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Oligomers Modulate Interfibril Branching and Mass Transport Properties of Collagen Matrices

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Abstract

Mass transport within collagen-based matrices is critical to tissue development, repair, and pathogenesis as well as the design of next generation tissue engineering strategies. This work shows how collagen precursors, specified by intermolecular cross-link composition, provide independent control of collagen matrix mechanical and transport properties. Collagen matrices were prepared from tissue-extracted monomers or oligomers. Viscoelastic behavior was measured in oscillatory shear and unconfined compression. Matrix permeability and diffusivity were measured using gravity-driven permeametry and integrated optical imaging, respectively. Both collagen types showed an increase in stiffness and permeability hindrance with increasing collagen concentration (fibril density); however, different physical property-concentration relationships were noted. Diffusivity wasn't affected by concentration for either collagen type over the range tested. In general, oligomer matrices exhibited a substantial increase in stiffness and only a modest decrease in transport properties when compared to monomer matrices prepared at the same concentration. The observed differences in viscoelastic and transport properties were largely attributed to increased levels of interfibril branching within oligomer matrices. The ability to relate physical properties to relevant microstructure parameters, including fibril density and interfibril branching, is expected to advance the understanding of cell-matrix signaling as well as facilitate model-based prediction and design of matrix-based therapeutic strategies.

Keywords

collagen matrix; oligomer; monomer; mass transport; permeability; diffusivity

INTRODUCTION

The architecture and material properties of the structural elements (e.g., collagens) that make up natural and engineered tissues determine physical attributes of the cellular microenvironment, including adhesion site distribution, spatial constraints, matrix

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viscoelasticity, fluid and solute transport, and biodegradability. It is now apparent that these physical features of cell-matrix interactions, together with traditional biochemical signaling modalities, work synergistically to direct fundamental cell behaviors as well as more complex tissue processes (Gjorevski *et al.*, 2010, Discher *et al.*, 2009). To date, there has been considerable focus in defining how collagen microstructure and fluid components of the extracellular matrix (ECM) contribute to the mechanical properties of tissues and tissue equivalents as well as associated mechanisms of energy storage, transfer, and loss (Sacks *et al.*, 2003, Sander *et al.*, 2008, Silver, 2008, Roeder *et al.*, 2009, Susilo *et al.*, 2010). Less is known regarding the manner in which specific attributes of the collagen fibril matrix regulate interstitial mass transport, despite the fact that fluid transport has been identified as a major determinant of cell survival and fate. This gap in understanding of hierarchical structure-function relationships precludes the rational design of collagen-based microenvironments for research and regenerative medicine strategies, including engineered tissue constructs, implantable biomaterials, and vehicles for controlled delivery of therapeutic molecules or cells.

Mass transport within tissues in vivo is facilitated by both hydraulic permeability and solute diffusivity. Permeability (K , units of m^2) measures the ease by which fluids flow under an applied pressure gradient through the porous structure. Experimental approaches routinely used for quantifying permeability involve the application of a pressure gradient to tissue explants or reconstituted polymeric matrices with defined dimensions and determination of the average flow rate. With this measured flow rate, permeability can be calculated according to Darcy's law. One of the few studies to quantify permeability in collagen-based matrices demonstrated that cell-induced contraction of collagen matrices decreased permeability from its nominal levels by 2–3 orders of magnitude (Gentleman *et al.*, 2004).

The transport of nutrients and waste products within tissues is also influenced by matrix diffusivity (D , units of m^2/s), which quantifies the random motion of solutes driven in response to concentration gradients. Various experimental and theoretical approaches have been developed to quantify solute diffusivity in collagen-based tissues and constructs. In addition to dye penetration methods (Deorosan *et al.*, 2011), two of the most widely used methods include fluorescent recovery after photobleaching (FRAP; (Axelrod *et al.*, 1976)) and integrative optical imaging (IOI; (Nicholson *et al.*, 1993)). The FRAP method involves uniform distribution of a dilute fluorescent tracer within the specimen. A well-defined concentration gradient then is induced by laser-induced photobleaching and relaxation of the tracer concentration profile is analyzed to yield the diffusion coefficient. Alternatively, IOI involves creation of a point source of fluorescent tracers via microinjection followed by image-based measurement of their temporal and spatial distribution.

Collectively, these experimental methods, together with the development of predictive models, have assisted in the identification of a number of compositional and structural parameters that modulate tissue mass transport including collagen concentration (Levick, 1987, Netti *et al.*, 2000), charged species (e.g., glycosaminoglycan and proteoglycan) content (Levick, 1987), as well as porosity (volume not occupied by solid fibrils divided by sample specimen volume; (O'Brien *et al.*, 2007)), fibril radius (Levick, 1987), and tortuosity (Maroudas, 1970).

The ECM of tissues represents a composite material consisting of a collagen fibrous meshwork surrounded by an interstitial fluid. For the majority of tissues, fibrillar type I collagen serves as the primary structural and mechanical determinant. Collagen fibrils exhibit a hierarchical organization defined by the staggered assembly and intermolecular cross-linking between triple-helical collagen molecules or monomers. Other collagen types (e.g., type III), elastin, and various accessory molecules also contribute to the tissue

specificity of these fibrous networks by influencing the molecular packing and therefore fibril morphology. Interspersed among the insoluble fibril component are various glycosaminoglycans, proteoglycans, and other soluble molecules (e.g., growth factors). Negative charges associated with glycosaminoglycan and proteoglycan components work to define the water content and viscosity of the interstitial fluid as well as its interaction with solid fibril components. In large part, tissue diffusivity and permeability are determined by the molecular composition and collagen fibril architecture of the ECM, and therefore values vary widely amongst tissues (Levick, 1987, Swartz *et al.*, 2007). Furthermore, variation in ECM composition and structure within an individual tissue or organ contributes to anisotropic and inhomogeneous transport behavior (Travascio *et al.*, 2009).

Collagen intermolecular cross-links have been implicated in the diversification of tissue microstructure and physical properties, collagen metabolism, and therefore tissue-specific cellular function (Knott *et al.*, 1997, Eyre *et al.*, 2005). In fact, intermolecular cross-links, which are formed during collagen assembly via an enzyme (lysyl oxidase) mediated process, represent chemistries and distributions that are tissue specific. Different extraction and purification procedures can be applied to tissues to yield collagen formulations that are primarily composed of either monomers (single collagen molecules) or oligomers (at least two collagen molecules joined by a covalent cross-link; (Kreger *et al.*, 2010, Bailey *et al.*, 2011)). In fact, we recently documented that stable, mature cross-links or oligomer precursors not only alter assembly kinetics but also substantially stiffen and strengthen polymerized collagen matrices (Kreger *et al.*, 2010, Bailey *et al.*, 2011). Qualitative evaluation of the fibril microstructure, as imaged using confocal reflection and transmission electron microscopy, suggested that the observed improvement in mechanical integrity over conventional monomer matrices was due to enhanced interfibril branching without correlative effects on fibril density or diameter (Kreger *et al.*, 2010, Bailey *et al.*, 2011). Unfortunately, at the time no reliable or direct methods existed for quantifying and comparing the number of interfibril branches within matrices. Oligomer-dependent changes in the collagen fibril microstructure and physical properties were also found to have important biological consequences on lineage specific differentiation of multipotential mesenchymal stem cells (Kreger *et al.*, 2010) and vessel morphogenesis by endothelial colony forming cells (Critser *et al.*, 2010, Bailey *et al.*, 2011).

The present study extends this work by defining how specific collagen building blocks, namely monomers and oligomers, affect interstitial transport properties of polymerized matrices. More specifically, using a new 3D microstructural analysis technique in which interfibril branch points and fibril length can be quantified, this work demonstrates for the first time that, in addition to fibril density, interfibril branching or connectivity is an important fibril microstructure determinant of both hydraulic permeability and molecular diffusivity. The ability to relate transport properties to relevant collagen fibril microstructure parameters is expected to advance the understanding of cell-matrix signaling as well as facilitate model-based prediction and design of matrix-based therapeutic strategies and functional engineered tissues.

MATERIALS AND METHODS

Preparation of Purified Monomeric and Oligomeric Collagens

All type I collagens were derived from the dermis of market weight pigs. Oligomeric collagen was prepared as described previously (Kreger *et al.*, 2010). Monomer-rich collagen was prepared by extracting pig skin with 0.5 M acetic acid followed by salt precipitation, which selectively eliminates oligomers from solution (Brennan *et al.*, 1980). Purified oligomeric and monomeric collagens were lyophilized from 0.1M acetic acid solutions. Lyophilized collagens were dissolved in 0.01 N HCl. Collagen concentration was

determined using a Sirius Red (Direct Red 80) assay as previously described (Brightman *et al.*, 2000).

The molecular composition of collagen formulations was assessed as described previously (Kreger *et al.*, 2010, Bailey *et al.*, 2011). In brief, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for detection of non-collagenous proteins. SDS-PAGE in interrupted and uninterrupted formats 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). Sulfated glycosaminoglycan (GAG) content was measured using an established alcian blue assay.

Preparation of 3D Collagen Matrices

All collagens were polymerized under identical reaction conditions to produce 3D matrices as described previously (Pizzo *et al.*, 2005). Collagen solutions were diluted with 0.01 N HCl and neutralized with 10X phosphate buffered saline (PBS, 1X PBS had 0.17 M total ionic strength and pH 7.4) and 0.1 N sodium hydroxide to achieve neutral pH (7.4). Neutralized collagen solutions were kept on ice prior to the induction of polymerization by warming to 37°C. Due to the increased viscosity of collagen solutions, positive displacement pipettes (Microman, Gilson, Inc., Middleton, WI) were used to accurately pipette all collagen solutions.

Analysis of Collagen Fibril Microstructure

Collagen matrices were polymerized at desired collagen concentrations or stiffness (shear storage modulus) in Lab-Tek chambered coverglass slides (Nunc, Thermo Fisher Scientific, Rochester, NY), fixed with 4% paraformaldehyde in phosphate buffered saline, and blocked with 1% bovine serum albumin. Matrices then were immunostained with monoclonal anti-collagen type I (Sigma-Aldrich, St. Louis, MO) followed by AlexaFluor546 donkey anti-mouse secondary antibody (Life Technologies, Grand Island, NY). Confocal microscopy 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). Images of the fibril microstructure were collected in combined reflection (fibril-based back-scattered light) and fluorescence (immunolabeled type I collagen) modes. Image stacks (52.736 $\mu\text{m} \times 52.736 \mu\text{m} \times 10 \mu\text{m}$; 101 slices at 0.10 μm thickness per slice) were collected from at least three random locations within each of three independent matrices per matrix formulation at a distance of 10–30 μm from the bottom of the coverglass (Supplementary Movies 1A and 1C). Image files of merged reflection/fluorescence (Figure 1A) were imported into Imaris (Bitplane, St. Paul, MN) for 3D reconstruction. Image signals were merged, processed, and thresholded (Figure 1B) prior to being analyzed using Filament Tracer software to generate filamentous structures that approximated the fibril architecture of the matrices (Figure 1C). Filament Tracer provided measurements for total fibril length, average fibril length, and number of interfibril branches. Fibril volume fraction (fibril density) was calculated by dividing the total fibril volume by the total image volume. Fibril diameter measurements were made using an Imaris measurement tool as described previously (Kreger *et al.*, 2010).

Oscillatory Shear and Unconfined Compression Testing

Viscoelastic properties of polymerized collagen matrices were measured in oscillatory shear and unconfined compression formats on a stress-controlled AR2000 rheometer (TA Instruments, New Castle, DE) using a stainless steel 40-mm diameter parallel plate geometry as previously described (Kreger *et al.*, 2010). Viscoelastic property-collagen concentration relationships were generated for four different monomer and oligomer

preparations (N=4). Each sample was polymerized (30 minutes) on the rheometer to standardize sample geometry and adherence to plates. A shear strain sweep from 0.01% to 5% strain at 1 Hz (chosen from predetermined linear viscoelastic response regions) was used to measure shear storage modulus (G') and shear loss modulus (G'' ; reported values are at 1% strain). For compressive testing, the normal force was measured in response to depressing the geometry at a rate of 20 $\mu\text{m/s}$ (strain rate 2.76%/s). Stress-strain plots were generated for each sample, with compressive strain calculated as $1 - L/L_o$ (Cauchy or engineering strain, L = height, and L_o = initial height) and stress calculated as normal force divided by plate area. The compressive modulus (E_c) was calculated using linear regression of the slope of the stress-strain curve from ~20 to 60% strain. Shear and compression testing was performed on three independent matrices per matrix formulation (n=3).

Permeability Analysis

Permeability of collagen matrices prepared within an open cell scaffold (26) was measured using a custom-designed, gravity-driven permeameter. Collagen solutions were polymerized (30 minutes at 37°C) within disks (0.125" thick, 0.5" diameter) of hydrophilic polyethylene foam (15–45 μm pore size; Small Parts Incorporated, Miami, FL). Silicone rings fitted around the polyethylene disks formed a tight seal between the disk and walls of the permeameter. A specified volume (approximately 15 mL) of PBS, pH 7.4 was added on top of each collagen/polyethylene composite and fluid flow monitored over time. Permeability measurements for each collagen formulation were conducted at 37°C for 8 to 10 independent matrices (n=8–10). Permeability for the collagen/polyethylene composite ($K_{\text{collagen+PE}}$) was calculated based on Darcy's Law:

$$K_{\text{collagen+PE}} = \frac{Q\mu L}{A\Delta P}$$

where Q is the time-averaged volumetric flow rate, A and L are the cross sectional area and thickness of the composite disk, P is the pressure gradient across the disk, and μ is the viscosity of the fluid. The pressure gradient was calculated from the height of the fluid column above the membrane. Permeability of the collagen matrix alone (K_{collagen}) was calculated using,

$$K_{\text{collagen}} = K_{\text{collagen+PE}} * (1 - \alpha_{PE})$$

where α_{PE} represents the volume fraction of the polyethylene foam. α_{PE} was measured using ImagePro software (Media Cybernetics, Bethesda, MD) by applying a contrast-threshold mask to images of thin cryotome sections (30 μm) of the polyethylene foam.

Diffusivity Analysis

Diffusivity within collagen matrices was measured for fluorescein (FITC) molecules and FITC-conjugated dextrans (10kDa, 40kDa, 500kDa, and 2MDa MW; Molecular Probes, Carlsbad, CA) using established IOI and FRAP methodologies.

IOI involved injection of fluorescent tracer (25–50 pL) into a polymerized matrix using the MINJ-D-BDCI microinjection system (Tritech, Los Angeles, CA). Immediately following injection, the temporal and spatial changes in fluorescent intensity were monitored with a CCD camera at 1 second intervals. The intensity profiles were fit to an analytic solution of diffusion from a point source to obtain an estimated value of the diffusion coefficient,

$$I(r, \gamma_i) = E_i(\gamma_i) \exp[-r/\gamma_i]^2$$

Here, E_i is an amplitude term compensating the de-focused point spread function, r represents the radius, and the term γ_i is given by,

$$\gamma_i^2 = 4D(t_i + t_o)$$

The measured intensity profile at each time step was curvefit for γ_i and subsequently the diffusion coefficient, D , was determined. In order to compensate the finite size of the injection, a virtual time, t_o , was added while evaluating the diffusion coefficient D . Diffusion coefficients were measured based on at least 5 replicate analyses ($n = 5$) within each of at least 3 independent samples ($N = 3$) for all cases.

FRAP measurements involved the addition of fluorescent tracer at 5 nM final concentration to neutralized collagen solutions prior to polymerization. A volume of collagen (20 μ l) was aliquoted onto a microscope slide, covered with a glass coverslip, and polymerized at 37°C for approximately 30 minutes. Resulting matrices were 40–100 μ m thick. Each sample then was subjected to brief localized 488 nm irradiation with a krypton-argon laser using a FluoView confocal system (Olympus America, Central Valley, PA) connected to a TE2000 inverted microscope (Nikon, Tokyo, Japan), which resulted in bleaching of fluorescence within a focal spot. Images were collected every 2.7 seconds as the bleached spot recovered fluorescence. Matrices polymerized with FITC alone or small FITC-conjugated dextrans (10 kDa, 40 kDa) were viewed at 20x magnification and the diameter of the bleached region was 90 μ m. Matrices embedded with large FITC-conjugated dextrans (500 kDa, 2 MDa MW) were observed at 60x magnification and the diameter of the bleached region was 30 μ m. The average pixel intensity as a function of radius was monitored over time for up to 20 different positions identified on concentric rings made around and within the bleached region and normalized as described previously (Axelrod *et al.*, 1976). A transient diffusion model based on Fick's Second Law,

$$\frac{\partial C}{\partial t} = D_{P,M} \frac{\partial^2 C}{\partial x^2}$$

was analyzed in COMSOL at specified diffusivity values, where C is the concentration of the diffusing species, t is time, x is position (distance), and $D_{P,M}$ is the diffusion coefficient, which is a function of both the diffusing species and the medium. Application of this model to the diffusive transport of fluorescent molecules in this experiment involved the following assumptions: 1) molecular transport occurs in a stationary fluid through a homogeneous continuum with constant diffusivity; 2) diffusing species is present at a very low concentration; and 3) diffusing species is passively contained within the collagen matrix (Lehner, 1979). Diffusivities based on experimental FRAP data were determined from model data through minimization of least squared residuals. Measurements were made in 5 to 10 regions for at least two independent matrices for each collagen formulation.

Statistical Analysis

All measured values are reported as mean $\pm$ SD. Statistical analyses were performed using SAS v. 9.2 (SAS Institute, Cary, NC). The general linear model procedure was used to

conduct analysis of variance (ANOVA) and perform multiple comparisons of least squares means using the Tukey-Kramer method. Pairwise comparisons were performed using two-tailed t-test assuming unequal variances. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Collagen matrix transport properties depend on both fibril density and interfibril branching

Traditionally, confocal reflection microscopy has been used to visualize the three-dimensional (3D) fibril microstructure of polymerized collagen matrices since it requires no sample processing or staining (Voytik-Harbin *et al.*, 2001). Such images have provided the basis for quantification of a subset of microstructure parameters including fibril volume fraction and fibril diameter (Kreger *et al.*, 2009) and even fibril-level strains (Roeder *et al.*, 2009). Unfortunately, image quality is often insufficient for reliable detection of other relevant microstructure features such as interfibril branch points and fibril length (Bailey *et al.*, 2011). Here, immunofluorescence and confocal reflection signals were merged to produce high quality 3D reconstructed images with improved signal to noise ratio and fibril boundary definition (Supplementary Movies 1B and 1D). Filament tracer software then could be applied for improved detection and a more detailed analysis of the fibrous microstructure. In this way, monomer and oligomer matrices produced at matched collagen concentrations (1.5 mg/ml) or stiffness (292 Pa) were independently compared to determine how differences in fibril microstructure contribute to differences in mechanical and transport properties. Figure 1 shows the strong similarity between the fibril architecture visualized using confocal microscopy and that detected by the filament tracer program. From this analysis, an expanded set of fibril microstructure parameters including fibril volume fraction, average segment length, number of branch points, and number of branch points per total fibril length were readily quantified and compared as shown in Figures 2A–D. Results show that as monomer concentration increased from 1.5 mg/ml to 3.1 mg/ml, fibril volume fraction increased 1.6-fold from about 20% to 32% ($p < 0.05$). This positive correlation between collagen concentration and fibril density is consistent with previous reports (Roeder *et al.*, 2002, Kreger *et al.*, 2010, Bailey *et al.*, 2011). Although there was a slight increase in the total number of branch points at a higher concentration of 3.1 mg/ml monomer compared to 1.5 mg/ml, it was not statistically significant, and there was little to no change in the number of branch points per total fibril length or average fibril segment length. On the other hand, comparison of monomer and oligomer matrices prepared at matched concentrations showed no difference in the fibril volume fraction, but oligomer matrices possessed a greater number of total interfibril branch points ($p < 0.05$), a greater number of interfibril branch points per total fibril length ($p < 0.05$), and a lower average fibril segment length ($p < 0.05$). Comparison of monomer and oligomer matrices prepared at matched stiffness (292 Pa) showed that monomer matrices had a significantly greater fibril density ($p < 0.05$) and a significantly lower number of total interfibril branch points, and interfibril branch points per total fibril length ($p < 0.05$). Fibril diameters were statistically similar for monomer and oligomer matrices prepared at matched concentration or stiffness, which is consistent with our previous findings (Roeder *et al.*, 2002, Kreger *et al.*, 2010, Bailey *et al.*, 2011).

Crosslink content impacts viscoelastic behavior of collagen matrices

Traditional collagen monomer preparations are routinely produced by acetic acid extraction of tissues followed by salt precipitation. Alternatively, extraction methods involving citric acid yield oligomer-rich formulations, which are characterized by a significantly greater intrinsic viscosity (average molecular weight) and intermolecular cross-link content (Bailey *et al.*, 2011, Kreger *et al.*, 2010). Upon polymerization, monomer and oligomer collagens exhibit different molecular assembly kinetics and mechanisms (Kreger *et al.*, 2010, Bailey *et*

al., 2011). This was evident from the fact that monomer and oligomer collagens yielded dramatically different matrix shear storage modulus (G' , stiffness) versus collagen concentration relationships as shown in Figure 3A. Results showed that oligomer matrices were significantly stiffer ($p < 0.05$) than monomer matrices polymerized at the same collagen concentration of 1 mg/ml or greater. Oligomer matrices also covered a much broader range of G' values, 24.9 to 971.6 Pa, compared to 7.5 to 313.0 Pa for monomer matrices.

Matrix viscoelasticity was also measured in unconfined compression. Compression properties are dependent upon hydraulic permeability and involve rate measurement of fluid extrusion from materials subjected to compression. In the case of collagen-based matrices, compressive loads are supported primarily by the interstitial fluid phase. Consistent with our previous reports, unconfined compression curves were biphasic in nature with distinguishable behavior noted within low-strain and high-strain regions (Kreger *et al.*, 2010). Here, compressive stiffness (E_c) was measured in the linear, low strain region to demonstrate the effects of collagen concentration and intermolecular cross-link content. At all concentrations, oligomer E_c was significantly higher ($p < 0.05$) than monomer values (Figure 3B). In addition, oligomer matrices covered a broader range of E_c values, 5.62 kPa–31.9 kPa, compared to 1.0 kPa–9.4 kPa for monomer matrices.

Increasing collagen concentration or intermolecular cross-link content decreases matrix permeability

Measurement of Darcy permeability for oligomer and monomer matrices prepared at different concentrations showed that permeability decreased with increasing concentration for both formulations (Figure 4A). The observed inverse relationship between collagen concentration and permeability is largely owing to the associated increase in fibril density (Kreger *et al.*, 2010). Monomers were more soluble than oligomers largely owing to their smaller molecular size. As such, monomers could be reliably tested at concentrations up to 15 mg/ml while the maximum oligomer concentration tested was 6.3 mg/ml. Permeability of monomer matrices prepared at 1.5–10 mg/ml decreased from $3.1 \mu\text{m}^2$ to $0.43 \mu\text{m}^2$ and then plateaued. There was no significant difference ($p > 0.05$) between 10 mg/mL and 15 mg/mL monomer matrices with permeability values of $0.43 \pm 0.19 \mu\text{m}^2$ and $0.32 \pm 0.21 \mu\text{m}^2$, respectively. Permeability values for oligomer matrices prepared at 0.7 to 4.4 mg/ml progressively decreased from $2.0 \mu\text{m}^2$ to $0.32 \mu\text{m}^2$. Permeability plateaued for oligomer concentrations of 4.4 mg/ml and above. There was no significant difference ($p > 0.05$) in permeability measurements for matrices prepared between 4.4 mg/mL and 6.3 mg/mL matrices.

In addition to collagen concentration (fibril density), intermolecular crosslink content was found to affect permeability of matrices. In fact, the relationships between permeability and concentration were significantly different for monomer and oligomer when analyzed statistically using a polynomial regression ($p < 0.05$; Figure 4A). When matched at concentrations of less than 10 mg/ml, monomer matrices were significantly more permeable than oligomeric matrices ($p < 0.05$). More specifically, 1.5 mg/ml monomer matrices were about 3 times more permeable than oligomer matrices prepared at the same concentration. Interestingly, when permeability was plotted as a function of matrix stiffness (G'), there was no significant difference between monomer and oligomer matrices (Figure 4B). Permeability values decreased as stiffness increased from 40 Pa to 200 Pa, and then plateaued.

Cross-link content but not concentration affects matrix diffusivity

Diffusivity of FITC-dextran of molecular weights 10 kDa, 40 kDa, 500 kDa, and 2 MDa was measured in monomer and oligomer matrices prepared at 1, 3, and 5 mg/ml. Free diffusivity was measured in matrices prepared with a low collagen concentration (0.3 mg/

ml). Here, an inverse relationship between molecular size and free diffusivity was observed and ranged from $22.2 \times 10^{-11} \text{ m}^2/\text{s}$ to $2.4 \times 10^{-11} \text{ m}^2/\text{s}$ for 10 kDa to 500 kDa FITC-dextran particles. These free diffusivity values were similar to those reported previously by Nicholson and co-workers, where IOI measurements were made in 0.3% agar gels (Nicholson *et al.*, 1993). An inverse relationship between molecular size and diffusivity was also observed for both matrix types at all measured concentrations (Figure 5). There were no significant differences observed between diffusivity values for monomer and oligomer matrices prepared at 1.5 mg/ml (Figure 5A). At 3 mg/ml, diffusivity was significantly ($p < 0.05$) higher in monomer matrices for 10 kDa and 40 kDa dextrans while no statistical differences were noted for 500 kDa and 2 MDa dextrans (Figure 5B). The general tendency for monomer to show greater diffusivity compared to oligomer continued to be observed for matrices prepared at 5 mg/ml (Figure 5C), where p-values of 0.04, 0.006, and 0.06 were observed for molecular sizes of 10 kDa, 40 kDa, and 2 MDa respectively. It is also important to note that collagen concentration had no significant effect on diffusivity values for any molecular size dextran within either collagen type over the range tested. Similar diffusivity values and corroborating results were obtained using FRAP measurement techniques (data not shown).

DISCUSSION

Fluid and solute transport within tissues, implantable collagen-based biomaterials, and engineered tissue constructs is important for many research and clinical applications. Fluid transport plays a critical role in guiding tissue morphogenesis and repair, maintenance of tissue structure-function, and the pathogenesis of several diseases (e.g., cancer). It does so by modulating the cell microenvironment through 1) delivery of nutrients and waste removal 2) spatial and temporal distribution of soluble factors (e.g., growth factors), and 3) physical cues (e.g., shear forces). In addition, mass transport has been recognized as an important design consideration for local, biomaterial-based delivery of therapeutic cells or molecules (e.g. bioactive factors, antibodies). Previous studies have documented that convective and diffusive transport within collagenous-based tissues and matrices depends on a number of factors including microstructure parameters, matrix composition, and extent exogenous cross-linking (Ramanujan *et al.*, 2002, O'Brien *et al.*, 2007, Stewart *et al.*, 2009). Here we show that the inclusion of intermolecular cross-links as a component of the fundamental collagen polymer building blocks affects the functional viscoelastic and transport properties of polymerized matrices in large part by regulating the hierarchical assembly and interfibril branch formation. As such, addition of oligomers and their associated intermolecular cross-links provides a new means to independently control viscoelastic and transport properties of polymerizable collagen matrices. Furthermore, a new 3D fibril microstructure analysis approach facilitated quantification and comparison of fibril-level parameters and ultimately identification of relationships between fibril microstructure and functional matrix physical properties.

Results showed that experimentally measured permeability values decreased with increasing collagen concentration (fibril density) for both monomer and oligomer matrices. This observed inverse correlation between permeability and collagen content is consistent with previous data collected for several intact tissues (Levick, 1987) and purified collagen matrices (Ramanujan *et al.*, 2002). Permeability values for monomer matrices ranged from $3 \mu\text{m}^2$ to $0.32 \mu\text{m}^2$ over a concentration range of 1.5 to 15 mg/ml, while values for oligomer matrices ranged from $2 \mu\text{m}^2$ to $0.32 \mu\text{m}^2$ for matrices prepared at 0.7 to 6.3 mg/ml. In general, monomer matrices were more permeable than their oligomer counterparts when prepared at matched collagen concentrations up to 6 mg/ml. As concentrations were increased further, permeability values plateaued at $0.32 \mu\text{m}^2$ for both matrix types. Comparison of compressive modulus values for the two collagen types, another routine

measure of hydraulic permeability (Serpoooshan *et al.*, 2010), corroborated findings that monomer and oligomer matrices exhibited different permeability-concentration profiles.

Measured permeability values for both collagens were within the range of previous published values for polymerized collagen matrices. Ramanujan reported values in the range of $0.01\text{--}1\times 10^{-3}\text{ }\mu\text{m}^2$ for collagen matrices prepared at 10–40 mg/ml with commercial pepsin-digested collagen (atelocollagen; (Ramanujan *et al.*, 2002)). The higher collagen concentrations used in this study were achieved by ultracentrifugation of atelocollagen solutions. It should be noted that atelocollagen represents a monomer form of collagen in which telopeptide ends have been removed. Telopeptide regions are known to be important in collagen assembly (Kuznetsova *et al.*, 1999) and are present in the pig skin monomer and oligomer formulations used in the present study. Measured permeability values for pig skin monomer and oligomer collagens were also in agreement with published work by Gentleman and co-workers, who reported permeability values of $35\text{ }\mu\text{m}^2$ for 10 mg/ml rat tail monomer matrices (Gentleman *et al.*, 2004). Permeability values for purified collagen matrices were generally higher than those reported for intact tissues, including cortical bone ($2.7\text{--}7.9\times 10^{-2}\text{ }\mu\text{m}^2$; (Li *et al.*, 1987)) and meniscus ($0.36\text{--}1.3\times 10^{-6}\text{ }\mu\text{m}^2$; (Proctor *et al.*, 1989)), and the ECM component of small intestinal submucosa ($1.7\times 10^{-5}\text{ }\mu\text{m}^2$; (Beatty *et al.*, 2002)). Such discrepancies are expected since intact tissues generally have a higher collagen content (>40 mg/ml) and other matrix components (e.g., glycosaminoglycans and proteoglycans) known to affect permeability. Permeabilities for polymerized collagen were closer to values reported for tissue microenvironments consistent with tissue repair and development (e.g., blood clot, $K\approx 1\text{--}100\text{ }\mu\text{m}^2$; fibrin matrix, $K\approx 1\text{--}100\text{ }\mu\text{m}^2$; (Diamond, 1999)).

Differences were also identified between monomer and oligomer matrices in terms of molecular-level transport or diffusivity. Diffusivity values for monomer and oligomer matrices decreased with molecular radius and ranged from about $100\text{ }\mu\text{m}^2/\text{s}$ for 10 KDa dextran to $10\text{ }\mu\text{m}^2/\text{s}$ for 2 MDa dextran. These values were similar to previously reported measurements for polymerized atelocollagen matrices, polymerized fibrin matrices, and cartilage tissue (Table 1). Molecular diffusivity for all particle sizes tested was statistically similar for low collagen concentration (1 mg/ml) monomer and oligomer matrices. However, oligomer matrices were found to offer progressively greater hindrance to diffusion as collagen concentration was increased. Although diffusivity values showed no significant dependence on collagen concentration for either collagen type, a gradual decline in diffusivity values was observed as collagen concentration was increased from 1 to 5 mg/ml. Inverse relationships between diffusivity and the collagen content of tissues and matrices have been reported previously (Ramanujan *et al.*, 2002, Erikson *et al.*, 2008).

Although monomer matrices generally showed higher permeability and diffusivities compared to oligomer matrices over the concentration range tested, measured values for both collagen types fell well within the physiological range. The different transport property-collagen concentration profiles identified for the two collagen precursors provided further evidence that their molecular assembly mechanisms and therefore fibril microstructures were different. In fact, transport property measurements have traditionally been used to probe fibrous structures for purposes of estimating specific microstructure features including pore size and fibril/fiber diameter (Diamond, 1999). As part of the present work, a detailed quantitative analysis of the 3D fibril microstructures was performed to further define structure-function relationships. A modified confocal imaging approach, where collagen type I immunofluorescence and reflected light signals were merged, provided high resolution images with improved signal to noise ratio and fibril boundary definition. Filament tracer software then could be applied for detection and quantitative analysis of an expanded set of microstructure parameters including fibril volume fraction,

total fibril length, number of interfibril branches, fibril diameter, and average segment length.

Detailed microstructure analysis showed that monomer and oligomer matrices prepared at matched concentration had similar fibril volume fractions (fibril density); however, oligomer matrices had a substantially greater number of interfibril branches. On the other hand, when matched on matrix stiffness, oligomer matrices exhibited significantly lower fibril densities and greater number of interfibril branch points. Interfibril branches serve to substantially enhance matrix stiffness and strength independent of fibril density. In fact, oligomer matrices are generally 4 times stiffer than their monomer counterparts and cover a much broader range of matrix stiffness values. Collectively, these results illustrate that naturally-occurring intermolecular cross-links represent another physiologically-relevant collagen assembly parameter that can be systematically controlled independent of collagen concentration to tune and improve matrix stiffness properties without compromising interstitial transport. Typically, the high interconnected matrix/scaffold microstructures necessary to maintain sufficient mechanical integrity of other biomaterials limit porosity and therefore cell viability and function. Interestingly, the microstructure-physical properties of oligomer matrices have been shown to offer improved cell instructive capacity over traditional monomer formulations in terms of matrix-guided differentiation of multipotential mesenchymal stem cells in vitro (Kreger *et al.*, 2010) and matrix-induced ECFC vessel formation and persistence in vitro and in vivo (Critser *et al.*, 2010, Bailey *et al.*, 2011).

Several predictive methods have been developed to relate specific microstructure features to fluid transport, specifically permeability, through porous medium and include those based on hydraulic radius theories (flow through channels) or drag theories (flow around solid objects). Both the Carmen-Kozeny equation (hydraulic radius theory; (Carman, 1937)) and the Happel equations (drag theory; (Happel *et al.*, 1965)) have been used to relate permeability to specific microstructure features (Serpooshan *et al.*, 2010). Both of these approaches are based on fibril volume fraction (pore volume fraction) and fiber radius. The Kozeny model also includes a correction factor or constant to account for pore shape and tortuosity. In terms of diffusivity, the movement of solutes in free solution routinely is estimated using the Stokes-Einstein equation. However within tissues or engineered matrices/scaffolds diffusivities may be altered significantly by steric hindrance due to the microstructure of the porous medium and/or solute-medium interactions. As such predictive relationships have been developed to relate matrix diffusivities to fibril volume fraction alone (Ogston *et al.*, 1973) or in combination with fibril radius (Kosto *et al.*, 2004). In fact, current models would be unable to predict the observed differences in measured transport properties between monomer and oligomer matrices since they do not account for effects of interfibril branching. Interesting, when permeability was plotted as a function of stiffness the two curves converged implying that stiffness, which is a function of both fibril density and interfibril branching, is a better predictor of collagen matrix permeability than collagen concentration.

To date the mechanisms by which intermolecular cross-links and oligomers modulate the hierarchical molecular assembly of collagen to give rise to distinct fibril architectures and matrix physical properties remain to be elucidated. Conventional monomer formulations represent individual rod-shaped collagen molecules measuring approximately 300nm in length and 1.5 nm in diameter. During fibril assembly, monomers align in a staggered array to create entanglements of long fibrils (Kadler *et al.*, 1996). In contrast, oligomers comprise small aggregates of collagen molecules (e.g., trimers) which retain their covalent intermolecular cross-link (Bailey *et al.*, 2011). The prominent cross-link in skin is histidinohydroxylysinonorleucine which links 1-Hyl 87, 2-His 92, and 1-Lys^{ald} 16c residues thereby creating a prong-shaped building block (Mechanic *et al.*, 1987; Yamauchi

et al., 1987; Yamauchi *et al.*, 1996). We postulate that this oligomer configuration supports molecular assembly at the carboxy ends of two collagen molecules (prongs) and the amino terminus of the third molecule (base), thereby yielding branched fibrils rather than individual lengthy fibrils. Further studies are necessary to confirm this supposition and determine its relevance to fibril formation in vitro and in vivo.

In conclusion, findings of these studies underscore the importance of including structural parameters beyond fibril density and radius in the definition of collagen matrix mass transport properties. Without the inclusion of more detailed hierarchical assembly and architectural information, only the bulk permeability of a material can be predicted which can overshadow important structural variations that impact cellular activity. Here, inclusion of oligomer precursors provides control over the extent of interfibril branching, which ultimately affects matrix physical properties (e.g., stiffness and mass transport) and therefore cell-matrix signaling. Future work targeting more advanced quantitative structure-function relationships and the development of mechanistic models that account for actual 3D nanoscale and microscale features of collagen matrices will continue to advance knowledge of cell-matrix signaling and ultimately improve the design of functional collagen-based materials for research and clinical applications.

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Figure 1.

Fibril microstructure of monomeric and oligomeric collagen matrices polymerized at matched concentration (1.5 mg/ml) and stiffness (292 Pa) as visualized (A) as unprocessed confocal image collected in combined immunofluorescence and back-scattered (reflected) light modes, (B) as processed image following signal merging and image thresholding, and (C) following filament detection. Image stacks were $52.736\ \mu\text{m} \times 52.736\ \mu\text{m} \times 10\ \mu\text{m}$ (101 slices at $0.1\ \mu\text{m}$ thickness per slice). Scale bar = $10\ \mu\text{m}$.

Figure 2.

Quantified microstructure parameters including (A) fibril volume fraction, (B) number of branch points, (C) branch points/fibril length, and (D) fibril segment length for monomer and oligomer collagen matrices prepared at matched concentration (1.5 mg/ml) and stiffness (292 Pa). Asterisks indicate statistically different pairwise comparisons ($p < 0.05$).

Figure 3.

Shear storage modulus (G' ; A) and compressive modulus (E_c ; B) values for monomer and oligomer matrices prepared at collagen concentrations from 0.5 to 3 mg/ml. Data points represent mean $\pm$ SD for 4 different monomer and oligomer preparations ($N=4$; $n=3$). G' and E_c , which is inversely related to hydraulic permeability, increased with increasing collagen concentration for both monomer and oligomer matrices but in a collagen type specific fashion. G' values were significantly higher ($p<0.05$) for oligomer matrices at collagen concentrations of 1 mg/ml and higher. E_c values were significantly higher ($p<0.05$) for oligomer matrices at all concentrations.

Figure 4.

Permeability values (mean $\pm$ SD) for monomer and oligomer matrices as a function of collagen concentration (A) and matrix stiffness (B). Permeability decreased with increasing collagen concentration for both collagens, but showed significantly different relationships ($p < 0.05$). In general, monomer matrices showed higher permeabilities when compared to oligomer matrices prepared at matched concentrations of about 10 mg/ml or less. Permeabilities for monomer and oligomer matrices showed similar relationships when plotted as a function of matrix stiffness.

Figure 5.

Diffusivity values (mean $\pm$ SD) for dextrans of various molecular sizes within monomer and oligomer matrices prepared at 1 mg/ml (A), 3 mg/ml (B), and 5 mg/ml (C) as determined using IOI. Diffusivity decreased with increasing molecular size for both monomer and oligomer matrices. Diffusivity values for oligomer matrices were generally lower than those for monomer matrices, especially when prepared at collagen concentrations greater than 1 mg/ml. Statistically determined p-values for monomer versus oligomer comparisons are included.

Table 1

Summary of diffusivity values for various tissues and polymerized matrices.

Tissue/Matrix	Probe Size	D ($\mu\text{m}^2/\text{s}$)	Reference
Pig Skin Monomer (1–5 mg/ml) polymerized	10KDa–2MDa	15–128	Present Study
Pig Skin Oligomer (1–5 mg/ml) polymerized	10KDa–2MDa	16–109	Present Study
Calf Skin Atelocollagen (2.4–45 mg/ml) polymerized and ultracentrifuged	4KDa–2MDa	1–100	(Ramanujan <i>et al.</i> , 2002)
Bovine Skin Atelocollagen (2–20 mg/ml) polymerized and ultracentrifuged	2MDa	2–6	(Erikson <i>et al.</i> , 2008)
Bovine Skin Atelocollagen (2.4 mg/ml) polymerized	10KDa–2MDa	10–70	(De Rosa <i>et al.</i> , 2006)
Cartilage	70KDa	20–40	(Leddy <i>et al.</i> , 2003)
Fibrin (70–110 mg/ml) polymerized	70kDa	100–200	(Leddy <i>et al.</i> , 2004)