Planar Biaxial Behavior of Fibrin-Based Tissue-Engineered Heart Valve Leaflets

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To design more effective tissue-engineered heart valve replacements, the replacement tissue may need to mimic the biaxial stress-strain behavior of native heart valve tissue. This study characterized the planar biaxial properties of tissue-engineered valve leaflets and native aortic valve leaflets. Fibrin-based valve equivalent (VE) and porcine aortic valve (PAV) leaflets were subjected to incremental biaxial stress relaxation testing, during which fiber alignments were measured, over a range of strain ratios. Results showed that VE leaflets exhibited a modulus and fiber reorientation behavior that correlated with strain ratio. In contrast, PAV leaflets maintained their relaxed modulus and fiber alignment when exposed to nonequibiaxial strain, but exhibited changes in stress relaxation. In uniaxial and equi-biaxial tension, there were few observed differences in relaxation behavior between VE and PAV leaflets, despite differences in the modulus and fiber reorientation. Likewise, in both tissues there was similar relaxation response in the circumferential and radial directions in biaxial tension, despite different moduli in these two directions. This study presents some fundamental differences in the mechanical response to biaxial tension of fibrin-based tissue-engineered constructs and native valve tissue. It also highlights the importance of using a range of strain ratios when generating mechanical property data for valvular and engineered tissues. The data presented on the stress-strain, relaxation, and fiber reorientation of VE tissue will be useful in future efforts to mathematically model and improve fibrin-based tissue-engineered constructs.

Introduction

REPLACEMENT HEART VALVES RESTORE FUNCTION with great short-term success. However, they suffer from limited lifetimes, and the recipients often require revision surgery and ongoing medical treatment. A need exists for a fully biologic, living replacement valve that can self-maintain and, as appropriate, grow with a patient. This need is being met by several approaches to functional tissue engineering of heart valve replacements.^{1–6} As these technologies progress, it is likely important that replacement tissues mimic the multi-axial mechanical behavior of native aortic valve tissues.

Planar biaxial testing yields relevant data on the mechanical response of a tissue because it can probe the effects of mechanical coupling between axes—that is, the effect of the stress or strain state along one axis affecting the stress-strain response of the other axis—as is known to occur in heart valve leaflets.^{7–9} Most investigators have used equi-biaxial loading, controlled by either displacement or load. While this yields important information, the aortic valve leaflet has been shown to be strained nonequibiaxially *in vivo*, with the radial strains as much as three times the circum-

ferential strains.^{10,11} Some investigators have used constant ratio loading, with the ratios of strain or load in the two axis directions ranging from 1:1 to 6:1.^{7,8} These data have been used to qualitatively compare native and bioprosthetic valve leaflets⁷ and as input to constitutive models of aortic valve function.^{12,13}

Further, the biaxial viscoelastic properties of heart valve leaflets may have an important role in valve mechanics. Valvular durability may be linked to the ability of the leaflets to dissipate stress during the cardiac cycle. It has been postulated that one reason for calcification and failure of bioprosthetic valves is the loss of natural leaflet relaxation behavior caused by chemical fixation.^{14,15}

The objective of this study was to characterize and compare the planar biaxial properties of tissue-engineered valve leaflets and native aortic valve leaflets. This study follows our previous success of creating functional tissue-engineered valve replacements using cells entrapped in fibrin gel as the basis for a completely biologic replacement construct termed a “valve equivalent” (VE).⁶ VE leaflets were shown to have a size, shape, and unstrained fiber alignment pattern similar to porcine aortic valve (PAV) leaflets. In this study, planar

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biaxial testing was performed on VE and PAV leaflets over a range of circumferential to radial strain ratios. Incremental stress relaxation steps were measured, and polarimetry was used to determine fiber alignment changes. The response of the relaxed moduli, stress relaxation, and fiber alignment to strain ratio in both leaflet types was compared as an investigation of differences in tissue behavior. This study highlights some fundamental differences in planar biaxial viscoelastic behavior of PAV and VE leaflets as well as structure–function relationships in both these tissues. The data will be useful in designing next-generation VEs and building constitutive models of engineered tissue.

Materials and Methods

All reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. All calculations and image analysis were performed using custom software built in Matlab (Mathworks, Inc., Natick, MA).

Tissue sources

Bi-leaflet valve equivalents (VEs, $n=6$) were fabricated from human dermal fibroblasts in bovine fibrin gel cast in a valvular mold as previously described and characterized.⁶ The VEs possessed circumferential fiber alignment in the leaflets, similar to PAV leaflets and were approximately 10% collagen by weight.⁶ One leaflet from each VE was resected and used for testing.

Porcine hearts ($n=7$) were obtained from the Minnesota Department of Agriculture. One aortic valve leaflet was resected from each heart and subjected to the same testing as VE leaflets.

Biaxial testing

Cruciform specimens were cut from VE and PAV leaflets such that one set of arms was parallel to the circumferential direction and the other set parallel to the radial direction. The cruciform geometry was chosen because of the inability of the weaker VE tissue to support the point-loads of the hook-and-suture method that has become more standard for biologic soft tissue.^{7,9,16–19} Based on previous studies that showed that observed stress–strain characteristics in cruciform specimens can vary with specimen arm length,^{20,21} care was taken to maintain the same arm lengths for each specimen tested. Using a clamped cruciform specimen likely causes an overestimation of the stress and, therefore, the stiffness in the material.²⁰ However, these effects would be the same for all tests, not affecting the relative comparisons made in this study. Four dots were placed in the central area of each cruciform using Verhoeff's stain. The dots were equally spaced approximately 5 mm apart, forming the corners of a square gauge area of tissue with sides corresponding to the circumferential and radial directions (Fig. 1). Thickness within the gauge area was measured with a low-force thickness probe²² using a minimum of three randomly chosen locations. Specimens were placed in compressive grips in a planar biaxial materials testing device (8848, Instron) and preloaded to 0.01 N tension. Specimens were subjected to a series of biaxial incremental stress relaxation tests comprised of 10 consecutive steps, each step composed of a displacement over 0.1 s followed by a 120 s hold (Fig. 1).

FIG. 1. Biaxial testing. Example load–time trace of an equibiaxial test. Shown above is a typical valve equivalent (VE) cruciform test specimen and gauge area (box) for measurement of strain and fiber orientation. Strain and orientation are calculated at the end of every relaxation period (only two such steps shown).

Preliminary data showed at 120 s the relaxation was at least 90% of the value when specimens were allowed to relax for 3600 s, at which time there was no discernable change in load. Additionally, relaxation parameters estimated from reduced relaxation functions (see below) were no different using data from 120 s and data from 3600 s. The displacement of each step was one-tenth of the overall displacement of the actuators prescribed for each direction. For each leaflet, four tests were performed in succession using circumferential to radial ($c:r$) strain ratios of 1:1, 1:3, 1:10, and 1:1. The tests were run using actuator displacement control, where the maximum displacement in any test was set to give a maximum strain in the gauge region of $\sim 20\%$ in either direction (e.g., a 1:3 test had a circumferential strain of 7% and a radial strain of 20%). The 1:1 and 1:3 ratios and 20% maximum strain were chosen to match the strains in native aortic valves during the cardiac cycles as observed by previous investigations.^{10,11,23,24} The higher strain ratio (1:10) was added as an extreme condition to assess fiber alignment effects. High-resolution images of the cruciform were taken at preload and after each step and used to calculate average engineering strain in the gauge area using bilinear interpolation of the marker locations²⁵ posttest. The width of the cruciform arms was measured optically using the preload image. Engineering stress in the circumferential and radial directions was calculated as the average load in each direction divided by the product of the initial arm width and specimen thickness. Data from the first and last 1:1 tests were checked for similarity to ensure that the sample was not mechanically compromised during testing. For all tests, the data from the first and last 1:1 tests were similar, indicating

no tissue damage occurred during testing. Data from the first 1:1 test was used in subsequent analysis. All testing was performed in phosphate buffered saline at room temperature.

Relaxed stress-strain curves were constructed by pairing the relaxed stress at each step with the optically measured strain at that step, in both the circumferential and radial directions. For each relaxed stress-strain curve, the tangent modulus was calculated by finding the slope of the linear least-squares fit through a hand-chosen linear region of the curve. This yielded a relaxed tangent modulus in both the circumferential and radial direction for each set of strain ratios.

Data from the last four steps (corresponding to the linear region) were used to calculate the relaxation properties of the tissues. Percent relaxation was calculated as the difference in peak and relaxed load divided by the peak load for each step and in each direction. The load-time data for each step in each direction was normalized by the peak load and fit to the reduced relaxation function of the form²⁶

$$G(t) = \frac{1 + C[E(t/\tau_2) - E(t/\tau_1)]}{1 + C \ln(\tau_2/\tau_1)}, E(x) = \int_x^\infty \frac{e^{-t}}{t} dt \quad \text{Eq. (1)}$$

using an interior-reflective Newton method of least squares optimization²⁷ to estimate the parameters C , τ_1 , and τ_2 . For all mechanical properties, preliminary studies were performed to assess dependence on strain level in the linear region, and none was observed.

Uniaxial testing

After biaxial testing, strips of tissue were cut from the specimens in the circumferential and radial directions. Strips were placed in compressive grips, attached to the actuator arms of the testing system, and straightened to a load of 0.01 N. After 10 cycles of 0–5% strain at 0.3%/s, samples were allowed to equilibrate for 60 s then subjected to a stress relaxation test at ~5% strain for 300 s. After relaxation, a quasi-static ramp test was performed at a rate of 0.3%/s. Strain, stress, tangent modulus, and relaxation properties were measured as described in the biaxial testing methods above. Preliminary data showed no difference in tensile properties between strips tested from fresh leaflets and strips tested from leaflets that were biaxially tested using the protocols above.

Fiber alignment analysis

During biaxial testing, fiber alignment imaging was conducted using the method of Tower *et al.*,²⁸ which has been shown to accurately determine the alignment direction and retardation of a known standard.²⁹ Alignment information was recorded at preload and during the last 2 s of each of the relaxation steps. In this method, a mean fiber alignment direction is generated for each pixel in the alignment image (the gauge areas were approximately 200×200 pixels spanning 5 mm by 5 mm). Distributions of the alignment directions, created using 5° increments, for all pixels were fit to a wrapped normal distribution of the form^{30,31}:

$$f(\theta) = \frac{1}{\pi} \left(1 + 2 \sum_{k=1}^{\infty} \rho^k \cos k2(\theta - \mu) \right) \quad \text{Eq. (2)}$$

The fit was performed using an interior-reflective Newton method of least squares optimization²⁷ to estimate the parameters of mean angle, μ , and distribution parameter, ρ , which is related to the standard angular deviation (s) by $\rho = \exp(-0.5s^2)$. The distribution mean and angular deviation were used as relative measures of changes in the fiber orientation during biaxial testing.

Statistics

Moduli and relaxation parameter values for the different biaxial strain ratios and uniaxial tests within each tissue type (VE and PAV) were compared by one-way ANOVA followed by Fisher LSD *post hoc* test. Differences between circumferential and radial properties in all cases were determined using a paired *t*-test. VE and PAV data from similar tests were compared by unpaired *t*-tests. In the biaxial tests, relaxation parameters were checked for correlation with strain using one-way ANOVA on data pooled from all tests of each strain ratio and tissue type. It is important to note that for many of the comparisons the data are paired, giving a high statistical power even though the sample number is low ($n = 6-7$). In all cases, significance was set at $p < 0.05$.

Results

Both VE and PAV leaflets exhibited typical nonlinear stress-strain behavior in both biaxial and uniaxial tests. All tests reached an apparent linear region of the stress-strain curves (Fig. 2). The tests were controlled with actuator displacements based on development with PAV tissue to set the actuator displacement such as to achieve the desired strain level and ratio in the gauge region. Because of an increased amount of stretch in the gauge region of the more compliant VE tissue, the actual strain ratios achieved were different than estimated when using the same actuator displacements. Especially during nonequibiaxial stretching, the circumferential strains were slightly higher than expected in the VE gauge region compared to PAV (Fig. 2), causing lower *c:r* ratios in the VE tissue. Actual strain ratios achieved in VE tissue for the three biaxial tests were 1:1, 1:2, and 1:5 as compared to 1:1, 1:3, and 1:10 for PAV leaflets. Therefore, only the 1:1 data were used for comparison between tissue types. Some variation in the absolute strains and ratios existed between samples, as strain control was not used in these tests because of technical incompatibility with the polarimetric method. This variation was a maximum 0.03 mm/mm strain at the highest strain levels, translating to a maximum error of 0.1 for each strain ratio. For example, the 1:3 data encompasses actual strain ratios of 1.0:2.9–1.0:3.1.

Relaxed modulus

Differences in the relaxed modulus were observed between tissue types and directions. In both VE and PAV leaflets, the circumferential modulus was larger than the radial modulus for all strain ratios (Fig. 3). The PAV leaflets displayed a higher degree of anisotropy than VE leaflets in that the circumferential modulus in the 1:1 tests was on average 4.3 times higher than the radial modulus for PAV leaflets, versus 1.7 times higher for the VE leaflets (Fig. 3). The PAV leaflets had higher moduli than VE leaflets in 1:1 tests in both the circumferential (6557 ± 3467 kPa, PAV vs.

FIG. 2. Typical relaxed biaxial data. Plot of typical relaxed stress-strain curves for VE (left) and porcine aortic valve (PAV) (right) leaflets tested in tension with varying $c:r$ strain ratios. Solid lines are circumferential data; dashed lines are radial data. VE tests resulted in $c:r$ ratios of 1:1 (black lines), 1:2 (dark gray lines), and 1:5 (light gray lines). PAV tests resulted in $c:r$ ratios of 1:1 (black lines), 1:3 (dark gray lines), and 1:10 (light gray lines). Asterisks on lines denote approximate location of tangent modulus calculation.

844 ± 299 kPa, VE; $p = 0.007$) and radial (1838 ± 960 kPa, PAV vs. 523 ± 258 kPa, VE; $p = 0.019$) directions.

The modulus was correlated to strain ratio in VE leaflets but not in PAV leaflets. In VE leaflets, as the $c:r$ ratio decreased, there was a stiffening in the circumferential direction and softening in the radial direction (Fig. 4). The circumferential modulus increased with decreasing $c:r$ ratio ($p < 0.001$), whereas the radial modulus decreased with decreasing $c:r$ ratio (Fig. 4, $p = 0.03$). This effect was not observed in PAV leaflets, where the moduli in both directions remained the same regardless of strain ratio ($p > 0.2$).

Stress relaxation

In VE leaflets, there were no differences in relaxation between strain ratios or between directions (Fig. 5). In the circumferential direction, the relaxation in biaxial tests was less than in uniaxial tests. For PAV leaflets, there were no differences between the circumferential and radial relaxations. The relaxation in the 1:10 tests was less than the relaxation in the 1:1 and 1:3 tests. The biaxial relaxations were less than uniaxial relaxations in both directions. In 1:1 and uniaxial tests, no difference in relaxation was observed between VE and PAV leaflets.

The reduced relaxation function fit the relaxation data well (average R^2 of 0.98 ± 0.02) for both biaxial and uniaxial

tests (Fig. 6). The relaxation parameter C was observed to be different between strain ratio and direction in both leaflet types (Fig. 7). It was lower in biaxial tests than uniaxial tests and higher in the 1:1 tests than the non-1:1 tests. In both the 1:1 and uniaxial tests, there were differences in C between directions. These differences were opposite in the two tissues, with C being lower in the circumferential direction in VE but higher in the circumferential direction in PAV. Between VE and PAV leaflets, the only difference in C was observed in the radial direction uniaxial tests, with the PAV leaflets having a lower value than VE leaflets ($p < 0.001$).

For the relaxation time constants τ_1 and τ_2 , there were no differences between circumferential and radial values in any test except the uniaxial test in PAV leaflets where τ_2 was higher in the radial direction (Fig. 7). There were no differences in τ_1 or τ_2 across strain ratio or between biaxial and uniaxial tests in VE leaflets. In PAV leaflets, τ_1 was lower in 1:10 tests than 1:1, 1:3, and uniaxial tests. PAV leaflet τ_2 was higher in the radial direction uniaxial tests compared to all biaxial test radial values.

Fiber alignment

The response of fiber alignment to biaxial stretching was different between VE and PAV leaflets. In both tissues, no

FIG. 3. Relaxed moduli. Relaxed moduli from each biaxial test (mean $\pm$ standard deviation [SD]). The "+" sign indicates that in all cases, the circumferential modulus was larger than the radial modulus ($p < 0.02$).

FIG. 4. Relaxed moduli ratios. Relaxed moduli from each nonequibiaxial test are normalized by their corresponding equibiaxial test moduli (=1.0) and plotted versus their *c:r* strain ratio. VE leaflets showed a significant negative correlation of circumferential modulus with increasing strain ratio and a significant positive correlation of radial modulus with increasing strain ratio. As the strain is increased in one direction, the orthogonal direction becomes apparently stiffer. No such effect was observed in PAV leaflets.

consistent shifting of mean fiber angle was observed with biaxial stretching at any strain ratio (Table 1). No difference in the change in mean angle was observed when comparing the nonequibiaxial (1:2, 1:3, 1:5, 1:10) strain tests to the equibiaxial (1:1) strain tests ($p > 0.1$). In VE leaflets, angular deviation increased significantly with decreasing *c:r* ratio (Table 1). On average, the angular deviation at the last step (highest strain values) was 14 times larger in the 1:2 tests and 20 times larger in the 1:5 tests than the 1:1 tests (Fig. 8, $p < 0.05$). No significant change in the angular distribution parameters was observed in PAV leaflets (Table 1 and Fig. 8, $p > 0.3$). VE and PAV leaflets had similar initial fiber mean angle (-1 ± 5 , VE; -3 ± 6 , PAV) and angular deviation (11 ± 2 , VE; 8 ± 3 , PAV) as measured at the preload state.

Discussion

In this study, fibrin-based tissue-engineered heart valve leaflets and PAV leaflets were tested in uniaxial tension and biaxial tension with multiple strain ratios. Results showed a correlation with strain ratio for relaxed modulus and fiber alignment distribution in engineered leaflets, but not in porcine leaflets. Some differences in relaxation behavior were observed with decreasing circumferential to radial strain ratios, especially at the extreme (1:10) ratio in PAV leaflets. Relaxation behavior was also different in biaxial tests compared to uniaxial tests. In uniaxial and equi-biaxial tension, there were few observed differences in relaxation behavior between VE and PAV leaflets, despite differences in the

FIG. 5. Relaxed stress. Relaxation percentages of VE and PAV leaflets biaxial and uniaxial tension (mean $\pm$ SD). The "+" sign indicates that uniaxial relaxation is significantly higher than corresponding biaxial relaxations ($p < 0.04$); Asterisk (*) indicates that 1:10 ratio relaxation is less than relaxation in 1:1 and 1:3 ratios.

FIG. 6. Reduced relaxation function (G) fits. Plot of reduced relaxation data for typical biaxial (left) and uniaxial (right) data along with fits (solid lines) of the relaxation function (G). In biaxial tests, fits were performed on eight incremental relaxation curves (dotted lines) four circumferential (black lines) and four radial (gray lines), chosen in the linear region of the relaxed stress-strain curve for each specimen at each strain ratio. In general, there was no apparent difference in the relaxation behavior with incremental strain, seen by the overlapping relaxation curves.

modulus. Likewise, in both tissues there was an overall similar relaxation response in both directions in biaxial tension, despite different moduli. To our knowledge, this is the first study of the dependencies of relaxation, modulus, and fiber orientation with biaxial strain ratio in engineered and PAV leaflets.

Differences in structure and composition exist between PAV and VE leaflets that correlate with the observed differences in their relative responses to biaxial stretching. PAV leaflets consist of three layers, a highly collagenous layer and an elastin-rich layer with a glycoprotein-rich layer in between.²⁴ The collagenous layer, the fibrosa, is undulated and contains distinct fiber bundles, both of which allow for large radial strains. The glycoprotein-rich layer may also allow for shear between the layers and reduction of leaflet stresses. VE leaflets are composed of collagen, residual fibrin, and other cell-produced ECM arranged in a more homogenous single-layered fiber network.⁶ In a previous study,⁶ PAV leaflets were observed to contain 95 mg/mL collagen and 3 mg/mL elastin, while VEs contained 12 mg/mL collagen and no measurable elastin. Over the strain range tested, PAV leaflets allowed for increasing radial strain without a commensurate increase in circumferential stress. In contrast, there was a stronger stress coupling in VE leaflets, where increasing radial strain caused a significant increase in circumferential stresses to create an apparent stiffening in the circumferential direction (Fig. 4). The microstructure of PAV leaflets appears designed to mitigate effects of nonequibiaxial loading, an important difference from the VE leaflets fabricated for this study and a consideration in heart valve replacement design and modeling. Similar to the modulus results, fiber reorientation in the VE leaflets was correlated to strain ratio, whereas PAV leaflets experienced little change in fiber alignment (Fig. 8). The broadening of the fiber alignment distributions with nonequibiaxial strain testing indicates that VE leaflets allowed more fiber reorientation with strain than PAV leaflets. Again, this is likely caused by the microstructural differences between the two tissues. The lack of

large changes in the distribution in PAV leaflets observed here are consistent with observations made by Billiar and Sacks³² using a different imaging technique. Vesely³³ observed the role of elastin in PAV leaflets to be to maintain the collagen fiber alignment during stretching, which may explain the differences reported here because there was relatively little elastin in the VE leaflets. For both modulus and fiber alignment, even though engineered tissue may be similar to the target tissue when unloaded, these properties can change significantly during mechanical loading. This is an important factor in design and modeling of engineered tissue.

The apparent stiffening of VE leaflets is likely a property of the bulk network as a whole. Intuitively, the change in fiber alignment in VE leaflets and lack of change in PAV leaflets with strain ratio might explain the observed modulus dependence. However, the apparent stiffening was opposite of the alignment change for VE leaflets. When the circumferential-to-radial strain ratio was decreased, the fiber alignment became less dominant in the circumferential direction. Assuming that all stress is conveyed along fibers, this would predict a decrease in the stiffness in the circumferential direction and increase in the radial direction. Observation of the opposite behavior points to a bulk network property causing an increasing volumetric stress with increasing strain. This bulk property could be a function of fiber-fiber interaction and/or involve components other than the fibrin and collagen fibers, such as fibronectin and proteoglycans. Further experimentation should be aimed at relating engineered tissue microstructure to the macroscopic mechanical response, such as coordinating immunolabeled EM imagery with stretch or loading states. The contribution of bulk material to the biaxial mechanics of engineered tissue may therefore be an important component in modeling these tissues in addition to fiber alignment, requiring the addition of an isometric stress term.³⁴

The time-dependent properties measured here were, in general, consistent with those measured in similar tissues by

FIG. 7. Relaxation parameters for biaxial and uniaxial tests. Parameters from the reduced relaxation function estimates of VE and PAV leaflets (mean $\pm$ SD). The “+” sign indicates that uniaxial value is higher than corresponding biaxial value ($p < 0.05$); Asterisk (*) indicates that C-value is less than corresponding 1:1 value ($p < 0.03$); The “#” sign indicates that circumferential value is different from radial value ($p < 0.05$). Percentage symbol (%) indicates that τ_1 1:10 test value is less than corresponding value in 1:1 and 1:3 test ($p < 0.01$).

different means by other investigators.^{35–38} The percent relaxation, and G-function parameters C and τ_2 , measured in PAV leaflets in uniaxial tension were similar to values reported for PAV leaflets by Sauren *et al.*³⁶ and Rousseau *et al.*³⁵ Our τ_1 values were larger than those of Sauren *et al.*, likely because of differences in testing method. The relaxation and G-function parameter values obtained in the equibiaxial strain tests for PAV leaflets (Figs. 6 and 8) were similar to the range of values measured in equibiaxial strain tests of the mitral valve leaflet by Grashow *et al.*³⁹ Likewise, the equibiaxial relaxation percent in PAV leaflets was similar to previously reported values obtained from equibiaxial tests on PAV leaflets.⁴⁰

The comparison of the relaxation behavior between PAV and VE leaflets and the behavior of both these tissues in nonequibiaxial strain are of particular interest because they have not been characterized in previous studies to our knowledge. In comparing PAV and VE leaflets in uniaxial and equibiaxial strain, there were very few differences in the relaxation behavior. The only observed differences were a lower C and higher τ_2 in uniaxially tested PAV leaflets in the radial direction. The similarities in relaxation behavior contrast to differences in the modulus and the fiber alignment behavior discussed above. This suggests that the tissue viscoelastic properties are mainly because of isotropically acting components, such as glycoproteins and fluid-matrix

TABLE 1. FIBER ALIGNMENT CHANGES WITH BIAxIAL STRAIN

		Change from preload to last step	
Strain ratio (c:r)		Mean angle (°)	Angular deviation (°)
VE	1:1	-1.8 ± 5.6	1.3 ± 1.0
	1:2	9.0 ± 8.2	14.5 ± 8.0^a
	1:5	2.4 ± 1.4	20.5 ± 10.0^a
PAV	1:1	2.3 ± 4.1	1.5 ± 3.5
	1:3	1.3 ± 10.1	1.4 ± 3.2
	1:10	8.1 ± 10.9	2.1 ± 6.3

Values shown are mean $\pm$ SD.

^aSignificant change between preload and last step ($p < 0.01$).

interaction. The observations of similar relaxation behavior yet markedly different modulus and fiber alignment behavior between VE and PAV leaflets suggest that tissue relaxation in these tissues is not dominated by fiber

alignment or directly related to the relaxed modulus. This finding corresponds to the observations made by Liao *et al.*⁴¹ that valvular collagen fibers are not intrinsically elastic.

While opinions differ on the role of stress relaxation in valve functionality *in vivo*, it has been shown that stress relaxation is important in healthy valve function.⁴² A limitation of the data in this study is that the timescale of stress relaxation is much shorter *in vivo* than in these experiments. This study focuses on nonfunctional tests to determine and compare the behavior of the two tissues and characterize mechanical properties of the material as opposed to providing functional data. As the technology for producing functional tissue-engineered constructs progresses, it will be important to test these responses on a more functionally relevant time scale. Then, these tests can be compared to the current data to draw correlations between the material mechanical properties measured commonly *in vitro* and functional performance assessments.

FIG. 8. Fiber orientation during biaxial testing. Typical fiber angle distribution changes with biaxial stretching for VE (left) and PAV (right) leaflets. In VE leaflets there was a significant fiber distribution change, evidenced by a broadening of the distribution (top graphs) from the preload (solid black line) distribution to the 1:2 (dashed black line) and 1:5 (solid gray line) distributions, plotted at point of maximum strain, where $x:y$ denotes the circumferential-to-radial strain ratio. This was observed quantitatively by plotting the angular deviation of the distribution with areal strain (lower graphs). In PAV leaflets, no significant change in the angular distribution was observed with biaxial stretching.

This study highlights some fundamental differences in planar biaxial viscoelastic behavior of PAV and VE leaflets as well as structure–function relationships in both these tissues. PAV leaflets were used as a homolog to human aortic leaflets owing to their mechanical similarity^{36–38} and use in current on the market heart valve replacements. While leaflets from the statically incubated VEs used in these studies had about an eightfold lower modulus than the PAV leaflets (Fig. 3 currently), leaflets from cyclically stretched VEs only have about a fourfold lower modulus (unpublished studies), and this may be reduced with further optimization. Moreover, the VE, which differs in many respects from the PAV microstructure and composition, will likely have different mechanical properties than PAV properties for optimal performance as a valve. The data will be useful in designing next-generation VEs and building constitutive models of engineered tissue. This study showed the PAV leaflets to maintain their apparent modulus and fiber alignment when exposed to nonequibiaxial strain, but to vary in stress relaxation properties. In contrast, fibrin-based engineered tissue exhibited a modulus and fiber alignment dependent on strain ratio, highlighting the importance of using a range of strain ratios when generating mechanical property data for native and engineered tissues. While the underlying causes of these structure–function differences are yet unclear, they are important considerations when building constitutive models from structural and mechanical property data, models that are needed in fluid–structure interaction simulations of valve function.^{43,44} These results underscore the value of building fiber-structure–based microscopic–macroscopic models to aid in rational design of engineered tissues. Ultimately, when designing engineered tissue, it may be important to mimic not only the initial fiber structure and properties of native tissue, but also the response of the native tissue to a nonequibiaxial loading environment.

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Disclosure Statement

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