Blayne A. Roeder
Student Mem. ASME
School of Mechanical Engineering,
Purdue University,
West Lafayette, IN 47907-1288
e-mail: blayne@ecn.purdue.edu

Klod Kokini
Mem. ASME
School of Mechanical Engineering/
Department of Biomedical Engineering,
Purdue University,
West Lafayette, IN 47907-1288
e-mail: kokini@ecn.purdue.edu

Jennifer E. Sturgis
Department of Basic Medical Sciences,
Purdue University,
West Lafayette, IN 47907-1515
e-mail: jennie@flowcyt.cyto.purdue.edu

J. Paul Robinson
e-mail: jpr@flowcyt.cyto.purdue.edu

Sherry L. Voytik-Harbin[1]
e-mail: voytik@ecn.purdue.edu

Department of Basic Medical Sciences/
Department of Biomedical Engineering,
Purdue University,
West Lafayette, IN 47907-1515

Tensile Mechanical Properties of Three-Dimensional Type I Collagen Extracellular Matrices With Varied Microstructure

The importance and priority of specific micro-structural and mechanical design parameters must be established to effectively engineer scaffolds (biomaterials) that mimic the extracellular matrix (ECM) environment of cells and have clinical applications as tissue substitutes. In this study, three-dimensional (3-D) matrices were prepared from type I collagen, the predominant compositional and structural component of connective tissue ECMs, and structural-mechanical relationships were studied. Polymerization conditions, including collagen concentration (0.3–3 mg/mL) and pH (6–9), were varied to obtain matrices of collagen fibrils with different microstructures. Confocal reflection microscopy was used to assess specific micro-structural features (e.g., diameter and length) and organization of component fibrils in 3-D. Microstructural analyses revealed that changes in collagen concentration affected fibril density while maintaining a relatively constant fibril diameter. On the other hand, both fibril length and diameter were affected by the pH of the polymerization reaction. Mechanically, all matrices exhibited a similar stress-strain curve with identifiable "toe," "linear," and "failure" regions. However, the linear modulus and failure stress increased with collagen concentration and were correlated with an increase in fibril density. Additionally, both the linear modulus and failure stress showed an increase with pH, which was related to an increased fibril length and a decreased fibril diameter. The tensile mechanical properties of the collagen matrices also showed strain rate dependence. Such fundamental information regarding the 3-D microstructural-mechanical properties of the ECM and its component molecules are important to our overall understanding of cell-ECM interactions (e.g., mechanotransduction) and the development of novel strategies for tissue repair and replacement.
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Keywords: Collagen, Extracellular Matrix, Three-Dimensional, Microstructure, Confocal Reflection Microscopy, Mechanical Properties

Introduction

One of the most critical aspects of tissue engineering is the ability to mimic extracellular matrix (ECM) scaffolds that naturally serve to organize cells and regulate their behavior [1]. The ECM provides relevant micro-environmental information to the cells biochemically through soluble and insoluble mediators and biophysically through imposition of structural and mechanical constraints (see Chiquet for review [2]). To date, significant advances have been made in our understanding of how specific molecules of the ECM affect fundamental cellular responses. However, less is known regarding the mechanisms by which specific geometric and mechanical properties of the ECM influence cell behavior. Likewise, the mechanisms by which the ECM transduces force and deformation from the macro-level (tissue-organ) to the micro-level (cell-ECM) remain to be elucidated. In this way, the physical state of the ECM, not just its molecular composition, provides the basis of cell-ECM interactions and must be considered in the design of new and improved scaffolds (biomaterials) for tissue repair and replacement.

To further the understanding of ECM biomechanics and its role in cell and tissue dynamics, we have developed an experimental approach in which micro-structural and subsequent mechanical properties of a simplified model of the ECM can be controlled. Our approach involves preparation of three-dimensional (3-D) matrices from purified type I collagen. Of the many component molecules of the ECM, type I collagen is the most abundant within connective tissue structures, including tendon, ligament, dermis, and blood vessel, and is the primary determinant of tensile properties [3]. In addition, type I collagen exhibits the ability to polymerize and form complex, 3-D supramolecular assemblies in vitro, a process known as ''self-assembly.'' Collagen fibrils formed in vitro have structural similarities (e.g., axial periodicity) to those formed in vivo and have been used extensively as a model system for understanding the collagen assembly process [4]. Collagen fibril dimensions and organization can be varied by adjusting parameters of the polymerization reaction (i.e., collagen concentration, pH, and ionic strength) [5]. Taken together, these characteristics of type I collagen make it an ideal biologically derived polymer for scaffold design. In fact, a number of biomaterials have been fashioned from type I collagen for restoration and reconstruction of specific tissues and organs. Likewise, 3-D collagen matrices have long been used as scaffolds for the culture of cells in vitro. Such in vitro systems have been shown to support a more in vivo like cellular phenotype and function and are instrumental in the study of the mechanisms involved in cell-ECM interactions [6].

Proposed relationships between collagen fibril morphology and the mechanical behavior of collagen-based tissues, scaffolds, extruded fibers, and matrices have been documented in a variety of contexts. Parry correlated specific structural features of collagen

[1]Corresponding author

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fibrils (e.g., total collagen content, fibril length, fibril diameter distribution, and fibril orientation) within intact tissues to tissue-specific mechanical properties [7]. Another series of studies has focused on the structural-mechanical properties of collagen scaffolds for the primary purpose of designing functional biomaterials for clinical applications. For example, the influence of specific glycosaminoglycans and cross-linking agents on type I collagen scaffold structure and mechanics has been evaluated in the development of artificial skin replacements [8–11]. Specifically, the glycosaminoglycans chondroitin-6-sulfate and dermatan sulfate increased the mechanical strength of a collagen based artificial skin, but did not cause micro-structural modification that could be appreciated with scanning electron microscopy [9]. In addition, extensive studies defining the structural-mechanical relationship of extruded collagen fibers have been performed with hopes of developing replacement constructs for tissues such as tendon and ligament [12–17]. The diameter of fibril subunits within extruded collagen fibers was correlated with low strain modulus but not ultimate tensile strength or high strain modulus [17].

Less is known, however, regarding the structural-mechanical properties of low concentration collagen matrices or gels. These matrices constitute collagen concentration of 5 mg/mL or less rather than 30-40 mg/mL characteristic of connective tissues in vivo [18]. Since low concentration matrices are routinely used for the culture and study of cells in vitro, determination of such fundamental structural-mechanical properties is necessary for predicting and interpreting the overall cellular response. In addition, knowledge of these collagen properties will contribute to the basic principles that will drive the engineering and development of clinically useful biomaterials.

A purely mechanical study performed by Özerdem and Tözeren demonstrated the viscoelastic behavior (e.g., stress-relaxation) of type I collagen matrices by imposing quick stretches [19]. In a second study, the rheological behavior of type I collagen matrices was described using confined compression testing [20]. Finally, Osborne et al. have reported the tensile properties of type I collagen matrices in the presence and absence of specific glycosaminoglycans [21]. In addition, they investigated the effects of various cross-linking techniques on the mechanical behavior of these matrices. To date, the only published study evaluating both the structural and mechanical properties of type I collagen matrices was performed by Hsu and co-workers [22]. In this particular study, collagen matrices were formed at various ionic strengths and with differing glycosaminoglycan contents, and the relationship between the rheological behavior and fibril morphology was investigated. For the structural analysis in this study as well as in others, traditional electron microscopy techniques were utilized. Limitations of these techniques include derivation of structural information in a 2-D rather than 3-D format and induction of structural artifacts due to extensive specimen processing. To our knowledge, the relationship between the 3-D microstructure and the tensile mechanical properties of low-concentration, type I collagen matrices has not been previously reported.

In the present study, we report polymerization of purified type I collagen at different collagen concentrations and pH values to form 3-D matrices differing both structurally and mechanically. Confocal reflection microscopy was used to establish and compare 3-D micro-structural differences between the matrices in their native, hydrated state. This information was then correlated with features of the stress-strain relationship, including linear modulus, failure stress, and failure strain derived from tensile mechanical tests.

Materials and Methods

Collagen ECM Preparation. Type I collagen prepared from calf skin was obtained from Sigma Chemical Co., St. Louis, MO, and dissolved in 0.01 M hydrochloric acid (HCl) to achieve desired concentrations. Three-dimensional collagen matrices were prepared by neutralizing the collagen solutions in 10X phosphate buffered saline (PBS) followed by incubation at 37°C in humidified chambers. To determine the effect of collagen concentration on collagen matrix structure and mechanical properties, solutions varying in collagen content were neutralized to achieve final collagen concentrations of 0.3, 1.0, 2.0 and 3.0 mg/mL while maintaining the same total phosphate, ionic strength (0.14 M), and pH (7.4). The effect of pH was studied by neutralizing collagen solutions with 10X PBS buffers with altered ratios of Na_2HPO_4 and KH_2PO_4. 10X PBS buffers of pH 6.0, 7.0, 7.4, 8.0, and 9.0 were used for these experiments.

Confocal Reflection Microscopy. Unstained 3-D matrices of type I collagen were polymerized on coverglasses and imaged using confocal reflection microscopy as previously described [23,24]. In brief, confocal reflection microscopy was performed using a BioRad (Hercules, CA) MRC1024 confocal on a Diaphot 300 (Nikon Corp., Tokyo, Japan) microscope equipped with a 60×, 1.4 NA oil immersion lens (Nikon) via a quarter wave plate. Specimens were illuminated with 488 nm light generated by an Innova Enterprise argon ion laser (Coherent Laser Group, Santa Clara, CA) and the reflected light was detected with a photomultiplier tube using a blue reflection filter (488 nm). A *z*-step of 0.2 μm was used to optically section the samples to depths of up to 300 μm. When necessary, nonuniform background caused by interference and reflection from the optical pathway was removed from the images using standard rank leveling procedures on each *x-y* section as described previously [24]. Three-dimensional images of the matrices were either compiled into a single view projection using Laser Sharp (BioRad) image-processing software or compiled into a 3-D projection using Voxel-View reconstruction software (Vital Images, Inc., Plymouth, MN).

At present, high-throughput computer algorithms are being developed to facilitate the quantification of specific structural parameters of collagen matrices, including fibril diameter, fibril density, fibril length, and fibril orientation. As a first approach to quantitatively assess fibril diameter, a *z*-series of images representing a collagen matrix was divided into quadrants and 10 fibrils within each quadrant were randomly selected (i.e., all fibrils weighted equally) for fibril diameter quantification. Using Voxel-View reconstruction software, a threshold was determined for the *z*-series and the images binarized. Five lines were then drawn perpendicular to the long axis of each fibril using the trace feature of the software. The average number of white pixels traversed by the trace lines (representing the fibril diameter) was determined and converted into nanometers based upon pixel dimensions.

Measurement of Mechanical Properties. Samples for mechanical testing were prepared by polymerizing the neutralized collagen solution in a "dog bone" shaped mold (Fig. 1(*a*)). The mold consisted of a glass plate and a piece of flexible silicone gasket. The gauge section of the mold measured 10 mm in length, 4 mm in width, and approximately 1.8 mm in thickness (Fig. 1(*b*)). Neutralized collagen solution (1 mL) was added to each mold and the mold incubated at 37°C in a humidified environment. Polypropylene mesh was embedded in the ends of the matrices to facilitate clamping of each specimen for mechanical testing. Following polymerization of the collagen matrix, both thickness and width measurements were made on the gauge section of the specimens using dial calipers. Total thickness measurements representing the width of both the specimen and glass plate were made by lowering the calipers until they touched the top of the matrix; this event was marked by the liquid being pulled into contact with the caliper edge via surface tension. Specimen thickness was calculated by subtracting the thickness of the glass plate from the total thickness (matrix+glass plate) measurement. To measure specimen width, the collagen matrix was placed under a Zeiss Stemi 2000 (Carl Zeiss, Jena, Germany) stereo microscope (2.5× magnification) and the caliper tips were matched to the width of the matrix. The dial caliper measurements are accurate to 0.02 mm.

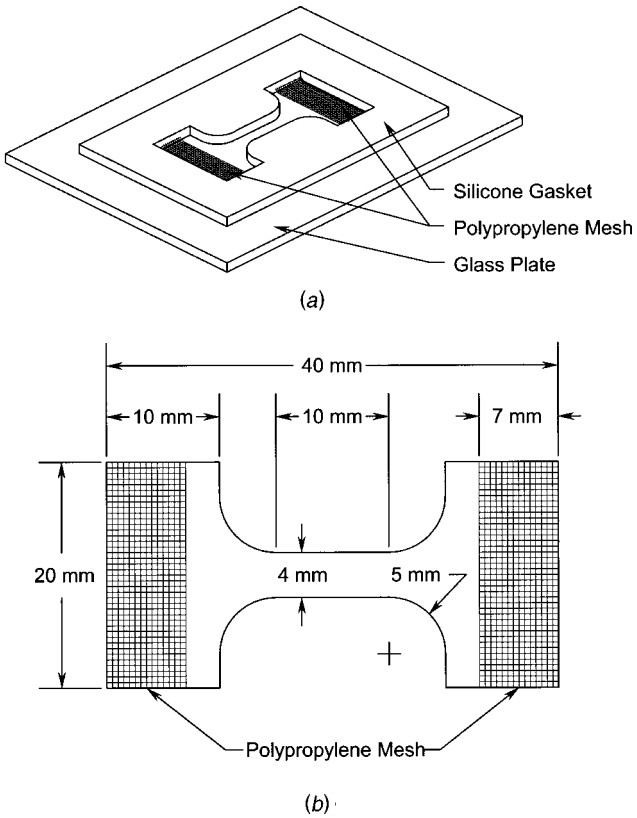

Fig. 1 Construction of the mold used to prepare collagen matrix test specimens (*a*). Schematic showing the dimensions of the collagen matrix test specimen with polypropylene mesh reinforcement (*b*).

Tensile properties of 3-D collagen matrices were measured using a modified Minimat 2000 miniature materials tester (Rheometric Scientific, Inc., Piscataway, NJ) and standard testing procedures for matrices of low mechanical strength (Fig. 2) [19,25–27]. One end of each specimen was attached to a stepper motor controlled linear actuator and the other end was attached to a load cell. For these experiments, a load cell designed with a sensitivity of 0.0003 N was utilized. Clamps offset the loading axis and allowed the matrix to be submerged longitudinally within a bath of PBS, pH 7.4, at approximately 37°C. For most experiments, an extension rate of 10 mm/min (38.5 percent/min strain rate) was

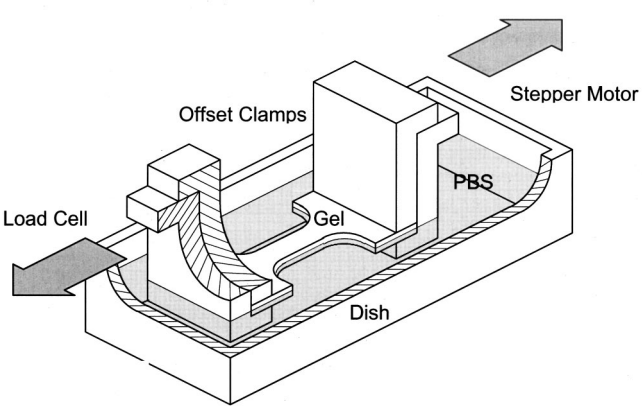

Fig. 2 Cut away view of the experimental setup used for mechanical testing of collagen matrices. Collagen matrices were tested while submerged in PBS, pH 7.4 at 37°C.

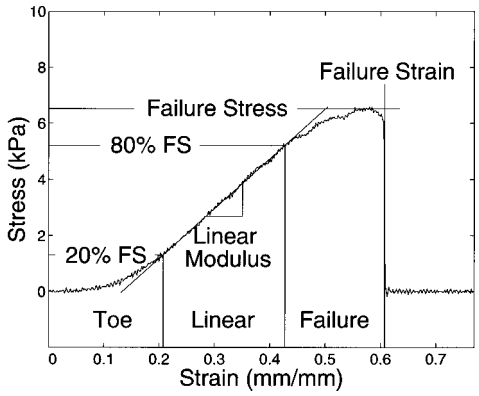

Fig. 3 Representative stress-strain curve of a collagen matrix (2 mg/mL, pH 7.4) tested at a strain rate of 38.5 percent/min. The stress strain curve can be separated into three distinct regions designated "toe," "linear," and "failure." Determinations of linear modulus, failure stress and failure strain are demonstrated.

employed. For experiments investigating the effect of strain rate on the mechanical properties, strain rates of 19.2, 192, and 385 percent/min were applied. For all mechanical tests, no preconditioning was applied. Engineering stress and strain were calculated from the load displacement data from the experiments. Engineering stress (σ_e) was calculated as

$$\sigma_e = \frac{F}{A_0} \tag{1}$$

where F is the force recorded by the Minimat and A_0 is the initial cross-sectional area (width × thickness) of the gauge section of the matrix specimen. Engineering strain (ε_e) was calculated as the change in length or cross-head displacement (Δl) divided by the original length (l_0).

$$\varepsilon_e = \frac{\Delta l}{l_0} \tag{2}$$

In preliminary experiments, 20 μm polystyrene beads were embedded into matrices and used as markers to determine longitudinal strain. Relative displacements of the beads were imaged, utilizing a Zeiss Stemi 2000 stereo microscope (2.5× magnification), and strains extracted. Experimentally determined strain measurements were found to correlate with cross-head displacement divided by the distance between the mesh (26 mm). Accordingly, for all the experiments, the distance between the mesh was used as the unstretched or original length of the specimen. The linear modulus was defined as the slope of the "linear" region between 20 percent and 80 percent of the maximum stress as determined by linear regression analysis. Failure stress represented the maximum stress value achieved during loading and failure strain was the strain at which each collagen matrix experienced total failure (Fig. 3). Each experiment (a particular collagen concentration, strain rate and pH combination) was repeated in at least triplicate, with each repetition including three to six samples.

Statistical Analysis. An analysis of variance was conducted on structural and mechanical property data obtained for matrices using the SAS Statistical Software package (SAS Institute Inc., Cary, NC). The Student Neuman Keuls method for multiple comparisons ($p<0.05$) was then applied.

Results

3-D Structural Properties. Adjustment of collagen polymerization conditions, including collagen concentration and pH, generated 3-D collagen matrices (Fig. 4) with distinct structural prop-

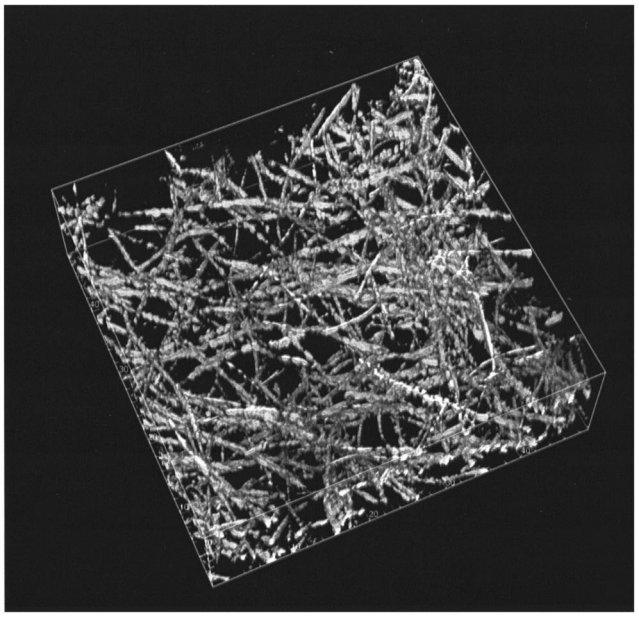

Fig. 4 Representative 3D reconstructed confocal reflection image of a matrix prepared from purified type I collagen (1 mg/mL, pH 7.4)

erties. Confocal reflection microscopy revealed that changes in collagen concentration affected fibril density while maintaining a relatively consistent fibril diameter (Fig. 5). An increase in collagen fibril density was observed with increasing collagen concentration. Fibril diameter measurements for matrices prepared at different collagen concentrations were not significantly different ($P < 0.05$) and are summarized in Table 1. Based upon qualitative assessment, both fibril length and diameter were affected by the pH of the polymerization reaction (Fig. 6). Fibrils formed at pH 6.0 were visually shorter and thicker, while those formed at pH 9.0 were longer and thinner. Fibril diameter measurements as a function of pH are provided in Table 1.

Table 1 Summary of fibril diameter measurements (mean ±standard deviation) in nanometers derived from the confocal images

		Collagen Concentration (mg/ml)			
		0.3	1.0	2.0	3.0
pH	6.0	-	-	490 ± 96	-
	7.0	-	-	469 ± 73	-
	7.4	418 ± 121	446 ± 65	435 ± 61	430 ± 71
	8.0	-	-	421 ± 62	-
	9.0	-	-	392 ± 65	-

Mechanical Property Measurements. When tested mechanically, all matrices exhibited a similar stress-strain relationship. All stress-strain curves showed identifiable ''toe,'' ''linear,'' and ''failure'' regions characteristic of intact biological tissues (Fig. 3). Time-lapse photographs of a characteristic tensile test are shown in Fig. 7. In addition to large longitudinal displacement imparted by the testing equipment, substantial deformation in the lateral direction was also noted. The majority of specimens failed at or near the middle of the gauge section. Collagen matrices that did not exhibit midgauge failure were not included in the data analysis.

Mechanical properties of 3-D collagen matrices were found to be dependent upon polymerization time. The variation of mechanical properties with polymerization time for 1.0, 2.0 and 3.0 mg/mL (pH 7.4) collagen matrices is shown in Fig. 8. Similarly the variation of mechanical properties with polymerization time for pH 6.0, pH 7.4 and pH 9.0 (2.0 mg/mL) collagen matrices is shown in Fig. 9. Collagen solutions were neutralized, aliquoted into the dog bone-shaped mold, and incubated in a humidified

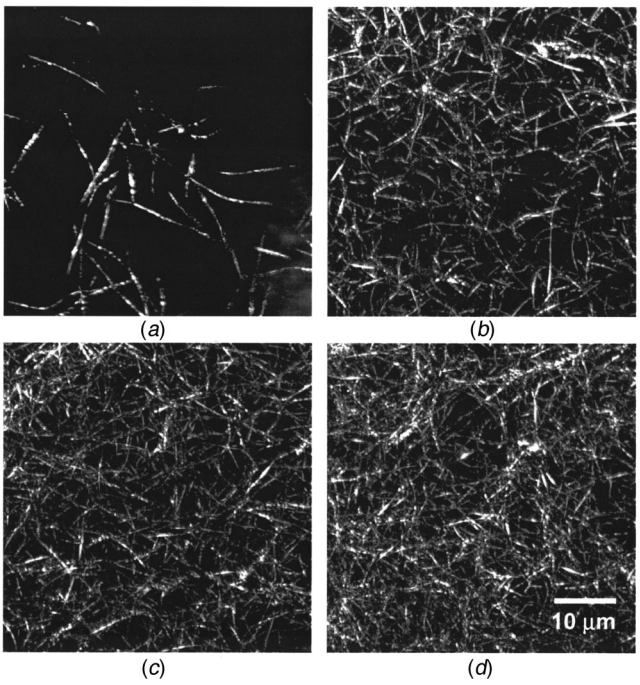

Fig. 5 Confocal reflection images comparing the microstructure of collagen matrices prepared at concentrations of 0.3 mg/mL (*a*), 1 mg/mL (*b*), 2 mg/mL (*c*), and 3 mg/mL (*d*). An increase in fibril density was observed with increasing collagen concentration (10 μm bar is applicable to all images).

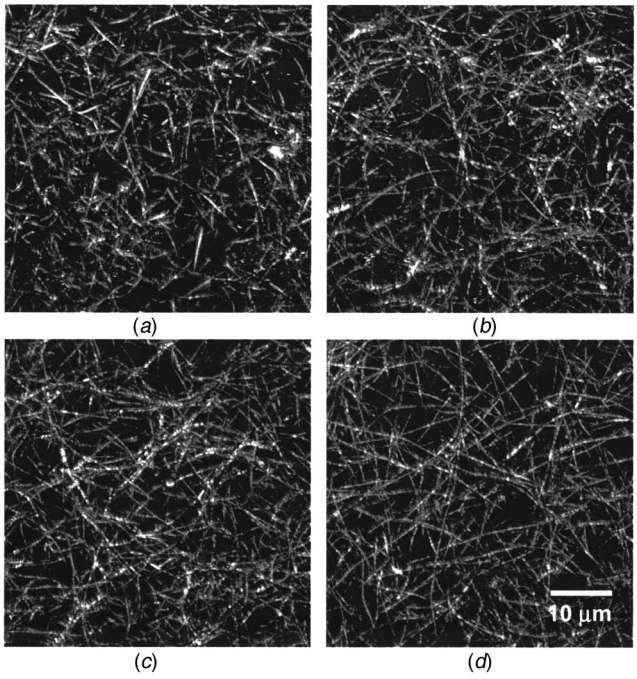

Fig. 6 Confocal reflection images comparing the microstructure of collagen matrices (2 mg/mL) prepared at pH of 6.0 (*a*), 7.0 (*b*), 8.0 (*c*), and 9.0 (*d*). Both fibril diameter and length were affected by pH. Fibrils formed at lower pH were shorter and thicker than those produced at higher pH (10 μm bar is applicable to all images).

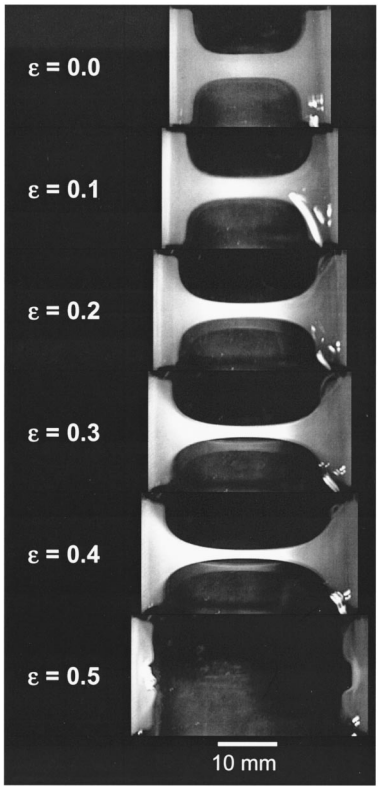

Fig. 7 Time-lapse images demonstrating the tensile testing of a collagen matrix (2 mg/mL, pH 7.4) at a strain rate of 38.5 percent/min

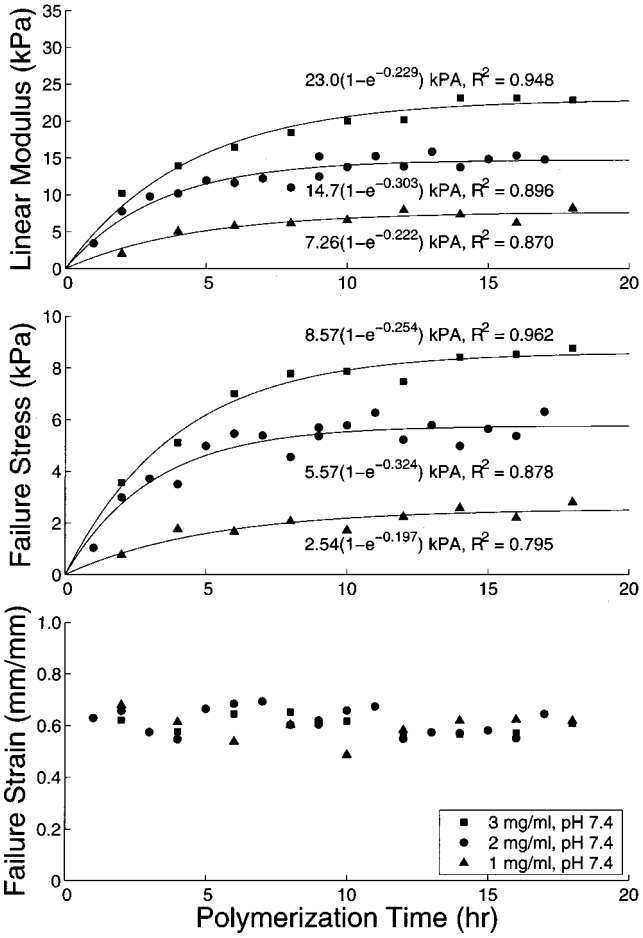

Fig. 8 Effect of polymerization time on the linear modulus, failure stress and failure strain of 1.0 mg/mL (▲), 2.0 mg/mL (●), and 3.0 mg/mL (■) collagen matrices (pH 7.4) tested at a strain rate of 38.5 percent/min. Exponential curve fits are shown for the linear modulus and failure stress data.

chamber at 37°C for periods of time ranging from 1 to 18 h. A progressive increase in failure stress and linear modulus was observed with matrices polymerized for up to 10 h. After that time, these parameters did not change significantly. Failure strain was found to be independent of polymerization time. Based upon these data, a polymerization time of at least 15 h was established and applied for subsequent experiments.

Variation of collagen concentration while all other polymerization reaction variables (e.g., pH and ionic strength) were held constant significantly affected the mechanical integrity of the 3-D collagen matrices (Fig. 10). Collagen matrices suitable for tensile testing using our experimental setup could be reproducibly prepared at collagen concentration 0.3 mg/mL and greater. Linear modulus and failure stress values increased linearly over the range of collagen concentrations evaluated (0.3-3 mg/mL). Linear modulus values ranged from 1.5 kPa for 0.3 mg/mL collagen matrices to 24.3 kPa for 3 mg/mL collagen matrices. Likewise, a 10-fold increase in collagen concentration resulted in a nearly 18-fold increase in failure stress. Except for 0.3 mg/mL, the failure strain of the collagen matrix was independent of collagen concentration ($p<0.05$).

Differences in the mechanical properties of collagen matrices were also noted when the pH of the polymerization reaction was adjusted between 6 and 9 (Fig. 11). Collagen matrices polymerized at normal physiologic pH (7.4) had a linear modulus and failure stress of 16.6 and 6.0 kPa, respectively. Matrices formed under increasingly acidic conditions showed a progressive decrease in failure stress and linear modulus values ($p<0.05$). The tensile strength of collagen matrices was enhanced by polymerization under basic conditions, with the greatest mechanical integrity being exhibited by matrices formed at pH 9.0. Failure strain remained relatively constant (approximately 0.6 mm/mm) over the range of pH values tested.

Finally, the mechanical properties of the collagen matrices were found to be dependent upon strain rate (Fig. 12). Over the range of strain rates tested, failure stress increased from 5.4 to 8.7 kPa and the linear modulus increased from 15.7 to 25 kPa. Failure strain was independent of strain rate ($p<0.05$). A summary of the dependence of tensile mechanical properties of collagen matrices as a function of collagen concentration, pH, and strain rate is provided in Table 2.

The length of the ''toe'' region (defined as the distance between zero strain and the intersection of the linear fit and the strain axis) was quantified for each mechanical test. Upon statistical analysis, no correlation was identified between ''toe'' region length and polymerization conditions. Characterization of a low strain modulus (the slope of the ''toe'' region) was not completed due to the low signal to noise ratio in this region.

Discussion

In the present study, the 3-D microstructure of type I collagen matrices prepared in vitro under different assembly conditions was determined and correlated with tensile mechanical properties. Within the ECM of connective tissues in vivo, collagen molecules are organized into hierarchical structures consisting of highly ordered fibrils that subsequently are aggregated into fibers or fiber bundles. Depending upon the specific tissue studied, collagen fibrils have been identified with diameters in the range of 25–500 nanometers [28,29]. On the other hand, fiber bundles have been

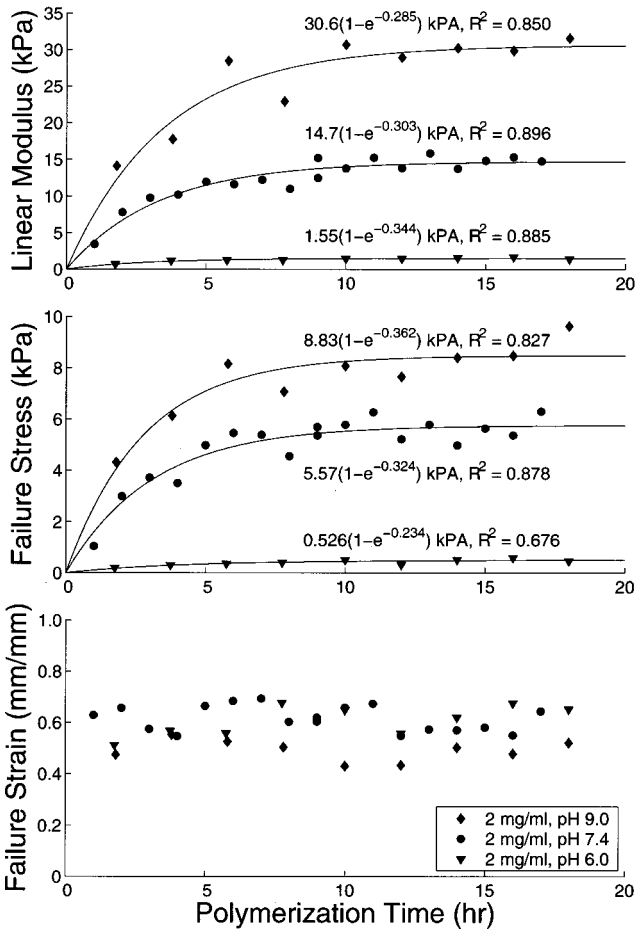

Fig. 9 Effect of polymerization time on the linear modulus, failure stress and failure strain of pH 6.0 (▼), pH 7.4 (●), and pH 9.0 (♦) collagen matrices (2.0 mg/mL) tested at a strain rate of 38.5 percent/min. Exponential curve fits are shown for the linear modulus and failure stress data.

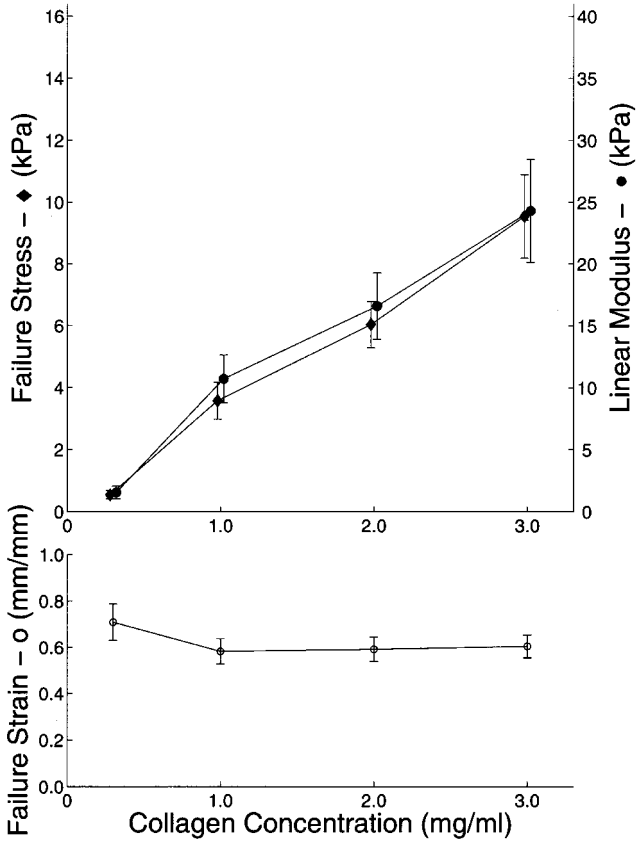

Fig. 10 Effect of collagen concentration (0.3–3.0 mg/mL) on the linear modulus (●), failure stress (♦), and failure strain (○) of collagen matrices (pH 7.4) tested at 38.5 percent/min

reported as large as several hundred micrometers [28,29]. It has been established that the distribution of collagen fibril sizes (i.e., length and diameter) together with their specific organization is an important determinant of the mechanical properties of tissues. In particular, it has been suggested that the creep-inhibition property of a tissue, i.e., its ability to resist plastic deformation, is directly related to the percentage of small diameter fibrils present [7]. On the other hand, the ability of the tissue to withstand high stress levels is related to the percentage of large diameter fibrils in the tissue. Differences in the mechanical behavior of tissues can also be attributed to variation in content and composition of noncollagenous components present in the ECM, including proteoglycans, glycosaminoglycans, and elastic fibers [7]. The tremendous diversity of ECM components and their complex organization in vivo make it difficult to decipher the importance of individual molecules to the ECM structural and mechanical properties that ultimately regulate cellular behavior. Herein, a simplified model of the ECM represented by 3-D, self-assembled matrices of type I collagen were used to determine the relationship between collagen fibril microstructure and mechanical behavior.

Herein, polymerization reaction conditions including collagen concentration and pH were varied to obtain 3-D collagen matrices with distinct microstructures. A key feature of this study was the application of confocal reflection microscopy to collect and analyze 3-D micro-structural details. This technique allowed collagen fibril parameters and organization to be visualized and quantified in a 3-D format in the absence of specimen processing (e.g., fixation, dehydration, and staining). Traditionally, structural information regarding collagen fibrils both in vivo and in vitro has been collected using light and electron microscopy techniques. These techniques provide structural information in a 2-D rather than 3-D format and require extensive specimen processing that often results in structural artifacts [17,30,31]. Three-dimensional images of collagen matrices in their native state allow for more accurate measurement of fibril parameters (i.e., diameter) and inspection of interfibril relationships.

Confocal reflection microscopy revealed that varying the collagen concentration of the polymerization reaction affects the fibril density of the collagen matrices. Quantitative analyses showed that fibril diameter did not vary significantly with collagen concentration. Fibril diameters for these matrices were approximately 450 nm, which falls into the range of fibril diameters of intact tissues. The dependence of fibril density but not fibril diameter on collagen concentration is consistent with previous studies by Wood and Keech [5].

Acidic conditions produced fibrils that were increased in diameter and decreased in length as compared to fibrils produced at basic conditions. The effect of pH on fibril diameter, but not length, has been previously shown in studies on matrices and extruded collagen fibers [5,17]. This study is the first to show the effect of pH on the length of collagen fibrils.

The fact that type I collagen is a major determinant of the mechanical properties of the ECM was apparent in that the stress-strain curves for in vitro assembled matrices were similar in shape to those for intact tissues. As observed with intact tissues [32], 3-D collagen matrices exhibited nonlinear stress-strain curves with three distinct regions. The region of small strains (''toe'' region) corresponds to the removal of a crimp in the collagen fibrils first at the fibrillar level and then at the molecular level

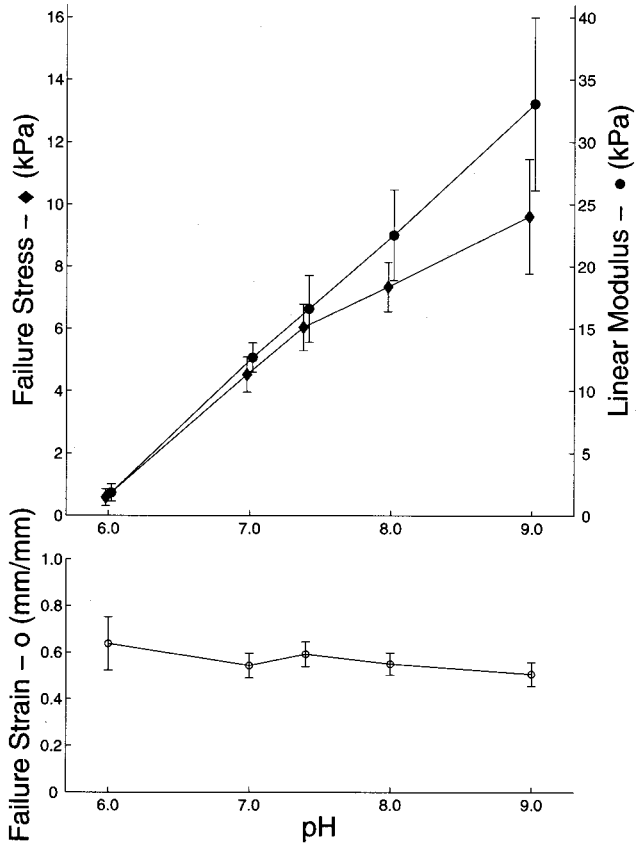

Fig. 11 Effect of phosphate buffer pH (6.0–9.0) on the linear modulus (●), failure stress (♦), and failure strain (○) of collagen matrices (2 mg/mL) tested at a strain rate of 38.5 percent/min

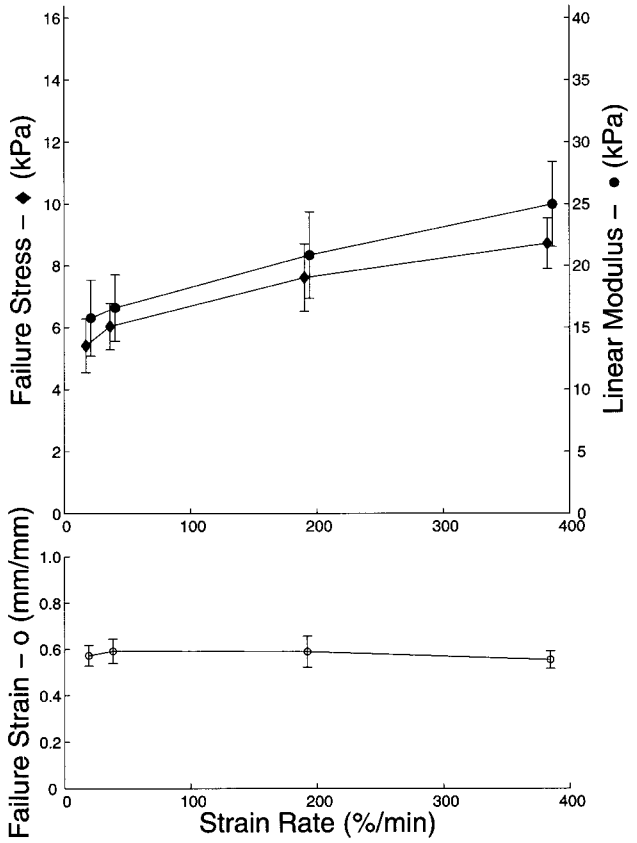

Fig. 12 Effect of strain rate (19.2–385 percent/min) on the linear modulus (●), failure stress (♦), and failure strain (○) of collagen matrices (2 mg/mL, pH 7.4)

[33]. In the ''linear'' region, the stiffness of the collagen fibrils increases considerably with extension. This region has been associated with stretching of the collagen triple helices or of the cross-links between the helices, implying a side-by-side gliding of neighboring molecules [34]. Finally, the ''failure'' region represents disruption of fibril structure. Additionally, the stress-strain response of the 3-D collagen matrices was shown to be sensitive to strain rate, a characteristic of viscoelastic materials. Nearly all living tissues show some level of viscoelasticity [35]. Specifically, the viscoelastic material properties of both collagen fibers derived from rat-tail tendon [36] and extruded collagen fibers have been demonstrated [37]. Again, the mechanical nature of the collagen matrices is shown to be consistent with properties of their main structural component, collagen fibrils.

Interestingly, the stress-strain behavior of the collagen matrices was found to be dependent upon the time of polymerization. The mechanical integrity of the matrices increased with polymerization time and then stabilized after 10 h. This result was somewhat surprising since the bulk of the visible fibril formation events in the self-assembly process are thought to be complete after 30–120 min, based upon traditional spectrophotometric (turbidity) [38] and time-lapse confocal reflection microscopy experiments [24]. Changes in the intermolecular hydrophobic interactions might have contributed to this observation. Comparison of mechanical data obtained from the present experiments was facilitated by using a polymerization time in which the mechanical integrity of the collagen matrices was determined to be stable (greater than 15 h).

As observed previously with intact tissues, mechanical behavior of in vitro assembled collagen matrices was dependent upon structural properties. As expected, the linear modulus and failure strength increased linearly with increasing collagen concentration, and therefore collagen fibril density. As a first approach, the collagen matrix can be represented as a composite consisting of an interstitial fluid reinforced with randomly oriented collagen fibrils. Parry presented a theoretical model for a randomly oriented fibrous composite that predicted that the Young's modulus was proportional to the volume fraction of collagen fibrils [7]. Additionally, he showed that higher collagen concentrations correlate with increased tensile strength.

Table 2 Results of the tensile tests

Variation of Collagen Concentration

Collagen Concentration mg/ml	Strain Rate %/min	pH	Linear Modulus (mean ± std) kPa	Failure Stress (mean ± std) kPa	Failure Strain (mean ± std) mm/mm	n*
0.3	38.5	7.4	1.54 ± 0.507	0.541 ± 0.138	0.708 ± 0.0787	7
1.0	38.5	7.4	10.7 ± 1.93	3.58 ± 0.598	0.583 ± 0.0546	15
2.0	38.5	7.4	16.6 ± 2.68	6.04 ± 0.750	0.592 ± 0.0530	20
3.0	38.5	7.4	24.3 ± 4.16	9.54 ± 1.35	0.605 ± 0.0496	15

Variation of pH

Collagen Concentration mg/ml	Strain Rate %/min	pH	Linear Modulus (mean ± std) kPa	Failure Stress (mean ± std) kPa	Failure Strain (mean ± std) mm/mm	n*
2.0	38.5	6.0	1.84 ± 0.701	0.586 ± 0.274	0.637 ± 0.115	8
2.0	38.5	7.0	12.7 ± 1.18	4.52 ± 0.567	0.543 ± 0.0535	13
2.0	38.5	7.4	16.6 ± 2.68	6.04 ± 0.750	0.592 ± 0.0530	20
2.0	38.5	8.0	22.5 ± 3.65	7.34 ± 0.797	0.549 ± 0.0474	13
2.0	38.5	9.0	33.0 ± 6.93	9.61 ± 1.84	0.506 ± 0.0515	13

Variation of Strain Rate

Collagen Concentration mg/ml	Strain Rate %/min	pH	Linear Modulus (mean ± std) kPa	Failure Stress (mean ± std) kPa	Failure Strain (mean ± std) mm/mm	n*
2.0	19.2	7.4	15.7 ± 3.07	5.41 ± 0.871	0.573 ± 0.0449	11
2.0	38.5	7.4	16.6 ± 2.68	6.04 ± 0.750	0.592 ± 0.0530	20
2.0	192.0	7.4	20.9 ± 3.49	7.61 ± 1.09	0.589 ± 0.0671	11
2.0	385.0	7.4	25.0 ± 3.44	8.72 ± 0.819	0.554 ± 0.0389	11

*n values are totals for at least 3 repetitions of each data set

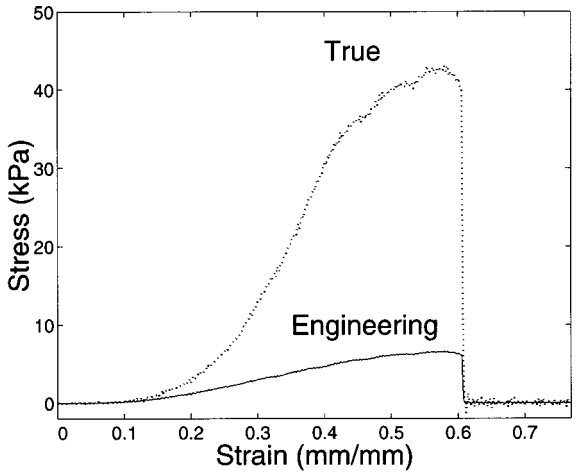

Fig. 13 Comparison of "true" stress (approximated) and "engineering" stress (calculated) as a function of strain for a collagen matrix (2mg/mL, pH 7.4)

In this study, collagen matrices prepared at 3 mg/mL showed the greatest failure stress (9.54 kPa) and linear modulus (24.3 kPa). Comparable tensile mechanical properties for this specific collagen concentration were also obtained by Osborne et al. [21]. In contrast, mechanical property data obtained in the present study was significantly greater than those reported by Özerdem and Tözeren [19]. This discrepancy can best be explained by their use of pepsin treated rather that acid solubilized collagen [39].

The dependence of collagen matrix mechanical properties on structure was also observed when the pH of the polymerization reaction was changed. Linear modulus and failure stress increased for matrices prepared under increasingly basic conditions up to pH 9.0. The increase in mechanical integrity was correlated with fibrils that were increased in length but decreased in diameter. Assuming a constant fibril diameter, an increase in length of the fibrils will increase the mechanical integrity (linear modulus and failure stress) of a fibrous composite system [40]. The increase in mechanical properties with increased fibril length and decreased fibril diameter suggests that fibril length has a greater effect on the mechanical properties of the 3-D collagen matrix construct than does the fibril diameter. This apparent trade-off between fibril diameter and length in dictating the mechanical properties of collagen based materials has not been previously reported.

The calculation of "engineering" rather than "true" stress and strain is consistent with previous studies involving collagen matrices [19,21] and facilitated data comparison. However, the photograph in Fig. 7 shows that the cross-sectional area of the specimen does experience a significant reduction. Thus, the "true" stress within the specimen is clearly greater than the calculated "engineering" stress values. Consistent with previous studies, this area reduction was not systematically measured in this study. However, in order to estimate the difference between "true" stress and "engineering" stress values, the area reduction in the specimens of Fig. 7 was approximated. We assumed that the specimen behaved isotropically and that the relative changes in width and thickness measurements at a given load were the same. Based upon these assumptions, "true" stress values were approximated over the various regions of the stress-strain curve and compared with calculated "engineering" stress (Fig. 13). The "true" stress is indicated as a dashed line since it is an approximate and not a directly measured quantity. Note that the largest difference between "engineering" and "true" stress values is observed in the "failure" region. At present, more sophisticated methods are needed for determining changes in cross-sectional area of specimens during mechanical loading so that "true" stress can be calculated accurately. Such methods are currently being investigated in our laboratory.

Our results represent the first extensive study on the effects of pH and collagen concentration on the mechanical behavior of collagen matrices. In turn, mechanical properties have been correlated to micro-structural changes in the collagen matrices. The ability to specifically modify ECM structure to yield predictable and reproducible mechanical properties will provide a useful tool for the study of cell-ECM interaction. In particular, the influence of ECM structure-mechanics on cell behavior can be explored. Structural variation will elicit an array of loading and deformation conditions on cells seeded within the matrices. Specific cellular responses to the different micromechanical environments can then be studied and mechanisms derived. This simplified system also can be used to determine specific molecular interactions (e.g., type I collagen plus specific glycosaminoglycans) that influence ECM structure-mechanics. Thus, experiments that provide quantitative as well as qualitative information regarding the ECM and its influence on cells will ultimately elucidate the basic principles that will drive the next generation of biomaterials and devices for tissue engineering applications.

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