4 Contribution of fiber microstructure to fibrin network mechanics

Supramolecular assembly is a powerful strategy that is widely used in nature to build fibrous networks with superior mechanical properties. Protein polymers like fibrin fibers have a complex internal architecture that provides remarkable fiber extensibility and contributes to the highly nonlinear elasticity of blood clots. Here we investigated with the aid of rheology the contribution of the internal architecture of fibrin fibers to the mechanics of networks in the limit of so-called “fine clots”. In this regime, fibrin forms thin double-stranded filaments known as protofibrils. We show that increasing levels of shear stress result in a strain-stiffening response with a complex functional dependence on stress. We can quantitatively account for this stiffening using an affine model for crosslinked semiflexible polymers that assumes uniform network deformation. Moderate stresses induce stiffening due to entropic chain stretching, followed by backbone extension, and shear-induced alignment of the protofibrils at higher stresses. These different levels of structural hierarchy render networks of fibrin protofibrils highly resilient, enabling them to withstand large deformations. In addition, these results lend support the loose bundle model proposed in Chapter 3 to account for the nonlinear elasticity of coarse clots, which are composed of thick bundles of protofibrils.

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4.1 **INTRODUCTION**

Living systems employ supramolecular assembly to build materials with complex architectures and remarkable mechanical properties [5]. The cytoskeleton that structures cells, and the extracellular matrix that structures tissues, are both examples of fibrous materials built of stiff protein polymers. These biopolymer materials are elastic, strain-stiffening, and mechanically resilient [46, 285, 151, 94]. The protein filaments that form the structural backbone of these materials are themselves also supramolecular assemblies made up of protein building blocks. Recent studies of the mechanical properties of protein fibers at the single filament level show rich mechanical behavior that is not captured by continuum elastic models [111]. Fibrin fibers [191, 82] and intermediate filaments [163] can, for instance, be extended by up to 4 times their original length, whereas collagen fibers are rather inextensible, but very tough owing to viscous sliding of subunits within the fiber [45].

In this chapter, we focus on fibrin, which is the main structural protein in blood clots [169]. Even though fibrin fibers in clots have a rather large diameter of around 100 nm, they are still remarkably flexible. This unexpectedly high flexibility can be accounted for by the loose coupling between the constituent protofibrils that build the individual fibers, as demonstrated in Chapter 3. The fibers consist of dozens of protofibrils, which themselves are composed of two half-staggered strands of fibrin monomers connected via flexible αC-regions (Fig. 4.1 A) [111, 88]. This complex hierarchical structure endows fibrin fibers with a built-in stretch-stiffening response that involves entropic stretching of thermal fluctuations of fibers between network crosslinks, followed by entropic stretching of flexible regions within the fibers (see Chapter 3). This can include the protofibrils themselves, but also the disordered and flexible αC-domains [82] or flexible domains caused by forced unfolding [44, 43, 179, 239].

Given the complex internal structure of fibrin fibers, it still remains unclear how the individual protofibrils contribute to the elastic response of fibrin clots, on scales ranging from the fiber to the whole network. To address this puzzle, experiments on the mechanical properties of networks of single (unbundled) protofibrils are crucial. A series of experiments performed in the 1980’s showed that the bundle size of fibrin fibers can be varied over a wide range by tuning the polymerization conditions [211, 154]. Fibrin networks are generally prepared in one of two rather different structural limits. Physiological pH and low ionic strength favor **coarse fibrin clots**, made up of fibers that contain tens of protofibrils. This is the limit studied in Chapter 3. In contrast, high pH (8.5) and high ionic strength (0.45) favor **fine fibrin clots** with fibers composed of one or a few protofibrils [85, 149, 211, 95, 247, 13, 213, 254]. Thus, rheology measurements on fibrin networks assembled under different solution conditions may provide critical insights into the effect of filament bundle size on network mechanics.

Here we report rheology experiments on **fine fibrin clots** composed of thin fibers made of three protofibrils, according to turbidimetry. We show that increasing levels of shear stress cause network stiffening with a complex functional dependence on stress. We can quantitatively account for this stiffening using
an affine model for crosslinked semiflexible polymers. The protofibril networks resist large deformations by a combination of entropic stretching, backbone stretching, and alignment of protofibrils in the shear direction. We compare the rheology data with the rheology of coarse clots reported in Chapter 3, in order to further examine the validity of the loose bundle model that we propose there.

4.2 Sample preparation

Fibrinogen from Enzyme Research Laboratories in a pH 7.4 buffer of 20 mM sodium citrate was first dialyzed for 2 days at 4°C against a buffer of pH 8.5 containing 50 mM Tris-HCl and 400 mM NaCl [211, 149, 13]. The dialyzed fibrinogen was centrifuged for 20 minutes at 9000 rpm to remove any aggregated material. The final protein concentration was determined by measuring light absorbance at 280 nm with correction for scattering at 320 nm, using an extinction coefficient of $\varepsilon_{280} = 1.6 \text{ ml/(mg cm)}$ [13, 215]. The dialyzed fibrinogen stock was frozen in liquid nitrogen and stored at −80°C until use. Assembly of fine clots was initiated by addition of 0.5 U/ml thrombin (Enzyme Research Laboratories) in the presence of 3.2 mM Ca²⁺ in dialyzing buffer, after which the sample was quickly transferred to the preheated plates of the rheometer. For fluorescence confocal microscopy, Alexa488-labeled fibrinogen was mixed with unlabeled fibrinogen in a molar ratio of 1:40. Labeled fibrinogen for fine clots was dialyzed in the same way as unlabeled fibrinogen. Turbidity measurements on fine gels were performed using cuvettes with a path length of 1 cm. All components, except thrombin, were mixed directly in the cuvette, and the mixture was degassed for 5-10 minutes to remove air bubbles. Thrombin was carefully added, and the networks were polymerized for 1 hour at 37°C taking care to prevent solvent evaporation.

SDS polyacrylamide-gel electrophoresis (SDS-PAGE) analysis for the determination of the crosslinking pattern caused by FXIIIa was performed with 7% polyacrylamide gels. The fibrin samples and a molecular marker (Kaleidoscope, Bio-Rad) were diluted in a 1:1 volume ratio with Laemmli Sample Buffer (Bio-Rad), heated up to 95°C, and run on gel for 40 minutes at 220V. Protein bands were visualized using GelCode Blue Stain Reagent (Thermo Scientific).

Coarse fibrin clots that were used to compare data with fibrin fine gels, were prepared as described in Chapter 3.

4.3 Results and Discussion

4.3.1 Network and bundle structure

Fibrin networks assembly in a complex manner. In the first step of polymerization process fibrin monomers always first assemble into double-stranded protofibrils that are held together by noncovalent interactions. This initial assembly process is triggered by the enzyme thrombin, which cleaves off two fibrinopeptides (A and B) from the central domain of the fibrinogen molecule. The
exposed sites bind to complementary sites in the distal end domains of adjacent fibrin molecules. Next, the protofibrils laterally associate to form thicker fibers. The final bundle size $N$ (i.e. the number of protofibrils per bundle) depends sensitively on pH, ionic strength and thrombin concentration [154]. In this study, we varied the pH and ionic strength, but used a fixed thrombin concentration (0.5 U/mL). We polymerized networks in two different limits. In the coarse clot limit, under near-physiological conditions, fibrin forms thick bundles composed of tens of protofibrils (Fig. 4.1 A Top). In the fine clot limit, at pH 8.5 and an ionic strength of 0.45, fibrin forms much thinner bundles, composed of 1-3 protofibrils only (Fig. 4.1 A Bottom).

To measure the bundle size of the fibers in the two structural limits, we performed turbidity measurements on gels with different fibrin concentrations, $c_p$. The wavelength dependence of the turbidity provides both the mass/length ratio, $\mu$, and the diameter, $d$, of the fibers in their native, hydrated state [327] (see Chapter 2). The number of protofibrils can be calculated from $\mu$ using the mass/length ratio of single protofibrils, $\mu_{pf} = 1.5 \cdot 10^{11}$ dalton/cm, which follows from the molecular weight, $M$ (340 kDa), and length, $L$ (45 nm), of fibrin monomers: $\mu_{pf}=2M/L$ [52]. The fibers in coarse clots consist on average of 87 protofibrils (open symbols in Fig. 4.1 B). The bundle size is independent of protein concentration up to 10 $\mu$M (1 mg/ml fibrin corresponds to a molar concentration of 2.94 $\mu$M), and then decreases slightly. Interestingly, the diameter of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.1.png}
\caption{Two different structural limits of fibrin clot assembly. (A) Schematic showing fibrin fibers in coarse and fine clots, which differ in the number of protofibrils per cross-section, $N$, as indicated. The protofibrils are laterally associated by long, flexible $\alpha C$-domains that protrude from their surface (yellow chains in zoom-in). (B) Bundle size and (C) diameter obtained by turbidimetry of fibrin gels assembled at different molar concentrations, $c_p$. Open symbols: coarse clot limit, solid symbols: fine clot limit.}
\end{figure}
the fibers is constant, with an average $d=120$ nm, over the entire concentration regime (open symbols in Fig. 4.1 C). This suggests a slightly reduced protein density in fibers formed at fibrin concentrations above 10 $\mu$M. However, over the entire concentration range, the protein density within the fibers is rather low, being of order 0.18 g/cm$^3$ until 10 $\mu$M (see Chapter 3, Fig. 3.3 C). This is a consequence of the large spacing between protofibrils, mediated by the long and flexible $\alpha$C-domains. In the fine clot limit, the number of protofibrils per fiber was much reduced, decreasing from $N=4$ at 4.41 $\mu$M to $N=1$ at 14.7 $\mu$M (closed symbols in Fig. 4.1 B). The fiber diameter was close to 100 nm, except at 15 $\mu$M, where the diameter was 10 nm (closed symbols in Fig. 4.1 C). We suspect that this surprisingly large diameter may be an artifact arising from scattering by bundles or aggregates.

To obtain a more direct measurement of the fiber diameter, we imaged fibrin clots prepared at a concentration of 0.5 mg/ml by AFM in a dry state (Fig. 4.2 C). Fibrin networks polymerized at physiological conditions consist of thick fibers with diameter of $\sim$130 nm and length exceeding tens of $\mu$m. In contrast, under higher pH and ionic strength, formation of much thinner filaments takes place. Occasionally, filaments bundle to create much thicker fibers.

To compare the structure of fine and coarse clots at the network scale, we performed confocal fluorescence microscopy. Both types of clots were homogeneous and isotropic. However, the thick fibers in the coarse clots could be resolved individually (Fig. 4.2 A-B Bottom panels), whereas the thinner fibers in the fine clots could not (Fig. 4.2 A-B Top panels). At the same mass density, the fine clots have much smaller voids between filaments than the coarse clots. This is in line with the much smaller mass/length ratio of the thin clot fibers. The mesh size, $\xi$, of an isotropic network of rigid fibers can be estimated from the total fiber length per volume, $\xi = 1/\sqrt{\rho}$. The length density follows from the known molar concentration of protein, $c_p$, and from the fiber mass/length ratio measured with turbidimetry: $\rho = Lc_pN_A/2N$, where $N_A$ is Avogadro’s number and $N$ is the number of protofibrils in a fiber ($N=\mu_{fiber}/\mu_{pf}$). For coarse clots, $\xi$ is in the order of microns, well above the optical diffraction limit. In contrast, $\xi$ in fine clots is close to 0.1 $\mu$m, which is substantially smaller than the optical diffraction limit, making it difficult to resolve individual filaments. The spatial resolution is further reduced due to thermal fluctuations of the thin fibers during image acquisition.
Figure 4.2. Structure of fibrin clots on the network and filament scale visualized by microscopy. (Top panels): Confocal (A, B) and AFM (C) images of fine fibrin clots polymerized at pH 8.5 and an ionic strength of 0.45 with 0.5, 1 and 0.5 mg/ml protein content, respectively. Confocal images are z-projections of 100 confocal planes over a 10µm-thick section of the gel with scale bars, 10µm. AFM images show 10x10 µm area. (Bottom panels): Corresponding confocal and AFM images of coarse fibrin clots assembled at physiological solution conditions.

Lateral aggregation of fibrin protofibrils is known to be promoted by the long, carboxy-terminal extensions of the α-chains (αC-domains) that protrude from the protofibril surface [102, 64, 187, 141]. The α-chains associate non-covalently [88], and these bonds are further reinforced by covalent crosslinking by the enzyme fibrinoligase factor XIII (FXIII) [193]. FXIII also generates covalent crosslinks between the γ-module of adjacent molecules. These dimeric γ-γ-crosslinks can be orientated longitudinally, or transversely between molecules of different strands [208, 214, 258, 305]. We quantified covalent crosslinking levels for the coarse and fine gels by SDS polyacrylamide-gel electrophoresis (SDS-PAGE). Uncleaved fibrinogen displays three characteristic bands on gel originating from its three constituent polypeptide chains: α (molecular weight: 63 kDa), β (56 kDa) and γ (47 kDa) [201] (Fig. 4.3 A-a). In coarse clots, the α- and γ-chains become cross-linked during polymerization, as evidenced by the disappearance of the α- and γ-chain band and the appearance of a major, high-molecular weight band at the top of the gel (Fig. 4.3 A-b). This high molecular weight material represents αₙ-polymers of >250 kDa (multiple bonds between the αC-regions of adjacent molecules) and γ-γ dimers of 100 kDa. In contrast, in fine clots, we do not observe any conversion of α chains to αₙ-polymers (Fig. 4.3 A-c). It is possible that a small fraction of αₙ-polymers is present in a concentration that is too low to be observed on gel. Only formation of γ-γ dimers and compounds with molecular weights between 150-250 kDa can be seen. These compounds may correspond to hybrid α-γ bonds between protofibrils, which have also been reported for coarse clots (Fig. 4.3 B) [258]. The gel results suggest that the protofibrils in fine clots are not (significantly) coupled
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Figure 4.3. Quantification of covalent crosslinks within fibrin gels. (A) SDS-PAGE gel of fibrinogen (a), fibrin coarse (b) and fibrin fine (c) gels, (d) molecular weight marker. (B) Schematic representation of possible ligation pathways between fibrin molecules within/among protofibrils.

by αC-linkers, in contrast to protofibrils in coarse clots, which form distinct bundles held together internally by αC-αC crosslinks.

4.3.2 Low-strain mechanics of fibrin gels

We monitored fibrin polymerization in the rheometer, by subjecting polymerizing fibrin solutions to an oscillatory shear strain with a small enough amplitude to ensure linear response. Specifically, care was taken to avoid either stiffening or irreversible deformation of the network (see Chapter 2). Both fine and coarse clots showed an immediate rise in their linear elastic modulus upon initiation of network polymerization by addition of thrombin (Fig. 4.4 A). However, fine clots reached a constant stiffness within 10 minutes (closed symbols), whereas the stiffness of coarse clots continued to increase gradually for another few hours (open squared symbols). It has been reported that αC-crosslinks are formed slowly, whereas γ-γ crosslinks are formed within the first few minutes of polymerization [111, 280, 200]. To test whether slow formation of αC-crosslinks was responsible for the slow polymerization of coarse clots, we polymerized coarse clots in the presence of a specific inhibitor of γ-glutamyl-ε-lysine linkage formation (50 mM hydroxylamine) [269, 99]. In the presence of this inhibitor, the stiffness reached a constant value within 10 minutes (open circles in Fig. 4.4 A), supporting the hypothesis that slow polymerization of coarse clots is caused by slow formation of αC-crosslinks. The short polymerization time of the fine clots, on the other hand, suggests that there is negligible formation of αC-crosslinks, consistent with the SDS-PAGE results.

Covalent crosslinks are important for stabilizing contacts between protofibrils within fibers, and also between fibers. To test for the presence of intra-fiber crosslinks, we probed the frequency dependence of the linear viscoelastic moduli at small strain amplitudes. Both, coarse and fine networks behaved as almost perfectly elastic solids over the entire frequency range, independent of protein concentration (Fig. 4.4 B). The storage modulus, $G'$, was independent of fre-
Figure 4.4. Linear viscoelastic behavior of coarse (open symbols) and fine (closed symbols) gels. (A) Polymerization of 3 mg/ml fibrin networks observed as an increase in the elastic shear modulus, $G'$, over time. In the presence of 50 mM hydroxylamine, 2 mg/ml fibrin network reaches a steady elasticity after only 10 minutes (circles). (B) Frequency dependence of $G'$ for 0.5 (squares) and 3 mg/ml fibrin gels (circles). (C) Corresponding loss tangents, $\tan\delta = G''/G'$.

frequency, $\omega$, down to at least 0.02 rad/s. Moreover, the elastic modulus was always larger than the viscous modulus by a factor 10 for dilute gels (0.5 mg/ml) and a factor 100 for dense gels (3 mg/ml), indicating that there was little viscous dissipation (Fig. 4.4 C). Thus, even though $\alpha_n$-polymer crosslinks were absent in fibrin fine gels, there were apparently still other types of crosslinks present to prevent structural relaxation. The fine clots at the same protein weight concentration always had a lower elastic modulus than the corresponding coarse clot. However, the loss tangents of coarse and fine clots at the same protein concentration were remarkably similar.

4.3.3 Nonlinear Rheology of Fine Clots

To test how reduced bundle size affects the nonlinear elastic behavior of fibrin networks, we subjected fine clots to different levels of a steady prestress, $\sigma_0$, and measured the tangent modulus $K^*(\sigma_0, \omega)$ at this prestress by superposing a small amplitude oscillatory stress and measuring the oscillatory strain response (see Chapter 2). Remarkably, the networks displayed no creep up to absolute strains of 200%, and only a small amount of creep at even larger strains of 250% (Fig. 4.5 A). $K^*(\sigma_0, \omega)$ was therefore time-independent. The elastic tangent modulus, $K'$, and the viscous tangent modulus, $K''$, were independent of applied prestress up to a certain characteristic stress value, $\sigma_{crit}$ (Fig. 4.6 A, regime 1). In this linear elastic regime, $K'$ and $K''$ correspond to the linear shear moduli, $G'$ and $G''$. Increasing $\sigma_0$ beyond $\sigma_{crit}$ caused a strong increase in network stiffness, $K'$, with a complex dependence on stress. At all fibrin concentrations, there was an initial fast rise in stiffness (regime 2), followed by a slower rise (regime 3), and then another fast rise just before network rup-
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Figure 4.5. Creep response of 3 mg/ml fibrin gel under different levels of prestress (A) and reproducibility of stiffening behavior for a 4 mg/ml fibrin network (B), which is subjected to three consecutive steady shear ramps with the steady shear going up.

ture (regime 4). The total extent of stiffening before rupture, relative to the initial elastic modulus, decreased with increasing protein concentration: dilute gels (0.5 mg/ml) stiffened 1000-fold, whereas dense gels (3 mg/ml) stiffened only 100-fold. The strain-stiffening curves showed no hysteresis in the nonlinear regime during repeated stress sweeps, even when the stress had been increased close to the rupture stress (Fig. 4.5 B). However, the initial (linear) network stiffness increased with each cycle. This work-hardening phenomenon is reminiscent of observations for F-actin networks crosslinked with filamin [189, 263]. Actin-filamin networks have similarly large rupture strains as the fibrin gels. At large strains, the filaments will align, and this alignment may lead to partly irreversible bundling, as shown in the case of actin-filamin gels by fluorescence confocal microscopy during shear [189].

Stress-stiffening behavior is a common feature of many different types of biopolymer networks, including networks of fibrin, collagen, actin, and intermediate filaments [285, 151, 94]. In case of actin and intermediate filaments, this behavior was shown to be well described by an affine entropic model for semiflexible polymers. This model assumes that stiffening is caused by stretching of the thermally undulating polymer segments between crosslinks [195]. Moreover, it assumes that the network deforms in a uniform (affine) manner, so that the macroscopic elastic modulus can be calculated by performing an orientational average over all filaments [285]. Semiflexible polymers have a highly nonlinear force-extension behavior, with an entropic stretch modulus that diverges once the stored length is pulled out and the end-to-end length reaches the contour length. The model predicts for the linear elastic modulus, \( G_0 \), and critical stress, \( \sigma_{\text{crit}} \), the following dependencies on filament persistence length, \( l_p \), and crosslink distance \( l_c \):
\[ G_0 = 6\rho k_B T l_c^{1/2} \]
\[ \sigma = \rho k_B T l_p^{1/2}, \]

where \( k_B T \) denotes thermal energy and \( \rho \) is the contour length density. In the nonlinear regime, at stresses above \( \sigma_{\text{crit}} \), the stiffness is expected to scale with stress as \( K' \sim \sigma^{3/2} \). Stiffening curves measured at different protein concentrations are therefore expected to collapse onto a single master curve when rescaled by \( G_0 \) and \( \sigma_{\text{crit}} \) [94]. The stiffening curves of fine fibrin clots for concentrations between 0.5 and 5.8 mg/ml do indeed collapse onto one mastercurve when the modulus is normalized by \( G_0 \) and the stress is normalized by \( \sigma_{\text{crit}} \) (Fig. 4.6 B). This suggests that the linear modulus and the onset of stress-stiffening are indeed well described by the affine entropic model. However, the collapse only works up to stresses that are 10-fold larger than \( \sigma_{\text{crit}} \). At larger stress, there are noticeable differences between the curves. Moreover, the rise in stiffness is weaker than the 3/2-increase predicted for inextensible semiflexible polymers (solid line). This behavior is in contrast with the stiffening behavior of rigidly crosslinked actin networks, which do stiffen as \( K' \sim \sigma^{3/2} \), but it is similar to the stiffening behavior observed in previous studies of coarse fibrin clots (see Chapter 3), fish protofibril gels [285], and intermediate filament networks [180]. The deviation from the affine entropic model in those studies was ascribed to extensibility of the filament backbone and the stiffening curves were well described by the affine entropic model when enthalpic stretching was included [285, 222, 180]. The difference between the rheology of fibrin and intermediate filaments as compared to actin is in line with force-extension measurements on single fibers: fibrin fibers and intermediate filaments have a much smaller stretch modulus and larger extensibility [191, 153, 299] than actin filaments [192].

To test whether the affine entropic model extended with backbone extensibility can fit the nonlinear elastic stiffening of fine clots, we fitted the model to the measured stiffening curves over the entire stress range. We used three fit parameters, the crosslink distance \( l_c \), persistence length \( l_p \), and stretch modulus \( \kappa_{\text{stretch}} \). We find excellent agreement of the theory with the experimental data for all protein concentrations, as exemplified for two protein concentrations (0.5 and 3 mg/ml) in Fig. 4.6 A (solid lines are theory curves). The theory captures the initial stiffening, caused by stretching of the thermally undulating fibrin fibers, as well as the slower stiffening at large stress. By itself, backbone extensibility leads to a saturation of the elastic modulus if the fibers have a linear stretch modulus. However, fiber alignment at large strains (regime 3) causes weak network stiffening simply by a geometrical effect. At high stress, just before network rupture (regime 4), the model underestimates the stiffness of the fine clots. This small discrepancy may indicate an intrinsic nonlinearity in the backbone elasticity of protofibrils. In fibrin coarse clots, we also identified stiffening at high stress due to an intrinsic nonlinearity in fiber stretching [232] (see Chapter 3). However, this nonlinearity was much more pronounced for coarse clots than it is for fine clots.

To thoroughly test the validity of the affine entropic model, we checked
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Figure 4.6. Nonlinear elastic response of *fine fibrin* networks with increasing levels of prestress (bar). (A) Stress-stiffening behavior of 0.5 (squares) and 3 mg/ml (circles) gels. Solid lines represent the affine entropic model for semiflexible polymers with a finite stretch modulus. The model was fitted to the data using three fit parameters, the crosslinking distance, $l_c$, the filament persistence length $l_p$, and stretch modulus, $K_{stretch}$. (B) Stress-stiffening curves rescaled by $G_0$ and $\sigma_{crit}$, using values calculated from the best-fit values of $l_c$ and $l_p$ according to Eq. (4.1). Data are shown for 0.5 (squares), 2 (circles) and 4 mg/ml (diamonds) fibrin networks. Solid line indicates 3/2 power-law stiffening expected for inextensible semiflexible chains. Cartoons above the graphs sketch the proposed mechanism underlying the 4 distinct regimes in the stress-stiffening curves: (1) entropic stretching of filaments between network crosslink points, (2) enthalpic stretching of filament backbones, (3) shear-induced alignment of filaments, and (4) forced unfolding of fibrin monomers within the filaments.

whether the fits returned physically reasonable values for the fit parameters. When we assumed an average bundle size of $N=3$, based on the turbidimetric data, we found unphysically small values for $l_c$ and $l_p$. The apparent persistence length, for instance, was only 50 nm ($l_p=0.048 \mu m$ and $l_c=0.055 \mu m$ for 1 mg/ml fibrin network), which is inconsistent with values estimated from electron microscopy and atomic force microscopy of fish fibrin protofibrils (500 nm, [285, 307]), and by light scattering for human fibrin protofibrils (200 nm, [22]). If we instead assumed a bundle size of $N=1$, we obtained much more reasonable values for $l_p$, around 150 nm (inset of Fig. 4.7 C) and also for $l_c$. The crosslink distance decreased from 0.25 \mu m at 0.5 mg/ml fibrin to 0.05 \mu m at 3 mg/ml (symbols in Fig. 4.7 C). These values are similar to $l_c$ values reported for intermediate filaments, which have a similar diameter (~10 nm) and persistence length (0.5-1 \mu m) as fibrin protofibrils [182]. The values are close to their lower
Figure 4.7. Concentration dependence of rheological and structural parameters derived from the rheology data in the context of an affine entropic model of semiflexible polymers. (A) Scaling of the elastic modulus with protein concentration. Data obtained in this study (closed symbols) agree well with data from other studies (open symbols) [307, 213] and with theory (line). (B) The critical stress (squares) also agrees well with theory (line). (Inset): Corresponding critical strain, which scales as $c_p^{-0.4}$. This scaling is consistent with theory, but the experimental values are 2-fold larger than predicted (line). (C) The crosslink distance decreases with concentration, in agreement with theory when assuming $l_c = \xi$ (solid line). The values are close to their lower bound, $l_c = \xi$ (dashed line). (Inset): the persistence length is 0.15 $\mu$m, independent of concentration. (D) The plateau modulus and critical stress are internally consistent as predicted by the theory, showing a 3/2 power-law slope (solid line) in the rescaled form of Eq. (4.3). Dashed line represents best-fit power-law slope of 1.4.

bound, corresponding to dense crosslinking, where the crosslink distance is comparable to the mesh size, $l_c = \xi$ (dashed line). The crosslink distance for semiflexible polymers is expected to be proportional to the entanglement length, $l_e$, which scales as $l_e \sim l_p^{1/5} \rho^{-2/5}$ [195, 94, 221]. Our measured values for $l_c$ agree remarkably well with this prediction, with a proportionality constant of 1 (solid line in Fig. 4.7 C represents $l_c = l_e \sim l_p^{1/5} \rho^{-2/5}$). This proportionality constant is comparable to the value of 0.35 that we found for coarse clots (see Chapter 3).

The fact that all three fit parameters have realistic values when we assume a bundle size of $N=1$ suggests that the network behaves mechanically as if it is composed of protofibril filaments that are crosslinked at intersections. If the protofibrils within a fiber are loosely associated, then it seems reasonable that one cannot distinguish between crosslinks within or between fibers. The
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protofibrils effectively behave independently from each other and contribute individually to the mechanical response of the network. This is different from coarse clots, where the fibers are distinct bundles consisting of a much larger number of protofibrils (N=87).

With the values for $l_c$ and $l_p$ obtained from the fits of the affine entropic model to the data, we can compute the linear elastic modulus, $G_0$, and critical stress, $\sigma_{\text{crit}}$, using Eq. (4.1). We find excellent agreement of the experimental data and the calculated values in both cases. $G_0$ increases with fibrin concentration according to a power-law with exponent 2.2, in agreement with the predicted $c_p^{11/5}$-scaling (Fig. 4.7 A). This exponent also falls well within the range of 2.1-2.3 reported previously for ligated fibrin coarse clots [270, 91, 213, 95, 232], and the absolute magnitude of the moduli agree well with prior measurements on fine clots (open symbols in Fig. 4.7 A) [307, 213]. The critical stress increases with concentration according to a power-law with exponent 1.8 in accordance with the predicted $c_p^{9/5}$-scaling (Fig. 4.7 B). The corresponding critical strain, $\gamma_{\text{crit}}$, scales as $\gamma_{\text{crit}} \sim c_p^{-0.4}$, consistent with the theoretical prediction,

$$\gamma_{\text{crit}} = \frac{1}{3} \frac{l_c}{l_p}$$

(4.2)

However, the measured strain values are 2-fold larger than predicted. This small discrepancy suggests that the network deformation may be somewhat nonaffine due to bending or rotation of filaments; simulations show that nonaffinity postpones the onset of nonlinearity [224, 144, 40]. However, nonaffinity, if indeed present, is small, given that the linear modulus agrees well with theory. It is important to note that the affine model for $G_0$ that we use here represents an upper bound on the shear modulus for given values of the various parameters in the model, all of which except $l_c$ are known independently. Furthermore, since our inferred values of $l_c$ are close to their lower bound of $\xi$, if the network deformation in our samples were to be predominantly nonaffine, a significantly smaller value of $G_0$ would be expected. This conclusion is consistent with an earlier direct mapping of the strain field in sheared gels of fish fibrin protofibrils, which showed only slight nonaffinity at small strain, which decreased with increasing strain [307].

As a final test of the internal consistency of our data, we checked whether we could collapse all data onto a single master curve using the theoretically predicted rescaling form:

$$c_p^{1/2}G_0 \sim \sigma_{\text{crit}}^{3/2}$$

(4.3)

which follows from Eq. (4.1). As shown in Fig. 4.7 D, the data are indeed in excellent agreement with Eq. (4.3), showing a power-law scaling close to 3/2 (best-fit value is 1.4, dashed line). Together with the fact that we obtain physically meaningful values of $l_c$ and $l_p$ from the affine model, this scaling provides strong evidence that the linear elasticity of fibrin fine gels is entropic in origin.

The affine entropic model gives a good description of the stress-stiffening behavior of fibrin gels over nearly the entire stress range. Even the upturn in
4.3 The stress-stiffening curves at large stress is rather well captured by the model, which predicts slight stiffening due to shear-induced alignment of the protofibrils (regime 3 in Fig. 4.6 A). This alignment may also account for the work-hardening phenomenon observed during repeated stress sweeps (Fig. 4.5 B). The shear cell described in Chapter 8 may permit us in future to quantify the degree of alignment as a function of strain, and confirm whether it is indeed consistent with the affine prediction.

Just before rupture, the fine clots stiffen slightly stronger than captured by the affine entropic model (regime 4 in Fig. 4.6 A). This additional stiffening may reflect intrinsic nonlinearities in the protofibril stretch modulus occurring at large strain. Such nonlinearities may arise from forced unfolding of fibrin monomers or from entropic stretching of the flexible αC-domains. Forced unfolding has been shown to occur in coarse fibrin clots under tensile load [44], in single stretched fibers [44, 179], and in single molecules [43, 179]. Stretching of αC-domains was demonstrated in single-stretching experiments of coarse clot fibers [82]. To confirm whether unfolding occurs also in fine clots, complementary measurements will be needed, for instance using small-angle X-ray scattering (SAXS) [44] or by staining with dyes that bind to unfolded, β-sheet rich structures [239]. To test the role of the αC-domains, the rheology could be repeated with networks prepared from derivatives of human plasma fibrinogen whose αC-domains are enzymatically removed [203], or with fibrinogen from different species, which varies in the length of the αC-domains [82].

Forced unfolding of molecules or stretching of flexible αC-domains may contribute to the remarkable resilience of fibrin networks against large strains. Fibrin fine gels can survive strains of up to at least 250% (Fig. 4.5 A). The rupture stress, $\sigma_{\text{max}}$, is always at least 1000-fold larger than $\sigma_{\text{crit}}$, and it increases with protein content according to a power-law with exponent 1.2 (Fig. 4.8)

**Figure 4.8.** Maximum (rupture) stress of fine clots as a function of protein concentration. Dashed line indicates power-law fit with exponent 1.2.
4.3.4 **Comparison between fine and coarse clot rheology**

In Chapter 3, we proposed that the rheology of fibrin coarse clots can be described by an affine entropic model, where the fibers behave as semiflexible bundles of $N=87$ loosely coupled protofibrils. If this is true, then the coarse clot data should be consistent with the fine clot data upon rescaling of the linear modulus, critical stress, and stretch modulus with the bundle-size dependent persistence length, $l_p = N l_p^f$, where $l_p^f$ is the persistence length of a single protofibril. To test this prediction, we first plot the linear modulus rescaled by $l_p^{7/5}$ as a function of contour length density (Fig. 4.9 A). We indeed find rather good agreement between the datasets for coarse and fine clots: the rescaled moduli nearly fall onto mastercurve with a power-law slope of 1.6 (close to the expected 11/5 slope). We next test the correspondence of the critical stress (rescaled by $l_p^{3/5}$) and critical strain (rescaled by $l_p^{-4/5}$) of fine and coarse clots. As shown in Fig. 4.9 B-C, both the stress and strain of the coarse clots is higher than expected based on the fine clot data, though they exhibit similar scaling with filament concentration.

The larger critical stress and strain of the coarse clots compared to the fine clots suggests a higher degree of nonaffinity in the coarse gels. Indeed, the coarse clots are expected to be less affine than fine gels. Based on recent lattice simulations, a phase diagram was proposed which maps the affinity as a function of the network connectivity, $z$, and the ratio of bending/stretching rigidities, $\kappa_{bend}/\kappa_{bend} l_c^2$ [40]. The local connectivity in fibrin networks is expected to be on average between $z=3$ (corresponding to a branch) and $z=4$ (corresponding to two fibers crosslinked at a crossing). For $z=3$, the defor-
mation field is expected to crossover from nonaffine to affine at a dimensionless ratio $\kappa_{\text{bend}}/\kappa_{\text{bend}}l_c^2$ of $10^{-2}$. Below this crossover ratio, fiber bending dominates, while above it, affine stretching. If we assume that the stretch modulus has an entropic origin, then [195]:

$$\kappa_{\text{stretch}} = 90\kappa_{\text{bend}} \frac{l_p}{l_c^3}$$  \hspace{1cm} (4.4)

For fine clots, the crosslink distance $l_c$ is comparable to the persistence length, $l_p$ (Fig. 4.7 C), so the dimensionless crossover ratio is $10^{-2}$. In coarse clots, the protofibrils are bundled, so the mesh size is larger than in fine gels. At a constant molar concentration of fibrin, the average crosslink distance increases with $N$ as:

$$l_c = N^{1/2} l_c^f$$  \hspace{1cm} (4.5)

where $l_c^f$ is the crosslink distance for the protofibril gel. The persistence length of the coarse clots, for completely loosely coupled bundles, is defined as:

$$l_p = N l_p^f$$  \hspace{1cm} (4.6)

The crossover ratio scales therefore as $N^{-1/2}$:

$$\frac{\kappa_{\text{bend}}}{\kappa_{\text{stretch}}l_c^2} = \frac{1}{90} \frac{l_c^f}{l_p^f} N^{-1/2}$$  \hspace{1cm} (4.7)

For coarse clots with $N=87$, this implies a ratio of $10^{-3}$, 10-fold smaller than for fine clots. This implies that coarse gels are indeed expected to be less affine than fine gels. This nonaffinity could explain the enhancement of the critical stress and strain of coarse clots over the affine predictions. Yet, the linear modulus of the coarse clots agrees well with the affine prediction, suggesting that the degree of nonaffinity in coarse networks is still rather small.

Finally, we compared the enthalpic stretch moduli of the protofibrils as inferred from the rheology of coarse clots (normalized by $N=87$) and fine clots. We find remarkably good agreement between the stretch moduli obtained from coarse and fine gels rheology data (Fig. 4.10). This agreement corroborates the proposition in Chapter 3 that the coarse clots stiffen by an entropic mechanism combined with backbone extensibility, analogously to the fine clots. The stretch modulus extracted from the rheology data varies between 175 and 300 pN (solid squares in Fig. 4.10). These values are consistent with the stretch modulus of fish fibrin protofibrils (50-100 pN) [285]. The Young’s modulus, assuming cylindrical fibers with a diameter of 9 nm and homogeneous elasticity, is 3-5 MPa. This value is consistent with single-fiber stretching and bending experiments on fibers within coarse fibrin clots (14 MPa) [65].

The most striking difference between the stiffening behavior of coarse and fine clots is that the coarse clots show a more pronounced nonlinearity at large
stress, with a distinct stiffening regime that scales as $K' \sim \sigma^{3/2}$, as expected from an entropic mechanism. Single fiber stretching experiments with coarse clot fibers also demonstrated intrinsically nonlinear stretching [143]. We proposed in Chapter 3 that this entropic stretching regime corresponds to stretching of flexible domains inside fibers. This interpretation relies on having distinct bundles, with a separation of length scales between crosslinks of protofibrils inside bundles and crosslinks between the surfaces of different bundles. Such a separation of scales is reasonable for the coarse clots, where the fibers have a large bundle size. In contrast, the protofibrils in fine clots behave as mechanically individual filaments.

4.4 Conclusion

Fibrin clots are among the most resilient protein networks found in the human body. Their supramolecular, internal structure assures high extensibility of the individual fibrin filaments and contributes to the rich nonlinear response of fibrin networks to large deformations. Fibrin fibers are composed of thin, semiflexible protofibrils that are laterally connected by flexible polypeptide linkers. The bundle size is sensitive to the solution conditions, including pH and ionic strength. In this chapter, we focused on the so-called “fine clot” regime at high pH and high ionic strength, where the bundle size is minimal. Turbidity measurements indicate a bundle size of 3. We showed that fine clots behave rheologically as networks of semiflexible polymers with a fine backbone extensibility. The clots strongly stress-stiffen, and stiffening is accurately described by an affine entropic model of polymers with a linear backbone elasticity over nearly the entire range of stress. Only at the highest levels of stress, just before network rupture, we see some evidence of intrinsic nonlinearity in the protofibril backbone extensibility that is not captured by the model.

The rheology of fine clots also provides key insights into the basis of the
stress-stiffening behavior of so-called “coarse fibrin clots” that form at near-physiological conditions. Coarse clots consist of fibers that are thick bundles of protofibrils. We compared the stress-stiffening behavior of fine clots with that of coarse gels with a bundle size of $N=87$. At small to moderate stress, both fine and coarse clots stiffen in accordance with the affine entropic model including backbone extension. The linear stretch modulus of the fine clot fibers agrees well with that of the coarse gels upon rescaling by bundle size. However, the coarse clots show a distinct stiffening regime at high stress that points to an intrinsically nonlinear entropic elasticity of the fiber backbone. This regime is not seen for fine gels. This difference from the fine clot rheology corroborates our proposition in Chapter 3 that the intrinsic nonlinear rheology of the coarse clot fibers derives from their loose bundle structure.

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