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A Microstructurally Motivated Model of the Mechanical Behavior of Tissue Engineered Blood Vessels

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Abstract

Mechanical models have potential to guide the development and use of engineered blood vessels as well as other engineered tissues. This paper presents a microstructurally motivated, pseudoelastic, mechanical model of the biaxial mechanics of engineered vessels in the physiologic pressure range. The model incorporates experimentally measured densities and alignments of engineered collagen. Specifically, these microstructural and associated mechanical inputs were measured directly from engineered blood vessels that were cultured over periods of 5–7.5 weeks. To the best of our knowledge, this is the first successful application of either a phenomenological or a microstructurally motivated mechanical model to engineered vascular tissues. Model development revealed the need to use novel theoretical configurations to describe the strain history of engineered vessels. The constitutive equations developed herein suggested that collagen remodeled between 5 and 7.5 weeks during a 7.5-week culture period. This remodeling led to strain energies for collagen that differed with alignment, which likely resulted from undulations that varied with alignment. Finally, biaxial data emphasized that axial extensions increase stresses in engineered vessels in the physiologic pressure range, thereby providing a guideline for surgical use: engineered vessels should be implanted at appropriate axial extension to minimize adverse stress responses.

Keywords

Tissue engineering; Blood vessels; Arteries; Mechanics; Model; Collagen; Microstructural influences

Introduction

Engineered vessels show great promise as a future source of vascular grafts for the 500,000 coronary artery bypass procedures or the 55,000 peripheral artery bypass procedures performed each year in the USA alone.^{1,27} Models of the mechanical behavior of engineered tissues may be used to guide surgical implantation, to predict *in vivo* behavior, including potential remodeling following implantation, and to guide the design of future culture protocols. To the

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best of our knowledge, such models have yet to be developed for tissue engineered blood vessels. In this study, we use models of native soft tissues as guidance for the development of a model for the biaxial mechanical behavior of engineered vessels.

Background

Constitutive relations (or, models) that describe the passive mechanical behavior of native arteries often assume hyperelasticity^{16,17,37,38} or pseudoelasticity,^{6,13,15} both of which can be used with good success depending on the particular application. The three-dimensional behavior of a nonlinear, hyperelastic medium under isothermal conditions can be described by the constitutive equation

$$\mathbf{t}_{\text{model}} = \frac{2}{\det \mathbf{F}} \mathbf{F} \cdot \frac{\partial \mathbf{W}}{\partial \mathbf{C}} \cdot \mathbf{F}^T, \quad (1)$$

where $\mathbf{t}$ is the true (or Cauchy) stress tensor, $\mathbf{F}$ is the deformation gradient tensor, $\mathbf{W}$ is the scalar strain energy function, and $\mathbf{C}$ is the right Cauchy-Green deformation tensor ($\mathbf{C} = \mathbf{F}^T \cdot \mathbf{F}$).¹⁹

The deformation gradient tensor, $\mathbf{F}$, transforms a differential position vector that connects two points in one configuration to a differential position vector that connects the same two points in a new configuration (or, strain state). Let $\mathbf{F}_1$ be the deformation gradient tensor that takes a differential position vector from a stress-free to an unloaded configuration (Fig. 1). Likewise, let $\mathbf{F}_2$ take a differential position vector from an unloaded to a loaded configuration. The deformation gradient tensor that describes the total motion from the stress-free to the loaded state is then $\mathbf{F} = \mathbf{F}_2 \cdot \mathbf{F}_1$. For an artery without shearing motion, we typically assume $\mathbf{F}_1 = \text{diag}(\Lambda_r, \Lambda_\theta, \Lambda_z)$ and $\mathbf{F}_2 = \text{diag}(\lambda_r, \lambda_\theta, \lambda_z)$, where Λ_i and λ_i are principal stretches in radial, circumferential, and axial directions.²¹

Theoretical Framework

A strain energy function describes the energy stored per unit volume of tissue during deformation. Lanir²⁵ proposed a microstructurally motivated strain energy function of the form

$$W_k = \sum S_k \cdot R_k(N) \cdot W_k(\alpha) \cdot \Delta\Omega, \quad (2)$$

where k is the type of fiber, S is the volume fraction of fibers, $R(N)$ is a distribution function for the alignment of fibers, N is a unit vector that describes the direction of each fiber, $w(\alpha)$ is the strain energy of an individual fiber as a function of fiber stretch (α), and $\Delta\Omega$ is a small unit of volume determined by a small change in angle. Note that the contribution of ground substance (e.g., glycosaminoglycans and water) in the extracellular matrix is often assumed to be negligible compared to that of fibers.^{22,25} This strain energy function (Eq. 2) has been applied to skin,²⁴ lung tissue,²⁶ and pericardium,³² but has not been applied previously to native or engineered blood vessels. The mechanical properties of native arteries are often ascribed to contributions from collagen, elastin, and smooth muscle.^{2,4,5} Engineered blood vessels, however, typically lack functional elastin^{7,28} and contain only partially contractile smooth muscle.⁷ Thus, for most engineered vessels, it is reasonable to focus on collagen as the only type of fiber (k in Eq. 2) that contributes to mechanical behavior. It should be noted that using the same w_k for each “family of fibers” tacitly implies that the stress-free state contains collagen that is undulated to the same extent regardless of its alignment within the tissue. We shall relax this assumption below.

The strain energy of an individual collagen fiber is often described by an exponential function, such as:

$$w=c_1(e^{c_2(\alpha-1)^2}-1), \quad (3)$$

where c_1 and c_2 are best-fit parameters,^{20,22,39} and the undulation is accounted for implicitly. Stretch of a collagen fiber is given by:

$$\alpha=\sqrt{N^T \cdot C \cdot N}, \quad (4)$$

where N is a unit vector having components in the R, Θ , and Z directions, respectively. Undulated fibers ($\alpha < 1$) do not contribute to resisting load, but “straightened” fibers ($\alpha \geq 1$) can resist loads. For each collagen fiber at an angle γ (Fig. 2) in the Θ -Z plane,

$$N=(0,\cos \gamma,\sin \gamma). \quad (5)$$

Methods

Experimental Methods

Vessel Culture—Engineered vessels were cultured as described previously.²⁹ Briefly, porcine carotid artery smooth muscle cells were seeded onto polyglycolic acid scaffolds (Albany International, Mansfield, MA) that were sewn into tubular shapes with 6-0 Dexon suture (United States Surgical, Norwalk, CT). For luminal support, vessels were cultured around a silicone tube having an outer radius of 1.5 mm. Vessels were then exposed to cyclic circumferential classical strains of 1.5%³⁴ ($\epsilon = 0.015$; $\lambda = 1.015$) at a rate of 2.75 Hz²⁹ for 5, 6, or 7.5 weeks by cyclically pressurizing the silicone tube, but were not exposed to flow-induced shear stresses. Note further that the vessels were fixed at both ends along the axial direction in the bioreactor such that cyclic strains were applied only circumferentially. To harvest engineered vessels from bioreactors, axial connections were severed. Although vessels were placed into the bioreactor without axial tension prior to culture, axial tension developed during culture. Release of axial tension upon excision from bioreactors caused vessels to shorten approximately 10% in length. Vessels were fixed for both TEM and histology on the silicone tubing used for support during culture. This non-pressurized silicone tubing held radii and circumference constant during fixation. Vessels were fixed in an axially unloaded configuration, and no changes in length were observed during fixation.

Structural Measurements—The density of collagen in each vessel was calculated as 10 times the amount of measured hydroxyproline.³⁰ Hydroxyproline content was measured spectrophotometrically at 550 nm after digestion of tissue samples in papain and 6 N HCl, oxidation of hydroxyproline with Chloramine T (Mallinckrodt, Hazelwood, MO), and color development with *p*-dimethylaminobenzaldehyde (Fisher Scientific, Hampton, NH).⁴⁰

Transmission electron microscopy (TEM) images of engineered vessels were used to measure alignments of collagen fibrils (γ 's in Fig. 2), as previously described.⁸ To incorporate these measurements within the framework of Eqs. (3)–(5), we assumed that groups of collagen fibrils at each alignment behaved as collagen fibers (i.e., a collagen fiber is a group of aligned collagen fibrils). Thus, measurements of fibrils are more sensitive than measurements of fibers, but alignments are similar. We observed very few radially aligned collagen fibrils, and thus, our study focused on measurement of collagen in the θ -Z plane.⁸ We assumed that alignment was symmetrical around the θ - and Z-axes,⁸ and fibril alignment was binned into groups of 10° between 0° and 90° (e.g., 0–10°, 10–20°, etc.). Thus, each vessel had 9 unit vectors (N 's in Eqs. 2, 4, and 5) describing alignments of collagen fibers at the mean angle of each bin (e.g.,

$N = (0, \cos 5^\circ, \sin 5^\circ)$ for the bin of $0\text{--}10^\circ$, etc.), and 9 corresponding relative frequency of observation values that describe the percentage of collagen fibers at a given alignment ($R(N)$ in Eq. 2).

Freshly harvested engineered vessel segments were also fixed for 1 h in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sliced into $5\text{ }\mu\text{m}$ -thick sections, and stained with H&E. Wall thicknesses were measured from histological cross sections.

Mechanical Measurements—During culture, tissue engineered blood vessels were axially secured in bioreactors to maintain a length of 8 cm. Prior to mechanical testing, vessel segments were removed from bioreactors, and the concurrent release of axial tension resulted in an axial contraction of approximately 10%. Note that silicone tubes used as luminal supports during culture were removed prior to mechanical testing. Upon loading into the biaxial testing system, vessel segments were elongated to accurately match the state of axial stretch imposed by the bioreactor. For example, if a vessel segment allocated for biaxial testing was 4 cm in length in the bioreactor, and contracted to 3.6 cm upon excision from the bioreactor, it was stretched to 4 cm (bioreactor length) upon placement in the biaxial testing system. The axial configuration of tissue engineered blood vessels that matched axial stretch in the bioreactor (no added elongation or contraction compared to the axially secured length in the bioreactor) is hereafter referred to as “bioreactor length”. As previously described for native arteries,¹⁸ a segment of each vessel (3–4 cm in length) was mounted on cannulae and immersed in Krebs buffer (in mM: 116.5 NaCl, 4.5 KCl, 22.5 NaHCO₃, 1.2 NaH₂PO₄, 2.4 Na₂SO₄, 1.2 MgSO₄ · 7H₂O, 1.5 CaCl₂, 5.6 Dextrose) perfused with 95/5% O₂/CO₂. Internal pressures, axial forces, and coordinates of 12 markers located on the surface of each vessel were recorded at a frame rate of 30 Hz throughout testing.^{11,18} Reference coordinates of markers were recorded at a baseline luminal pressure of 0.5 mmHg and at bioreactor length. Three loading protocols were utilized: cyclic inflation (0–120 mmHg) at bioreactor length, axial extension from bioreactor length to 10% above bioreactor length at a constant luminal pressure of 60 mmHg, and cyclic inflation (0–120 mmHg) at 10% above bioreactor length. As expected, analysis of the motions of the 12 surface tracking markers during biaxial testing revealed negligible shearing strains relative to the circumferential and axial extensional strains. Although we could not measure possible reaction torques, consistent with the assumed symmetry of the helical collagen fibers, we assumed that shearing stresses were likewise negligible in the data analysis. Ten inflation or extension cycles were performed for each testing protocol. Given that two or three cycles of preconditioning were sufficient to achieve consistent mechanical behavior, models were developed with data from the fourth loading cycle of each testing protocol.

From the experimental mechanical data, mean true circumferential stresses,

$$\langle t_{\text{exp},\theta} \rangle = \frac{Pr_i}{h}, \quad (6a)$$

and mean true axial stresses,

$$\langle t_{\text{exp},Z} \rangle = \frac{P\pi r_i^2 + L}{\pi h(2r_i + h)}, \quad (6b)$$

were computed, where P was the internal pressure, r_i was the internal radius in the loaded configuration, h was the wall thickness in the loaded configuration, and L was the axial load.²¹ We assumed that vessels were thin-walled (mean value $h/r_i \sim 0.1$), thereby allowing a 2D analysis. Statistical significance was determined by one-way ANOVA and the Tukey–Kramer post-hoc multiple comparison test. All data are presented as the mean $\pm$ standard error of the mean (SEM).

Functional Forms of the Model

Three cases were evaluated to understand the biaxial mechanical behavior of engineered vessels within the framework of Eqs. (1) and (2). *Case 1* assumed that the strain state of vessels was similar in the unloaded and stress-free configurations ($\mathbf{F}_1 = \mathbf{I}$); see Fig. 1. This assumption was based in part on pilot measurements that showed no change in radii between the stress-free and unloaded reference configurations. In addition, the assumption was supported by observations that engineered vessels contained smooth muscle cells that were significantly less contractile than cells in native arteries,⁷ and lacked functional elastin.^{7,28} Contractile smooth muscle cells and elastin contribute to residual stresses in normal arteries. Circumferential and axial components of the deformation gradient $\mathbf{F}_2$ (Fig. 1) were calculated based on changes in distance between markers on the vessel surface during biaxial testing. The radial component of $\mathbf{F}_2$ was then calculated by enforcing incompressibility. Equation (3) was used to model the strain energy of collagen fibers. Given that multiple combinations of c_1 and c_2 could fit data equally well, these parameters were not averaged across vessels.

In *Case 2*, we also assumed that the strain state was similar in the unloaded and stress-free configurations (same assumption as in *Case 1*), but we expanded the strain energy function for collagen fibers (Eq. 3) such that c_1 (and hence w) depended on the alignment of each collagen fiber (N):

$$W_a = c_{1-a}(e^{c_2(a-1)^2} - 1), \quad (7)$$

where ‘a’ was “circ” (circumferential for $\gamma \in [0-30^\circ]$), “hel” (helical for $\gamma \in [30-60^\circ]$), or “axial” ($\gamma \in [60-90^\circ]$). With c_2 held fixed for each vessel, we could directly compare c_{1_circ} , c_{1_hel} , and c_{1_axial} as an “effective stiffness” of collagen fibers in the circumferential, helical, or axial directions, respectively. To compare trends across vessels, a relative percent contribution for collagen in each alignment was calculated:

$$\%c_{1-a} = \frac{c_{1-a}}{c_{1_circ} + c_{1_hel} + c_{1_axial}} * 100. \quad (8)$$

In *Case 3*, we assumed that the strain energy of collagen was independent of alignment (as in *Case 1*), but we expanded the possible configurations of an engineered vessel to include (Fig. 3): stress-free, unloaded (circumferentially and axially unloaded), silicone tube (circumferentially loaded and axially unloaded), reference (axially loaded), and biaxially loaded (circumferentially and axially loaded). Hence, $\mathbf{F} = \mathbf{F}_5 \cdot \mathbf{F}_4 \cdot \mathbf{F}_3 \cdot \mathbf{F}_2 \cdot \mathbf{F}_1$. Using experimental data from the silicone tube, reference, and loaded configurations, we determined new components of the deformation gradients to predict a strain history for engineered vessels that was more thorough than that used in *Case 1* or *2*. Notationally, the circumferential component of $\mathbf{F}_2 \cdot \mathbf{F}_1$ was denoted as $F_{\theta 12}$ and similarly for other components. Given that the stress-free and silicone tube configurations were both axially unloaded, we let the axial component of $\mathbf{F}_2 \cdot \mathbf{F}_1$ be unity, which by incompressibility required the radial component of $\mathbf{F}_2 \cdot \mathbf{F}_1$ to be $F_{\theta 12}^{-1}$. $F_{\theta 12}$ was used to project experimental measurements of radii, wall thicknesses, and alignments of collagen fibers in the silicone tube configuration to corresponding values in the stress-free configuration. Additional components, $F_{\theta 1234}$ and $F_{z 1234}$, accounted for circumferential and axial deformation, respectively, between the stress-free and reference configurations. $\mathbf{F}_5$ in *Case 3* was calculated from surface marker data during biaxial testing, and was equivalent to $\mathbf{F}_2$ in *Case 1*.

Numerical Implementation

A random number generator was used to create 10 initial guesses for each parameter (material parameter in a strain energy or unmeasured component of $\mathbf{F}$ in Fig. 3), as described previously.

⁴¹ To accelerate computations, the model was fit first to a reduced number of data points for each initial guess.¹⁰ The best initial guess was then used to fit the model to every 5th data point (using every 5th data point, an average of 140 data points for each pressure, load, and stretch were evaluated per loading protocol), yielding final parameters for the model. Cases 1 and 2 were fit to data to minimize the objective function e ,

$$e = \sum_i \sum_{j,k} \left(\frac{t_{\text{model},ijk} - t_{\text{exp},ijk}}{\text{mean}(t_{\text{exp},ij})} \right)^2, \quad (9)$$

for each direction, i (circumferential or axial), within each testing protocol, j , and the error was summed across fit data points, k , within each testing protocol. Case 3 was fit to data by minimizing the error between experimental and modeled pressure and load,

$$e_3 = \sum_i \sum_{j,k} \left(\frac{P_{\text{model},jk} - P_{\text{exp},jk}}{\text{mean}(P_{\text{exp},j})} \right)^2 + \sum_i \sum_{j,k} \left(\frac{L_{\text{model},jk} - L_{\text{exp},jk}}{\text{mean}(L_{\text{exp},j})} \right)^2. \quad (10)$$

Error was minimized via the Marquardt–Levenberg method. A fitting parameter normalized each objective function by the total number of fit data points (k) summed for fit testing protocols (j) and fit directions (i),⁴¹ namely

$$\Phi = \frac{1}{\sum_i \sum_j k_{ij}} \sum_i \sum_{j,k} \left(\frac{t_{\text{model},ijk} - t_{\text{exp},ijk}}{\text{mean}(t_{\text{exp},ij})} \right)^2. \quad (11)$$

The final value for Φ was calculated by comparing the fit of the model to all data points rather than to every 5th data point. For example, a biaxial fit of the model to three loading protocols may have included 700 data points for inflation at bioreactor length for each the circumferential and axial fit, 711 data points for axial extension for each the circumferential and axial fit, and 689 data points for inflation at 10% above bioreactor length for each the circumferential and axial fit. Thus, the total number of data points for a biaxial fit to three loading protocols would be 4200 in this example. In contrast, the total number of data points for a circumferential fit to inflation at bioreactor length would be only 700 in this example. Without normalization by the total number of fit data points, the error, as calculated by Eq. (9), would appear much higher as the number of fit data points increased. Thus, the fitting parameter, Φ , allowed comparison of the quality of fit across data sets with different numbers of data points, fit testing protocols, and fit directions.

Results

Microstructural inputs for models of the mechanical behavior of 12 engineered vessels included densities of collagen, wall thicknesses, and alignments of collagen (Table 1).

During inflation at bioreactor length, Case 1 consistently fit uniaxial data well (Circumferential Data: $\Phi = 0.040 \pm 0.015$; Axial Data: $\Phi = 0.043 \pm 0.016$; $n = \text{four 7.5-week vessels}$), but fit biaxial data poorly (Figs. 4a and 4b, Table 2).

In contrast, Case 2 successfully described biaxial mechanical behavior during inflation (Fig. 4c, Table 2) by allowing strain energies of collagen fibers to depend on alignment. Parameters obtained by fitting Case 2 to biaxial data from inflation at bioreactor length (Table 3) or 10% above bioreactor length predicted changes in the effective stiffnesses of collagen with alignment and length of culture (Fig. 5a). In vessels cultured for 7.5 weeks and tested at

bioreactor length, the predicted stiffness of helical collagen was significantly greater than that of circumferential or axial collagen. At bioreactor length, parameters suggested an increasing trend in the contribution of helical collagen ($p < 0.06$ for 5-week vs. 7.5-week vessels) and an associated decreasing trend in the contribution of axial collagen with length of culture ($p < 0.07$ for 5-week vs. 7.5-week vessels). Importantly, Case 2 predicted a decreasing trend for the contribution of axial collagen with length of culture, which mimicked a decreasing trend in the experimentally measured axial forces required to maintain the bioreactor length (Fig. 6; $p < 0.06$ for 5-week vs. 7.5-week vessels).

At 10% above bioreactor length, Case 2 predicted significantly greater stiffnesses for axial collagen and significantly lower stiffnesses for helical collagen than at bioreactor length for 6- and 7.5-week vessels (Fig. 5b). Furthermore, at 10% above bioreactor length, the predicted stiffness of axial collagen was significantly higher than that of circumferential or helical collagen in vessels cultured for 5 or 6 weeks (Fig. 5b). Fitting Case 2 simultaneously to biaxial data from all three loading protocols yielded parameters that weighted the contribution of helical and axial collagen significantly more heavily than that of circumferential collagen ($\%c_{1_circ} = 2.3 \pm 1.2$; $\%c_{1_hel} = 43.5 \pm 16.7^*$; $\%c_{1_axial} = 54.2 \pm 17.2^*$; $n = \text{four 7.5-week vessels}$; * indicates $p < 0.05$ compared to $\%c_{1_circ}$).

Case 3 assumed that collagen fibers in all directions contributed equally to the mechanical properties. During inflation at 10% above bioreactor length, Case 3 successfully fit biaxial mechanical data (Fig. 4d, Tables 2 and 4) and generated a mean fitting parameter comparable to that of Case 2. No significant differences were detected between the best-fit parameters of Case 3 with length of vessel culture at 10% above bioreactor length (Fig. 7). Note that an axial extension from bioreactor length to 10% above bioreactor length increased stresses dramatically in engineered vessels (Figs. 4c and 4d). At bioreactor length, circumferential stresses consistently exceeded axial stresses (e.g., Fig. 4c). In contrast, axial stresses consistently exceeded circumferential stresses in axially extended engineered vessels, and axially extended vessels had higher overall stresses at physiologic pressures (e.g. Fig. 4d). Although Case 3 nicely fit biaxial data from inflation at 10% above bioreactor length, it fit biaxial data of 7.5-week vessels at bioreactor length poorly (Table 2). Interestingly, at bioreactor length, Case 3 fit biaxial data from inflation of 5-week vessels better than that of 6-week vessels or 7.5-week vessels (Table 2).

Deformations predicted by Case 3's best-fit parameters suggested an absence of circumferentially aligned collagen ($0\text{--}30^\circ$) in the stress-free and reference configurations (Fig. 8). Rather, Case 3 predicted a shift in collagen towards the helical and axial directions for the stress-free and reference configurations.

Discussion

Lanir proposed in 1979 that the alignment of collagen fibers impacts the form of the strain energy for a soft tissue, but little experimental data for collagen alignment was available at that time. Historically, therefore, models with a microstructural framework have employed statistical distributions, such as Gaussian functions, to describe the alignment or undulation of collagen.²⁴ Using experimental measurements for alignments of collagen eliminates the need to utilize statistical distributions, and thereby yields more accurate best-fit parameters.³² Sacks *et al.*^{32,33} have experimentally measured alignments of collagen with small angle light scattering (SALS) and incorporated them into structurally motivated mechanical models for native tissues. In this study, we input experimentally measured collagen alignments⁸ into models for engineered tissues.

Notwithstanding the advances achieved via the present biaxial testing and constitutive formulation, there is need for improvements in both experimental methods and modeling. First, we assumed that the collagen fibers could be classified into three groups: circumferentially, helically, and axially oriented fibers, with the further assumption of symmetry of the helical fibers consistent with prior work on native vessels.^{3,16} There is a need to quantify the full transmural distribution of the collagen fibers, however, and to incorporate this information into a structural model. Given the possibility that the fibers may not have a symmetrical distribution, there is also a need to measure possible reaction torques (in the absence of applied torsion) during extension and inflation testing and thereby to assess possible contributions due to shear stresses. Indeed, torsion tests would provide additional important information for modeling, particularly for engineered grafts intended for use as coronary bypasses. We also modeled only the mechanical behavior of the “developed tissues” at 5–7.5 weeks of culture. There is a need to model the developmental process, that is, the growth and remodeling of the collagenous tissue and the degradation of the polymeric scaffold, for such information could be very helpful in designing improved culture conditions. Another limitation of our model is the use of random numbers to generate initial guesses for parameters. Initial estimates of parameter values impact the estimated best-fit values, which is a common observation for models that seek simultaneous fits to multiple parameters.⁴¹ To address this issue, we used 10 initial random guesses to minimize error in the model fit. Overall conclusions were based on the fit of the model, not absolute parameter values. Solutions were robust enough to produce trends for the fit of each case of the model to the data. In Case 2, we drew additional conclusions based on relative parameter values, rather than absolute parameter values, which allowed multiple solutions to produce similar relative outcomes. Although our model had limitations, development of three cases identified useful parameters that impact the mechanical behavior of tissue engineered blood vessels.

Case 1 successfully fit the uniaxial mechanical behavior of engineered vessels, but it failed to describe their biaxial behavior. Thus, it was necessary to develop a novel model for the biaxial mechanical properties of engineered vessels. First, we developed Case 2, which assumed that the mechanical properties of collagen differed for circumferential, helical, and axial alignments. To our knowledge, Case 2 is the first successful phenomenological fit of a nonlinear elastic model to engineered vascular tissue; it is consistent philosophically with multi-fiber family models for native tissues.^{3,16} In addition, Case 2 fit biaxial mechanical properties better than Case 3 for the majority of applications. Case 3 outlined a novel set of kinematic considerations to describe the strain “history” of engineered vessels, yet several of the deformation gradient tensor values predicted by Case 3 may exceed realistic deformation values for tissue engineered vessels. For example, Case 3's predicted deformation gradient tensor values for 7.5-week vessels were surprisingly high (Table 4 and Fig. 7), which led to high and possibly unrealistic values for circumferential stretch (Fig. 4d). However, the Case 2 and Case 3 models revealed consistent structural and mechanical insights that have been largely neglected by tissue engineers to date.

Both Case 2 and Case 3 suggest that helically and axially oriented collagen contribute to the mechanical behavior of engineered vessels more than circumferentially oriented collagen. This observation must be interpreted, of course, within the framework of the culture system. During culture, only circumferential strains were applied to engineered vessels. Axial dimensions were held constant during culture, which may have created static axial strains. Results from modeling with Case 2 suggested that helical and axial collagen had higher strain energies than circumferential collagen in engineered vessels; Case 3 predicted a shift of collagen towards helical and axial alignments to explain their increased contribution to the mechanical behavior of engineered vessels. Although both Cases 2 and 3 emphasize contributions of helical and axial collagen to overall mechanical behavior, ultrastructural analysis showed collagen fibers in circumferential alignments in all vessels. Thus, it may be that engineered vessels contain

circumferentially aligned collagen in the reference configuration, but this collagen is less stiff than corresponding helical or axial collagen. Currently, the causes for this difference in collagen stiffness remain unclear, though the applied strain regimen during culture may certainly contribute.

The extent of fiber undulation/straightening will affect the contribution of collagen to load bearing at each alignment. It is thought that native vessels remodel over time to achieve “normal” wall stresses^{12,36} or strains.²³ It is possible therefore that the undulation of collagen differs with length of culture and with alignment as a result of strain or tension patterns during culture. As engineered vessels matured, the axial load required to maintain bioreactor length decreased, which suggested that collagen aligned in the axial direction may have become increasingly undulated with length of culture. Case 2 supported the possibility that axial collagen became more undulated with time by predicting a decrease in the percent contribution of axial collagen with length of culture. Furthermore, both Cases 2 and 3 predicted that the contribution of axial collagen increased with axial stretch, which may be explained by straightening of undulated collagen between bioreactor length and 10% above bioreactor length. Although we were unable to experimentally quantify changes in undulation of collagen with alignment using traditional TEM, it may be possible to measure undulation in future studies using three-dimensional ultrastructural reconstruction techniques such as automated electron tomography.¹⁴ Measuring the undulation of collagen fibers at each alignment would provide additional useful additional input data for future microstructurally motivated models.

Collagen at a given alignment also may appear weak if it stress relaxes or creeps more quickly than collagen in other alignments. For example, concentrated regions of scaffolding remnants in engineered vessels cause local stress concentrations,⁷ which may lead to accelerated stress relaxation in the circumferential direction during preconditioning. In addition, collagen fibrils may slip past each other to achieve stress-relaxation³¹ during culture, especially in response to applied cyclic strain. Diameters of collagen fibers, along with densities of collagen fibrils within collagen fibers, also may differ with alignment in response to culture conditions.

Finally, the experimental data emphasized the impact of axial extension on maximum stresses of engineered vessels in the *in vivo* pressure range, thereby raising our awareness of the attention that must be paid to the axial extension of an engineered vessel in the clinical setting. Specifically, our models and experimental observations suggest that engineered vessels should be implanted at bioreactor length to minimize wall stresses and thereby minimize the risk of graft failure. Increased wall stresses associated with axial elongation may increase the potential for dilation and rupture of engineered vessels *in vivo*. Furthermore, increased stiffnesses may exacerbate the mismatch in compliance between an implanted engineered vessel and the adjacent native vasculature. Compliance differences, in turn, may cause disturbances in blood flow and thereby increase the potential for graft failure via thrombosis or intimal hyperplasia.^{9,35}

The models developed herein provided valuable insights into parameters that impact the mechanical behavior of engineered tissues, and thus, into inputs that should be included in the next generation of models for engineered tissues. First, future models should include an appropriate set of configurations to describe the strain history of an engineered tissue. Second, precise measurements of physical dimensions and collagen alignments should be taken at each configuration. Third, strain energies for collagen should be allowed to vary with fiber alignment, and these strain energies may depend on additional microstructural inputs such as undulation of collagen, densities and diameters of collagen fibers, and scaffold parameters. Finally, future models should include shear stresses and strains. These suggestions may allow future models to predict the mechanical behavior of engineered tissues with one universal set of best-fit functions. A predictive model, in turn, will be able to guide experimental design of

engineered tissue culture, provide additional guidance for surgical handling, and predict behavior and remodeling *in vivo*.

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FIGURE 1.

Experimentally achievable configurations of a native artery, and the coordinates used to describe each configuration. In the stress-free configuration, an artery is cut to relieve residual stresses. The stress-free configuration is mapped to an unloaded configuration (e.g., an excised artery), which is then mapped to a loaded configuration with applied stresses (e.g., luminal pressure, axial tension, etc.).

FIGURE 2.

Illustration of the unit vector, N , associated with collagen fiber alignment. (a) Collagen fibers are aligned primarily in the θ - Z plane of a vessel. (b) The unit vector associated with the alignment of collagen fibers is described by Eq. (5).

FIGURE 3.

Novel configurations for theoretical analysis of the mechanics of engineered vessels. The unloaded configuration appears twice in this schematic to emphasize that an engineered vessel passes through the unloaded configuration between the stress-free and silicone tube configurations, and between the silicone tube and reference configurations.

FIGURE 4.

Representative fit of Case 1 to (a) circumferential or (b) biaxial experimentally measured stress-stretch behavior of a vessel during inflation at bioreactor length. (c) Representative fit of Case 2 to biaxial stress-stretch behavior of an engineered vessel during inflation at bioreactor length. (d) Representative fit of Case 3 to biaxial data from inflation at 10% above bioreactor length. Note that a, b, and c described behavior at bioreactor length, and stretch (λ_θ) was calculated using the framework presented in Fig. 1 (e.g., $\lambda_\theta = F_{1,\theta\theta} F_{2,\theta\theta}$, for F_1 and F_2 as shown in Fig. 1, where $F_1 = I$). In d, however, data are presented for behavior at 10% above bioreactor length, and stretch (λ_θ) was calculated using the framework presented in Fig. 3 (e.g., $\lambda_\theta = F_{\theta 1234} F_{5,\theta\theta}$, for F_1, F_2, F_3, F_4 , and F_5 , as shown in Fig. 3, where $F_{\theta 1234}$ was a best-fit parameter for circumferential stretch between the stress-free and reference configurations).

FIGURE 5.

Percent contribution of circumferential (%c_{1_circ}), helical (%c_{1_hel}), and axial (%c_{1_axial}) collagen predicted by the fit of Case 2 to biaxial data from vessels cultured for 5 ($n = 4$), 6 ($n = 4$), or 7.5 ($n = 4$) weeks (a) during inflation at bioreactor length and (b) during inflation at 10% above bioreactor length. * and ** denote statistical differences ($p < 0.05$) compared to the %c_{1_circ} or %c_{1_hel} values, respectively, of vessels with the same length of culture that were tested at the same axial strain. # Denotes a statistical difference ($p < 0.05$) between predicted parameters for inflation at bioreactor length and inflation at 10% above bioreactor length.

FIGURE 6.

Mean force required to maintain bioreactor length for vessels cultured 5 ($n = 4$), 6 ($n = 4$), or 7.5 ($n = 4$) weeks.

FIGURE 7.

Best-fit parameters for Case 3 during inflation at 10% above bioreactor length for vessels cultured 5 ($n=4$), 6 ($n=4$), or 7.5 ($n=4$) weeks. $F_{\theta 12}$ described the circumferential deformation from the stress-free configuration to the silicone tube configuration, $F_{\theta 1234}$ described the circumferential deformation from the stress-free configuration to the reference configuration, and $F_{z 1234}$ described the axial deformation from the stress-free configuration to the reference configuration (recall Fig. 3).

FIGURE 8.

Relative frequency of observation for the alignment of collagen fibers during inflation at 10% above bioreactor length. Functions were experimentally measured in the silicone tube configuration, or calculated using Case 3's predicted deformation parameters for the stress-free and reference configurations. * Denotes a statistically significant ($p < 0.05$) difference between both (1) the stress-free and silicone tube configurations and (2) the silicone tube and reference configurations.

Experimentally measured densities of collagen as a percent of dry tissue weight, wall thicknesses, and relative frequency of observation for the alignment of collagen in vessels cultured for 7.5, 6, or 5 weeks

Culture (weeks)	Collagen (% dry wt.)	Thickness (μm)	Relative frequency of observation for alignment of collagen (%)								
			0-10°	10-20°	20-30°	30-40°	40-50°	50-60°	60-70°	70-80°	80-90°
7.5	46.84	186	16.93	10.70	5.64	13.55	16.87	7.85	0.87	6.34	21.23
7.5	43.33	196	26.44	9.95	1.52	0.66	9.91	4.02	2.61	9.83	35.07
7.5	42.50	154	22.49	4.27	3.97	5.28	30.77	4.78	2.11	5.19	21.14
7.5	45.95	103	11.51	6.83	1.66	5.05	22.00	7.91	3.28	6.27	35.50
6	40.07	169	13.32	7.68	3.57	13.87	34.83	4.59	3.06	5.40	13.69
6	45.50	167	7.23	1.31	0.25	2.34	37.80	12.60	7.49	7.58	23.40
6	43.11	133	14.84	5.29	3.22	4.43	27.63	7.59	4.99	7.07	24.94
6	38.05	129	23.93	3.71	1.64	4.12	27.85	6.50	5.14	4.97	22.14
5	22.93	99	18.85	4.53	2.53	5.49	34.00	8.71	4.13	5.47	16.28
5	25.77	73	18.26	4.61	2.42	2.66	32.49	7.87	4.87	6.72	20.10
5	34.17	194	14.82	4.27	3.42	3.86	39.95	8.02	3.06	7.29	15.31
5	33.91	158	16.01	5.86	2.89	12.31	31.06	6.23	2.56	4.95	18.13

Table 2
Fitting parameter (Φ) values for the fit of Cases 1, 2, and 3 to biaxial data*

Loading protocols	Vessel culture length	Case 1 Φ values	Case 2 Φ values	Case 3 Φ values
Inflation at bioreactor length	7.5 weeks ($n = 4$)	0.405 ± 0.068	0.048 ± 0.014	0.524 ± 0.271
	6 weeks ($n = 4$)	0.444 ± 0.031	0.037 ± 0.007	0.102 ± 0.022
	5 weeks ($n = 4$)	0.442 ± 0.020	0.046 ± 0.008	0.077 ± 0.022
Inflation at 10% above bioreactor length	7.5 weeks ($n = 4$)	0.310 ± 0.063	0.039 ± 0.011	0.036 ± 0.018
	6 weeks ($n = 4$)	0.394 ± 0.033	0.058 ± 0.003	0.065 ± 0.015
	5 weeks ($n = 4$)	0.331 ± 0.086	0.045 ± 0.009	0.023 ± 0.010
Inflation at bioreactor length, axial extension, and inflation at 10% above bioreactor length	7.5 weeks ($n = 4$)	0.353 ± 0.049	0.129 ± 0.021	0.691 ± 0.296
	6 weeks ($n = 4$)	0.440 ± 0.023	0.120 ± 0.033	0.624 ± 0.235
	5 weeks ($n = 4$)	0.416 ± 0.032	0.157 ± 0.040	0.336 ± 0.130

* Data are presented as the Mean $\pm$ SEM.

Table 3
Parameter values generated for the fit of Case 2 to biaxial data from four 7.5-week vessels

Vessel	c_{1_circ} (Pa)	c_{1_hel} (Pa)	c_{1_axial} (Pa)	c_2	Φ
1	<<1	615	<<1	331	0.050
2	4.28	2410	<<1	773	0.085
3	73.69	3444	<<1	789	0.027
4	0.35	2878	<<1	301	0.028

Table 4
Parameter values generated for the fit of Case 3 to biaxial data from four 7.5-week vessels at 10% above bioreactor length

Vessel	c_1 (Pa)	c_2	$F_{\theta 12}$	$F_{\theta 1234}$	$F_{\theta 1234}$	Φ
1	<<1	15.09	1.33	1.93	1.97	0.020
2	0.015	13.98	1.27	2.00	1.91	0.088
3	0.001	45.87	1.48	1.68	1.43	0.023
4	<<1	15.82	1.41	1.90	1.92	0.012