Strain Stiffening in Collagen I Networks

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ABSTRACT:

Biopolymer gels exhibit strain stiffening that is generally not seen in synthetic gels. Here, we investigate the strain-stiffening behavior in collagen I gels that demonstrate elasticity derived from a variety of sources including crosslinking through telopeptides, bundling through low-temperature gelation, and exogenous crosslinking with genipin. In all cases, it is found that these gels exhibit strain stiffening; in general, onset of strain stiffening occurs earlier, yield strain is lower, and degree of strain stiffening is smaller in higher concentration gels and in those displaying thick fibril bundles. Recovery after exposure to high strains is substantial and similar in all gels, suggesting that much of the stiffening comes from reversible network deformations. A key finding of this study is that collagen I gels of identical storage and loss moduli may display different nonlinear responses and different capacities to recover from high strain. © 2012 Wiley Periodicals, Inc. Biopolymers 99: 35–46, 2013.

Keywords: *collagen; confocal microscopy; gel; rheology; semi-flexible polymer*

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

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INTRODUCTION

Intracellular and extracellular biopolymer gels, from the highly dynamic actin cytoskeleton to more static extracellular networks such as basement membrane, are ubiquitous in animals. These gels provide cells and tissues with high sensitivity to local deformations that allows for cell shape change on the intracellular level and matrix remodeling on the extracellular level. On the other hand, biopolymer networks also provide significant structure and strength to cells and tissues. One property of biopolymer gels that contributes to cell and tissue integrity is their tendency to strain stiffen at low-to-intermediate strains.[1–6] The strain-stiffening response reinforces these materials in the presence of forces exerted from both internal and external sources.

Understanding the origin of strain stiffening in biopolymer networks is of great importance from both fundamental and applied perspectives. From a biomedical engineering point of view, enhanced understanding of strain stiffening can aid development of tissue replacements that allow for cell remodeling while providing resistance to failure at moderate strains. Materials that are relatively soft but stiffen under strain satisfy these requirements and best recapitulate the behavior of native tissues. From a fundamental point of view, understanding strain stiffening and its ubiquity in biological polymers and general absence in synthetic ones is also of interest. Although it was originally assumed that the complex nonlinear mechanical behaviors of tissue were integrally related to the organizational hierarchy commonly found in tissue, recent experiments investigating a variety of purified intracellular and extracellular biopolymer gels in vitro have shown that such behavior may emerge generically and not require particular microscopic and/or macroscopic organization.[2] Although the generic nature of strain stiffening of biopolymer networks is now accepted, the origin of the behavior is not agreed upon. Indeed, it is not yet clear whether the strain hardening behavior seen in these materials emerges from stretching out individual filaments in a manner that limits their thermal fluctuations (entropic strain stiffening) and/or from inhomogeneous, nonaffine deformations of sets of filaments (one source of enthalpic strain stiffening).[2,4,6–12]

Indeed, differences in particular biopolymer filament microscopic structure and stiffness as well as overall network architecture may influence the type of deformations that contribute most substantially to the strain-stiffening behavior.[13] Among semi-flexible polymers, those composed of filaments with relatively low stiffness and short persistence lengths are most likely to undergo the deformations driven by entropy as described by Storm et al.[2] This is especially true in dense, isotropic networks. In networks composed of stiffer filaments and/or in the presence of sparse, anisotropic networks, details of the network topology, and nonaffine network rearrangements, as described by Onck et al.,[7] may dominate the strain hardening behavior.[13]

Even for a given biopolymer, preparation conditions of the gel may subtly affect microscopic organization within the individual filamentous structures and/or macroscopic organization of the network, which in turn may affect origin and details of the strain-stiffening behavior. In this study, we investigate whether this is the case for the strain stiffening in collagen I gels. Collagen I forms fibrils with long persistence lengths and, as such, one would expect collagen I network elasticity to be inconsistent with that derived from entropic origins[13]; however, mechanical behavior of collagen I has been shown to be similar to that of less stiff semi-flexible biopolymers and consistent with at least some predictions of elasticity derived from entropic origins.[14,15] Aspects of the nonlinear mechanical response of collagen I networks have been investigated with uni- and bi-axial testing as well as with simple and oscillatory shear.[4,6,16–22] Here, imaging during extension and shear, as well as the studies of realistic model networks, has suggested that the strain stiffening in collagen I gels is consistent with enthalpically driven nonaffine deformations and rearrangements within the gel[4,6,7,16,20] though at least one study also points to the importance of the extension of individual collagen fibrils.[16]

In this study, we investigate the strain-stiffening behavior of collagen I gels with different microscopic and/or macroscopic organizations. In particular, linear and nonlinear rheological responses to oscillatory shear are measured in acid-solubilized (AS) collagen prepared at physiological conditions and AS collagen in which fibril bundling has been induced via low-temperature gelation. In addition, AS collagen stiffened with an exogenous crosslinker and pepsin-solubilized (PS) collagen is also investigated. The collagen gels investigated exhibit different types of intra and interfibrillar interactions: PS collagen lacks the telopeptides present in vivo, whereas AS collagen retains these telopeptides. In vivo, telopeptides allow for crosslinking via the enzyme lysyl oxidase. In vitro, a key difference between AS and PS collagen preparations is that high-molecular-weight oligomers crosslinked through telopeptides are present in AS collagen solutions. As such, reconstituted AS collagen fibrils likely possess intrafibrillar crosslinks absent in reconstituted PS fibrils.[23,24] AS collagen prepared at low temperature develops thick fibril bundles lacking in both PS and AS networks formed at physiological conditions. Finally, AS collagen stiffened with the exogenous crosslinking agent genipin develops additional crosslinks beyond any already present in the AS preparation. Although AS and PS collagen networks have been compared previously in terms of structure and certain mechanical properties,[22,25] detailed comparison of their nonlinear rheological response has not been undertaken. Investigating the strain-stiffening behavior of this set of collagen networks with interactions ranging from minimal (only entanglements, no crosslinks) to substantial (intrafibrillar cross-linking in addition to interfibrillar bundling and/or cross-linking) will reveal how these factors contribute to strain stiffening in collagen networks and help clarify the relative importance of entropic and enthalpic contributions to strain stiffening in collagen I gels.

MATERIALS AND METHODS

Materials

AS type I collagen extracted from rat tail tendon was obtained from BD Biosciences (San Jose, CA). The solution was delivered at ~10 mg/mL in acid. PS type I collagen extracted from bovine hide (Nutragen) was obtained from Advanced BioMatrix (San Diego, CA). The solution was delivered at ~6 mg/mL in acid. 10× Dulbecco's Modified Eagle's Medium (DMEM) solution, sterile NaOH (1*N*), and genipin were purchased from Sigma Aldrich (St. Louis, MO). Gibco 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (1*M*) was obtained from Invitrogen (Carlsbad, CA).

Preparation of Collagen Gels

Collagen gel solutions from 0.5 to 4.0 mg/mL were prepared by diluting the high-concentration collagen stock solutions. Appropriate amounts of high-concentration collagen, depending on the final concentration desired, were mixed with 10% (v/v) 10× DMEM, 2.5% (v/v) HEPES buffer, and distilled, deionized water to reach the final desired volume. The solution was prepared at 4°C to prevent self-assembly of collagen monomers. NaOH was added to neutralize the solution just before the temperature was raised to effect collagen fibrillogenesis. Final pH of the solutions was 7.4 and final ionic strength of the solutions was $I = 0.13$.

For imaging, collagen solutions were gelled by neutralizing them as described above and immediately placing them in an incubator at 37 or 24°C for at least 1 h. For rheological experiments, collagen solutions were prepared in situ: they were neutralized as described above and immediately placed on the rheometer. To avoid potential perturbation of the system during gelation, solutions were allowed to gel on the rheometer for 30 min prior to rheological

measurements. Fixed frequency oscillatory shear measurements on gels constructed in this manner displayed identical storage and loss moduli to networks gelled for longer times in situ. Except for low-collagen content PS gels, these gels also displayed storage and loss moduli identical to those gelled during rheological time sweeps in which oscillations were imposed during gelation.

Genipin-stiffened gels were prepared either by adding genipin solution atop a formed collagen network as described previously[26,27] or by adding genipin to the collagen solution before gelation. The second approach allowed genipin-stiffened gels to be prepared in situ on the rheometer in the same manner as the other collagen gels investigated in this study. For genipin-stiffened gels investigated with microscopy, genipin dissolved in DMSO at 4 or 10 m*M* was either added atop the collagen gel or to the collagen solution before neutralization. When added to the solution, gels were then formed as described above for gels prepared for imaging. Genipin-stiffened gels were prepared for rheological studies solely through adding genipin to the collagen solution. Control experiments on collagen gels with the same amount of DMSO without genipin appeared structurally and rheologically the same as collagen gels prepared in the usual manner (data not shown).

Confocal Microscopy

Confocal reflectance microscopy (CRM) images were recorded with an inverted confocal laser scanning microscope (Olympus Fluoview 300) equipped with a 60×, NA = 1.2 water objective. An Ar^+ laser at 488 nm was used to illuminate the sample, and the reflected light was detected with photomultiplier tube detectors.

Rheology

Rheological experiments were conducted on an AR-2000 rheometer with built-in temperature and gap calibration (TA Instruments). A 1° acrylic cone geometry (diameter 60 mm, 26-μm truncation gap) with a solvent trap was used. Fixed strain experiments (time sweeps and frequency sweeps) were conducted in oscillatory mode at a fixed frequency of 1.0 Hz with controlled strain amplitude (γ) of 0.01. In all cases, 1.0 mL of collagen solution was neutralized and applied to the measuring stage at 4°C. After applying the sample, the solvent trap was added, and the tool was heated to the desired temperature (37 or 24°C). The measurement began 30 min after the tool reached that temperature.

For strain-stiffening measurements, both individual and consecutive strain sweeps were performed. For single-strain sweep measurements, the elastic and loss moduli were measured by applying strains from $\gamma = 0.001$–2.0 (or less if the material broke at $\gamma < 2.0$) using a logarithmic ramp at $\omega = 1.0$ Hz. For consecutive measurements, strain was ramped from 0.001 to strains above the critical strain but below the yield strain of each type of gel. The onset of strain stiffening, critical strain (γ_c), was characterized as the strain at which the stress, σ exceeds $G'_0\gamma$ by more than 10%, where G'_0 is the storage modulus in the small-strain linear regime. The yield strain (γ_y) was characterized as the maximal strain before network weakening or breakage was seen, as characterized by a (usually sharp) decrease in storage modulus. Maximum strain imposed in the consecutive strain measurements varied from $\gamma = 0.2$ for high-concentration AS gels formed at 24°C to $\gamma = 1.0$ for low-concentration AS gels formed at 37°C. Elastic and loss moduli were obtained using standard TA Instruments software. Although characterizing the nonlinear response of materials in this manner can overestimate critical strain and underestimate maximal stiffening, this approach has been used previously for studying collagen gels and is suitable for comparison of nonlinear response across a set of collagen gels.[4,6,19,28] Sample Lissajous curves in the linear and nonlinear regime are provided in the Supporting Information.

All measurements were repeated at least three times except for the sets of consecutive strain measurements, which were repeated at least two times.

RESULTS

Gel Structure

We prepared gels of AS collagen at 37°C, AS collagen at 24°C, and PS collagen at 37°C at 0.5, 1.0, 2.0, and 4.0 mg/mL. AS collagen at 4.0 mg/mL gelled at 37°C and treated with genipin is also prepared. Figure 1 shows representative CRM images of 0.5 and 4.0 mg/mL PS gels prepared at 37°C and AS gels at those concentrations prepared at both temperatures of 37 and 24°C. As we and others have noted previously, collagen fibril morphology as revealed by CRM in pure AS collagen gels is distinct from that in pure PS collagen gels.[22,29] This can be appreciated more fully in lower collagen content gels in which fibrils are well separated. In the 0.5 mg/mL images shown in Figure 1, it is apparent that the PS gel is more homogeneous than the AS gel. Visual inspection also suggests that the PS gel has fewer fibrils than the AS gel and that the fibrils are longer and straighter than those in the AS gel. To further investigate this, wavelength-dependent turbidity measurements were performed, which revealed that the fibrils in the AS and PS gels are of nearly the same diameter, ~100 nm (details are provided in the Supporting Information). For preparations of a particular concentration, given fibrils of the same diameter, gels with fewer fibrils will have longer fibrils than those with more numerous fibrils, consistent with the images of AS and PS collagen shown in Figure 1. At 4.0 mg/mL, both the AS and the PS gels are filled with a dense, homogeneous network of fibrils, though it still appears that the individual PS fibrils are longer on average than are those in the AS gels.

AS collagen gels at 0.5, 1.0, 2.0, and 4.0 mg/mL are also prepared at 24°C. As we have noted previously, AS collagen induced to self-assemble at temperatures below 37°C formed fibril bundles, or fibers.[15] This is shown in Figures 1c and 1f, where thick fibers composed of individual fibrils (straighter and longer than those apparent in the AS gels prepared at 37°C) are evident. Unlike AS collagen, PS collagen does not form thick fibers at low temperature though fibrils that are somewhat longer and thicker than those produced at 37°C do form (data not shown).[30]

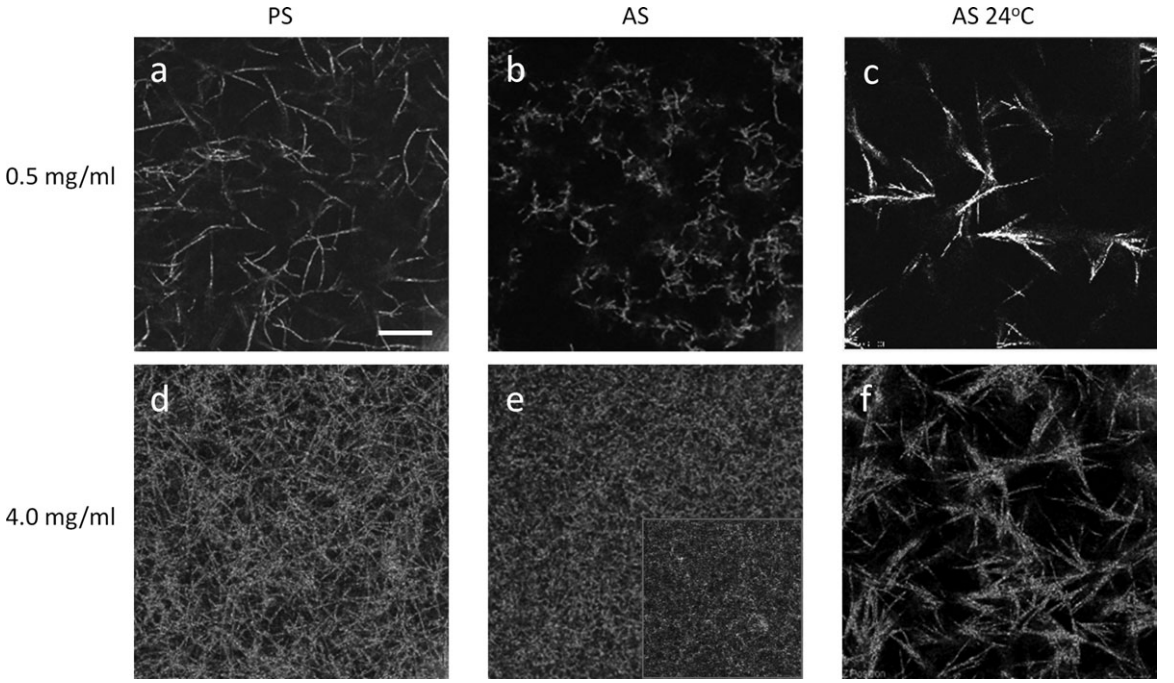

FIGURE 1 (a–c) 0.5 mg/mL and (d–f) 4.0 mg/mL (left) PS collagen prepared at 37°C, (middle) AS collagen prepared at 37°C, and (right) AS collagen prepared at 24°C. Inset in (e) is from a 4.0 mg/mL AS collagen gel formed at 37°C with 10 m*M* genipin in the solution during gelation. Scale bar is 20 μm and is the same in all images.

AS collagen gels formed at 37°C and stiffened with 4 and 10 m*M* genipin are also investigated with CRM. As described in **MATERIALS AND METHODS** these gels are prepared in two ways: placing genipin solution atop formed collagen networks and placing genipin within collagen solutions prior to gelation. CRM reveals no differences in these gels (data not shown). Further, CRM images of AS gels with and without genipin added appear very similar (Figure 1e and inset). Some subtle differences are apparent, with the genipin-stiffened gel images displaying somewhat lower signal:noise than the images of untreated AS collagen.

Linear Rheological Response

AS collagen gelled at 37 and 24°C and PS collagen gelled at 37°C have been characterized by their equilibrium storage (G'_0) and loss (G''_0) moduli in the small strain regime. This is done using oscillatory shear measurements at $\omega = 1.0$ Hz and $\gamma = 0.01$ following 30 min of gelation on the rheometer. Elastic and loss moduli are also quantified from the strain-sweep measurements, averaging measured moduli at strains in the linear regime, from $\gamma = 0.003$ to 0.03. Both methods yield the same results to within error (data not shown).

Equilibrium storage and loss moduli in gels of 0.5–4.0 mg/mL AS collagen as well as 2.0 mg/mL PS collagen gelled at 37°C obtained from the linear portion of the strain-sweep measurements are shown in Figure 2. The AS collagen gel storage moduli over this concentration range are found to scale with concentration as $G' \sim c^{2.2}$, similar to that measured previously in AS collagen ($G' \sim c^{2.1}$).[15] AS collagen gels formed at 24°C display qualitatively similar structure to AS

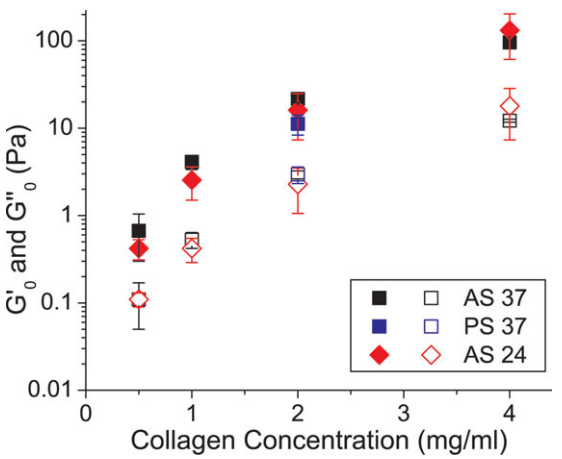

FIGURE 2 Plateau storage modulus, G'_0, (solid symbols) and loss modulus, G''_0, (open symbols) for AS collagen gelled at 37°C (black squares), PS collagen gelled at 37°C (blue squares), and AS collagen gelled at 24°C (red diamonds) obtained from the small strain regime of strain-sweep measurements. Averages and standard deviations are obtained from three to four samples.

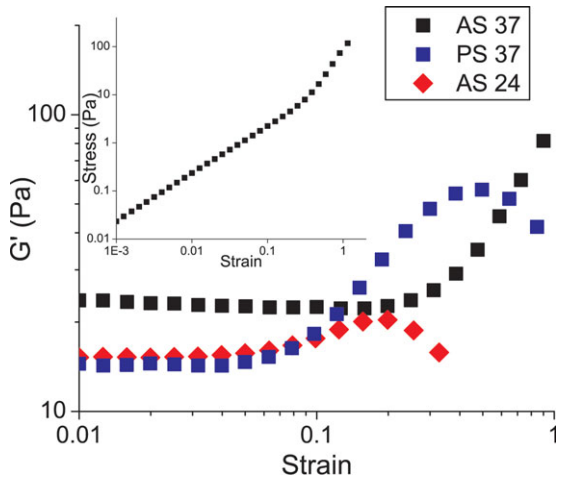

FIGURE 3 Storage modulus as a function of strain for single representative trials of 2.0 mg/mL AS collagen gelled at 37°C (black squares), PS collagen gelled at 37°C (blue squares), and AS collagen gelled at 24°C (red diamonds). Inset shows stress vs. strain for the 2.0 mg/mL AS collagen gel formed at 37°C.

collagen gels formed at 22°C but have somewhat lower storage moduli.[15] The power law scaling of the storage moduli with concentration is consistent with that measured at 22°C previously. Here, $G' \sim c^{3.0}$ compared with $G' \sim c^{2.8}$ measured in an earlier study.[15] Power law fits to the data are shown in the Supporting Information.

Nonlinear Rheological Response

Individual Strain Sweeps. It has been shown previously that, like other biopolymers, type I collagen networks strain stiffen. We investigate this behavior as a function of collagen concentration, collagen solubilization technique, and gelation temperature. Figure 3 shows representative strain sweeps for 2.0 mg/mL AS collagen gelled at 37°C, PS collagen gelled at 37°C, and AS collagen gelled at 24°C. Consistent with strain sweeps of other biopolymer gels, each of these measurements reveals a linear regime in which the gel exhibits the equilibrium storage modulus, G'_0, followed by an increase in storage modulus representing the nonlinear portion of the response. In these gels, the nonlinear regime is followed by a sudden decrease in modulus, indicating network yield. For the 2.0 mg/mL gels shown in Figure 3, the AS collagen network formed at physiological temperature displays the latest onset of strain stiffening, the greatest amount of strain stiffening, and the highest resistance to yield, in that network breakage is not seen until strain applied reaches more than $\gamma = 1.0$. The PS network at this concentration exhibits an earlier onset of both strain stiffening and yield. Yield strain is even lower in the AS collagen formed at 24°C, and in this gel rather limited strain stiffening is seen.

For AS collagen gelled at 37 and 24°C, we investigated the strain-stiffening response as a function of concentration. Figure 4 shows strain sweeps averaged over three to four trials at 0.5, 1.0, 2.0, and 4.0 mg/mL. In all cases, the averages are constructed to strains below the yield strain seen in any of the individual strain sweeps for a given type of gel and preparation temperature. In AS collagen gelled at 37°C, measurements are highly reproducible except in the 0.5 mg/mL case, whereas for gels constructed at 24°C there is somewhat less reproducibility as reflected in the error bars. In general, the greater the mesh size in the system (as occurs both at low collagen content and low gelation temperature), the more

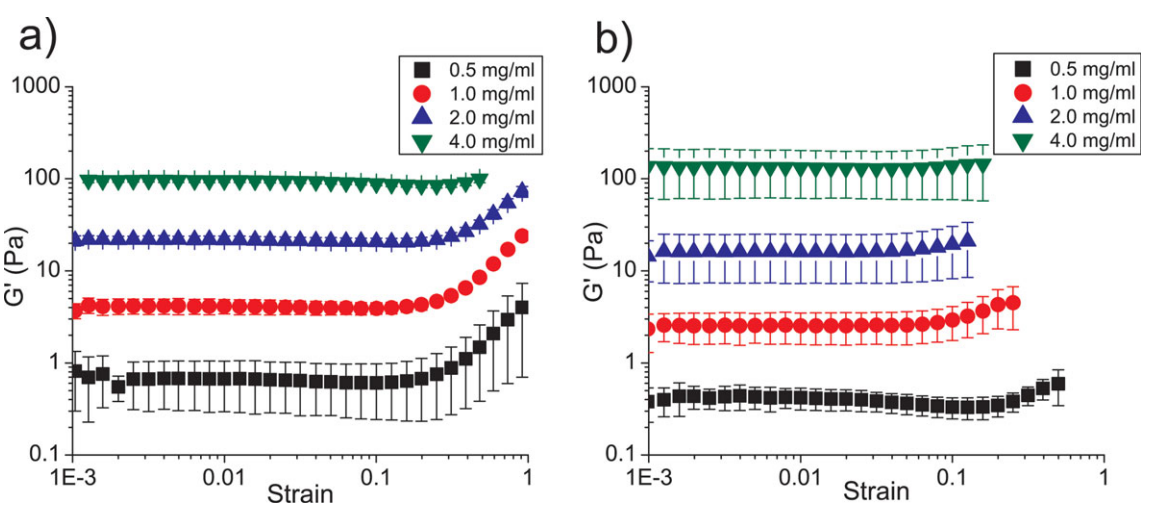

FIGURE 4 Storage modulus as a function of strain for AS collagen gelled at (a) 37°C and (b) 24°C for gels of 0.5 mg/mL (black squares), 1.0 mg/mL (red circles), 2.0 mg/mL (blue triangles), and 4.0 mg/mL (green inverted triangles).

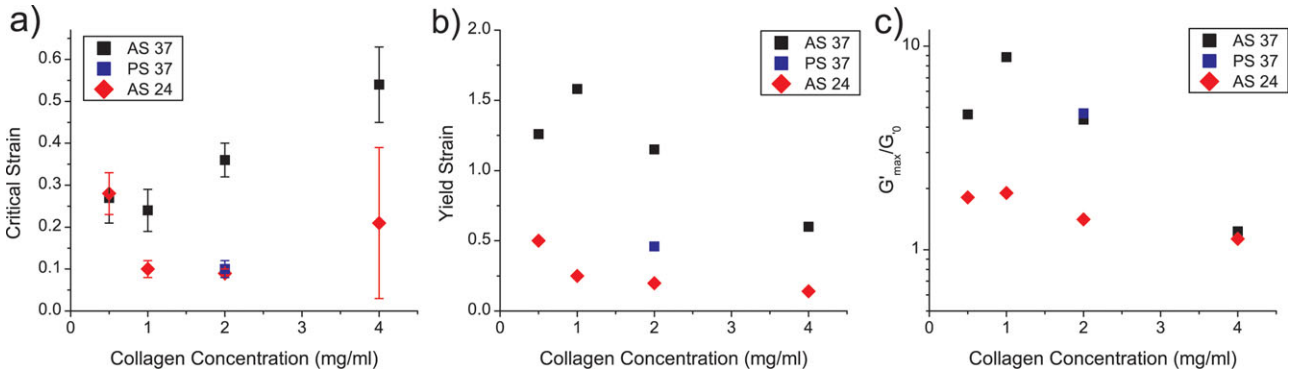

FIGURE 5 (a) Critical strain, (b) yield strain, and (c) storage modulus at yield strain normalized by plateau storage modulus as a function of collagen concentration. In (a) averages and standard deviations are over three to four runs. In all cases, black squares represent AS collagen gelled at 37°C, the blue square represents PS collagen gelled at 37°C, and red diamonds represent AS collagen gelled at 24°C.

varied is network structure as well as linear and nonlinear rheological behavior. In gels constructed at both 37 and 24°C, the largest degree of strain stiffening relative to initial modulus is seen at the lowest concentration and decreases as concentration increases. At 4.0 mg/mL, there is a slight strain weakening of the system before strain stiffening occurs. Strain weakening has been noted previously in 4.0 mg/mL AS collagen gelled at 37°C, though in that case no eventual stiffening was seen even at strains up to $\gamma = 1.0$.[19] Strain weakening followed by stiffening was recently seen by Kurniawan et al. in 3.5 mg/mL AS collagen formed at 37°C.[28] The decrease in degree of strain stiffening with increasing collagen content is owing, at least in part, to the fact that yield occurs at higher strains at the lower concentrations; thus, at these concentrations the strain sweeps continue to higher strains, allowing for enhanced stiffening. For a given concentration, a gel formed at 37°C tends to show greater strain stiffening before yield than the same collagen content gel formed at 24°C.

The nonlinear rheological response of AS collagen gels as well as that of 2.0 mg/mL PS collagen formed at 37°C is shown in Figure 5. Here, critical strain (γ_c), yield strain (γ_y), and maximal modulus before yield relative to plateau modulus (G'_{max}/G'_0) are shown as a function of gel type, gelation temperature, and collagen content. Critical strain increases as a function of collagen content for AS collagen gelled at 37°C but not for AS collagen gelled at 24°C. In all cases except 0.5 mg/mL AS collagen, the gels formed at 37°C display a higher critical strain than those formed at 24°C. The 2.0 mg/mL PS collagen gelled at 37°C exhibits a critical strain lower than that of AS collagen at the same collagen content and gelation temperature and very similar to that of 2.0 mg/mL AS collagen gelled at 24°C. Critical strain values are highly reproducible for 37°C gels of 1.0 mg/mL and higher and somewhat less reproducible for the other gels, which are less homogeneous and display more diverse mechanical properties. Yield strain shows somewhat different behavior than critical strain. For AS collagen gelled at 37 and 24°C, there is a decrease in yield strain with increasing collagen content. As for critical strain, PS collagen gelled at 37°C exhibits yield strain behavior more similar to AS collagen gelled at 24°C than that gelled at 37°C.

In part owing to differences in yield strain, there are also differences in the maximum modulus achieved in the gels relative to their plateau storage moduli (Figure 5c). The value of G'_{max}/G'_0 is greater in gels constructed at 37°C than in the gels constructed at lower temperature. For AS collagen gelled at 24°C, G'_{max}/G'_0 is small and quite consistent across collagen concentration. In these gels, the network yields when strain stiffening of less than a factor of two has occurred. The gels formed at 37°C achieve more stiffening before yield, with increase in storage modulus up to a factor of 10. Among the gels constructed at physiological temperature, the least stiffening is seen in the highest concentration gel, which displays an increase in modulus of a factor of approximately 2, similar to that in the AS gel formed at lower temperature.

Collagen stiffened through use of the natural product genipin was also investigated rheologically. Genipin is a non-zero-length crosslinker of collagen that has been shown to be suitable for use in vivo.[26,31,32] Figure 6 shows a strain sweep of a representative 4.0 mg/mL AS collagen network gelled at 37°C as well as a 4.0 mg/mL AS collagen gel formed with 10 m*M* genipin in the solution. Time between the beginning of gelation and the measured strain sweeps is 30 min in both cases. In the genipin-treated gel, additional stiffening continues to occur for many hours after gelation is complete; for consistency with the other gels and to avoid difficulties of gel drying, we performed rheological studies following 30 min of gelation rather than upon ultimate stiffening. Though the

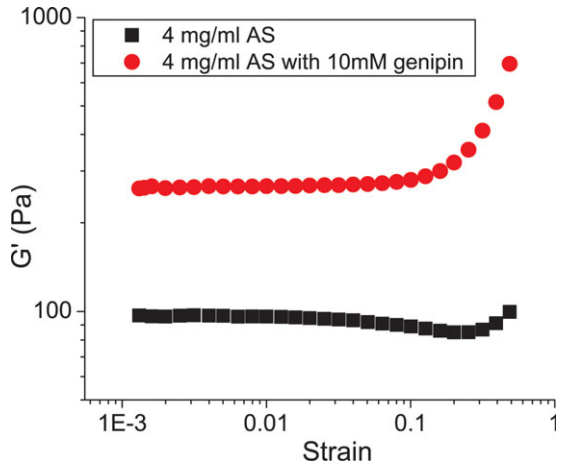

FIGURE 6 Strain-sweep measurement of 4.0 mg/mL AS collagen gelled at 37°C (black squares) and 4.0 mg/mL AS collagen gelled at 37°C stiffened with 10 m*M* genipin (red circles).

gel is stiffening over time, this stiffening is sufficiently slow so that it does not significantly affect individual strain sweep measurements, as can be appreciated from the fact that the storage modulus does exhibit a plateau in the linear regime of the strain sweep. The genipin-treated network exhibits a plateau storage modulus that is greater than that of the untreated gel by a factor of approximately 3, consistent with the treatments of collagen at this concentration via the more typical post-gelation approach.[27] Differences are apparent not only in the plateau modulus but also in the strain-stiffening behavior of the genipin-treated network. In the stiffened network, $\gamma_c = 0.16$, lower than that seen in any of the AS gels formed at 37°C and much lower than the $\gamma_c = 0.54$ measured in the untreated AS gel at this concentration. At $\gamma = 0.5$, where the untreated gel has not even reached critical strain, the genipin-stiffened gel has demonstrated an increase in modulus of more than a factor of 4. Additionally, the gel does not undergo the intermediate strain weakening seen in the 4.0 mg/mL unstiffened collagen gel that precedes the strain stiffening. The gel also remains intact at strains well beyond the yield strain of the untreated 4.0 mg/mL AS gel.

Consecutive Strain Sweeps. In addition to single-trial strain sweeps, consecutive strain sweeps were performed to investigate network ability to recover after subjection to strains sufficient to alter storage modulus. In these measurements, strain was increased to $\gamma = 1.0$ for 0.5–2.0 mg/mL AS gels and to $\gamma = 0.5$ for 4.0 mg/mL AS gels constructed at 37°C. These strains are three to four times the critical strain and ≈80% of the yield strain for 0.5–2.0 mg/mL gels and are close to both critical and yield strain for the 4.0 mg/mL gel, where the values of critical and yield strain are relatively small and similar to each other. For gels constructed at 24°C, the consecutive strain sweeps extend to $\gamma = 0.5$, 0.2, 0.2, and 0.15 for gels of 0.5, 1.0, 2.0, and 4.0 mg/mL, respectively. In all cases, this is a factor of approximately 2 above critical strain and just below yield strain except for the 4.0 mg/mL gel where variability in yield strain requires only low levels of strain to be applied to avoid breakage.

Figure 7 shows an example of five consecutive strain sweeps for 1.0 mg/mL AS collagen gels formed at 37 and 24°C. In all measurements of this type, the initial strain sweep is consistent with single-strain-sweep measurements for gels of given type, concentration, and preparation temperature. For subsequent strain sweeps, behavior is very similar in AS gels formed at both temperatures. In the examples shown in Figure 7, the second strain sweeps look similar to the first except the values of G' at all strains are lower. Critical strain occurs at nearly the same point, and ultimate storage

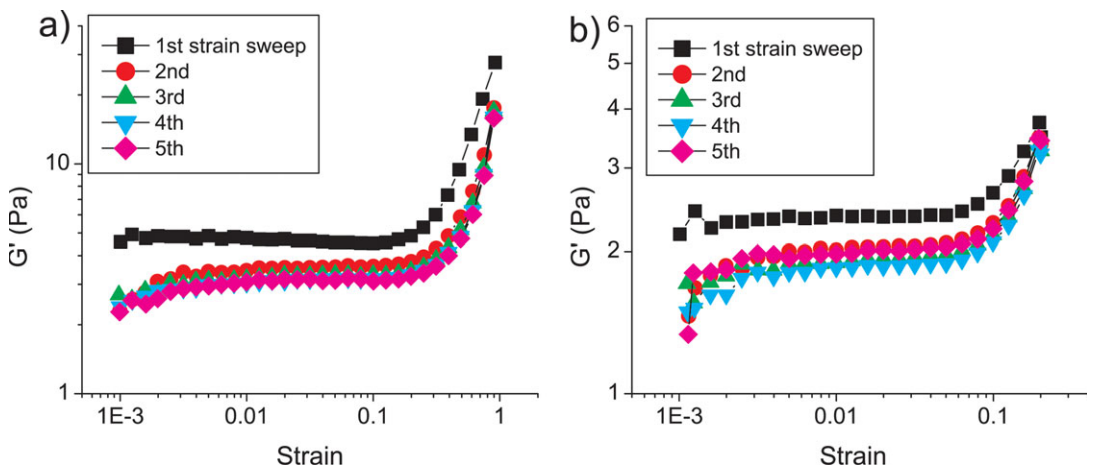

FIGURE 7 Five consecutive strain sweeps for 1.0 mg/mL AS collagen gelled at (a) 37°C and (b) 24°C.

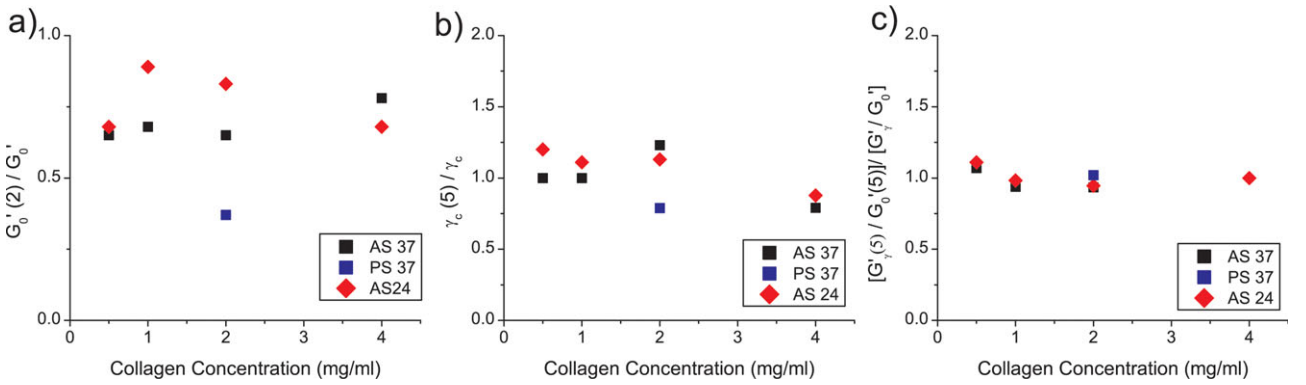

FIGURE 8 (a) Plateau modulus of the second strain sweep relative to the first ($G'_0(2)/G'_0$), (b) critical strain of the fifth strain sweep relative to the first ($\gamma_c(5)/\gamma_c$), and (c) relative value of modulus in the strain-stiffening region (at $\gamma = 0.1$ for PS collagen and AS collagen gelled at 24°C and $\gamma = 1.0$ for AS collagen gelled at 37°C) of the fifth strain relative to the first strain $(G'_\gamma(5)/G'_0(5))/(G'_\gamma/G'_0)$, all as a function of collagen concentration. In all cases, black squares represent AS collagen gelled at 37°C, the blue square represents PS collagen gelled at 37°C, and the red diamonds represent AS collagen gelled at 24°C.

modulus relative to plateau modulus is very similar in the first and second sweeps. The third through fifth strain sweeps show little additional change. The behavior of collagen gels subjected to consecutive strain sweeps is shown in Figure 8. In all AS gels investigated, decrease in storage modulus in the linear regime between the first and the second strain sweeps is similar and rather insensitive to collagen concentration or gelation temperature (Figure 8a). Similarly, critical strain value changes little with additional strain sweeps—all AS collagen gels start to strain harden at approximately the same strain independent of how many times the strain ramp has been applied so long as yield strain has not been reached (Figure 8b). Finally, modulus at particular strains in the nonlinear regime relative to plateau modulus does not vary with subsequent strain sweeps and is similar in all AS gels tested (Figure 8c). PS collagen was only investigated at 2.0 mg/mL. It shows similar response to consecutive strain sweeps as AS collagen but with a steeper drop in storage modulus between the first and the subsequent sweeps. Consecutive strain sweeps of genipin-stiffened collagen were not analyzed as the continued stiffening occurring during the experiment complicates interpretation of the data.

DISCUSSION

The study presented here investigates structure, linear rheological behavior, and nonlinear rheological behavior of collagen gels of different solubilization techniques, concentrations, and preparation temperatures. These gels exhibit a range of interactions: AS gels prepared at 37°C have intrafibrillar crosslinks and interfibrillar entanglements, AS gels formed at 24°C have intrafibrillar crosslinks, interfibrillar entanglements, and interfibrillar bundling, PS gels formed at 37°C have only interfibrillar entanglements, and AS gels treated with genipin are expected to have native intrafibrillar crosslinking as well as additional crosslinking owing to genipin activity. Although the degree of each type of crosslinking has not been quantified, measurements on these systems can suggest the relative importance of intra and interfibrillar crosslinks on gel structure and mechanical properties, including on strain-stiffening behavior.

CRM reveals that collagen fibril and network structure vary as a function of collagen solubilization technique and preparation conditions (Figure 1). Consistent with other studies, we find PS collagen forms fibrils that are longer, straighter, and more homogeneous than those of AS collagen at the same concentration and gelation temperature.[22,29] We also find that AS collagen, unlike PS collagen, forms thick fiber bundles at low gelation temperature. We hypothesize that fibril bundling occurs in AS but not PS collagen formed at low temperature because in AS collagen residual telopeptides are present along the outside of AS collagen fibrils, allowing for interfibrillar associations; fibril interactions of this type occur preferentially at low temperatures, where gelation is slow, allowing for both larger nucleation centers and increased chance of fibril encounters in advance of arrest of the network.[15,30]

Although fibril microstructure is not investigated in this study beyond that which is revealed via visual inspection of CRM images, collagen microstructure may vary somewhat as a function of solubilization technique, species and tissue source, and gelation conditions.[22,23,29,33] PS collagen is

thought to be less well organized than AS collagen, though it does generally retain the 67 nm banding seen in vivo.[34,35] Additionally, collagen's microstructure is dependent on gelation temperature, and AS collagen at 37°C is expected to have somewhat less regular banding and thinner individual fibrils than gels constructed at lower temperature.[15,36,37] Genipin-stiffened AS collagen has been shown to have a significant proportion of fibrils with non-native banding. Additionally, nonfibrillar aggregates have been seen in these preparations.[26] Both factors may contribute to the lower signal:noise seen in these gels relative to those formed in the absence of genipin (Figure 1e and inset).

Just as the collagen network structure measured in this study is largely consistent with previous measurements, the linear and nonlinear rheology of collagen described here is consistent with previous findings. First, the measured values of plateau storage and loss moduli are similar to those ascertained via earlier shear and microrheological measurements in both AS and PS collagens.[4,21,22,30,33,38] In our measurements on AS collagen, we find as $G' \sim c^{2.2}$, similar to that measured previously by us in AS and PS collagens ($G' \sim c^{2.1}$).[15,30] AS collagen gels formed at 24°C display qualitatively similar structure to AS collagen gels formed at 22°C, but they have somewhat lower storage moduli.[15] This difference may be due to the higher temperature of gelation, which results in less fibril bundling than in the gels constructed at the lower temperature. Despite small differences in moduli values, the power law scaling of the storage moduli with concentration is consistent with that measured at 22°C previously. Here, $G' \sim c^{3.0}$ compared to $G' \sim c^{2.8}$ measured previously.[15] Similar studies of PS collagen formed at 37°C have yielded $G' \sim c^{2.1}$ and $G' \sim c^{2.7}$.[6,30]

The general strain-stiffening behavior measured here is similar to that seen in other studies of biopolymer gels.[1–4,6,19,21,22,28] Although our findings are largely in accordance with the previous measurements on collagen I gels, some differences are seen. In large amplitude oscillatory shear measurements similar to those performed here, AS collagen was found to strain stiffen and exhibit critical strain at γ_c = 0.20–0.30 for concentrations up to 2 mg/mL but to strain weaken at 4.0 mg/mL.[19] For AS collagen gels constructed at 37°C, we find somewhat higher critical strains (γ_c = 0.24–0.54), and we find strain stiffening even in the highest concentration gel investigated. This is consistent with the recent study of Kurniawan et al. on 3.5 mg/mL AS collagen, a study which also reveals very similar Lissajous curves to those we measure (Supporting Information Figure 1) and similar response to repeated application of strain.[28] Another study found critical strain in PS collagen to be in the range of γ_c = 0.05–0.15 for gels of 0.5–5.0 mg/mL.[4] These measurements are in accordance with our finding of γ_c = 0.10 for PS collagen of 2.0 mg/mL.

Yield strain has also been investigated previously: In continuous shear measurements, AS collagen was found to have a gap-dependent yield strain that increased with decreasing gap size.[21] In that study, regardless of gap, yield strain decreased with increasing collagen content from 1.0 to 3.0 mg/mL.[21] For the smallest gap measured (50 μm), $\gamma_y \approx 1.20$ at 1.0 mg/mL and $\gamma_y \approx 0.45$ at 3.0 mg/mL. This is consistent with our measurements of $\gamma_y \approx 1.60$ at 1.0 mg/mL and γ_y = 0.60 at 4.0 mg/mL for an even smaller gap, though direct comparison is complicated by the fact that that study used a parallel plate tool while we employed a cone and plate. AS and PS collagen gel yield has also been measured using uniaxial testing, which revealed no clear difference between AS and PS collagen and no clear trend with concentration from 0.5 to 2.0 mg/mL.[22] In that study, across collagen types and concentrations, yield strains were in the range of γ_y = 0.40–0.60, somewhat higher than those found in oscillatory rheology done with the largest gaps (300 μm).[21,22] Finally, strain-stiffening behavior in collagen gels treated with genipin, which has not been measured previously, displays some similarities to collagen stiffened with other crosslinkers. In PS collagen stiffened with glutaraldehyde, critical strain measured with two-point stretching was found to be γ_c = 0.10.[4] This critical strain was generally lower than that measured for collagen without glutaraldehyde as determined via either two-point stretching or oscillatory rheology.[4] The finding of earlier onset of strain stiffening for glutaraldehyde-treated collagen relative to native collagen is consistent with our finding on genipin-treated AS collagen.

Comparison of strain-stiffening behavior across the different types of collagen gels investigated here allows us to speculate on the relative importance of fibrillar structure, network structure, and crosslinking in setting nonlinear rheological behavior. Trends found in this study include that AS collagen gels formed at 37°C display increasing critical strain with increasing concentration, with critical strains in the range of γ_c = 0.24–0.54 for collagen of 0.5–4.0 mg/mL. Significantly lower critical strains are found in gels of the same type and collagen content formed at low temperature, where fibril bundling and large network pore size exist. Yield strain is also much higher in the AS gels formed at 37°C than at 24°C, as is the ultimate modulus attained in the 37°C gels relative to the 24°C gels of the same concentration. Additionally, we find that PS gels act more similarly to AS gels constructed at 24°C than to those constructed at 37°C in terms of their critical and yield strain.

The key difference between AS collagen gels of a given concentration formed at 37 and 24°C is network

organization, with AS gels formed at 24°C exhibiting fibril bundling that leads to a network of thick struts and large pores, as opposed to a network of thinner struts and smaller pores as is seen for AS gels of the same collagen content formed at 37°C. The key difference between AS and PS collagen gels formed at the same temperature is less clear: both types of networks are composed of thin struts and relatively small pores but some network structural differences are evident (Figure 1); moreover, intrafibrillar interactions are expected to differ between the two preparations with AS collagen fibrils bearing some intrafibrillar crosslinking. We note that the AS gels formed at 37°C, the AS gels formed at 24°C, and the PS gels of a given collagen content have very similar linear rheological response (Figure 2) but distinct nonlinear mechanical responses. We suggest that differences in nonlinear rheological response between AS gels formed at 37 and 24°C are caused by differences in network organization, whereas differences in response between AS and PS gels may be owing to the differences in intrafibrillar interactions or network organization.

The clear differences in critical and yield strain for AS collagen gels of a particular concentration constructed at 37 and 24°C are consistent with the nonlinear mechanical response of these systems being very sensitive to network architecture. For gels of similar linear elastic storage and loss modulus, those gels with thin unbundled fibrils and relatively small pore size exhibit higher critical strains, higher yield strains, and a greater degree of strain stiffening than do those gels with bundled fibers and larger pore size. Lower critical strain in gels with large pore size and relatively few total fibrils or fibers (i.e., gels of low concentration and/or low gelation temperature) is consistent with strain stiffening owing to nonaffine deformations: such deformations are more likely in open, inhomogeneous systems, where few fibrils and entanglement points are present. The more fibrils and entanglement points, the more resistant the gel is to the substantial deformations that lead to this type of enthalpic strain stiffening. The fact that PS collagen, with much more similar network organization to AS collagen of the same concentration, also exhibits low critical strain suggests this property can also emerge from a second mechanism associated with lack of telopeptides. Although telopeptides are primarily thought to lead to intrafibrillar crosslinks, we have suggested that they may also reinforce entanglement points; this is consistent with the presence of bundles in low-temperature AS collagen gels but not low-temperature PS collagen gels.[29] If this is the case, PS collagen of the same network organization as AS collagen would be more likely to rearrange under shear and thus would be expected to strain stiffen at a lower strain than does AS collagen. The fact that strain stiffening sets in earlier in the high-concentration genipin-stiffened AS gel relative to the untreated one is not immediately consistent with the view of strain stiffening being dominated by athermal nonaffine deformations. Indeed, one would expect gels reinforced with crosslinks at entanglement points to be more resistant to the types of nonaffine deformations that we suspect lead to strain stiffening in most AS collagen gels.[20,39] Genipin, however, effects many changes in the collagen network and may even limit the proportion of collagen molecules present in fibrils, complicating interpretation of the results.[26]

The fact that we find a decrease in yield strain with increasing collagen concentration as occurs in AS gels constructed at both temperatures 37 and 24°C is not intuitive, as it may be expected that with fewer fibrils failure of any given fibril or interfibrillar entanglement or crosslink would be more likely to disrupt the spanning structure, breaking the gel. However, measurement of decreased yield strain with increasing concentration is in accordance with the other measurement of yield strain as a function of collagen concentration as well as similar measurements in actin gels.[21,39] Indeed, Gardel et al. showed that for entropic elasticity, it is expected that yield strain will decrease weakly with number of fibrils for a fixed concentration of crosslinks.[39] Although some features of the elasticity of collagen networks have been shown to be consistent with entropic elasticity,[15] the relatively stiff fibrils of collagen make elasticity emerging solely from thermal, entropic sources unlikely in these networks. Similarly, comparison to actin results is complicated by the fact that in collagen networks crosslink density varies with collagen content. Although the origin of the decreasing yield strain with increasing concentration may differ for collagen and actin, the observation is similar.

The response of the gels to consecutive strain sweeps also sheds light on the origins of strain stiffening in these gels. So long as yield strain is not reached, changes to AS collagen gels' mechanical properties induced by high strains are generally found to be reversible. Although there is some drop in storage modulus after the initial strain sweep, no further changes are seen with subsequent sweeps, and critical strain and modulus at a given strain relative to that in the initial strain sweep remain constant for gels of all concentrations and gelation temperatures (Figure 8). These results are consistent with strain-stiffening behavior being largely set by reversible nonaffine deformations of the network. The initial drop in modulus after the first strain sweep may be owing to irreversible deformations of individual fibrils. This effect is greatest in PS collagen, where the modulus decreases to approximately half of the original modulus after the first strain sweep, suggesting that the telopeptides in the AS collagen that allow for intrafibrillar crosslinking limit irreversible

changes to individual fibrils. The fact that subsequent strain sweeps in AS and PS collagen are consistent with the previous strain sweeps in terms of onset and degree of strain stiffening suggests the majority of the strain hardening is owing to reversible network reorganization. The suggestion of reversible deformations is inconsistent with the direct microscopy performed during two-point stretching of PS collagen gels performed by Vader et al.[4] In this study, permanent alignment was seen after strains in the nonlinear regime were imposed on untreated PS gels through reversible alignment was seen in glutaraldehyde-treated samples.[4] On the other hand, Tower et al. found that prealigned untreated PS collagen matrices do exhibit reversible alignment in both cross and parallel-loaded uniaxial tests.[16] Differences in the details of the sample configuration as well as the particular stresses applied may influence the reversibility of network deformations in these two studies as well as in the study presented here.

The picture beginning to emerge is that of initial strain stiffening occurring from small individual fibril stretching deformations followed by more substantial stiffening emerging from nonaffine (potentially reversible) fiber rearrangements. This is consistent with a recent finding tracking birefringence and storage modulus of fibrin gels under strain, which showed that initial strain stiffening proceeds alignment.[5] Although both entropic and enthalpic contributions likely play a role in collagen strain stiffening, their relative importance likely depends sensitively on the degree of organization and strength of the individual fibrils as well as the interfibrillar interactions and initial network organization.

CONCLUSIONS

Collagen gels of different solubilization technique and gelation temperature were prepared: these gels display similar storage moduli in the linear regime but exhibit different mechanical responses in the nonlinear regime. Those gels formed from monomers with telopeptides exhibit higher critical and yield strains. Similarly, gels prepared at physiological temperature, where the network is organized as a relatively dense set of thin fibrils, display higher critical and yield strains than the gels constructed at low temperature, where fibril bundles dominate the network architecture. These results suggest that the difference in strain-stiffening behavior is owing largely to differences in network architecture. We speculate that initial strain stiffening at low strains emerges from stretching deformations of individual fibrils and that these deformations are most prominent in gels with thin fibrils, no crosslinking, and/or poor individual fibril organization. The remaining strain stiffening is likely due to nonaffine deformations of the network including reversible fibril alignment. The fact that collagen I gels with very similar linear storage and loss moduli may have significantly different nonlinear mechanical responses in terms of critical strain, yield strain, and degree of strain stiffening is an important consideration for applications requiring recapitulation of native tissue response to both cell-driven and external strains.

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