Chapter 6

Decoupling of shear and Young’s moduli in extracellular networks


Chapter 6 Decoupling of shear and Young’s moduli in extracellular networks

Abstract

Gels formed by semiflexible fibers such as most biopolymers exhibit non-linear behavior in their response to shear deformation, e.g., with a pronounced strain stiffening and negative normal stress. These negative normal stresses suggest that networks would collapse axially when subject to shear stress. This coupling of axial and shear deformations can have particularly important consequences for extracellular matrices and collagenous tissues. Although measurements of uniaxial moduli have been made on biopolymer gels, these have not directly been related to the shear response. Here, we report measurements and simulations of axial and shear stresses exerted by a range of hydrogels subjected to simultaneous uniaxial and shear strains. These studies show that, in contrast to volume-conserving linearly elastic hydrogels, the Young’s moduli of networks formed by the biopolymers are not proportional to their shear moduli and both shear and uniaxial moduli are strongly affected by even modest degrees of uniaxial strain.

6.1 Introduction

Networks formed by filamentous biopolymers intracellular proteins like actin and vimentin, as well as extracellular proteins like collagen and fibrin show distinct nonlinear viscoelastic mechanical responses when deformed in shear. The shear storage modulus $G_s$ of such networks is higher than that of flexible polymer networks with the same mass density [191]. The storage modulus increases with concentration as $G_s \propto c^x$, where $x \approx 2$ to 2.5 for both intracellular and extracellular networks [49, 63, 64, 66, 75, 97, 192]. The large elastic moduli and their strong dependence on polymer density occur even though biopolymer networks fall below the isostatic threshold. This threshold corresponds to a 6-fold connectivity for minimal mechanical stability of 3D networks with only central-force (i.e., stretching) interactions [123]. In these 3D biopolymer networks, the nodes consist of either two cross-linked fiber segments with coordination number $z = 4$, or branching points with $z = 3$. Thus, the stability of biopolymer networks must be due to other factors, such as the bending rigidity of fibers, or internal stresses such as those applied by motor proteins [113, 125, 145, 193].

Both biological and synthetic semiflexible polymer networks also show dramatic nonlinear elastic effects, including strain stiffening at relatively low shear strains, depending on network density and polymer stiffness [76, 87]. Strain-stiffening can be understood either by the nonlinear force extension relation of semiflexible polymers due to thermal undulations at the filament level, or by collective rearrangements and alignment of filaments at the network level [72]. When sheared, these networks also exhibit large
negative axial (or normal) stress; in contrast, flexible elastomers exhibit a much smaller positive (compressive) normal stress. Therefore, biopolymer networks would tend to collapse upon applying shear, whereas linear elastic polymer networks would expand [78]. This effect might complicate the measurement of shear moduli in stress-controlled shear rheometers that automatically alter plate spacing to maintain a constant (usually zero) axial stress.

Nearly all measurements of the non-linear rheology of semiflexible polymer networks have been done under shear deformation, with the assumption that the Young’s modulus is two to three times larger, depending on the Poisson’s ratio of the materials. Young’s moduli have been directly measured for collagen and fibrin gels, but not directly related to rheology under shear strain.

Interest in biopolymer mechanics has increased since it has been shown that mechanical properties of cell substrates or extracellular matrix (ECM), influence cell functions [194]. Changes of the ECM have been linked to, or even precede common pathologies such as cancer [195], atherosclerosis [196] and fibrosis [197]. Tissue engineering requires detailed knowledge of the mechanics of the materials used as scaffolds to replace tissues. For this purpose, reconstituted networks and tissues have been characterized with either shear or uniaxial testing methods. However, most tissues are subjected to multiaxial mechanical stimuli at a variety of time scales.

This chapter is based on a collaborative work with A. van Oosten and P. Janmey [110], presenting experiments on reconstituted collagen and fibrin networks, which are directly relevant to the kinds of theoretical models that form the focus of this thesis. Common rheological techniques are adapted to provide a mechanical characterization of these extracellular networks undergoing multiaxial deformations that mimic strains occurring in vivo. Rheological tests were done on networks with concentrations ranging from 2 mg/mL to 10 mg/mL, which spans the concentrations at which these biopolymers are found in vivo [198]. We show that shear moduli decrease to an equilibrium value when networks are compressed but show a constant increase when samples are extended. Young’s moduli are significantly lower in compression compared to extension. When comparing apparent Young’s and shear moduli over a range of axial strains, the networks’ Young’s moduli are not linearly related to, and in fact, decoupled from their shear moduli. In addition, we also include simulation results in collaboration with M. Vahabi et al [199], that point to the stiffening mechanism of fibrous networks undergoing multiaxial deformations.
Figure 6.1: (a) A shear rheometer is used with a parallel plate geometry. Biopolymer samples are polymerized between the plates; after polymerization buffer is added around the sample in order to prevent drying and allow free fluid flow to in and out of the sample. The gap between the plates is changed to apply axial strain; the lower plate is rotated to apply shear strain. The torque and axial force are recorded. (b) The storage moduli measured with a shear rheometer and axial stress measured with a tensile tester of polyacrylamide gels (PAA). The PAA gel results show that for pure elastic linear materials the storage modulus is independent of the gap height and that the axial stress-strain curve is linear and symmetrical in compression and extension. The Young’s modulus is calculated from the slope of the stress-strain curve as $E = 2.8 \times G$. (Schematic and data by A.S.G. van Oosten.)
6.2 Materials and Methods

Strain-stiffening and negative normal stress of semiflexible polymer gels suggest that shear moduli might be altered by internal stresses, generated by axial strain orthogonal to the shear plane. Figure 6.1a shows the experimental system that allows compression or extension to be applied to disk-shaped samples, while their shear modulus is measured by oscillatory shear displacements. A strain-controlled rheometer with parallel-plate geometry was used to apply axial strain by changing the gap after the network was fully formed. The parallel plate configuration results in a shear strain field that is zero in the centre and increases proportionally with the radius of the plate. A reservoir of solvent surrounds the sample to allow volume change by fluid flow across the free edges. As a control, linearly elastic polyacrylamide gels (PAA) were tested. This reveals the storage moduli of PAA are independent of the level of axial strain. The axial stress is linear with axial strain (Figure 6.1b). The Young’s modulus of PAA is $E = 2.8 \text{ Pa}$ over the entire range of deformations, and is in close agreement with the reported Poisson’s ratio of 0.486 [200].

Networks of collagen at 2.5 mg/mL and fibrin at 10 mg/mL were subjected to increasing shear strain amplitudes after static compression or extension. An oscillatory shear strain of constant frequency and increasing magnitude was applied. The shear storage moduli of networks under axial stress were compared to their strain-stiffening without axial strain. The axial stress consists of both the negative contribution in response to the shear deformation and the applied axial stress. If the sample is extended, the axial stress is positive while a compressed sample shows negative axial stress. This sign convention is used for consistency with axial strain measurements.

6.3 Modeling and Rheology Simulation

To model networks of fibrin and collagen, we generated disordered, lattice-based networks such that the average coordination number (connectivity) $z = 3.4$ is consistent with direct observations of collagen networks [122]. In order to model networks with local connectivity of less than four, our 3D networks are generated by dilution of a phantomized FCC structure, in which the six fibers crossing at a node are separated randomly into three cross-linked pairs [115], using freely-hinged joints. The 3D structure fills up a total volume $V = W^3$, where $W$ is the linear dimension of the lattice. To reduce any edge effects, periodic boundaries are used for networks under deformation. Lees-Edwards boundary conditions are used to calculate lengths of fibers for networks under
deformation [131]. We treat intersections of fiber segments as freely-hinged permanent crosslinks.

The network connectivity is well below the point of marginal stability for pure stretching interactions [123] suggesting that the stability (finite shear modulus) of such networks arises from additional stabilizing interactions, such as bending and applied stress. We model the total elastic energy $\mathcal{H}$ of the network by combining bending and stretching contributions of all the fibers $f$: 

$$
\mathcal{H} = \sum_f \left[ \int \kappa \left( \frac{dt}{ds_f} \right)^2 ds_f + \int \frac{\mu}{2} \left( \frac{d\ell}{ds_f} \right)^2 ds_f \right].
$$

(6.1)

Here, $\kappa$ is the bending rigidity of the individual fibers and $\mu$ is their stretch modulus (see SI). The dimensionless bending rigidity $\tilde{\kappa} = \frac{\kappa}{\mu_0^2}$, where $l_0$ is the lattice spacing. In our 3D lattice-based networks, $l_0$ is the same as $l_c$, which is the spacing between adjacent cross-linkers. We vary $\kappa$, keeping $\mu = 1$ fixed. Assuming that the network is made up of $N$ fibers which can be treated as homogeneous cylindrical rods of radius $a$, length $L$, and Young’s modulus $E$, from classical beam theory [60] $\mu = \pi a^2 E$ and $\kappa = \frac{\pi}{4} a^4 E$. From this $\tilde{\kappa} = \frac{a^2}{4l_0^2}$, which is smaller than but proportional to the volume fraction $\varphi = \frac{\pi a^2 NL}{V}$ of fibers in the network, since there will be at least one fiber strand of volume $\pi a^2 l_0 \leq \varphi \sim \pi a^2 / l_0^2$. For our collagen networks with concentration 2.5 mg/mL, the volume fraction $\varphi \simeq 0.2\%$. We therefore use $\tilde{\kappa} = 10^{-4}$ to compare with collagen experiments, an order of magnitude smaller than $\tilde{\kappa} = 10^{-3}$ for the 10 mg/mL fibrin networks.

The simulated networks were subjected to increments $\delta \varepsilon$ of axial compression or extension using fixed lateral boundaries, while measuring the linear shear modulus at shear strains of $\gamma = 1\%$ (Figure 6.2). At each $\delta \varepsilon$ step, the network is allowed to relax, the energy is minimized using a conjugate-gradient numerical scheme [130] before...
applying the next axial strain step. The linear shear modulus is defined as $G = \sigma_S / \gamma$ where $\sigma_S$ is the shear stress and $\gamma$ is the shear strain. The shear stress is calculated as $\sigma_S = (1/V) \delta H / \delta \gamma$. Axial stresses are calculated from the energy changes due to the infinitesimal axial deformation as $\sigma_N = (1/V) \delta H / \delta \varepsilon$.

6.4 Results

Collagen and fibrin networks stiffen with increasing shear strain, both when extended and compressed, although the storage modulus at low shear strains is altered dramatically by application of axial strain, it is lower in compression and larger in extension compared to uncompressed samples (Figure 6.3). For collagen, the onset of strain-stiffening occurs at larger shear strains under compression, and the critical strain at which strain-softening is observed is higher. Under extension, collagen networks show a similar onset of strain-stiffening, but the critical strain is lower compared to the samples without axial strain (Figure 6.3a). In fibrin networks, the onset of strain-stiffening is higher under extension than under compression. However, the absolute peak values of $G$ are similar for the three levels of axial strain. (Figure 6.3b)

6.4.1 Shear response under external axial stress

The simulated networks were first compressed or extended axially before applying an increasing simple shear strain. This enables comparison with the experimental results shown in Figure 6.3a and Figure 6.3b. As a reference, we also show experiments and simulations without axial strain. Figure 6.3c and Figure 6.3d show the results from 3D network simulations with parameters quantifying the fiber rigidity and network mesh size comparable to experiments. As in the experimental results, applying axial stress changes the storage modulus.

The effects of axial strain on the shear storage moduli of fibrin and collagen networks are shown in more detail in Figure 6.4a. Networks were subjected to an incremental series of compressions or extensions, while simultaneously measuring the dynamic shear moduli at an amplitude of 2% shear strain and frequency of 10 rad/s. Between steps, the networks were allowed to relax for a duration of 100 to 1200 seconds (depending on step size and sample) before applying the next step of axial deformation. The relaxed values of the storage moduli were plotted for every level of axial strain.

The storage moduli in the absence of axial strain were 1138 Pa ± 160 Pa (SD) and 458 Pa ± 23 Pa (SD) for the fibrin and collagen, respectively (Figure 6.4b). Both storage
moduli increase steadily when extended, with collagen networks stiffening faster than fibrin. In compression, the storage modulus shows a decrease, which levels out between 5-10% compression (Figure 6.4a). At compression levels higher than 10%, the storage modulus becomes constant within the error of measurement Figure 6.4b.

A tensile tester was used to measure the axial stress after relaxation for similar levels of axial strain, as were used with the shear rheometer. From this, axial forces can be measured more accurately than with the shear rheometer. The slope of the axial stress-strain curve defines an apparent Young’s modulus. Extension is defined as a positive axial strain whereas compression is a negative axial strain. In order to attain a positive
6.4 Results

Figure 6.4: (a) Axial stress $\sigma_N$ and storage modulus $G$ as a function of axial strain $\varepsilon$ for 10 mg/ml fibrin and 2.5 mg/ml collagen. Both quantities are normalized by the modulus $G_0$ at which $\varepsilon = 0$. The samples subjected to a series of step-wise increases of the axial strain and were allowed to relax between subsequent steps. For both networks, their storage moduli decrease with compression and increase with extension in an asymmetric manner. The axial stress response is equally asymmetric. (b) The storage modulus is shown over a range of 20% compression up to 3% extension. The data between -10% and 3% are the same as shown in (a). The compression was continued up to 20% which shows that $G$ levels off between 10-20% compression within the error of measurement. In both (a) and (b), extension and compression series were performed on separate samples, with the mean of 3 samples shown with ± SD error bars. (Data by A.S.G. van Oosten.)

A positive axial stress is defined as the sample pulling on the upper plate in extension.

Collagen and fibrin networks show a similar trend. In extension, the axial stress increases approximately linearly with the axial strain over the entire range. By contrast, the stress-strain relationship is nonlinear up to $\sim 4\%$ compression, with a significant reduction in the slope. At larger compression, the response becomes linear Figure 6.4a. We determine the Young’s modulus in this linear region, between 4% and 10% compression, where it is much smaller than what we measure under extension. For collagen, the apparent Young’s modulus in compression is 29.1 Pa and 6.52 kPa in extension. For fibrin in compression it is 128 Pa in compression, and 10.7 kPa in extension. The ratio of the apparent $E$ in extension to the apparent $E$ in compression is 224 for collagen and 84 for fibrin.

In contrast to polyacrylamide gels, the relationship between the storage and Young’s moduli of fibrin and collagen gels varies with the axial strain. In extension the Young’s modulus stays constant within the tested range, whereas the storage modulus continues to increase. When compressed, both the storage and Young’s moduli approach new limiting values at large compressions. However, between 4% and 10% compression the shear storage modulus of collagen is an order of magnitude larger than the Young’s
Figure 6.5: Axial stress and storage modulus as a function of axial strain for platelet-poor plasma (PPP) and platelet-rich plasma (PRP). For PRP, only one exemplar is shown, which was compressed first, returned to 0% axial strain and subsequently extended. For PPP, the mean of 2 samples is shown with ± SD error bars. One sample was axially strained in the same manner as PRP while the second was extended first, returned to 0% axial strain and subsequently compressed. As in fibrin and collagen networks, the storage modulus decreases with compression, and increases with extension. For PPP this response is asymmetric, while for PRP the response is symmetric. The axial stress response is equally asymmetric for PPP, while PRP shows fairly symmetric response. (Data by A.S.G. van Oosten.)

modulus. This result means that the resistance of the network to compression is limited, while having a higher resistance to shear deformation.

6.4.2 Shear response under internal contractile stresses

The strong effects of external stress on the shear modulus of these networks suggest that generation of internal stress within the network, such as those generated in vivo, would alter the effects of axial strain on the shear modulus. To determine the effect of internal stress on fibrin networks, blood plasma containing fibrinogen as its major polymerizing protein was prepared with and without platelets. These cells bind the fibrin strands in a clot formed after activation of fibrinogen by thrombin and put them under tension [188]. Platelet-rich (PRP) and platelet-poor plasma (PPP) clots were subjected to similar multiaxial tests. Because of the rigid adhesive boundaries the parallel plates provide, the fibrin network is put under significant stress. This is confirmed by the development of a significant axial pre-stress of 46 Pa for PRP, whereas the axial stress in PPP is positive but small, on the order of single Pa. The platelet contractility causes the moduli of the PRP to increase drastically. Also, the response of the shear modulus is now symmetric over the tested range of axial strains; the axial stress shows less...
asymmetry between compression and extension compared to the unstressed networks (Figure 6.5). By contrast, PPP clots show very similar behavior as collagen and fibrin networks showing the characteristic asymmetric response to axial strain (Figure 6.4).

### 6.4.3 Stiffening mechanism

Figure 6.6 shows simulations of networks in the absence of pre-stress, as well as with an imposed 10% extension and 10% compression pre-stress. Without pre-stress, the simulations (Figure 6.6a) agree well with the measured dependence of $G$ on axial strain as seen in Figure 6.4. Our model can account for the features observed in the experiments, including the qualitative differences between collagen and fibrin, i.e., the sharp asymmetry of collagen networks relative to fibrin in response to axial strain.

It is important to mention that normal stresses measured experimentally have an offset, which is set to zero. This suggests performing simulations on networks which are initially pre-stressed. The results with pre-stress are also compared in Figure 6.6b. It can be seen how pre-stress delays (compression cases) or hastens (extension cases) the stiffening. Both the storage moduli and axial stresses show a 10% shift in their response. The compressed networks stiffen later, the extended networks stiffen earlier. As a result, the curves corresponding to networks under extension show a symmetric response around 0% axial strain. Such symmetry in the axial response is qualitatively similar to PRP (Figure 6.5), suggesting that platelets put the network under isotropic extension.

We now propose the following mechanism to explain the features observed in both experiment and simulations. The asymmetry seen in the $\sigma_N$ and $G$ response to compression and extension is caused by much lower fiber bending contributions relative to fiber stretching. Indeed, in networks dominated by fiber bending such as collagen, $G \propto \tilde{\kappa} \rho \sim c^2$ [88, 103]. In other words, it is easier to compress or buckle a network of floppy fibers than it is to remove undulations by stretching the network ([102]). As the network is compressed, it introduces a higher level of fiber undulations and buckling. Because of their much softer response, the buckled fibers do not contribute to the network stability which results in a lower shear modulus and thus a decreased stability under axial load. By contrast, extension removes undulations and will result in a higher shear modulus, i.e., an increased stability in response to axial stress. From this picture, we expect that the storage modulus relative to the unstressed network (i.e., at $\varepsilon = 0$) should grow linearly with the applied axial stress.

Indeed, the stabilizing effect of axial stress as proposed in [88] is consistent with our experiments and simulations [199]. In particular, it can be observed in Figure 6.4, Figure 6.5, and Figure 6.6 that the storage modulus response is proportional to the
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Figure 6.6: (a) Axial strain simulations without pre-stress for a diluted 3D phantomized FCC lattice-based network with \( L/l_0 = 6.67 \) (local coordination number \( z = 3.4 \)) and two different values of \( \tilde{\kappa} \). The difference in the sharpness of the asymmetric response with respect \( \tilde{\kappa} \) is comparable to experiments on fibrin and collagen (Figure 6.4a) (b) Simulation for the same network with varying pre-stress shows the same trend for axial stress and storage modulus. The dimensionless bending rigidity is fixed at \( \tilde{\kappa} = 10^{-3} \). Under compressive axial pre-stress, strain hardening is delayed (i.e., occurs at higher axial strains). By contrast, extensional pre-stress hastens strain hardening. (Data by M. Vahabi.)
Figure 6.7: Normalized storage modulus vs axial stress from (a) simulations for different fiber bending rigidities and (b) experiments from fibrin networks at different concentrations. The simulation results shown here are from 2D lattice-based networks with $L/l_0 = 6.67$ (local coordination number $z = 3.4$), the same local geometry as we have in our 3D networks. It has been shown in a recent study that the nonlinear mechanics of networks below the isostatic threshold is independent of dimensionality provided they have the same local coordination number \cite{103}. In (b), as in all other experiments we show in this work, $\sigma_N$ is already corrected for the offset value at zero axial strain. (Experimental data by A.S.G. van Oosten and simulations by M. Vahabi.)

negative axial stress response. This is clearly seen when we plot $G$ vs $\sigma_N$ normalized by concentration squared in experiments or fiber rigidity in simulations after subtracting their respective values at $\varepsilon = 0$ (Figure 6.7).

6.5 Discussion and Implications

Polymer networks in soft biological materials are subjected to simultaneous axial and shear deformation. For example, blood vessels are subjected to shear strain from fluid flow and extensional and compressive strains from dilation and constriction. Adipose tissue is statically sheared and compressed at long intervals when sitting. Analysis of such materials generally assumes a simple relationship between the resistance to axial compression and extension, characterized by the Young’s modulus $E$, and the resistance to shear deformation, characterized by the shear modulus $G$. For elastic solids, these quantities are generally related by $E = 2G(1 + \nu)$, where $\nu$ is Poisson’s ratio defined as the ratio of transverse strain to axial strain, which also quantifies the extent to which the sample maintains its volume when it is deformed. Nearly all simple materials have Poisson’s ratios $0 < \nu < 0.5$, which means $2G < E < 3G$, with the upper limits indicating incompressible systems. This assumption underlies all measurements of elastic moduli by atomic force microscopes or other indentation probes, which produce strains that are combinations of simple shear and uniaxial compression as well as extension.

The experiments reported here show that the shear moduli of collagen and fibrin networks decrease under compression and increase when under extension. The Young’s
moduli are an order of magnitude lower in compression compared to extension. The change in the shear modulus is decoupled from the change in the Young’s modulus during compression and extension: in extension $E$ is nearly constant while $G$ continues to increase (Figure 6.4). Furthermore, when networks are compressed by only a small fraction of the original sample height, the Young’s modulus drops below the shear modulus. When subjecting the samples to increasing shear strain under axial pre-stress, the strain-stiffening behavior is altered both in terms of the onset of nonlinearity and amplitude (Figure 6.3).

Overall, the simulations capture the behavior of the networks well, but several assumptions are made that might be more applicable either to collagen or fibrin. There are subtle differences between the response of fibrin and collagen when the networks are subjected to an increasing shear strain after axial pre-stress. For example, the shift of the strain onset is more pronounced for collagen than fibrin, which is not seen in the simulations. The structures of fibrin and collagen are also different, especially at the fiber level. In reconstituted collagen networks, there is no enzymatic cross-linking whereas the fibrinogen monomers within fibers are cross-linked with factor XIIIa [110]. Moreover, the ability of single fibrin fibers to increase in length after straightening of the undulations due to monomer stretching and sliding, is much greater than that of collagen fibers. At the network level, the nodes of collagen are entangled, whereas in fibrin networks branch points form, and loose fiber ends are rarely found in a fibrin network. While collagen and fibrin have different structures in detail, there are reasons to apply the same model to them. Prior studies of similar models have shown a rather surprising degree of insensitivity to network structure in the predicted mechanics. For instance, Licup et al demonstrated very close correspondence in the form of the nonlinear mechanics of lattice-based networks in both 2D and 3D, as well as off-lattice Mikado models [88].

The increase in storage moduli of fibrin networks due to active pre-stress generation has been described before using conventional shear rheometry [188, 190, 201]. The multiaxial rheology of pre-stressed networks reveals several interesting features. Even though the networks still soften under compression and stiffen under extension, the symmetry of the response is greatly enhanced. The simulations reveal that a 10% extensional pre-stress results in a symmetric response from 7% compression throughout the whole range of extension (Figure 6.6). Internal pre-stresses can be estimated in experiments from the development of axial stress during polymerization, while the gap is kept at a constant height. For PRP, the internal pre-stresses due to cell contractility yields $G \simeq 199 \text{Pa}$, and the axial stress that develops during polymerization is $-46 \text{ Pa}$, which is 23% of the initial shear modulus. Although this number cannot be compared directly to the amount of pre-stress used in the simulations, it indicates that there is a pre-stress higher

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than 10%. It is therefore likely that the symmetry seen over the whole test range for PRP is due to the large pre-stress imposed on the network.

The simulations show a simple shift in the response of the network to axial strain with imposed pre-stress. This is not seen when comparing the unstressed networks of collagen, fibrin, and PPP with PRP. However, there are several parameters that could contribute to these differences. The platelets not only put tension on the networks, they also aggregate and form additional cross-links in the network [202]. Collagen, fibrin and PPP are either entangled or branched (in the case of fibrin and PPP). Blood proteins other than fibrin like albumin, globulins and fibronectin are present in plasma and are known to influence fibrin structure and mechanics [203].

It is important to note that in the experiments described here, the samples are completely surrounded by buffer, and fluid is allowed to flow freely in and out of the network when axially strained, allowing the sample volume to change. This change was visually observed by photographing the boundaries of the gels after axial strain, which remain the same for collagen and fibrin. Thus, our results are consistent with a vanishing Poisson’s ratio. Here, the Young’s modulus coincides with the longitudinal modulus, in which the lateral dimensions of the sample do not change. For this reason, we impose fixed lateral boundaries in our simulations. The in/outflow of fluid and resulting compressibility of collagen has been described previously [204]. We also verified the water flow out of a collagen network by incorporating food dye in a collagen network and measuring the dye in the surrounding buffer after compression as a function of time [110]. By contrast, in our experiments on PAA gels, we observe boundaries that bulge under axial compression and are concave in extension, consistent with the near incompressibility of PAA.

The volume change of collagen results in higher (lower) protein concentrations in compression (extension). The changes in the storage modulus are opposite to what would be expected from the change in polymer concentration due to the change in volume. The increased polymer mass in compression and its dilution in extension make the effects of axial strain on storage moduli even more striking.

The few studies directly comparing mechanical responses of soft hydrogels after deformation in different directions show results consistent with our findings. In one study, the compressive moduli of collagen gels measured with atomic force microscopy are between 2.85 Pa and 23.1 Pa, and the shear moduli measured with a rheometer are between 19.9 Pa and 152 Pa respectively, for collagen isolated from rats of increasing age [205]. Another study compared creep in confined compression and shear and concluded that the shear modulus was higher in shear than in compression [206]. It has been shown for fibrin gels that when compressed, the shear modulus drops initially. The same study
also imaged the networks during compression and showed directly that filaments buckle when compressed [207].

The relation between $E$ and $G$ in viscoelastic solid has previously described to be extremely time dependent, with a Poisson’s ratio ranging from $-1 < \nu < 0.3$ [208, 209]. However, in our experiments, the networks are allowed to relax considerably and show even larger variability in the relationship between $E$ and $G$. Thus, the decoupling of $E$ and $G$ is not merely an issue of temporal relaxation but an intrinsic material property. Fluid flow into the gel might account for the differences observed in previous studies, where extension of these gels is often described as viscoelastic. A logical continuation of this particular work is to analyze the time dependency of these systems.

Fibrin and collagen gels have often been suggested as scaffolds for cells in tissue engineering purposes. The mechanical adequacy has often been debated due to the variability of the reported moduli. In this study it becomes apparent that the ability of these networks to withstand compression and the combination of shear and compression is limited, which might pose a problem when these gels are used to replace damaged tissues. It is vital to further study the mechanics of whole tissues and cell-seeded biopolymer networks to ascertain whether this improves the ability of such constructs to resist mechanical loads.
6.6 Appendix: Materials and Methods

Preparation of fibrin

Fibrinogen (Fbg), isolated from human plasma and plasminogen depleted, (CalBioChem, EMD Millipore, Billerica, MA, USA) was dissolved in 1× T7 buffer (50mM Tris, 150 mM NaCl at pH 7.4). Thrombin (Thr) isolated from salmon plasma (SeaRun Holdings, Freeport, ME, USA) was diluted in ddH2O at 1000 U/mL. Solutions were aliquoted and snap frozen for future use. Salmon thrombin clotting properties for human fibrinogen were checked previously [210, 211] and were found to be near identical to human thrombin at the Fbg and Thr concentrations used in this study. Factor XIII cross-linking was checked with a SDS PAGE gel and found identical between human and salmon thrombin (Figure ??).

To prepare fibrin networks, solutions were warmed to room temperature; fibrinogen stock solution, 1× T7 buffer, CaCl2 stock and thrombin were added at appropriate ratios to yield 10 mg/mL fibrinogen, 30 mM Ca2+ and 0.5 U thrombin/mg Fbg. The samples were polymerized at 25°C.

Preparation of collagen type I

Collagen type I isolated from calf skin (MP Biomedicals, Santa Ana, CA, USA) was dissolved in 0.02 N acetic acid. To prepare collagen networks 10× PBS, 0.1M NaOH and ddH2O were warmed to room temperature and added in appropriate ratios to yield a 2.5 mg/mL collagen concentration in 1X PBS solution with a pH between 7 and 7.5. The samples were polymerized at 37°C.

Preparation of platelet-rich and platelet-poor plasma

A blood donation was conducted in accordance with all appropriate guidelines and regulations and with approval of the Internal Review Board at the University of Pennsylvania (protocol no. 805305). Human blood was drawn with informed consent from a healthy volunteer. Whole blood was drawn via venipuncture from in K3EDTA. To obtain platelet-rich plasma the whole blood was centrifuged for 15 minutes at 120×G, from which the supernatant was removed and used. Platelet-poor plasma was prepared by spinning whole blood at 2200×G for 15 minutes. To prepare plasma clots salmon thrombin and calcium were added to yield 2 U thrombin/mL and 30 mM Ca2+. The samples were polymerized at 37°C.
Preparation of soft polyacrylamide gels

Forty percent acrylamide and 2% bis-acrylamide solutions (Bio-Rad, Hercules, CA, USA) were mixed with ddH2O to yield a 7.5% acrylamide and 0.01% bis-acrylamide solution. Polymerization was initiated by adding ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). Gels were polymerized at room temperature in a well, allowed to swell in ddH2O, and cut into shape with a circular punch prior to measurements.

Rheometry

A strain-controlled rotational rheometer (RFS3, TA Instruments, New castle, DE, USA) was used with a parallel plate of 8 mm for 10 mg/mL fibrin; 25 mm diameter for 2.5 mg/mL collagen, both with a gap of 1 mm. PRP and PPP clots were measured with a 50 mm diameter plate with a 400 µm gap. The bottom plate incorporated a Peltier plate, allowing to control the sample temperature, 25°C for 10 mg/mL fibrin and 37°C for the other samples. The biopolymer samples were pipetted between the plates prior to polymerization. After polymerization, the appropriate buffer was pipetted around the free edge of the sample to prevent drying and allow free fluid flow in and out of the sample. The shear moduli of the samples were measured by applying a low oscillatory shear strain of 2% at a frequency of 10 rad/s. Axial strain was applied by changing the gap between the plates.

Some samples were subjected to a fixed step compression or extension after which a shear strain sweep was performed. Strain sweeps were performed in absence of axial strain and after applying 20% compression, 12.5% extension (for fibrin) and 2.5% extension (for collagen). The shear strain was increased from 2% up to the point of breakage, which depends on the sample type and the level of axial strain, at a frequency of 1 rad/sec. This lower frequency was necessary to observe the response of the axial stress to the applied shear stress. Other biopolymer samples were subjected to either an incremental compression or extension series. The step-size was optimized for axial strain in each direction, separately for collagen, fibrin and plasma, to yield optimal resolution and prevent the sample from tearing (in extension). During the axial strain series the samples were allowed to relax between 100 and 1200 seconds before continuing to the next level of axial strain. For a precise application of axial strain the plate was moved at a low speed of 2 µm/s.
6.6 Appendix: Materials and Methods

**Tensile testing**

The mechanical properties of collagen and fibrin networks were measured under axial deformation using a tensile tester (Instron 5564) with parallel platens at a gap of 1 mm, using similar volumes of fluid as for rheometry. To obtain data on fully relaxed networks, samples were subjected to 10 µm steps of compression or extension at 2 µm/s, which were allowed to relax for 15 minutes between consecutive steps.