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Collagen Oligomers Modulate Physical and Biological Properties of Three-Dimensional Self-Assembled Matrices

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Abstract

Elucidation of mechanisms underlying collagen fibril assembly and matrix-induced guidance of cell fate will contribute to the design and expanded use of this biopolymer for research and clinical applications. Here, we define how Type I collagen oligomers affect in-vitro polymerization kinetics as well as fibril microstructure and mechanical properties of formed matrices. Monomers and oligomers were fractionated from acid-solubilized pig skin collagen and used to generate formulations varying in monomer/oligomer content or average polymer molecular weight (AMW). Polymerization half-times decreased with increasing collagen AMW and closely paralleled lag times, indicating that oligomers effectively served as nucleation sites. Furthermore, increasing AMW yielded matrices with increased interfibril branching and had no correlative effect on fibril density or diameter. These microstructure changes increased the stiffness of matrices as evidenced by increases in both shear storage and compressive moduli. Finally, the biological relevance of modulating collagen AMW was evidenced by the ability of cultured endothelial colony forming cells to sense associated changes in matrix physical properties and alter vacuole and capillary-like network formation. This work documents the importance of oligomers as another physiologically-relevant design parameter for development and standardization of polymerizable collagen formulations to be used for cell culture, regenerative medicine, and engineered tissue applications.

Keywords

collagen; oligomer; intermolecular crosslink; extracellular matrix (ECM); physical properties; endothelial colony forming cells (ECFC)

INTRODUCTION

Type I collagen is a natural polymer and the predominant molecular component that defines the microstructure-mechanical properties of connective tissue extracellular matrices (ECMs). Collagen molecules, also known as monomers, exist as coils of three intertwined helical polypeptides. These fundamental building blocks self-assemble in a hierarchical fashion to form tissue-specific networks of micro-fibrils, fibrils, fibers, and fiber bundles. Mechanical and chemical stability of the fibril network, in part, is derived from intra- and inter-molecular cross-links.¹ In addition, these networks are specialized in terms of their fibril architecture and physical properties to support tissue-specific cellular function. While much of the information necessary to drive assembly of individual fibrils is inherent to the collagen molecule, the mechanisms underlying diversification of ECM fibril networks and therefore tissue form and function have yet to be fully elucidated.

Synthesis and assembly of collagen represents a complex cellular process involving a myriad of posttranslational events such as hydroxylation, glycosylation, trimerization, and cross-linking. In fact, it is for this reason that production of functional collagen using standard recombinant technology has been so challenging.² Major events following ribosomal production of individual procollagen alpha (α) chains, include hydroxylation of specific proline and lysine residues which contributes to triple helix stabilization³⁻⁵ and molecular cross-linking,^{6,7} respectively. Processed polypeptides then undergo trimerization to form heterotrimeric procollagen molecules consisting of two $\alpha 1(I)$ and a single $\alpha 2(I)$ chains. Upon extrusion into the extracellular space, both amino- and carboxy-terminal propeptides are enzymatically cleaved rendering the collagen monomer capable of fibril formation.^{8,9} As prefibrillar aggregates of staggered monomers assemble, lysyl oxidase binds to and catalyzes formation of covalent cross-links to create oligomers (dimers or trimers).¹⁰ In turn, these early oligomer precursors are believed to direct the progressive molecular packing and assembly that eventually gives rise to tissue-specific fibril architecture and matrix function.

Naturally-occurring collagen cross-links that give rise to oligomers have been extensively studied since the 1960s. In fact, it is now evident that cross-link chemistries are more tissue-specific rather than species-specific.¹¹ Major cross-links are derived from oxidative deamination of ϵ -amino groups of specific lysine and hydroxylysines by lysyl oxidase within the nontriple helical telopeptide regions of the molecule. In turn, the resulting aldehydes react with lysine or hydroxylysine residues within the central triple-helical region to form intermediate divalent cross-links of the aldol, hydroxyaldol, or ketoimine varieties. Upon maturation these divalent cross-links convert into more stable trivalent cross-links such as the histidine derivative histidinyl-hydroxylysinonorleucine (HHL) which is prominent in skin and hydroxylysyl pyrrole which is prevalent in bone.^{12,13} Evidence that cross-link content is a critical determinant of tissue physical properties is derived from numerous in-vitro and in-vivo studies where specific cross-linking enzymes (e.g., lysyl oxidase or lysyl hydroxylase) are selectively inhibited or genetically knocked out.¹⁴⁻¹⁷

Cross-linked oligomers or molecular aggregates that accompany monomers upon extraction from tissues have historically been viewed as undesirable by-products, especially for traditional studies of collagen fibril assembly.¹⁸ In fact, these molecular species are routinely minimized or eliminated from collagen preparations via enzymatic digestion, secondary purification strategies, or young or lathritic animals, where tissue cross-link content is decreased.¹⁹⁻²² The few studies performed to date documenting the effects of oligomeric moieties on in-vitro fibril assembly have reported increased intrinsic viscosities of collagen solutions,²³ decreased polymerization times,¹⁸ and increased thermal melting temperatures of polymerized products.²³ While clearly, collagen cross-links and their

associated oligomers impact assembly and mechanics of fibrils, their role in defining fibril-level microstructure and physical properties of supramolecular collagen assemblies in vivo or in vitro remains unclear.

Recently, we reported that acid-solubilized Type I collagen derived from pig skin (PSC) possessed a dramatically different polymerization potential compared to three commercial monomeric collagen sources. PSC consistently displayed rapid polymerization, and when polymerized at the same collagen concentrations and reaction conditions as the other collagen sources yielded matrices with the greatest mechanical integrity (stiffness) and broadest range of mechanical properties as characterized in oscillatory shear, uniaxial extension, and unconfined compression.²⁴ The observed fibril microstructure-mechanical relationships, together with molecular composition, polymerization kinetics, and intrinsic viscosity results, suggested that the polymerization potential of PSC may be attributed to its unique intermolecular cross-link and oligomer content. We now extend these findings by testing the hypothesis that the oligomer content of PSC, as quantified by average polymer molecular weight (AMW), is positively related to polymerization rate, interfibril interaction, and matrix stiffness. To test this hypothesis, PSC was fractionated into monomer- and oligomer-rich solutions using established techniques. These fractions then were mixed in different proportions to systematically vary monomer/oligomer content or AMW. In turn, the effects of AMW were determined on polymerization kinetics as well as the fibril microstructure and mechanical properties of in-vitro polymerized matrices. Finally, cytoplasmic vacuole number and area of cultured endothelial colony forming cells (ECFCs), known to be important parameters for vascular lumen formation, were found to be significantly influenced by the monomer/oligomer content, thus providing proof-of-concept as to the biological significance of collagen AMW modulation. Results from this work are important because they document, for the first time, the role of oligomers in defining the hierarchical structure–function properties of in-vitro assembled collagen matrices.

MATERIALS AND METHODS

Collagen Separation into Monomer- and Oligomer-Rich Fractions

Type I collagen, comprising monomers and oligomers, was acid solubilized and purified from the dermis of market weight pigs as described previously.²⁴ Subsequent fractionation of PSC into monomer- and oligomer-rich formulations was performed using two established methods: (1) polymerization in 0.03M sodium phosphate buffer, pH 7.0 containing 1M sodium chloride, and 0.6M glycerol²² and (2) selective salt precipitation at 3% sodium chloride.¹⁹ To demonstrate reproducibility of the glycerol-based protocol, collagen was obtained from a single pig hide (single source) or a pooled source representing three different pig hides (pooled source). Monomer- and oligomer-rich fractions were recombined at different ratios to create collagen formulations that varied in monomer/ oligomer content and thus AMW.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the purity and molecular composition of the collagen formulations. The 12% Novex Tris-Glycine gels (Invitrogen, Carlsbad, CA) were used for identification of noncollagenous proteins and small molecular weight contaminants. SDS-PAGE (4%) in interrupted and uninterrupted formats²⁵ and western blot analysis using mouse monoclonal antibodies specific for Type I (AB6308, Abcam, Cambridge, MA) and Type III (MAB 1343, Chemicon, Temecula) collagen were used for analysis of collagen type content (e.g., Types I, III, and V). According to manufacturer's specification, the antibody for Type I collagen was developed against the full length native protein. However, in the present study this antibody showed specificity to the $\alpha 1(I)$ chain when applied to denatured collagens as part of a Western blot. Gels were stained with Coomassie Blue or silver nitrate and imaged using

a digital camera and light box. An alcian blue assay was used as previously described²⁶ to assess sulfated glycosamino-glycan (GAG) content. Heparin derived from porcine intestinal mucosa (Sigma-Aldrich, St. Louis, MO) was used to prepare a standard curve (1–20 heparin U ml⁻¹).

Preparation of Three-Dimensional (3D) Collagen Matrices

All collagen formulations were polymerized under identical reaction conditions to produce 3D matrices. Lyophilized collagens were dissolved and diluted in 0.01N HCl and neutralized with 10× phosphate buffered saline (PBS, 1× PBS had 0.17 M 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 2.75 mg ml⁻¹. Neutralized collagen solutions were kept on ice prior to 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.

Quantification of Intrinsic Viscosity and AMW

The AMW of the collagen formulations was determined by intrinsic viscosity measurements as described previously.²⁷ In brief, apparent viscosities of collagen solutions in 0.01N HCl were measured on an AR2000 rheometer (TA Instruments, New Castle, DE) with a cone geometry (40 mm, 2° cone angle). Viscosities for solutions representing collagen concentrations of 0.1–0.3 mg ml⁻¹ were measured at shear rates of 100–1500 s⁻¹ at 10°C. The AMW was calculated using the Mark-Houwink equation, $|\eta| = kM^a$, where $a = 1.8$ and k was determined for each shear rate assuming a monomer-rich AMW of 282 kDa.^{28,29} AMW was extrapolated to zero shear rate. Three replicates were performed at the three concentrations.

Analysis of Collagen Polymerization Kinetics

A turbidimetric assay was used to analyze the polymerization kinetics of each collagen formulation.^{30,31} Neutralized collagen solutions at 0.5, 0.7, and 1 mg ml⁻¹ concentrations were transferred to a prewarmed (37°C) 384-well plate. Absorbance at 405 nm (A405) was measured using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) every 30 s for 2 h at 37°C unless specified otherwise. A405 values were background corrected by subtracting the absorbance value measured at $t = 0$ min time point. Kinetic parameters calculated from the sigmoidal-shaped turbidity curves included lag time (x -intercept of line formed from one-quarter and one-half final absorbance values), polymerization rate during growth phase (growth rate; slope of the line formed from previous points), and polymerization half-time (time at which absorbance equals half the final absorbance value). At least four replicates were performed at each concentration tested.

Analysis of Collagen Fibril Microstructure

Confocal reflection microscopy (CRM) was used to collect high-resolution, 3D images of the matrices in their native, hydrated state.³² Matrices were polymerized in Lab-Tek chambered coverglass slides (Nunc, Thermo Fisher Scientific, Rochester, NY) at concentrations of 0.5, 0.7, and 1.0 mg ml⁻¹ for 2 h at 37°C. CRM was performed on an Olympus Fluoview FV1000 confocal system adapted to an Olympus IX81 inverted microscope with a 60× UPlanSApo water immersion objective (Olympus, Tokyo, Japan). Image stacks were collected at five random locations within each of five independent matrices per formulation ($n = 25$). Three microstructure parameters, fibril density, fibril diameter, and pore size were quantified from the images. Fibril density and diameter were measured as described previously.³³ In brief, for fibril density measurements, images were read into Matlab (Mathworks, Natick, MA) and binarized based upon a mathematically

determined threshold value. Fibril density then was calculated as the percentage of fibril-containing voxels to total image voxels. Diameter measurements were made on randomly chosen single, nondividing fibrils (10 fibrils per each of five independent matrices; $n = 50$). Pore size was calculated from 2D projections of 50-slice image stacks representing a total thickness of 5 μm using Image-Pro Plus 5.1 software (Media Cybernetics, Bethesda, MD). The images were binarized by thresholding³³ and pore size was quantified as the cross-sectional areas encompassed by collagen fibrils.

Imaging via transmission electron microscopy (TEM) was performed on aliquots of the collagen polymerization reaction taken at various periods of time. Samples were placed on copper support grids with carbon-coated formvar films and allowed to settle for 30 s. Next, the samples were stained using 1% phosphotungstic acid (pH adjusted to 7.2 with potassium hydroxide), air-dried, and imaged using a Philips CM-100 TEM (FEI Company, Hillsboro, OR) with 80 kV accelerating voltage. Images were captured on Kodak SO-163 Electron Image film and scanned into digital format at 600 dpi.

Analysis of Matrix Mechanical Properties in Shear and Unconfined Compression

Viscoelastic properties of polymerized collagen matrices (0.5, 0.7, and 1 mg ml^{-1} final collagen concentration) were measured in both oscillatory shear and unconfined compression using a stress-controlled AR2000 rheometer (TA Instruments, New Castle, DE) adapted with a stainless-steel, 40-mm diameter, parallel-plate geometry as described previously.²⁴ A shear strain sweep from 0.01 to 5% strain at 1 Hz was used to measure the shear modulus (reported values 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 $\tan(\delta) = G''/G'$. Following the strain sweep, normal force was measured in response to compressive strain generated by depressing the measurement head at a rate of 20 $\mu\text{m s}^{-1}$ (strain rate 2.76%/s). The resultant compression stress-strain curve was biphasic in nature with distinguishable linear behavior within low-strain and high-strain regions as described previously.²⁴ The compressive modulus (E_c) was calculated by performing a regression analysis of the linear, low-strain region from 10 to 30% strain. Shear and compression tests were performed on at least four independent matrices per formulation ($n = 4$).

Preparation and Analysis of 3D Cellularized Tissue Constructs

Human umbilical cord blood ECFCs were obtained from EndGenitor Technologies, (Indianapolis, Indiana) and cultured according to established methods.³⁴ ECFCs represent an EPC population defined by high proliferative potential and an ability to vascularize collagen-based matrices in vivo.^{34,35} ECFCs were used between Passage 6 and 9 for all experiments. Cellularized tissue constructs were prepared as previously described^{24,36} with minor modifications. ECFCs (5×10^5 cells ml^{-1}) were suspended in neutralized solutions of monomer-rich or PSC (oligomer-rich) formulations that were matched on final collagen concentration or matrix stiffness as measured by G' . Collagen-cell suspensions were pipetted into either 24-well (0.5 ml/well; morphological evaluation), or 96-well plates (58 μl /well; vacuole number and area quantification), allowed to polymerize at 37°C, and then cultured in complete endothelial cell growth medium (EGM-2, Lonza, Walkersville, MD) at 37°C and 5% CO_2 . For morphological evaluation, tissue constructs were fixed and stained with FITC-conjugated Ulex Europaeus Agglutinin 1 (UEA1) lectin (Sigma-Aldrich) and DRAQ5 deoxyribonucleic acid dye (Biostatus Limited, Leicestershire, United Kingdom). Tissue constructs were imaged using confocal microscopy in combined fluorescence and reflection modes for visualization of ECFC and collagen fibrils, respectively. For visualization and quantification of vacuole number and area, in-vitro tissue constructs were

stained with toluidine blue O (1% in 39% methanol). Quantification was performed on bright field images (100 $\times$) representing the central 9–16 fields within each of three different *z*-planes per construct. MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) was used to trace vacuoles to yield both vacuole density and area. Total vacuole area was calculated as the product of vacuole density and vacuole area.

Statistical Analysis

All measured values are reported as mean $\pm$ standard deviation (SD). Statistical analyses were performed using SAS v. 9.1 (SAS Institute, Cary, NC). To determine differences among treatment groups the general linear model (GLM) procedure was used to conduct unbalanced analysis of variance (ANOVA, in some cases a Kruskal-Wallis ANOVA for nonparametric distributions) and perform multiple comparisons of least squares means using the Tukey-Kramer method. In some cases, pairwise comparisons were made using Student *t* tests. Differences were considered statistically significant when $P < 0.05$.

RESULTS

PSC Comprises Monomers and Oligomers

The starting material, acid-solubilized PSC represented a highly purified Type I collagen. Alcian blue analysis of solutions with collagen concentrations between 5 and 6 mg ml⁻¹ showed no measurable glycosaminoglycans. SDS-PAGE using a 12% gel showed no detectable noncollagenous proteins (data not shown). The absence of contaminating collagen Types III and V was verified by SDS-PAGE in an interrupted format and Western blotting with an antibody specific for collagen Type III (data not shown). Interestingly, SDS-PAGE (4% gel) showed that in addition to the $\alpha 1(I)$, $\alpha 2(I)$, $\beta 1(I)$, $\beta 2(I)$, and $\gamma(I)$ bands routinely observed in denatured purified collagen Type I preparations, PSC contained a prominent band corresponding to molecular weight of 260 kDa as well as high molecular weight (HMW) components with molecular weights greater than 300 kDa (Figure 1, Lane 1). $\alpha 1(I)$ and $\alpha 2(I)$ bands, which are present at a ratio of 2 to 1 respectively, represent component polypeptide chains (~100 kDa) within a single triple helical collagen molecule. β , Oligo260, γ , and HMW bands represent two or more α chains that are covalently linked by natural collagen cross-link chemistries. Western blot analysis confirmed that $\alpha 1(I)$, $\beta 1(I)$, $\beta 2(I)$, Oligo260, and HMW components contained the epitope for collagen $\alpha 1(I)$ (Figure 1, Lane 4). These molecular purity results for PSC were consistent with previous published observations.²⁴ Subsequent polymerization of PSC in the presence of glycerol yielded separate fractions designated monomer- and oligomer-rich, which differed in their SDS-PAGE protein banding pattern (see Figure 1). Compared to its monomer counterpart, the oligomer fraction showed higher levels of 260 kDa and HMW proteins suggesting that these components represent oligomer derivatives with intermolecular cross-links. Intrinsic viscosity determinations, which provide a measure of the AMW of collagen polymer solutions in their native, undenatured state, indicated that monomer- and oligomer-rich fractions had AMW of 282 kDa and 603 $\pm$ 92 kDa, respectively. These results confirmed the prominence of cross-linked collagen monomers within the oligomer-rich fraction. PSC yielded roughly four times more oligomer-rich collagen compared to monomer-rich collagen, based upon dry weight. Fractionation results were similar for single and batched PSC sources.

Oligomer-Rich Collagen Shows Rapid Polymerization That is Relatively Independent of Collagen Concentration

Turbidimetric analysis was used to compare polymerization kinetics of glycerol-derived monomer- and oligomer-rich collagens as a function of collagen concentration. All formulations yielded expected sigmoidal-shaped relationships between A405 and time with

definable “lag,” “growth,” and “plateau” phases (Figure 2A). Interestingly, all measured kinetic parameters, including polymerization half-time (Figure 2B), lag time (Figure 2C), and growth rate (Figure 2D), for monomer- and oligomer-rich formulations, showed distinct dependence on collagen concentration as determined from statistical analysis of best-fit lines ($P < 0.05$). The monomer-rich fraction showed a nearly 50% reduction in polymerization half-time and a 30% reduction in lag time as collagen concentration was increased from 0.5 to 1.0 mg ml⁻¹ (Figures 2B and 2C). Half-time and lag time values for the oligomer-rich fraction remained relatively constant over the concentration range tested, with values of 3 and 1 min, respectively. For all collagen concentrations tested, half-time and lag-time values for the oligomer-rich fraction were statistically shorter ($P < 0.05$) than those obtained for the monomer-rich fraction. Furthermore, the oligomer-containing formulations exhibited a progressive increase in growth rate as a function of concentration, which was not observed with the monomer-rich fraction (Figure 2D). The growth rates observed for the oligomer-rich fraction were statistically greater ($P < 0.05$) than those for the monomer-rich at all concentrations tested. However, these differences had only minor effects on the overall polymerization half-time, which, in general, appeared to more closely parallel lag phase duration.

Oligomer-Rich Collagen Matrices Show Increased Mechanical Integrity Compared to Monomer-Rich Collagen Matrices

Further insight into the assembly properties of monomer- and oligomer-rich collagens as a function of collagen concentration was gained through mechanical testing of polymerized matrices. Since collagen matrices represent composite viscoelastic materials, mechanical properties were determined using oscillatory shear and unconfined compression loading formats, as described previously.²⁴ Although matrix stiffness measures G' and E_c were positively correlated with concentration for both monomer- and oligomer-rich matrices, matrices formed with the oligomer-rich fraction showed a significantly greater increase in G' and E_c over the concentration range tested ($P < 0.05$; Figures 3A and 3C). Phase shift δ , an indicator of matrix fluidity, decreased with concentration for monomer-rich matrices. In contrast, δ values remained relatively low and constant for oligomer-rich matrices (Figure 3B). The best-fit lines for δ as a function of concentration were found to be statistically different ($P < 0.05$) for monomer- and oligomer-rich matrices.

Polymerization Half-Time Increases with Collagen AMW

Turbidimetric analysis also was used to determine how collagen polymerization kinetics varied with monomer/oligomer content or AMW. Here, AMW was systematically adjusted by mixing monomer- and oligomer-rich fractions in different proportions. As AMW was increased from 282 to 306 kDa a significant step change in mean polymerization half-time from 55 ± 9 min to 10 ± 1 min was observed ($P < 0.05$; Figure 4A). Polymerization half-times remained consistently short and were statistically similar ($P > 0.05$) for solutions with AMW between 306 and 603 kDa. A similar trend was observed for lag time as a function of AMW (Figure 4B) again demonstrating lag phase is a primary determinant of the overall polymerization half-time. Growth rate increased monotonically with increasing AMW (Figure 4C).

Collagen AMW Affects Interfibril Branching

The variation of AMW and therefore monomer/oligomer content was found to influence not only polymerization kinetics but also the overall fibril microstructure of polymerized matrices. CRM was used to visualize the 3D fibril microstructure of unprocessed, hydrated specimens (see Figure 5) and provided the basis for quantification of fibril diameter, fibril density, and projected pore size. Average fibril diameters measured 341 ± 32 nm to 370 ± 50 nm for the range of collagen AMW tested (Table I). While statistical differences were noted

between some groups based upon the experimental design and statistical analysis applied, no general trends or predictive relationships between fibril diameter and collagen AMW were identified. Fibril density varied between $8.1\% \pm 2.6\%$ and $14.6\% \pm 1.6\%$ for the collagen formulations tested (Table I). Unlike collagen concentration and fibril density,²⁴ no positive correlation was observed between AMW and fibril density. Finally, while mean pore size and its associated variance showed a general decline with increasing AMW, only 282 kDa was found to be significantly different from the rest of the formulations ($P < 0.05$; Table I). Visualization of single image slices as well as image stacks suggested that fibril persistence length (or distance between interfibril branch points) decreased with increasing AMW. In fact, matrices prepared from 282 kDa AMW collagen appeared as entanglements of long fibrils. Interestingly, time-based analysis of fibril assembly using high resolution TEM indicated that banded fibrils formed more rapidly and with a larger number of interfibril branches within oligomer-rich fractions compared to their monomeric counterparts (see Figure 6). Banded fibrils appeared to form and elongate via microfibril condensation at their tapered ends (Figure 6A, black arrows). In contrast, lateral associations between developing fibrils appeared to contribute to fibrils with increased diameters as well as interfibril branch formation (Figure 6A, white arrows). Branched fibrils were identified based on the intertwining of the molecular structure of adjacent fibrils (Figure 6B),³⁷ events which also have been documented during in-vivo tendon development.^{38,39}

Matrix Stiffness is Positively Related to Collagen AMW

The functional significance of increased interfibril branching as occurs with increasing collagen AMW was documented by measurement of matrix viscoelastic properties. Despite collagen concentration remaining constant, G' increased and δ decreased progressively with increasing AMW (Figures 7A and 7B). This enhanced ability to store elastic energy was further corroborated by an increase (nearly seven-fold) in E_c as a function of AMW (Figure 7C). Values ranged from 3.3 ± 0.9 kPa for 282 kDa matrices to 20.8 ± 5.4 kPa for 603 kDa matrices, indicating that hydraulic conductivity or permeability of matrices was reciprocally related to oligomer/ monomer content. It should be noted that independent modulation of AMW (Figure 7A) and oligomer concentration over the ranges tested (Figure 5A) yielded a similar fold-change in G' . On the other hand, modulation of oligomer concentration resulted in only a three-fold increase in E_c (Figure 5C) compared to a seven-fold increase observed with variation of AMW (Figure 7C). Collectively, these results demonstrate that the underlying mechanism by which fibril microstructure-mechanical properties are altered with modulation of AMW is different from that achieved with collagen concentration. In general, the highly consistent mechanical properties established previously for PSC lots prepared from different pig hides²⁴ translated into good reproducibility of mechanical testing results obtained as a function of AMW for both single and pooled source batches.

Salt Fractionation as an Alternative Method for Separating PSC Monomers and Oligomers

PSC monomers and oligomers also could be separated from PSC based upon differential salt precipitation. However, the fractionation efficiency of this method was less than that obtained with the glycerol-based procedure as evidenced by monomer yield and AMW for the resulting fractions. Monomer yield from salt precipitation was 10% or 2.5 times less than that achieved with glycerol. Furthermore, salt precipitation produced monomer- and oligomer-rich fractions with AMW of 290 ± 37 kDa and 394 ± 48 kDa, respectively. This range was substantially less than that obtained with the glycerol-based method where the monomer fraction was 282 kDa and the oligomer fraction was 603 kDa. However, when salt separated fractions were used to generate collagen formulations that varied in AMW, similar trends in polymerization kinetics (Figure 8A), fibril microstructure (Figure 8B), and matrix mechanical properties (Figures 8C–8E) were observed. The slight discrepancies noted between the visco-elastic properties obtained with salt and glycerol methods can likely be

attributed to molecular differences (e.g., extent of denaturation) induced during secondary processing.

Systematic Variation of Collagen AMW Modulates ECFC Vacuole and Capillary-Like Network Formation In Vitro

Recently, it has been established that in-vitro and in-vivo vessel morphogenesis by differentiated endothelial cells or endothelial progenitor cell (EPC) populations can be modulated by varying collagen concentration, and therefore stiffness, of the surrounding polymerized matrix.^{35,40} The increase in matrix stiffness that accompanies increased collagen concentrations has been attributed to an increase in collagen fibril density. In the present study, collagen AMW was varied and provided independent control of collagen concentration and matrix stiffness. Based upon fibril microstructure-mechanical analyses, AMW appears to modulate matrix stiffness in part by increasing the extent of interfibril branches. ECFCs were seeded at the same cell density and cultured in vitro within pig skin derived monomer or oligomer collagen matrices. Whether matched in either collagen concentration or matrix G' , formulation-dependent differences in the time course and extent of ECFC vacuolization and multi-cellular, capillary-like network formation were immediately apparent upon morphological evaluation (see Figure 9). ECFC vacuolization and limited cell-cell associations were noted within both 1 mg ml⁻¹ and 200 Pa monomer matrices over the first 48 h, after which time they began to regress. In contrast, oligomer matrices prepared at 1 mg ml⁻¹ and 200 Pa supported early vacuolization followed by formation of extensive multi-cellular, capillary-like networks. These vessel networks had obvious lumens (Figure 9D, insert) and persisted well beyond 7 days in culture. It should be noted that although significant reorganization and remodeling was noted at the collagen fibril level during vacuole and multi-cellular network formation, no significant contraction was observed at the construct level for any of experimental conditions studied. In fact, all tissue constructs had fixed geometries (adherent to the sides of the culture well) for the duration of the experiment. As such cell-induced matrix compaction or reduction in construct volume did not contribute to observed differences in cell density.

It is well established that endothelial cell vacuole formation and fusion drive the process of vascular network formation not only in vitro within 3D collagen matrices but also in vivo during development.^{41,42} Therefore, a more in-depth study involving quantification of vacuole density and area was conducted after 48 h of culture and yielded several interesting and corroborating findings (see Figure 10). As part of this study we noted that vacuole density and vacuole area appeared to be independently and differentially regulated as a function of collagen concentration and matrix stiffness for monomer- and oligomer-rich matrices. As such, total vacuole area, which represents the product of these two parameters, was used to evaluate collectively the consequences of the observed changes in these two parameters. All results were analyzed as a function of collagen concentration (Figures 10D–10F, upper panel) and G' (Figures 10D–10F, lower panel) since monomer and oligomer collagens possess different concentration- G' (fibril microstructure-mechanical properties) relationships.

At both collagen concentrations tested (1 and 2.75 mg ml⁻¹), the oligomer yielded the greatest vacuole density (Figure 10D, upper panel), similar vacuole areas (Figure 10E, upper panel), and the greatest total vacuole area (Figure 10F, upper panel) compared to the monomer. Interestingly, both formulations showed a decrease in ECFC vacuole area as a function of collagen concentration (Figure 10E, upper panel) which can likely be attributed to spatial constraints imposed by the associated increase in fibril density. Interestingly, the observed increase in vacuole density with monomer concentration was greater than the associated decrease in vacuole area. As such, these changes resulted in an overall increase in total vacuole area with monomer concentration. Alternatively, as oligomer concentration

increased, little to no change was observed in ECFC vacuole density and vacuole area decreased. In this case, total vacuole area was found to decrease with increasing oligomer concentration.

When analyzed based on matrix stiffness or G' , additional insight was gained since stiffness is dictated by not only fibril density (collagen concentration) but also the extent of inter-fibril branching (collagen AMW). When matched in stiffness (136 Pa), vacuole density within the oligomer was nearly three-fold higher than that observed for the monomer (Figure 10D, lower panel). At lower matrix G' levels of 24 Pa, which corresponded to 1 mg ml⁻¹ monomer, little to no vacuole formation was noted. Those that did form had relatively large vacuole areas (roughly 70 μm^2 ; Figure 10E, lower panel). At relatively high matrix G' levels of 300 Pa, as produced by 2.75 mg ml⁻¹ oligomer, vacuole density was similar to that observed for the 136 Pa oligomer matrix and vacuole area was relatively small (roughly 50 μm^2 ; Figures 10D and 10E, lower panels). While vacuole area declined with increasing G' for both formulations, the vacuole area- G' relationship for the oligomer showed a rightward shift compared to that of the monomer (Figure 10E, lower panel). Finally, the collective changes in vacuole density and area as a function of G' resulted in an increase in total vacuole area with increasing monomer G' as shown in the lower panel of Figure 10F. In contrast, total vacuole area decreased as a function of oligomer G' over the range tested. In summary, the fact that these two formulations yielded different cell fates when evaluated based upon either concentration or G' , suggests that both fibril density and interfibril branching work cooperatively as critical determinants of the ECFC vasculogenic response. Furthermore, these results were consistent with findings made when ECFCs were cultured and compared in PSC and commercial monomeric collagen preparations (Becton Dickinson rat tail collagen; data not shown).

DISCUSSION

A more in-depth understanding of mechanisms innate to collagen assembly and matrix-induced guidance of cells will support the rational design and expanded use of this natural polymer for research and medical applications. It is increasingly apparent that the fibril microstructure of collagen-based ECMs determines not only tissue-level mechanical properties but also provides instructive physicochemical features of the local cellular microenvironment. Cells sense and respond to the spatial distribution of fibrils largely by forming cell-matrix adhesions through integrin-mediated binding of collagen adhesion domains. A mechanical force balance then is established as cells exert cytoskeletal-based contraction which is resisted by the stiffness of the surrounding collagen fibrils. The resultant physical and biochemical based signal transduction reactions ultimately guide fundamental cellular behaviors, including proliferation, migration, and differentiation.^{43,44} In addition, the fibril microstructure as well as the type and extent of intermolecular cross-linking determine matrix remodeling and degradation, known regulators of normal and disease processes including tissue (e.g., vascular) morphogenesis,⁴⁵ wound healing,⁴⁶ and cancer cell metastasis.^{47,48}

We and others have documented that matrix assembly is dependent upon a number of polymerization parameters including buffer composition, pH, and ionic strength, presence of copolymers (e.g., other collagen types) or accessory molecules (e.g., proteoglycans), as well as collagen molecule integrity (e.g., presence or absence of telopeptides).^{21,24,30,33,49-52} As such systematic variation of specific polymerization parameters can be used to predictably control relevant physical properties (e.g., fibril density, fibril diameter, interstitial fluid viscosity, and matrix stiffness) of polymerizable collagen-fibril matrices.^{24,30,33,51} Herein, we demonstrate that modulation of another physiologically-relevant collagen assembly parameter, namely naturally-occurring intermolecular cross-links in the form of oligomers,

alters polymerization kinetics as well as fibril microstructure-mechanical properties of polymerized matrices. From this work, it is evident that oligomers serve as preformed nucleation sites that effectively decrease lag time and increase the growth rate of the assembly process. In addition, data show, for the first time, that oligomers, as distinct collagen building blocks, yield more elastic and stiffer fibrillar networks by fostering formation of interfibril branches rather than increasing fibril density, which has major implications on tissue-level mechanical behavior as well as cell mechanotransduction.

Intermolecular cross-link chemistries are known to vary amongst tissues and have been implicated in the development of tissue-specific form and function⁶; however, their role in guiding fibril assembly in vivo or in vitro has not been extensively studied. Oligomers created by these covalent cross-links, together with monomers, are natural outcomes of collagen isolation procedures that maintain the integrity of telopeptides regions (e.g., acid solubilization). Polymerization in the presence of glycerol, salt precipitation, and chromatographic methodologies have been used to further fractionate monomer and oligomer components from crude collagen mixtures.^{19,53,54} In the present paper, both glycerol and salt precipitation methods were used to generate monomer- and oligomer-rich fractions from PSC, verifying that PSC contained significant amounts of both components.²⁴ Interestingly, PSC oligomers were distinct in molecular composition compared to those generated previously from calf skin by Na et al.¹⁸ In addition, to γ and HMW bands, which Na used as indicators of oligomers, PSC contained a prominent 260 kDa component, Oligo260. Such molecular discrepancies can likely be attributed to differences in acid extraction protocols used to generate the initial collagen. Evidence that this 260 kDa component and associated HMW moieties represent oligomers includes their selective elimination upon pepsin digestion, positive staining for the Type I collagen epitope, and increased intrinsic viscosity and AMW of the oligomer-rich fraction compared to the monomer-rich fraction. Based upon molecular attributes of Oligo260, it is plausible that it represents a trimer formed specifically by the acid-stable, HHL cross-link, which is known to be prevalent in skin.¹³ More in-depth molecular analyses are currently underway to fully characterize this component.

Despite the noted compositional differences between pig skin and calf skin oligomers, similar effects on polymerization kinetics were observed when monomer/oligomer content was varied. Consistent with previous reports¹⁸ and the accepted nucleation-growth theory of fibril assembly,⁴⁹ we found that polymerization half-time decreased with increasing AMW. Both a reduction in lag time and a moderate increase in growth rate contributed to this enhanced polymerization efficiency. While the monomer-rich fraction displayed concentration-dependent decreases in polymerization half-time and lag time, no significant changes were observed in growth rate as a function of concentration. In contrast, the oligomer-rich fraction maintained short and relative constant polymerization half-times and lag times over the concentration range evaluated with a concentration-dependent increase in growth rate. Such findings support the notion that oligomers serve as critical nucleation sites that progress into fibrils through linear and lateral addition of monomers and association with other nuclei.⁵⁵ From the standpoint of research (cell culture) and medical (injectable, polymerizable biopolymer) applications, a short polymerization time is an important design requirement to ensure rapid and localized matrix formation and uniform distribution of suspended cells.

It is well established that an increase in collagen concentration is correlated with an increase in fibril density and a concomitant increase in matrix stiffness.^{24,36,51} In the case when total collagen concentration of a specific collagen formulation is altered the monomer/oligomer ratio remains constant. In the present study, monomer- and oligomer-rich PSC fractions independently showed an expected increase in matrix G' with increasing collagen

concentration. However, when polymerized at the same collagen concentration, the oligomer-rich fraction yielded stiffer matrices (increased G' and E_c) compared to its monomeric counterpart. Such results indicate that G' -concentration relationships are dependent upon the collagen molecular composition and are consistent with those obtained previously when oligomer-containing PSC was compared to commercial monomeric collagens.²⁴

Systematic variation of monomer/oligomer ratio or collagen AMW effectively allowed the uncoupling of changes in fibril density and matrix stiffness that accompany collagen concentration modulation. Since collagen self-assembly depends upon selective binding between different regions of collagen molecules,⁵⁶ it is plausible that oligomer amount and type promote specific types of molecular assemblies and give rise to different fibril morphologies and microstructures. Based upon the fibril microstructure parameters measured (e.g., fibril density and diameter), there were no obvious correlative relationships with collagen AMW identified for collagen formulations derived by either the glycerol or salt precipitation methods. Interestingly, a general trend noted was that projected pore size decreased with increasing AMW suggesting that interfibril spacing was greatest for monomeric matrices. This supposition was further corroborated by an increase in interfibril branching for oligomer matrices as documented using TEM. It should be noted that the interfibril branching observed by TEM in the present study showed strong similarities to those documented previously *in vivo* during early tendon development.^{38,39} Further evidence that oligomers modulated molecular assembly and interfibril interactions was inherent in the observed increases in G' and E_c and associated decreases in δ as a function of AMW. Collectively, these observations support the theory that an increase in oligomer nucleation sites would foster not only lateral and linear addition of monomers to individual developing fibrils but also associations between growing fibril precursors. Furthermore, the pattern of such molecular associations would be dictated by the specific staggered arrangement of molecules within the oligomer. As such, fibril networks produced by high AMW (oligomer) collagens would represent more highly branched networks with relatively short interfibril lengths and high stiffness.⁵⁷ While matrices produced with low AMW (monomer) collagen represent entanglements of lengthy fibrils and provide low stiffness. Unfortunately, resolution limitations and specimen processing requirements of state-of-the-art imaging technologies precluded accurate quantification of interfibril branches within polymerized collagen matrices.

The biological implication of modulating the monomer/ oligomer content or AMW of polymerizable collagen matrices was illustrated by the differential vasculogenic response of cultured ECFC. We and others have documented that alteration of matrix physical properties, including apparent matrix stiffness and fibril (ligand) density, by varying collagen concentration effectively modulates early vacuolization as well as the number and morphology of vessel networks formed by ECFC and differentiated endothelial cells *in vitro* and *in vivo*.^{35,40} In the present study, we vary collagen AMW and effectively modulate matrix stiffness by altering another critical fibril microstructure property independent of fibril density, the extent of interfibril branching. Results show that modulation of AMW allowed tuning of interfibril branching and therefore matrix stiffness to support the necessary ECFC traction forces required for vacuole formation.⁵⁸ This modulation of matrix stiffness independent of fibril density allowed preservation of the necessary fibril spatial distribution (fibril density) to foster vacuole expansion and formation of multi-cellular networks. In fact, monomer matrices require a substantially higher fibril density to generate the same matrix stiffness as oligomer matrices,²⁴ which can constrain cell spreading and cell-cell associations necessary for vessel formation. Vacuole density was always highest for oligomer matrices at the matched concentrations and G' values tested, indicating that the fibril microstructure-mechanical properties of these matrices supported the necessary

traction forces and cell extension. Vacuole area was inversely related to collagen concentration and therefore fibril density for both collagen formulations. However, the apparent rightward shift in the vacuole area- G' curve for the oligomer formulation indicated that vacuole areas similar to those in monomer matrices could be achieved with oligomer matrices at higher stiffness values. Finally, the independent changes in both vacuole number and area induced by matrix physical properties could be captured in the single parameter total vacuole area. Just as total vessel area was identified as a useful metric for elucidating how matrix physical properties influence functional vessel formation in vivo,³⁵ total vacuole area may serve as a useful predictive measure of functional vessel network formation in vitro and in vivo.

Differences were also noted in the ability of the two matrix formulations to support persistent multi-cellular, capillary-like network formation. Consistent with previous reports,^{59–61} rapid regression of the ECFC vessel morphogenic response was noted within monomer matrices after 48 h. On the other hand, extensive capillary-like networks with obvious lumens persisted within oligomer matrices beyond 7 days in culture, indicating that fibril microstructure-mechanical properties and associated microenvironmental signals provided by these matrices are more conducive to network stabilization. Interestingly, independent studies have investigated the effects of matrix physical properties on capillary network outgrowth by HUVEC-coated microcarrier beads cultured within fibrin matrices. In these cases, increasing matrix stiffness by increasing fibrin concentration⁶² or increased factor VIII cross-linking⁶³ was found to reduce capillary network length. A unique contribution of our study is the finding that suggests a cooperative interplay between fibril density (collagen concentration) and interfibril branching (collagen AMW), two determinants of matrix stiffness, in terms of optimizing the ECFC vessel forming response within polymerizable collagen matrices. Further, the ratio of stiffness to collagen concentration (fibril density) may serve as a better predictor of vessel formation rather than collagen concentration (fibril density) and matrix stiffness as independent parameters.

It should also be noted that modulation of oligomer and therefore intermolecular cross-link content likely also influences matrix degradability, which has recently been implicated as another matrix characteristic that regulates vessel morphogenesis. Specifically, Sacharidou and co-workers recently documented involvement of multi-molecular signaling complexes involving $\alpha 2\beta 1$ -integrin and the membrane-anchored collagenase membrane type 1-matrix metalloproteinase (MT1-MMP).⁶⁴ Interestingly, MT1-MMP involvement in endothelial cell vessel formation⁶⁵ as well as other cellular differentiation and morphogenesis responses (e.g., adipocyte differentiation, tumor cell invasion)^{47,66} has been shown to occur within 3D matrices but not on 2D matrix surfaces, thus indicating the physiologic significance of the physical context of cell-matrix interactions. Additional studies are needed to further define which matrix features of the collagen ECM microenvironment including stiffness, degradability, and transport properties (permeability and diffusion) work independently and integrally to control vessel formation.

Collectively, this work provides the first detailed report of how the monomer/oligomer content of soluble collagens contributes to the assembly kinetics, fibril microstructure-mechanical properties, and cell instructive capacity of polymerizable collagen matrices. It is apparent that specific molecular-level polymerization reaction conditions, whether collagen assembly takes place in vivo or in vitro, define fundamental fibril-level properties, including fibril density, degree of interfibril branching, fibril diameter, fibril stiffness, and interstitial fluid phase viscosity. In turn, these fibril-level features dictate matrix-level physical, degradation, and functional properties.⁶⁷ Further elucidation of the hierarchical relationships of collagen assembly is essential to the rational design and standardization of polymerizable collagen formulations for in-vitro cell culture model development, regenerative medicine

strategies (e.g., ECFC-based cellular therapies), and engineered tissue constructs. Furthermore, insights gained from in-vitro collagen assembly may contribute to advancing the current understanding of how development and diversification of tissue-specific ECM fibril networks occurs in vivo.

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FIGURE 1.

PSC starting material (Lanes 1 and 4) as well as glycerol-derived oligomer- (Lanes 2 and 5) and monomer-rich (Lanes 3 and 6) fractions represent different molecular compositions of Type I collagen as demonstrated by SDS-PAGE (4%, Lanes 1–3) and Western blot (collagen $\alpha1(I)$ antibody, Lanes 4–5). In addition to $\alpha1(I)$, $\alpha2(I)$, $\beta11(I)$, $\beta12(I)$, and $\gamma(I)$ bands routinely observed in denatured collagen preparations, PSC contained prominent bands corresponding to molecular weights of 260 kDa (Oligo 260) and greater than 300 kDa (HMW). Oligo260 and HMW components (arrows) were retained at significant levels in the oligomer-rich fraction and found at substantially reduced levels in the monomer-rich fraction. Western analysis with an antibody specific for collagen $\alpha1(I)$ verified that Oligo260 and HMW bands contained the type I collagen epitope. Molecular weight markers are indicated in left margin.

FIGURE 2.

Representative turbidity plots (A) showing time-dependent changes in A405 during polymerization of monomer- (gray triangles) and oligomer-rich (black squares) fractions (0.7 mg mL^{-1} collagen concentration) derived from the glycerol-based protocol. Data was collected at 10-s intervals. To determine how polymerization kinetics varied with collagen concentration, formulations were polymerized using identical reaction conditions and collagen concentrations of 0.5, 0.7, and 1 mg mL^{-1} . Kinetic parameters, including half-time (B), lag time (C), and growth rate (D) were calculated from the resultant turbidity curves. Both single (squares) and batched (circles) PSC sources were used to generate monomer- (open shapes) and oligomer-rich (solid shapes) fractions. Data points represent mean $\pm$ SD ($n = 4$) with associated best-fit lines calculated for single and batched PSC sources collectively. In some cases, error bars are smaller than the data symbols. Best-fit lines obtained for monomer- (dashed lines) and oligomer-rich (solid lines) fractions were statistically different ($P < 0.05$) for all measured kinetic parameters. Oligomer-rich displayed the shortest polymerization half-time, shortest lag times, and fastest growth rates for each concentration tested ($P < 0.05$).

FIGURE 3.

Shear storage modulus (G' , A), phase shift (δ , B), and compressive modulus (E_c , C) for monomer- (open shapes, dashed lines) and oligomer-rich (solid shapes and lines) fractions prepared from single (squares) and batched (circles) PSC sources. Data points represent mean $\pm$ SD ($n = 4$) with associated best-fit lines. Monomer- and oligomer-rich fractions demonstrated statistically different ($P < 0.05$) best-fit lines for each of the measured mechanical parameters G' , δ , and E_c as a function of concentration.

FIGURE 4.

Polymerization kinetic parameters, including polymerization half-time (A), lag time (B), and growth rate (C), as measured via a turbidimetric assay for collagen formulations with AMW ranging from 282 to 603 kDa. Glycerol-derived monomer- and oligomer-rich fractions were generated from both single (squares) and batched (circles) PSC sources. These fractions were combined in different proportions to generate different AMW. Polymerization was conducted using identical reaction conditions and a collagen concentration of 0.7 mg ml^{-1} . Data points represent mean $\pm$ SD ($n = 4$). Polymerization half-time and lag time decreased significantly ($P < 0.05$) as AMW was increased from 282 to 306 kDa and then remained relatively constant for AMW up to 603 kDa. In contrast, growth rate increased monotonically with increasing AMW.

FIGURE 5.

Collagen fibril microstructure of matrices prepared with collagens with AMW ranging from 282 to 603 kDa. Representative CRM images are shown for matrices polymerized at the same collagen concentration (0.7 mg ml^{-1}) and under the same conditions. The 2D projections represent a total image thickness of $10 \mu\text{m}$ (101 slices, scale bar = $10 \mu\text{m}$). Low AMW collagens produced entanglements of lengthy fibrils and the distance between interfibril branch points appeared to decrease with increasing AMW. Quantified microstructure parameters and associated statistical analyses are summarized in Table I.

FIGURE 6.

Hierarchical matrix assembly for oligomer-rich collagen. Fibrils and their associated intermediates were negatively stained and observed by TEM early during the assembly process (A, scalebar = 500 nm). Longitudinal growth of larger fibrils was apparent as the branched network of microfibrils which were 5–25 nm in diameter coalesced at the tapered ends (A, black arrows). Lateral associations between growing fibril segments contributed to fibrils which were larger in diameter (up to 250 nm) and possessed identifiable branch points (A, white arrows). Branched fibrils were identified by the intermingling or entanglement of the molecular structure of adjacent fibrils, which was readily visible at higher magnifications. Fibril branching event identified by the white box in upper image is shown at higher magnification (B, scalebar = 3 μ m).

FIGURE 7.

Shear storage modulus (G' , A), phase shift (δ , B), and compressive modulus (E_c , C) for matrices prepared with collagen AMW ranging from 282 to 603 kDa. Both single (squares) and batched (circles) source formulations were polymerized using identical reaction conditions and a collagen concentration of 0.7 mg ml^{-1} . Data points represent mean $\pm$ SD ($n = 4$). G' and E_c increased linearly while δ decreased with increasing AMW.

FIGURE 8.

Similar trends in polymerization half-time (A), fibril density (B), and viscoelastic properties (C–E) as a function of AMW were observed with monomer- and oligomer-rich fraction generated by differential salt precipitation of PSC. Data points represent mean $\pm$ SD ($n = 3$).

FIGURE 9.

Tuning AMW or monomer/oligomer content of 3D collagen matrices directed different cellular and vessel morphogenesis responses by cultured ECFCs. ECFCs (5×10^5 cells ml^{-1}) were cultured for 7 days within matrices prepared with monomer- (A and C) or oligomer-rich (B and D) collagen formulations. Matrices were matched in terms of collagen concentration (1 mg ml^{-1} , A and B) or G' (200 Pa, C and D). Constructs were stained with FITC-conjugated UEA1 lectin (green) and DRAQ5 nuclear stain (red) and imaged in combined fluorescence and reflection modes. Panels A–D represent z-stack projections (21 slices at $5 \mu\text{m}$ thickness each) showing combined lectin and nuclear staining. Insert in lower left corner of Panel D represents a cropped single slice image showing both lectin and collagen fibril microstructure to document that multi-cellular, capillary-like networks possessed lumens. Scale bar = $100 \mu\text{m}$.

FIGURE 10.

Modulation of matrix stiffness (G') and collagen concentration of 3D matrices prepared from monomer- and oligomer-rich collagens induced formation of different vacuole densities (D), vacuole areas (E), and total vacuole areas (F) by entrapped ECFCs. ECFCs (5×10^5 cells ml^{-1}) were cultured for 2 days within monomer and oligomer matrices that were matched in collagen concentration (1 or 2.75 mg ml^{-1}) or matrix stiffness (136 Pa). Panels A through C provide representative images of ECFC-derived vacuolated structures observed within toluidine blue stained tissue constructs. Inserts in bottom right corner depict the cell membrane (black) and vacuoles (white areas surrounded by black cell borders). Scale bar = 50 μm .

Table 1

Summary of Microstructural Properties of 3D Matrices Prepared with Collagen Formulations of Varying AMW or Monomer/Oligomer Content

Collagen AMW (kDa)	Fibril Diameter (nm)	Fibril Density (%)	Pore Size (μm^2)
282	$370 \pm 50^{\text{A}}$	$8.1 \pm 2.6^{\text{D}}$	$15.1 \pm 13.8^{\text{A}}$
306	$352 \pm 31^{\text{B}}$	$11.5 \pm 2.3^{\text{C}}$	$2.7 \pm 1.4^{\text{B}}$
424	$349 \pm 31^{\text{B}}$	$13.6 \pm 1.7^{\text{A,B}}$	$1.9 \pm 0.8^{\text{B}}$
521	$377 \pm 29^{\text{A}}$	$14.6 \pm 1.6^{\text{A}}$	$1.7 \pm 0.7^{\text{B}}$
603	$341 \pm 32^{\text{B}}$	$13.1 \pm 2.3^{\text{B}}$	$1.8 \pm 0.8^{\text{B}}$

All matrices were polymerized under similar conditions at 0.7 mg ml^{-1} collagen concentration. Letters indicate statistically different groups for each parameter based upon Tukey-Kramer range testing ($P < 0.05$).