Polymerization and Matrix Physical Properties as Important Design Considerations for Soluble Collagen Formulations

S. T. Kreger,1 B. J. Bell,1 J. Bailey,1 E. Stites,1 J. Kuske,1 B. Waisner,2 S. L. Voytik-Harbin1,2
1 Weldon School of Biomedical Engineering, College of Engineering, Purdue University, West Lafayette, IN 47907
2 Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907

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ABSTRACT:
Despite extensive use of type I collagen for research and medical applications, its fibril-forming or polymerization potential has yet to be fully defined and exploited. Here, we describe a type I collagen formulation that is acid solubilized from porcine skin collagen (PSC), quality controlled based upon polymerization potential, and well suited as a platform polymer for preparing three-dimensional (3D) culture systems and injectable/implantable in vivo cellular microenvironments in which both relevant biochemical and biophysical parameters can be precision-controlled. PSC is compared with three commercial collagens in terms of composition and purity as well as polymerization potential, which is described by kinetic parameters and fibril microstructure and mechanical properties of formed matrices. When subjected to identical polymerization conditions, PSC showed significantly decreased polymerization times compared to the other collagens and yielded matrices with the greatest mechanical integrity and broadest range of mechanical properties as characterized in oscillatory shear, uniaxial extension, and unconfined compression. Compositional and intrinsic viscosity analyses suggest that the enhanced polymerization potential of PSC may be attributed to its unique oligomer composition. Collectively, this work demonstrates the importance of standardizing next generation collagen formulations based upon polymerization potential and provides preliminary insight into the contribution of oligomers to collagen polymerization properties. © 2010 Wiley Periodicals, Inc. Biopolymers 93: 690–707, 2010.

Keywords: collagen; ECM; mechanical properties; fibril microstructure; polymerization; 3D matrix; mesenchymal stem cell differentiation

INTRODUCTION

Purified type I collagen is routinely used for basic research including creation of physiologically relevant three-dimensional (3D) culture models, investigation of cell-extracellular matrix (ECM) interactions, and, more recently, development of engineered tissue constructs.1–5 Furthermore, collagen has been processed into various biomaterial forms (e.g., films, fibers, sponges, and fibers) for numerous clinical applications including corneal shields, dermal repair, plastic surgery, drug delivery, and hemostasis.6 Collagen is an ideal candidate polymer for such research and medical applications, because it serves as the predominant component and primary structural-mechanical determinant of most tissue ECM.7,8 Here, it exists as a composite material consisting of an insoluble fibril network surrounded by interstitial fluid. The material
properties and microstructure of this fibrous matrix endow tissues with critical physical attributes (e.g., diffusional, mechanical, and degradation/remodeling properties). In addition, collagen imparts its biological activity through cell surface-receptor-mediated signaling, primarily through integrin receptors.\textsuperscript{8} Integrins mediate the physical connectivity between the cytoskeletal elements of cells and their surrounding ECM. In fact, the mechanical force balance that results between a cell and its ECM has been shown to be a critical determinant of cell morphology and fate.\textsuperscript{9–11}

Despite the significant advantages offered by this natural polymer, a number of factors hinder its more wide-spread use, especially for the creation of 3D culture systems. At present, both commercial and laboratory-grade collagens undergo cursory characterization, primarily focused on molecular mass of constituent polypeptide chains and purity. Furthermore, there is a lack of standardization amongst the various commercial and laboratory-generated collagens, which ultimately contributes to significant lot-to-lot and product-to-product variation and makes comparisons between studies difficult.\textsuperscript{1} Soluble collagens traditionally have been used to coat surfaces (e.g., tissue culture plastic or scaffolds) to promote cell adhesion; therefore, little focus has been given to polymerization as a functional collagen property. Because collagen fibrils are indeed the fundamental unit of the ECM, elucidation of the mechanisms underlying collagen polymerization and how it contributes to the fibril organization and mechanical properties of collagen matrices are critical to the study of cell-ECM interactions. In addition, such information will contribute to the rational design of next generation collagen polymer formulations that can recapitulate and facilitate precision-tuning of the multiple, dynamic signaling modalities (biochemical and physical) inherent to 3D cellular microenvironments.

Unfortunately, due to collagen’s complexity in design and relatively unique protein biochemistry, the term collagen is often generally applied to preparations that differ significantly in molecular composition and ability to form supramolecular structures (aggregates versus fibrils). Collagen molecules are \(~300\) kDa in size and exist as coils of three intertwined helical polypeptides consisting generally of two alpha 1(I) [\(\alpha_1(1)\)] and a single \(\alpha_2(1)\) chains. Collagen molecules self-assemble in a hierarchical fashion to form microfibrils, fibrils, fibers, and fiber networks or bundles. Mechanical and chemical stability of the fibril network is further derived from cross-links between polypeptide chains within the same molecule (intramolecular) and between different molecules (intermolecular).\textsuperscript{2} Type I collagens routinely used for basic research and tissue-engineering applications are solubilized from different source tissues and animals (e.g., rat-tail tendon and calf skin) using a variety of methods involving neutral salt, dilute acid solutions, or enzymes (e.g., pepsin and pronase). It is important to note that the purity and integrity of the collagen polypeptides and molecules (e.g., presence of telopeptides, cross-links, and monomer/oligomer fractions), kinetics of polymerization, and structural–mechanical properties of polymerized fibrils differ based upon the isolation technique used.\textsuperscript{12–17} For example, partial loss of telopeptides from collagen molecules, as occurs with pepsin or pronase digestion,\textsuperscript{16} has major effects on fibril growth, including loss of diameter uniformity, loss of unidirectional packing, and changes in the fibril assembly pathway.\textsuperscript{17}

Over the last several years, we have attempted to use commercial collagens for correlating physical-design properties of 3D collagen matrices with cellular responses. The overarching goal of these efforts was to develop a soluble collagen formulation designed to polymerize in situ to form a 3D fibril-based microenvironment, serving to both entrap cells when forming tissue constructs in vitro or used as an injectable cell-delivery vehicle or biomaterial in vivo. Furthermore, this 3D microenvironment would be tunable in terms of relevant biochemical and biophysical cues such that the survival and fate of embedded cells could be predictably modulated. Unfortunately, use of such collagens in this capacity yields matrices that possess low reproducibility, slow polymerization rates, low-mechanical integrity, and a limited range of fibril microstructures and mechanical properties. To remedy these problems, we developed a new soluble collagen formulation derived from porcine skin collagen (PSC). Acid solubilization was used to maintain the integrity of telopeptide regions, which are known to be critical elements of the assembly process. In addition, isolation and characterization of this collagen focused not only on purity and molecular composition but also on polymerization potential (e.g., kinetics of matrix assembly and fibril microstructure and mechanical properties of formed matrices). Here, we present results from studies where PSC and three common commercial sources were compared in terms of molecular composition, polymerization kinetics, 3D fibril network organization (microstructure), and mechanical properties. Matrices were tested in shear, compression, and uniaxial extension to fully characterize mechanical properties and gain insight into important relationships between fibril microstructure and mechanical properties.\textsuperscript{18} Collagen concentration was covaried with source to correlate changes in matrix properties between these two variables. Finally, we document the ability of cultured mesenchymal stem cells (MSC) to differentially sense and respond to the range of physical properties provided by PSC matrices. Results from this work are important, because defining the parameters that control the polymerization
kinetics of collagen as well as the functional microstructure-mechanical relationships that result upon polymerization is an essential first step in the design and standardization of collagen formulations to be used for future in vitro cell culture, tissue engineering, and regenerative medicine applications.

**MATERIALS AND METHODS**

**Preparation and Analysis of Purified Type I Collagens**
PSC was acid solubilized from the dermis of market weight animals according to a modified protocol from Gallop and Seifter. Before use, lyophilized PSC was dissolved in 0.01 N hydrochloric acid (HCl) and rendered aseptic by exposure to chloroform overnight at 4°C. PSC was compared to three commercial grade type I collagen sources. PureCol, previously marketed as Vitrogen (Inamed, Fremont, CA), represents a pepsin digested type I collagen harvested from bovine dermis and was purchased as a sterile solution (BD-RTC and referred to as BD-rat tail collagen (BD-RTC)). Collagenous proteins and small molecular weight contaminants. Neutralized collagen preparations to be used for future in vitro cell culture, tissue engineering, and regenerative medicine applications.

Preparation of 3D Collagen Matrices
All collagen preparations were polymerized under identical reaction conditions to produce 3D matrices. Collagen solutions were diluted with 0.01 N HCl and neutralized with 10X phosphate-buffered saline (PBS, 1X PBS had 0.17M total ionic strength and pH 7.4) and 0.1N sodium hydroxide to achieve neutral pH (7.4) and final collagen concentrations ranging from 0.5 to 4 mg/ml. PureCol matrices were limited to ≤2.0 mg/ml due to the available stock concentration of ~3.0 mg/ml. Neutralized collagen solutions were kept on ice before the induction of polymerization by warming to 37°C.

Because of the increased viscosity of collagen solutions, positive displacement pipettes (Microman, Gilson, Middleton, WI) were used to accurately pipette all collagen solutions.

Analysis of Collagen Polymerization
A turbidimetric assay was used to analyze the polymerization (fibrillogenesis) kinetics of each collagen source. Neutralized collagen solutions prepared from each source at both 0.5 and 1 mg/ml collagen concentrations were transferred into prechilled (4°C) 96-well plates before polymerization. Plates were loaded into a preheated (37°C) Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA), and absorbance at 403 nm (A403) was measured at 10-s intervals for 2 h. Kinetic parameters calculated from the sigmoidal-shaped turbidity curves included lag time (x-intercept of line tangent to the inflection point of the sigmoidal turbidity curve), polymerization rate during growth phase (slope averaged around inflection point), maximum absorbance value, and polymerization half-time (time at which absorbance equals half the maximum absorbance value). Turbidity measurements were performed on three independently prepared matrices per matrix formulation (n = 3).

Analysis of Collagen Fibril Microstructure
Collagen matrices prepared from each collagen source at concentrations ranging from 0.5 to 3 mg/ml were polymerized (2 h in 37°C humidified incubator) in Lab-Tek IV chambered coverglass slides (Nunc, Thermo Fisher Scientific, Rochester, NY) and overlaid with PBS. Confocal reflection microscopy (CRM) was used to collect high-resolution 3D images of the matrices in their native, hydrated state. Confocal imaging was performed on an Olympus Fluoview FV1000 confocal system adapted to an Olympus IX81 inverted microscope with a 60X UPlanSApo water immersion objective (Olympus, Tokyo, Japan). Image stacks were collected at three to five random locations within each of two independent matrices (n = 6–10 images) per matrix formulation. The 3D fibril network was visualized using Imaris 5.0 software (Bitplane, St. Paul, MN). Two microstructural parameters, fibril volume fraction (fibril density) and fibril diameter, were quantified from images. For fibril volume fraction, images were read into Matlab (Mathworks, Natick, MA) and binarized by thresholding. Each threshold value was chosen mathematically as the center of the concave bend in the sigmoidal decay curve of fibril volume fraction versus threshold value for that image. Fibril volume fraction was calculated as the percentage of fibril-containing voxels to total image voxels. Fibril diameters were measured from image stacks in random order using a measurement tool in the Imaris software. Sixteen random fibrils (defined as single, nondividing fibrils) were measured from three different images of each treatment group (n = 48 fibrils). Each fibril diameter
represents the average of three diameters measured along the major axis of an individual fibril. Fibril measurements were validated by a blinded observer.

**Analysis of Matrix Mechanical Properties in Shear, Compression, and Uniaxial Extension**

Viscoelastic properties of polymerized collagen matrices were measured in both oscillatory shear and compression on a stress-controlled AR2000 rheometer (TA Instruments, New Castle, DE) using a stainless steel 40-mm diameter parallel plate geometry. Each sample was polymerized on the rheometer to standardize sample geometry and adherence to plates. The neutralized collagen solution (1 ml) was pipetted onto the bottom plate and the geometry lowered to the gap distance (725 μm). A peltier heater in the base plate was used to induce polymerization and maintain temperature at 37°C. Humidity was maintained using a solvent trap. Samples were allowed to polymerize for 30 min, which was sufficient time for polymerization due to conductive (rather than convective) heating. Following polymerization, a shear strain sweep from 0.01% to 5% strain at 1 Hz (chosen from predetermined linear viscoelastic response regions) was used to measure the shear modulus (reported values are at 1% strain). The controlling software calculated shear storage (G', elastic/solid component representing stored, recoverable energy) and loss (G″, viscous/fluid component representing energy permanently lost during deformation) moduli, which are related by phase shift (δ) as tan(δ/G′/G″).

Following the strain sweep, compressive behavior of each sample was evaluated in an unconfined format. Normal force was measured in response to compressive strain generated by depressing the geometry lowered to the gap distance (725 μm). A peltier heater in the base plate was used to induce polymerization and maintain temperature at 37°C. Humidity was maintained using a solvent trap. Samples were allowed to polymerize for 30 min, which was sufficient time for polymerization due to conductive (rather than convective) heating. Following polymerization, a shear strain sweep from 0.01% to 5% strain at 1 Hz (chosen from predetermined linear viscoelastic response regions) was used to measure the shear modulus (reported values are at 1% strain). The controlling software calculated shear storage (G', elastic/solid component representing stored, recoverable energy) and loss (G″, viscous/fluid component representing energy permanently lost during deformation) moduli, which are related by phase shift (δ) as tan(δ/G′/G″).

Preparation and Analysis of 3D Tissue Constructs

MSCs (D1) derived from mouse bone marrow were obtained from American Type Culture Collection (Manassas, VA). MSCs were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 1.5 g/l sodium bicarbonate, 10% fetal bovine serum (FBS, Sigma-Aldrich), 100 U/ml penicillin (Gibco, Carlsbad, CA), and 100 μg/ml streptomycin (Gibco). MSCs were grown and maintained in a humidified environment of 5% CO2 at 37°C. To ensure multipotency, MSCs were kept below 80% confluence and used in experiments at passage six to nine.

3D tissue constructs consisting of MSCs embedded within PSC matrices were prepared as described previously. Collagen fibril density and matrix shear storage modulus (G′, stiffness) were systematically varied by adjusting the collagen concentration. MSCs were harvested in complete medium and added at a relatively low cell density (5 × 10^4 cells/ml) as the last component before polymerization within a humidified environment at 37°C. Following polymerization, complete medium was added, and the tissue constructs were maintained in a humidified environment at 37°C and 5% CO2. The day the cells were seeded within the PSC matrices was denoted as Day 0. On Day 1, tissue constructs were treated with one of two different medium formulations: "regular" consisted of DMEM containing 4 mM l-glutamine, 1.5 g/l glucose, 1.5 g/l sodium bicarbonate, and 1.0 mM sodium pyruvate and supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. "Adipogenic" consisted of "regular" medium supplemented with 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 1 μg/ml insulin. MSCs were seeded at 1.25 × 10^4 cells/cm² and cultured in parallel fashion on tissue-culture plastic.

Tissue constructs were harvested at designated time points up to 14 days and fixed in 4% formaldehyde. To visualize lipid-filled vacuoles, a hallmark of adipogenesis, cells and tissue constructs were stained with Oil Red O (Sigma-Aldrich). Alizarin red S (Sigma-Aldrich) was used to stain for calcium, a marker of osteogenesis. Cells cultured on plastic or within collagen constructs were viewed using an inverted microscope (Nikon Eclipse TE2000-S) and photographed (QImaging Retiga 4000R; Vancouver, BC). For each experiment, the mean and standard deviation (SD) of adipocyte and calcified nodule counts were determined for three replicate wells. Each experiment was repeated three times.

Tissue constructs were cryopreserved, and total RNA was isolated using established methodologies with modifications. Total...
RNA was treated with Turbo DNA-free (Ambion, Austin, TX) and quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription was performed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Relative quantification of gene expression was performed using the ABI 7500 real-time polymerase chain reaction system (Applied Biosystems). TaqMan® Gene Expression Assay kits (Applied Biosystems) were used to identify transcript levels of tissue-specific genes. The endogenous control was 18S, and core binding factor alpha 1 (CBFA1 or runx2) was used to assess early osteogenesis and lipoprotein lipase (LPL) was an indicator of adipogenesis (Assays on Demand: 18S: Hs99999901_s1, CBFA1: Mm00501578_m1, and LPL: Mm00434764_m1; Applied Biosystems). Relative gene expression was calculated using the comparative Ct method relative to undifferentiated MSCs. Total messenger RNA was isolated from four to six replicate wells for each experiment. Each experiment was repeated at least three times.

Statistical Analysis
All measured values are reported as mean ± SD. Statistical analyses were performed using SAS v. 9.1 (SAS Institute, Cary, NC). To determine differences among treatment groups, the general linear model procedure was used to conduct unbalanced analysis of variance (ANOVA, in some cases, a Kruskal–Wallis ANOVA for non-parametric distributions) and perform multiple comparisons of least squares means using the Tukey–Kramer method. In cases of covaried collagen source and concentration, data were routinely grouped by source or concentration for single-factor ANOVA (e.g., compare means of all concentrations of a single source or all sources at a single concentration). Differences were considered statistically significant when \( P < 0.05 \).

RESULTS

PSC Has a Unique Molecular Composition Compared to Commercial Collagen Sources
SDS–PAGE analysis under denaturing conditions showed that PSC and commercial collagen sources contained highly purified type I collagen protein. Each collagen source displayed the expected banding patterns for type I collagen including individual \( \alpha \), beta (\( \beta \)), gamma (\( \gamma \)), and high-molecular weight components (Figure 1A). Interestingly, PSC contained prominent banding between the \( \beta \) and \( \gamma \) components with molecular weights in the range of 250–280 kDa (arrow Figure 1A). Western blot analysis confirmed that the band contained the type I collagen epitope (Figure 1B). Although \( \beta \) and \( \gamma \) bands were identifiable within PureCol, they showed noticeable differences in their mobility and/or proportions compared to those of acid-solubilized collagens. Such observations are expected, because PureCol is isolated using limited proteolytic digestion, thereby retaining some telopeptides and level of molecular cross-linking. Western blot analysis as well as interrupted gel electrophoresis confirmed that PSC and the other commercial collagens did not contain significant levels of collagen types III and V (data not shown). Furthermore, significant levels of GAGs or noncollagenous proteins were not identified within any of the sources.

On the basis upon the results from the compositional analyses, we speculated that PSC contained a unique composition of oligomers. Oligomers represent at least two collagen molecules (monomers) that are covalently cross-linked and, together with monomers, are inherent outcomes of collagen isolation from tissues. As such, follow-up studies were performed to determine the intrinsic viscosity as a means of estimating the average molecular weight of the polymeric solutions. As expected, PSC exhibited a significantly higher intrinsic viscosity (and therefore average molecular weight) at 2.85 ± 0.07 ml/mg compared to the commercial collagens (\( P < 0.05 \)). Intrinsic viscosity measurements for PureCol, Sigma, and BD-RTC were 2.50 ± 0.06, 2.38 ± 0.02, and 1.15 ± 0.02 ml/mg, respectively.

PSC Exhibits Rapid Polymerization
Upon neutralization, PSC transitioned within minutes from its solution form to an insoluble, fibril-based matrix. Turbidimetric analysis showed that all collagen sources displayed sigmoidal-shaped polymerization curves with characteristic
lag, growth, and plateau regions. Quantified kinetic parameters, including lag time, polymerization rate during growth phase, final absorbance, and polymerization half-time are listed in Table I. Importantly, each collagen source had distinct polymerization kinetics (see Figure 2), suggestive of differences in fibril formation and growth mechanisms, which ultimately affect fibril structure and fibril–fibril interactions. Polymerization half-times ranged widely from 8 to 52 min. Half-times were shortest for PSC and BD-RTC (statistically same, \( P = 0.35 \)) and longest for PureCol (\( P < 0.05 \)). Lag times and polymerization rates followed same trends. Increasing collagen concentration primarily affected final \( A_{405} \) values while slightly decreasing lag and half-times. Interestingly, BD-RTC and PureCol had significantly larger final \( A_{405} \) values than PSC and Sigma, indicating that absorbance values were also dependent on source and not directly relatable to collagen concentration.

### PSC Forms 3D Fibril Networks with Tunable Fibril Density

CRM analysis showed that matrices produced with PSC and other collagen sources generally appeared as highly porous 3D networks of collagen fibrils linked and entangled together (see Figure 3). The structural organization of fibril networks showed subtle variations with collagen source. Specifically, each source appeared to produce different fibril–fibril interactions, such as fibril coalescence or bundling events (Figure 3, arrows). PureCol matrices were most dissimilar displaying less fibril coalescence and appearing more homogeneous, possibly relating to being pepsin digested. Increasing collagen concentration resulted in similar network organizations with increased fibril density for each source. Fibril volume fraction (fibril density) was quantified from CRM images and found to increase linearly with collagen concentration (Figure 4A, Table II catalogs all measured microstructure and mechanical properties for comparison and reference). PSC, Sigma, and BD-RTC fibril volume fractions were not significantly different at any concentration (\( P \geq 0.13 \) at each concentration). PureCol was only significantly different from other sources at 2 mg/ml (\( P < 0.05 \)). Thus, in general, fibril density was not dependent on collagen source. Sigma, BD-RTC, and PSC fibril volume fractions increased at a level of 3.09% ± 0.44%/ (mg/ml) with collagen concentration (mean ± SD of three sources), whereas PureCol’s level of increase, 4.69%/ (mg/ml), was different.

Measurement of fibril diameters from CRM images revealed that collagen source had a small, but significant effect on average fibril diameter (Figure 4B). As an important distinction, fibril diameters here represent single fibrils that did not appear to further divide, meaning larger coalesced/bundled fibrils (Figure 3, arrows) were not measured. At a given collagen concentration, average fibril diameters were significantly different between collagen sources (\( P < 0.05 \)). Specifically, Sigma produced the smallest fibrils, PSC and BD-RTC intermediate-sized fibrils, and PureCol the largest fibrils. Note that SDs (range, 30–50 nm) are reported to

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**Table I  Collagen Polymerization Kinetic Parameters for PSC and Commercial Collagen Sources**

<table>
<thead>
<tr>
<th>Collagen Source</th>
<th>Concentration (mg/ml)</th>
<th>Lag Time (min)</th>
<th>Polymerization Rate During Growth Phase ((10^{-3}/\text{min}))</th>
<th>Final Absorbance Value</th>
<th>Polymerization Half-Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PureCol</td>
<td>0.5</td>
<td>38.9 ± 4.3</td>
<td>21.5 ± 6.2</td>
<td>0.303 ± 0.040</td>
<td>40.9 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>50.7 ± 2.4</td>
<td>36.8 ± 0.7</td>
<td>0.504 ± 0.015</td>
<td>52.3 ± 2.1</td>
</tr>
<tr>
<td>Sigma</td>
<td>0.5</td>
<td>36.0 ± 5.6</td>
<td>9.9 ± 1.9</td>
<td>0.171 ± 0.011</td>
<td>38.3 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>33.6 ± 1.0</td>
<td>20.5 ± 1.1</td>
<td>0.314 ± 0.010</td>
<td>35.5 ± 0.8</td>
</tr>
<tr>
<td>BD-RTC</td>
<td>0.5</td>
<td>10.1 ± 2.1</td>
<td>56.4 ± 1.1</td>
<td>0.289 ± 0.016</td>
<td>12.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7.4 ± 1.2</td>
<td>130.9 ± 15.0</td>
<td>0.561 ± 0.042</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td>PSC</td>
<td>0.5</td>
<td>7.9 ± 1.1</td>
<td>56.8 ± 1.9</td>
<td>0.182 ± 0.008</td>
<td>9.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.1 ± 1.1</td>
<td>102.9 ± 18.8</td>
<td>0.322 ± 0.021</td>
<td>7.7 ± 0.9</td>
</tr>
</tbody>
</table>

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**Figure 2** Polymerization kinetics of PSC and commercial collagen sources measured spectrophotometrically at 405 nm. Sources were polymerized at concentrations of 0.5 and 1.0 mg/ml (lower and upper curves for each collagen source, respectively). Curves represent mean ± SD \((n = 3)\) with SD plotted at 15-min intervals for clarity. Kinetic parameters calculated from curves are summarized in Table I.
describe fibril populations, but due to large \( n \)-value standard error of the means were 5–8 nm. Interestingly, the larger average diameter of PureCol fibrils was consistent with the qualitative appearance of thicker, more homogenous fibrils. In general, average fibril diameters for each collagen source did not significantly change with collagen concentration.

**PSC Matrices Show High-Mechanical Integrity with Tunability Across a Broad Range of Mechanical Properties**

Because collagen matrices represent composite viscoelastic materials, the mechanical properties of PSC were determined using multiple loading formats, including oscillatory shear, compression, and uniaxial extension and compared to that of commercial collagens. This comprehensive mechanical properties evaluation provided critical insight into the disparate fibril microstructure–mechanical behavior of each collagen source.

**Shear.** Oscillatory shear testing showed that PSC matrices had distinct viscoelastic responses compared to commercial collagen matrices through both their elastic and viscous-phase components. At each collagen concentration, PSC showed the highest \( G' \) \((P < 0.05)\) followed by Sigma \((P < 0.05)\) and then BD-RTC and PureCol. PureCol and BD-RTC were not significantly different at any concentration \((P = 0.97)\). Matrix \( G' \) (elastic modulus) significantly increased with increasing collagen concentration, but to dramatically different magnitudes depending on the collagen source (Figure 5A, Table II). Overall, PSC matrices covered a much broader \( G' \) range (25–1450 Pa) than achievable with other sources (1–350 Pa) at the concentrations tested. \( G' \) and collagen concentration \(([C])\) data were best fit with quadratic rather than linear equations. Equation coefficients varied largely between sources; for PSC \( G' = 2230[C]^2 + 2020[C] - 136, R^2 = 0.993\), for Sigma \( G' = 4.88[C]^2 + 75.2[C] - 35.54, R^2 = 0.984\), and for PureCol and BD-RTC combined \( G' = 8.22[C]^2 + 1.57[C] - 0.772, R^2 = 0.981\).

In comparison with \( G' \), matrix shear loss modulus \((G''; viscous modulus)\) was small and, in general, followed the same statistical trends. Small \( G'' \) translated to small \( \delta \) and indicated, as expected, that matrix shear response was dominated by the collagen fibril network (solid/elastic phase). At each concentration tested, PSC matrices showed the lowest \( \delta \) with values around 4°. Matrices prepared with Sigma, BDRTC, and PureCol possessed progressively larger \( \delta \) values that were significantly different \((P < 0.05)\) at each concentration with the exception of 0.5 mg/ml where BD-RTC and Sigma were similar \((P = 0.661; Figure 5B)\). Interestingly, \( \delta \) was independent of collagen concentration.
Unconfined Compression. In contrast to shear, compressive loads are supported by the interstitial fluid phase and depend on hydraulic permeability. In general, unconfined compression stress–strain curves were biphasic in nature with distinguishable behavior noted within low-strain and high-strain regions (Figure 6A). $E_C$ was measured in the linear, low-strain region of the curves (as indicated in Figure 6A) to compare differences in matrix compressive resistance. At all concentrations, PSC $E_C$ was significantly higher ($P < 0.05$) than those obtained for other sources (Figure 6B, Table II). In addition, PSC matrices covered the broadest range of $E_C$ values, 5.3 kPa–42.8 kPa. Significant differences between other sources did not emerge until $\geq 2$ mg/ml concentrations ($P < 0.05$). $E_C$ and collagen concentration were linearly related with slopes of 5.69, 1.20, and 10.5 kPa/(mg/ml) for PureCol, Sigma, and PSC, respectively. On the other hand, BD-RTC $E_C$ was not dependent upon concentration ($P = 0.056$).

Uniaxial Extension. Uniaxial extension testing of collagen matrices causes a dramatic reorganization and alignment of fibril architecture.\cite{18,33} Therefore, this loading format allowed determination and comparison of PSC and other commercial collagen matrices to develop tension within the aligned microstructure. To measure $E_T$ and $\sigma_U$, stress was calculated as “engineering” or “true” to account for assumptions of constant cross-sectional area or actual cross-sectional area, respectively.\cite{27,29} “Engineering” assumptions are widely used for traditional materials (low-Poisson’s ratio) and/or in situations where real-time cross-sectional area measurements are difficult. On the other hand, “true” stress calculations account for the significant cross-sectional area changes (high-Poisson’s ratio) observed with biological-based materials and tissues.

Despite the stress definition used, PSC matrices showed the highest $E_T$ and $\sigma_U$ for each collagen concentration tested (Figures 7A–7D, Table II). In fact, both “engineering” $E_T$ and $\sigma_U$ were found to be dependent upon source at each concentration with the following statistically significant relationships:

\begin{align*}
\text{PSC} &> \Sigma > \text{BD-RTC} = \text{PureCol} (P < 0.05 \text{ except for BD-RTC and PureCol where } P \geq 0.5). \\
\end{align*}

In general, “true” $E_T$ and $\sigma_U$ values were significantly higher and showed greater variation than their “engineering” counterparts largely owing to the significant specimen compaction with loading and the associated error in cross-sectional area measurements. However, these parameters also were found to be dependent upon source with statistical trends similar to those specified for “engineering” values. Importantly, the dependence of $E_T$ and $\sigma_U$ on collagen concentration varied largely between sources. More specifically, for PSC, “engineering” $E_T$ increased with concentration ($C$) in logarithmic fashion ($E_T = 117 \times \ln(C) + 107$, $R^2 = 0.958$), whereas “engineering” $\sigma_U$ showed more linear dependence (Figures 7A and 7B). “Engineering” $E_T$ and $\sigma_U$ for PureCol and Sigma also showed moderate increases with concentration, but linear fits were poor ($R^2 = 0.5$). On the other hand, “engineering” $E_T$ and $\sigma_U$ for BD-RTC matrices actually decreased with increasing concentration, a contradiction to common theory.\cite{18} Similar relationships between “true” $E_T$ and concentration were observed for each source (Figures 7C–7D); how-

**FIGURE 4** Fibril volume fraction (fibril density, A) and average fibril diameter (B) measured from CRM images of matrices prepared with different collagen sources and concentrations. Fibril volume fraction increased linearly with collagen concentration and was not significantly dependent on source [with the exception of PureCol at 2 mg/ml ($P < 0.05$), $n = 6$–10 images per matrix formulation]. Average fibril diameter showed a small, but significant dependence on collagen source ($P < 0.05$ at each concentration, $n = 48$ fibrils per matrix formulation). In general, fibril diameters did not change with collagen concentration.
Table II  Summary of Mechanical and Microstructural Properties of 3D Collagen Matrices Prepared With PSC and Commercial Collagen Sources

<table>
<thead>
<tr>
<th>Collagen Source</th>
<th>Concentration (mg/ml)</th>
<th>Microstructure</th>
<th>Shear</th>
<th>Uni-Axial Tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fibril Volume Fraction (%)</td>
<td>Avg. Fibril Diameter (μm)</td>
<td>G′ (Pa)</td>
</tr>
<tr>
<td>PureCol</td>
<td>0.5</td>
<td>9.21 ± 0.782</td>
<td>436 ± 52.8</td>
<td>2.33 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11.4 ± 0.589</td>
<td>420 ± 49.4</td>
<td>9.77 ± 4.95</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>13.4 ± 2.14</td>
<td>410 ± 61.8</td>
<td>20.9 ± 8.78</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.3 ± 1.67</td>
<td>435 ± 55.5</td>
<td>37.2 ± 17.5</td>
</tr>
<tr>
<td>Sigma</td>
<td>0.5</td>
<td>7.71 ± 0.609</td>
<td>339 ± 35.5</td>
<td>8.96 ± 2.07</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11.0 ± 1.05</td>
<td>340 ± 33.8</td>
<td>35.9 ± 2.29</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.1 ± 0.764</td>
<td>320 ± 29.1</td>
<td>142 ± 11.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.2 ± 0.902</td>
<td>313 ± 29.0</td>
<td>221 ± 12.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>— —</td>
<td>743 ± 27.9</td>
<td>398 ± 1.40</td>
</tr>
<tr>
<td>BD-RTC</td>
<td>0.5</td>
<td>8.65 ± 0.907</td>
<td>390 ± 38.8</td>
<td>1.71 ± 0.226</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.99 ± 1.69</td>
<td>381 ± 50.5</td>
<td>7.33 ± 0.106</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.5 ± 1.86</td>
<td>411 ± 62.8</td>
<td>34.8 ± 1.66</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.1 ± 1.92</td>
<td>401 ± 57.4</td>
<td>75.7 ± 6.01</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>— —</td>
<td>138 ± 0.611</td>
<td>20.1 ± 0.206</td>
</tr>
<tr>
<td>PSC</td>
<td>0.5</td>
<td>8.06 ± 0.980</td>
<td>391 ± 50.6</td>
<td>27.3 ± 3.51</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.18 ± 1.30</td>
<td>367 ± 49.6</td>
<td>126 ± 8.74</td>
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<tr>
<td></td>
<td>2</td>
<td>13.3 ± 2.55</td>
<td>381 ± 41.6</td>
<td>491 ± 12.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.9 ± 2.98</td>
<td>379 ± 54.8</td>
<td>1010 ± 69.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>— —</td>
<td>1440 ± 54.3</td>
<td>881 ± 4.21</td>
</tr>
</tbody>
</table>

* Failure occurred in attachment block region for >50% of matrices; 4 mg/ml matrix CRM data were not collected.
ever, statistically significant differences were less largely owing to large variance in “true” $\varepsilon_T$ values. In general, the greatest increase in “true” $\varepsilon_T$ and $\sigma_U$ as a function of concentration again was observed for PSC. In fact, “true” $\varepsilon_T$ and $\sigma_U$ values for PureCol and Sigma did not change significantly with concentration while those for BD-RTC decreased with increasing concentration. It should be noted that PSC matrices prepared at concentrations greater than 2 mg/ml maxed out the load cell, and Sigma matrices prepared at concentrations less than 2 mg/ml failed outside the gauge section; therefore, these groups were excluded from $\sigma_U$ analyses.

Interestingly, matrix $\varepsilon_F$ showed no consistent dependence on collagen source or concentration (Figure 7E). PSC and BD-RTC $\varepsilon_F$ were not significantly different at any concentration ($P > 0.9$) despite their dramatically different $\varepsilon_T$ and $\sigma_U$. These results implied that matrices produced by the four sources differed in their ability to store energy, which was corroborated by visual observations of matrix failure (see Figure 8). At failure, stiffer PSC and Sigma matrices appeared to stretch like a band, then abruptly tear, and snap with elastic recoil (Figures 8A–8C). In contrast, BD-RTC and PureCol

![Figure 5](image1.png)

**Figure 5** Shear storage modulus ($G'$, A) and phase shift ($\delta$, B) of matrices prepared with PSC or commercial collagen sources at varied concentrations (values reported at 1% strain, 1 Hz frequency, $n = 3$). PSC had the largest $G'$ and lowest $\delta$ at each concentration ($P < 0.05$). $G'$ increased with increasing collagen concentration, but with significantly different relationships for each source ($P < 0.05$ at each concentration with the exception of PureCol and BD-RTC ($P > 0.9$ for all comparisons)). $\delta$ was significantly different for each source ($P < 0.05$) and did not change with collagen concentration.

![Figure 6](image2.png)

**Figure 6** Unconfined compression stress–strain curves (A) and modulus ($E_C$, B) for matrices prepared with PSC or commercial collagen sources at varied concentrations. Representative stress–strain curves for each source prepared at 2 mg/ml are shown to illustrate source-dependent compressive behavior [curves are mean ± SD ($n = 3$) with SD plotted at 10% strain intervals for clarity]. $E_C$, measured in a linear region of curves from 15 to ~60% strain as indicated in (A), increased with collagen concentration in a source dependent fashion. PSC exhibited the largest $E_C$ at each concentration ($P < 0.05$).
matrices appeared to plastically dissociate, developing fibrous connections that eventually fell apart (Figures 8D–8F).

PSC Matrix Physical Properties Are Sufficient to Guide Lineage-Specific Differentiation of MSCs In Vitro

To demonstrate biocompatibility of PSC matrices as well as the biological relevance of their broad range of physical properties, MSCs were entrapped within PSC matrices in which fibril density and matrix stiffness \((G)\) were varied by modulating the collagen concentration of the polymerization reaction. 3D tissue constructs were classified based upon their measured matrix \(G\) values as either 45 Pa (45 ± 8 Pa) or 695 Pa (695 ± 53 Pa). Because of the high-differentiation potential of MSCs, the effects of modulating fibril density and matrix stiffness were immediately apparent on the in vitro cell response. MSCs cultured in regular medium and entrapped within 45 Pa-matrices showed numerous adipocytes with no identifiable calcified nodules, whereas those within 695 Pa-matrices showed calcified nodules with little to no identifiable adipocytes as observed qualitatively (see Figure 9).
Expression levels of LPL and CBFA1, molecular indicators of adipogenesis, and osteogenesis, respectively, corroborated these findings (see Figure 10). More specifically, MSCs cultured in regular medium and 45 Pa-matrices showed greatest LPL expression at both timepoints (Figure 10A). In contrast, matrix-dependent differences in CBFA1 expression were identifiable at 14 days only, with greatest levels observed for 695-Pa matrices (Figure 10B). As expected, MSCs cultured in regular medium on the surface of tissue-culture plastic showed no evidence of lineage-specific differentiation [Figures 9C (upper panel), 10A, and 10B].

When 3D tissue constructs and cells on plastic were cultured in the presence of adipogenic medium, increases in adipogenic differentiation were noted in all cases but in a culture-format-dependent fashion. As shown in Figure 9 (lower panel), MSCs cultured within 45 Pa-matrices, and adipogenic medium showed a ninefold increase in adipocyte number compared to those cultured in regular medium. Interestingly, MSCs cultured within 695 Pa-constructs in the presence of adipogenic medium showed not only a 32-fold increase in the number of adipocytes but also an eightfold increase in the number of calcified nodules [Figure 9 (lower panel)]. Interestingly, similar LPL expression profiles were observed for the two matrices when cultured in the presence of adipogenic medium, with greatest levels observed for 45-Pa cultures at 14 days (Figure 10C). On the other hand, matrix-dependent differences in CBFA1 expression persisted even in the presence of soluble adipogenic factors, with 695-Pa matrices showing the highest levels at both time points (Figure 10D). MSCs cultured on plastic in the presence of adipogenic medium showed high levels of adipogenesis and no osteogenesis as evidenced by the numerous adipocytes [Figure 9D (lower panel)] and expression of LPL (10). Collectively, these results indicate that (1) MSCs are able to sense and respond to the broad range of physical properties provided by PSC matrices, and (2) modulation of fibril density and matrix stiffness is sufficient to guide lineage-specific differentiation of MSCs. Furthermore, it is evident that soluble-factor signaling is not sufficient to overcome collagen matrix-induced MSC differentiation, but rather soluble factors work synergistically with physical signals to drive stem-cell phenotype and function.

**PSC Preparations Show Low Intra- and Interhide Variability**

Lots prepared from the hide obtained from the same (intra-hide) or different (interhide) market weight pigs have shown high reproducibility in terms of molecular composition, polymerization potential (e.g., kinetics and fibril microstructure–mechanical properties), and therefore biological response. The low intra- and interlot variability of PSC is best documented by comparing the functional polymerization properties as measured by matrix viscoelastic properties (e.g., $G'$) as a function of the collagen content of the polymerization reaction (see Figure 11). For cell culture and other applications, collagen matrix formulations are routinely prepared and classified based upon matrix $G'$ as pre-
dicted from the quantified relationship between $G'$ and collagen content.

**DISCUSSION**

As the natural, fibrous polymeric substrate for cells in vivo, collagen will play an important role as the base component of physiologically relevant, fibril-based microenvironments that predictably direct cell fate both in vitro and in vivo. Indeed, the architecture of the collagen fibrils as well as the fibril material properties serves as critical determinants of the physicochemical properties of the matrix including interstitial fluid flow,

\(^5\) tensile mechanical properties,

\(^72\) and hierarchical 3D strain transfer properties.

\(^35,34\) In this way, an applied deformation whether externally applied or cell-induced may deviate substantially across the different size scales (construct or tissue level down to cellular level).

\(^35\) We and others have shown cells embedded within 3D collagen matrices that are able to sense and respond to the modulation of these physical parameters, resulting in instructive changes to cell morphology, cytoskeletal organization, contractility, differentiation, and proliferation.

\(^30,36,37\) As such, next generation collagen formulations should facilitate multifactorial investigations of cell–collagen interactions in which both biochemical and physical features can be user-control and defined. Indeed, it has long been known that purified collagen polymerizes in vitro to form hierarchical fibrillar structures that exhibit strong similarities to those found in vivo.

\(^38\) In addition, it is well documented that the nature of the fibrils (e.g., diameter, length, and density) formed is highly sensitive to polymerization reaction conditions, including temperature, phosphate ion concentration, ionic strength, and structural features of collagen molecules (e.g., presence of telopeptides).

\(^39–42\) Despite this extensive body of literature, collagen formulations produced commercially or within individual laboratories are not routinely standardized in terms of their functional polymerization properties. Furthermore, implantable biomaterials have not included the polymeric and fibril self-assembly properties of collagen as part of their design features and therapeutic strategy. Herein, we describe a type I collagen formulation that is acid-solubilized from pig skin and specifically developed for engineering collagen fibril-based matrices. Characterization and comparison of PSC were expanded beyond traditional purity and polypeptide analyses to include relevant performance properties focused on polymerization kinetics as well as the fibril microstructure and mechanical properties of formed matrices, a perspective not established previously.

Despite the fact that type I collagen is highly conserved amongst animal species,

\(^43\) results obtained both in vitro and in vivo using animal derived collagens depend critically on the animal source (e.g., species and age), tissue source (e.g., skin and tendon), and isolation procedure used.

\(^44\) In fact, the production and deposition of collagen in vivo involve a complex series of intracellular and extracellular events. Immediately following synthesis, procollagen-$\alpha$ chains undergo post-translational modification including hydroxylation and glycosylation. Propeptide regions on both ends of the procollagen-$\alpha$ chains then guide the formation of a procollagen tri-
The procollagen molecule is then secreted into the extracellular space, where cleavage of the propeptides ends produces a molecule with a central triple-helical domain flanked by non-helical telopeptide domains. Removal of propeptide ends enables collagen molecules (monomers) to undergo spontaneous self-assembly in a staggered conformation to form hierarchical fibril structures. Cross-linking reactions, which are regulated primarily by the hydroxylation pattern and aldehyde formation within telopeptide and triple-helix domains, contribute to the formation of covalent bonds between $\alpha$-chains within the same and different molecules. These intra and intermolecular cross-links are thought to contribute to the variations in structural and mechanical properties amongst different connective tissue types. Based upon collagen’s hierarchical design, intramolecular cross-links are thought to contribute primarily to the stiffness of individual collagen molecules, whereas intermolecular cross-links between laterally associated molecules contribute more significantly to the stored energy and strength of assembled collagen fibrils. Although the classes of cross-links may differ between connective tissue sources, published reports indicate...
cate that the allysine pathway predominates in skin and rat-tail tendon; however, the extent is known to vary with age and specific disease states. Collagen isolation from tissues is routinely carried out under acidic conditions in the presence or absence of proteolytic enzymes such as pepsin. Acid solubilization was selected as the isolation method for PSC so as to maintain the integrity of the telopeptide regions of the collagen molecule, which are known to be critical elements of polymerization and important sites for cross-linking. Similarly, rat-tail collagen and calf-skin collagens from BD and Sigma, respectively, represent acid-solubilized preparations. However, according to manufacturer’s specifications, different isolation protocols and acids are used to generate these two collagen formulations. In contrast, PureCol is derived from calf skin using limited pepsin proteolysis. Pepsin cleaves telopeptide regions of the collagen molecules, rendering it more soluble and improving yield. Despite the solubilization method used, cross-linked collagen molecules persist giving rise to a soluble mixture comprising monomers (single collagen molecules) and oligomers (two or more covalently cross-linked collagen molecules). Pepsin treatment is known to dramatically reduce the oligomer content largely owing to its cleavage of the telopeptide regions (primary sites for intramolecular and intermolecular cross-linking). Interestingly, the commercial collagen sources used herein specify that they are primarily monomeric in composition; however, no specific postprocessing details to minimize or eliminate the oligomer component are provided.

In the case of PSC, no specific processing was used to minimize the contribution of oligomers. Following SDS-PAGE, a procedure most commonly used to assess collagen source purity and polypeptide chain composition, expected similarities between PSC collagen and commercial sources were observed in terms of α, β, and γ banding, with β and γ resulting from inherent intramolecular cross-linking. Little to no contaminating GAGs and noncollagenous proteins were identified within PSC. Furthermore, significant levels of type III or type V collagens, which are also present in skin, were not apparent based upon interrupted gel electrophoresis analyses. The most striking difference was that PSC contained a prominent protein band between 250 and 280 kDa. The band stained positively for the collagen type I epitope, suggesting that PSC contained a unique composition of cross-linked α chains. These findings and the fact that PSC had the greatest intrinsic viscosity and therefore average molecular weight, suggested that PSC possessed a unique and likely greater oligomer content compared to commercial sources. Analyses of polymerization kinetics and microstructure–mechanical properties of formed matrices further support this notion.

In addition to routine compositional analysis, detailed studies focused on the polymerization kinetics as well as the fibril microstructure and mechanical properties of polymerized matrices were performed. Collectively, results from these analyses clearly showed that commercial collagen sources were dissimilar with respect to polymerization potential. While all sources were polymerized under identical reaction conditions, including collagen concentration, each source demonstrated a unique turbidity profile and kinetic parameters. These results imply that the molecular composition of these sources exhibits different underlying assembly mechanisms as they transition from a soluble to an insoluble fibrillar state. PSC showed rapid polymerization, which is a critical design requirement that facilitates rapid and homogenous cell entrapment, and clinically useful injectable formulations. The short polymerization half-time coupled with the low-net change in turbidity (A405) further supported the notion that PSC maintained its telopeptides and possessed a significant oligomer content. On the other hand, PureCol, a collagen formulation where telopeptides have been partially eliminated by limited pepsin digestion, exhibited long polymerization half-times (41–53 min). Such findings are consistent with previous reports, documenting the importance of telopeptide regions in the initiation phase or nucleation steps of fibril formation. It should be noted that, if present, small amounts of mucusaccharides or other contaminants can affect the polymerization kinetics and may also have contributed to observed differences between the sources. Interestingly, application of the acid-solubilization methodology used to produce PSC to other connective tissues (e.g., bone and tendon) does not yield collagens with the same polymerization properties (data not shown). This is not surprising, because the number and type of cross-links differ amongst tissues, thereby endowing them with specific fibril architectures and mechanical properties. As such, it is expected that collagen extraction from different tissue sources would yield different collagen polymer compositions (e.g., types and amounts of oligomers). More in-depth characterization of the collagen polymer compositions derived from pig skin and other tissue sources is currently underway.

Although turbidity analysis provides insights into kinetics of the sol–gel transition, no specific information regarding molecular assembly and fibril microstructure can be derived from this data. Therefore, CRM was used to assess 3D fibril microstructure of polymerized matrices in their native, hydrated state. Fibril density, as measured, did not change with source (except maybe slightly in the case of PureCol) and was linearly related to collagen concentration (for 0.5–3 mg/ml range). This linear relationship has been observed before, but not shown to correlate between varied sources.
Here, we also found that the average diameter of individual fibrils did not vary significantly with source or concentration. Although PSC and BD-RTC showed evidence of enhanced fibril–fibril coalescence or bundling, these events were not quantified. The range of fibril diameters measured (270–500 nm) and their concentration independence are consistent with the previous reports (290–800 nm). It should be noted that CRM diameters may overestimate diameters due to diffraction artifacts at fibril edges and have resolution limitations comparable to fibril sizes (lateral 150–300 nm, axial 500 nm). The limited resolution of CRM also does not account for relevant structural differences that may exist on smaller size scales (e.g., microfibrils). Our results suggest that additional differences in the hierarchical fibril morphology and organization may be detectable using more sensitive and alternative imaging techniques (e.g., SEM and AFM); however, many of these require extensive specimen processing, which can induce artifacts.

Functional assessment of the fibril networks was obtained through comprehensive mechanical analysis of polymerized matrices, which is not traditionally included as part of collagen characterization or comparison studies. Subjecting polymerized matrices to different loading formats, oscillatory shear, unconfined compression, and uniaxial extension allowed differential assessment of solid (fibril) and fluid phases and their associated interactions. From these results, differences in the polymerization potential of PSC and the other collagen sources were most evident. Overall, PSC showed the greatest mechanical integrity, which overrides a major limitation to the handling and application of commercial collagens to date. PSC matrices prepared over the range of concentrations tested (0.5–4 mg/ml) covered the broadest range with stiffness values ranging from 27–1440 Pa in shear ($G'$) and 33–277 kPa in extension ("engineering" $E_T$). These values were significantly greater than those for other sources as measured here (2–343 Pa $G'$ and 1–57 kPa “engineering” $E_T$) or reported elsewhere for similar collagen concentrations (0.1–150 Pa $G'$ and 1–60 kPa $E_T$). These published ranges include values obtained previously for PureCol, Sigma, and BD-RTC. It is interesting to note that both $G'$ and $E_T$ are primarily governed by the solid fibril phase of the matrix. In contrast, $\delta$, which is used as a measure of the fluidity of a material, was lowest for PSC matrices. Unconfined compression testing, as measured here, provided insight into the hydraulic permeability of the matrix. For purposes of comparison, $E_C$ was calculated within the low-strain region of the biphasic stress–strain curve. For all sources, $E_C$ increased with collagen concentration, and these relationships were described by different linear relationships. The fact that $E_C$ increased with increased collagen concentration is not surprising, considering the concomitant increase in fibril density. However, PSC yielded the broadest range of $E_C$ values, a linear relationship between $E_C$ and concentration with the greatest slope, and fibril densities similar to the other collagen sources. These data imply that the stiffness of the fibrils themselves and/or interfibril connectivity is responsible for these significant differences.

Collectively, the source-dependent differences in $E_T$ as well as $G'$, $E_C$, $\sigma_U$, and $\varphi_p$ share a common theme that PSC matrices, compared to matrices produced with commercial sources, show an enhanced ability to store elastic energy. This was obvious from the mode of failure observed during uniaxial extension. At failure, PSC matrices snapped and recoiled, whereas BD-RTC/PureCol matrices merely dissociated/fell apart with no recoil. Although fibril diameter and length have been related to changes in tensile mechanical properties, fibril rearrangements (e.g., sliding and realigning) are thought to be primarily responsible for shear and tensile responses. Fibril branching (interfibril cross-links) and strong fibril–fibril interactions, which resist fibril slipping, are responsible for large elastic energy storage in tendons and collagen fibers. In fact, the abrupt failure in extension relates to elastic energy storage in intermolecular cross-links not achievable in entanglements. Here, altered packing and alignment of collagen molecules that occurs in the presence of covalently cross-linked molecules (oligomers) during self-assembly may result in an overall stiffening of an individual collagen fibril or more likely distinct hierarchical fibril architectures (e.g., increased fibril–fibril branching or fibril–fibril bundling). This hypothesis is also supported by the fact that commercial collagens had a much lower Poisson’s ratio than PSC (responsible for the difference between “engineering” and “true” stress and stiffness values), indicating that fibril–fibril interactions acted more like hinge points in PSC (leading to increased off-axis strain) rather than entanglements. Although both fibril stiffening and connectivity would strengthen the matrix, our collective experience with different collagen sources and engineered collagen matrices strongly support the latter and will be the subject of future investigations.

We and others have found that commercial collagens are insufficient for precision-tuning “instructional” cell microenvironments because of the significant lot-to-lot variability in polymerization capacity as well as their ability to produce only a limited range of physical properties. Here, proof-of-concept experiments in which MSCs were embedded within PSC matrices prepared with varied physical properties documented that PSC not only was biocompatible but also provided a range of physical properties to which cells could sense and respond in a predictable and reproducible manner. In
fact, it was apparent that modulation PSC matrix physical properties were sufficient to direct lineage-specific differentiation of MSCs down adipogenic and osteogenic pathways. Although specific soluble factor cocktails were not sufficient to overcome matrix-induced MSC differentiation, it was apparent that both soluble factor and physical-based signaling worked synergistically to guide lineage specific differentiation of MSCs. Such observations are consistent with the Engler and coworkers’ work who showed that modulation of the elasticity (stiffness) of collagen-coated polyacrylamide gels was sufficient to direct differentiation of MSCs cultured on their surface. It is important to note that the in vitro response of MSCs as well as other cell types to PSC matrices shows low intra- and interhide variability when lots are quality controlled on polymerization potential and matrix physical properties (e.g., $G'$).

In conclusion, this work demonstrates that PSC has many critical design features that make it amenable as a collagen polymer platform for customizing and engineering 3D culture models as well as injectable or implantable cellular microenvironments that are precision-tuned in terms of molecular composition and relevant physical properties so as to yield desirable and predictable cell fates. It is clear that polymerization properties amongst collagen sources are vastly different, thereby yielding 3D matrices that are dramatically different in physical features, namely their fibril microstructure–mechanical properties relationships. This is an important problem, because it is now increasingly recognized that physical features of matrices, specifically fibril density and stiffness, can serve as potent signals that direct the morphology, mechanics, and fate of embedded cells. Therefore, it is not surprising that conventional collagen formulations, which are often poorly characterized, show significant lot-to-lot and product-to-product variability, thereby precluding meaningful comparisons of results between studies and laboratories. Furthermore, this study establishes that collagen concentration is a misleading descriptor for predicting and comparing performance of collagen preparations for these applications. We found that quality controlling PSC based upon polymerization potential, as determined by $G'$ and fibril density, has provided a very effective means for obtaining robust collagen polymer formulation with low intrahide and interhide variability in terms of physical properties, therefore contributing to high predictability and reproducibility in terms of cell response. Furthermore, this work suggests that the oligomeric content may be a critical design feature that effectively broadens the range of fibril microstructures and mechanical properties that are achievable with polymerization. Experiments to demonstrate a direct mechanistic linkage between oligomers and functional collagen properties are currently underway.

At present, cell biology is transitioning from conventional 2D to emerging 3D in vitro cell and tissue-culture formats, which will drive significant advances in basic and translational research related to cell biology (e.g., stem cell and tumor biology), tissue engineering, and drug development. It is evident that new perspectives into collagen chemistry and collagen polymer engineering will allow this natural polymer to reach its potential as an effective 3D matrix that will advance basic research and next generation tissue engineering and regenerative medicine strategies.

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