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Structural basis for the nonlinear mechanics of fibrin networks under compression

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Abstract

Fibrin is a protein polymer that forms a 3D filamentous network, a major structural component of protective physiological blood clots as well as life threatening pathological thrombi. It plays an important role in wound healing, tissue regeneration and is widely employed in surgery as a sealant and in tissue engineering as a scaffold. The goal of this study was to establish correlations between structural changes and mechanical responses of fibrin networks exposed to compressive loads. Rheological measurements revealed nonlinear changes of fibrin network viscoelastic properties under dynamic compression, resulting in network softening followed by its dramatic hardening. Repeated compression/decompression enhanced fibrin clot stiffening. Combining fibrin network rheology with simultaneous confocal microscopy provided direct evidence of structural modulations underlying nonlinear viscoelasticity of compressed fibrin networks. Fibrin clot softening in response to compression strongly correlated with fiber buckling and bending, while hardening was associated with fibrin network densification. Our results suggest a complex interplay of entropic and enthalpic mechanisms accompanying structural changes and accounting for the nonlinear mechanical response in fibrin networks undergoing compressive deformations. These findings provide new insight into the fibrin clot structural mechanics and can be useful for designing fibrin-based biomaterials with modulated viscoelastic properties.

Introduction

The fibrin network is an end product of blood clotting and a major structural component of protective hemostatic clots and pathological obstructive thrombi that largely determines their mechanical stability [1]. Molecular mechanisms of fibrin formation and its basic

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structural characteristics have been extensively studied [2–5]. Normally, fibrin networks form at sites of vascular injuries and perform a mechanical task of stemming blood flow by forming a gel, which also incorporates platelets and red blood cells [6]. Fibrin networks have been also used for numerous purposes of surgical repairs and tissue engineering as a biodegradable tissue adhesive or sealant to stop or control bleeding [7–9] or to form a provisional fibrin matrix for growing blood vessels and tissue repair [10]. Additionally, fibrin has been utilized for drug delivery applications [11], when drug molecules or factors are loaded in the fibrin gel via impregnation and tethering to the gel through covalent linkages or affinity-based systems.

Fibrin clots must withstand deformations and stresses generated in the blood stream as they are exposed to different external forces including pulsatile hydrodynamic stresses induced by oscillating blood flow, forces resulting from fluctuations of the blood vessel wall or due to platelet contraction leading to clot retraction [1]. Understanding how the mechanical response of the fibrin network is related to the network structural topology can provide the structural basis for biomechanics of fibrin-based blood clots and thrombi as well as engineered biomaterials.

The aggregate of forces that act on fibrin clots under various dynamic conditions *in vivo* can be segregated into shear, tensile, and compressive types with shear forces representing a complex combination of tension and compression [12, 13]. Shear stresses acting on a clot originate from the velocity gradient of the blood flow across the vessel lumen and have been shown to affect fibrin network structure [14, 15]. When exposed to shear or tensile stresses, fibrin networks display nonlinear mechanical responses [16 - 18] manifesting as a strain-stiffening behavior, i.e. an increase of the elastic modulus measured under shear or stretch as the magnitude of deformation increases.

Dynamic shear moduli of fibrin clots measured under moderate and large oscillatory deformations were systematically studied for clots with or without covalent ligation [19, 20]. These studies showed that the differential shear storage modulus can increase by a factor of 20 when shear strain increases from 1% to 50%. Strain-stiffening of plasma clots was addressed in [21], where it was demonstrated that the presence of platelets in fibrin gels decreased the degree of strain stiffening although significantly increased the storage modulus at low strains. The phenomenon of strain-stiffening was demonstrated not only at the whole clot level, but also at the level of individual fibers [22, 23]. It has been recently shown that nonlinear mechanical responses of networks formed from un-cross-linked fibrin continually change under repeated large-strain loading [12, 24]. Remarkably, the imposed shear loading resulted not in weakening of the underlying matrices but rather in delayed occurrence of the strain stiffening. Another common feature of fibrin clots is their negative normal stress response when exposed to the shear stress [25].

Fibrin clots have a number of remarkable mechanical properties that make them very different from other proteinaceous biopolymers [1]. Tensile experiments have shown that fibrin clots are highly extensible and can be stretched more than four times their relaxed length before breaking [26]. Moreover, stretching of fibrin networks resulted in fiber densification and aggregation accompanied by expulsion of water. These properties were

shown to result from protein unfolding [27], which was not observed in other matrix proteins such as collagen.

Despite the fact that a compressive deformation is inherent to (patho)physiological conditions, such as blood flow, vasoconstriction (contraction of the wall of the vessel), and clot retraction induced by platelets [28] and myofibroblasts [29], changes in clot elasticity and plasticity under compression and their structural origin are largely unknown. Currently, there are only few studies on how the structural and mechanical properties of blood clots are affected by compressive load. Litvinov *et al.* [30] recently demonstrated that compression as well as stretching of fibrin fibers was accompanied by transition of fibrin network microstructure and molecular unfolding followed by an increase in the fraction of β -sheets and a corresponding reduction of α -helices.

The goal of the present paper is to establish correlations between the mechanical response and corresponding structural changes of fibrin networks exposed to compression. We hypothesized that dynamically compressed polymeric fibrin, similar to shear- or stretch-induced deformations, have nonlinear mechanical behavior caused by structural rearrangements of the entire fibrin network as well as alterations of individual fibers. This hypothesis was tested by using a custom-built experimental setup, which combines a confocal microscope and a rotational rheometer. This instrument enabled us to explore shear viscoelastic properties of dynamically compressed fibrin clots in parallel with simultaneous structural changes at the levels of the entire filamentous network as well as single fibrin fibers. This combined approach revealed complex non-linear mechanical properties of fibrin gels originating from their structural rearrangements in response to compressive deformations.

Materials and Methods

Formation of Fibrin Clots

Fibrin clots were prepared by mixing pooled human citrated platelet-poor plasma with calcium chloride (40 mM final concentration) and human thrombin (from 0.1 to 1 U/mL). Alexa-Fluor 488-labeled human fibrinogen (Molecular Probes, Grand Island, NY) was added to the plasma samples (5% of total volume, 0.08 mg/mL final concentration) before clotting to visualize fibrin structure in a fluorescence confocal microscope. Plasma clots were allowed to form at the room temperature for 30 to 100 minutes in a gap between horizontal rheometer plates separated by a distance of 500–750 μm . Sample volume varied from 300 to 375 μL . The upper rheometer plate was a 20-mm-diameter acrylic disc and the lower plate comprised a 22-mm-diameter microscope glass cover slip permitting confocal imaging of the network in combination with rheological measurements. During clotting and measurements, a piece of wet filter paper was placed around the rheometer plates and periodically (each 20–30 minutes) sprayed with water to prevent sample drying. Before each experiment the surfaces were thoroughly cleaned and dried to assure firm attachment of fibrin clots. 30 fibrin clot samples were prepared as described and analyzed at various experimental conditions.

Compressive Deformations of Fibrin Clots

The 500–700- μm -thick fresh hydrated fibrin clots formed between horizontal plates of the rheometer were compressed vertically stepwise up to 1/10 of their initial thickness. Compression was performed in 5–25 μm steps at the rates of 10–30 $\mu\text{m/s}$, as the upper rheometer plate exerted an axial force on the upper surface of the clot. The degree of compression (compressive strain) was defined as the absolute fractional decrease in fibrin clot thickness, $\gamma = \left| \frac{\Delta L}{L_0} \right|$, where $L = L_0 - \Delta L$, and L_0 and L are the initial and reduced thickness dimensions of the uncompressed and compressed clots, respectively.

Rotational Rheometry of Fibrin Clots

Viscoelastic properties of fibrin clots were characterized using a Bohlin Gemini rheometer (Malvern Instruments, Westborough, MA). During a strain-controlled test, the rheometer imposed a sinusoidal oscillatory shear strain on the clot sample in the form of $\gamma = \gamma_0 \sin(\omega t)$, where ω is the frequency and γ_0 is the strain amplitude, and the shear stress σ required to impose such a deformation was measured. For a linear viscoelastic material, shear stress is a sinusoidal function with some phase shift δ , i.e. $\sigma = \sigma_0 \sin(\omega t + \delta)$, where σ_0 is the shear stress amplitude. The elastic response of the clot is characterized by the shear elastic modulus, G' , corresponding to the part of shear stress, which is in phase with strain and is calculated as $G' = (\sigma_0/\gamma_0)\cos(\delta)$. The viscous response of the clot to applied shear is measured by the shear loss modulus, G'' , calculated as the out-of-phase part of the stress as $G'' = (\sigma_0/\gamma_0)\sin(\delta)$.

To probe a clot's shear stress-strain response, the shear strain of 0.005 and oscillatory frequency of 1 Hz were used to produce a linear stress-strain response. The linearity was assured by running a shear strain and frequency sweep tests from 0.005 to 0.03 shear strain at 1 Hz and over the frequency range from 0.5 to 10 Hz. Oscillation tests started immediately after each subsequent step of deformation (compression or decompression) and were run for 60 s to 300 s depending on the normal force relaxation period. During each test, the storage modulus G' and the loss modulus G'' as well as the normal compression force were measured in combination with confocal microscopy of the compressed/decompressed clot. This allowed us to quantify the effect of fibrin clot deformations on its mechanical properties and structure for a single deformation event as well as for a multi-step deformation.

Confocal Microscopy of Fibrin Clots

To follow simultaneously and correlate structural and rheological changes of fibrin networks during deformations in the same clot sample, the oscillatory strain measurements and fluorescent confocal microscopy were performed using a custom-built “confocal rheoscope” which combined a Bohlin Gemini rheometer with a Nikon Eclipse 200/Confocal VT Eye microscope [31]. The experimental setup is shown schematically in Figure 1. It consists of two independent computer-controlled data acquisition systems for rheological measurements and confocal microscopy. The rheometer and confocal microscope are both mounted on a damped optical table, with the rheometer placed over the microscope on the horizontal rail mounting platform supported by four vertical damped posts. To allow for sample loading and coarse alignment of the two systems, the rheometer plate is allowed to move on rails

horizontally. To perform fine alignment of the rheometer plate, the sample holder, and microscope objective, the horizontal position of the sample holder is controlled by a two-axis ministage, while the tilt angle of the glass cover slip is tuned by means of a three adjuster kinematic mount. Confocal images of fibrin clot in z-stacks were collected after each compression/decompression step before each subsequent rheological measurement. We used Nikon CFI Plan 100x oil immersion objective lens (NA 0.5–1.3). A round 22-mm in diameter glass cover slip (thickness N1, 0.13–0.17 mm) was used as a bottom plate glued to a round aluminum holder. A laser beam with $\lambda = 488$ nm was used for fluorescent imaging. An objective piezo-actuator permitted collection of up to 30- μ m-thick z-stacks.

In a separate series of experiments, uncompressed and compressed fibrin clots were analyzed using the Zeiss LSM510 META NLO laser scanning confocal microscope to get higher resolution images with z-stacks spanning the entire clot thickness.

Image Analysis

We adopted and improved the image segmentation and analysis approach from [32] for identifying the topological structure of fibrin networks in z-stacks of confocal microscopy images. We designed an effective image segmentation scheme as well as additional centerline pruning steps to improve the robustness and accuracy of segmentation. As in [32], our algorithm consists of five main steps: Segmentation, 3D Thinning, Centerline Pruning, Branch Point Identification, and Network Analysis. Among these, 3D Thinning and Network Analysis in our algorithm are the same as the corresponding steps in [32], while other steps have some key differences. For details of the image analysis algorithm see Supplementary Information (Text S1).

Results

Mechanical Response of Fibrin Networks to Compression

Changes in fibrin network shear viscoelasticity with compression—To study the shear viscoelastic properties of uncompressed and compressed fibrin networks, we performed rotational rheometry of fibrin networks while they were under various degrees of compression. Changes in the viscoelastic properties of fibrin clots are shown in Figure 2A, in which relative shear elastic and loss moduli are plotted as a function of the compressive strain. Relative shear moduli (G'^* and G''^*) have been normalized by the initial values of G'_0 and G''_0 , i.e., $G'^* = G'/G'_0$ and $G''^* = G''/G''_0$. Figure 2A shows that both G'^* and G''^* display a characteristic non-linear behavior in response to compression. Typically, the shear elastic modulus (G'^*) of a fibrin network gradually decreased two-fold by $\gamma = 0.6$ – 0.8 compressive strain followed by a dramatic increase of the elasticity at the compressive strains $\gamma > 0.8$. At the maximal compressions ($\gamma > 0.9$), fibrin networks displayed more than a 10-fold increase in the shear elastic modulus compared to their uncompressed states. The loss modulus (G''^*) followed the same trend. First, G''^* exhibited four-fold decrease at $\gamma = 0.6$ – 0.8 compressive strains and then underwent up to 100-fold dramatic increase at higher degrees of compression ($\gamma > 0.9$).

To quantify the relative changes in viscous and elastic components of fibrin clot mechanics, we calculated the relative phase angle δ^* defined as $\delta^* = \delta/\delta_0$, where $\delta = \text{atan}(G''/G')$ and $\delta_0 = \text{atan}(G_0''/G_0')$. This parameter changed with compression nonlinearly, indicating an increase in the contribution of energy dissipative mechanisms of the network with compressive strains (Figure 2B). There are three regimes that can be distinguished in the plot of phase shift versus compressive strain: 1) a monotonic increase up to compressive strains of $\gamma = 0.10$ – 0.12 ; 2) a relative quasi-plateau over compressive strains of $\gamma = 0.12$ – 0.6 followed by 3) rapid up to 5-fold growth in δ^* at the compressive strain reaching $\gamma = 0.9$.

To study the effect of fibrin covalent cross-linking on the network response to compression, we repeated fibrin network compression experiments with inhibited factor XIIIa cross-linking transglutaminase activity. Factor XIIIa was inhibited by adding iodoacetamide (1 mM final concentration) to plasma before triggering fibrin polymerization with thrombin. Our results indicated no substantial difference in the behavior of cross-linked versus uncross-linked fibrin networks, revealing a similar softening-hardening transition of network shear stiffness in both cases (not shown).

Compressive (normal) stress-strain response of fibrin networks—As fibrin networks were compressed, a normal force was measured for each degree of compression and a corresponding normal stress was calculated as the force distributed over the smaller (upper) rheometer plate area. The typical plots of normal stress, σ , as a function of compressive strain, γ , (Figure 3) revealed three characteristic portions: 1) a nearly linear elastic regime at the low degrees of compression ($\gamma < 0.05$ – 0.15); 2) an elastoplastic plateau or softening up to $\gamma = 0.3$ – 0.5 ; and 3) a non-linear regime of stiffening observed at the higher degrees of compression ($\gamma > 0.5$).

Our experiments demonstrated that the compressive stress-strain curve depended on the initial clot stiffness as well as on the rate of compression (Figures 3B, C). An increase in both the initial clot stiffness (Figure 3B) and the size of each compression step (Figure 3C) resulted in a steeper growth of normal stress, σ , with compression. Increasing the initial clot stiffness G'_0 from 26 Pa to 90 Pa and the compression step from 20 μm to 50 μm , yielded 6 and 1.7 times higher average stress values, respectively. In addition, the data presented revealed a non-monotonic compressive stress-strain response at low compressive strains $\gamma < 0.2$, which was influenced by the initial clot stiffness as well as by the rate of compression. We noticed that increasing both clot elasticity (Figure 3B) and compression rate (Figure 3C) resulted in a more rapid upturn in normal stress curve, accompanied by shortening of the clot softening response, and earlier onset of the network stiffening regime. In addition, increasing the initial clot stiffness G'_0 from 26 Pa to 90 Pa, as well as compression step from 20 μm to 50 μm resulted in a 7 and 2.2 times shorter softening portions of the stress-strain curve, respectively, and consequently the clot stiffening started at lower compressive strains.

Thus, our findings demonstrated that variations in the initial (intrinsic) elasticity of fibrin clots, G'_0 , and the regime of externally applied compression can lead to quantitatively different normal stress-strain responses affecting the rate and the onset of clot compressive strain-softening and strain-hardening behavior. In other words, the clot normal stress-strain

behavior strongly depends on both initial elasticity and compression rate (Supplementary Information: Figure S2, Figure S3).

Stiffening of the fibrin network and hysteresis of elasticity revealed in repeated compression/decompression cycles

—To assess the mechanical response of clots exposed to repeated compressive/decompressive loads, we determined changes in the shear elastic modulus during repeated compression/decompression cycles. 650- μm -thick clots were vertically squeezed (pre-compressed) to various degrees of pre-compression, D , followed by their decompression (positive strain). Here, the degree of pre-compression, D , was defined as the ratio of the initial unperturbed clot thickness L_0 to its pre-compressed thickness L_{PC} , and the strain was defined as $\gamma^* = L/L_0$, where $L = L - L_0$ was the relative change in clot thickness with respect to its uncompressed thickness dimension. For each pre-compression degree D , we also introduced the normalized decompression strain $\gamma^{**} = \gamma^* - \gamma_{\text{PC}}$, where $\gamma_{\text{PC}} = (L_{\text{PC}} - L_0)/L_0 = 1/D - 1$, was the fibrin network pre-compression strain. To quantify shear elasticity we used the relative shear modulus $G'^* = G'/G'_0$, where G'_0 is the elastic modulus of an uncompressed clot. Additionally, to quantify the rate of clot hardening, we introduced the non-dimensional rate of stiffening, defined as the ratio of change in clot elastic modulus to change in strain, $q = G'^*/\gamma^*$.

After each compression/decompression step, continuous small-strain shear oscillations were imposed and the shear viscoelastic properties were measured. In a number of experiments, decompression was followed by a repeated compression (re-compression) to the thickness of the pre-compressed clot. An example of a typical compression/decompression cycle is shown in the Supplementary Information (Figure S4).

Repeated decompression-recompression cycles of pre-compressed fibrin clots revealed several trends (Figure 4). In each cycle, as the pre-compressed clot was stepwise decompressed, the relative elastic modulus, G'^* , gradually increased. At low strains, the elastic modulus was rather insensitive to decompression, hence reflecting a linear elastic response of pre-compressed fibrin networks to strain (Figure 4B). Further increase in decompression produced a rapid upturn of the elastic modulus of the fibrin clot, corresponding to the strain-stiffening regime. As the strain decreased during the recompression portion of the cycle, the relative elasticity of the clot gradually dropped and resulted in a closed hysteresis loop. This hysteresis behavior indicated energy dissipative mechanisms occurring in the fibrin network under cyclic loading.

We found that the elasticity of a clot as well as the degree of clot stiffening during the decompression portion of the cycle strongly depended on clot pre-compression and progressively increased with the pre-compression degree, D . In particular, as the degree of pre-compression rose from $D = 2.17$ to $D = 13$, the relative elastic modulus of the fibrin clot, G'^* , assessed at $\gamma^* = -0.5$ (Figure 4A) increased 44-fold. Additionally, the non-dimensional stiffening rate, q , evaluated for a 10% strain following the onset of hardening, increased 8.4-fold from $q=10$ at $D = 1.44$ to $q=84$ at $D = 13$. Moreover, the analysis of the initial portion of the normalized decompression-recompression cycles indicated (inset Figure 4B), that the onset of stiffening also depended on the pre-compression degree D and shifted to smaller normalized decompression strains, from $\gamma^{**}=0.2$ to 0, as D increased from 1.44 to 13.

Thus, our results revealed that each subsequent pre-compression/decompression/recompression cycle caused stiffening of the fibrin clot, accompanied by hysteresis behavior of the clot elastic modulus, increase in the clot stiffening rate, and shifting the onset of clot hardening to smaller strains.

Structural Changes in Fibrin Networks during Compression

To elucidate the origin of the unusual non-linear rheology of fibrin networks exposed to compressive loads, we followed structural changes of fibrin networks during deformations in the same clot samples that were subjected to rheometry. Structural transitions of the networks in response to compression were analyzed after reconstituting the three-dimensional structure of the networks obtained using a rheometer-coupled fluorescent confocal microscope [31]. We implemented a network analysis algorithm [32] extended and described in detail in the Supplementary Information (Text S1). The dynamic structure of the 3D fibrin network was characterized using the following set of parameters: network node density, ρ_n , fiber density, ρ_f , distributions of fiber segment length, $P_n(L)$, and fiber diameter, $P_n(d)$, fraction of bent fiber segments, α_b , fiber bending degree χ , and fiber network orientation tensor, Ω .

Fiber buckling and bending—Direct visual observations and analysis of the 3D structures of fibrin networks at different compressive strains revealed buckling of fibrin fibers along the direction of compressive force and increasing fiber bending as compression proceeded (Figures 5, see also Supplementary Video). The deformation of an individual fiber greatly depended on its orientation in the network. Fibers buckled and bent when oriented in the direction of compression, but remained straight when oriented in the compression plane.

Figure 5A shows reconstructed shape changes in the 3D traces of several selected fibers, oriented predominantly along the z axis and deformed as a result of applied compression from $\gamma = 0$ to $\gamma = 0.33$. It is seen from this figure that clot compression can result in formation of curved fibers. Bending of individual fibers in the network was accompanied by their re-orientation, sometimes leading to alignment of a fiber in the plane of compression. Figure 5B–E exemplifies an individual fiber experiencing progressive buckling and bending during increasing compressive deformation of the entire fibrin network.

To quantify bending of many fibers in the compressed fibrin network, we calculated the fraction of bent fibers and the degree of bending of individual fibers (Figure 6). The latter parameter was defined as the ratio of a fiber contour length and the shortest distance between the fiber ends (branch points). The fraction of bent segments first increased, reaching a peak of approximately 50% at $\gamma = 0.4$, and then dropped due to an apparent increase in the number of less curved fiber segments caused by fiber crisscrossing during network compression. The degree of fiber bending increased monotonically with compressive strain. However, the major changes in the degree of bending were observed when the compressive strain exceeded $\gamma = 0.4$ – 0.5 and reached a 5-fold increase at $\gamma > 0.8$.

Fiber network densification—To quantify changes of the density of the fibrin network during compression, we measured fiber and node numbers per volume at different

compressive strains. Structural analysis of deformed fibrin networks revealed that during progressive clot compression, both the network node and fiber densities increased nonlinearly (Figures 7A, B). As the fibrin clot was compressed from $\gamma = 0$ to around $\gamma = 0.4$, the network node density, ρ_n , changed slightly, while at larger compressions ($\gamma > 0.4$), ρ_n gradually increased, reaching 10-fold higher values at $\gamma > 0.8$ as compared to the uncompressed network node density (Figure 7A). Fiber density as a function of compressive strain exhibited a similar trend: ρ_f did not change significantly until $\gamma = 0.4$, but became increasingly pronounced at larger strains ($\gamma > 0.4$) demonstrating 7- to 8-fold increase at $\gamma > 0.8$ (Figure 7B).

As a result of network densification, there was a remarkable change in the apparent degree of branching upon compression, i.e. in the number of nodes connected with 3 or 4 fibrin fibers or their segments, named 3- and 4-degree nodes, respectively. However, it should be noted that compression of the 3D fibrin network could result in mechanical criss-crossing of fibrin fibers that would show up as an additional increase in the fraction of 4-degree nodes. We found that as the network was subjected to compressive deformations, the degree of branching did not essentially change at the compressive strains $\gamma < 0.4$, but underwent gradual changes at the larger degrees of compression, $\gamma > 0.4$. Particularly, we quantified variations in the number of branched and criss-crossed fibers by measuring the corresponding density of 3- and 4-degree nodes of the network. Our analysis revealed a significant increase in the formation of criss-crossing fibers at $\gamma > 0.4$. As evident from Figure 7C, the 3- and 4-degree node densities increased at different rates, which led to intersection of density curves at around $\gamma = 0.7$. Importantly, this compressive strain corresponded to the dramatic viscoelastic changes revealed by the rotational rheometry (Figure 2). Further prevalence of the 4-degree over 3-degree node density at $\gamma > 0.7$ likely represents multiple oblique contacts of fibers as the network densification proceeds.

Fibrin network orientation—To quantify the fibrin network orientation and how it changes with compression, we calculated the network orientation tensor based on individual fiber orientation vectors (Table 1). The initially uncompressed sparse fibrin network was composed of nearly straight fibers having a small degree of orientation anisotropy, which was probably due to the wall effects of glass-polymer interfaces (see Table 1, $\gamma = 0$). As the network was compressed, the orientation tensor reflecting the network structure orientation changed: as compressive strain increased from $\gamma = 0$ to $\gamma = 0.88$, the value of the principal component along the direction of compression dramatically decreased about 2-fold from 0.41 to 0.21, while values of the two other principal components increased 1.3 and 1.4 times from 0.27 to 0.36 and from 0.32 to 0.44, respectively (see also Supplementary Information Text S2). To assess how network orientation changes with respect to the direction of compression, we calculated a mean angle between fibers and the direction of compression, $\langle \theta \rangle$, which increased from 50.2° ($\gamma = 0$) to 60.3° ($\gamma = 0.8$). Taken together, our results showed that compression resulted in re-orientation of fiber network towards a planar structural configuration perpendicular to the direction of compression.

Fiber thickness and length distributions—Fibrin network image analysis showed that the thickness of fibrin fibers had a log-normal distribution and that variations of the most

probable fiber diameter from 220 to 230 nm (peak positions) with compression were within an experimental error (Figure 8A). Fiber diameter histograms are shown in Figure S5 (Supplementary Information).

The length of fibrin fibers or their segments in the uncompressed and compressed fibrin networks also followed a log-normal distribution, $P_n(L)$ (Figure 8B). As the network deformed, the segment length distribution narrowed with the increasing compressive strain as the tail of the distribution was pushed to smaller segment lengths. The most probable segment length, corresponding to the peak in the distribution, $P_n(L)$, shifted from 1.32 μm to 1.02 μm when γ changed from 0 to 0.88. This shift to shorter segments likely resulted from formation of buckled and bent fibers as well as multiple oblique contacts between fibers.

Thus, our measurements indicated that compression of a fibrin network over compressive strains from $\gamma = 0$ to $\gamma = 0.9$ did not result in any significant variations in the distribution of fiber thickness, but caused shortening of fibrin network segments, which was reflected in shifting the peak and the tail of the segment length distribution to shorter segments (Figure 8B).

Discussion

Fibrin networks display non-linear softening-stiffening in response to compression

We have shown that a fibrin network exposed to external compression revealed a non-linear shear viscoelastic response manifested by the network softening followed by its dramatic stiffening. In a stress-softening regime, occurring at low and intermediate compressions, the elastic and loss moduli of the network gradually decreased two- and four-fold, respectively. In the stress-stiffening regime, at the higher compressions, the elasticity of the fibrin network increased with applied compression and achieved an almost 10-fold increase at compressive strains exceeding 0.8. Meanwhile, the loss modulus increased almost 100-fold with respect to the modulus of the uncompressed network (Figure 2).

Previous studies suggested that stress-stiffening of fibrin clots under shear and stretching loads originate from single fiber stiffening accompanied by redistribution of load over the network structure [32 – 35]. In addition, stress-softening under transverse shear was attributed to network rupture and fiber lengthening due to slippage between protofibrils or the breakage of knob-hole bonds [19, 36, 37]. Unlike the shear deformation, compressive stress-softening of the fibrin network at low strains cannot be attributed to network damage and fiber lengthening because these would reflect permanent alterations in the network structure leading to irreversibility of changes in the elasticity (plasticity) of the network. In contrast, our fibrin network compression-decompression experiments revealed reversible softening of networks exposed to low strains (Figure 4A). In addition, compressive stress-stiffening at high strains can be only partially explained by individual fiber stiffening in the plane normal to compression. To elucidate the origin of the fibrin network softening-stiffening transitional response to compression, we performed structural analysis of reconstructed 3D fibrin networks whose viscoelastic properties were simultaneously measured with a rheometer.

The observed compressive normal stress-strain behavior resembles the compressive response of polymeric foams [38] and liquid crystal elastomers [39]. As compressive strain increases, they consequently reveal the linear regime, viscoelastic plateau, and stiffening regime. Some polymeric foams exhibit certain similarities in structure [40]; therefore buckling can be expected. However, these systems are different from fibrin gels. Polymeric foam combines a solid and a gas phase and has open-cell morphology. Polydomain liquid crystals behave like a liquid with some degree of ordering of its molecules. Our results at low compressive strains are consistent with those obtained for actin filament networks [41], demonstrating a non-linear response to compression reflecting stiffening-softening behavior. As in compressed actin networks, it is likely that both entropic and enthalpic resistance components of fibrin filaments play important roles as compressed fibrin networks undergo a softening-stiffening transition.

Our experiments also revealed that repeated compression-decompression cycles resulted in hysteresis of the fibrin network elasticity and intensified stiffening of the network exposed to intermediate and large degrees of pre-compression and subsequent extension and re-compression (Figure 4). A plausible explanation of formation of hysteretic loops of the network elasticity is that the network fibers undergo transition from a loose to a tightened state, due to fiber straining, and back to a loose state as interfiber connections dissociate under progressive loading. Intensified stiffening can be attributed to crisscrossing and tangling of fibers with subsequent network pre-compression, which result in a new network structure of increased connectivity and enhanced elasticity.

Structural alterations underlying softening-stiffening transition in compressed fibrin networks

Our structural observations demonstrate that upon compression, individual fibrin fibers in the network begin to buckle and bend in the direction of compression. This is consistent with a model assuming that the observed mechanical properties are the result of the combination of compression, extension and bending (Figure 9). As compression proceeds over small and intermediate compressive strains, more fibers of the network buckle and bend, thus reducing the amount of load-sustaining network elements. Upon buckling and subsequent bending, fibers become more compliant [42], and as a result, the elasticity of the network in the transverse shear direction gradually decreases with compression. Since the interconnected structure of the network does not allow individual fibers to completely collapse upon buckling, fibers can unbuckle after recompression resulting in a reversible softening at low strains, justified by our experiments on decompression-recompression of the network (Figure 4A).

At large degrees of compression the number of criss-crossed fibers dramatically increases (Figure 7C). In addition, fibers undergo re-orientation, transforming the whole network architecture to a planar-like structure aligned in a plane normal to the compression direction (Table 1). A new structure composed of shorter fiber segments and aligned in a shear plane has larger shear elasticity, since the averaged strain of individual fibers is larger compared to the strains induced in an uncompressed sparse network composed of fibers oriented randomly in a three dimensional space. The shear loss modulus increases at large

compressions due to an increase in the number of fibers sliding over each other at the cross points, thus inducing intra- and inter-fiber friction and, therefore, increasing the energy dissipation revealed in oscillatory shear stress measurements.

As compression proceeds and the fibrin network density increases, fibers reorient in the compression plane and shorter fiber segments, experiencing higher strains, are formed as a result of crisscrossing. At higher compressions, fibers are further forced into each other, enhancing their linkage at crisscrossing points. Thus, the elasticity increases as a result of entropic resistance to fiber extension in the direction normal to the direction of compression and owing to resistance to indentation of fibers into each other.

Previous studies demonstrated that covalent cross-linking of fiber networks with factor XIIIa controls whether the mechanical bulk properties of fibrin networks shift under shear loading or remain fixed. Factor XIIIa activity is associated with increased stiffening of individual fibers and the whole clot and with improved resistance of fibrin clot to lysis. In addition, it was recently shown [12] that factor XIIIa might have another function of modulating the workability of uncompressed fibrin exposed to large amplitude shear strains. Our results reveal that in compressed fibrin networks, covalent cross-linking did not affect the qualitative behavior of softening-stiffening transition of shear elastic modulus. This allows us to infer that the qualitative fibrin network mechanical response to compression at low and intermediate compressive stresses is predominantly modulated by the fibrin network structural architecture as a whole rather than by the mechanical properties of individual fibers. Meanwhile, one can expect increased structural changes occurring at the intrafiber level at very high compressions, when the structure of an individual fiber undergoes significant alterations accompanied by protein unfolding and transition from α -helices to β -sheets [30].

Structural metrics reveal log-normal distributions of fiber thickness and length

Previous studies on fibrin clot structural morphology considered unperturbed clots and provided different type of metrics extracted either from confocal microscopy [32, 43] or stereoscopic intermediate electron microscopy [44] images. These metrics included fractal dimensions of static fibrin structures [43], average length and thickness of fibers and network porosity. However, detailed quantification of a fibrin network structural dynamics has not been performed. In the present study we used a constitutive set of metrics to characterize in detail dynamic structural changes occurring in fibrin networks under compression. These metrics included: the length and thickness of fibers, the number and type of network nodes, the bending degree, and the orientation tensor.

Our fibrin network structural analysis provided experimental evidence of a log-normal fiber length distribution, which is consistent with fiber length measurements performed by [43] for unperturbed fibrin networks. In addition, we found that fiber thicknesses are also log-normally distributed. Moreover, for both metrics, the type of distributions was conserved with compression. Perhaps, the observed log-normal distributions characterizing the networks originate from fibrin polymerization and might be related to the interplay in population of different types of trifunctional branches associated with tetramolecular and trimolecular branchpoints [45]. The ubiquity of the log-normal distributions can be a solid

basis for models of fibrin network structural mechanics, and emphasizes the unacceptability of a Gaussian type distribution of fibrin fiber lengths and thicknesses.

In summary, getting insight into the mechanical behavior and properties of fibrin networks under compression is important for understanding clot dynamics when exposed to various physiological and pathological conditions. Our results provide direct evidence of the structural origin of fibrin clot mechanical response to compressive loads, which have not been considered so far. Our findings are also important for understanding mechanisms of cell-fibrin interaction during normal and impaired clot formation. How the stiffness of the fibrin network affects cellular contractile strength is not well addressed [46 - 48]. Our results suggest that depending on the degree of compression, deformation of the fibrin matrix can either slacken or improve the mechanical functioning of platelets during clot retraction. Thus, alteration of fibrin properties may potentially provide a means for intervention into thrombosis and development of design principles for enhanced biomaterials.

Conclusions

In this study, we have shown that *in vitro* human plasma clots exhibit a non-linear mechanical response to external compression. Using a combination of confocal microscopy and rheological measurements in the same clot samples, we demonstrated that these non-linear mechanical properties originated from structural rearrangements of the entire fibrin network, as well as alterations of individual fibers including fiber buckling, bending and reorientation. Measurements of elastic and loss shear moduli of fibrin networks revealed dual softening-hardening transitions as the networks were exposed to compressive loads, with softening occurring at small and intermediate compressive strains, while hardening developing at larger degrees of compression. We demonstrated that the softening of fibrin network occurred as a result of buckling and bending of individual fibers upon compression. The network hardening strongly correlated with an increase in the number of intersecting fibers, resulting from densification of the compressed network and reorientation of the whole fibrillar network toward a planar structural architecture perpendicular to the direction of negative strain. Our findings suggest that entropic and enthalpic responses of fibrin fibers to compression play a significant role in elastic, softening and stiffening regimes occurring during compressive deformations of fibrin clots.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

1. Viscoelastic properties of fibrin networks under compression reveal dual softening-hardening transitions.
2. Structural modulations underlie nonlinear mechanical responses of compressed fibrin networks

Figure 1.

Experimental setup combining rheometry and confocal microscopy to measure structural mechanics of human plasma fibrin clots. Rheological and structural data were simultaneously collected and stored independently by the coupled systems of data acquisition.

Figure 2.

(A) Relative elastic and loss moduli, G'^* and G''^* , respectively, as a function of compressive strain. Here, $G'^* = G'/G'_0$ and $G''^* = G''/G''_0$, where G'_0 and G''_0 are the initial elastic and loss moduli of uncompressed clots, while G' and G'' comprise the elastic and loss moduli, respectively, at different degrees of compression. The inset figure represents the relative moduli-compressive strain dependence in a semi-logarithmic scale for compressive strains from $\gamma = 0.75$ to $\gamma = 0.9$. (B) A measure of the viscosity/elasticity ratio of fibrin clots, the relative phase angle, $\delta^* = \delta/\delta_0$, as a function of compressive strain γ , where $\delta = \text{atan}(G''/G')$ for the compressed and $\delta_0 = \text{atan}(G''_0/G'_0)$ for the uncompressed clots, respectively ($M \pm \text{SD}$). Each plot shown in Figure 2 is averaged over 3 fibrin clot samples prepared under the same conditions and having very similar initial values of G'_0 and G''_0 ($M \pm \text{SD}$).

Figure 3.

(A) Normal stress, σ , as a function of compressive strain, γ , of fibrin clots corresponding to the changes in viscoelasticity shown in Figure 2. (B) Normal stress, σ , as a function of compressive strain, γ , for two different fibrin clots with the initial elasticity (G'_0) of 26 and 90 Pa. The inset bars show the mean normal stress values for the clots with two different G'_0 values averaged over compressive strains from $\gamma = 0.4$ to $\gamma = 0.6$ ($n=4$). (C) Normal stress, σ , as a function of compressive strain (γ) for two different compression steps (Δh) of 20 and

50 μm . The inset bars show the mean normal stress values for two compression steps averaged over compressive strains from $\gamma = 0.4$ to $\gamma = 0.6$ ($M \pm SD$, $n=4$).

Figure 4.

Fibrin clot pre-compression/decompression/recompression cycles performed for different decompression amplitudes and for various degrees of clot pre-compression, D . Here, $D = L_0/L_{PC}$, where L_0 and L_{PC} are the thickness dimensions of uncompressed and pre-compressed clots, respectively. The relative elastic modulus of fibrin networks (**A**) as a function of decompressive strain, γ^* , and (**B**) as a function of shifted (normalized) decompressive strain (γ^{**}). The different pre-compression degrees are shown by different colors and symbols (upper right inset key). Arrows show the directions of decompression and re-compression in a single cycle. **Figure B**, left inset, shows the relative elastic modulus, G^{**} , non-dimensionalized with respect to fibrin clot pre-compression thickness in each cycle, versus shifted (normalized) strain γ^{**} . The arrows show direction of decompression stiffening for different cycles.

Figure 5.

(A) Examples of fiber buckling and bending in Z direction as a result of the fibrin network vertical compression. Four reconstructed individual fibers from different parts of the network are shown in different colors before ($\gamma = 0$, dashed lines) and after ($\gamma = 0.33$, solid lines) the compression. (B–E) Structural changes of a fibrin network under compression shown as a z-projection of confocal images of the network z-stack for different compressive strains: $\gamma = 0$ (B), $\gamma = 0.18$ (C), $\gamma = 0.38$ (D), and $\gamma = 0.53$ (E). The blue boxes highlight an individual fiber experiencing a progressive buckling and bending deformations. As the degree of network compression increases, the individual fiber appears increasingly bent and the entire network becomes denser. All images are x-y projections of z-stack images of a clot volume of $35.8 \times 35.8 \times 25.5 \mu\text{m}$.

Figure 6.

Fraction of bent fibers and their segments, α_b , and the fiber bending degree, χ , as a function of compressive strain γ . Here, α_b is defined as the ratio of the number of bent fibers to the total number of fibers analyzed, and $\chi = l_c/l_f$, where l_c is a fiber contour length and l_f is the shortest distance between the fiber end-nodes.

Figure 7.

Densification of the fibrin network upon compression. (A) Fibrin network node density, ρ_n , and (B) fibrin network fiber density, ρ_f , as a function of compressive strain, γ , ($M \pm SD$, $n = 3$). (C) Increasing branching in the fibrin network upon compression. 3-degree (circles) and 4-degree (squares) node densities in fibrin networks as a function of compressive strain. The corresponding types of fiber connectivity are schematically shown.

Figure 8.

Fibrin fiber diameter (**A**) and fibrin network segment length (**B**) probability distribution functions, $P_n(d)$ and $P_n(L)$, for various applied compressive strains, γ . Fiber length histograms and length distributions of three- and four-degree connectivity fibers and are provided in Figure S6 and Figure S7 in Supplementary Information.

Figure 9.

The stress-softening in fibrin networks exposed to compression can originate from fiber buckling and bending shown here schematically (A→B). Stress-hardening can arise owing to fiber resisting extension and buckling of filaments resisting normal compression as well as due to densification of the network resulting in the increase of criss-crossing fibers (B→C). Here, σ is the normal compression stress, γ is the compression strain, and γ' is the compression strain characterizing softening-hardening transition.

Table 1

Fibrin network orientation tensor for different compressive strains visualized by an orientation ellipsoid. The shape of the ellipsoid is defined by principal components and principal values, calculated for each of the fibrin network structural configuration (see Supplementary Information Text S2). As compression proceeds from $\gamma = 0$ to 0.88, fibrin network undergoes structural transitions resulted in planar re-orientation of the network architecture.

Compressive Strain, γ	0	0.38	0.53	0.88
Fibrin Network Orientation Tensor
$\langle \cos^2\theta \rangle$	0.41	0.39	0.38	0.21