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Recombinant fibrinogen reveals the differential roles of α- and γ-chain cross-linking and molecular heterogeneity in fibrin clot strain-stiffening

I. K. Piechocka,*[,†,¶] N. A. Kurniawan,*[,‡,¶] J. Grimbergen,[§] J. Koopman[§] and G. H. Koenderink*

* Department of Systems Biophysics, FOM Institute AMOLF, 1098 XG Amsterdam, The Netherlands

[†] ICFO-Institut de Ciencies Fotoniques, 08860 Castelldefels (Barcelona), Spain

[‡] Department of Biomedical Engineering & Institute Complex Molecular Systems, Eindhoven University of Technology, 5600 MB Eindhoven, The Netherlands

[§] ProFibrix BV, 2333 CR Leiden, The Netherlands

[¶] Authors contributed equally

Essentials

- Fibrinogen circulates in human plasma as a complex mixture of heterogeneous molecular variants.
- We measured strain-stiffening of recombinantly produced fibrinogen upon clotting.
- Factor XIII and molecular heterogeneity alter clot elasticity at the protofibril and fiber level.
- This highlights the hitherto unknown role of molecular composition in fibrin clot mechanics.

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Summary

Background: Fibrin plays a crucial role in haemostasis and wound healing by forming strain-stiffening fibrous networks that reinforce blood clots. The molecular origin of fibrin's strain-stiffening behavior remains poorly understood, primarily because plasma fibrinogen is a complex mixture of heterogeneous molecular variants and is often contaminated by plasma factors that affect clot properties.

Objectives and Methods: To facilitate mechanistic dissection of fibrin nonlinear elasticity, we produced a homogeneous recombinant fibrinogen corresponding to the main variant in human plasma, termed rFib610. We characterized the structure of rFib610 clots using turbidimetry, microscopy, and x-ray scattering. We used rheology to measure the strain-stiffening behavior of the clots and determined the fiber properties by modeling the clots as semiflexible polymer networks.

Results: We show that addition of FXIII to rFib610 clots causes a dose-dependent stiffness increase at small deformations and renders the strain-stiffening response reversible. We find that γ-chain cross-linking contributes to clot elasticity by changing the force–extension behavior of the *protofibrils*, whereas α-chain cross-linking stiffens the *fibers*, as a consequence of tighter coupling between the constituent protofibrils. Interestingly, rFib610 protofibrils have a 25% larger bending rigidity than plasma-purified fibrin protofibrils and a delayed stretch-stiffening, indicating that molecular heterogeneity influences clot mechanics at the protofibril scale.

Conclusions: Fibrinogen molecular heterogeneity and FXIII affect the mechanical function of fibrin clots by altering the nonlinear viscoelastic properties at the protofibril and fiber scale. This work provides a starting point to investigate the role of molecular heterogeneity of plasma fibrinogen in fibrin clot mechanics and haemostasis.

Keywords: blood coagulation; elasticity; fibrin; polymers; rheology; turbidimetry

Running title: Recombinant fibrinogen and fibrin clot mechanics

Correspondence:

Gijsje H. Koenderink

FOM Institute AMOLF, Science Park 104, 1098 XG Amsterdam, The Netherlands.

Tel.: +31 20 7547100; fax: +31 20 7547290. E-mail: gkoenderink@amolf.nl

Introduction

Fibrinogen is a fibrous protein that assembles into an elastic fibrin network upon vascular injury [1]. Biophysical studies have shown that fibrin networks can stiffen up to 1000-fold when sheared or stretched [2,3] and the constituent fibers can be stretched up to four-fold their original length without breaking [4]. These extraordinary nonlinear elastic properties appear to be crucial for clot function, since mutations in fibrinogen or its regulatory factors that affect fibrin mechanics are associated with severe clotting disorders [5].

The structural origin of fibrin's nonlinear elastic behavior has been difficult to elucidate due to fibrin's complex hierarchical structure. Fibrinogen *monomers*, which are built from Aα, Bβ and γ chains, assemble into double-stranded *protofibrils* by non-covalent interactions [6], and these bundle into *fibers* that branch and percolate to form space-spanning *networks* (Figs. 1A,2A). The key challenge is therefore to separate contributions from the molecular, protofibril, fiber and network scales to fibrin clot mechanics. This challenge has been partly met by characterizing individual fibrin fibers and molecules. Tensile tests on single fibers revealed that their extensibility [7,8] and elasticity [9] is correlated with the length of αC connectors,

suggesting that the elastomeric behavior of fibrin clots originates from the composite structure of the fibers, with rigid, folded domains interconnected by flexible linkers [10]. However, force spectroscopy [11,12] and molecular simulations [13] of fibrin molecules showed that forced unfolding of the coiled-coil domains and the γ chains can also contribute. Further, fibrin's mechanical properties *in vivo* crucially depend on the presence of Factor XIII (FXIII), which catalyzes covalent cross-linking between monomers [14]. Cross-linking increases the stiffness of fibrin networks [15-17] and prevents irreversible deformation of the fibers [18]. Since FXIII forms γ-chain cross-links within protofibrils as well as α-chain cross-links between protofibrils [19], it probably changes both the properties of the protofibrils and the interactions between them.

An important obstacle in tracing fibrin clot mechanics down to the molecular scale is that fibrinogen purified from human plasma, used in most studies, has heterogeneous molecular composition arising from partial proteolytic degradation of the Aα chains, resulting in three main fibrinogen variants [20-22], and alternative splice variations [23,24]. These variants result in clots with marked differences in fiber diameter and network structure [22,25,26]. Purified plasma fibrinogen furthermore often contains variable amounts of other plasma proteins such as FXIII and fibronectin [27].

To provide a more controllable experimental system, following the pioneering work of Gorkun *et al* [28], we here report the production and characterization of recombinant human fibrinogen having intact Aα chains with 610 amino acids, corresponding to the main constituent of plasma fibrinogen. We for the first time produce the recombinant protein, termed rFib610, at a scale large enough to allow systematic study of the nonlinear viscoelasticity of reconstituted clots, thus complementing previous studies that focused on the linear (micro)rheology of

recombinant fibrin networks [29] and the mechanics of single fibers [10,18]. We demonstrate that FXIII-mediated γ- and α-chain cross-linking differentially act at the protofibril and fiber levels to stiffen fibrin clots and suppress plastic deformation. Additionally, we uncover the influence of molecular heterogeneity on the force–extension behavior of individual protofibrils.

Material and Methods

Recombinant fibrinogen production

cDNAs for the individual fibrinogen chains were cloned in separate pcDNA3.1(+) expression vectors. The Aα-chain cDNA (629 residues), Bβ-chain cDNA (491 residues) and γ-chain cDNA (437 residues, isoform γA) resulted in the production of mature Aα, Bβ and γ chains as found in circulation (610, 461 and 411 residues, respectively). rFib610 was produced in PER.C6® cells using a fed-batch procedure in a stirred-tank reactor with CDM4PERMAb/PERMAab feed, as described previously for IgM [30]. During expansion period, the culture supernatants were screened for fibrinogen with intact Aα chains [31], with plasma-derived fibrinogen (pFib) from Enzyme Research Laboratories (ERL; Swansea, UK) as reference. Recombinant fibrinogen (rFib610) was purified from the clarified cell-culture medium using cationic and anionic column chromatography (details are not disclosed for proprietary reasons) and was concentrated to 15–25 mg mL^{-1} in PBS by diafiltration [32], with a typical yield of 0.3–0.4 g per liter of cell culture supernatant. Both rFib610 and pFib were dialyzed into HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) against 100× sample volume. The final protein concentration was measured by spectrophotometry and confirmed by total protein measurements with a modified Biuret method using Pierce BCA Protein Assay kit (Thermofisher). Glycosylation and gel filtration analyses

reveal similar extents of N-linked glycosylation and a small (6–9%) fraction of oligomers in rFib610 and pFib (Figs. S1,S2).

Fibrinogen molecular composition, fibrin formation and cross-linking

Fibrin clots were obtained by adding 1 U mL^{-1} human α-thrombin (ERL) to fibrinogen in HBS buffer supplemented with 2 mM $CaCl_2$ and incubating for 4 h at 37°C unless indicated otherwise. To access the response of fibrin protofibrils, we also assembled clots under *fine* clot buffer conditions (50 mM Tris-HCl, pH 8.5, 400 mM NaCl) [33] in the presence of 3.2 mM $CaCl_2$ and 0.5 U mL^{-1} thrombin at 37°C for 4 h. The molecular composition and degree of cross-linking of the samples were evaluated by sodium-dodecyl-sulfate polyacrylamide-gel-electrophoresis (SDS-PAGE) on 8% gels. Cross-linked rFib610 clots were obtained by adding purified human FXIII zymogen (ERL) to the assembly solution containing thrombin and calcium. We used reversed-phase high-performance liquid chromatography (RP-HPLC) to analyze fibrinopeptide release upon thrombin activation [34].

Multi-scale structural characterization

We characterized the *network* structure of fibrin clots by imaging clots with a confocal laser scanning microscope (Nikon Eclipse Ti, Amsterdam, Netherlands) using 488 nm laser (Coherent Inc., Utrecht, Netherlands) for illumination and photomultiplier tube detector. The clots were fluorescently labeled by co-polymerizing fibrinogen with 10 mole% Oregon-Green-488-labeled fibrinogen. Three-dimensional z-stack images were obtained with 100× oil-immersion objective (NA = 1.49). The network structure was examined in higher resolution by scanning electron microscopy (SEM; Zeiss Supra 25; Fig. S3).

We characterized fibrin *fiber* structure by turbidity measurements using UV1 Spectrophotometer (Thermo Optek). We computed the average diameter, d, and mass/length ratio, μ, of the fibers from the data using a theoretical model for light scattering from isotropic networks of rigid rod-like particles [15,35,36], with correction for wavelength dispersion [37] (see Fig. S4). The samples were polymerized for 4 h in humid, 37°C conditions in cuvettes (UV-Cuvette micro, Plastibrand) with an optical path length of 1 cm. The early-stage kinetics of fibrin polymerization was monitored using an Ultrospec 2100 Pro (Amersham Biosciences, NY) spectrophotometer. Comparative tests with colloidal samples and different optical path lengths confirmed that multiple scattering was negligible in our measurements.

The axial *molecular* packing order of the fibrin fibers was examined by small angle X-ray scattering (SAXS) measurements at the DUBBLE beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) on fibrin samples polymerized in 2-mm diameter glass capillaries (Hilgenberg, Malsfeld, Germany). Incoming x-ray beam (12 keV) was focused to a 300×350 μm spot and scattering patterns were collected with 1 min exposure at r.t. using a Pilatus 1M detector at a sample-to-detector distance of 2.8 m. The patterns were background-corrected for glass capillary and buffer and radially-averaged to obtain scattering curves of intensity I as a function of wavevector q. The q-range was calibrated using silver behenate powder as reference.

Rheology

Fibrin samples were polymerized *in situ* in the steel cone–plate geometry (40 mm diameter, 1° cone angle) of a rheometer (Physica MCR 501, Anton Paar, Graz, Austria) preheated to 37°C. The linear shear moduli of fully-formed clots were measured by applying 0.5% oscillatory strain

at a frequency of 0.5 Hz. To quantify the nonlinear response, we utilized differential measurements [38]: the sample was held at constant stress, τ, and the differential strain response, $\delta\gamma$, to a superposed stress oscillation of amplitude $|\delta\tau| = 0.1\tau$ was measured. To test the elastic limits of the samples, we performed (i) *consecutive differential measurements* by iteratively increasing the prestress τ to a maximum value τ_m and releasing it and (ii) *consecutive creep tests* by monitoring the strain, γ, in response to a constant stress for 60 s followed by stress release.

Results

Compositional and structural characterization of rFib610 fibrin

We first validate the homogeneous composition of rFib610 fibrinogen using SDS-PAGE. As expected, we observe three bands corresponding to the Aα (66 kDa), Bβ (52 kDa) and γ (47 kDa) chains (Fig. 1B). Addition of thrombin causes a slight shift of the Aα- and Bβ-chain bands towards lower MW, indicating successful cleavage of FpA and FpB. A weak band also appears below the main α-chain band, indicating a small degree of proteolytic degradation [28]. There are no high MW bands, proving the absence of FXIII. In contrast, fibrin prepared by thrombin activation of plasma fibrinogen (pFib) displays clear high MW bands originating from covalently cross-linked α-chain polymers and γ-dimers [39,40] and two minor bands that are absent in rFib610.

Next, we analyzed the fibrinopeptides released from fibrinogen E-region using RP-HPLC. The HPLC profile of rFib610 (Fig. 1C, *red trace*) shows the main FpA and FpB peaks at retention times of 38 and 55 min, respectively, and a minor FpB desArg peak eluting at 48 min. These peaks are identical to those found with pFib (Fig. 1C, *black trace*) [41]. However, rFib610 is free of the two FpAP and FpAY minor peaks commonly seen with pFib [34]. Thus, rFib610

possesses high degree of molecular homogeneity, in contrast to the heterogeneity of pFib. Kinetic analysis of fibrinopeptide release shows a fast release of FpA followed by slower release of FpB (Fig. S5A), consistent with prior reports on plasma-derived fibrinogen [42,43]. FpA release allows for the formation of double-stranded protofibrils, followed by non-covalent protofibril bundling into thicker fibers facilitated by αC connectors that dissociate from fibrinogen E-region upon release of FpB [44,45]. Indeed, upon thrombin addition the turbidity of rFib610 quickly rises (Fig. S5B), reflecting the bundling of protofibrils into thick fibers that strongly scatter light.

We then performed a multi-scale structural analysis of rFib610 clots over a range of concentrations, from 0.8 to 5 mg mL^{-1}, spanning the physiological levels of fibrinogen in plasma (2–4 mg mL^{-1}). At the *network* scale, confocal microscopy and SEM show homogeneous, isotropic networks (Figs. 2B,S3). The average pore diameter determined from analysis of the confocal images monotonically decreases with increasing fibrin concentration, from 4.7±0.4 μm at 0.8 mg mL^{-1} to 1.5±0.2 μm at 5 mg mL^{-1} (Fig. S6). This 3.1-fold reduction is larger than the 2.5-fold reduction expected from the inverse-square-root dependence of pore size on concentration if concentration only affects the fiber number density [46], suggesting increased protofibril lateral association with increasing fibrin concentration, perhaps due to the changing thrombin/fibrinogen ratio. This hypothesis is supported by turbidity measurements of the average *fiber* mass/length ratio, μ (Fig. 2E). Given a protofibril $\mu = 1.44\times10^4$ Da nm^{-1} [47], the average number of protofibrils within each fiber, N_p, can be calculated to increase from 60 at 0.8 mg mL^{-1} to 95 at 5 mg mL^{-1}, accompanied by an increase of fiber diameter from 130 nm to 180 nm (Fig. 2D). At the *molecular* scale, SAXS measurements show that rFib610 fibers exhibit a peak at $q \sim 0.85$ nm^{-1} that corresponds to the 3rd order reflection of the axial repeat distance of 22.5

nm (Figs. 2A,2C,S7) [2]. Thus, rFib610 fibers possess the same half-staggered molecular packing order as pFib fibers.

Nonlinear rheology of uncross-linked rFib610 clots

The purity of rFib610 preparation makes it an ideal model system to study the rheological behavior of fibrin clots in the strict absence of FXIII-mediated cross-linking, providing a more stringent benchmark than obtainable for plasma-derived fibrinogen that requires FXIII removal [48,49] or inhibition [15,50-52]. We first characterized the viscoelastic response of uncross-linked rFib610 clots at small deformations. The elastic modulus G' is nearly independent of frequency and the ratio between the viscous and elastic moduli, $\tan \delta = G''/G'$, is much less than 1 for all fibrin concentrations studied (Fig. 3A). The linear modulus G_0 strongly increases with increasing fibrin concentration, from 10 Pa at 0.8 mg mL^{-1} to 500 Pa at 5 mg mL^{-1} (Fig. 3B), while $\tan \delta$ decreases from 0.10 to 0.02, indicating suppression of viscous dissipation (Fig. 3C). Thus rFib610 clots behave like elastic solids despite the absence of covalent cross-links. The non-covalent interactions responsible for fibrin self-assembly, with measured binding energies of 20–50 k_BT per molecule [11,45,53-55], are therefore sufficient to provide an elastic response at small deformations.

To test the response of rFib610 clots to large deformations, we applied increasing levels of shear stress and measured the change in the differential elastic, K', and viscous, K'', moduli. Both moduli strongly increase as stress is ramped up (Fig. 3D). This response is reversible up to strains of ~50%, while beyond that the clots become softer with each repeated loading (Fig. 3E). Moreover, the clots exhibit creep and incomplete strain recovery when stress is removed (Fig. 3F). The non-covalent interactions are therefore not sufficiently strong to prevent irreversible

(viscoplastic) changes at large deformations. This finding is consistent with conclusions from recent studies of plasma fibrin clots, which showed that FXIII removal caused irreversible behavior at large stresses [49,52].

The effect of FXIII-mediated cross-linking

The absence of FXIII in rFib610 is also ideal for testing how controllable levels of FXIII-mediated cross-linking (Fig. 4A) affect fibrin rheology, providing a more precise approach than chemical- or antibody-mediated FXIII inhibition used in previous studies of plasma fibrinogen [15,40,48,51,52]. SDS-PAGE analysis shows that when we add 5 μg mL^{-1} FXIII to 2 mg mL^{-1} rFib610, the γ chains fully dimerize, stabilizing the protofibrils, whereas the α chains connecting adjacent protofibrils are <60% cross-linked (Fig. 4C–D). Increasing FXIII concentration leads to increasing multimerization of α chains until complete α-chain cross-linking with 20 μg mL^{-1} (or 40 Loewy U mL^{-1}) FXIII. Confocal and SEM imaging show no obvious effect of FXIII on rFib610 network structure (Figs. 4B,S3), consistent with recent reports on plasma fibrin [15,48]. Indeed, turbidimetry reveals that cross-linking results in only a slight decrease of fiber diameter (Fig. S8).

Rheology measurements show that FXIII-mediated cross-linking strongly enhances clot stiffness at small deformations: G_0 progressively increases from 90 Pa for 2 mg mL^{-1} uncross-linked rFib610 to 150 Pa at 5 μg mL^{-1} and 560 Pa at 20 μg mL^{-1} FXIII (Fig. 4E). In contrast, viscous dissipation, as measured by tan δ, immediately drops from 0.045 to 0.016 upon addition of 5 μg mL^{-1} FXIII and is not further reduced with higher FXIII (Fig. 4F). These results suggest that viscous dissipation in fibrin clots is mainly determined by γ-chain cross-linking, which controls molecular connectivity within *protofibrils*, whereas clot stiffness is determined by a

combination of γ-chain cross-linking within protofibrils and multimeric α-chain cross-linking between protofibrils, which acts at the *fiber* level.

At large stresses, we find similar strain-stiffening behavior in both cross-linked and uncross-linked rFib610 clots (Fig. 4G). Strikingly, cross-linking leads to a completely reversible response up to ~200% strains (close to breakage) in the presence of only 5 μg mL^{-1} FXIII, even though α-chain cross-linking is incomplete at this FXIII level. Moreover, cross-linking suppresses creep during stress application and incomplete strain recovery after stress removal (Fig. S9).

Structural basis of fibrin nonlinear rheology

Both uncross-linked and cross-linked rFib610 clots show a triphasic strain-stiffening response (Figs. 5A,B), similar to that observed for plasma fibrin [50,56]. The first (linear) regime corresponds to entropic elasticity associated with the resistance of the fibers to straighten out their thermal bending fluctuations, as validated previously by optical tweezer microrheology [56]. This entropic regime transitions to an enthalpic regime when the fibers are thicker or at high fibrin concentration [57,58]. Once the fibers are straightened, the network stiffness increases (regime 2) until the stress is large enough to axially stretch the fiber backbone (regime 3). Prior studies on plasma fibrin clots showed excellent agreement of the fiber mechanical properties inferred from the triphasic strain-stiffening response [50,56,57] and from light-scattering and single-fiber experiments [3,10,18,59]. Below we will use the same theoretical framework to interrogate rFib610 fibrin mechanics.

Fiber bending rigidity is typically quantified by the persistence length, which represents the length over which the filament deviates from straight conformation. By modeling fibrin fiber as semiflexible bundle of N_p protofibrils, each having a persistence length of l_p, we can estimate its effective persistence length as $l_p N_p^x$ [60]. The factor x quantifies the strength of coupling between the protofibrils, which should depend on the extent of α-chain cross-linking between protofibrils and vary between 1 for loose coupling and 2 for tight coupling. Entropic elasticity models predict that the linear modulus of the network (regime 1), G_0, is directly related to l_p and N_p via [50]:

$$G_0 = 6k_B T \rho^{11/5} \left(l_p N_p^x\right)^{7/5}. \quad (1)$$

Here $k_B T$ is the thermal energy and $\rho = c/\mu$ denotes fiber contour length density, where c is fibrin concentration. Knowing G_0 from rheology and N_p from turbidimetry, we can therefore use Eq. (1) to infer the strength of protofibril bundling within rFib610 fibers, provided l_p is known. We estimated l_p by fitting measured strain-stiffening curves for *fine* fibrin clots, which show suppressed protofibril bundling (Figs. S10,S11), to the theoretical prediction of entropic stiffening of semiflexible filament networks [56] (Fig. S12). Assuming N_p = 2 (based on turbidimetry data) and x = 1, like in plasma fine clots [50], we infer l_p of rFib610 protofibrils to be 75 nm. Using this l_p value, we find that x for (bundled) rFib610 clots varies between 1.90±0.22 in the absence of FXIII and 2.25±0.15 with 20 μg mL^{-1} FXIII (Figs. 5C). The latter value exceeds the tight coupling limit (x = 2), likely due to the uncertainty in l_p, as EM imaging shows that N_p varies between 1 and 2 within the network (Fig. S11). Similar analysis assuming other l_p estimates consistently yields similar trend of increasing x with increasing FXIII concentration (Fig. S13). FXIII-mediated α-chain cross-linking therefore increases clot stiffness by strengthening protofibril associations and stiffening the fibers.

To extract the force–extension behavior of the fibers, we consider the strain-stiffening behavior in regime 3 and rescale the stress-stiffening curves by the protofibril length density, $N_p\rho$. The curves for uncross-linked and cross-linked rFib610 clots overlap in regime 3 (Fig. 5F), indicating that the protofibrils contribute independently to network elasticity. The scaled curves initially show a plateau, from which we can infer the linear stretch rigidity of the protofibrils (Table 1), followed by a strain-stiffening regime (Fig. 5B). The fibers themselves apparently stiffen when they are sufficiently stretched, consistent with AFM studies on single plasma fibrin fibers that demonstrated strain-stiffening above strains of 100% [10,61]. Interestingly, cross-linked rFib610 fibers show more pronounced stiffening (~2-fold larger slope) than their uncross-linked counterparts once the average force per protofibril $t/(N_p\rho)$ exceeds ~1 pN, suggesting that cross-linking changes the force–extension behavior of the fibers. To distinguish the roles of γ- and α-chain cross-linking in this fiber stiffening, we analyzed the nonlinear behavior of rFib610 *fine* clots where cross-linking occurs primarily through γ-chains (Fig. S10). Clots of cross-linked rFib610 protofibrils stiffen in a similar manner to bundled rFib610 clots (Fig. 5D–E, *triangles*). Surprisingly, uncross-linked rFib610 protofibrils (Fig. 5E, *circles*) show an extended stiffness plateau that remains constant until the average force per protofibril reaches ~4 pN (Fig. 5F). This is in contrast to both cross-linked protofibrils and fibers, which already stiffen at ~1 pN. From the network stiffness at this plateau, we estimate the stretch modulus of the protofibrils to be ~100 pN, similar to previously reported values of 50–100 pN for fish fibrin protofibrils [3].

We finally test the role of molecular composition in protofibril mechanics by comparing fine rFib610 clots with their pFib counterparts. At low stress, fine rFib610 clots are always stiffer than fine pFib, regardless of cross-linking (Fig. S14). Using the polymer elasticity model explained above, we infer that rFib610 protofibrils have a 25% larger bending rigidity than pFib

protofibrils (Table 1 and Fig. S15). Moreover, cross-linking increases the stretch modulus of rFib610 protofibrils by 50% (Table 1), whereas it does not affect the stretch modulus of pFib protofibrils [50]. Thus, molecular heterogeneity directly affects the mechanical properties of fibrin protofibrils.

Discussion

Understanding the molecular basis of fibrin elasticity is a long-standing challenge that is difficult to address due to fibrin's multiscale structure and molecular heterogeneity. We here show that recombinant fibrinogen provides an ideal model system to systematically probe the role of molecular composition and plasma factors in clot mechanics.

Our results show that the *viscoelasticity* of fibrin clots is determined by intermolecular cross-links both within and between protofibrils. At low strain, the influence of α-chain cross-linking on the stiffness of fibrin clots [15,17,62] and fibers [10,18,61] can be explained by strengthening of protofibril association within each fiber. At high strain, where the network response is dominated by the force–extension behavior of individual fibers, we observe nonlinearity regardless of protofibril bundling, suggesting a much more limited role of α-chain cross-linking in fiber stretching. Since the protofibrils are stretched independently at high axial loads [63], forced-unfolding of the constituent monomers is likely the main source of the intrinsic nonlinearity of protofibril (and fiber) stretching [8,64]. Interestingly, protofibrils have been proposed to undergo hyperbranching within fibers [65], which may potentially modify fiber force–extension response. Further investigation into the secondary structure and monomer packing within strained fibers is necessary to precisely resolve this regime.

Our results also indicate that γ-chain cross-linking contributes to clot viscoelasticity by rendering the network response more reversible and by directly changing the force–extension behavior of protofibrils. These findings significantly expand our understanding on the elusive role of γ-chain cross-linking in fibrin mechanics [17,51,62,66]. The structural origin of the reduced onset force for stretch-stiffening from 4 to 1 pN for uncross-linked rFib610 protofibrils remains unknown. This force level is much lower than the critical forces required to unfold fibrinogen's coiled-coil region (125 pN) or γ nodule (74 pN), but could be related to entropic stretching of αC connectors [9], suggesting an entropic origin of protofibril strain-stiffening. Subtle changes in intermolecular connectivity, for instance due to hyperbranching [65], may potentially shift the onset of unfolding of the different domains of fibrin molecules within the protofibrils. Our data therefore establishes that γ-chain and α-chain cross-linking have distinct effects on the linear viscoelasticity of fibrin clots as well as the strain-stiffening response at the network, fiber and protofibril levels.

Despite the difference in molecular composition and probably also in posttranslational modifications, rFib610 displays similar early kinetics of thrombin-mediated fibrinopeptide release, protofibril formation and bundling as pFib, resulting in similar clot and fiber structure. However, the nonlinear rheological response of uncross-linked rFib610 protofibrils shows a larger bending rigidity and an extended linear response compared to pFib protofibrils. These observations suggest that molecular composition influences fiber and network mechanics by modulating properties at the protofibril scale. At the fiber level, changes in molecular composition can potentially influence the lateral packing of protofibrils within the fibers, which has been recently linked to fibrin fiber stiffness [67] and cell mechanosensing [68]. It will be interesting to probe the effect of molecular and structural heterogeneity on protofibril and fiber

stiffness directly by AFM-based nanoindentation and pulling [10,61]. These experiments can also help in independently quantifying protofibril persistence length. Here we report l_p = 75 nm, which is smaller than l_p values predicted by modeling protofibrils as homogeneous elastic rods [3,65,69]. However, recent atomistic Molecular Dynamics simulations point to the existence of hinge points within fibrinogen coiled-coil regions [70] and in the D:D interface between fibrin monomers within single-stranded oligomers [71] that may make protofibrils more flexible than anticipated.

Our study underlines the importance of understanding how, during coagulation, different fibrinogen variants co-assemble into protofibrils and fibers and how FXIII-mediated cross-linking contributes to clot mechanics at different levels of mechanical stress. The importance of these findings is highlighted by the various bleeding disorders associated with inherited FXIII deficiency [72], thrombosis associated with altered clot stiffness [73] and abnormal fibrinogen variants found in patients with diabetes, cancer or immune diseases [74]. A promising step is to investigate the properties of fibrin fibers and networks made of controlled mixtures of different fibrinogen variants, especially those varying in the α-chain length [7] and lateral association [75].

Addendum

J. Koopman and G. H. Koenderink conceived the research. I. K. Piechocka, N. A. Kurniawan and J. Grimbergen performed the research and contributed analytic tools. I. K. Piechocka, N. A. Kurniawan and G. H. Koenderink analyzed the data and wrote the manuscript. All authors revised the manuscript.

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Disclosure of Conflict of Interests

J. Grimbergen and J. Koopman were full-time employees of ProFibrix BV, Leiden, The Netherlands and are now employed with Fibriant BV, Leiden. The other authors have no conflict of interests to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Glycosylation analysis of rFib610 and pFib fibrinogen preparations.

Fig. S2. Aggregation analysis of rFib610 and pFib fibrinogen preparations.

Fig. S3. SEM images of rFib610 fibrin network with and without FXIII.

Fig. S4. Turbidity of rFib610 fibrin clots at different fibrinogen concentrations.

Fig. S5. Kinetics of rFib610 fibrin formation.

Fig. S6. Average pore diameter of rFib610 fibrin networks at different fibrin concentrations.

Fig. S7. SAXS spectra of rFib610 clots at different fibrin concentrations.

Fig. S8. Dependence of the diameter and packing density of rFib610 fibers on FXIII concentration.

Fig. S9. Creep behavior of cross-linked rFib610 fibrin clots.

Fig. S10. Reducing SDS-PAGE gel of rFib610 and pFib fibrinogen and fibrin in fine clot buffer.

Fig. S11. Fine rFib610 fiber morphology.

Fig. S12. Fit of experimental nonlinear rheology data for fine rFib610 clot to the theoretical prediction for cross-linked semiflexible polymers.

Fig. S13. Influence of the assumed persistence length of rFib610 fibrin protofibrils on the extracted coupling exponent x

Fig. S14. Comparison of the nonlinear rheology of fine rFib610 and pFib fibrin clots.

Fig. S15. Fits of the nonlinear rheological response of cross-linked and uncross-linked fine rFib610 and pFib clots.

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Figure legends

Fig. 1. Comparison of the molecular composition of recombinant rFib610 and plasma pFib fibrinogens. (A) Schematic representation of fibrinogen structure. Fibrinogen is a hexamer composed of two sets of three polypeptide chains, referred to as Aα, Bβ and γ. The carboxy-terminal portion of each Aα chain forms an αC region that is connected by an unstructured αC connector to the E region. Fibrinogen is converted to fibrin by enzymatic cleavage of the fibrinopeptides FpA and FpB by thrombin, initializing the polymerization reaction. (B) Reducing SDS-PAGE of rFib610 and pFib before and after addition of thrombin show the main Aα, Bβ and γ chains bands. In addition, pFib contains bands corresponding to degraded Aα chains ("dAα") and to γ' chains, confirming its known molecular heterogeneity. (C) RP-HPLC analysis of fibrinopeptide release from rFib610 (*red*) and pFib (*gray*) fibrinogens show peaks corresponding to FpAP, phosphorylated FpA; FpA, fibrinopeptide A; FpAY, FpA lacking the N-terminal Alanine residue; FpB desArg, FpB lacking the C-terminal Arg residue; FpB, fibrinopeptide B.

Fig. 2. Multi-scale characterization of the structure of fibrin clots reconstituted from rFib610 fibrinogen. (A) Schematic representation of a network of fibrin fibers, which are bundles of semiflexible protofibrils associated via flexible αC chains. The protofibril itself is a double-stranded filament of half-staggered fibrin monomers held together by non-covalent knob–hole interactions. (B) Confocal microscopy images, showing isotropic and homogeneous rFib610 networks at different fibrin concentrations. The fibers appear straight although they do experience thermal fluctuations (leading o entropic elasticity) since the amplitude of the transverse bending fluctuations is expected to be only ~26 nm, which cannot be resolved by

diffraction-limited fluorescent microscopy. The images are z-projections of 129 frames over a 25.6 μm-thick z-stack. Scale bars, 10 μm. (C) SAXS analysis of the axial packing order of fibrin monomers for rFib610, with and without FXIII, and for pFib at 2 mg mL^{-1} fibrin concentration. The peaks in the shaded (gray) region coincide with the expected third reflection of the half-staggered axial periodicity (22.5 nm) of fibrin fibers, suggesting a similar axial packing arrangement in the three networks. The curves are shifted along the *I*-axis with the indicated prefactors. (D–F) Turbidimetry analysis of the structure of the fibers as a function of fibrin concentration, showing (D) the average fiber diameter, d , (E) mass/length ratio, μ, and number of protofibrils per fiber, N_p, and (F) protein density.

Figure 3. Linear and nonlinear viscoelastic properties of uncross-linked rFib610 fibrin clots measured using shear rheology. (A) Frequency dependence of the linear elastic modulus, *G*' (solid symbols), and viscous modulus, *G*'' (open symbols), at 5 (blue), 2 (red) and 0.8 mg mL^{-1} (black) fibrin. Concentration dependence of (B) the linear elastic modulus, G_0, and (C) loss tangent, tan $\delta = G''/G'$. (D) Stress-stiffening behavior of 2 mg mL^{-1} rFib610 clot measured by applying different levels of a constant shear stress τ and superposing a small stress oscillation to obtain the differential elastic (*K*') and viscous (*K*") moduli. (E) Sequential stress ramps up to different maximum values, as specified in the legend, show irreversible clot softening when the maximum applied stress exceeds ~100 Pa. (F) Slowly increasing strain (creep) during prolonged application of a constant shear stress (see legend) together with residual strain after stress removal demonstrate viscoplastic behavior.

Fig. 4. The effect of FXIII-induced intermolecular cross-linking on the rheology of 2 mg mL^{-1} rFib610 fibrin clots. (A) Schematic illustration of α and γ chain cross-linking with γ–γ cross-links stabilizing intermolecular contacts within protofibrils and α–α cross-links reinforcing lateral bundling of the protofibrils into fibers. Short purple lines denote knob–hole interactions. (B) Confocal microscopy images of rFib610 clots without and with 20 μg mL^{-1} FXIII. Scale bars, 10 μm. (C) Reducing SDS-PAGE analysis of degree of cross-linking of rFib610 clots with varying concentrations of FXIII. (D) Densitometric quantification of the percentage of cross-linking of α and γ chains with band intensities normalized by that of the β chain. (E) Linear elastic shear modulus, G_0, and (F) loss tangent, tan δ, as a function of FXIII concentration. $^*p < 0.05$. (G) Strain-stiffening response of rFib610 clots cross-linked with 5 μg mL^{-1} FXIII measured by repeated stress sweeps up to increasing levels of maximum stress as indicated in the legend. In all cases, the clots were polymerized for 4 h before measurement.

Fig. 5. The contributions of fiber and protofibril mechanics to the nonlinear rheology of cross-linked and uncross-linked rFib610 clots at 2 mg mL^{-1} fibrin concentration. (A and D) Schematic explanation of the entropic elasticity regime (regimes 1 and 2; pulling out of thermal bending fluctuations) and the enthalpic elasticity regime (regime 3; fiber backbone stretching). (A) Fibrin clots formed under physiological conditions consist of bundles of protofibrils, whereas clots formed under fine buffer conditions consist of protofibrils that are virtually unbundled (D). (B) Clots of bundled rFib610 protofibrils without and with 20 μg mL^{-1} FXIII show a triphasic strain-stiffening response. The differential elastic modulus, K', is plotted as a function of shear strain, γ. (C) The exponent x quantifying the degree of coupling of protofibrils within fibers plotted as a function of FXIII concentration. The difference between different FXIII concentrations is not

statistically significant ($p > 0.05$). The horizontal dashed lines depict the tight ($x = 2$) and loose ($x = 1$) coupling limits. (E) Nonlinear rheology of fine (unbundled) rFib610 clots without and with 20 μg mL^{-1} FXIII. (F) The nonlinear mechanical response of these clots at different levels of stress τ. K' and τ are normalized by protofibril length density, $N_p\rho$, to remove the influence of bundle size on the network response in the enthalpic regime (above 1 pN). In all cases, the clots were polymerized for 4 h before measurement.

Table 1. Summary of structural and mechanical properties of 2 mg mL^{-1} rFib610 and pFib fibrin clots at different hierarchical levels of organization.

Scale	Properties	rFib610	x-linked rFib610	pFib
Network[a]	G'	84.1 ± 11.2 Pa	564 ± 51 Pa	114.7 ± 27.3 Pa
	G''	3.8 ± 1.3 Pa	7.1 ± 0.5 Pa	2.0 ± 0.5 Pa
	Elastic limit	47–59%	181–214%	158–174%
Fiber[b]	Diameter	138 ± 7 nm	119 ± 3 nm	151 ± 11 nm
	N_p	75 ± 9	60 ± 2	123 ± 24
	Protein density	0.12 ± 0.02 g mL^{-1}	0.13 ± 0.01 g mL^{-1}	0.25 ± 0.07 g mL^{-1}
Protofibril[c]	l_p	75 nm	75 nm	60 nm
	Stretch modulus	66 ± 3 pN	98 ± 4 pN	95 ± 4 pN

[a]Network mechanics data measured by shear rheology.

[b]Fiber structure data obtained from model-based interpretation of turbidimetry measurements.

[c]Protofibril data obtained from model-based interpretation of the rheology on fine fibrin clots.