6. Architecture and Normal Stress Govern Collagen Network Mechanics

Collagen is the most abundant protein in the human body and its architecture is tailored to serve tissue-specific purposes. Recent computational studies predicted that collagen can be considered as an athermal polymer network, whose elastic properties are governed by the network geometry since the average coordination number is less than the critical connectivity needed for mechanical stability. It was proposed that the linear modulus is governed by fiber bending, while strain-stiffening occurs as a consequence of a transition to a stretching-dominated regime. In the limit of vanishing bending rigidity, this strain-controlled bend-to-stretch transition is a mechanical phase transition with a critical point. Here we show experimentally that reconstituted collagen networks show signatures of critical behavior in vitro. By systematically performing non-linear rheology measurements on collagen networks whose architecture is controlled through temperature and concentration, we show that the mechanical properties are controlled by the network architecture, consistent with the theoretical predictions. To characterize the network architecture, we combine confocal and electron microscopy with turbidity measurements. We show that the networks are stabilized by bending elasticity in the linear regime, and by a shear-induced normal stress in the nonlinear regime. In summary, we now have a quantitative theoretical framework to predict the mechanical properties of collagen networks as a function of architecture, which provides a powerful tool to design collagen networks with certain mechanical properties.

Collagen is the most important structural molecule in the mammalian body, providing mechanical strength to all connective tissues and making up about 30% of the total protein content \[301,580,581\]. The collagen family consists of 29 genetically distinct members, but the predominant member in most tissues is type I \[582–584\]. Collagen Type I monomers consist of a long (300 nm) and thin (1.5 nm) triple helix of three polypeptides, flanked by two non-helical telopeptides \[584\]. Cells secrete collagen molecules with protective propeptides, which are removed in the extracellular space by enzymatic cleavage \[584\]. This process triggers spontaneous formation of fibrils. The diameter and higher-order organization of the fibrils are controlled in a tissue-specific manner by supporting ECM components, such as minor fibrillar collagens like collagen V and small leucine rich proteoglycans \[127\]. The monomers within a collagen fibril form a densely interconnected rope-like structure with a precise axial stagger of 67 nm, referred to as the "D-period" \[316,580\]. Reinforcement by permanent intermolecular crosslinks provides the fibrils with a high tensile strength \[585–587\].

The mechanical properties of collagen are intricately linked to tissue function: Abnormalities in collagen organization and mechanics due to mutations in genes encoding for collagen can cause severe diseases characterized by weak and fragile tissues \[581,588\]. Excessive crosslinking of collagen creates abnormally stiff tissues, which also hamper normal tissue function \[3,587\]. The mechanics of collagen is thus essential for normal tissue functioning.

In addition to being responsible for the structural integrity of tissues, collagen also contributes to the functional architecture of tissues by providing anchoring support to cells in the tissue. The structural as well as the mechanical properties of the collagen matrix are known to impact cell behavior. Matrix mechanics has been shown to influence cell spreading, migration, proliferation and cell fate decisions \[41,43–47\] (see Chapter 1.3 section 1.3.1 for a more extensive overview). Also, abnormal changes in the collagen matrix \textit{in vivo} have been shown to trigger tumor cell behavior and tumor-like behavior of non-tumorigenic cells \[3–5\].

In view of the crucial role of collagen mechanics in cell and tissue biology and function, there is a long history of research on its mechanical properties, dating back to at least 1904 \[589\]. But despite this long history, the physical basis of collagen mechanics remains poorly understood. Mechanical measurements on whole tissues have demonstrated intriguing material properties, including a pronounced strain-stiffening response to an applied mechanical load \[301,590–593\]. This strain-stiffening response is thought to protect tissues from mechanical damage and to tailor their biological functions \[594\]. However, the hierarchical architecture and complex molecular composition of collagenous tissues have made it difficult to reveal the origin of this remarkable mechanical
behavior. *In situ* X-ray scattering studies on mechanically stretched tendons suggest that all hierarchical levels contribute to the macroscopic response [301]. Due to this complexity, increasing focus is on reconstituted model systems of purified collagen. Here one can control the molecular and structural complexity, facilitating a quantitative comparison of experiments with predictions from theoretical or computational models.

*In vitro* collagen gels are made by self-assembly, where one typically starts with an acidic aqueous solution of collagen monomers. When the pH is raised to physiological values, an homogeneous network of collagen fibrils is formed, which show a comparable 67 nm axial repeat in electron microscopy images and X-ray scattering as seen *in vivo* [303]. The diameter of the fibrils and the structure of the networks they form can be tuned by changing the solvent pH [307–310], salt conditions [308] or the polymerization temperature [90,305,306]. Using these control parameters, the network structure can be varied from a highly porous mesh work with a pore size of a few tenths of microns and thick fibers (order microns) to networks with thin collagen fibers (~100 nm) and small pore sizes on the order of 1 µm or less [90,306,307,309,310]. A number of experimental studies have shown that these purified collagen network stiffen when strained by shearing or stretching, in a similar manner as whole tissues [251,253,272,279].

In the past few years, there has been tremendous progress in computational modeling of collagen mechanics. Due to collagen’s complex hierarchical structure, it is difficult to model it while taking into account all hierarchical levels. The most detailed models, based on atomistic simulations, are limited to single collagen molecules and microfibrils [595–597] and require coarse-graining approaches to reach to the fibril level [588]. In contrast, models aimed to describe collagen at the network level usually treat the fibrils as simple polymers and ignore the internal molecular packing structure [279,598,599]. These models treat the fibrils either as (athermal) elastic beams, or as semiflexible polymers, depending on their bending rigidity $\kappa$. The bending rigidity is characterized by the polymer’s thermal persistence length, $l_p = \kappa/k_B T$, which quantifies the distance over which angular correlations along the polymer decorrelate as a consequence of thermal fluctuations, where $k_B$ is Boltzmann’s constant and $T$ is temperature [278]. There is strong evidence that collagen fibrils can be considered as essentially athermal, meaning that their persistence length is much larger than their contour length. The Young’s modulus of a collagen fibril in the hydrated state is on the order of 0.1 GPa [251,284–288]. With a typical diameter of 200 nm, this implies that $l_p$ is on the order of a meter, while the typical fibril length is of the order of microns.

Computer simulations of athermal fibrous networks have shown that the elasticity of such networks critically depends on its local connectivity [279]. ECM networks such as interstitial collagen have an average connectivity be-
between 3 and 4 [279, 390, 600], which implies a sub-marginal state: This connectivity is lower than the Maxwell criterion and the network is expected to be unstable. This criterion states that for a network of springs to be stable, the average connectivity (in 3D) should be at least 6 [601]. Collagen networks are nevertheless stable, due to the large bending rigidity of the filaments [279, 280]. Several computational studies suggest that mechanically shearing an athermal fibrous networks will cause strain-stiffening by inducing a transition from a soft, bending-dominated regime at low strain to a stiffer, elastic stretching-dominated regime at high strain [277, 425]. However, a recent computational model of Licup and co-workers [279] suggests that collagen networks already start to stiffen well before the stretch-dominated regime sets in. It was proposed that normal stress builds up, which stabilizes the sub-marginal network as it strain-stiffens.

The recent computational model of Licup et al. [279] makes several predictions for the rheology of collagen networks that can be tested experimentally. First, it predicts that the network stiffness in the small strain regime scales a power law in collagen concentration with an exponent of 2, assuming that the network architecture is constant. Experimentally, a wide range of exponents has been observed, often between 2 and 3 [253, 272, 395, 602, 603], though sometimes smaller exponents have been reported (between 1 and 2) [92, 251, 527, 603]. The reason behind this variability is not well understood. Second, the model predicts that the initial strain-stiffening response is governed by normal stress buildup. In particular, the elasticity $K'$ is predicted to increase with normal stress $\sigma_N$ as: $K' \simeq G_0 + \chi |\sigma_N|$, where $G_0$ is the linear (low strain) modulus and $\chi$ the susceptibility. This prediction has not been tested yet in experiments. Third, the model predicts that collagen networks undergo a mechanical transition from a floppy, bend-dominated regime to a rigid, stretch-dominated regime, which is governed by an underlying critical point determined by the network architecture [604, 605]. Also this prediction has not been tested yet in experiments.

In this chapter, we measure the rheological properties of reconstituted collagen networks as a function of network architecture. We vary the architecture by tuning collagen concentration over a wide range, and by varying the polymerization temperature. The fibril diameter, the network connectivity and pore size are characterized by light scattering, scanning electron microscopy and confocal reflectance microscopy. To interpret our findings, we compare the experimental results with predictions of the computational model of Licup et al. [279], which treats the collagen networks as a disordered lattice of rigid (athermal) beams. We show that the rheology of collagen networks is in quantitative agreement with the model. First, the concentration dependence of the linear elastic modulus of the networks is consistent with the model by taking into account a subtle change in network architecture with changing collagen
concentration. Second, we are able to confirm the theoretical prediction that
the initial strain-stiffening of collagen networks is governed by normal stress.
Third, we are able to confirm the theoretical prediction that collagen networks
exhibit critical behavior. We show that the entire strain-dependence of the
elastic modulus can be described by an analytical expression based on a me-
chanical equation of state. Our findings provide a firm basis to tie together
the different hierarchical levels of collagen structure into a unifying model.
Moreover, our work provides a quantitative approach to relate collagen net-
work structure to mechanics, which is essential to understand the influence
of collagen network architecture on migration, spreading, and proliferation of
healthy and tumorigenic cells [90,310,606].

6.2 Materials and Methods
6.2.1 Sample Preparation
To reconstitute collagen networks, we polymerized purified rat tail collagen
type I (high concentration rat tail collagen type I in 0.02 N acetic acid, BD
Biosciences, Breda) under solution conditions compatible with in vitro cell
culture. Cell culture medium (DMEM10x without phenol red), antibiotics
(penicillin/streptomycin, pen/strep), sodium bicarbonate (7.5% sterile solu-
tion) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were
all obtained from Sigma and stored at 4°C, except for pen/strep, which was
stored in aliquots at -20°C. Fetal bovine serum (FBS) was obtained from Gibco
and stored in aliquots at -20°C. DMEM10x, antibiotics and FBS solutions were
divided in aliquots in a sterile environment, which were used only for 1 day
after thawing.

Collagen samples were obtained by mixing all components on ice. First,
collagen solution was pipetted into a precooled 2 ml Eppendorf tube on ice.
Due to the high viscosity of the collagen stock, we weighted the tube to deter-
mine the exact amount of collagen. The sample was then centrifuged at 4°C
to transport the collagen to the bottom of the tube. Next, the solution ingre-
dients were added in the following sequence: DMEM10x, HEPES, antibiotics,
FBS, sodium bicarbonate and sterile milliQ water. The final gel conditions
are: DMEM 1x, 1% FBS, 50 mM HEPES, 1.5 mg/ml sodium bicarbonate and
0.1% pen/strep. The pH was adjusted to 7.3–7.4, by the addition of NaOH,
with the help of a pH meter (Hanna Instruments, Germany) equipped with a
micro-electrode. The time between setting the pH with NaOH and transport-
ing the sample to a sample holder was always kept close to 10 min, in order
to minimize potential variations due to premature polymerization on ice [607].
Bubbles were removed by centrifuging the samples before polymerization for
10 seconds at room temperature for samples of 2–4 mg/ml collagen, and for
a few minutes at 4°C for samples of ≥ 5 mg/ml.

The following sample holders were used: a cuvette (turbidity), a flow cham-
ber (light microscopy), a 5 ml Eppendorf tube (SEM) or a pre-cooled glass-bottom petridish (Mattek, light microscopy). In all cases, the sample holders were pre-cooled (on ice) and the collagen gels were polymerized in a humid atmosphere to prevent solvent evaporation. After the addition of sample, the sample holders were rapidly warmed to the desired temperature. The polymerization temperature was varied between 22–37°C to achieve different network architectures. The collagen concentration was varied between 0.2 mg/ml up to 6 mg/ml. For rheology, samples were polymerized in situ using a cone-plate geometry that was preheated to the desired temperature.

6.2.2 Rheology

Rheology tests were performed with a stress-controlled rheometer (Physica MCR 501, Anton Paar, Graz, Austria). We used a stainless steel cone-plate geometry with 40 mm diameter and 1° cone angle, which was prewarmed to the desired temperature (22 – 37°C) in the presence of water in the wells. We note that we performed preliminary tests using a 40 mm cone-plate with 2° cone angle of either polycarbonate or stainless steel, which gave similar results to the stiffening curves shown in this chapter (not shown). Neutralized cold collagen solution was added to the plate and the cone was lowered immediately. The sample was trimmed while the angular position of the cone was kept constant, a solvent trap was added to further prevent evaporation. The time between adding the sample, trimming the sample and adding the solvent trap was typically around 20 seconds. The collagen solution was not probed for 6 hours, to allow for unperturbed polymerization. Since collagen gels are known to polymerize rather slowly at low temperatures [304, 306, 608], we checked the polymerization time at 22°C (see turbidity section 6.2.3). For all collagen concentrations studied at 22°C, the network was fully formed after ~200 minutes polymerization (Fig. 6.20 in SI).

Once the network was formed, we probed its linear viscoelastic moduli by performing oscillatory shear tests with a small (0.5%) strain amplitude and frequencies ranging from 10 to 0.05 Hz. Next, a prestress protocol was performed to determine the nonlinear mechanical properties. First, a creep test was performed by applying a constant shear stress, $\sigma$, for 30 seconds and measuring the resulting strain $\gamma$. The creep rate was defined as the slope of a linear fit over the last 20 seconds of the time-dependent $\gamma$. Then a small oscillatory stress, $\delta \sigma$, was superposed on top of the constant prestress, where $\delta \sigma$ was 10 times lower than $\sigma$, while monitoring the differential oscillatory strain, $\delta \gamma$. The frequency was set to 0.5 Hz. In total, 6 oscillations were performed per prestress value and the differential modulus $K' = \delta \sigma / \delta \gamma$ was determined as an average over the last 5 oscillations. The onset of strain-stiffening was defined as the local minimum of $K'/\sigma$ (see Fig. 6.21 in the SI). The critical strain, $\gamma_c$, was obtained as the inflection point of the log($K'$) versus log($\gamma$) curves in the strain-stiffening regime. Per shear stress decade, 11 prestress data points were
collected, equally spaced on a logarithmic scale. To characterize the nonlinear regime, we determined the maximal power law slope of the strain-stiffening curve ($K'$ versus $\sigma_0$) from its first derivative. This slope is referred to as the stiffening exponent $\beta$.

For plotting the mean quantities, the average values $\pm$ the standard deviation are shown for three independently prepared samples, unless stated otherwise. In case of fits, the errors in the best-fit values are expressed as the standard error, unless stated otherwise.

6.2.3 Turbidity

Turbidity measurements were performed using a Cary300 UV-Vis spectrophotometer (Agilent Technologies, Amstelveen, Netherlands). Samples were polymerized inside disposable plastic cuvettes (UV-Cuvette micro, Plastibrand, Germany) for at least 6 hours and up to overnight at temperatures in the range of $22 - 37^\circ$C. The blank for background correction was prepared in the same way as the collagen samples, but with collagen replaced by a corresponding volume of 0.02 N acetic acid. The optical density $I_0$ of both sample and blank were measured over a range of wavelengths $\lambda$ of 350–900 nm. The turbidity $\tau$ follows from $I_0$ as:

$$\tau = \frac{I_0 \ln(10)}{L}$$

where $L$ is the optical path length. If one assumes that there is no light absorbed, and that the collagen fibers can be modeled as randomly oriented, monodisperse rod-like particles with small radius and long length compared to the wavelength used, then the wavelength dependence of the turbidity can be rescaled to reveal the the mass-length ratio $\mu$ and the radius $a$ of the fibers [295,296]:

$$\tau \lambda^5 = A\mu(\lambda^2 - Ba^2)$$

$A$ and $B$ are constants and respectively equal to $(88/15)c_p\pi^3n_s(dn/dc_p)^2/N_A$ and $(184/154)\pi^2n_s^2$. Here $c_p$ is the collagen concentration in g/ml, $n_s$ is the solvent refractive index (which is 1.33), $dn/dc_p$ is the specific refractive index increment (which is 0.186 cm$^3$/g for collagen [609]) and $N_A$ is Avogadro’s constant. The turbidity data were replotted according to eq. 6.2 and a linear fit was made between 890 nm and 650 nm. This regime was chosen to limit the chance of signal saturation of the spectrometer at low wavelengths and noise at high wavelengths. The slope and intercept were used to calculate the mass-length ratio, $\mu$, and the radius, $a$. The number of monomers $N_p$ in a cross-section was determined from $\mu$ based on the known quarter-staggered molecular packing arrangement of collagen molecules within the fibrils [302]:

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\[ N_p = \frac{4.6D \cdot \mu}{M} \]  

where \( D \) is the axial periodicity (67.2 nm) and \( M \) is the molecular mass of a collagen monomer (290 kDa [610]).

In some cases, the solution turbidity was measured as a function of time during polymerization, in order to quantify the kinetics of fibril nucleation and growth. Here, the Cary300 instrument was preheated to the desired temperature using a Cary temperature controller (Agilent Technologies) and measurements were started directly after sample preparation. Every two minutes during polymerization, a wavelength scan was taken from 900 nm to 350 nm. We observed a typical sigmoidal dependence of the turbidity on polymerization time, with a lag phase in which the turbidity remains close to zero, a growth phase in which the turbidity rises, and a plateau phase in which the turbidity saturates to a constant level (see Fig. 6.20 in SI). The lag time, \( t_\tau \), that characterizes the duration of the lag phase, was determined from turbidity measurements at \( \lambda = 600 \) nm by normalizing \( I_0 \) by the maximum scattered intensity reached in the plateau phase, \( I_{max} \), and fitting the time dependence to the following functional form [611]:

\[ \frac{I_0}{I_{max}} = a - b \cdot \ln(t + c) \]  

where \( t \) is time, and \( a, b \) and \( c \) are fitting constants. \( I_0/I_{max} \) was fitted over the range 0.25 to 0.75. The fit was extrapolated to the time point where \( I_0/I_{max} = 0 \), which was defined as the lag time \( t_\tau \).

### 6.2.4 Imaging

Confocal reflectance images of collagen gels polymerized at different collagen concentrations and different polymerization temperatures were collected using an inverted Eclipse Ti microscope (Nikon) with an 488 Ar laser (Melles Griot, Albuquerque, NM) for illumination. We obtained z-stacks by recording confocal slices over a total distance of 20 \( \mu m \) for the 100x (N.A. 1.49) objective with 0.2 \( \mu m \) z-spacing and 40 \( \mu m \) with step size 0.2 \( \mu m \) for the 40x (N.A. 1.30) objective, starting at least 10 \( \mu m \) away from the coverslip surface. For display purposes, the confocal stacks were summed to give an impression of the 3D network structure and the typical mesh size.

For scanning electron microscopy (SEM), 4 mg/ml collagen gels (50-100 \( \mu l \)) were polymerized overnight inside 5 ml Eppendorf tubes at different temperatures (22 – 37 °C, water bath) in humid conditions. Humid conditions were obtained by adding a small wet tissue inside the Eppendorf tubes. Samples were prepared for SEM using a protocol adapted from the group of John Weisel [600,612]. After polymerization, samples were washed three times with sodium cacodylate buffer (50 mM cacodylate, 150 mM NaCl, pH 7.4) for 30–60
min each, at their polymerization temperature. Samples were fixed with 2.5% glutaraldehyde in the same buffer for at least 2 hours. Next, samples were washed three times with sodium cacodylate buffer (room temperature) and dehydrated with an increasing percentage of ethanol in milliQ water (30%, 50%, 70%, 80%, 90%, 95%, and finally 3 times 100% v/v). After complete dehydration, 50% hexamethyldisilazane (HMDS) in ethanol was added (twice), left for 30 min, and then removed by pipetting under the hood and afterwards replaced by 100% HMDS. The HMDS was removed after 30 min and the samples were left to dry overnight in the hood. The next day, the dry samples were transported to a stub with carbon tape and sputter coated using a K575X sputter coater (Quorum Technologies, Gouda, The Netherlands). A layer of 15.4 nm of Au/Pd was sputtered using a current of 80 mA. The SEM samples were visualized using a STEM setup (Verios 460, FEI Company, Eindhoven, the Netherlands) using 50 pA, 5 kV and 4 mm working distance, in immersion mode. The average connectivity of the networks was determined by subdividing the SEM images taken at a magnification of 20,000 up to 50,000 magnification (depending on polymerization temperature) into 5x5 squares. In each other square, we manually determined the number of fibers at each junction in focus. We analyzed more than 100 junctions per sample. In total, we analyzed 4 samples for $T = 26^\circ C$, 3 for $T = 30^\circ C$, 2 for $T = 34^\circ C$ and 3 for $T = 37^\circ C$. Due to the inhomogeneous nature of the $T = 22^\circ C$ collagen gels, we could not determine an average connectivity for these samples.

### 6.3 Computational Model of Collagen Network Rheology

Since previous studies have convincingly shown that collagen I networks behave as networks of rigid rods for which thermal fluctuations are negligible [279], we compare our data to computational models that treat the fibers as athermal, elastic beams. Simulations have shown that the elastic properties of an athermal fibrous network are primarily determined by the local connectivity [280]. Collagen networks are inherently sub-marginal: the number of fibers meeting at each junction is less than the isostatic limit needed to ensure a stable network if the filaments were springs. As shown by Maxwell [601], spring networks require a local coordination number, or connectivity, $z$ of at least $z_{crit} = 2d$, where $d$ is the dimensionality, for mechanical stability. At the isostatic (or marginal) point, corresponding to $z = 6$ for a 3D network, the number of degrees of freedom is just balanced by the number of constraints so that the system is marginally stable to small deformations. Upon increasing the connectivity from $z < z_{crit}$ through the $z_{crit}$ point, spring networks undergo a mechanical phase transition from a floppy to a rigid phase. Collagen networks have a typical average connectivity between 3 (local branching) and 4 (binary crosslinking), placing them well below both 2D and 3D isostatic...
thresholds [279, 390]. Such sub-isostatic networks can, nevertheless, exhibit a finite elasticity as a result of other mechanical constraints. One such constraint is that the fibers have a finite bending rigidity [280, 613, 614]. An externally applied strain can also stabilize fiber as well as spring networks [615].

Two classes of models have been used to analyze the mechanical properties of submarginal fibrous networks. The first class of models, referred to as Mikado models, represents the fiber network as a collection of rigid rods that are randomly deposited in a 2D box until the desired connectivity is obtained [613, 614]. The second class of models represents the network by a 2D or 3D disordered lattice-based structure [280]. Lattice models have the advantage that they are more computationally tractable, making it easier to simulate large networks. Moreover, lattice models are versatile, since the network architecture and connectivity can be easily tuned. In this chapter, we will therefore compare our experimental data primarily to a computational model of (mostly 2D) disordered lattices. The model makes several quantitative predictions that we will test in this chapter via experiments on collagen networks.

We will focus specifically on a recent computational model that was developed by Albert Licup, Abhinav Sharma, Robbie Rens, Misha Sheinman and Fred MacKintosh at the VU University (Amsterdam, the Netherlands). All simulation data shown in this chapter (Fig. 6.2, Fig. 6.12, Fig. 6.14A, Fig. 6.18B, Fig. 6.19B and Table 6.1) were kindly provided by these authors. The details of the model are described in detail elsewhere [279, 604, 605], but will be briefly summarized here. Networks are modeled as 2D triangular lattices. The size of the networks is $W \times W$, where $W$ is the linear dimension while the lattice spacing is $l_c$ and $W = 50l_c$. In 2D, local 4-fold connectivity is enforced by randomly selecting two of the three fibers at each vertex and forming a binary cross-link between them. The remaining fiber crosses this vertex as a phantom that does not interact with the other two fibers. After this phantomization procedure, the average connectivity is further reduced to a value characteristic of collagen networks (3.2) by randomly removing segments (random bond dilution) with a probability $q = 1 - p$, where $p$ is the probability of an existing bond. This procedure reduces the average fiber length to $L = l_c/q$. Thus, the networks are, by construction, sub-isostatic and floppy in the absence of bending interactions.

Each filament is assigned a stretching modulus, $\mu_s$, and a bending modulus, $\kappa$. These two parameters define a dimensionless measure of the relative bend-stretch stiffness:

$$\tilde{\kappa} = \kappa / \mu_s l_c^2$$

For fibers behaving as homogeneous elastic beams with a cylindrical cross-section, $\mu_s = \pi a^2 E$, and $\kappa = \frac{1}{4} \pi a^4 E$ [616]. Here, $a$ is the fibril radius and $E$ is the Young’s modulus. Thus, $\tilde{\kappa} = \frac{1}{4} \frac{a^2}{l_c^2}$. Note that $\tilde{\kappa}$ is determined purely by
the geometrical parameters $a$ and $l_c$, while being independent of the material properties of the fibers.

The networks are subjected to a simple shear strain $\gamma$ and allowed to relax by minimization of the total elastic energy per unit volume, $H$, which is calculated using a discrete form of the extensible wormlike chain Hamiltonian [279,605]. The stress follows from the minimum energy $H$ as: $\sigma = dH/d\gamma$, while the differential elastic shear modulus follows as: $K = d^2H/d\gamma^2$. Stress and stiffness are measured in units of $\mu/l_c^{d-1}$, where $d = 2$ is the dimensionality.

6.4 Results

6.4.1 Impact of Polymerization Conditions on Collagen Elastic Properties

The aim of this chapter is to relate the rheological properties of reconstituted collagen networks to the underlying network architecture. Since collagen networks are formed by a self-assembly process driven by noncovalent interactions, the final architecture is highly sensitive to the solution conditions (i.e. pH and ionic conditions), protein concentration and temperature. Here, we employ two control parameters to tune network architecture and mechanics: We vary the collagen concentration over a wide range (0.2–6 mg/ml at 37°C, 1–5 mg/ml at 22°C and 2–5 mg/ml at 30°C), and we vary the polymerization temperature (from 22°C to 37°C) for a fixed collagen concentration of 4 mg/ml. The pH and ionic conditions are chosen to be close to physiological conditions, which are compatible with cell culture (DMEM at pH 7.3–7.4, supplemented with 50 mM HEPES, 1.5 mg/ml sodium bicarbonate and 1% serum).

We first tested the dependence of collagen network elasticity on protein concentration by performing shear rheology measurements on networks polymerized at 37°C. We employed a differential prestress protocol, whereby a constant prestress, $\sigma$, was applied to the sample with a small superposed oscillatory stress to probe the differential stiffness at this prestress value, $K'(\sigma)$. For each sample, the prestress was increased until network rupture occurred. As shown in Fig. 6.1A, the networks strongly strain-stiffen at all concentrations, until rupturing at strains of around 50% (see also Fig. 6.33 in supplementary information (SI)). At low strains, the networks exhibit a linear response characterized by a constant elastic shear modulus, denoted as $G_0$. However, after an onset strain $\gamma_0$ (red symbols in Fig. 6.1A), the stiffness markedly increases with increasing strain. In a few cases, the nonlinear regime showed an initial strain-softening, which was followed by strain-stiffening, similar to earlier findings with large amplitude oscillatory shear [252]. An example of this behavior is shown in Fig. 6.22 in the SI. We speculate that these samples may be prestressed, causing them to have a lower low-strain modulus than the majority of samples, which do not show this initial stiffening. Since this
Figure 6.1: Rheological properties of collagen networks measured for different polymerization conditions. (A) Strain-dependence of the differential elastic modulus measured at 37°C and at collagen concentrations of 0.7 (squares), 1 (triangles up), 2 (triangles down), 3 (stars), 4 (circles) and 5 mg/ml (triangles right), in increasing shades of gray. The onset of strain-stiffening is depicted by red symbols. (B) Concentration dependence of the low strain (linear) plateau modulus, $G_0$, at 22°C (triangles down), 26°C (stars), 30°C (circles), 34°C (triangle up) and 37°C (squares) in increasing shades of gray. (inset) Concentration dependence of the strain at the onset of strain-stiffening, $\gamma_0$, as depicted by the red points in panel (A), at 26°C (stars), 30°C (circles), 34°C (triangle up) and 37°C (squares) in increasing shades of gray. The 37°C data show a weak power law dependence on $c_p$ with an exponent of -0.25 ± 0.03. In (A), the symbols show every 5th data point, while the lines show the full dataset.

non-monotonic nonlinear behavior was rare, these samples were excluded from further analysis.

With increasing collagen concentration, the stiffening curves do not change in overall shape, although they shift up to larger moduli. This is in qualitative agreement with simulations results for fibrous networks with an average coordination of 3.2 shown in Fig. 6.2A. Here, the unit-less bending rigidity $\tilde{\kappa}$ is progressively increased from $10^{-6}$ to $10^{-3}$, which is equivalent to an increase in concentration by a factor 1000, since $\tilde{\kappa}$ is proportional to collagen concentration. Similar to our experimental findings, increasing $\tilde{\kappa}$ causes the linear modulus to shift up, while the stiffening curves do not change their overall shape.

Experimentally, we find that the linear elastic modulus $G_0$ of the networks increases with collagen concentration, $c_p$, as a power law with an exponent of 2.5 (with fitting error ± 0.05) as shown in Fig. 6.1B (black squares). However,
this dependence only holds for \( c_p > 0.6 \text{ mg/ml} \). Below 0.6 mg/ml, \( G_0 \) shows a weaker, close to linear, dependence on concentration. Coincidently, at these low concentrations, the collagen networks are very inhomogeneous, as seen in confocal reflectance microscopy (CRM) (Fig. 6.23 in SI). At the lowest concentration tested, namely 0.2 mg/ml, the network is barely connected, and the fibrils exhibit thermal fluctuations indicative of the presence of dangling ends (not shown). It should be noted that at an even lower concentration of 0.1 mg/ml, there is no network formed at all. Based on these observations, we hypothesize that the fibrils in networks formed at low concentrations (<0.6 mg/ml) are not fully connected and thus do not all contribute to the network stiffness. We therefore exclude these low density gels from the analysis in the rest of this chapter. At concentrations above 0.6 mg/ml, the network do look homogeneous and well connected. The power law exponent 2.5 is comparable to values found in several earlier studies [253, 272, 395, 602, 603]. But we note that this exponent is higher than the exponent of 2 predicted by simulations of fibrous networks when we assume that the geometry of the network in terms of \( z \) (or \( L/l_c \)) is concentration independent. Thus, the concentration dependence of \( G_0 \) we observe suggests that the architecture of the networks changes with collagen concentration.

Next, we tested the dependence of collagen network rheology on network architecture by varying the polymerization temperature, prompted by earlier work demonstrating a strong effect of this parameter on network structure [305, 602]. When we polymerize collagen networks at 22°C, we found a somewhat stronger increase of \( G_0 \) with collagen concentration (power law exponent 3±0.1) than at 37°C, as shown in Fig 6.1B (light gray triangles down). This is consistent with earlier findings [602]. The magnitude of the modulus depends non-monotonically on polymerization temperature (see Fig. 6.1B and 6.29A in SI): at a fixed collagen concentration, the stiffest gel is formed at 26°C, while the softest one is formed at 30°C. This finding is consistent with an earlier study that demonstrated a stiffness maximum at 27°C [602]. However, another study reported a monotonic increase of stiffness with increasing polymerization temperature between 4°C and 37°C [305]. These contradictory findings may be due to slight differences in salt conditions, which are known to affect the stiffness dependence on temperature [617]. It should be anyhow noted that the variations in network stiffness with temperature are rather small, remaining within a factor 3 (Fig. 6.29A in SI).

The onset strain for strain-stiffening, \( \gamma_0 \) (red symbols in Fig. 6.1A) decreases with collagen concentration according to a weak power law with an exponent of -0.25 for networks formed at 37°C (Fig. 6.1B inset, black squares). This dependence is consistent with earlier reports [251, 272], where the onset strain also decreased with increasing collagen concentration and varied by about a factor 2 within a similar concentration range. Note, however, that
Figure 6.2: Simulation results for a 2D network showing the elastic properties of athermal fibrous networks subjected to a shear deformation. (A) Strain-stiffening response of networks with varying dimensionless bending rigidity, $\tilde{\kappa}$, and $L/l_c = 3.1$ ($z = 3.2$). (B) The onset strain for strain-stiffening depends significantly on network architecture $l_c/L$. For both (A) and (B), bending rigidities were varied between $10^{-6}$ (triangles down), $10^{-5}$ (triangles up), $10^{-4}$ (circles), and $10^{-3}$ (squares).

Figure 6.3: Stress-stiffening behavior of collagen networks measured for different polymerization conditions. (A) The differential elastic modulus $K'$ for $22^\circ$C (triangles down), $26^\circ$C (stars), $30^\circ$C (circles), $34^\circ$ (triangle up) and $37^\circ$C (squares) in increasing shades of gray. (B) The stiffening exponent, $\beta$, defined as the maximum power law slope of the stress-stiffening curves in the nonlinear regime, measured for $4$ mg/ml collagen as a function of polymerization temperature.
Figure 6.4: Stiffening exponent $\beta$ for 2D simulated networks shows a dependence on network architecture and the dimensionless bending rigidity $\tilde{\kappa}$. (A) The stiffening exponent $\beta$ with varying dimensionless bending rigidity, $\tilde{\kappa}$. Bending rigidities were varied between $10^{-6}$ (triangles down), $10^{-5}$ (triangles up), $10^{-4}$ (circles), and $10^{-3}$ (squares). (B) The stiffening exponent $\beta$ for strain-stiffening depends on $\tilde{\kappa}$ at fixed network architecture. Data are shown for $L/l_c = 3.1$.

different definitions for the onset strain were used, so the absolute magnitudes of $\gamma_0$ cannot be directly compared. Consistent with the concentration dependence of $G_0$, the weak concentration dependence of $\gamma_0$ also suggests that the network geometry changes somewhat with collagen concentration. Simulations for fibrous networks assuming a concentration-independent architecture namely predict that $\gamma_0$ should be insensitive to concentration, as shown in Fig. 6.2B (where $\tilde{\kappa} \sim c_p$ and $l_c/L$ is a measure for architecture). Instead, $\gamma_0$ is sensitive to changing architecture. When the architecture of the triangular lattices is varied by changing $l_c/L$, the simulations reveal a linear increase of $\gamma_0$ with increasing $(l_c/L)^2$ for small $\tilde{\kappa}$ ($10^{-6}$ up to $10^{-4}$) and a somewhat weaker increase of $\gamma_0$ with increasing $(l_c/L)^2$ for larger $\tilde{\kappa}$ ($10^{-3}$), as shown in Fig. 6.2B. We note that we expect $\tilde{\kappa} \sim 10^{-4}$ to be relevant for collagen networks [279] (see also Fig. 6.15 in section 6.4.4). As summarized in Table 6.1 in the SI, $\gamma_0$ is predicted to go down when the average connectivity goes up from 3 to 3.87, i.e. with a decreasing degree of branching. Thus, the concentration dependence of $\gamma_0$ we observe experimentally indicates that the collagen networks polymerized at 37°C become less branched with increasing collagen concentration. When we vary the polymerization temperature between 22 and 37°C at a fixed collagen concentration of 4 mg/ml, we find a slight decrease of $\gamma_0$ with decreasing temperature (see Fig. 6.29B in SI). In view of the simulation results for networks with varying $l_c/L$ (or equivalently, $z$), this finding suggests that networks formed at lower temperatures are less branched.
The extent of strain-stiffening that is reached before the network ultimately breaks is dependent on the polymerization temperature. Networks polymerized at temperatures between 30°C and 37°C stiffen about 100-fold before breakage (Fig. 6.1A). In contrast, networks formed at 22°C or 26°C stiffen only about 3-10 times their original stiffness before rupture and they rupture at a smaller shear stress (Fig. 6.1A). To characterize the stiffening response, we determine the power-law slope of the $K'(\sigma)$ response at high stress, which we denote as $\beta$. As shown in Fig. 6.3B, $\beta$ increases monotonically with increasing polymerization temperature, from a value close to 1 at 22°C to 1.6 at 37°C. Note that the value of $\beta$ measured at 22°C may not be fully representative of the elastic limit since these networks exhibit creep (see section 6.4.2). As shown in Fig. 6.31 in the SI, $\beta$ exhibits a weak dependence on collagen concentration. For networks formed at 37°C, $\beta$ increases from $\sim 1.4$ at 0.7 mg/ml to 1.6 at 4 mg/ml. Consistent with the concentration dependence observed for $G_0$ and $\gamma_0$, these results suggest that the network geometry changes with collagen concentration. Simulations of networks with constant $L/l_c$ (or equivalently $z$) and varying $\tilde{\kappa}$ namely predict a weak decrease of $\beta$ for increasing $\tilde{\kappa}$ (or equivalently, $c_p$) (Fig. 6.4B). However, $\beta$ also heavily depends on varying architecture (i.e. $z$ or $L/l_c$), as shown in (Fig. 6.4A), where $\tilde{\kappa}$ is
kept constant. Thus, the model predicts that the stiffening exponent is mainly
dictated by geometry and is also dependent on $c_p$. We also note that the sim-
ulations predict $\beta \sim 1.2 - 1.5$ for $\tilde{\kappa} \sim 10^{-4}$ and $z = 3.2$, which is in line with
the stiffening exponent reported for the higher temperature data (Fig. 6.3B).
We conclude that the architecture of the collagen networks must change with
concentration as well as with polymerization temperature.

We further quantify the extent of strain-stiffening by plotting the ratio of
the modulus at the breakage point, $K_{max}$, over the linear modulus, $G_0$. In
the absence of architecture changes with varying $c_p$, we expect from simula-
tions that all stiffening curves measured at different collagen concentrations
should go to the same affine, stretch-dominated limit, whereas the low strain
modulus should scale with $\tilde{\kappa}$. This means that we expect for experiments that
this maximum stiffness $K_{max}$ (which should be close to the stiffness in the
stretch-dominated limit) is dependent on protein content, and thus $c_p$. From
simulations, we also expect the linear modulus to scale as $G_0 \sim c_p^2$ in the
absence of changes in architecture. Thus, the ratio $K_{max}/G_0$ should scale as
$\sim c_p^{-1}$ in the absence of architecture changes. However, we have seen that
the linear modulus shows a larger concentration dependence of $c_p^{2.5}$. Thus, we
expect that the extent of stiffening scales as $c_p^{-1.5}$. As shown in Fig. 6.5A,
we indeed observe that $K_{max}/G_0$ measured at 37°C decreases as a power law
in concentration with an exponent close to -1.5 (best-fit value -1.7±1.3), but
only above 0.7 mg/ml. At 0.7 mg/ml, $K_{max}/G_0$ exhibits a maximum. The
breakage stress likewise shows a concentration dependence with two regimes
(Fig. 6.5B). Below 0.7 mg/ml, the breakage stress increases as a power law
with exponent close to 3, while above 0.7 mg/ml, it increases nearly linearly
with concentration (fit gives 1.2 ± 0.5). This linear dependence is expected
when the network failure is due to failure of the stretched fibers. The presence
of two regimes for $K_{max}/G_0$ and for $\sigma_{max}$ again indicates that the networks
are inhomogeneous below 0.7 mg/ml. The network is likely not fully connected
at low concentrations, i.e. there are dangling ends that do not contribute to
the network response.

6.4.2 Impact of Polymerization Conditions on Collagen
Viscoelastic Properties

Given that collagen networks are expected to behave as viscoelastic solids
[72, 413, 500], we also tested whether variations in collagen concentration or
polymerization temperature have an impact on the ratio of the elastic and
viscous shear moduli. We quantified this via the loss tangent, which is defined
as the ratio between the viscous and elastic shear modulus, $\tan(\delta) = G''/G'$. As shown in Fig. 6.6, $\tan(\delta)$ changes little with concentration and with temper-
(C)ture, varying between 0.1 and 0.2. Thus, the collagen networks behave as elastic solids when probed at a frequency of 0.5 Hz. Frequency-dependent
Figure 6.6: Viscoelastic behavior of collagen networks in the linear regime for different polymerization conditions. (A) Concentration dependence of the loss tangent, $\tan(\delta)$, at $22^\circ$C (triangles down), $26^\circ$C (stars), $30^\circ$C (circles), $34^\circ$ (triangle up) and $37^\circ$C (squares) in increasing shades of gray. (B) Temperature dependence of the loss tangent for networks of 4 mg/ml collagen (same color coding as in panel A). The loss tangent is in all cases smaller than about 0.2, indicating predominantly elastic behavior ($G' \gg G''$). In both (A) and (B), the loss tangent was determined at 0.5 Hz.

Figure 6.7: Creep response of a 4 mg/ml collagen polymerized at $37^\circ$C at increasing constant shear stress levels: from light blue to pink (bottom to top) the shear stress is 18.7, 23.1, 28.5, 35.1, 43.3, 53.4, 65.8, 81.1, 100, 123, 152 and 187 Pa respectively. The last stress level was just before apparent sample breakage and determined to be $\sigma_{max}$ for this particular sample.
Figure 6.8: Inelastic behavior of 4 mg/ml collagen gels formed at different polymerization temperatures. The shear stress was increased up to a certain level (vertical dashed lines) and then decreased back down to the linear regime. This procedure was repeated, with increasing maximum stress level. Gray lines are increasing stress, while red dotted lines are decreasing stress. The polymerization temperature was 22°C (A), 26°C (B), 30°C (C), 34°C (D) and 37°C (E). Inelastic behavior is observed only for networks formed at 22°C.
oscillatory measurements show that the samples remain solid-like down to frequencies of at least 0.05 Hz (see Fig. 6.25, Fig. 6.26 and Fig. 6.27 in SI).

To test whether viscous dissipation becomes important at longer time scales, we also measured the creep response of collagen networks by applying a constant shear stress and measuring the time-dependent strain. As shown in Fig. 6.7, the samples show a quick rise of the strain upon application of stress, characteristic of an elastic solid, followed by a gradual increase in the strain, characteristic of a viscous fluid. To quantify this dissipative response, we determined the creep rate as the slope of the strain versus time curves (in units of %/s) as a function of strain (taken at $t = 30$ s). As shown in Fig. 6.28C in the SI, the creep rate is independent of the polymerization temperature at small strains of 1%. However, at strains above $\sim 10\%$, samples prepared at 22°C exhibit substantially more creep than samples polymerized at higher temperatures (Fig. 6.28A in the SI). This temperature dependence is more obvious when we directly compare the strain dependence of the creep rate measured at different polymerization temperatures while keeping the collagen concentration fixed at 4 mg/ml, as shown in Fig. 6.28B in the SI. The same curves are shifted for clarity in Fig. 6.28D in the SI. At strains below $\sim 3\text{−}5\%$ the creep curves all overlap. Above $\sim 5\%$, the 22°C sample continues to creep at the same rate. For temperatures between 26°C and 37°C, the creep rate first rises with strain, then plateaus, and finally increases sharply just before breakage (Fig. 6.28D).

To test whether network creep influences the determination of $K'$ [345], we checked whether the strain-stiffening behavior of the collagen networks was reversible. We first ramped the prestress up in a stepwise manner, and then ramped it back down to the linear regime, as shown Fig. 6.8. For samples polymerized at temperatures of 26°C and above the strain-stiffening curves showed hardly any hysteresis and the network stiffness returned to its original value (Fig 6.8(B-E)). In contrast, gels polymerized at 22°C showed a pronounced hysteresis between the upward and downward curves and significant softening (Fig. 6.8A). This hysteresis prevented a quantitative comparison of the data obtained at 22°C to the simulations, which consider perfectly elastic networks with permanent crosslinks.

### 6.4.3 Impact of Polymerization Conditions on Network Architecture

The rheology data (in particular $G_0$, $\gamma_0$ and $\beta$) suggest that the architecture of the collagen networks changes with polymerization temperature as well as collagen concentration. However, this is a somewhat indirect conclusion, since it is based on a comparison of the data with simulation results for disordered lattice-like networks. To directly test how the network structure changes
with assembly conditions, we visualized collagen networks formed at different temperatures directly using Confocal Reflectance Microscopy (CRM), as well as scanning electron microscopy (SEM) (see Fig. 6.10). CRM is convenient to characterize the network homogeneity and porosity on length scales of $\sim 10 - 100 \mu m$ under hydrated conditions without sample labeling, but it does not provide enough spatial resolution to determine fiber diameter and connectivity. In contrast, SEM provides high resolution images that give insight into the network connectivity and fiber diameter, but it requires intensive sample preparation and dehydrated conditions. Previous studies on collagen have shown that SEM shows qualitatively the same trend as seen with optical microscopy techniques (i.e. a decrease in mesh size in confocal microscopy accompanies a decrease in diameter in SEM) [90,602,618]. We expect that the fiber diameter as seen with SEM is an underestimate, however, due to loss of water [619].

CRM reveals that the collagen networks are rather homogeneous and dense at 34°C and 37°C, while at lower temperatures, the network become increasingly inhomogeneous. In the most extreme case, at room temperature (22°C), we observe fan-shaped bundles of collagen fibrils. Similar structures have been seen before in other studies for collagen at low polymerization temperatures (4–27°C) [305,306,577,606,620]. With CRM, not all fibers are detected [621]. In particular, fibers that are orientated (close to) parallel to the imaging axis will not be visible. Furthermore, fibers that are thin compared to the imaging
wavelength will be missed using this technique [608]. It is therefore difficult to identify how the fan-shaped structures are connected to form a 3D network. Using SEM however, it is possible to see that the ‘fans’ are connected by a bundle of collagen fibrils (Fig. 6.10). The fans are composed of bundles of thinner fibrils, which splay out on one end. We speculate that these fan-shaped bundles may arise as a consequence of kinetic arrest when the growing fibrils meet another fibril and adhere [577]. These fan-shaped bundles disappear when the polymerization temperature is raised to 26°C or 30°C. Consistent with CRM, the SEM images show that the microstructure at these higher temperatures is rather homogeneous, though some bundling is still visible. This bundling is further reduced at 34°C and 37°C, where the networks look homogeneous both in SEM and CRM images. From the SEM images, we can estimate the average connectivity $z$ of the networks by counting the number of fibrils/bundles crossing each junction. We did not attempt this analysis for networks formed at 22°C, in view of the inhomogeneous network structures. As shown in Fig. 6.9, $z$ is around 3.1 for temperatures of 26°C and 34°C, and is around 3.3 at temperatures of 30°C and 37°C. This implies that the network junctions are mainly provided by branches (see images on the right in Fig. 6.9). However, we do observe also examples of junctions made of two adjoining fibers with $z = 4$ (see images on the right in Fig. 6.9). We note that these values for the average connectivity are in excellent agreement with an earlier study of collagen [390] and fibrin networks [600].

The CRM images clearly reveal a decreasing pore size with increasing polymerization temperature, which is accompanied by a decrease in bundling, as seen by SEM. To quantify the degree of bundling in the hydrated state, we measured the turbidity of collagen polymerized at different temperatures. In case the collagen networks can be considered as mesh works of rod-like particles, where the rods are long compared to their diameter and to the wavelength of the incoming light, rescaling the turbidity according to eq. 6.2 should give a straight line, from which the mass-length ratio, $\mu$, and fiber radius, $a$, can be readily extracted. Fig. 6.11A shows that this rescaling indeed holds for collagen networks polymerized at temperatures between 26–37°C. However, at 22°C this rescaling does not result in a straight line. This is probably due

Figure 6.10 (facing page): Network architecture of 4 mg/ml collagen networks polymerized at different temperatures (22–37°C), as indicated by the (rotated) columns (in units of °C). (row 1) Confocal reflection images, showing at 22°C an open network of ‘fan-shaped’ fibril bundles, while with increasing temperature the networks look increasingly homogeneous and dense. (row 2–4) Scanning Electron Microscopy (SEM) images at increasing magnification. Scale bar denotes 20 µm for rows 1 and 2, 2 µm for row 3, and 200 nm for row 4.
Figure 6.11: Light scattering measurements of the fibril diameter and mass-length ratio for collagen networks polymerized under different conditions. (A) Turbidity, $\tau$, as a function of wavelength, $\lambda$, of 4 mg/ml collagen networks, rescaled according to eq. 6.2. The curves are straight for polymerization temperatures between 26°C and 37°C, whereas they deviate from the model for the more inhomogeneous networks formed at 22°C. (B-D) The number of monomers in a fibril cross-section, $N_p$, the radius of a collagen fibril, $a$, and its mass-per-length ratio $\mu$, calculated from eq. 6.2 and 6.3. In all panels, the polymerization temperature was 22 (light gray triangles down), 26 (gray stars), 30 (gray circles), 34 (dark gray triangles up) or 37°C (black squares). In panel (A), data points were taken every 2 nm wavelength, and the symbols are for clarification only. In panel (D), the solid line represents a power law fit with exponent $-0.34 \pm 0.49$ to the 37°C dataset (shifted for clarity).
to the large inhomogeneity in fiber structure and non-random arrangements of the fibers in these networks. Thus, the numbers extracted from scattering data for the 22°C networks are indicative only. As shown in Fig. 6.11, the mass-length ratio (panel D), the number of monomers in a cross-section (panel B), \( N_p \), and radius \( a \) (panel C) of the collagen fibrils all depend on polymerization temperature as well as collagen concentration. At fixed concentration, the fibers become thinner and are of smaller mass with increasing temperature, which is consistent with the imaging data shown in Fig. 6.10. Previous studies also report an increase in mesh size with decreasing polymerization temperature [90,305].

### 6.4.4 Collagen Mechanics Shows Signatures of Critical Behavior

In section 6.4.1 of this Chapter, we have shown that collagen networks stiffen when subjected to an increasing shear strain. Simulations of sub-marginal networks with a similar connectivity (\( z = 3.2 \)) as the collagen networks predict that the strain-stiffening is accompanied by a continuous transition from a bending dominated elastic regime to a stretching dominated elastic regime at a critical strain \( \gamma_c \) [605], as shown in Fig. 6.12A. Below \( \gamma_c \), the network elasticity is dominated by bending modes. For a rope-like network, for which the dimensionless fiber rigidity \( \tilde{\kappa} = 0 \), the network is floppy and there is no linear regime below a strain threshold \( \gamma_c \) (red dashed line). In the other extreme limit of \( \tilde{\kappa} \to \infty \), the network already has a high modulus in the linear regime, because deformation is dominated by affine fiber stretching (full red line). In this case only little stiffening occurs above \( \gamma_c \), which reflects shear-induced fiber alignment along the shear direction. For networks with a finite \( \tilde{\kappa} \), the rope limit is approached as \( \tilde{\kappa} \) decreases. However, even below the critical point of the rope-like networks, these networks are mechanically stable. The linear modulus \( G_0 \) increases linearly with increasing \( \tilde{\kappa} \) (see inset), clearly demonstrating that \( G_0 \) is determined by fiber bending. Irrespective of \( \tilde{\kappa} \), above \( \gamma_c \), all curves converge onto the affine limit, where the elasticity is proportional to the enthalpic stretch modulus of the fibers. Since the simulation data for \( K \) are in units of \( \rho \mu \), and since \( \tilde{\kappa} \sim \rho \), linear scaling of \( G_0 \) with \( \tilde{\kappa} \) implies that \( G_0 \sim \rho^2 \sim c_p^2 \), where \( \rho \) is the total length per volume. Indeed, a nearly quadratic power law dependence has been seen before for collagen gels [253, 272, 395, 602, 603], although stronger and weaker dependencies have also been reported [92,251,527,603].

If strain-stiffening involves a continuous transition from a bend-dominated to a rigid stretch-dominated state at \( \gamma_c \), we expect that the stress-stiffening curves can be collapsed unto one master curve via critical scaling [280,605]:

\[
K \sim |\Delta \gamma|^f \mathcal{G}_\pm \left( \frac{\tilde{\kappa}}{|\Delta \gamma|^\phi} \right)
\]  

(6.6)
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Figure 6.12: Simulation data showing critical scaling of the elastic modulus of athermal fibrous networks of varying \( \tilde{\kappa} \) and fixed architecture of \( L/l_c = 3.1 \) (corresponding to \( z = 3.2 \)) with strain. (A) Dimensionless differential elastic modulus \( K \) for fibrous networks with \( \tilde{\kappa} = 0 \) (dotted red line), \( 10^{-6} \) (triangles down), \( 10^{-5} \) (triangles up), \( 10^{-4} \) (circles), \( 10^{-3} \) (squares) and \( \sim \infty \) (red solid line). (inset) The linear modulus, \( G_0 \), shows a linear dependence on \( \tilde{\kappa} \) (dashed line), where \( G_0 \simeq \tilde{\kappa} \). (B) The data from panel (A) collapsed according to eq. 6.6 using scaling exponents \( f = 0.75 \) and \( \phi = 2.1 \). The lines show power law scaling with exponents of 1 for the lower critical branch, and \( f/\phi \) for the upper branch.

Here, \( \Delta \gamma = \gamma - \gamma_c \) denotes the proximity to the critical point and \( G_{\pm} \) is a scaling function to capture the asymptotic behavior of \( K(\gamma) \) in the limits \( \gamma \to 0 \) and \( \gamma \to \infty \). \( G_{\pm} \) has a negative branch that corresponds to networks below the critical point (\( \Delta \gamma < 0 \)) and a positive branch that corresponds to networks above the critical point (\( \Delta \gamma > 0 \)). Simulations of networks with varying \( z \) indicate that the critical exponents \( f \) and \( \phi \) are set by the architecture (i.e. \( L/l_c \) or equivalently \( z \)) of the network (see Table 6.1 in SI). As shown in Fig. 6.12B, the simulation data for \( K(\gamma) \) in Fig. 6.12A with varying \( \tilde{\kappa} \) can indeed be collapsed onto one mastercurve according to eq. 6.6, using \( f = 0.75 \) and \( \phi = 2.1 \). Note that we determined the critical point, \( \gamma_c \), as the inflection point of the \( \log(K) \) versus \( \log(\gamma) \) curves in the strain-stiffening regime. In the limit where \( \gamma \to 0 \), we observe the expected linear dependence of \( G_0 \) on \( \tilde{\kappa} \) (solid line in Fig. 6.12B). This regime corresponds to the negative branch of \( G_{\pm} \), for \( (\Delta \gamma < 0) \). When \( \gamma \) approaches \( \gamma_c \), the scaling shows a power law with exponent that is proportional to \( f/\phi \) (dashed line in Fig. 6.12B). Since \( K \) must be finite at \( \Delta \gamma = 0 \), we expect \( K \sim \kappa^{f}\mu^{1-f/\phi} \). When \( \gamma \) increases above \( \gamma_c \), all rescaled prestress curves converge to a plateau value that is independent of
κ. We note that this critical scaling, though obtained for an athermal system, is analogous to the critical scaling of the magnetization in the presence of an applied field for a ferromagnet at varying temperatures [622].

In experiments, we can test whether our collagen systems also show a continuous transition from a stretch-dominated to a bend-dominated regime at γ_c, by using a slightly modified expression for the critical scaling [605]:

\[ K' \sim c_p |\Delta \gamma|^f G_\pm \left( \frac{c_p}{|\Delta \gamma|^{\phi}} \right) \]  

As shown in Fig. 6.13A, the prestress data measured at 37°C and at varying collagen concentrations (0.7–5 mg/ml) nicely collapse to one universal curve when we use scaling exponents of \( f = 0.8 \) and \( \phi = 2.2 \). For networks polymerized at 30°C (2–5 mg/ml), we obtain data collapse with comparable exponents, \( f = 0.8 \) and \( \phi = 2.1 \). In simulations, these exponents are consistent with \( \langle z \rangle = 3.3 \) (Table 6.1 in SI). This \( z \)-value is consistent with our determined average \( \langle z \rangle \) for both 37°C and 30°C (Fig. 6.9). Moreover, this \( z \)-value is close to a previously reported value of 3.4 [390] and interestingly, it is also close to reported values for fibrin networks [600]. Thus, our data show that the nonlinear elastic response of collagen networks can be understood as a mechanical transition from a soft, bending-dominated state, to a rigid, stretching-dominated state. In the limit where \( \tilde{\kappa} = 0 \), this is a mechanical phase transition with a critical point \( \gamma_c \).

We will now more closely examine the concentration dependence of the critical scaling obtained at 37°C and 30°C. In the linear elastic regime, where \( \gamma \ll \gamma_c \), we expect from eq. 6.7 a concentration dependence of \( K' \) according to:

\[ \frac{K'}{c_p} \sim c_p^{\gamma_c(f-\phi)} \]  

If we assume that \( \gamma_c \) only varies due to architecture changes, then we can write:

\[ \frac{K'}{c_p} \sim c_p^{1+\alpha} \]  

Experimentally we find \( K'/c_p \sim c_p^{1.25} \) and \( K'/c_p \sim c_p^{1.1} \) for the lower branch of the critical scaling for 37°C and 30°C respectively (see solid lines in Fig. 6.13A and B). The bottom insets of Fig. 6.13A and B show that this scaling is consistent with the measured concentration dependence of \( G_0 \). Note that the scaling is also close to the scaling of \( G_0 \) with \( c_p \) shown earlier (Fig. 6.1B), where we found \( G_0 \sim c_p^{2.5\pm0.05} \) for 37°C and \( G_0 \sim c_p^{1.9\pm0.49} \) for 30°C.

Experimentally, we also observed a weak concentration dependence for the critical strain:
Figure 6.13: Critical rescaling for collagen networks polymerized at 37°C (A) and 30°C (B) according to eq. 6.7. The lines show power law scalings with exponents close to 1 for the lower critical branch (1.25 and 1.1) and $f/\phi$ for the upper branch. (bottom insets) The linear modulus $G_0$ (solid symbols) is consistent with the concentration scaling observed in the lower critical branch, where $\gamma \rightarrow 0$ and $\gamma < \gamma_c$ (dashed lines). (top insets) Open and closed symbols correspond to $\gamma_0$ and $\gamma_c$ respectively. In panel (A) the lines are best power law fits with exponents -0.11 ± 0.03 and -0.25 ± 0.03 for $\gamma_c$ and $\gamma_0$ respectively. In panel (B), the expected concentration dependences are shown in the insets, based on the critical rescalings. In panel (A), the expected dependence for $G_0$ is shown in the inset (2.25). The critical exponents are (A) $f = 0.8$ and $\phi = 2.2$, (B) $f = 0.8$ and $\phi = 2.1$. Increasing concentration is indicated in increasing shades of gray (0.7–5 mg/ml for (A) and 2–5 mg/ml for (B)).
where $\epsilon \sim 0.1$ at $37^\circ$C as well as $30^\circ$C. This concentration dependence suggests that the network architecture changes with concentration, since simulations of networks with constant architecture predict a concentration-independent $\gamma_c$ (see Fig. 6.12A). By combining eq. 6.8 and eq. 6.9, we find that the exponents $f$, $\phi$, $\alpha$ and $\epsilon$ should be internally consistent:

$$\gamma_c \sim c_p^{-\epsilon}$$  \hspace{1cm} (6.10)

Based on our data, $\epsilon = \alpha/(\phi - f) \simeq 0.2$ for $37^\circ$C and $\epsilon \simeq 0.1$ for $30^\circ$C, which is indeed nicely consistent with the observed dependence of $\gamma_c$ on $c_p$ as shown in the insets in Fig. 6.13A and B.

For the upper critical branch corresponding to the limit of $\gamma \to \gamma_c$, we expect that $K'/c_p|\Delta\gamma|^{-f}$ should increase as a power law in $c_p/|\Delta\gamma|^{-(\phi - f)}$ with a power law exponent of $f/\phi$ (dashed lines in Fig. 6.13). Experimentally, we find $f/\phi \simeq 0.36$ for $37^\circ$C and $f/\phi \simeq 0.38$ for $30^\circ$C. In the limit where $\gamma$ is much larger than $\gamma_c$, simulations predict that all rescaled prestress curves should converge to the same constant level (see Fig. 6.12B). We do observe an approximate convergence of data obtained at different concentrations at both $37^\circ$C and $30^\circ$C. Interestingly, this plateau value has the same order of magnitude for both temperatures (i.e. $K'/c_p|\Delta\gamma|^{-f} \sim 10^4$). This is expected, since in this high strain regime, the affine elastic stretching limit, architecture no longer plays a role since the fibers in the network become more aligned with shear direction in the affine limit.

On the basis of the successful critical scaling presented in Fig. 6.13, the internal consistency of the scaling exponents, and the agreement of the exponent with predictions of simulations of sub-marginal fibrous networks, we can conclude that the nonlinear elasticity of collagen gels polymerized at $37^\circ$C and $30^\circ$C is well-explained by a strain-induced transition from a soft to a rigid state. However, we find clear evidence that the network architecture changes with concentration, $c_p$: Both $G_0$ and $\gamma_0$ show a dependence on collagen concentration that deviates from the dependencies expected in case of scale-invariant networks. This is especially obvious for $\gamma_c$, which decreases with increasing concentration, whereas it is expected to be constant in case of scale-invariant networks. The onset strain for strain-stiffening likewise shows a power law dependence on collagen concentration, as shown in the top insets of Fig. 6.13A and B:

$$\gamma_0 \sim c_p^{-\alpha},$$  \hspace{1cm} (6.12)
Figure 6.14: Internal consistency of the critical exponents. (A) Simulation data for the dependence of $\gamma_0$ (open symbols) and $\gamma_c$ (closed symbols) on the average network connectivity, $\langle z \rangle$, for athermal fibrous networks. Data for $\gamma_0$ are consistent with estimates from eq. 6.14 (crosses), which assumes that $\gamma_0$ and $\gamma_c$ are interrelated via the critical scaling exponents $f$ and $\phi$. (inset) Consistent with eq. 6.14, $\gamma_0$ increases linearly with $\gamma_c^{\phi-f}$ (full line). For each data point in panel (A), the individual $f$ and $\phi$ values were used as summarized in Table 6.1 in SI. (B) Experimental data for $\gamma_0$ and $\gamma_c$ likewise show a linear dependence according to eq. 6.14. Data are shown for 0.7–5 mg/ml for 37°C (squares), 4–5 mg/ml for 26°C (stars), 1–5 mg/ml for 30°C (circles) and 4 mg/ml for 34°C (triangle up) in increasing shades of gray. For the 30 and 37°C datasets, we used the exponents from the critical rescaling (Fig. 6.13, $f = 0.8$ and $\phi = 2.1$ for 30°C and $f = 0.8$ and $\phi = 2.2$ for 37°C). For the 34°C and 26°C data sets, we assumed $f = 0.75$ and $\phi = 2.1$, based on simulation data for an average connectivity $\langle z \rangle \simeq 3.2$ (Table 6.1 in SI) as measured under these conditions (Fig. 6.9). The full line denotes the expected linear scaling.
write the following [623]:

$$\frac{\log \gamma_0}{\log \gamma_c} \sim \frac{-\alpha \log c_p}{f - \phi \log c_p} \sim \phi - f$$  \hspace{1cm} (6.13)

Thus if changes in architecture are the only source of the concentration dependence for $\gamma_0$ and $\gamma_c$, these two quantities should be related by:

$$\gamma_0 \sim \gamma_c^{(\phi - f)}$$  \hspace{1cm} (6.14)

We checked this relation both for simulations and experiments in Fig. 6.14. As shown in panel A, the simulations demonstrate that $\gamma_0$ indeed scales linearly with $\gamma_c^{(\phi - f)}$ (see inset), and that $\gamma_0$ calculated from $\gamma_c$ (crosses) agree with values of $\gamma_0$ directly measured from the simulated stiffening curves (open squares).

To test eq. 6.14 for the experimental data, we used the critical exponents as determined in Fig. 6.13 for the 30°C and 37°C data sets, and expected values of $f$ and $\phi$ for the 26°C and 34°C data sets as predicted (Table 6.1 in SI) for networks having the same connectivity as measured in SEM images (Fig. 6.9). We find that also in case of the experiments, $\gamma_0$ increases linearly with $\gamma_c^{(\phi - f)}$, and the data measured for temperatures between 26–37°C collapse onto a single curve with exponent 1 (Fig. 6.14B). This strongly indicates that changes in architecture provide the main source of the concentration dependence of $\gamma_0$ and $\gamma_c$.

The turbidity measurements indeed corrobore the conclusion that the architecture of the collagen networks changes with concentration. These data show that the fibril mass-length ratio, and consequently $N_p$, weakly decreases as a power law with increasing concentration for collagen networks formed at the 37°C (Fig. 6.11B and D). Intriguingly, this power law exponent is close to the concentration exponent of $\gamma_0(c_p)$ (Fig. 6.13A top inset). This correspondence suggests that $\mu$ and network architecture may be related. One possible way to explain this, is that fibers with a higher mass-length ratio are less likely to branch, thereby increasing $z$, while fibers with lower mass-length ratio branch more frequently. However, this effect is apparently small, since the experimental stiffening curves still show a good collapse onto a mastercurve, using a single set of critical exponents (albeit on a log-log scale). It will be interesting to test this hypothesis in the future by determining the connectivity as a function of collagen concentration.

In view of the evidence for critical behavior, we can write down an equation of state for fiber networks under shear deformation [605] in analogy to ferromagnetism [622]. By numerical inversion of this equation of state, an approximate scaling function $G_{\pm}$ can be computed that holds over the entire strain range:
Figure 6.15: Quantitative comparison of the measured strain-stiffening behavior of collagen networks polymerized at different collagen concentrations with scaling theory for fibrous networks undergoing a mechanical transition from bend-dominated to stretch-dominated elasticity, measured at (A,B) 37°C, and (C,D) 30°C. In panels (A,C) experimental data (symbols) are compared with fits (red lines) to the scaling prediction in eq. 6.15, using $\tilde{\kappa}$ as the only fitting parameter. The collagen concentration was 0.7 (squares), 1 (triangles up), 2 (triangles down), 3 (stars), 4 (circles) and 5 mg/ml (triangles right), in increasing shades of gray. In panels (B,D) the best-fit values for $\tilde{\kappa}$ are shown as a function of collagen concentration. The red lines denote the expected linear scaling, while the dashed black lines denote a power law fit with exponents of $1.15 \pm 0.09$ in (B) and $1.19 \pm 0.02$ in (D).
Using eq. 6.15, we can fit the experimental $K'$ versus $\gamma$ data, using $\tilde{\kappa}$ as the sole fitting parameter. For this, we need to convert the dimensionless modulus, $K'$, from simulations to physical units by $K' = \mu \rho K$, where $K$ is a function of $z$, $\tilde{\kappa}$ and $\gamma$. A change in architecture gives an extra $\sigma_p^0$ dependence in the low strain regime. If we assume we can write $K' = A_T c_p^{1+\epsilon} K$, where $A_T$ is a constant per polymerization temperature containing the Young’s modulus of a fiber among other geometric factors. Using this $K'$ relation in eq. 6.15, and $\alpha = 0.25$ for 37°C and 0.1 for 30°C, we can fit our experimental curves. As shown in Fig. 6.15A and C, eq. 6.15 (red lines) indeed accurately captures the entire stiffening curves of 37°C and 30°C collagen gels for all concentrations tested.

As a consistency check, we show that $\tilde{\kappa}$ extracted from the fits indeed scales approximately linearly with collagen concentration as expected from simulations in Fig. 6.15B and D. For the 37°C dataset, $\tilde{\kappa}$ increases from $\sim 8 \cdot 10^{-5}$ to $\sim 7 \cdot 10^{-4}$ as $c_p$ increases from 0.7 to 5 mg/ml. For the 30°C data, $\tilde{\kappa}$ is slightly lower at the same collagen concentration, increasing from $\sim 8 \cdot 10^{-5}$ to $\sim 2 \cdot 10^{-4}$ as $c_p$ increases from 2 to 5 mg/ml. These values for $\tilde{\kappa}$ are consistent with earlier estimations of $\tilde{\kappa} \leq 10^{-3}$, based on measurements on rat tail collagen I gels polymerized under different buffer conditions (pH 9.5 instead of pH 7.3) [279].

In this section, we have shown for the first time that collagen shows signatures of critical behavior, as shown in Fig. 6.13, and that collagen mechanics can be described by eq. 6.15 (Fig. 6.15). Also, the concentration dependences of the linear modulus, the onset of stiffening and the critical strain are related via their critical exponents $f$ and $\phi$ (Fig. 6.14), which are governed by architecture changes.

### 6.4.5 Quantitative Prediction of Collagen Network Stiffness

In this section, we will make a quantitative comparison between simulations and experiment. For this, we first need to estimate the total amount of fiber length per unit volume, $\rho$, in the 3D FCC lattice. The total fiber length in an FCC lattice is $L_{\text{total}} = 6N^3 l_c$, where $N^3$ is the total number of vertices and $l_c$ is the spacing between vertices. The factor 6 originates from the fact that every lattice site is the intersection of 6 fibers. The total volume of the lattice is given by $V = v_0 N^3$, where $v_0$ is the volume of a unit cell. To identify $v_0$, we enclose every vertex by a sphere of radius $r_0 = l_c/2$, such that we have a close packing of spheres. Each sphere then encloses a total fiber length of $6l$. Thus, the fiber length per volume within one sphere is $6 \times 2R_0/V_{\text{sphere}} = 9/(\pi R_0^2)$,
where the sphere volume is $4\pi R_0^3/3$. The maximum length of fiber per volume (i.e. before random dilution) in the full packing is smaller than this by a factor $\phi_{\text{max}}$, where $\phi_{\text{max}} = \pi/\sqrt{18}$ is the volume fraction of the FCC lattice. Thus, $\rho_{\text{max}} = 3/\sqrt{2}l_c^{-2}$.

Since the lattice spacing $l_c = 2R_0$, $\rho_{\text{max}} = 12/\sqrt{2}l_c^{-2}$. This expression provides an overestimate of the density of an actual network, since the FCC lattice is highly ordered. Thus, we did not allow for bond dilution yet.

To obtain an average connectivity $\langle z \rangle \approx 3.4$ that is comparable to the typical connectivity of a collagen network, typically 20% of fiber segments are removed in the simulations. Even then, the simulated networks are likely still denser than actual networks [624]. Thus, henceforth, we will assume that the actual fiber length per volume is about 50% of the maximum length per volume in the FCC lattice, so $\rho = 4l_c^{-2}$. The polymer volume fraction is proportional to $\rho$ according to:

$$\phi_{\text{vol}} = \frac{\pi a^2}{4\rho} = 4\pi a^2 l_c^{-2}.$$

To allow a quantitative comparison between simulations and experiment, we next need to map the dimensionless bending rigidity from the simulations onto actual network parameters. The filaments have a stretching modulus, $\mu_s$, and bending modulus, $\kappa$. Assuming that the collagen fibrils behave as tight bundles with a cylindrical cross-section, $\mu_s = \pi a^2 E$, and $\kappa = \frac{1}{4} \pi a^4 E$ [616]. Here, $a$ is the fibril radius and $E$ is the Young’s modulus. Combining these expressions, we obtain $\tilde{\kappa} = a^2/l_c^2$.

Note that $\tilde{\kappa}$ is determined purely by the network geometry, and is independent of the material properties of collagen. We can rewrite this expression for $\tilde{\kappa} = 1/16\pi\phi_{\text{vol}}$, where $\phi_{\text{vol}}$ is the fiber volume fraction.

Micromechanical measurements on bovine collagen fibrils and tendons under hydrated conditions indicate values of $E$ between 0.03 and 0.6 GPa [251,284–288], though larger values were found in one study of human collagen fibrils (~3 GPa [625]).

The simulations show that at low stress, the elastic energy of the network is governed by soft bending modes. Thus, the linear elastic modulus is proportional to the fiber bending rigidity:

$$G_0 = F(z)\mu_s/l_c^2\tilde{\kappa} = F(z)\frac{1}{4}\phi_{\text{vol}}E\tilde{\kappa} = F(z)\frac{1}{64\pi}\phi_{\text{vol}}E$$ (6.16)

The prefactor $F$ depends on the coordination number $z$. If $z$ is independent of concentration, as assumed in the simulations, then this model predicts a quadratic increase of $G_0$ with protein concentration. Indeed an approximate quadratic dependence has been observed in several [602,603], but not all [251, 602, 603], experimental studies of collagen. We can thus directly estimate $G_0$ based on the Young’s modulus of a collagen fiber and the fiber volume fraction, if we assume that the factor $F(z)$ is close to unity. The fiber volume fraction we can estimate using the turbidity results (Fig. 6.11), where the total length per volume is $c_pN_A/\mu$, where $N_A$ is Avogadro’s number. For a 1 mg/ml collagen gel polymerized at 37°C, where we have shown that $\alpha = 0.25$ (see section 6.4.4 and Fig. 6.13A), $\mu = 2.1 \cdot 10^{13}$ Da/cm (Fig. 6.11D) and taking $E \approx 0.1$.
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Figure 6.16: Concentration dependence of the low strain (linear) plateau modulus calculated according to eq. 6.16 (using \( F(z) = 1 \)) (open symbols) compared with measured \( G_0 \) values (closed symbols) at a polymerization temperature of 37°C (A) or 30°C (B).

GPa [251, 284–288], we obtain \( G_0 \simeq 13 \) Pa. This value is indeed reasonably close to the measured value of 5 Pa measured by rheology.

In Fig. 6.16, we checked the concentration dependence of \( G_0 \) estimated following the above reasoning. The estimated \( G_0 \) values indeed show a similar concentration dependence as the experimental data, both for networks gelled at 37°C (panel A) and at 30°C. However, the calculated values are systematically somewhat larger than the measured values, except in the 30°C case (panel B). Potentially, the Young’s modulus of the reconstituted collagen fibrils is lower than the literature values, which were mostly measured for fibrils extracted from tissue. Note that the elastic moduli are several orders of magnitude lower than the affine limit, where \( G_0 \) is dominated by the mechanical resistance of the fibers to axial stretch. For an isotropic network of rods, the elasticity in the affine limit is \( G_0 = \frac{1}{15} \rho \mu_s \) [613]. At 1 mg/mL collagen concentration, \( \mu_s \) is \( \sim 1800 \) nN (taking \( a = 80 \) nm and \( E = 0.1 \) GPa), so \( G_0 \) is 34 kPa, which grossly overestimates the measured stiffness. All in all, these considerations strongly suggest that the low strain elastic modulus is governed by fiber bending and can be predicted based on geometrical arguments. We also note that we find a somewhat stronger concentration dependence for the linear modulus with a power law of 2.25 for 37°C and 2.1 for 30°C, in contrast to what this model predicts (power law of 2). These findings suggest that the average coordination number \( \langle z \rangle \) of collagen networks does depend on concentration, at least under the self-assembly conditions used here. This can be accounted for by assuming that the factor \( F(z) \) can be expressed by a factor \( c_p^{\alpha} \). With this assumption, the linear modulus is also in quantitative agreement with the 37°C data set (see Fig. 6.32 in SI).
6.4.6 Normal Force Tunes Network Stiffness

We have presented the nonlinear elasticity of collagen gels polymerized at different temperatures in section 6.4.1. In section 6.4.4 we showed that the nonlinear elasticity of collagen networks is consistent with multiple predictions made by computational studies of sub-marginal fibrous networks. In particular, collagen networks exhibit mechanical critical behavior, which explains the concentration dependence of the linear elastic modulus, the strain at the onset of stiffening, the critical strain $\gamma_c$, and the full strain-dependence of $K'$.

In this section we will check another prediction of the athermal network model. When a fibrous network is sheared between two plates with a fixed gap, it will develop a negative (contractile) normal stress [255,454,626]. Recent simulation data of athermal fibrous networks using the same model as described in this Chapter showed that $K$ grows in direct proportion to the normal stress, $\sigma_N$ [279,604]:

$$K' \simeq G_0 + \chi|\sigma_N|$$  \hspace{1cm} (6.17)

Here $\chi$ is a constant that will be referred to as the susceptibility. Based on
Figure 6.18: The susceptibility of collagen networks exhibits a linear dependence on the inverse of the onset strain for strain-stiffening, $1/\gamma_0$, in experiments as well as simulations. (A) Measured susceptibility as a function of onset strain, which was varied by varying the polymerization temperature and collagen concentration (see Fig. 6.1B). Data are shown for 0.7–5 mg/ml collagen gels polymerized at 37°C (squares), 4 mg/ml gels at 34°C (triangles up), 1–5 mg/ml gels at 30°C (circles) and 4–5 mg/ml networks at 26°C (stars), in decreasing shades of gray. (B) Simulation data for the susceptibility as a function of the onset strain, which was varied through $L/l_c$ (see Fig. 6.14A). Simulations were performed for $\tilde{\kappa}$ values of $10^{-6}$ (triangles down), $10^{-5}$ (triangles up), $10^{-4}$ (circles), and $10^{-3}$ (squares). The solid lines indicate the expected linear scaling.

this observation, it was hypothesized that normal stresses can stabilize submarginal collagen networks at strains below $\gamma_c$. However, this hypothesis was not tested experimentally until now. To test it, we measured the normal stress for collagen gels polymerized at different temperatures and collagen concentrations. As shown in Fig. 6.17A, we indeed observe a negative normal stress upon shearing, as expected for biopolymer networks [255,454,626]. We can observe a negative normal stress in all gels having collagen concentrations above $\sim$1 mg/ml, except in the 22°C case, where the data are too noisy and the strain-stiffening regime is rather small (not shown). We always observe an offset for the normal force $F_n$ in the linear regime, which is likely dominated by the surface tension of the sample at the outer edge of the cone-plate. Since this effect is not related to the collagen network itself, and since we expect $F_n$ to be close to zero when there is no shear stress applied [626], we subtracted the offset from the normal stress data.

Based on eq. 6.17, we expect to find a linear dependence of $K'$ on $\sigma_N$. We checked this for varying collagen temperatures and collagen concentrations (Fig. 6.17B). Indeed, we find a linear dependence of $K'$ on $\sigma_N$, from which we
can in principle determine $\chi$ by linear fitting. However, since it was difficult to measure $\sigma_N$ reliably for the lower collagen concentrations (i.e. $c_p < 2$ mg/ml), we employed an alternative way to determine $\chi$.

Simulations previously showed that at the onset of stiffening, the shear stress and the normal stress are equal in magnitude [255, 604, 626]. Thus, eq. 6.17 can be rewritten as $K'/\sigma_0 \simeq \chi$, where $\sigma_0$ is the onset stress. We used this relation to determine $\chi$ for all collagen experiments. The resulting susceptibility data are depicted in Fig. 6.18A and compared with simulations in panel B. In both cases, we find that the susceptibility increases linearly with $1/\gamma_0$, meaning that it depends on network architecture. We next checked whether $K'$ follows the prediction made in eq. 6.17. For this we focused on collagen gels with $c_p \geq 2$, where $F_n$ could be measured over more than half a decade of $\sigma$. As shown in Fig. 6.19, eq. 6.17 provides a reasonable prediction of $K'$, especially in terms of slope, for all collagen concentrations tested, both at $37^\circ$C (panel A) and at $30^\circ$ (panel C). This observation suggests that normal force indeed governs the nonlinear elasticity of collagen gels. The consistent underestimation of $K'$ by a factor of about 2 could be due to a residual offset in $F_n$. However, for the simulation data, $K'$ at high stresses is underestimated by the theoretical prediction as well. Indeed, eq. 6.17 is no longer expected to hold when $\sigma$ approaches the critical stress $\sigma_c$.

### 6.5 Discussion and Conclusion

Collagen is the most important structural and mechanical component of the ECM [301]. For this reason, collagen has been a popular model system for in vitro studies of cell in a 3D ECM environment in the context of mechanobiology, and normal and tumor cell migration [90, 271, 606, 627, 628]. There has been quite a large number of publications reporting on the linear and nonlinear rheological properties of collagen [92, 251–253, 272, 279, 395, 413, 527, 602, 603], with some attempts to quantitatively relate the rheology to the underlying network structure [251, 252, 272, 413, 602]. However, a consensus about the physical basis of the nonlinear mechanics of collagen networks has been lacking.

Here we have shown for the first time that the mechanics of collagen gels is governed by a mechanical transition from a floppy, bend-dominated elastic regime to a rigid, stretch-dominated elastic regime. This is a consequence of the gels being sub-marginal, with an average connectivity of $\sim 3.4$, which is below the isostatic limit of $z = 6$ in 3D. The development of rigidity is characterized by a strain-controlled continuous phase transition with signatures of criticality. The critical exponents $f$ and $\phi$ are set by the network architecture, in particular the average connectivity $\langle z \rangle$. We showed that the strain-stiffening behavior of collagen networks is well described across the entire accessible strain range by an approximate scaling function (eq. 6.15) derived in analogy to ferromagnetism [605, 622], as shown in Fig. 6.15. Also, the concentration dependencies
Figure 6.19: Experiments and simulations demonstrate that the strain-stiffening response of collagen gels is governed by normal stress. (A,C,D) Measured $K'/G_0$ (lines) for collagen gels polymerized at different concentrations and at fixed temperatures of 37°C (A) or 30°C (C), and for gels polymerized at different temperatures ranging between 26°C and 37°C and at a fixed concentration of 4 mg/ml (D), compared with predictions made using eq. 6.17 (red-outlined symbols), using measurements of the normal stress and the susceptibility (see Fig. 6.18) as input. In (A) and (B) the collagen concentration was 2 (triangles down), 3 (stars), 4 (circles) and 5 mg/ml (triangles right), in increasing shades of gray. In (D), the symbols denote collagen polymerized at 26°C (stars), 30°C (circles), 34°C (triangles up) and 37°C (squares) in increasing shades of gray. (B) Corresponding simulation data for athermal fibrous networks of varying normalized bending stiffness, $\tilde{\kappa}$ and fixed $L/l_c = 3.1$, where the gray lines are the rescaled $K'/G_0$ data and the red dotted lines are the predictions of eq. 6.17. In increasing shades of gray, $\tilde{\kappa}$ is $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$. 

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of the linear modulus, the onset of stiffening, and the critical strain are interrelated via the critical exponents $f$ and $\phi$ (Fig. 6.14). Experimentally, we could vary these exponents by inducing architecture changes by varying the collagen concentration and the polymerization temperature over a wide range. Between 22°C and 37°C, the collagen architecture could be changed from a very open and heterogeneous mesh work of thick collagen fibril bundles to a homogeneous dense network of collagen fibrils with small pore size.

Earlier computational studies had suggested that strain-stiffening of athermal fiber networks originates from a transition from bending-dominated elasticity at low strain to a stretch-dominated elasticity regime at high strain [425]. However, here we showed that the networks already stiffen substantially even well before the strain-controlled transition to the stretch-dominated state. Thus, the strain-stiffening that sets in at $\gamma_0$ is actually not caused by a bend-to-stretch transition. The simulations suggest that stiffening in the bend-dominated stiffening regime is induced by a build-up of normal stress. When a fibrous network is sheared between two plates with a fixed gap, the networks develop a negative normal stress [255, 626]. Based on simulation data, it was hypothesized that this normal stress can stabilize sub-marginal collagen network for strains below $\gamma_C$. Here we could support this hypothesis by our experimental data showing that $K'$ grows in direct proportion to the normal stress $\sigma_N$.

Now that we have a complete quantitative understanding of the nonlinear elastic properties of collagen, we can predict the elastic stiffening of a given collagen network based its connectivity and the fiber volume fraction. This gives us a powerful tool to predict the elastic mechanical properties of more physiologically relevant collagen systems, where the mass-length ratio of the fibers is regulated by accessory ECM molecules such as the minor fibrillary collagens type V or III [251].

We found that collagen gels exhibit significantly more viscous dissipation than the fibrin networks presented in Chapter 2. The loss tangent for collagen is typically around 0.1, whereas it is $\sim 0.01$ for fibrin [66, 297]. The microscopic basis for this viscous behavior is still unknown. It may originate at the level of fiber-fiber contacts, or at the level of the fibers themselves. It has been proposed in prior studies of single collagen fibril mechanics that viscous dissipation may be mediated by sliding between monomers [287, 629]. We discovered a significant dependence of the inelastic behavior on polymerization temperature. Collagen gels polymerized at 22°C were clearly inelastic, whereas collagen gels polymerized at temperatures of 26°C were more elastic. This observation is consistent with a picture where sliding between or within fibrils contributes to viscous dissipation, since increasing temperatures should strengthen the hydrophobic interactions that hold collagen monomers together [630, 631]. Several recent studies showed that viscous dissipation of cell substrates can have
a major influence on cell behavior \cite{69, 70, 500}. It was suggested that viscous dissipation is especially significant at the slow time scales relevant for cells (\sim min) \cite{72}. In terms of experiments on cellular mechanosensing, the viscous and inelastic properties of collagen networks should be considered for cell tests inside 3D collagen gels.

6.6 Acknowledgments

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Figure 6.20: Time dependence of the scattered light intensity measured at a wavelength of 600 nm for polymerizing collagen gels at room temperature, at varying collagen concentrations. Concentrations are 1 (triangles up), 2 (triangles down), 3 (stars), 4 (circles) and 5 mg/ml (triangles right), in increasing shades of gray. Every 2 minutes, a data point was collected. The vertical dotted line indicates the time when rheology measurements were always started. The curves exhibit a typical sigmoidal shape with a characteristic lag time before the turbidity starts to increase. The lag time decreases with concentration, whereas the final turbidity reached in the plateau phase increases with concentration. (inset) Concentration dependence of the lag time, $t_{\tau}$. The gray line denotes a power law fit with exponent $-0.65\pm0.08$. 


Figure 6.21: Definition of the onset strain, $\gamma_0$, of stiffening as the minimum of $K'/\sigma$. (A) Experimental data for collagen gels polymerized at 37°C and at varying collagen concentrations. Concentrations are 0.7 (squares), 1 (triangles up), 2 (triangles down), 3 (stars), 4 (circles) and 5 mg/ml (triangles right), in increasing shades of gray. (B) Simulation data for fixed $L/l_c = 3.1$ and varying dimensionless bending rigidities $\tilde{\kappa}$: $10^{-6}$ (triangles down), $10^{-5}$ (triangles up), $10^{-4}$ (circles), and $10^{-3}$ (squares). Red symbols in (A) and (B) are taken as the onset of stiffening.

Figure 6.22: Example curve for the nonlinear elastic response of a 1 mg/ml collagen gel that initially shows softening before it starts to stiffen as the stress is further increased. This non-monotonic type of behavior was only seen for 1, 2 and 3 mg/ml gels and at 37°C. Since this behavior was rare, these gels were excluded from further analysis.
Figure 6.23: Confocal reflection images of collagen networks formed at different collagen concentrations and a fixed polymerization temperature of 37°C. Concentration is indicated in the images in mg/ml. Scale bar denotes 20 μm for all images. All images are summations over a distance of 20 μm in z, starting ~10 μm above the surface and using a z-spacing of 0.2 μm.

Figure 6.24: Confocal reflection images of collagen networks formed at different collagen concentrations and a fixed polymerization temperature of 22°C. Concentration is indicated in the images in mg/ml. Scale bar denotes 50 μm for all images. All images are summations over a distance of 40 μm in z, starting at least 10 μm away from the surface and using a z-spacing of 0.2 μm.
Figure 6.25: Frequency dependence of the viscoelastic shear moduli of collagen gels polymerized at 37° at different collagen concentrations. (A) Example curves of the elastic shear modulus, $G'$, and (B) the corresponding viscous shear modulus, $G''$. Symbols denote collagen concentration: 0.7 (squares), 1 (triangles up), 2 (triangles down), 3 (stars), 4 (circles) and 5 mg/ml (triangles right), in increasing shades of gray.

Figure 6.26: Frequency dependence of the viscoelastic shear moduli of collagen gels polymerized at 4 mg/ml and at different polymerization temperatures. (A) Example curves of the elastic shear modulus, $G'$, and (B) the corresponding viscous shear modulus, $G''$. Symbols denote collagen polymerized at 26°C (stars), 30°C (circles), 34°C (triangles up) and 37°C (squares) in increasing shades of gray.
Figure 6.27: The loss tangent determined at 0.05 Hz at different collagen concentrations for networks polymerized at 22°C (triangles down), 26°C (stars), 30°C (circles), 34°C (triangles up) and 37°C (squares) in increasing shades of gray.

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Table 6.1: Simulation results for the critical scaling exponents, \( f \) and \( \phi \), and the onset strain for strain-stiffening, \( \gamma_0 \), and critical strain, \( \gamma_c \), determined for disordered 2D triangular lattices of varying average coordination number, \( z \). Data were kindly provided by Albert Licup, Abhinav Sharma, Robbie Rens, Misha Sheinman and Fred MacKintosh at the VU University Amsterdam.
Figure 6.28: Creep behavior of collagen gels formed at different collagen concentrations and polymerization temperatures. (A and C) Creep rate evaluated at a strain of 12% (A) or 1% (C) for different collagen concentrations and temperatures. (B) The full creep behavior for 4 mg/ml collagen networks at varying strain levels. The creep curves overlap at low strains, but they deviate at high strains. (D) Same creep curves as in panel (B), but now shifted to better compare the shapes. For all panels, symbols denote 22°C (triangles down), 26°C (stars), 30°C (circles), 34°C (triangles up) and 37°C (squares) in increasing shades of gray.
Figure 6.29: Linear elastic behavior of 4 mg/ml collagen gels as a function of polymerization temperature. (A) Linear (low strain) modulus, $G_0$, and (B) onset strain for strain-stiffening, $\gamma_0$.

Figure 6.30: Stiffening behavior of 4 mg/ml collagen gels polymerized at different temperatures. Increasing gray-scale indicates increasing polymerization temperature. Symbols denote 22°C (triangles down), 26°C (stars), 30°C (circles), 34°C (triangles up) and 37°C (squares). The symbols show every 5th data point, while the lines represent the full data sets.
Figure 6.31: The stiffening exponent, $\beta$, in the high strain regime for 4 mg/ml collagen gels polymerized at varying temperatures. Increasing gray-scale indicates increasing polymerization temperature. Symbols denote $22^\circ$C (triangles down), $26^\circ$C (stars), $30^\circ$C (circles), $34^\circ$C (triangles up) and $37^\circ$C (squares).

Figure 6.32: Concentration dependence of the low strain (linear) plateau modulus calculated using eq. 6.16 (open symbols), using $F(z) = c_p^\alpha$, compared with measured $G_0$ values (closed symbols) at a polymerization temperature of $37^\circ$C ($\alpha = 0.25$) (A) or $30^\circ$C ($\alpha = 0.1$) (B).
Figure 6.33: Concentration dependence of the maximal strain, $\gamma_{\text{max}}$, reached before apparent network breakage, measured for collagen gels polymerized at different polymerization temperatures. Symbols denote 22°C (triangles down), 26°C (stars), 30°C (circles), 34°C (triangles up) and 37°C (squares) in increasing shades of gray.