Microstructural and mechanical differences between digested collagen–fibrin co-gels and pure collagen and fibrin gels

Victor K. Lai a, Christina R. Frey b, Allan M. Kerandi c, Spencer P. Lake b, Robert T. Tranquillo a,b, Victor H. Barocas b,c*

a Department of Chemical Engineering and Materials Science, University of Minnesota–Twin Cities, 421 Washington Avenue South East, Minneapolis, MN 55455, USA
b Department of Biomedical Engineering, University of Minnesota–Twin Cities, 7-105 Nils Hasselmo Hall, 312 Church Street South East, Minneapolis, MN 55455, USA
c Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota–Twin Cities, 6-155 Jackson Hall, 321 Church Street South East, Minneapolis, MN 55455, USA

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ABSTRACT

Collagen and fibrin are important extracellular matrix (ECM) components in the body, providing structural integrity to various tissues. These biopolymers are also common scaffolds used in tissue engineering. This study investigated how co-gelation of collagen and fibrin affected the properties of each individual protein network. Collagen–fibrin co-gels were cast and subsequently digested using either plasmin or collagenase; the microstructure and mechanical behavior of the resulting networks were then compared with the respective pure collagen or fibrin gels of the same protein concentration. The morphologies of the collagen networks were further analyzed via three-dimensional network reconstruction from confocal image z-stacks. Both collagen and fibrin exhibited a decrease in mean fiber diameter when formed in co-gels compared with the pure gels. This microstructural change was accompanied by an increased failure strain and decreased tangent modulus for both collagen and fibrin following selective digestion of the co-gels. In addition, analysis of the reconstructed collagen networks indicated the presence of very long fibers and the clustering of fibrils, resulting in very high connectivities for collagen networks formed in co-gels.

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1. Introduction

Naturally occurring biopolymers such as collagen and fibrin play an important role in the physiological and mechanical function of various systems and processes in the human body. Collagen, the most abundant protein in the human body, is divided into many different types that are structurally distinct from one another, with type I being most prevalent [1]. Fibrin is the main matrix component found in blood clots and early granular tissue during the wound healing process. These extracellular matrix (ECM) components are also commonly used as scaffolds for tissue engineering applications, including in combination with one another or other proteins, e.g. collagen–fibrin [2] and collagen–elastin [3]. Understanding how various ECM components interact with each other to confer the overall mechanical behavior is important in more rational design in tissue engineering. The choice of initial scaffold material(s) on which to seed cells is a critical aspect in fabricating tissues with structural integrity and biochemical functionality akin to those of native tissues because cells respond differently according to their mechanical and biochemical environments. For example, our group has found that seeding vascular smooth muscle cells (SMCs) on a fibrin gel promotes cellular production of collagen I, hence improving the mechanical strength of the overall tissue equivalent (TE) [4]. Regardless of the initial choice of scaffold material, engineered tissues, like native tissues, almost always contain a combination of different ECM components. For example, compositional changes in the ECM occur in fibrin-based constructs during the growth and remodeling processes, during which the fibrin matrix is gradually degraded and replaced by a cell-derived collagen matrix [5–7], a process akin to wound healing. While the structure and mechanics of individual ECM components (e.g. collagen, fibrin, and elastin) have been extensively studied [8–16], an understanding of composite ECM systems is still lacking. Previous studies on interpenetrating network systems, in both synthetic polymer blends [17,18] and natural biopolymer [19–21] mixtures, have shown complex mechanical responses, indicating complex interactions between the networks that are not well understood.

Specifically, for collagen–fibrin co-gels previous studies on interactions between these two ECM components have reported conflicting results, with some [22,23] refuting the presence of chemical bonding between these proteins as reported in Duckert and Nyman [24]. Our group is interested in understanding how
network architectures and interactions in interpenetrating ECM systems influence the overall mechanics and in developing a computational model that can adequately capture the mechanics of such systems based on their respective microstructural and mechanical details. That the network morphology and mechanical behavior of individual collagen and fibrin networks can be altered by varying the gelation conditions has been well documented. For fibrin, seminal studies by Ferry and co-workers investigated the effects of the fibrinogen and thrombin concentration as well as pH, temperature and ionic strength of the solutions [25]. This work was followed by similar studies conducted by other groups [26–30]. More recent work by our group [31] and others [32–35] has included studies with cells entrapped in a fibrin gel, which are more relevant to tissue engineering. Similarly, studies performed on collagen gels have given insight into the effect of gelation conditions (e.g. temperature and pH) on collagen microstructure and mechanics [8,36,37]. However, the mechanism by which the fibrin and collagen network microstructure and mechanical behavior are changed when formed in the presence of each other, as well as how these ECM networks interact with each other to confer overall mechanical properties to engineered tissue, remains poorly understood.

Collagen and fibrin have different gelation mechanisms. Fibrin assembly is initiated by thrombin-catalyzed cleavage of fibrinopeptides from fibrinogen, exposing sites for intermolecular associations between fibrinopeptides to form fbrils [38]. In contrast, collagen fbrils are formed by self-assembly of triple helical pro-collagen molecules (of the order of 300 nm in length) in a staggered formation [39]. Fibrin–collagen composite systems are relevant both physiologically (in the context of wound healing and atherosclerosis [40]) and in bioengineered tissues. Specifically, in wound healing collagen and fibrin play distinct roles: a fibrin clot forms a provisional matrix to stop bleeding, while the process of wound repair involves deposition of newly synthesized collagen by fibroblasts [41]. Our previous work on collagen–fibrin co-gels [20] focused on the composition–function relationship between the two networks, showing non-linearity in mechanical behavior with increasing collagen concentration. In addition, a comparison of experimental data and predictions by our computational model showed a transition in collagen–fibrin interactions from series behavior to parallel mechanical behavior as the volume fraction of collagen increased. While the previous study gave some insight into how collagen and fibrin interact in co-gels, those results also raised the question of whether the collagen and/or fibrin architecture changes when formed in the presence of each other, and how such changes in morphology alter the mechanics of the co-gels.

The purpose of the current study was to investigate the effect of altering network architecture on collagen and fibrin mechanical properties, thereby elucidating structure–composition–function relationships between collagen and fibrin in co-gels. We hypothesize that the overall mechanics of collagen–fibrin co-gels is influenced not only by their composition, but also by changes in the network architecture of both collagen and fibrin arising from gelation. Specifically, this study investigated changes in the microstructure of collagen and fibrin when gelled individually (i.e. in the pure form without a secondary network present) compared with gelation in the presence of each other, and sought to relate these microstructural changes to alterations in mechanical behavior. To do so collagen–fibrin co-gels were formed and subsequently subjected to digestion to remove either collagen (with collagenase) or fibrin (with plasmin) while leaving the other network intact. The microstructure and mechanical behavior of the resultant networks were compared with those of their pure counterparts. Scanning electron microscopy (SEM) and confocal microscopy to probe network morphology were coupled with tensile tests to failure to aid in understanding the mechanical behavior of these gels.

2. Materials and methods

2.1. Preparation and digestion of collagen, fibrin, and collagen–fibrin co-gels

The methods for casting collagen, fibrin, and collagen–fibrin co-gels were adapted from Cummings et al. [2]. Briefly, 1 ml of collagen formulation comprised 660 μl acid-solubilized rat tail collagen type I (Life Technologies Corporation–Invtitrogen, Grand Island, NY) neutralized with 26 μl of 1 M NaOH (Sigma-Aldrich, St Louis, MO), supplemented with 100 μl of 10−2 minimum essential medium (MEM) (Sigma-Aldrich), 60 μl of fetal bovine serum (Thermo Fisher Scientific–HyClone, Logan, UT), 10 μl of l-cysteine (Invtitrogen), and 1 μl each of penicillin/streptomycin (Invitrogen) and fungizone (Invitrogen). The fibrin formulation consisted of bovine fibrinogen (Sigma-Aldrich) dissolved in 0.02 M HEPES buffer (Meditech, Manassas, VA) in saline (constituting 66% by volume of formula- tion), polymerized by mixing with a solution containing 0.35 vol.% thrombin, 0.06 vol.% Ca2+ (Sigma-Aldrich), and 33 vol.% 1 × Dulbecco’s modified Eagle’s medium (Meditech). Collagen–fibrin co-gels (CG) were made by mixing 50 vol.% of the collagen and fibrin formulations. To make pure collagen gels (C) with the same collagen concentration as in the co-gels the above collagen formulation was diluted with an equal amount of 0.5 M HEPES buffer (Meditech). Similarly, pure fibrin gels (F) were made by diluting the fibrin formulation with an equal amount of 0.02 M HEPES buffer. The gel solutions were cast either into Teflon ring molds (15.5 mm OD, 11.5 mm ID), for mechanical testing, or Lab-Tek chambered coverglass slides (Thermo Scientific–Nunc, Rochester, NY), for confocal microscopy. Gels were incubated at 37°C overnight before application of different digestion media.

The digestion protocols are summarized in Table 1. Gels were treated with either 0.02 U ml−1 plasmin (Pn) (Sigma-Aldrich), 1 U ml−1 high purity collagenase (Cs) (Sigma-Aldrich) or 1 × phosphate-buffered saline (PBS) (Meditech). Positive controls to verify the efficacy of the enzymes were performed on pure collagen and pure fibrin gels. All digestion treatments were left overnight at 37°C before further testing or preparation.

2.2. Scanning electron microscopy (SEM)

Gels were prepared for SEM after digestion using the protocol described in Lai et al. [20]. Briefly, the gels were fixed with 2.5% glutaraldehyde (Electron Microscopy Services, Hatfield, PA), followed by post-fixation staining with 1% osmium tetroxide (Electron Microscopy Services) before sequential dehydration with increasing concentrations of ethanol. The dehydrated samples were freeze-fractured in liquid nitrogen before critical point drying (Tousimis 780A, Tousimis Corp., Rockville, MD) and sputter coating with platinum. Imaging was performed in a Hitachi S-900 field emission gun scanning electron microscope (Hitachi High Technologies America, Pleasanton, CA) using a beam voltage of 2 keV.

2.3. Mechanical testing

The gel rings were looped over T-bar grips and uniaxially stretched to failure in an Instron 8848 MicroTester (Instron, Norwood, MA) equipped with a 5 N load cell. Before stretch to failure each ring was stretched to a grip to grip length of 14.5 mm for 5 s. Tensile stress to failure was performed at a rate of 0.13 mm s−1. The first Piola–Kirchhoff stress (1st PK) was computed by dividing the force data by the cross-sectional area of each gel, estimated based on the volume of gel cast into each ring mold. The following material properties were calculated from plots of 1st PK versus Green strain: ultimate tensile stress (UTS), Green strain at failure,
The contribution of longer fibers in a non-normal distribution of fiber lengths. It should be noted that collagen fibrils cannot be distinguished from collagen fibers (from fibril bundling) in these confocal images, hence the term “fiber” refers to both collagen fibrils and fibers in these confocal results.

2.6. Statistical analysis

Statistical comparisons of the material properties and network parameters were performed using a one-way ANOVA F-test, coupled with multiple comparisons using the Bonferroni procedure. Analyses were carried out using the commercial statistical package in Origin (OriginLab Corp., Northampton, MA).

3. Results

3.1. Mechanical test results

Fig. 1 shows the average failure points of each gel condition after digestion treatment. The data show that failure points are grouped according to the respective network present in the gels: collagen gels (C in PBS, C in Pn, CG in Pn) clustered in the range of low Green strains around 0.5–1, while fibrin gels (F in PBS, F in Cs, CG in Cs) clustered at higher Green strains between 2.5
and 3.5. That the digested co-gels exhibited similar failure properties to their pure counterparts strongly suggests that the digestion treatments with collagenase and plasmin were effective in eliminating the target network, to the extent that any residual undigested fibrils could not contribute significantly to the overall mechanical behavior of the resultant network. The undigested co-gel (CG in PBS) exhibited a higher UTS compared with the negative controls (C in Pn). These comparisons are statistically significant at the 95% confidence level, with the exception of Green strain at failure for fibrin between the digested co-gel (CG in Cs) and PBS control (F in PBS) which is significant at the 90% level (P = 0.067).

### 3.2. Microstructural results

**Fig. 4** shows SEM images of the gels after digestion. Images of the negative controls of pure collagen (C in Pn) and pure fibrin (F in Cs) appear similar to their respective PBS controls (C in PBS and F in PBS), which suggests that the network microstructures were not affected by the digestion media. Comparison of the pure collagen and pure fibrin networks showed that both protein matrices qualitatively exhibited similar diameters. Closer inspection of these images (see Supplementary Material for larger images) revealed characteristic banding on the collagen fibrils, absent in the fibrin fibrils. The co-gel control (CG in PBS), however, exhibited distinct differences in fibril diameters: finer, wisp-like structures can be seen in addition to the fibrils of similar thickness observed in the pure collagen and fibrin images (representative fibril diameters shown by arrows → → ). These wispy, web-like structures are still prevalent after the collagen had been digested (CG in Cs), strongly indicating that these structures were fibrin fibrils. Qualitatively, the distribution of collagen fibril diameters appears similar in the digested co-gel (i.e. CG in Pn) compared with the pure collagen gels, although less bundling was observed (denoted by ‘’ in the images) between the fibrils, such that fiber diameters appeared smaller in these digested co-gels. Finer fibrils can also be seen in the CG in Pn co-gel, albeit much less prevalent than in the fibrin-containing co-gel (CG in Cs). While we were unable to discern whether these finer fibrils were collagen or fibrin, we believe that they were primarily residual fibrin fibrils that were not digested. Collectively, a comparison of SEM images suggests that both the collagen and fibrin network morphologies are altered when formed in the presence of each other: fibrin fibril diameter decreases and collagen fibrils exhibit less bundling.

To further analyze network differences in the collagen networks 3-D reconstruction of these networks using confocal image stacks of the collagen gels was performed. **Fig. 5** shows a representative 3-D image of a reconstructed collagen network (C in Pn) (top), as well as...
well as a comparison between the collapsed confocal image stack (bottom) before deconvolution (left) and after deconvolution (middle), and the reconstructed network (right). It should be reiterated that collagen fibrils cannot be differentiated from collagen fibers (bundled fibrils) in these confocal images, so the term “fiber” refers collectively to both fibrils and bundled fibers. Comparison of the collapsed images before and after deconvolution indicates that while the deconvolution process makes fibers more distinct by removing the spread in fluorescence, fibers also appear more fragmented as a result. These fragmented fibers are reconnected during the network reconstruction process via the FIRE algorithm, where fiber segments of similar orientation are extended as the same fiber. A comparison between the reconstructed network and deconvoluted network shows good qualitative agreement, in general, in network morphology. Results for the network morphologies under the different conditions are shown in Fig. 6, comparing total fiber length per volume (Fig. 6A), average connectivity around a cross-link (Fig. 6B), average segment length and tortuosity (Fig. 6C and D), and average fiber length and tortuosity (Fig. 6E and F). A segment is defined as the spacing between cross-links, while a fiber can be composed of multiple adjacent segments of similar orientation (see Fig. 7 for an illustration). The minimum value allowable between two segments for them to be considered part of the same fiber (i.e. angle \( \alpha \) in Fig. 7) was set at 120°. This value was determined via a sensitivity analysis examining the effect of varying \( \alpha \) on the total number of cross-links with a connectivity of only 2 (data not shown). Since cross-links of connectivity 2, formed by contact between two fibril ends, were expected to be very uncommon, a large jump in the number of such cross-links (which occurred between 120° and 130°) suggested that at \( \alpha \) values greater than 120° several fibers had been artificially broken up into two fibers. In general, no significant differences in network characteristics were observed between samples formed under the same gelation conditions but placed in different digestion media (i.e. CG in Pn vs. CG in PBS and C in Pn vs. C in PBS). Assuming the same collagen density for all samples, the total fiber length per volume (Fig. 6A) provides a crude measure of fiber diameter, with the higher values in the co-gels (CG in P, CG in PBS) compared with the pure gels (C in Pn, C in PBS). In addition, the collagen networks formed in the co-gels also

![Figure 3](image_url) Comparison of the material properties of (A) Green strain at failure, (B) ultimate tensile stress (UTS), (C) tangent modulus, and (D) transition strain between the collagen and fibrin networks under different digestion conditions. In general collagen and fibrin networks obtained from co-gel digestion exhibited similar UTS values, but higher failure strains and lower tangent moduli compared with their pure counterparts. The material properties of the undigested co-gel (CG in PBS) cannot be derived from the sum of the properties of the digested co-gels (CG in Pn, CG in Cs), showing the the rule of mixtures does not apply to these collagen–fibrin systems. Error bars represent 95% confidence intervals, *p < 0.05, statistical significance at the 95% level; #, statistical significance at the 90% level.
exhibited higher average connectivities (Fig. 6B) compared with the pure gels (\(^*\) indicates statistical significance at the 95% level). While no significant differences were observed in the average segment length (Fig. 6C) and average segment tortuosity (Fig. 6D) between the co-gels and pure gels, the collagen network in the co-gels exhibited longer average fiber lengths (Fig. 6E) but similar fiber tortuosities (Fig. 6F) than the networks in the pure gels.

To investigate these differences in connectivity and fiber length further their distributions were analyzed, as shown in Figs. 8 and 9, respectively. The distribution of connectivities comprises integer values which spanned a narrow range from 2 to 12. Fig. 8A shows similar overall distributions of connectivity across all samples, with more than 80% of cross-links having connectivities of 3 or 4. The collagen networks from co-gels (CG in Pn, CG in PBS) exhibited a slightly higher proportion of cross-links with connectivities of 5 or greater. The box-plot in Fig. 8B shows that key differences in the connectivity distributions occurred only above the 90th percentile (top of the whisker): the collagen networks in co-gels exhibited higher connectivities at the 99th percentile point (●) and had larger maximum connectivity values (▲). Collectively these results explain the higher average connectivities in the co-gels (CG in P, CG in PBS): these networks have more cross-links that have higher connectivities. Fig. 9 shows a similar analysis for the fiber length distributions in the collagen networks, with Fig. 9A showing an example of the non-normal distribution of fiber lengths. The right skewed distribution in all the samples is evident from the lower median value (middle line in the box) compared with the mean (■) in the box-plot in Fig. 9B. While all four samples showed similar fiber lengths at the 25th, 50th and 75th percentile points (i.e. in the box region), longer fiber lengths at the 90th (top of the whisker) and 99th percentile points (●) were observed for the networks in the co-gels compared with those in the pure gels. These results suggest that the longer average fiber lengths in the networks from co-gels (Fig. 6F) were not due to a general increase in length of all fibers, but due to the presence of very long fibers in the co-gel networks. A comparison of the length average fiber length (Fig. 9C) confirms this result, as the difference in fiber lengths between the networks in co-gels vs. networks in pure gels is further accentuated when longer fibers are weighted more heavily.

4. Discussion

This study has investigated the effect of collagen–fibrin co-gelation on the network morphologies of each network, and how such changes in network architecture influence the overall mechanical behavior. Upon digestion of the collagen–fibrin co-gels with either
plasmin or collagenase a network of undigested ECM remained in the gel. This observation suggests that extensive end-to-end cross-linking between collagen and fibrin fibrils or monomers did not occur. Instead, each biopolymer formed its own network that interpenetrated the other in a “parallel” fashion. In addition, the results showed effects on the microstructure and mechanics of both collagen and fibrin when these ECM proteins are co-gelated in the presence of each other (as summarized in Table 2).

4.1. Fibrin

Microstructural results from SEM images showed a finer network containing web-like/wispy structures, which apparently are fibrin fibrils, when the fibrin network was co-gelated with collagen. This observation is consistent with previous studies which showed that fibrin morphology is sensitive to gelation conditions such as the thrombin and Ca\(^{2+}\) concentrations [26,31,32]. Furthermore, we had also previously shown the existence of such finer fibrin fibrils in collagen–fibrin co-gels, although these were only observed at higher collagen concentrations [20]. Mechanically, fibrin formed in the co-gels exhibited a similar failure strength, lower modulus, but greater extensibility before failure. Correlation of the SEM results with these mechanical data for fibrin suggested that, in general, a decrease in fibril diameter resulted in a more compliant gel of similar tensile strength but greater extensibility before failure. This result is in contrast to previous findings by Rowe et al. [32], who found that a decrease in fibril diameter (by decreasing the thrombin concentration) produced an increase in UTS and modulus. It should, however, be noted that the microstructural changes caused by altering the thrombin concentration in that study were significantly more drastic. SEM images from Rowe et al. [32] show that as fibril diameter decreased with decreasing thrombin concentration the overall network became much more interconnected with smaller pores, leading to an increase in mechanical strength and stiffness. In contrast, our SEM images of fibrin networks under different conditions at lower magnifications of 10,000× (see Supplementary Material) showed no visible differences in network architecture. Previous studies on fibrin networks suggest that thicker fibrin fibrils are formed from lateral associations of thinner fibrils, with possible inter-fibril γ-chain cross-linking [38]. Conceivably, the smaller failure strain observed in the pure fibrin gels (F in PBS, F in Cs) is due to decreased extensibility of these thicker fibrils as the inter-fibril cross-links hinder the stretching of individual fibrils within these fibers. The lower stretching borne by these thicker fibrils necessitates that the thinner fibrils bear a larger proportion of the overall macroscopic stretching, causing catastrophic failure to occur at lower strains. In addition, these inter-fibril cross-links could also stiffen fibrin bundles compared with individual fibrils, resulting in an overall network with a slightly higher modulus.

4.2. Collagen

Similar trends in microstructure and mechanics were observed for our collagen networks when formed in the presence of fibrin (CG in Pn), compared with formation in its absence (C in Pn, C in PBS). SEM images showing a decrease in average collagen fibril and fiber diameter were accompanied by mechanical data showing a similar failure strength, a higher strain at failure and a lower tangent modulus. A similar explanation could be made to correlate the microstructure with the mechanics, that inter-fibrillar bonding between collagen fibrils in bundled fibers [43] (i.e. bonding between...
lateral fibrils within fibers) decrease the overall extensibility while increasing the stiffness. Further analysis into the collagen network from the reconstructed networks confirmed the presence of smaller average collagen fibril/fiber diameters (cf. Fig. 6A). In addition, while the collagen networks in co-gels showed higher average connectivities, this result was due to the presence of more cross-links with very high connectivities rather than a general increase in connectivity throughout all cross-links. In all cases connectivities of 3 or 4 around each cross-link were predominant. A connectivity of 3 suggests branching of collagen fibers from a bundled fiber, a phenomenon also observed by cryo-SEM of collagen networks [43]. A cross-link with connectivity 4 can be formed by two fibrils crossing in close proximity to each other and forming an inter-fibrillar chemical bond, or fibrils intertwining to form a mechanical bond. Connectivities above 4 are likely a result of clumping of fibrils, with fibrils radiating out from a central cluster as the FIRE code is unable to resolve fibrils within the cluster. The results from this study suggest that, in general, collagen networks formed in the presence of fibrin retain similar cross-link characteristics compared with collagen only networks, with similar spacings...
and tortuosities between cross-links (i.e. segment lengths) (cf. Fig. 6C and D), and predominant connectivities of 3 or 4. The very high connectivities observed for the collagen networks in co-gels (Fig. 8B) suggest more clustering of collagen fibrils when formed with fibrin.

Several groups have previously studied the correlation between collagen network microstructure and the overall mechanical behavior of collagen gels. Notable works include the study by Christiansen et al. [8], who investigated the effect of pH and temperature on collagen network formation. They reported a decrease in collagen fibril diameter with increasing pH from 5.0 to 9.0. A comparison with the mechanical properties showed that an increase in collagen fibril diameter was correlated with an increase in low strain modulus, but had no effect on UTS or high strain moduli. A similar study by Roeder et al. [37] confirmed the decrease in collagen fibril diameter with increasing pH, as well as an increase in fibril length. However, that group reported an accompanying increase in UTS and linear modulus, in contrast to the results of Christiansen et al. Roeder and co-workers concluded that collagen fibril length had a greater effect on the mechanical properties than fibril diameter. In the current study co-gelation of collagen with fibrin yielded longer but less bundled collagen fibers (i.e. smaller fiber diameters). However, like Christiansen et al., no significant increase in UTS was observed. In contrast to Roeder et al., the results from this study suggest a greater effect of collagen fibril diameter than fibril length: thicker fibers reduce macroscopic extensibility and increase overall gel stiffness, presumably due to inter-fibrillar bonding in fiber bundles. It should be noted that all three studies employed similar strain rates for mechanical testing, so that these differences are not due to the viscoelastic nature of these gels. Such discrepancies among reported results highlight the complexity of biological networks: changes in microstructure alone cannot fully account for the changes in macroscopic mechanical properties. Different gelation conditions, such as variations in pH and the presence of contaminants, could have affected fibrils at the molecular level, e.g. the degree of cross-linking and bonding between fibrils, factors that cannot be visualized using SEM or confocal microscopy.

Mechanical data for collagen networks revealed additional differences not present for fibrin networks. Firstly, the collagen PBS control (C in PBS) exhibited a higher UTS compared with gels placed in plasmin (C in Pn, CG in Pn). While positive controls were carried out to verify the efficacy of the enzymes used (pure collagen and fibrin gels were fully dissolved when placed in collagenase and plasmin, respectively) the plasmin could have caused slight degradation of the collagen network and reduced the strength of the gel, even though the collagen network remained largely intact. Secondly, the collagen gels formed by co-gelation with fibrin showed significantly larger transition strains, i.e. these gels have a longer toe region. Previous studies suggest that the toe region corresponds to straightening of undulating fibrils at the fibrillar [9] and the molecular [44] levels, as well as fibril reorientation in the direction of stretching [45,46]. The results of this study showed similar fiber and segment tortuosities between collagen networks in co-gels and in pure gels, which indicated that this longer toe region in co-gels could not be explained by more curved fibrils at the fibrillar level. Whether the longer toe region was caused by differences at the molecular level or was due to changes in network characteristics more conducive to fibril rearrangement remains unclear.

The combination of SEM and confocal imaging techniques allowed observation of collagen networks at both the fibril level and the network level. However, visual comparisons of images from these techniques showed straighter fibers and larger void spaces in the confocal images compared with SEM. We believe that these discrepancies are due to structural artifacts introduced during sample preparation for SEM; the drying process is known to cause slight network collapse resulting in smaller void spaces.

![Fig. 7](Image)

**Fig. 7.** Schematic representation of the definition of a segment and of a fiber in the FIRE algorithm. Each length between adjacent cross-links is defined as a segment; fibers can comprise several segments, where segments with a similar orientation are considered part of the same fiber. \( \alpha \) was set at 120° from sensitivity analysis.

![Fig. 8](Image)

**Fig. 8.** Comparison of connectivity distributions in collagen networks prepared under different casting and digestion conditions. (A) >80% of cross-links contain three or four connectivities in all samples. (B) Box plot showing that collagen networks from co-gels exhibit slightly higher maximum connectivities (\( \bullet \)) and higher connectivities at the 99% level (\( \circ \)).
Nevertheless, SEM images provide useful qualitative information on our networks at the fibrillar level, such as collagen fiber bundling and wispy fibrin fibrils. Previous studies have shown that gelation conditions of pH and ionic strength influence both fibrin [25] and collagen [36] fibril formation, resulting in varied microstructures. The formulations used to prepare the gels for these studies varied such that slight differences in pH and ionic concentration were observed between the formulations (Table 3). While the pH of all three formulations were maintained close to neutrality, there was a significant difference in the ionic strength of pure collagen (C) (0.12 M) compared with that in the co-gel (CG) (0.16 M). Wood and Keech [36] reported that an increase in ionic strength from 0.13 to 0.23 M resulted in an increase in collagen fibril diameter; more extensive fibril bundling was also observed. In contrast, an opposite trend for changes in collagen microstructure was observed in our gels: an increase in ionic strength from 0.12 (in pure collagen) to 0.16 M (in the collagen–fibrin co-gel) resulted in less fibril bundling and a decrease in average fibril diameter. These different findings highlight the intricate effects of the gelation conditions on collagen microstructure and warrants further investigation.

The results of this study indicate that while averaged characteristics are useful in quantifying gross differences in overall network architecture, they do not give the full picture of how the network morphology is changed. Thus these findings are also relevant to improving modeling approaches to understand the mechanics of...
certain native and bioengineered tissues for which a collagen–fibrin co-gel is a relevant experimental model. Different groups have used a variety of approaches to model fiber networks, ranging from periodic [48] or homogeneously distributed fiber models [49] to known models for disordered networks, e.g. the Poisson–Voronoi model [50]. To model the mechanical contribution of the ECM in tissues our group has employed a multi-scale framework using a representative volume element (RVE) containing a fiber network around each Gauss point [51,52]. This current method of network generation in the RVEs creates a randomly oriented network of elastic fibers connected to one another via rigid pin joints [51]. While our current network models are able to capture the inhomogeneous and anisotropic nature of ECM fiber networks, they suffer the same limitation as the afore-mentioned approaches: these networks do not accurately reflect the microstructure of the ECM networks in tissues. Here we utilized a method of characterizing ECM networks developed by Stein et al. via 3-D reconstruction of confocal z-stacks and fiber network analysis to obtain actual ECM network characteristics (connectivities, fiber length distributions, fiber tortuosities, etc.), which can be used to improve our network generation approaches to create fiber networks that more closely mimic the actual microstructure of the ECM in tissues.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–3 and 5–9, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.07.010.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2012.07.010.

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