worm-like chain (WLC) model fits the stress-strain behavior of individual fibrin fibers well[63] suggesting that the initial elastic response has an entropic origin.[67] This arises from the flexible polymer chain randomly fluctuating and adopting an equilibrium conformation and hence length, usually termed persistence length. If force is applied, random fluctuations are progressively restricted as chains elongate. At greater extensions, enthalpic bond stretching (as a simple spring) begins to dominate, and eventually fiber stiffening becomes significant. The WLC model suggests that the structure which bears the load at low strains is highly flexible with short persistence length.[58] The unstructured fibrin αC-region, which interconnects protofibrils within the fiber is a good candidate for this highly extensible entropic response[63] and molecular modelling supported the key contribution of αC-regions in fibrin stress-strain behavior.[68] This is further supported by data showing that shortening of the αC-region reduces fibrin extensibility.[69] Clots made with α251 fibrin showed decreased storage modulus (G') and increased tangent modulus (G"/G') indicating that α251 clots are less stiff and prone to plastic deformation.[26]

Fibrin fibers showed elastic stress-strain behavior (full recovery with no energy loss) below 100% strain[45] on a sub-millisecond timescale, which is too short for protein refolding,[70] suggesting that structural transitions taking place during stretching are quickly and fully recoverable, pointing to an origin in the unstructured αC-region. Further supporting these single fiber data, fibrin networks showed elastic behavior at lower strain, while viscous behavior mainly occurred at higher strain in the stiffening regime, not long before network failure.[47] However, another study showed viscous behavior of individual fibers already at low (<110%) strain, or even below 50% strain for fully crosslinked fibers with a combination of fast (~2s) and slow (~52 s) stress relaxation.[66] This suggests that different processes like refolding and structural transitions other than stretching of the disordered αC-region may also contribute to the mechanical behavior of fibrin fibers and network. This could include α-helix to β-strand transition of the coiled coils[68,71] or partial unfolding of the γ-chain C-terminal domain.[64,72] A previous study has observed α-helix to β-strand transition in clots,[73] which showed spatial heterogeneity[74] suggesting non-homogeneous strain distribution in the clots. Energy dissipation and increased stiffness, both observed in fibrin at high strains, are characteristic for α-helix to β-strand transitions.[75]

The mechanical behavior of fibrin likely involves complex interplay between the reported structural transitions.[68,76] A recent study combined shear rheology with small angle X-ray scattering (SAXS) following the structural transitions happening at increasing shear strains. The 22.5 nm monomer length is unchanged at strains up to 100% indicating that the level of the spatial order was maintained,[54] suggesting that longitudinally bonded fibrin monomers do not unfold below 100% strains, however αC-regions binding laterally can uncoil.[54] An earlier paper showed that even though the monomer length is maintained, the structural order decreases, possibly due to coiled-coils acting like springs at low strains or stochastically unfolding at large strains,[53] while lateral fiber pulling also emphasized the importance of the unfolding of the αC-region,[63] especially at low strains.[70] Above 100% strain, the 22.5 nm monomer length increased slightly, and the spatial order decreased.[54] Fibers likely start to stiffen above 100% strain and thus distribute stress in the network. In agreement with this, individual fibers were shown to redistribute strain in the network above 100% strain[65] as a consequence of individual fiber stiffening (seen from decreasing strain increment of the stretched fiber).

When crosslinked by factor XIII, clots show increased rigidity,[77-80] increasing linearly with the number of crosslinks.[81] In agreement with this, individual fibrin fibers also became stiffer when crosslinked,[66] even if activated factor XIII was added later to already formed uncrosslinked and mechanically manipulated fibers.[63] Uncrosslinked fibers extend 2–3-times their original length, whereas crosslinked fibers typically have lower extensibility.[62,66] An earlier study showed higher extensibility for crosslinked fibers,[45] which was later explained by the possibility that fibers were not fully crosslinked.[66]

3.3 | Rupture

The unique mechanical behavior of fibrin protects against rupture at many levels. First, due to the elastic deformation at low strains and low stresses, the structure can adapt to external loads while maintaining its structural integrity. Second, the structure can adapt to larger loads due to strain stiffening. In addition, as a consequence of strain stiffening, local strain is distributed throughout the network and protects against rupture.[65] Moreover, bonds were reported to form between adjacent fibrin fibers upon moderate strains, possibly leading to plastic remodeling of the fibrin network.[82] However, heterogeneities in the secondary structure were already present at 56% vertical strain,[74] which suggests that some fibers are heavily loaded and thus prone to rupture.

In agreement with this, a recent study of crack propagation in clots found that clot rupture does not involve diffuse damage, but rather the propagation of a 'critical flaw' in the network structure, where fibers break when experiencing a critical stretch ahead of the crack.[83] Thus, rupture happens at points of weakness or load

concentration and the high extensibility of individual fibers likely plays an important role against clot rupture. In individual fiber pulling experiments, all parameters like stiffness, rupture stress and rupture strain have an impact on the mechanical integrity of the fibers. Toughness, or the area under the stress-strain curve, which indicates the energy needed to rupture a fiber, could be an important parameter for future studies on the mechanical stability of the individual fibers.

It is important to note that viscoelastic properties of individual fibrin fibers, such as (non-linear) stiffness, cannot be directly related to the rupture properties. While stiffness is an inherent material property, rupture is a specific event on the stress-strain curve. However, recent studies from our lab showed associations between decreased average clot (and fiber) stiffness and increased occurrence of clot rupture.[55,84] The association between these properties requires further investigation.

4 | CLINICAL IMPLICATIONS OF FIBRIN MECHANICS

The importance of fibrin biomechanical properties for hemostasis and thrombosis can be easily understood from the remarkable elastic properties that fibrin fibers and clots possess. Fibrin is one of the most elastic and extensible biological polymers known in nature, and has been compared with other elastic biological polymers.[45] As with every other biological polymer, fibrin also displays viscous characteristics (i.e. is visco-elastic), which is also an important behavior as it means it can deform and absorb energy under impact.[85] But elasticity generally outweighs its viscous nature and is crucially important for hemostasis and thrombosis. During hemostasis, the fibrin clot functions as a temporary extracellular matrix when blood clots temporarily replace injured tissue. Clots sealing injury sites will be stretched upon movement, and thus it is clear that elasticity (the ability to return to original shape upon deformation) is critically important.

Also, in the setting of thrombosis or the occlusion of a major blood vessel due to vessel injury or blood stasis, the visco-elastic properties of fibrin likely play an important role. Visualize thrombi in deep veins of the legs, which are subjected to movement of the leg muscles.[86] There is a risk that parts of the clot may break off and lodge themselves in another blood vessel downstream, causing a blockage and tissue ischemia of a critical organ (e.g. the lungs). A similar situation occurs with clots that generate in the atrium due to arrythmia, or in the carotid artery due to local atherosclerosis, which may embolize to clot fragments occluding major brain blood vessels causing a stroke.[87] While as discussed above fibrin fiber breakage and thus clot rupture is not directly linked to fibrin elasticity, it is clear that fibrin biomechanical properties must be critical to prevent such events from happening.

Despite such clear plausible mechanistic connections between fibrin visco-elastic characteristics and thrombosis or hemostasis, relatively little is known about the topic. There are some studies using TEG or ROTEM in patients with thrombosis, but many of these are in the surgical setting and have been reviewed elsewhere.[88-90] Furthermore, thromboelastometry is limited by the relative arbitrary units as discussed above. We will therefore not discuss these papers further and instead focus on those studies that have provided more detailed analysis of the biophysical mechanical properties. In this regard, we are only beginning to understand how fibrin biomechanical characteristics may associate with or impact on thromboembolic diseases. Clots from patients with coronary artery disease were highly branched and showed increased stiffness.[91] Fibrin clots from patients with deep vein thrombosis and pulmonary embolism (PE) developed G' and G" upon blood clotting quicker, had reduced fibrin fiber density and increased susceptibility to fibrinolysis compared with patients with deep vein thrombosis only.[92] These findings suggest a role for fibrinolysis and biomechanical fibrin stability in thromboembolic disease. Another study using lateral force AFM spectroscopy found no difference in visco-elastic properties of fibrin fibers in 5 patients with diabetes and poor glycemic control compared to 5 patients with optimal glycemic control or 4 healthy individuals.[93] These findings are surprising as diabetes is known to increase the risk of thromboembolic disorders,[94] and is also known to impact on clot structure.[95,96] However, this lateral force AFM study was limited by a small number of patients and the experimental error could be relatively large. Interestingly, an association between thin fibers and increased stiffness was observed, suggesting that thin fibrin fibers are more densely packed than thick fibers.[93]

Recent studies from our lab showed two key new findings. The first is that fibrin clots from patients with recurrent venous thromboembolism showed reduced storage (elastic) moduli, thus were less stiff as analyzed in microrheology experiments using magnetic tweezers, compared with patients that had only one event, indicating that reduced elasticity may increase the risk of thromboembolism recurrence.[55] These differences were observed without changes in factor XIII or fibrinogen levels or significant changes in clot structure, suggesting that other mechanisms could determine clot elasticity. More recently we investigated the role of fibrin cross-linking by activated factor XIII in fibrin fiber stiffness, fiber strength and clot fragmentation in a novel murine model of pulmonary embolism using X-ray and fluorescence imaging, combined with light sheet microscopy of the lungs. We found a 40% increase in pulmonary embolism in a new genetically modified murine model where we deleted the fibrinogen γ-chain cross-linking sites for activated factor XIII, compared with wildtype control.[84] We also found that fibrin fibers of the γ-chain mutant ruptured at 40% less stress by lateral force AFM spectroscopy than wildtype control. These findings demonstrate a key role of fibrin γ-chain cross-linking in maintaining fiber integrity and recovery under strain, with consequences for fragmentation of venous clots and subsequent pulmonary embolism[84]

5 | CONCLUDING REMARKS

Fibrin demonstrates unique biomechanical properties that are key for its main function, i.e. the stemming of bleeds, while also impacting on

thrombosis and thromboembolism. We have learned that fibrin rheological properties are determined by characteristics of the protein itself, the manner in which protofibrils are packed into individual fibers, and the structural properties of the fiber network. Any changes to the protein, fibers and network may have critical impact on the biomechanical properties of fibrin. We have also learned that the elastic properties and the resistance of fibers to rupture and clots to fragmentation are important properties for the prevention of thromboembolic disorders such as pulmonary embolism and likely ischemic stroke. Fibrin γ-chain crosslinking by activated factor XIII appears indispensable in this regard.

Several open questions remain, however. With the improvement of resolution in many techniques (high resolution AFM,[97] cryo-electron microscopy, super-resolution microscopies), we are beginning to understand processes of early formation of fibrin (proto)fibers and how these early events determine structural and mechanical properties of clots. The protofibril arrangements and packing inside individual fibrin fibers are a good working model, but the precise structural arrangements and how they impact on fibrin biomechanics is still not fully understood. The Y-ladder model and the catch-slip behavior of the A:a knob-hole bonds can add further interesting perspectives to our recent understanding. The molecular and structural basis of the unique mechanical properties of fibrin, i.e. the remarkable extensibility, more than 3 times its original length and strain stiffening behavior is yet to be fully deciphered. Partially truncated fibrin variants can be great tools to further elucidate such mechanisms in the future. Future studies need to establish optimal visco-elastic properties of fibrin that are able to stem bleeding yet reduce the risk of thromboembolism. Clinical studies are needed to study associations between clot biomechanical properties, bleeding, thrombosis and thromboembolism. Further studies are required to treat thrombosis by reducing clotting and clot size, while maintaining clot stability and resistance to embolism during treatment. While substantial progress has thus far been made, these are some of the important future challenges in thrombosis and hemostasis research related to fibrin biology and biomechanics.

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CONFLICT OF INTEREST

None of the authors declare any conflict of interest with regards to this study.

AUTHOR CONTRIBUTIONS

TF and RASA wrote the initial draft of the manuscript. SDAC critically reviewed the draft. All authors read and commented on the paper and approved submission.

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