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Defining biomechanical endpoints for tissue engineered heart valve leaflets from native leaflet properties

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Abstract

The design and development of functional engineered tissues is dependent on multiple considerations, with biomechanics paramount for load-bearing constructs such as tissue engineered heart valves. As the cryopreserved allograft is the current standard for valve replacement in pediatric patients, identifying and quantifying essential structural–mechanical properties of the native valve leaflet is a crucial step in the engineered valve leaflet design process. Native valve leaflet properties provide an intuitive basis for assessing engineered valvular tissue performance, and can potentially be used as biomechanical endpoints for qualifying engineered leaflets prior to clinical applications. In this short review, we present three analysis techniques that have been used by our lab and others for characterizing heart valve leaflet biomechanical response and discuss the relevance of these properties as candidate endpoints for engineered leaflet tissues. The studies presented herein focused primarily on the aortic valve, which most frequently warrants repair or replacement in the general population and has been useful in our understanding of bioprosthetic heart valve mechanics. However, these analysis techniques are directly applicable for pulmonary and most engineered valve leaflets. Where data is available, initial studies applying these techniques for in vitro assessment of scaffolds and engineered valve leaflets are presented. The development of a tissue engineered heart valve for the pediatric population is conceptually appealing, since few options currently exist due to the lack of growth potential in non-viable prosthetics and size limitations. While significant challenges remain, we believe that a derivative of the current tissue engineered heart valve paradigm will ultimately yield a design suitable for clinical evaluation. The role of biomechanics in this process will be to identify and quantify the structural–mechanical endpoints essential for appropriate heart valve leaflet function, while guiding investigators prior to and during clinical evaluation.

Keywords: Valve leaflet biomechanics; Tissue engineering; Soft tissue mechanics; Congenital valve disease

1. Introduction

As with most engineered tissues designed for use in the surgical repair or replacement of load-bearing tissues, the desired endpoint of engineered heart valve leaflet tissue is functional equivalence to the native leaflet. Because of this, the development of a tissue engineered heart valve is complicated, in that it mandates extensive biomechanical design and characterization. Moreover, any tissue engineered heart valve design will need to meet appropriate biomechanical criteria, or endpoints, before clinical evaluation. While heart valves serve a deceptively simple physiologic function–preventing retrograde blood flow–the means to this end are accomplished by a non-linear, anisotropic, heterogeneous tissue adapted to withstand the time-varying stresses resulting from each cardiac cycle. Therefore, characterizing the performance and properties of native leaflets is not trivial and requires fundamental understanding of native valve leaflet structure–function correlates.

Mechanically, the leaflets of both native semilunar valves undergo similar passive loading regimes: flexure during opening and closing, shear stress from flowing blood when opened, and planar tension when closed. The atrioventricular valves are more complicated due to their asymmetrical shape

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and the tethering effect of the chordae tendinae; therefore, they will not be considered further in this manuscript. Aortic and pulmonary valve leaflets have an evolved, specialized architecture that allows for efficient opening and closing with slight pressure gradients while also withstanding large transvalvular pressures when apposed during closure. For example, the leaflets have a tri-layered structure, which is composed predominantly of fibrillar collagen (fibrosa layer), glycosaminoglycans (spongiosa), and elastin (ventricularis). It is the structural arrangement of the aligned fibrillar collagen network that largely defines the mechanics of the leaflet, thereby making it essential to recapitulate this response in the engineered tissue.

Interestingly, the ultimate tensile strengths of human aortic and pulmonary valve leaflets are very similar (1460 and 1450 kPa, respectively) [1]; however, the minimum required mechanical strength of an engineered heart valve leaflet will be dependent on the level of transvalvular pressure they must support (10 mm Hg for the pulmonary and 80 mm Hg for the aortic) [2]. While uniaxial testing of native leaflets is useful for failure strength measurements, this information does not complete our understanding of the tissue biomechanics, in that they rarely fail catastrophically. Of greater concern is their ability to remain sufficiently compliant and coapted when apposed, which is a bi-directional response. Because of these considerations, native leaflet response to diastolic pressure is best examined mechanically by biaxial testing of the belly region of the leaflets [3–6].

The ability of heart valve leaflets to open and close efficiently 3×10^9 times during a person's lifetime is also of great importance, and this motion is directly related to the bending stiffness of the tissue. Additionally, the bending stiffness of the leaflets can be used to assess damage mechanisms associated with calcification and flexural fatigue [7,8]. Bending tests are highly sensitive at low stress–strain values, and when combined with specialized imaging techniques and histology, they allow investigators to probe the individual layers of a leaflet tissue. Dictating both the biaxial and flexural responses is the aforementioned collagen architecture of the native leaflets, which can be analyzed and quantified by small angle light scattering (SALS) [9–11]. The sum of these analyses serves to describe the gross mechanical response resulting from the fibrous architecture of native heart valve leaflets. Finally, experimental results not only provide quantitative data for use in setting engineered tissue goals, but also allow for the development and implementation of constitutive models [12,13] which, when properly formulated and validated, can vastly expand the scope of variables that can be perturbed through analytical and numerical simulations.

The need for basic research to elucidate suitable biomechanical endpoints for engineered tissues is an underemphasized area, and the ability to direct the development of a tissue engineered heart valve will be hindered if it does not become a more pronounced theme. We believe that biomechanical approaches toward the design and assessment of engineered heart valve tissues complement the pragmatism and ingenuity of traditional surgical research approaches. Furthermore, future success of tissue engineered heart valves will be highly dependent on the ability to adequately characterize the final construct prior to pre-clinical animal studies or potential clinical evaluations. It is during both the iterative process of development and the final analysis of the functional engineered valve where biomechanics serves its primary role of defining necessary requirements. Though there are many current challenges, progress is being made towards a biomechanically functional tissue engineered heart valve, and herein, methods for analysis and milestones are reported on this progress.

2. Methods

2.1. Tissue preparation

Performing biomechanical analysis is largely dependent upon the size of the sample specimen, particularly regarding the ability to grip and apply mechanical loads to the tissue. Because of size limitations and our interest in bioprosthetic heart valve biomechanics, the majority of our native valvular research has dealt with porcine aortic valve leaflets. Leaflets are excised from the valve by cutting along the basal attachment. Typically, a square specimen (10×10 mm) from the central belly region of the leaflet is removed. Depending on what exactly is being analyzed, the specimen can be run through the following series of tests in sequential order: flexure testing, biax testing, and SALS analysis (Fig. 1). This sequence is important due to the application of markers with cyanoacrylate that would alter mechanical response if leaflets were tested otherwise (i.e. biax before flexure).

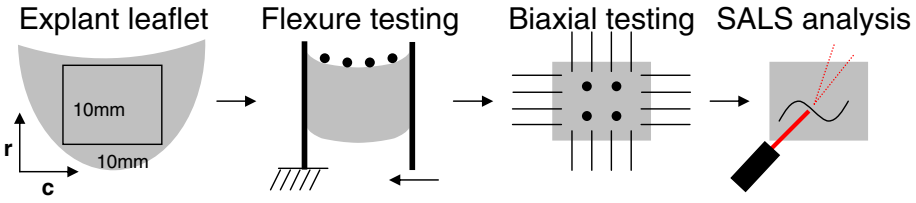

Fig. 1. Schematic of biomechanical analyses of heart valve leaflets. Note that the sample in the Flexure testing configuration is being bent and not compressed. Proceeding form the left to the right, the leaflet is excised, tested under flexure, tested biaxially, and then analyzed with SALS. The small black circles represent applied markers used for strain determination.

2.2. Flexure testing

Loads to achieve flexure are applied by stationary and translatable arms that are coupled to the ends of the tissue by short metal sleeves (attached by cyanoacrylate) that slide onto the arms of the bending device [7,8,14,15]. The translatable arm is a deformable bar that was previously calibrated to determine loads from deflection. Markers are attached to the top of the tissue nearest the free edge. Specimen deflection is recorded with a CCD camera and real-time, resulting moment, M (mN mm), and change-in-curvature, $\Delta\kappa$ (mm^{-1}). Values are determined at small time increments with a custom program by tracking the marker and bending bar positions. The applied moment versus the change-in-curvature is related by the Bernoulli–Euler moment–curvature equation, $M=E_{\text{eff}}\ I\ \Delta\kappa$, where $E_{\text{eff}}\ I$, termed flexural rigidity, corresponds to the slope of $M-\Delta\kappa$ curve, with I as the second moment of inertia calculated as $I={}^{1}/_{12}t^{3}w$. The terms t and w are the thickness and width of the sample, respectively. The physical meaning of E_{eff} is the instantaneous effective stiffness for a given $\Delta\kappa$, and is analogous to Young's modulus from uniaxial tension testing.

2.3. Biaxial testing

Biaxial testing of native and bioprosthetic leaflets has been described extensively in previous publications [6,16–18]. Briefly, tissue deformations are measured by monitoring the movements of four small graphite particles which are attached to the center of the specimen in a square configuration. Sutures (four per side) are attached to the sample to apply loads and are coupled to the actuating arm of the device via pulleys to allow equal stress distribution at each suture point. Additionally, it is essential to allow freedom for strain in the orthogonal direction during biaxial testing and this suturing method accomplishes this by not rigidly fixing each side of the tissue [19]. When compared to the reference state, these marker movements reveal the resulting orthogonal strains and in-plane shear from applied loads, which are simultaneously monitored with sensitive load cells. Typically, loads are applied in a quasi-static fashion which is not physiological for leaflets; therefore, we recently built a system that was capable of applying and tracking high-speed physiologic strain rates (500–1000%/sec) [20]. Both systems reveal the orthogonal, coupled stress–strain response which is of paramount importance for anisotropic planar tissues such as leaflets.

2.4. SALS analysis

SALS is an effective technique for the microstructural analysis of planar fibrous connective tissues, and we have used it previously for mapping the architecture of leaflets from normal, pressure fixed, and explanted bioprosthetic valves [9,10,21]. Briefly, a continuous unpolarized wave laser is passed through the tissue, which scatters light according to the internal planar fiber structure. The resulting angular distribution of scattered light intensity about the laser axis represents the distribution of fiber angles within the beam envelope at the current tissue location. Gathered information includes: 1) preferred fiber direction, 2) distribution skew, and 3) orientation index, which represents 50% of the total number of fibers.

2.5. Development of constitutive models

Utilizing experimental data to develop constitutive models that succinctly describe the mechanical response is a valuable tool that can be used to analyze native and engineered tissues. Previously, constitutive models have been developed for the aortic valve leaflet [12] and an experimentally derived fiber orientation model for planar collagenous tissues [13]. These serve as comparative models for not only engineered tissues, but also for initial scaffolds [22] that are being investigated for tissue engineered leaflets.

3. Results

3.1. Flexural response of native and engineered leaflets

Flexure of soft biological materials offers two distinct advantages over uniaxial mechanical testing: 1) the ability to discern slight changes in stiffness at low-stress strain levels that would not be appreciable in tension and 2) the ability to assess individual layers of multi-layered structures. Because of these advantages, flexure is ideal for analyzing slight changes to leaflet layer properties due to structural damage, cellular contraction, and ECM biosynthesis.

Aortic leaflets reveal a distinct bending response depending on the direction of bending. This is due to the ECM composition in the different outer layers. In the natural curvature state, the ventricularis is in tension and the fibrosa is in compression (Fig. 2a). The effective stiffness measured in the with-curvature direction is dominated by the tension in the ventricularis, with little contribution from the fibrillar collagen in the fibrosa, which is not designed to support compressive loads. Conversely, when the leaflet is bent against the natural curvature, the fibrosa is in tension and the ventricular is in compression. Here, we see the influence of the collagen fibers from the fibrosa; aortic valve leaflets are stiffer in the against-curvature direction compared to the with-curvature direction (Fig. 2b) [7]. Additionally, when the valve interstitial cells basal tonus is inhibited with thapsigargin (10 μm) treatment overnight, significant loss of stiffness is observed. This reveals the cellular mechanical contribution to the native leaflets at low stress–strain levels, further demonstrating the sensitivity of this testing method.

In tandem with flexural mechanical testing of native leaflets, we have also investigated the effects of flexural

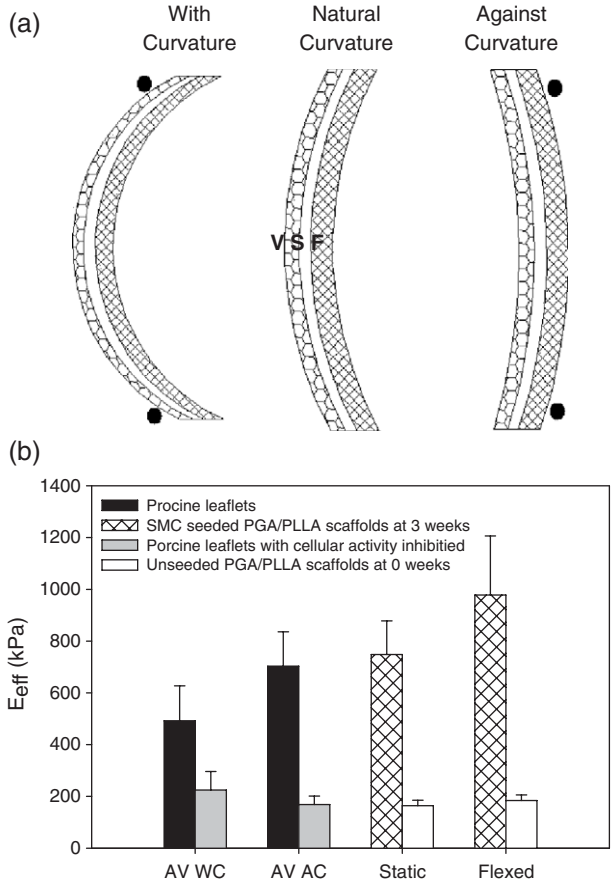

Fig. 2. Flexure testing. (a) Bending directions of the leaflet. Note that the ventricularis is in tension when bent 'with-curvature', and the fibrosa is in tension when bent 'against-curvature' (V=ventricularis, S=spongiosa, and F=fibrosa). (b) Compiled results from [7] and [14], reprinted with permission, demonstrating the bending stiffness of native porcine aortic leaflets (black), native leaflets with the cellular basal tonus inhibited (gray), cell-seeded PGA/PLLA scaffolds at three weeks (diagonal checkerboard), and unseeded PGA/PLLA scaffolds at zero weeks. This data is presented to show the sensitivity of flexure testing and the effects of cell seeding and mechanical training of engineered tissues on mechanical properties.

mechanical stimulation on engineered heart valve leaflet tissue formation. A novel bioreactor was developed which has the capacity to subject up to 12 rectangular strips of scaffold or tissue to cyclic flexure [15]. In a subsequent study, nonwoven scaffolds fabricated from 50% poly(glycolic acid) and 50% poly(L-lactic acid) fibers (PGA/PLLA) were seeded with vascular-derived smooth muscle cells and either maintained under static conditions (static group) or stimulated by unidirectional cyclic flexure for three weeks [14]. Compositional, histological, and mechanical analyses of the three week specimens revealed increased collagen concentration, enhanced vimentin expression, and a trend of increased E_{eff} (Fig. 2b) in the flex versus static groups. Note that the virgin unseeded PGA/PLLA scaffolds had significantly lower E_{eff} values than either the static or flex group specimens. Of potential utility in tissue engineered heart valve design, these results revealed that the virgin PGA/PLLA scaffolds had similar E_{eff} values to those of thapsigargin-treated native porcine leaflets (i.e., no cellular basal tonus) [7], and after smooth muscle cell seeding and three weeks incubation exhibited E_{eff} values comparable to freshly harvested native porcine leaflets (Fig. 2b). It should be noted, however, that these E_{eff} values are all significantly higher than E_{eff} of the native ovine pulmonary valve leaflets typically replaced in our large animal studies [23]. This was the first study to examine the flexural properties of a tissue engineered heart valve leaflet.

3.2. Biaxial response of native leaflets and scaffolds used in engineered leaflet fabrication

As mentioned above, native aortic and pulmonary valves have an aligned and organized collagen architecture that is primarily oriented in the circumferential direction of the leaflet. This fibrous architecture largely defines the response

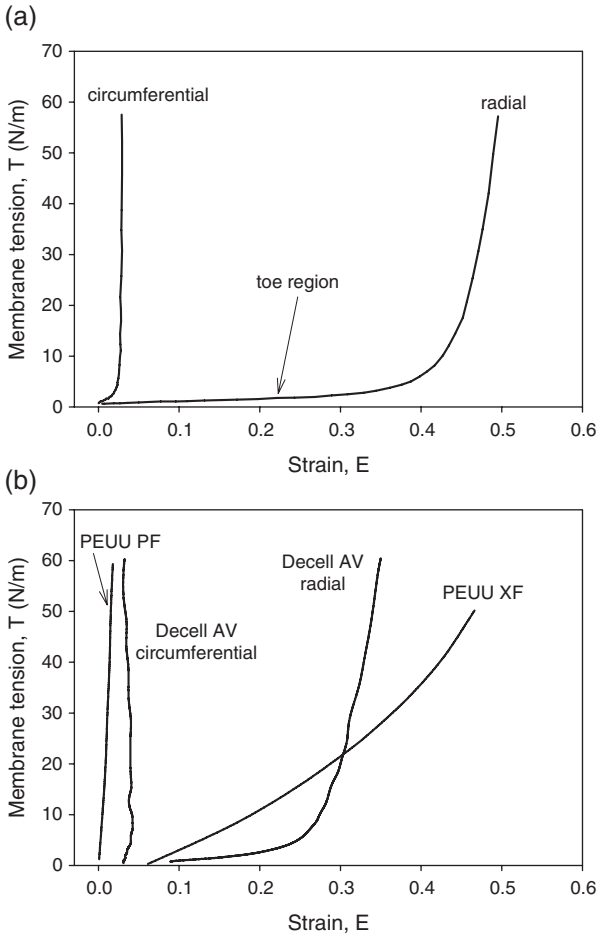

Fig. 3. Biaxial testing. (a) Biaxial response of a native porcine aortic leaflet. Note the anisotropic response of the tissue. 60 N/m membrane tension corresponds to in vivo diastolic pressures at rest. (b) Biaxial response of a decellularized porcine aortic valve leaflet and an electrospun polyurethane urea (PEUU) biopolymer fabricated at a spinning speed of 2300 rpm. PF and XF refer to the orientation of the electrospun fibers; PF is in the direction of fiber deposition and XF is in the orthogonal direction. Note the similar strain response from both materials compared with the native leaflet under the same loading regime.

to biaxial tension. As in other collagenous tissues with some degree of orientation, native leaflets have distinct responses in the circumferential and radial directions (Fig. 3a). At the beginning of the biaxial test, the collagen fibers become uncrimped in the circumferential direction and exhibit a sharp rise in tension with little increasing strain. This is not surprising since the robust collagen fibers are supporting the applied load and are straightened and taut. Radially, there is a much more gradual response since this axis has fewer aligned fibers. Initially, there is a large toe region where strain increases with little appreciable stress. Then there is a transition leading to a rise in stress where the tissue reaches maximum extensibility. The high compliance exhibited in this initial radial direction toe region allows the leaflet to stretch and remain coapted during diastole. Conversely, the stiff circumferential direction is necessary to support the large transvalvular pressure imposed on the tissue. These distinct directional responses are crucial for proper valve function and must be of the highest priority when analyzing engineering constructs.

To date, several candidate biomaterials for use as tissue engineered heart valve scaffolds have been analyzed biaxially. Among these are porcine derived small intestinal submucosa [24], decellularized aortic valves, and electrospun polyurethane urea [25]. Decellularized heart valves are conceptually appealing in that they already contain the necessary architecture to elicit an appropriate biaxial response as well as innate bioactivity for cell attachment. The novelty of the electrospun polymer scaffold lies in the ability to tune the biaxial properties of the material by controlling the deposition speed of the polymer and angular velocity of the rotating mandrel onto which the polymer is being deposited. It can be seen that there exist similarities in the decellularized aortic valve leaflet and the electrospun polymer compared with the native porcine aortic leaflet biaxial response (Fig. 3b); however, further studies will be necessary to understand resulting changes from scaffold degradation and extracellular matrix synthesis. While these materials are encouraging from a mechanics point of view, there still remain challenges of optimizing cellular penetration, viability, and biosynthesis. Recently, smooth muscle cells were integrated into the electrospun scaffold during the fabrication process [26], which may solve the problem of cellular penetration. Future work will determine what effect cell integration has on the mechanical properties and additionally, the long term viability of the integrated cells. Ultimately, biaxial testing will need to be done on explanted engineered heart valve leaflets and results compared with native leaflet properties. Unfortunately, there exists no biaxial data of explanted engineered leaflet tissues at the current time to the authors' knowledge.

3.3. SALS of native leaflets and scaffolds used in engineered leaflet fabrication

The mechanical response of the native semilunar leaflets is highly dependent on the state of the aligned collagen network, and while biaxial testing demonstrates this re-

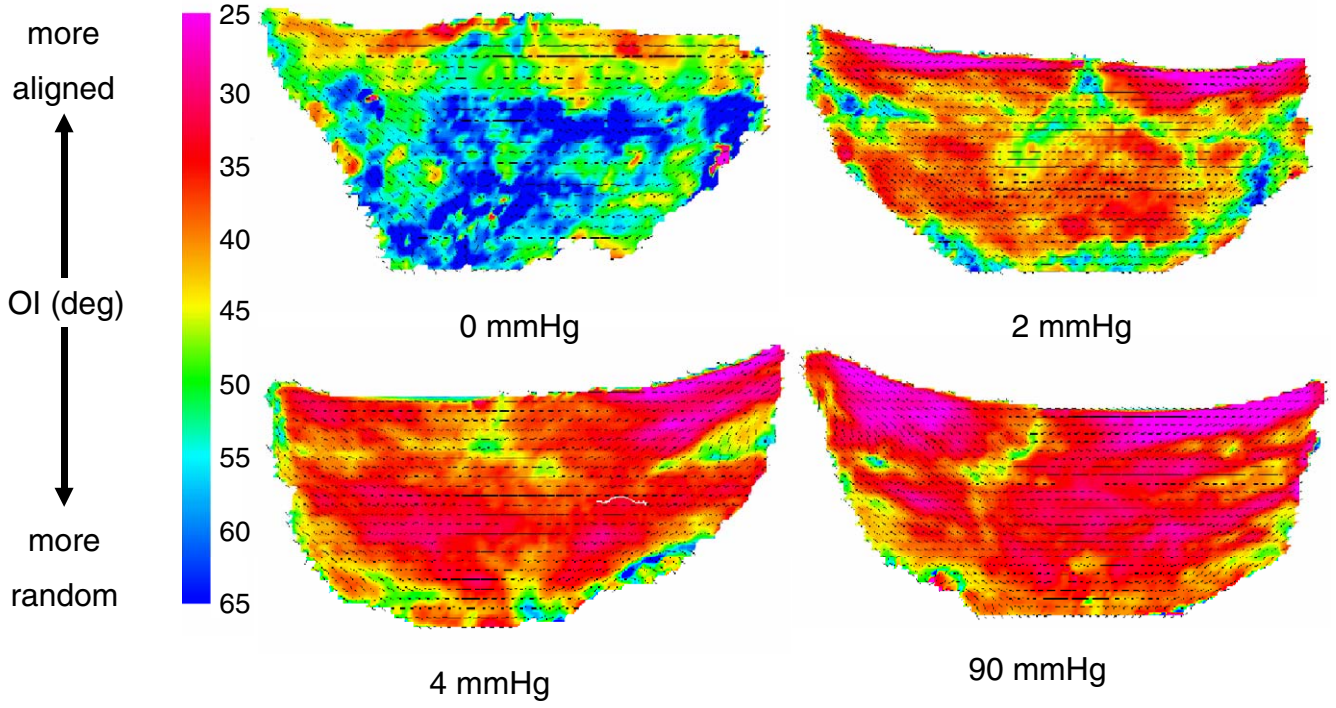

Fig. 4. SALS of the native porcine aortic valve leaflet reprinted with permission from [9]. Leaflets were fixed at increasing transvalular pressures and analyzed for collagen orientation. OI (deg) represents the orientation index and represents 50% of the total number of fibers in that area. Essentially, pink areas have a lower OI value and are more aligned whereas blue areas have higher OI values and are less aligned.

sponse, it does not reveal local variations in the architecture. Therefore, to identify regions of interest, SALS has been utilized to probe areas of collagen disruption and damage [21]. Additionally, SALS has been used to assess exactly how the aortic valve collagen fibers rotate due to applied transvalvular pressure [9]. It can be seen that increasing pressure on the leaflet induced the greatest changes in fiber alignment between 0 and 4 mm Hg, with no further change past 4 mm Hg (Fig. 4). Additionally, when the native leaflet outer layers are separated by dissecting through the spongiosa and rescanned, there is much higher degree of orientation in the fibrosa layer while the ventricularis appears to be randomly oriented. At higher pressures (>4 mm Hg), differences in each layer became less pronounced and indistinguishable at 60 mm Hg. These results further highlight the complexity of the leaflet structure, and demonstrate a sensitive response to low transvalvular pressures. Scaffolds and engineered leaflets may not need to exactly recapitulate this structural response, but it is believed that this should be of consideration.

More recently, the nonwoven PGA and PLLA scaffolds used in the development of engineered leaflet tissues have been analyzed using the SALS technique [22]. In contrast to previous reports of an isotropic structure, each of the commercially available nonwoven PGA, PLLA, and PGA/PLLA scaffolds tested exhibited distinct preferred and cross-preferred fiber directions. Flexural mechanical testing demonstrated that this unique fiber arrangement yielded an ~3-to-1 ratio in the value of E_{eff} measured for the preferred and cross-preferred fiber directions, respectively. While it remains unclear how this nonwoven fiber arrangement might influence the orientation of the cells and deposited collagen in an engineered heart valve tissue, we recently used the SALS technique to demonstrate that large-scale (~200 μm) open pore structures can be used to guide cell and collagen orientations in engineered tissues [27]. In light of the unique flexural mechanical requirements of the native valve for proper opening and closing, these findings could have profound implications on how scaffolds are fabricated and oriented in constructing an engineered heart valve leaflet.

3.4. Constitutive models of native leaflets and scaffolds used in engineered leaflet fabrication

A structural constitutive model has been developed for the aortic valve leaflet [12]. While this model makes some assumptions in simplifying the structure of the leaflet, it was able to account for the full spectrum of axial coupling exhibited under various loading protocols. The utility of this type of model lies in the ability to analyze an engineered leaflet tissue under a variety of biaxial protocols (as opposed to just equibiaxial, which is shown in Fig. 2). This exhaustive mechanical analysis reveals subtleties that may be overlooked with less thorough studies. While this level of understanding may not be required until late in the development process, it will likely be necessary for completion.

Toward developing a constitutive model to describe an engineered heart valve leaflet, we recently developed a structural model for simulating the flexural mechanics of the nonwoven PGA, PLLA, and PGA/PLLA scaffolds [22]. In the structural model we incorporated both the experimentally measured fiber orientation distribution measured by SALS, as well as the number of fibers and their spring-like tensile mechanical behavior. During nonwoven scaffold fabrication, the PGA and PLLA yarns are crimped to yield a sinusoidal curved structure [28]. We measured the geometry of these curved fibers using scanning electron micrographs and calculated effective fiber stiffnesses which, when incorporated into the structural model, yielded accurate simulations of the experimentally measured flexural response.

Perhaps the most intriguing finding of this study was that the cell-seeded nonwoven scaffolds, which are effectively composites dominated by extracellular matrix–scaffold interactions, cannot be described using traditional rule-of-mixtures approaches [29] in which E_{eff} of the composite is predicted from E_{eff} of the individual constituents and their respective volume fractions. The primary mechanism for stiffening in nonwoven scaffolds was found to be an increase in the number of fiber-to-fiber bonding points, and thus our structural model explained for the first time the large increases in E_{eff} observed in the three week, smooth muscle cell-seeded specimens discussed above (Fig. 2b).

4. Discussion

4.1. Native leaflet biomechanics and candidate endpoints for assessing engineered leaflets

From a biomechanics point of view, the important functional properties of heart valve leaflets are compliance to adeptly open and close, and structural integrity to withstand planar tension when apposed during diastole. Therefore, the bending stiffness and biaxial response of an engineered tissue will likely need to resemble that of the native leaflet. To achieve this desired response, it is intuitive that some collagenous or otherwise organized architecture, similar to that of the native leaflet, would be required; hence, SALS is useful in evaluating this. The difficulty that faces those analyzing the engineered leaflet tissue biomechanical response is determining how to successfully couple these analyses. Flexure data does not incorporate appropriate biaxial coupling, while biaxial testing does not yield transmural tissue mechanical properties. Additionally, SALS data can only serve to demonstrate where and how the collagen fibers are arranged and oriented; this information is independent of fiber mechanical properties, which can only be determined through structural constitutive modeling. While the methods and results presented here demonstrate an ability to describe most aspects of leaflet biomechanics, they can only be utilized in as much as they are applied in concert. Furthermore, one can easily envisage a case where an

engineered tissue responds as desired to one or more of these analyses, but ultimately does not function as a suitable valve replacement. Hence, we believe that these analyses are important and directive and should compliment surgical intuition derived from hands-on in vitro and animal model experience.

As mentioned previously, it is believed that these analyses are important prior to pre-clinical animal studies and clinical trials; however, once in these stages, an iterative process will likely be required. While quantifying pre-implant mechanics gives an initial starting point, effects of host cell remodeling, mechanical fatigue, and biodegradation will need to be assessed in the in vivo setting. Therefore, progress must be monitored after sequential improvements or longer implant durations. From previous experience with bioprosthetic valves, it is believed that monitoring is crucial as unforeseen events can arise during both early [30] and late implant times (mechanical fatigue independent of calcification [21]). Hence, biomechanical analysis should not be relegated to in vitro studies before moving to in vivo studies; the process will likely require multiple generations of engineered valves for continual improvement of the design.

4.2. Future directions for biomechanics of native and engineered valve leaflets

The methods presented here are not exhaustive, in that they examine the global mechanical response of the tissue. Biomechanical analysis at smaller scales (individual fibers, cells, and molecules) would undoubtedly yield useful information; however, we believe that initial and prolonged success of an engineered construct will ultimately be at the tissue level. Recently, we examined the mechanical properties of individual heart valve interstitial cells and their resulting biosynthetic response [2]. While the results were interesting, their mechanical contribution to leaflet mechanics is likely negligible, and we speculate that their true importance is in regard to protein production due to applied local tissue stress. This mechanical–synthetic relationship will certainly be of importance for engineered tissues and further work on smaller scales will be necessary to elucidate it; however, at this point much remains unclear as to how tissue stresses translate into functional cellular responses in native tissues.

The methods and results presented here are well documented for aortic valve leaflets due to their importance in understanding bioprosthetic heart valve development for adults and the resulting success and failure of these valves. However, the pediatric population would greatly benefit from an engineered heart valve due to lack of options at the present time, and the pulmonary valve will likely have better initial success due to the less demanding mechanical environment. To address this, we have begun to analyze the native porcine pulmonary valve leaflet with the same protocols presented here [31–33]. A previous study compared native and chemically fixed aortic and pulmonary leaflets under biaxial tension and found that they are quite different with respect to their response, suggesting that the collagen content was different between the leaflets [3]. It is essential to understand this difference and if this difference is important or inconsequential. For instance, clinical explants suggest that success of the Ross procedure may be due in part to adaptive remodeling [34]; however, would the reverse be true (i.e. aortic to pulmonary switch)? If not, this would indicate that the valve is capable of adapting to greater mechanical demands but not less. Therefore, leaflets may need to be specifically designed for loads just below what they will see in vivo and adapt once implanted.

5. Summary

This short review paper is meant to familiarize the general reader with current methods and progress in understanding native heart valve leaflet biomechanical analysis in order to define possible endpoints for engineered leaflets. It is believed that these analyses should serve as a minimum level of suitable qualification for engineered leaflet constructs, and development and implementation of additional analyses will likely be necessary as our understanding increases.

Acknowledgements

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